Phytochemical and Biological Studies on Selected Medicinal Plants Used In Indian Traditional System

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy (Ph.D.)

By

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March, 2015

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University of Hyderabad

(A Central University established in 1974 by act of parliament)

HYDERABAD– 500 046, INDIA

"DECLARATION"

I, **Zahoor Gul Mir**, hereby declare that this thesis entitled **"Phytochemical and Biological studies on selected medicinal plants used in Indian traditional system"** submitted by me is based on the results of the work done under the guidance and supervision of **Dr. Irfan A. Ghazi** at Department of Plant Sciences, School of Life Sciences, University of Hyderabad. The work presented in this thesis is original and plagiarism free. I also declare that no part or in full of this thesis has been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

Zahoor Gul Mir
(Research Scholar)

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"CERTIFICATE"

This is to certify that this thesis entitled **"Phytochemical and Biological studies on selected medicinal plants used in Indian traditional system"** is a record of bonafide work done by **Mr. Zahoor Gul Mir,** a research scholar for Ph.D. programme in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Dr. Irfan A. Ghazi
(Supervisor)

Prof. Ch. Venkataramana
HEAD
Department of Plant Sciences

Prof. P. Reddana
DEAN
School of Life Sciences

Dedicated to:

My Loving Mother
&
My Mentor - Malik Mehraj

ACKNOWLEDGEMENTS

In the first place, I offer my humblest thanks to **"Almighty Allah"** who enabled me to make some material contribution to the pre-existing ocean of knowledge and thoughts. All the praise and glory to **"HIM"** who blessed me with health, patience and strength to complete this project successfully. All blessings and respects are for our beloved Prophet Muhammad (PBUH) whose teachings guide us towards light of knowledge.

In pursuit of this academic endeavour, I feel that I have been especially fortunate as inspiration, guidance, direction, co-operation, love and care, all came in my way in abundance and it seems almost an impossible task for me to acknowledge the same in adequate terms. At the end of my thesis, it is a pleasant experience to express my gratitude to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

At this moment of accomplishment, I express my deepest gratitude to my supervisor, **Dr. Irfan Ahmad Ghazi**, Assistant Professor, Department of Plant Sciences, School of Life Sciences, University of Hyderabad for his esteemed supervision, incessant support, constructive comments and limitless patience throughout my research work. His mentorship was paramount in providing a well-rounded experience consistent my long-term career goal. He encouraged me to not only grow as an experimentalist but also as an instructor and an independent thinker. I am indebted to him for allowing me to work under his guidance at that time when others closed their doors.

I thank the present and the former Deans, School of Life Sciences, **Prof. P. Reddana, Prof. A. S. Raghavendra, Prof. R.P. Sharma, Prof. M. Ramanadham** and the present and former Heads, Department of Plant Sciences, **Prof. Ch. Venkatramana, Prof. A. R. Reddy and Prof. A. R. Podile** for allowing me to use the facilities of the School and the Department.

Grateful appreciation is also conveyed to my doctoral committee members, **Prof. A. S. Raghavendra and Dr. Sarada D. Tetali** for periodically evaluating my progress in research and their helpful suggestions and comments.

I thank **Dr. Insaf A. Qureshi** for his helpful suggestions and comments during the progress presentations and extending his lab facilities. I also thank **Dr. Radheysham Maurya** for providing his lab facilities for cell culture studies and his words of encouragement. I also extend my gratitude to **Dr. Ragiba** for her suggestions and encouragement.

I express my gratitude to all the faculty members of School of Life Sciences, University of Hyderabad for their words of encouragement.

I ardently extend my thanks to **Dr. Mushtaq Ahmad** (**Ex Director), Central Research Institute for Unani Medicine, Hyderabad** for generously providing the plant materials and his valuable suggestions and words of encouragement.

I take this opportunity to sincerely acknowledge the infrastructural support provided by **UGC-SAP, DST-FIST, DBT-CREBB**, to the Department of Plant Sciences and School of Life Sciences, University of Hyderabad and **DST, DBT** and **UPE-II** to IAG laboratory.

For financial support, I gratefully acknowledge the **University Grants Commission (UGC)** and the **Central Scientific and Industrial Research (CSIR)**, Govt. of India for providing financial assistance in the form of fellowships; (RFSMS) and (SRF) respectively which buttressed me to perform my work comfortably.

Most of the results described in this thesis would not have been obtained without a close collaboration of my friends from different laboratories at School of Life Sciences, University of Hyderabad.

I would also like to thank some people from early days of my research tenure. **Dr. Mohammad Bhakshu** and **Dr. Girish Kumar** were among those who kept me going at the beginning. I thank them for their advice and their willingness to share their bright thoughts with me which was very fruitful for shaping up my ideas and research. **Dr. Mohammad Bhakshu's** help in standardising the protocols and constant support deserves special mention here.

I owe deep and sincere appreciation to **Dr. K. Manjulatha**, Woman Scientist, Department of Biochemistry, University of Hyderabad for her love, care, guidance, fruitful discussions, encouragement and constructive advices and particularly for sharing her expertise in metabolomics analysis.

It is impossible to frame the help and co-operation of my friend, **Mr. Chandrasekaran S** in words. Thank you for always helping and assisting me in cell culture studies and sharing your expertise that greatly improved this study. This work would not have been possible without his active help and support. I owe my thanks to dear friend **Mr. Farhan Ahmed** for his help and cooperation during the research work.

My dear friend, **Mohammad Mujahid's** insightful comments, suggestions and guidance from the beginning till the end of this project were thought-provoking and helped me focus my ideas. I am also grateful to him for sharing good moments during my stay at the University of Hyderabad.

I would like to thank my lab-mates **Ms. Waikhom Bimolata, Mr. Anirudh Kumar, Mohd Yasin**. They have been great lab-mates which made the working in the laboratory more smooth and enjoyable.

I gratefully acknowledge **Mr. Prashant** and **Miss Nalini** for their amicable assistance in using metabolomics facility and confocal microscopy respectively.

I am thankful to **Dr. Roy and Dr. Venkat** for their valuable suggestions and sharing his expertise with me during the course of study.

I am indebted to my many student colleagues for providing a stimulating and fun filled environment in the School of Life Sciences, University of Hyderabad that helped me to stay sane through these difficult years. Their support and care helped me overcome setbacks and stay focussed on my graduate study. I greatly value their friendship and I deeply appreciate their concern that helped me adjust to a new place away from my home.

I acknowledge the guidance, help and appreciation of faculty and the staff of the Department of Plant Sciences and School of Life Sciences in my endeavours.

I owe a great deal of appreciation and gratitude to **Prof. R. L. Hangloo (Dept. of History, UoH) and Prof. S. N. Koul (Dept. of Physics, UoH)** for their affection and words of encouragement. It is impossible for me to forget these two people during my lifetime.

It's my fortune to gratefully acknowledge some special individuals. Words fail me to express my appreciation to **Dr. Gulfishaan Habib (Associate Professor, Moulana Azad National Urdu University, Hyderabad)** for her encouragement, generous care, love and the homely feeling at Hyderabad.

I would like to thank my friends, **Dr. Adil Hussain, Dr. Naseer Khan, Mr. Zameer Ahmad and Mr. Asif Khanday** who were always there when I really needed. Thank you doesn't seem sufficient but it is said with appreciation and respect to them for their support and care when I was ill, their encouragement, understanding and precious friendship. I am much indebted to **Mr. Danish Manzoor** for his friendship and spending his precious times with me.

I appreciate the discussions about my personal and professional life with my friend, **Dr. Faheema Khan.** She reminded me what could be the influence of the loving Allah through prayers and having faith in Him. This helped me to work hard to accomplish my project.

It is also a pleasure to mention my friends from my homeland, **Miss Iqra Bashir**, **Mr. Tahir, Mr. Rauf Mir, Mr. Taufeeq, Mr. Sajad, Mr. Fayaz, Mr. Tariq ul Islam, Mr. Amjad, Mr. Bilal Mir, Mr. Waseem Raja, Mr. Lateef, Mr. Rayees, Mr. Layeeq, Mr. Yawar** for creating a jovial and exhilarating atmosphere like home here in the University of Hyderabad.

I thank my lab attendees **Mr. Raju** and **Mr. Ranjeet** for their assistance in the lab.

I would like to extend my gratitude to my friends, at School of Life Sciences, UoH, **Miss Sonali Dey, Dr. Anjali, Miss Sravani, Miss Sarada, Mrs. Shreya, Mr. Tapas Mukherjee, Mr. Nasim Imam, Mr. Sumit, Mr. Naveen, Mr. Abhay, Mr. Subnarayan Das, Mr. Rajsheel, Mr. Parag, Mr. Arif, Mr. Majid, Mr. Nishant, Mr. Mr. Kishore, Mr. Arshad** for their cheerful and helpful moments during my stay.

I am eternally indebted to **Miss Malthi Bheri** for scrupulously correcting my chapters in the midst of her busy schedule. She has been a good friend.

Words fail me to express my appreciation to **Miss Nupur** for her encouragement, generous care and the homely feeling at Hyderabad.

I would also like to extend warm and heartfelt thanks to **Kalam Bhaijan** and **Sadaf Baji** for their love and encouragement.

I will be failing in my duties if I do not acknowledge the friendship of **Mr. Bilal Malik** and **Mubashir Ahmad**. I will always cherish the moments shared with them.

My sincere thanks go to **Miss Sana Firdaus Khan** for her love and care. Her separation from me at the end of my thesis became too excruciating for me to put up with.

I sincerely thank my wife, **Mrs. Amtul Baseer Quadri** for always being beside me during the inevitable ups and downs while conducting my research. She often reminded me life's true priorities to push me and motivate me. Her unwavering love was undeniably the bedrock upon which the past five years of my life have been built. Her tolerance of my occasional bad moods is a testament in itself of her unyielding devotion and love. I doubt, I will ever be able to convey my appreciation fully, but I owe her for being unselfishly let her intelligence, passions, and ambitions collide with mine.

I would take this opportunity to thank **Mr. Malik Mehraj**. I found guidance, friendship, discipline and love, everything in him. Above all and the most needed, he provided me unflinching encouragement and support in various ways in pursuit of my education. His exceptional and unmatched intuition remarkably inspired and enriched my growth as a student and a researcher. Without him, I would have been lost. He has been the mentor of life and shown me the right path. He triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. I thank him for inspiring me and whatever little I have achieved in my life is because of him. I will be grateful to him throughout my life.

I am grateful to all my teachers, who are instrumental in shaping up my life and taught me the ABCs of biology.

Saving the most important for the last, I owe my loving thanks and deepest gratitude to my family for encouraging me in the most important and difficult decisions throughout my life and always supported me in my endeavours. Leaving you for so long has been the hardest thing here.

My loving mother, **Mrs. Hafeeza Parveen** deserve special mention for her support, devout prayers and patience she has shown during this period. Thank you very much for being by my side, believing in me, and putting up with me during the good and bad times. I thank my father, **Mr. Gh. Nabi Mir**, for all what he has done to me. It is because of his upbringing, I stand here in my life. I am forever grateful to **Mrs Rumeena Gul, Miss Humaira Gul** and **Miss Sumaira Gul** for being supportive and caring siblings.

My genuine appreciation and sense of gratitude from the citadel of my heart to my younger sister **Miss Humaira Gul**, for relieving me from my responsibilities and taking care of family during my absence. I appreciate the love and care that I receive from my brother- in- law, **Dr. Jawaid Hassan**. This sojourn would have been more tiring had it not been filled with the loving memories of my niece and nephew, **Fadheelah Farheen** and **Fadhil Jawaid** which often gave me sense of relief after hours of tedious work in the laboratory.

I would also like to express my deep sentiments to my father-in-law, **Mr. Syed Sadiq Ali Quadri** and mother-in-law, **Mrs. Rafeeq Amena** for their affection and blessings.

There are too many names to list of the multitude that have helped to make this a reality, apologies to those whose names I have neglected or omitted here, please forgive. You are not forgotten and your aid and friendship are most appreciated.

Mir Zahoor Gul

ABBREVIATIONS	
°C	Degree centigrade/Degree celsius
µg	Micro gram
µL	Micro litre
µM	Micro molar
$^1O_2$	Singlet oxygen
a.m.u	Atomic mass unit
AAE	Ascorbic acid equivalent
ANA	*Artemisia nilagirica* alcohol extract
ANE	*Artemisia nilagirica* ethyl acetate extract
ANH	*Artemisia nilagirica* hexane extract
ANOVA	Analysis of variance
ANW	*Artemisia nilagirica* water extract
APA	*Abrus precatorius* alcohol extract
APE	*Abrus precatorius* ethyl acetate extract
APH	*Abrus precatorius* hexane extract
APW	*Abrus precatorius* water extract
ASE	Ascorbic acid equivalents
BD	Becton, Dickinson and Company
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
bp	Base pair
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CAT	Catalase
CHA	*Cocculus hirsutus* alcohol extract
CHE	*Cocculus hirsutus* ethyl acetate extract
CHH	*Cocculus hirsutus* hexane extract
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CO_2	Carbon dioxide
CPA	*Cissampelos pareira* alcohol extract
CPE	*Cissampelos pareira* ethyl acetate extract
CPH	*Cissampelos pareira* hexane extract
CPW	*Cissampelos pareira* water extract
DCF	Dichlorofluorescein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
Dnase	Deoxyribonuclease
DPPH$^\bullet$	2,2-diphenyl-1-picrylhydrazyl
dUTP	Deoxyuridine Triphosphate
dw	Dry weight
EA	Ethyl acetate
ECL	Electrochemiluminescent
EDTA	Ethylene diamine tetra acetic acid

ESI	Electrospray ionization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fig	Figure
FR	Free radicals
FRAP	Ferric reducing antioxidant power
g	Gram
GAE	Gallic acid equivalents
GPx	Glutathione peroxidase
GSH	Glutathione
H	Hour(s)
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HEK-293	Human Embryonic Kidney 293 cell line
HeLa	Human cervix adenocarcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	Human hepatocellular carcinoma cell line
HPLC	High pressure liquid chromatography
HPLC-ESI-MS-MS	High-performance liquid chromatography/electrospray ionization tandem mass spectrometry
HRMS-ESI	High resolution mass spectrometry- electrospray ionization
IC_{50}	Half maximal inhibitory concentration
IMDM	Iscove's Modified Dulbecco's Medium
JM-1	Human B-cell lymphoma leukaemia cell line
kDa	Kilo Dalton
L	Litre
LC-DAD-ESI-MS	Liquid chromatography, with diode array detection and electrospray ionisation mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LOO^-	Lipid peroxyl
LOOH	Lipid peroxide
M	Molar
m/z	Mass to charge ratio
MDA	Malondialdehyde
MDR	Multidrug resistance
mg	Milli gram
min	Minute(s)
mL	Milli litre
mM	Milli molar
MQ	Milli Q
MS/MS	Tandem mass spectrometry
MTT	3- (4, 5-dimethyl-thiazol-2-yl) -2, 5- diphenyltetrazolium bromide
MW	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCCS	National Centre for Cell Sciences
NIST	National Institute of Science and Technology

nm	Nanometre
$NO^{\bullet}$	Nitric oxide radical
N-terminal	Amino terminal
$O_2^{\bullet-}$	Superoxide anion
O_3	Ozone
OD	Optical density
PBS	Phosphate buffer saline
PI	Propidium iodied
PMS	Phenazine methosulfate
QE	Quercetin equivalents
QTOF-MS	Quadrupole time-of-flight mass spectrometer
RNase	Ribonuclease
RNS	Reactive nitrogen species
$RO_2^{\bullet}$	Peroxyl radicals
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium
RT	Retention time
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second(s)
SOD	Superoxide dismutase
SupT1	Human T-cell lymphoblastic lymphoma cell line
TAC	Total antioxidant capacity
TAE	Tris base, acetic acid and EDTA.
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reacting substances
TBS	Tris-buffered Saline
TBST	Mixture of Tris-buffered saline and Tween 20
TdT	Terminal deoxynucleotidyl transferase
TFC	Total flavonoid content
THP-1	Human acute monocytic leukaemia cell line
TIC	Total ion current chromatogram
TLC	Thin layer chromatography
TPC	Total phenolic content
Tris	Tris-(hydroxymethyl) aminoethane
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
V	Volts
v/v	Volume to volume ratio
WHO	World Health Organization
λ	Lambda

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Chapter I:

Introduction

The utilisation of plants with therapeutic properties in the treatment of certain human diseases is an evidence of man's ingenuity and have formed the basis of erudite traditional medicine system [De Pasquale, 1984]. The therapeutic effectiveness of these medicinal plants in traditional medicinal systems dating from around 2900-2600 B.C. The ancient civilisations of Chinese, Indians and North Africans provide documented evidence for the use of medicinal plants for curing various diseases to greater extent [Phillipson, 2001]. Additionally, during the course of development of different human cultures, the use of these plants has had magical-religious significance and different perceptions regarding the concepts of health and disease existed within each culture [Rates, 2001]. These plant based medicinal systems have not only been found useful in the ancient medical systems, they are still playing a crucial role in contemporary healthcare. The large majority of people on this word still rely on traditional material medica (medicinal plants and other organisms) for their day to day health care needs ranging from coughs and colds to parasitic infections and inflammation [Newman and Cragg, 2010]. It is also a believed that one quarter of all medical prescriptions are formulations based on ingredients obtained from plants or plant-derived synthetic analogues. According to the World Health Organization (WHO), before 1980, 80% of the world's population-primarily those of the developing countries, count on plant-derived medicines for their basic healthcare needs [Winston, 1999]. In the middle of the 20th century, this vast body of knowledge has been swept aside in favour of synthetic drugs, which while saving countless lives in certain fields, have not proved to be a universal panacea that researchers had hoped for.

1.1. NATURE: A THERAPEUTIC GOLDMINE:

Nature continuously stands as a golden mark to represent the outstanding phenomena of symbiosis. Since time immemorial, mankind have relied on nature to cater for their basic needs like shelter, foodstuff, clothing, means of transportation, fertilizers and not the least of which are medicines for the treatment of various ailments [Cragg and Newman, 2005]. Despite the scientific advances behind synthetic medicines, conventional medicine suffered from several shortcomings. The synthetic medicine was found to be inefficient (e.g. side effects, ineffective therapy and resistance) and abusive. Furthermore, a large percentage of the world's population does not have access to conventional pharmacological treatment and relatively unaffordable due to their high cost. However, natural products from medicinal

plants were found to be chemically balanced, with advantages like minimal toxicities**,** effectiveness in chronic conditions, widespread availability and lower cost [Jiang et al., 2006; Newman et al., 2003; Reddy et al., 2003; Tan et al., 2006]. Number of biologically active plant-derived secondary metabolites have found direct medicinal application as drugs, and many other bioactive plant compounds are useful as the leads or scaffolds for efficacious drugs for multitude of disease indications [Balandrin et al. 1993].

There are 46 - 126 nature-derived drugs approved in every 5-year periods since 1991, 7.1% - 14.5% of which are from previously untapped species (i.e. untapped before the specific 5 year period). These species represent 11.4% - 41.7% of the drug-productive species that have yielded approved drugs in 1991 - 2010. In contrast, there were 26 - 133 nature-derived drugs in every 5-year period of 1961 - 1990, 18.0% - 62.8% of which were from previously untapped species that represent 36.7% - 76.2% of the drug productive species in 1961 - 1990. This evidently establish that plant-derived products are still vital as medicinal agents and models for the designing, synthesis, and/or semi-synthesis of novel substances for the treatment of various ailments (Table 1.1). During 1991 - 2010, 59 new drug-productive species emerged, 33 (55%) of which are from existing drug-productive species families and another 22 (37%) are from new species families in existing drug productive clusters. These data suggested a great probability of finding new drug-productive species from existing drug-productive families or new families located in existing drug-productive clusters [Zhu et al., 2012].

Modern drug discovery approaches based on structure activity relationships, computer modelling, combinatorial chemistry, high throughput screening and spectroscopy (MS, NMR, and IR) have prompted and spearheaded the discoveries of many natural drugs for treatment of many human diseases. Additionally, it stimulated the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents [Baker et al., 1995]. Examples of important drugs obtained from plants are serpentine from *Rauwolfia serpentine*, digoxin from *Digitalis* spp., acetylsalicyclic acid (aspirin) from *Salix alba*, quinine and quinidine from *Cinchona* spp., vincristrine and vinblastine from *Catharanthus roseus*, paclitaxel from *Taxus brevifolia*, atropine from *Atropa belladonna* and morphine and codeine from *Papaver somniferum*, arteether and artemotil from *Artemisia annua* [Balunas and Kinghorn, 2005].

Table 1.1. Historic data of the numbers of nature-derived approved drugs from previously untapped and previous drug-productive species and the numbers of drug-productive species during every five-year period from 1961 to 2010

Period	Number of Nature-derived drugs in period			Number of drug productive species in period		
	Total No. of drugs in that period	No. & %age of drugs from previously untapped species	No. & %age of drugs from previous drug productive species	Total No. of species	No. & %age of previously untapped species	No. & %age of pervious drug productive species
1961–1965	37	22 (59.5%)	15 (40.5%)	42	32 (76.2%)	10 (23.8%)
1966–1970	26	10 (38.5%)	16 (61.5%)	26	10 (38.5%)	16 (61.5%)
1971–1975	36	8 (22.2%)	28 (77.8%)	30	11 (36.7%)	19 (63.3%)
1976–1980	78	49 (62.8%)	29 (37.2%)	80	56 (70.0%)	24 (30.0%)
1981–1985	108	30 (27.8%)	78 (72.2%)	68	31 (45.6%)	37 (54.4%)
1986–1990	133	24 (18.0%)	109 (82.0%)	63	30 (47.6%)	33 (52.4%)
1991–1995	117	17 (14.5%)	100 (85.5%)	60	25 (41.7%)	35 (58.3%)
1996–2000	126	9 (7.1%)	117 (92.9%)	58	10 (17.2%)	48 (82.8%)
2001–2005	124	12 (9.7%)	112 (90.3%)	70	19 (27.1%)	51 (72.9%)
2006–2011	46	5 (10.9%)	41 (89.1%)	44	5 (11.4%)	39 (88.6%)

Ref: Zhu et al., 2012

Medicinal plants continue to offer significant contribution in drug discovery programs of pharmaceutical industry and other research organizations as they provide the foundation to modern therapeutic sciences, thus, enabling the establishment of an empirical system of medicine. During the last century, various modern procedures have been adopted for the determination of biological properties of plant extracts and identification of active principles especially chromatographic, spectroscopic and high throughput bioassay techniques. Research in this direction has been greatly facilitated by the use of modern physio-chemical techniques of isolation and structural elucidation. Recent approvals of several new plant-derived drugs and semi-synthetic and synthetic drugs based on plant secondary compounds confirm the advancement of medicinal plants research. An important reason for the use of medicinal plants as an attractive source of therapeutic candidates is the tremendous variety of species found in nature and the resulting molecular diversity of the isolated compounds [Borris, 1996]. The significance of medicinal plants is clearly enormous, about 25% of the drugs prescribed worldwide come from plants. Out of which, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by WHO, 11% are exclusively from plant origin and a significant number are synthetic drugs obtained from natural precursors [Rates, 2001]. Furthermore, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies [Williamson et al., 1996].

1.2. TRADITIONAL MEDICINAL SYSTEM AND MEDICINAL FLORA OF INDIA:

India is one of the prominent countries in Asia in terms of the wealth of traditional knowledge systems related to the use of plant species. It has a concept of using plants for medicinal purpose dating back to as early as 2500 - 500 BC. It is one of the most important country in the world in term of floristic diversity. It is one of the world's 12 biodiversity centres and the rich heritage of flora is due to diversified and varied agro-climatic conditions. It is known as botanical garden of the world with a vast repository of medicinal and aromatic plants (approx. 15,000 -20,000 plant species) that are used in traditional medical treatments [Ahmadullah and Nayar, 1999; Chopra et al., 1994; Dev, 1997]. About 54% of the country's land is under cultivation for food, ornamental and medicinal plant crops and approximately 19% of area has varying degree of forest vegetation cover. It also has a very long, safe and

continuous usage of many herbal drugs for treating different ailments in the officially recognised alternative systems of medicines viz. Ayurveda, Yoga, Unani, Siddha and Homeopathy [Rabe and Van-Staden, 1997]. But only 7,000-7,500 plants are being used by 500 traditional communities for curing different diseases [Kamboj, 2000; Perumal and lgnacimuthu, 2000]. These plant species have a unique collection of secondary metabolites distributed throughout their tissues. A percentage of these metabolites are expected to respond positively to an appropriate bioassays, however, only a small percentage of them have been investigated for their potential value as drugs and much of the wealth is still unexplored. Besides their direct medicinal application, natural products especially from plants can also serve as pharmacophores for the design, synthesis or semi-synthesis of novel substances for medicinal uses. Therefore, the scientific study of traditional medicines, derivation of drugs through bio-prospecting and systematic conservation of the concerned medicinal plants are thus of great importance.

1.3. FREE RADICALS, OXIDATIVE STRESS AND ANTIOXIDANTS:

Free radicals (FR) or Reactive oxygen species (ROS) are vital to any biochemical process and signify an essential part of aerobic life and human metabolism. Free radicals can be defined as chemical species capable of independent existence (hence the term 'free') possessing an unpaired electrons which are extremely reactive, short lived and having a damaging activities towards the macromolecules like proteins, DNA and lipids [Yashikawa et al., 1997]. The ROS include hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), peroxyl radicals ($RO_2^{\bullet}$), lipid peroxyl (LOO^{-}), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), Ozone (O_3), lipid peroxide (LOOH) and as well as reactive nitrogen species (RNS) [Devasagayam et al., 2004]. They are ubiquitously produced as by products in the cells through frequent physiological and biochemical processes in the living systems. They are also generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air pollutants, pesticides, etc. (Fig. 1.1) [Halliwell and Gutteridge, 1990; Tiwari, 2001; Lee et al., 2004].

In the course of evolution, the human body has evolved a highly complicated defence system with endogenous antioxidants composed of enzymes and vitamins against oxidative stress to prevent deleterious effects. These defence systems are mainly classified as (i) suppression of generation of ROS, (ii) scavenging/quenching of ROS, (iii) clearance, repairing

and re-constitution of damage and (iv) induction of antioxidant proteins and enzymes [Noguchi et al., 2000; Nose, 2000]. This antioxidant system includes, antioxidant enzymes {e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx) and reductase, catalase CAT, etc.}, nutrient-derived antioxidants {e.g., ascorbic acid (vitamin C), tocopherol (vitamin E), glutathione (GSH), vitamin A, tocotrienols, carotenoids and lipoic acid}, metal binding proteins {e.g., ferritin, lactoferrin, albumin and ceruloplasmin} [Cheeseman and Slater, 1993]. However, these defence mechanisms present under normal physiological conditions are sufficient to cope up with the normal threshold of physiological rate of free-radical generation only.

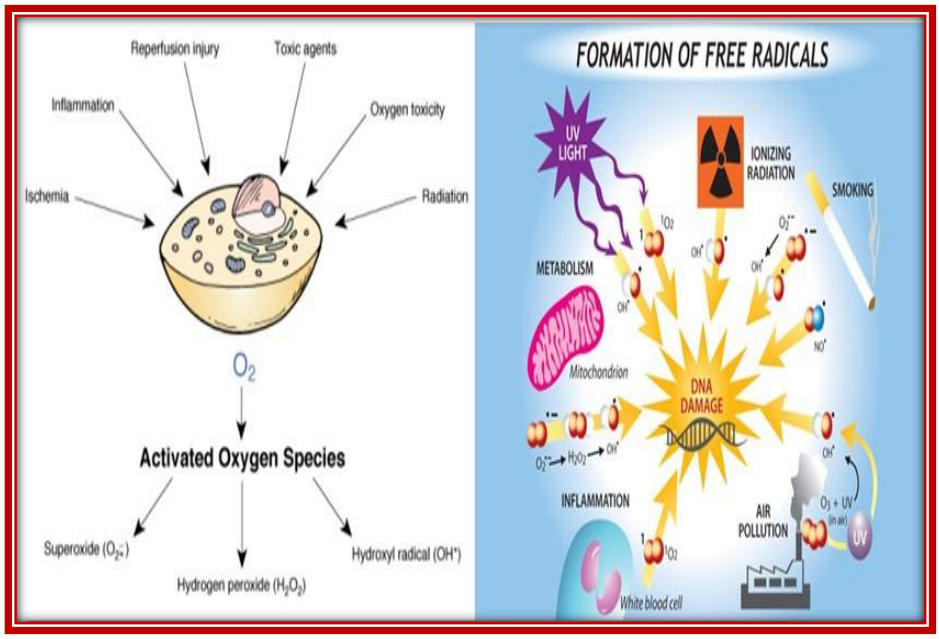


Fig. 1.1: Illustration of formation of Reactive Oxygen Species.
*(**Ref:** Sarma et al., 2010; www. vibrantlikeme.com).*

The over production of ROS creates a homeostatic imbalance between production of reactive species and antioxidant defence. These FR species attack cells, tearing through the cellular membranes to react and create havoc with the nucleic acids, proteins, carbohydrates and enzymes present in the body leading to potential damage. This situation is referred to as oxidative stress (Fig. 1.2). The oxidative stress and oxidative modification of biomolecules is associated with a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation, differentiation and apoptosis, as well as ischemia-reperfusion, inflammation and many neurodegenerative disorders which ultimately leads to the development of various health disorders such as Alzheimer's disease,

cancer, atherosclerosis, diabetes mellitus, hypertension and ageing (Fig. 1.3) [Ames et al., 1993; Bland, 1995].

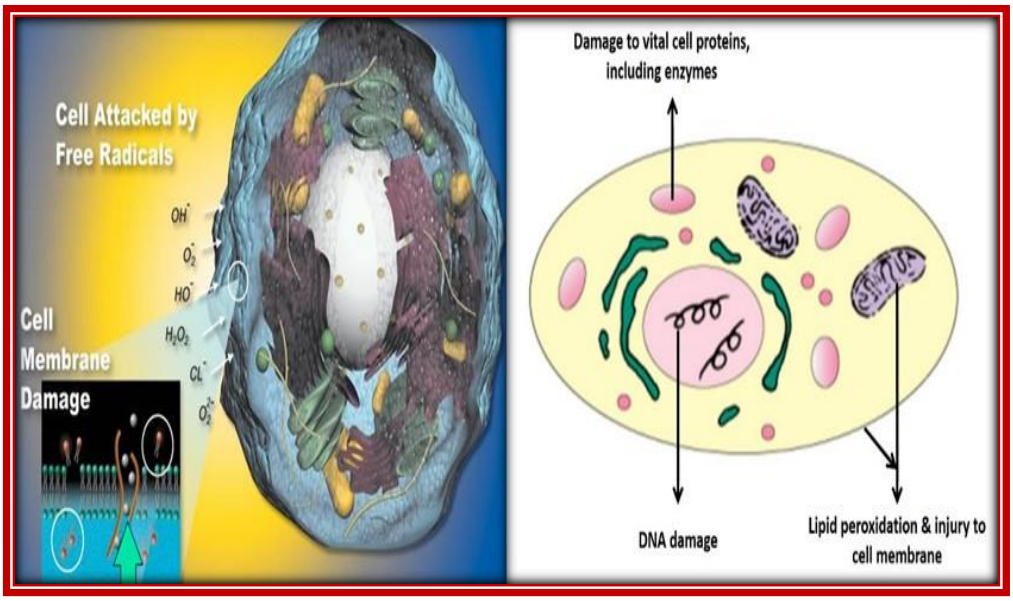


Fig. 1.2: Cellular damage (Oxidative stress) due to free radicals.
*(**Ref:** Sarma et al., 2010; www.h2-h2o.com).*

Fig. 1.3: Overview of organs affected by free radicals and different diseases.
*(**Ref:** www.miltonchiropractic.com)*

Therefore, in modern Western medicine, the balance between anti-oxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system

[Davies, 2000; Devasagayam et al., 2004; Finkel and Holbrook, 2000]. Any vitiation therefore, is understood to give rise to disorderliness in the physiological system leading to a variety of diseases depending upon the sensitivity and susceptibility of the organs. Thus, the antioxidants in humans reflect the dynamic balance between antioxidant defence and prooxidant conditions and have been suggested as a useful tool in estimating the risk of oxidative damage [Nose, 2000; Papas, 1996; Polidori et al., 2001; Tiwari, 2001]. Research in the recent past has accumulated enormous evidences revealing that enrichment of body systems with exogenous antioxidants may correct the vitiated homeostasis [Halliwell, 1994; Pietta, 2000; Tiwari, 1999] and can prevent the onset as well as treat diseases caused and/or fostered due to free-radical mediated oxidative stress.

Antioxidants can inhibit or delay the oxidation of an oxidisable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases [Halliwell et al., 1992]. Depending on the chemical structures, antioxidants have diverse mechanism of action. Preventive antioxidants like deferrioximine or desferal, are compounds which form chelates with metals, thereby, delaying or inhibiting the initiation or propagation of oxidative chain reactions. The second type of antioxidants known as chain-breaking antioxidants are the most important class of antioxidants, which can scavenge chain propagating free radicals like peroxyl radicals and convert them to inactive products, e.g., vitamin-E or α-tocopherol, curcumin. The synthetic antioxidants like butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective to prevent the oxidative stress, but they possess some side effects and toxic properties to human health [Anagnostopoulou et al., 2006]. These developments accelerated the search for new, effective and appropriate antioxidant principles aimed at minimizing the oxidative stress and providing defence against free radical induced stress in diverse clinical and pathological conditions. Therefore, in the present study, we made an attempt to screen different multi solvent extracts prepared from dried leaves of selected plants to evaluate the antioxidant potential on the basis of their phytochemical significance.

1.4. MEDICINAL PLANTS WITH ANTIOXIDANT POTENTIALS:

In recent years, much attention has been devoted to natural antioxidant and their association with health benefits. Plant kingdom is a noble source of natural preparations containing effective bioactive compounds which can be used for different applications,

particularly as food additives and health promoting ingredients in the formulations of functional foods and nutraceuticals. Dietary antioxidant intake has proven to be an important approach for inhibiting or delaying the oxidation of susceptible cellular substrates and is thus relevant to disease prevention in many models. Medicinal properties of plant species (fruits, vegetables, medicinal herbs, etc.) have made outstanding contribution in the origin and evolution of many traditional therapies. The interest in plant derived antioxidants has increased remarkably in the last decade because of their elevated capacity in scavenging free radicals associated with various diseases [Ali et al., 2008]. Large numbers of medicinal plants have been investigated for their antioxidant activities and the results have illustrated that either their raw extracts or their individual chemical constituents are more effective antioxidants (*in vitro*) than the synthetic antioxidants, e.g. butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) or vitamin E [Gordon and Weng, 1992; Gu and Weng, 2001; Pyo et al., 2004]. Moreover, these synthetic antioxidants may have carcinogenic and other harmful effects on the lungs and liver of human beings [Branen, 1975; Grice, 1986; Wichi, 1988]. Converging evidence from both experimental and epidemiological studies have demonstrated that myriad of phenolic compounds from medicinal plants possess multifunctional properties and are of great value in preventing the onset and/or progression of many human diseases because of their high redox potentials which allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators [Matkowski and Wołniak, 2005; Perron and Brumaghim, 2009].

During the last few years, investigations for phenolic compounds derived from medicinal herbs have gained much prominence due to their high antioxidative activity [Zhu et al., 2004]. Polyphenols such as phenolic acids, flavonoids, tannins, alkaloids, terpenoids, coumarins, stilbenes, anthocyanin etc. have many favourable effects on human health, like inhibiting the oxidization of low-density proteins [Frankel et al., 1993], thereby, decreasing the risk of heart disease [Williams and Elliot, 1997]. These compounds have anti-inflammatory and anti-carcinogenic properties [Carrol et al., 1999; Maeda-Yamamoto et al., 1999]. Also, these compounds of plant origin have been reported as scavengers of reactive oxygen species (ROS), and are regarded as promising therapeutic drugs for free radical pathologies [Parshad et al., 1998; Lee et al., 2000]. The exploration for potential and non-toxic antioxidants of plant origin which could prevent oxidative stress related disorders in human beings has gained importance over the years and also can replace the harmful

synthetic antioxidants [Branen, 1975; Grice, 1986; Wichi, 1988]. The natural antioxidants can be used either as dietary, food supplement or as a drug [Abdel-Hameed, 2009]. Therefore, the research into the determination of the natural anti-oxidant source is very important to uphold the public health and ethno-pharmacology plays a significant part in the search for interesting and therapeutically useful plants. Since preliminary antioxidant activities of selected medicinal plants have been reported previously, but extensive study with respect to their antioxidant activities were lacking. Therefore, in the present study, we have evaluated these four potent medicinal plants for their antioxidant activities by using series of *in vitro* methods.

1.5. CANCER – A DREADFUL DISEASE:

Cancer is one of the most serious health problems, affecting millions of individuals each year from different sexes, ages and races across the globe spreading further with continuance and increasing incidence annually [Globocon, 2012]. It is a vast and diverse spectrum of diseases, all of which are characterized by uncontrolled virtually autonomous growth of abnormal cells, often involving invasions into surrounding tissues and sometimes metastasis into distant organs or tissues of the body (Fig. 1.4A). It continues to represent the largest cause of mortality in the world. There were 14.1 million new cases and 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in the year 2012 worldwide, compared with 12.7 million and 7.6 million, respectively, in 2008. 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions (Table 1.2). By 2025, it has been estimated that mortality can reach as high as 12 million with a substantive increase to 19.3 million new cancer cases per year [Ferlay et al., 2013]. Among the different types of cancers (Fig. 1.4B), Leukaemia – a type of cancer that can affect the bone marrow, the blood cells, the lymph nodes and other parts of the lymphatic system accounts for over 3,52,000 newly diagnosed cases in the year 2012, accounted for 2.5% deaths of all cancer [Ferlay et al., 2013]. In Indian scenario, Cancer is also a major health issue and leading causes of mortality. In the year 2012; 10,14,934 new cases of cancer were reported and more than 6,82,830 patients died due to this fatal disease. There were around 32,532 new leukemic cancer cases with mortality of 26,712 reported in the same year [Globocon, 2012].

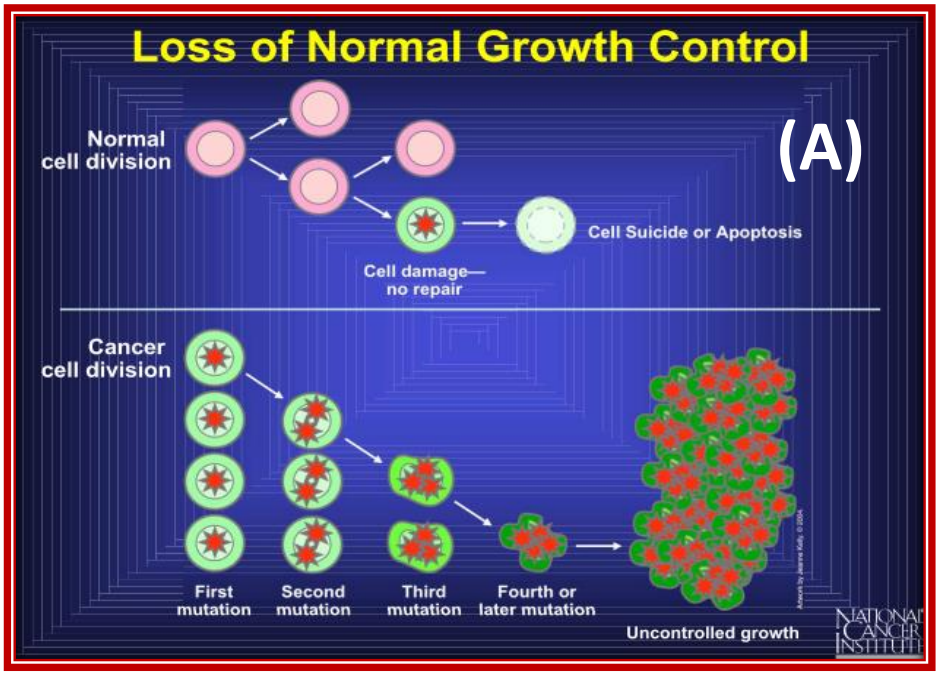


Fig. 1.4 (A): Uncontrolled virtually autonomous growth of abnormal cells *(**Ref:** www.cancer.gov/cancertopics)*

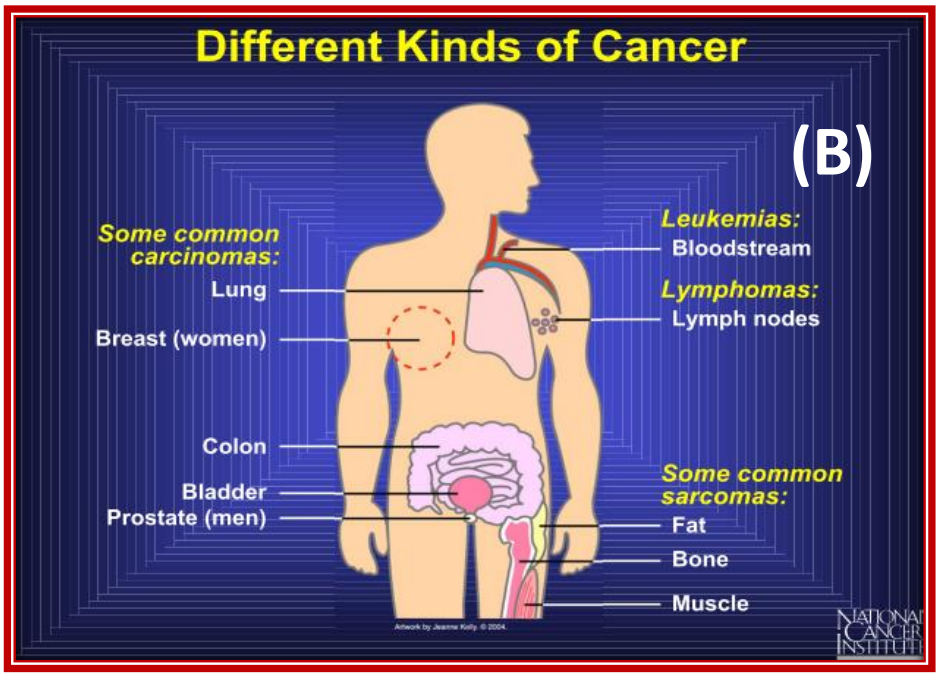


Fig. 1.4 (B): Different types of cancers prevalent in human beings. *(**Ref:** www.cancer.gov/cancertopics)*

Table 1.2: All Cancers (Excluding Non-Melanoma Skin Cancer) Estimated Incidence, Mortality and Prevalence Worldwide in the year 2012

Estimated numbers (in 1000's)	Men			Women			Both Sexes		
	Cases	Deaths	Previous 5 years	Cases	Deaths	Previous 5 years	Cases	Deaths	Previous 5 years
World	7427	4653	15362	6663	3548	17182	14090	8202	32545
More developed regions	3244	1592	8616	2832	1287	8297	6076	2878	16913
Less developed regions	4184	3062	6747	3831	2261	8885	8014	5323	15632
WHO Africa region (AFRO)	265	205	468	381	250	895	645	456	1363
WHO Americas region (PAHO)	1454	677	3843	1429	618	4115	2882	1295	7958
WHO East Mediterranean region (EMRO)	263	191	461	293	176	733	555	367	1194
WHO Europe region (EURO)	1987	1081	4857	1750	852	4933	3737	1933	9790
WHO South-East Asia region (SEARO)	816	616	1237	908	555	2041	1724	1171	3278
WHO Western Pacific region (WPRO)	2642	1882	4493	1902	1096	4464	4543	2978	8956
IARC membership (24 countries)	3706	1900	9259	3354	1570	9425	7060	3470	18684
United States of America	825	324	2402	779	293	2373	1604	613	4775
China	1823	1429	2496	1243	776	2549	3065	2206	5045
India	477	357	665	537	326	1126	1015	683	1790
European Union (EU-28)	1446	716	3759	1211	561	3487	2657	1276	7246

Ref: GLOBOCON, 2012 (IARC), Section of Cancer Serveillance (16-12-2014)

1.5.1. CONVENTIONAL CANCER THERAPY AND DRAWBACKS:

In the last two decades, there has been a lot of progress in the field of cancer research. Advances in cellular and molecular biology have helped us in understanding the different mechanisms of this disease. The principal modalities of cancer therapy, i.e. surgery, radiotherapy, immunotherapy and chemotherapy have been utilized separately or in combination. However, these therapies are associated with many drawbacks and hamper the desirable results. For instance, in surgery, the immune system is compromised due to the large amount of cortisole released subsequent to the surgery, which increases the probability of cancer relapse. Also, due to the infiltrative nature and the rapid recurrence of the malignant tumour, complete surgical resection of these tumours is typically not achieved [Scheck et al., 2006]. Radiotherapy which is widely used in the world is also accompanied by a great deal of side effects. External beams of radiotherapy are associated with unacceptably high levels of local-regional toxicity. Particularly, it affects the rapidly dividing cells of mucosa, causing irritative urinary and blood loss. Later toxic effects result from damage to the more slowly proliferating cells such as fibroblasts, endothelial, or paranchymal stem cells causing chronic fibrosis and vascular damage [O'connor and Fitzpatrick, 2005]. Chemotherapy is recognized as an important approach in the treatment of cancer, but due to low selectivity, different levels of toxicity to normal tissues and rapid development of multidrug resistance (MDR), the survival rate has not improved significantly. Moreover, the established drugs suffer from low specificity towards tumour cells leading to toxic effects on rapidly dividing tissues such as bone marrow suppression, deterioration of the gastrointestinal mucosa and alopecia. Other side effects like, bone necrosis, lung fibrosis, lower blood count, skin de-vascularization, ulceration, nausea, vomiting and renal damage are also associated with all types of conventional therapies [Le, 2004; O'connor and Fitzpatrick, 2005; Zhu et al., 2005]. Hence, the development and search for more effective and safe treatment modalities and/or adjuvant therapies for early and advanced stages of the disease have now become important research target.

1.5.2. DISCOVERY OF ANTI-CANCER AGENTS FROM MEDICINAL PLANTS:

Medicinal plants had an important place in the therapeutic armoury of mankind. They contain a wide variety of secondary metabolites, which can be used to prevent or cure diseases, or to promote general health and well-being [Calixto, 2000; Salim et al., 2008].

During the last decades, scientific evidences of the medicinal values of plant products (through *in vitro*/*in vivo* investigation) aroused public apprehensions about the conservation of such plants, in order to preserve their economic and therapeutic significance [Sharma and Sharma, 2001]. Isolation and identification of bioactive compounds present in a crude extract sample of medicinal plants, has begun as the major path of anti-cancer agent development. Chemo-diversity of medicinal plants has proven to be important in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last five decades have been applied towards combating the disease of cancer [Butler, 2004; Gullo et al. 2006; Mendonça-Filho, 2006; Newman et al., 2000; 2003]. Numerous useful anti-neoplastic drugs (e.g. taxoids, campthotecine, podophyllotoxin derivatives and vinca alkaloids) have been discovered in higher plants by following up ethno-medicinal uses or the results of anti-tumour screening [Conforti et al., 2008]. The plant kingdom still holds many species of plants containing substances of medicinal interest which are yet to be investigated. Therefore, the screening of medicinal plants plays a substantial role in the discovery of new biologically active compounds and hence in the development of drugs for cancer chemotherapy.

Hence, further evaluation of drugs derived from plants requires the screening of large numbers of plant extracts, isolation and identification of the active compounds, the study of their mechanism of action, as well as the proof of its non-toxicity to human cells. The rapid identification of these bio-active compounds, however, is critical if this tool of drug discovery is to compete with developments in technology.

1.5.3. BIOASSAY-GUIDED FRACTIONATION:

Bioassay-guided fractionation is a common approach for studying plants crude extracts and their fractions. With this procedure, crude extracts and their fractions are screened in quest of biologically active compound(s) present in them. This process includes the selection of plants for investigation, the primary screening of the plant extracts and the subsequent bioactivity-guided fractionation, comprising several consecutive steps of chromatographic separation, where each fraction obtained has to be submitted to mechanism-based bioassays in order to follow the activity (Fig. 1.5).

The identified active crude extracts/fractions are further progressed to isolate purified compound(s) and subsequently tested for activity and parallely characterised for its/their

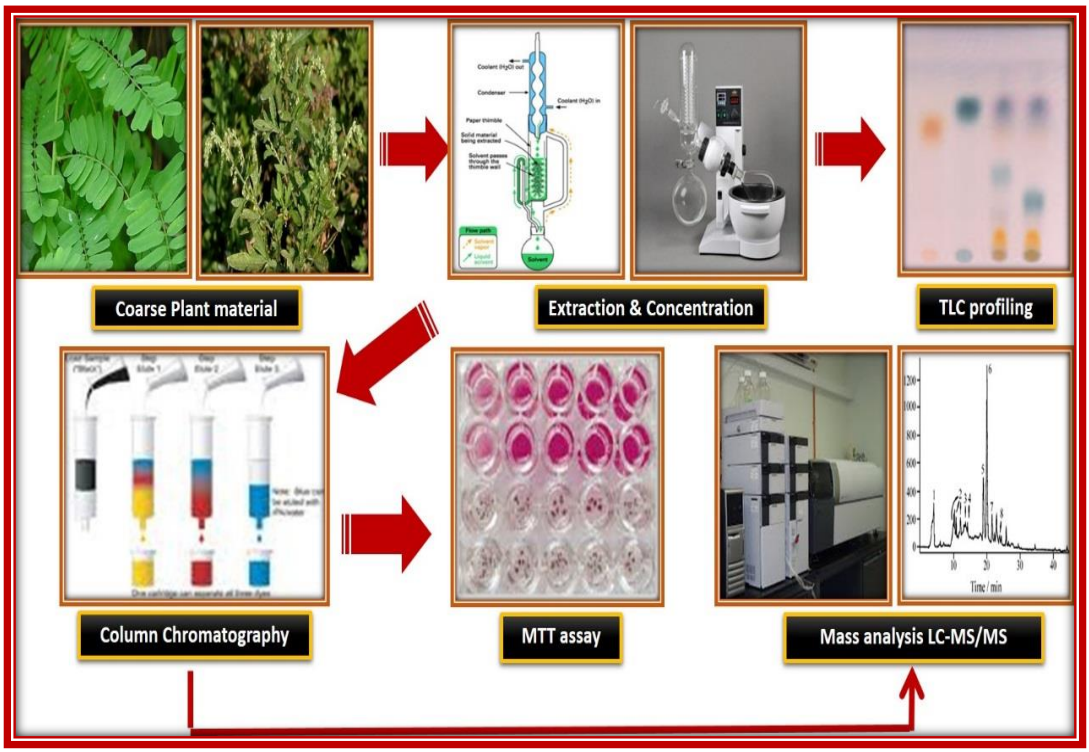


Fig. 1.5: Schematic representation of Bioassay guided fractionation which shows different steps involved in the process such as Collection, Extraction, Concentration, Separation, Bioactivity and Identification.

structure. A combination of chemical screening with biological screening is the fastest way to arrive at new lead compounds from plants. Bioassays plays an important role in the discovery of natural products. For the bioassays, a wide variety of cultured cancer cell lines of human or animal origin are available as targets [Hamburger and Hostettmann, 1991; Kintzios, 2004]. The desirable mechanisms of action are those which might allow a drug to target tumour cells selectively or specifically. Since tumour cells tend to replicate more regularly, and therefore, replicate their genomes repeatedly and DNA damaging agents are rather selective to tumour cells. An assay may be designed that can quantify DNA damage, even specific kinds of DNA damage, and the assay can be used to guide fractionation efforts. More recently, achievements in separation sciences propose much better solutions for the separation of the complex mixtures than it was attainable before. Specifically, the advances in the preparation of monolithic columns has enabled the separation of very complex mixtures due to the high number of theoretical plates of these columns [Tanaka et al., 2001]. Moreover, during the recent past, the advancement of ion hyphenated techniques such as LC-MS/MS, GC-MS/MS, CE-MS/MS, and LC-NMR have speed up the process of drug

screening and discovery of metabolites from crude/mixture of complex compounds [Niessen, 1998].

1.5.4. METABOLOMIC APPROACHES TO IDENTIFY BIOACTIVE COMPOUNDS:

Over the last decade or so, a rich array of analytical approaches have been developed for high throughput analysis of plant metabolites. Such approaches have led to a change of paradigm in the development and application of complex plant mixtures [Ulrich-Merzenich et al., 2007]. These approaches are typically referred to under the broader mantel of metabolomics. Metabolomics is an emerging and rapidly evolving science and technology system of comprehensive experimental analysis of metabolite profiles, either as a targeted subset of related chemicals or more globally, for diverse applications in diagnosis, toxicology, drug discovery and development and phytomedicine. A variety of detection procedures have been applied for metabolite analysis in metabolomics, comprising, Ultraviolet absorption Spectroscopy (UV), Infra-red spectroscopy (IR), Mass spectrometry (MS), Nuclear Magnetic Resonance spectroscopy (NMR), Mass spectrometry coupled to gas chromatography or liquid chromatography (GC-MS and LC-MS respectively), Direct injection mass spectrometry (DIMS), Fourier transform infrared spectroscopy (FT-IR), Capillary electrophoresis mass spectrometry (CE-MS), High-performance liquid chromatography with photodiode array detection (HPLC-PDA) and Thin layer chromatography with UV detection (TLC-UV) are also part of the metabolite analysis arsenal. Out of these techniques, hyphenated methods such as GC-MS-MS and LC-MS-MS, DIMS and HPLC-NMR-MS are likely the most commonly applied methods. The hyphenated techniques have received ever-increasing attention as the principal means to solve complex analytical problems. The power of combining separation technologies with spectroscopic techniques has been demonstrated over the years for both quantitative and qualitative analysis of unknown compounds in complex natural product extracts or fractions [Urban and Separovic, 2005]. In other words, chromatography produces pure or nearly pure fractions of chemical components in a mixture whereas, spectroscopy gives selective information for identification using standards or library spectra.

With the MS as the preferred detection method and single- and triple quadrupole, ion trap and time-of-flight (TOF) mass spectrometers as the instruments most frequently used, the LC-MS and GC-MS are the widespread hyphenated techniques that are used in

metabolite profiling now-a-days [Wilson and Brinkman, 2003]. Mass spectra obtained by this hyphenated technique offer more structural information based on the interpretation of fragmentations. The fragment ions with different relative abundances can be compared with library spectra. GC-MS, which is developed from the coupling of GC and MS, was the first of its kind to become useful for research and development purposes. It is an outstanding platform for the volatile metabolites or that could become volatile by means of derivatisation. But the ability of GC and CE in separating these specific classes of chemicals also restricts their application in analysing other classes of chemical constituents in plant extracts, animal and human samples.

At present, LC is the most commonly used platform in MS-based metabolomics owing to its good compatibility with the metabolites in biological system. LC-MS or HPLC-MS refers to coupling of an LC with a mass spectrometer (MS). Liquid chromatography can separate metabolites that are not volatile and have not been derivatised. As a result, LC-MS can analyse a much wider range of chemical species than GC-MS. LC-MS is best suited for a discovery based approach when researching unknown metabolites, or when many of the targeted metabolites are not readily amenable to GC-MS analysis due to volatility issues. An LC-MS combines the chemical separating power of LC with the ability of MS to selectively detect and confirm molecular identity. MS is one of the most sensitive and extremely selective approaches of molecular analysis, and offers information on the molecular weight as well as the fragmentation pattern of the analyte molecule. The information acquired from MS is invaluable for confirming the identities of the analyte molecules. This qualitative analysis makes it possible to reconstruct an unknown compound from MS data. The ionization techniques used in LC-MS are generally soft ionization techniques that mainly display the molecular ion species with only a few fragment ions. Hence, the information obtained from a single LC-MS run, on the structure of the compound, is rather poor. However, this problem has now been tackled by the introduction of tandem mass spectrometry (MS-MS), which provides fragments through collision-induced dissociation of the molecular ions produced [Herderich et al., 1997]. Hyphenated techniques such as HPLC coupled to UV and mass spectrometry (LC-UV-MS) have proved to be extremely useful in combination with biological screening for a rapid survey of natural products [Allwood et al., 2012; Chernushevich et al., 2001; Hopfgartner et al., 2004; Hu et al., 2005]. Therefore, in this study, Bioassay guided fractionation including Liquid Chromatography-Mass Spectrometry

(LC-MS/MS) technique were utilized to identify the bioactive compounds with anti-cancer activity from active fractions of *Abrus precatorius* and *Artemisia nilagirica*.

1.6. APOPTOSIS:

Apoptosis (or programmed cell death) is a fundamental and highly organised physiological process that enables an organism to eliminate the damaged or abnormal cells during any stage of development, therefore, it plays a crucial role in maintaining the integrity of multi cellular organisms [Dey et al, 2013]. The outstanding feature of apoptosis is its remarkable stereotyped morphology showing distinct cellular changes that include cell shrinkage, irregularities in cell shape, membrane blabbing, externalization of phosphatidyl serine in cell membrane, chromatin condensation, inter-nucleosomal DNA fragmentation and increased mitochondrial membrane permeability. These changes leads to release of pro-apoptotic proteins (such as Bad, Bax and Caspases) in the cytoplasm and consequently formation of 'apoptotic bodies' which are eventually phagocytosed by macrophages and other neighbouring epithelial cells activating immune response. Actually, the apoptotic process is functionally conserved and physiological forms of this type of cell death are genetically programmed [Arends and Wyllie, 1991; Dey et al., 2013; Wyllie et al., 1980]. In cancer therapy, one approach that overwhelms the growth of cancer cells is by activating the apoptotic machinery in the cells [Bold et al., 1997; Kiechle and Zhang, 2002; Lowe and Lin, 2000]. Besides, the apoptotic process includes a mechanism that controlled both the packaging and disposal of dead cells, thus preventing inflammation of the surrounding tissue [Zornig et al., 2001]. Various studies during the recent past established that a large majority of cancer chemotherapy agents affected tumour cell killing *in vivo* and *in vitro* by launching the apoptosis cascade [Makin and Dive, 2001]. Induction of apoptosis appears to be associated with their effectiveness in modulating carcinogenic processes [Sun et al., 2004; Thompson, 1995]. Extensive varieties of natural compounds possess significant cytotoxic as well as chemo-preventive activities, which act via apoptosis (Fig. 1.6). Consequently, in the past few years chemoprevention through medicinal plants has become an increasingly active area of research. Therefore, the study of apoptosis is significant, not only to understand the regulatory mechanisms of normal physiological processes, but also to define the patho-physiological mechanisms of many human diseases.

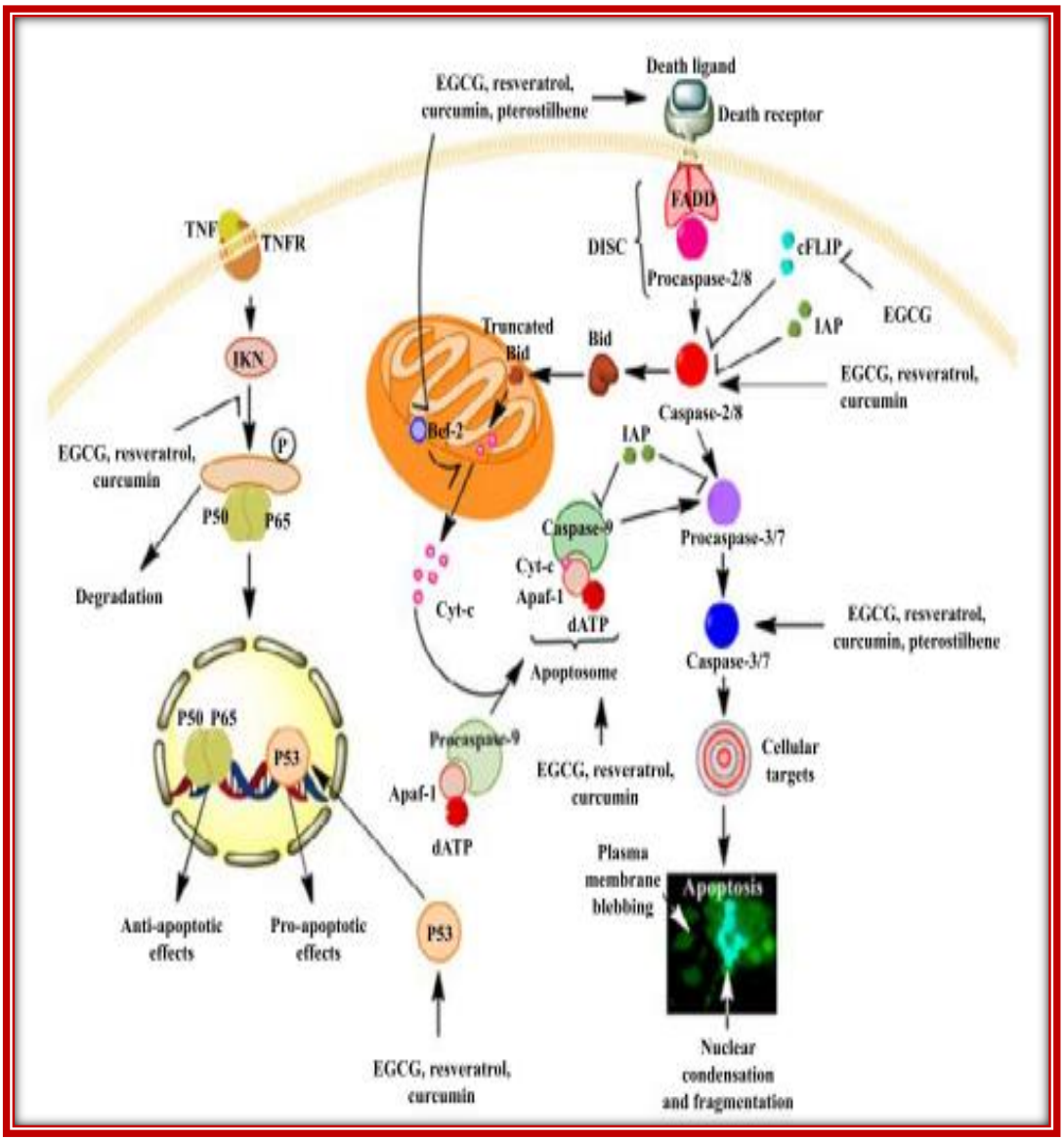


Fig. 1.6: Effects of natural polyphenols on the apoptosis pathways in cancer. *(**Ref:** Rodríguez et al., 2013)*

1.6.1. CASPASES:

The final pathway that leads to execution of the death signal is the activation of caspases, a family of cytosolic cysteine proteases present as inactive precursors in growing cells (Fig. 1.7). The mechanism of apoptosis is remarkably conserved across species, involving a cascade of sequential activation of initiator and effector caspases, cysteine proteases with aspartate substrate specificity [Riedl and Shi, 2004; Thornberry and Lazebnik, 1998]. The caspases that have been well described are caspases-3, -6, -7, -8, and -9. The initiator procaspase-8 is activated to caspase-8 as active form by self-processing due to

death inducing stimuli. On the other hand, procaspase-9 gets activated through mitochondrial pathway [Denecker et al., 2001]. Mitochondria too play a crucial role in cell death in response to death inducing signal, e.g., growth factor deprivation, ionizing radiation and some anti-neoplastic drugs, such as cyclophosphamide and etoposide [Wilson, 1999]. The mitochondrial pathway is facilitated by Bcl-2 family proteins. Anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein of the Bcl-2 family regulates the passage of cytochrome c from the mitochondria [Kelekar and Thompson, 1998]. In the cells that are undergoing apoptosis, cytochrome c is released from mitochondria into cytosol due to several apoptotic signals [Newmeyer and Ferguson-Miller, 2003]. Cytochrome c act together with dATP, apoptosis protease activating factor (Apaf1) and procaspase-9 within the apoptosome complex in the cytosol and consequently results in conversion of procaspase-9 to active form caspase-9 [Chandra et al., 2000] and afterwards this caspase-9 activates the executioner caspases like caspases-3, -6 and -7 [Salvesen and Dixit, 1997]. One of the best characterized members is caspase-3, which is activated by other caspases and can cleave many cellular targets, such as poly ADP-ribose polymerase (PARP), laminas and inhibitor of caspase activated DNase (ICAD) during the execution phase of apoptosis, resulting in characteristic morphological features of apoptosis such as chromatin condensation, DNA fragmentation and formation of apoptotic bodies. Activation of this caspase generally results in an irreversible commitment to cell death [Germain et al., 1999].

1.6.2. NEED FOR APOPTOTIC INDUCERS:

The accepted modality of cancer therapy are associated with many drawbacks and often provide temporary relief from symptoms, prolongation of life and occasionally cures. An efficacious anti-cancer agent/drug should execute or incapacitate cancer cells without causing excessive damage to normal cells. This ideal state is feasible by inducing apoptosis in cancer cells. The life span of both normal and cancer cells are significantly affected by the rate of apoptosis. Hence, modulating apoptosis may be advantageous in the management and therapy or prevention of cancer. The therapeutic application of apoptosis is at present being considered as a model for the development of effective anti-cancer drugs. A huge amount of synthetic work has contributed relatively small improvements over the prototype drugs. There is a constant need for new prototypes - new templates to use in designing of potential chemotherapeutic agents. Significantly, natural products are providing such

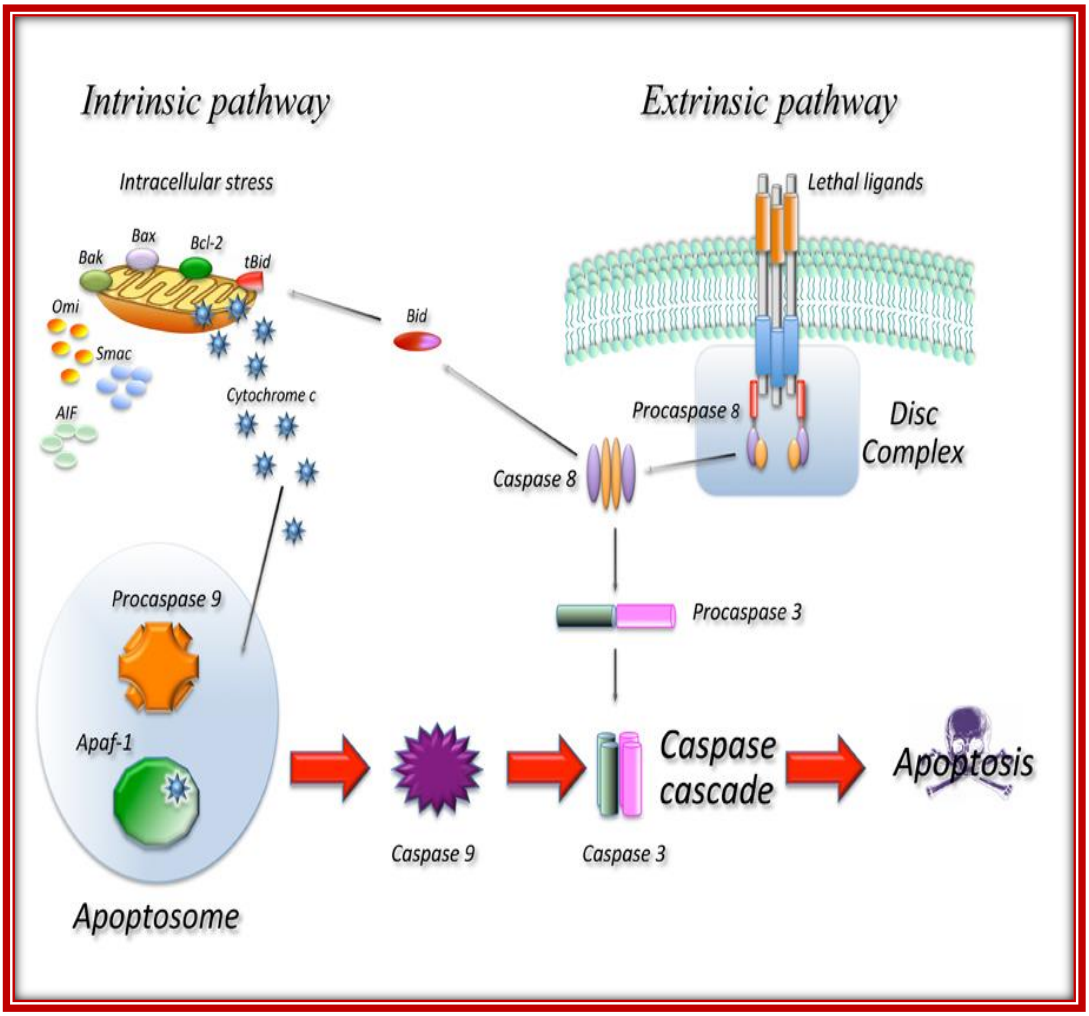


Fig. 1.7: A schematic diagram of two well-characterized apoptosis pathways-the extrinsic pathway and the intrinsic pathway.

*(**Ref:** Favaloro et al., 2012)*

templates. It is thus, considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. It is therefore, critical to identify novel apoptosis-inducing compounds that are candidate anti-tumour agents. In this context, we made an attempt to investigate the apoptotic induced cell death by selected plant fractions on different cancer cell lines. Understanding the modes of action of these fractions may provide useful information for their possible application in the cancer prevention and perhaps also in cancer therapy.

1.6.3. MEDICINAL PLANTS AND ANTI-CANCER DRUGS:

As the conventional cancer therapies failed to completely fulfill the criteria for successful cancer elimination, natural products played an important role in the development

of contemporary cancer chemotherapy due to their limited toxicities and high efficacy [Greenwald, 2002; Johnson, 2007]. Various studies have shown associations between apoptosis and cancer, in as much as the apoptosis-inducing agents are being appreciated as weapons for the management of cancer [Schmitt, 2003; Stewart, 2003]. Natural products are playing a cumulative role in the discovery of candidates for the development of new/novel chemotherapeutic agents [Lee, 1999; Schwartsmann, 2000]. In spite of competition from other drug discovery approaches, natural products are still providing their reasonable share of new clinical candidates and drugs. These compounds are still an important source of new drugs, especially in the anti-cancer therapeutic areas and some of them have since progressed further into clinical trials or onto the market [Butler, 2004]. They also offer a valuable source of compounds with a wide variety of chemical structures with biological activities, and provide important prototypes for the development of new and novel chemotherapeutics [Cragg, 1998; Verpoorte, 1998; Vuorelaa et al., 2004]. Modern technological advances and the development of innovative approaches have revolutionized the screening of natural products and offer distinctive opportunities to re-establish natural products as main sources of drug leads. Of the 79 Food and Drug Administration (FDA) approved anti-cancer drugs between 1940 and 2002, 9 of them were isolated directly from the natural products and 21 of them were natural product derivatives. Also among the 39 synthetic anti-cancer drugs, 13 of them were based on a pharmacophore originated from natural compounds (Fig. 1.8). Approximately 60% of all drugs now undergoing clinical trials for the multiplicity of cancers are either natural products or compounds derived from natural products [Butler, 2004; Newman et al., 2003].

Medicinal plants are a store house for substances that attract researchers in the quest for new and novel chemotherapeutics. The search for anti-cancer agents from plant sources started as early in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* (L.) (Apocynaceae), and the isolation of the cytotoxic podophyllotoxins. The discovery of cytotoxic agents from medicinal plants by following up ethno-medicinal uses or the results of previous anti-cancer screening has had a rich and fruitful past, with the identification of novel anti-cancer agents (Table 1.3). Modern technological advances and the development of innovative approaches have revolutionized the screening of medicinal plants and offer

distinctive opportunities to establish as main sources of drug leads [Corcoran and Spraul, 2003; Ganesan, 2002; Steinbeck, 2004]. Simultaneously, the synergistic effects of the

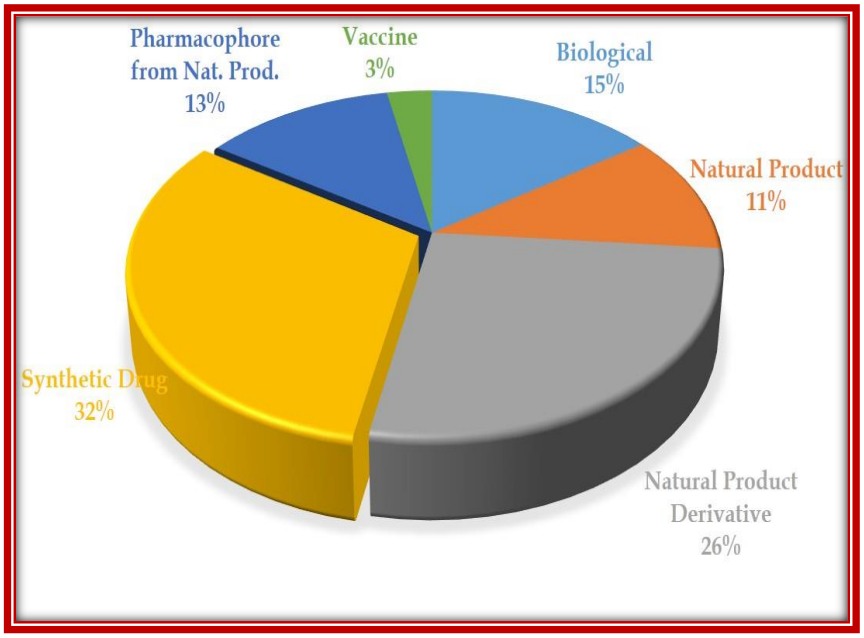


Fig. 1.8: Percentage of anti-cancer drugs derived from natural products between 1940's-2002).
*(**Ref:** Balunas and Kinghorn, 2005)*

cocktail of plant metabolites and the multiple points of intervention offer higher efficacy during chemoprevention regimens. A large number of plants used in the traditional medicine have now become a part of the modern world health care system [Fabricant and Farnsworth, 2001]. Medicinal plant based drugs provide outstanding contribution to modern therapeutics because of their wide range of mechanism of actions. The current studies on tumour inhibitory compounds derived from plants have yielded an impressive array of novel structures which have been applied towards combating cancer [Balunas and Kinghorn, 2005]. Research on bioactive compounds from medicinal plants continues to explore a diversity of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industry [Patwardhan et al., 2004]. These approved entities, representative of very wide chemical diversity, continue to demonstrate the importance of compounds from medicinal plants in modern drug discovery efforts. The identification of effective herbs and elucidation of their underlying mechanisms could lead to the development of an alternative and complementary method for cancer prevention and/or treatment [Nobili et al., 2009]. The Indian sub-continent has great botanical diversity and

Table 1.3: List of anticancer compounds from different medicinal plants

Medicinal plant	Family	Active constituent(s)	Class
Agapanthus africanus	Agapanthaceae	Isoliquiritigenin	Chalcone
Aglaila sylvestre	Meliaceae	Silvesterol	Alkaloid
Ailanthus altissima	Simaraubaceae	Ailnthone, Ailantenol	Quassinoids
Apium graveolens	Umbelliferae	Apigenin	Flavonoid
Betula species	Betulaceae	Betulinic acid	Triterpene
Bleckeria vitensis	Apocynaceae	Ellipticine	Alkaloid
Brucea antidysenterica	Simaraubaceae	Bruceantin	Quassinoid
Bursera microphylla	Burseraceae	Burseran	Lignan
Camptotheca acuminata	Nyssaceae	Campothecin	Alkaloid
Cassia quinquangulata	Fabaceae	Resveratrol	Flavonoid
Catharanthus roseus	Apocynaceae	Vincristine, Vinblastin	Alkaloid
Centaurea montata	Asteraceae	Montamine	Alkaloid
Centaurea schischkinii	Asteraceae	Schischkinnin	Alkaloid
Cephalotaxus harringtonia	Cephalotaxaceae	Homoharringtonine	Alkaloid
Cleistanthus collinus	Euphorbiaceae	Cleistanthin, Collinusin	Lignan
Combretum caffrum	Combretaceae	Combrestatins	Stilbenes
Croton lechleri	Euphorbiaceae	Taspine	Alkaloid
Diphylleia grayi	Berberidaceae	Diphyllin	Lignan
Dipteryx odorata	Fabaceae	Isoliquiritigenin	Flavonoid
Dysoxylum binectariferum	Meliaceae	Rohitukine	Alkaloid
Erythroxylum pervillei	Erythroxylaceae	Pervilleine A	Alkaloid
Euphorbia semiperfoliata	Euphorbiaceae	Jatrophane	Terpenoid
Fritillaria thunbergii	Liliaceae	Zhebeinone	Alkaloid
Gunnera perpensa	Gunneraceae	2-methyl-6(3-methyl 2-butenyl) benzo 1-4 quinone	Quinone
Hypericum perforatum	Clusiaceae	Hypericin	Anthraquinone
Hypoxis colchicifolia	Hypoxidaceae	Hypoxoside, Rooperol	Glycoside
Indigofera tinctoria	Leguminosae	Indirubins	Indigoids
Justicia procumbens	Acanthaceae	Justicidin A,B	Lignan
Lantana camara	Verbenaceae	Verbascoside	Glucoside
Larrea tridentate	Zygophyllaceae	Terameprocol	Lignan
Lonicera japonica	Caprifoliaceae	Luteolin	Flavanoid
Pestemon deustus	Serophulariaceae	Liriodendrin	Lignan
Phaleria macrocarpa	Thymelaeaceae	Pinoresinol, Laricinesinol	Lignan
Physalis philadelphica	Solanaceae	Ixocarpalactone A	Lectone
Podophyllum peltatum	Berberidaceae	Epipodophyllotoxin, Podophyllin	Alkaloid
Polygonum cuspidatum	Polygonaceae	Resveratrol	Flavanoid
Pteris multifida	Pteridaceae	Pterokaurane	Terpenoid
Pygeum africanum	Rosaceae	Amygdalin	Glycoside
Taxus brevifolia	Taxaceae	Paclitaxel	Terpenoid
Vitex rotundifolia	Verbenaceae	Casticin	Flavanoid
Wikstroemia viridi	Thymelaeaceae	Wikstromol	Caumarin

Ref: Balunas & Kinghorn, 2005; Kaur et al 2011

provides a rich pool of novel and efficacious agents for cancer prevention and treatment and there are huge number of molecules that still remain to be trapped. As a vast proportion of the available plant species have not yet been subjected to scientific evaluation for their potential anti-cancer principles. Anti-cancer drug discovery from plants still remains an essential component in the search for new medicines to combat cancer diseases, particularly with the development of highly sensitive and versatile analytical methods [Krishnaswamy, 2008]. Continuing searches among medicinal plants and the semi-synthesis of analogues of active principles will undoubtedly lead to further examples of novel plant derived anti-cancer agents. Therefore, based on these information, the present study aimed to investigate the cytotoxic effects of selected medicinal plants using different human cancer cell lines and to determine the possible mechanism of cell death.

1.7. RATIONALE OF THE STUDY:

As discussed, medicinal plants, remains the most successful source of new drugs, new drug leads, and new chemical entities (NCEs) due to their unique structural diversity in comparison to standard combinatorial chemistry which presents opportunities for discovering mainly novel low molecular weight lead compounds. In addition to the phyto-constituents which have found direct medicinal application as drug entities, many others can serve as chemical models or templates for new molecule(s) design, synthesis and semi-synthesis of novel substances for treating humankind diseases. Apart from that they can also lead to the discovery and improved understanding of the targets and pathways involved in disease processes [Tulp and Bohlin 2005]. Recent significant advances in the methodology and technology for separation, structure elucidation, screening and combinatorial synthesis have led to revitalization of plant products as sources of new drugs [Corcoran and Spraul, 2003; Ganesan, 2002; Steinbeck, 2004]. These modern techniques would be helpful in discovering new or novel drugs to combat different ailments of mankind. Therefore, studies on medicinal/herbal planta profoundly not only to discover active compounds but also to find the effective mechanism of them to develop into drugs for treatment of diseases. In order to maximize the use of such plants, it is desirable to screen the extracts for a variety of bioactivities. Invariably, bioassay techniques have to be developed for the high through-put-screening of the plant extracts for quicker results. Plants, the best combinatorial

chemists, still wait for us to discover the hitherto hidden secrets of their healing properties to unburden mankind from dreaded diseases such as cancer [Cragg and Newman, 2005].

In this context, we have undertaken a systematic evaluation of the chemo-preventive potential of four potent medicinal plants, viz., *Abrus precatorius, Artemisia nilagirica, Cissampelos pereira and Cocculus hirsutus* widely used in Indian traditional medicine by monitoring their antioxidant effects. In addition, we wanted to investigate the direct anti-proliferative effects of some extracts and their chromatographic fractions on the growth of different cancer cell lines, using a bioassay-guided fractionation method. These plants have been more or less documented chemically or pharmacologically, but presumably not completely exploited for their antioxidant and anti-proliferative properties. The results of this study will contribute towards the growing knowledge on Indian traditional system of medicine and will help to rationalise their therapeutic application/the development of effective therapeutic principles.

1.7.1. SCOPE OF THE PRESENT STUDY:

From the foregoing analysis of the challenges posed to the healthcare by diseases such as cancer, and the limited scope offered by the synthetic drugs, an acute need is felt to explore the alternative source of potential drugs from natural sources especially medicinal plants. Out of the estimated 2,50,000 - 5,00,000 species reported for possessing biological activity, only less than 5% of the plant species have been investigated. Hence, there is an ample scope for screening number of important herbs with chemical diversity. Therefore, it was proposed to screen the selected plants in order to validate their use in traditional medicine and to identify the active principle by employing modern scientific methods.

1.8. SELECTION OF PLANTS FOR PRESENT RESEARCH:

Four plant species from three different families were selected for preliminary screening based on the following criteria:

- Ethano-pharmacological use of plants in the management of various ailments.
- Preliminary pharmacological evaluation of these medicinal plants.
- Absence/limited availability of published literature describing biological properties
- Their availability of evaluation.

The literature review on the selected plants for antioxidant and anti-proliferative studies yielded little information and limited reports. The plants were *Abrus precatorius, Artemisia nilagirica, Cissampelos pereira* and *Cocculus hirsutus*.

1.8.1. *ABRUS PRECATORIUS* (L.):

❖ **TAXONOMIC HIERARCHY:**

- Kingdom: Plantae- Plants
- Division: Magnoliophyta – Flowering plants
- Class: Magnoliopsida – Dicotyledons
- Order: Fabales
- Family: Fabaceae - Pea family
- Genus: *Abrus*
- Species: *A. precatorius* L.
- English name: Rosary pea

❖ **BOTANICAL DESCRIPTION:**

Abrus precatorius (L.) is a branched, slender, perennial, woody twinning vine/creeper with characteristic red and black seeds. Stem is cylindrical, wrinkled with bark smooth-textured and brown in color. Leaves are pinnate and glabrous, resembling tamarind leaves with many leaflets (20 - 40 leaflets) arranged in pairs. The leaflets are oblong, measuring 2.5 cm in length and and 1.5 cm in width (Fig. 1. 9A). The plant bears orange-pink flowers, which occur as clusters in short racemes that are sometimes yellowish or reddish purple in colour, small and typically pea- like. The plant produces short and stout brownish pods, which curl back on opening to reveal pendulous red and black seeds with 4 to 6 peas in a pod [Ivan, 2003].

❖ **HABITAT AND GEOGRAPHICAL DISTRIBUTION:**

It is native to India and grows wild in thickets, farms, secondary clearings and sometimes in hedges and bushes in exposed areas at altitudes up to 1200 m on the outer Himalayas and down to Southern India and Ceylon [Nadkarni, 1999]. It is most common in rather dry areas at low elevations throughout the tropics and subtropics [Acharya et al., 2004].

Fig. 1.9 (A): *Abrus precatorius* (Fabaceae). Different parts of the plant such as seeds, leaves are used as medicinal purposes in different systems of medicines for treating different ailments.

❖ **TRADITIONAL MEDICINAL REPORTS:**

A. precatorius is considered as a potent medicinal plant and is widely used in Ayurveda, Folk, Homeopathy, Sidha, Tibetan and Unani traditional systems of medicine for over 2500 years to treat various diseases and human ailments. It is traditionally used to treat tetanus and to prevent rabies. In the Ayurvedic medicine, leaves of *A. precatorius* are laxative, expectorant, aphrodisiac and are used in treating urticaria, eczema, stomatitis, nerve tonic, conjunctivitis, alopecia areata, migraine, lymphomas/ leukaemia and dysmenorrhoea, itching and other skin diseases [Anonymous, 2004]. Decoction of leaves is widely used to cure fevers, cough, cold and colics. Juice of fresh leaves is employed as a cure for hoarseness, mixed with oil, applied externally to relieve painful swellings [Kirtikar and Basu, 2005].

The roots and leaves are also used as diuretic, diarrhoea, gastritis, heart diseases, kidney diseases, insomnia, Cancer and CNS sedative [Daniel, 2006; Kubiatowicz and Benson, 2003]. The leaves are sweeter and are equivalent in sweetness to sucrose [Anonymous, 2004; Dymock et al., 1893; Inglett, 1968]. The roots are considered emetic and alexiteric.

The roots are used to treat jaundice and haemoglobinuric bile. The watery extract is useful in relieving obstinate coughs cure, sore throat, abdominal pains and rheumatism. Root are chewed as a snake bite remedy. Hot water extract of fresh root is anti-malarial and anti-convulsant and is used to treat bronchitis and hepatitis in traditional medicine [Kirtikar and Basu, 2005].

Internally, the seeds are described as poisonous due to the presence of abrin, however, it is useful in the treatment of the nervous system after alleviation, and externally, in skin diseases, ulcers [Verma et al., 2011]. The seeds reduced to a paste are recommended to apply locally in the treatment of sciatica, stiffness of the shoulder joint, inflammation, paralysis and other nervous diseases. In white leprosy, a paste composed of the seed and plumbago root is applied as stimulant dressing. Taken internally by women, the seed disturbs the uterine functions and prevents conception [Kirtikar and Basu, 2005]. The alleviated roots are also used as diuretic, diarrhoea, gastritis, heart diseases, kidney diseases, insomnia, purgative, emetic, tonic, anti-phlogistic, aphrodisiac, anti-ophthalmic, cancer and CNS sedative [Kubiatowicz and Benson, 2003].

❖ **BIOLOGICAL ACTIVITIES:**

A. precatorius possesses wide variety of therapeutic properties which include abortifacient effect [Prakash et al., 1976], agglutinin activity [Krupe et al., 1968], anti-alzheimer effect [Zambenedetti et al., 1998], analgesic activity [Dhawan et al., 1977], anti-helmintic activity [Mølgaard et al., 2001], anti-bacterial activity [Desai et al., 1966; Dhawan et al., 1977], anti-convulsant activity [Dhawan et al., 1977; Adesina et al., 1982], anti-diarrheal activity [Nwodo and Alumanah, 1991], anti-estrogenic effect [Agarwal et al., 1970], anti-fertility effect [Zia-Ul-Haque et al., 1983], anti-fungal activity [Dhawan et al., 1977], anti-gonadotropin effect [Jadon and Mathur, 1984], anti-inflammatory activity [Dhawan et al., 1977; Anam, 2001; Sudaroli and Chatterjee, 2007], anti-malarial activity [Limmatvapirat et al., 2004; Menan et al., 2006], anti-spasmodic activity [Dhawan et al., 1977; Nwodo and Alumanah 1991; Wambebe and Amosun, 1984], anti-spermatogenic effect [Rao, 1987; Sinha, 1990], anti-viral activity [Dhawan et al., 1977; Otake et al., 1995], bronchodilator [Mensah et al., 2011]. CNS depressant activity [Adesina, 1982], cytotoxic activity [Bhutia et al., 2009; Hussein et al., 1982; Itokawa et al., 1990; Ramnath et al., 2002], hypoglycemic activity [Dhawan et al., 1977; Gbolade, 2009], immunomodulator [Tripathi and Maiti, 2005],

nephroprotective activity [Ae et al., 2009; Sohn et al., 2009; Sohn et al., 2009b], spermicidal effect [Rao, 1987; Ratnasooriya et al., 1991].

❖ **PHYTOCHEMISTRY:**

The literature survey on phytochemical reports of the *A. precatorius* reveals that this medicinal plant species contain wide array of phyto-constituents mainly alkaloids, flavonoids, steroids, triterpenoids, tannins, coumarins, phenolic compounds, protiens etc. Several compounds like abrin, trigonelline [Ibrahim, 1980], abruslactone A, hemiphloin [Ragasa et al., 2013], abrusoside A-D [Choi et al., 1989a; 1989b] glycyrrhizin [Akinloye and Adalumo, 1981] have been identified from the leaves of *A. precatorius*. Various chemical constituents such as abrol, abrasine, precasine and precol [Khaleqe et al., 1966; Willaman et al., 1970], choline, N, N dimethyl-tryptophan, N, N, dimethyl-tryptophan-metho-cation-methyl-ester, P coumaroylgalloyl-glucodelphinidin, pectin, pentosans, phosphorus, delphinidin, gallic-acid, picatorine, polygalacturonic-acids, precatorine [Ghosal and Dutta, 1971], isoflavonoids and quinones-abruquinones A-F [Kuo et al., 1995], abruslactone a, abrusgenic acid-methanol-solvate [Chang et al., 1982; 1983] are present in the roots of *A. precatorius*. Proteins such as abraline, abricin, abrusgenic acid, abrusgenic-acid-methyl-ester, abruslactone, abrussic acid, anthocyanins, campesterol, cycloartenol, delphinidin, trigonelline, hypaphorine have been reported from the roots of this plant [Ghosal and Dutta 1971]. Triterpenoids and saponins [Chang et al., 1982], glycyrrhizin [Akinloye and Adalumo, 1981] and oleanolic acid [Ross, 2003] have been established to be present in the aerial parts of *A. precatorius.* Seeds are rich in several essential amino acids like serine, abrusin, abrusin-2'-0-apioside, hederagenin, kaikasaponin III, sophoradiol, sophoradiol-22-0-acetate, tryptophan [Desai et al., 1971].

Seeds contain principle compounds such as, abrin [Ghosal and Dutta, 1971], abrin A-B [Lin et al., 1981], abrin C [Wei et al., 1974], abrin l-III, abrus agglutinin APA-l, II [Hegde et al., 1991]. Alkaloids and nitrogen compounds- methyl ester of N, N-dimethyl tryptophan metho cation (I) and precatorine (II), hypaphorine, trigonelline [Ibrahim, 1980], flavonoids and triterpenoids, steroids, saponins, flavones, flavonols glycosides, reducing sugars, phenolic compounds, glycosides [Devasagayam and Sainis, 2002; Govindarajan et al., 2005; Scartezzini and Speroni, 2000; Shatish et al., 2010] are present in the seeds and leaves. Flavonoids and anthocyanins-abrectorin, dimethoxycentaureidin-7-O-rutinoside,

precatorins I, II [Ghosal and Dutta 1971] and III, abrectorin, centaureidin, demethoxy 7-O-beta-d'rutinoside, luteolin, orientin and iso- orientin [Bhardwaj et al., 980] have been isolated from the seeds. A new triterpinoid saponin 3-O- β -D-glucopyranosyl-(1—>2)- β-D-glucopyranosyl subprogenin D together with six known terpinoids C-glucosylscutelarein 6,7-dimethylether (abrusin) and its 2"-O-apioside have been identified as minor components in the seeds [Xiao et al., 2011].

1.8.2. *ARTEMISIA NILAGIRICA* (CLARKE) PAMP:

❖ **TAXONOMIC HIERARCHY:**

- Kingdom: Plantae- Plants
- Division: Tracheophyta
- Class: Magnoliopsida – Dicotyledons
- Order: Asterales
- Family: Compositae (Asteraceae)
- Genus: *Artemisia*
- Species: *Artemisia nilagirica/ Artemisia vulgaris* (Clark)
- English name: Indian wormwood, Mugwort

❖ **BOTANICAL DESCRIPTION:**

Commonly known as Indian wormwood or "mugwort", *A. nilagirica (Synonym: A. vulgaris)* is an aromatic perennial shrub that can reach up to 60 - 160 cm high, with many thin lateral roots. The branched, purplish-brown stems are parallel grooved, with ascending twigs covered with short hairs. Leaves are papery, pubescent, dark green on the upper surface and have various shapes depending on their position on the plant. Head inflorescences are oblong, 2.5 – 3.0 mm in diameter, borne densely in a spike on the branched twigs as well as spreading panicles on the stems (Fig. 1.9B). There are seven to ten purple female flowers, with narrow tube-shaped corollas. Fruits, appearing from August to October together with flowers, are obovate or ovate achene's [Chopra et al., 1994].

❖ **HABITAT AND GEOGRAPHICAL DISTRIBUTION:**

A. nilagirica is a perennial weed, native in Asia, Europe and in eastern regions of Canada and North America growing wild and abundantly in temperate and cold temperature zones of the world [Cui, 1989]. It is found throughout the mountainous districts of India. It

grows in Mount Abu, Marwar and in the Western Ghats and parts of Southern India [Bhattacharjee, 2000].

Fig. 1.9 (B): *Artemisia nilagirica* (Asteraceae). It is described in Ayurveda and Siddha as a potent drug against a variety of ailments. Arial parts of this herb are used for wide variety of medicinal applications

❖ TRADITIONAL MEDICINAL REPORTS:

The plant *A. nilagirica* is described in Ayurveda and Siddha as a potent drug against a variety of ailments [Trivedi, 2006]. It is intended to stimulate gastric secretions in patients with poor appetite and is used against flatulence, distention, colic, diarrhea, constipation, cramps, worm infestations, hysteria, epilepsy, vomiting, menstrual problems, irregular periods, to promote circulation and as a sedative. The root of this plant have been used as tonic for psychoneuroses, neurasthenia, depression, autonomic neuroses, irritability, restlessness, insomnia and anxiety. It is also used as an anti-spasmodic, expectorant, stomachic, tonic, laxative, anti-hysteric and anti-helminthic. It is used for menstrual problems, metorrhagia and to prevent abortion. In children, it is used as a decoction against measles and as a leaf juice against whooping cough. Leaf powder is used against hemorrhage, dysentery, intestinal complaints, urinary tract problems and skin diseases [Kapoor, 2000].

❖ **BIOLOGICAL ACTIVITIES:**

Some reports have revealed that mugwort is a potent immunomodulatory [Schmid-Grendelmeier et al., 2003], anti-hypertensive [Tigno et al., 2000], anti-inflammatory [Tigno and Gumila, 2000], antioxidant [Luo et al., 2007] and hepatoprotective agent [Gilani et al., 2005]. Anti-tumoural activity has been reported to artemisic acid and artemisinin B extracted from mugwort [Sun et al., 1992]. Insect repellent and fumigant activity has been found in essential oils from mugwort [Wang et al., 2006]. The insecticidal activity of essential oils from mugwort has been evaluated on Aedes aegipti [Chantraine et al., 1998]. In addition, anti-viral activity has also been described to extracts of this plant [Tan et al., 1998].

❖ **PHYTOCHEMISTRY:**

Phytochemical investigation of *A. nilagirica* revealed the presence of various secondary metabolites including terpenoids, flavonoids, coumarins, sterols, caffeoylquinic acids, alkaloids, amino acids, quinines, tannins, sesquiterpene and lactones which are known to be biologically and medicinally important. The representative compounds that are reported in *A. nilagirica* are Terpenes- p-cymene, fenchone, α- and β-thujone, cineole, camphor, β-pinene, 4-terpinenol, borneol α-thujone, α-terpineol, geraniol, caryophyllene, coumarins sterols caffeoylquinic acids, eriodictyol and luteolin [Ahameethunisa and Hopper, 2010; Carnat et al., 2000; Govindaraj et al., 2008; Lee, 1998; Marco et al., 1990; Murray and Stefanovic, 1986; Ragasa et al., 2008; Trumpowskaand and Olszewski, 1968]. The plant contains many active compounds including the monoterpenes, eucalyptol, camphor, linalool, thujone, 4-terpineol, borneol, α-cadinol, spathulenol and many more. These monoterpenes are present in the essential oils that makes up 0.03% - 0.3% of the plant. The plant also contains sesquiterpenes and sesquiterpene lactones such as eudesmane, vulgarin, psilostachyin and psilostachyin C. Flavonol glycosides are present including quercitin 3-O-glucoside, rutin and isorhamnetin 3-O-glucoside. Different coumarins are also found in this plant such as aesculetin, aesculin, umbelliferone, scopoletin and 6-methoxy-7,8-(methylenedioxy)coumarin. Polyacetylenes, carotenoids and pentacyclic triterpenes are present in the form of sitosterol and stigmasterol [Bisset, 2000]. The main constituents of the essential oil of the *A. nilagirica* reported as camphor, β -eudesmol, 1,8-cineole, borneol, artemisia alcohol, camphene, α -gurjunene, p-cymene, terpinen-4-ol, α -pinene, borneol and inositol [Agrawal and Singh, 1994; Haider et al., 2007; Uniyal et al.,

1985]. The plant was also reported for β -caryophyllene, l-linalool, β -thujone, azulene, thujyl alcohol and an unidentified hydrocarbon. The plant contains a crystalline pentacyclic alcohol fernenol, stigmasterol, β -sitosterol, and α -amyrin and its acetate. The plants from lower altitude had more percentage of cineol, thujone, thujyl alcohol and citral whereas those from higher altitudes contained a higher percentage of limonene, tripinoline and aromadendrene [Anonymous, 2004; Asolkar et al., 1992; Leeja and Thoppil, 2004].

1.8.3. *CISSAMPELOS PEREIRA* (L.):

❖ **TAXONOMIC HIERARCHY:**

- Kingdom: Plantae- Plants
- Division: Tracheophyta
- Class: Magnoliopsida – Dicotyledons
- Order: Ranunculales
- Family: Menispermaceae
- Genus: *Cissampelos*
- Species: *C. pereira* L.
- English name: Velvet leaf, Abuta

❖ **BOTANICAL DESCRIPTION:**

C. pareira is a perennial climbing herb/shrub 2-5 m high with a thickened root. Leaves possess an orbicular shape and are 7-14 cm in diameter. They are membranous or leathery, veined, glabrous to densely pilose. Flowers are small greenish yellow, unisexual with 4-5n sepals, ovate to obovate, peltate or orbicular-reniform, ovate sub-reniform leaves with truncate cordate base, globrous or hairy above up to 3-12 cm long (Fig. 1.9C). It produces inediable, dark grape sized berries. Fruits are short hairy orange to red drupe. Seeds are horseshoe-shaped [Prasad et al., 1962; Smitin and Larsen, 1991].

❖ **HABITAT AND GEOGRAPHICAL DISTRIBUTION:**

The plant is commonly found in orchards, hedges, parks and gardens of moist soils either creeping or twinning around other plants. It is also common on the hilly tracts along water courses. *C. pereira* was first described from Latin America but actually occurs throughout tropics especially warm parts of Asia, East Africa. It is also found in the areas of Comoros, Madagascar and Islands.

Fig. 1.9 (C): *Cissampelos pereira* (Menispermaceae). In the traditional folk medicine, roots and leaves of *C. pereira* are used as one of the most important traditional ayurvedic herbal formulation and are prescribed for various ailments

❖ TRADITIONAL MEDICINAL REPORTS:

In the traditional folk medicine, *C. pereira* is used against various ailments. It is used as a diuretic, tonic, reduces fever and also act as pain killer. Roots of this plant are used as one of the most important traditional ayurvedic herbal formulation and are prescribed for diseases like rheumatism, ulcers, fevers etc. The decoction of roots is used in relieving menstrual cramps, difficult menstruation, threatened miscarriage, excessive bleeding during pre and post-delivery uterine haemorrhages in women, fibroid tumours, pre and postnatal pain, colic, constipation, poor digestion and dyspepsia [Feng et al.,1962; Mukerji and Bhandari,1959]. The roots are used in the treatment of dysuria and renal calculi. Decoction of the leaf and stem is used as an oral analgesic, asthma and for traumas [Gogte, 2001]. The leaves are used in the treatment of indolent ulcers [Kirtikar and Basu, 2005] and diarrhoea [Amresh et al., 2003]. The plant is considered to be anti-septic and on account of this property, it is used in the treatment of urinary tract infection [Dandiya and Chopra, 1970). Expressed juice of *C. pareira* is given in migraine [Singh and Gupta, 2005]. *C. pareira* also has

a long history for its uses in muscle inflammation, snake bite, rheumatism, diarrhoea, dysentery and menstrual problems, including asthma and for traumas [Mokkasmit, 1971].

❖ BIOLOGICAL ACTIVITIES:

Many pharmacological studies have been carried out extensively to investigate/validate the therapeutic properties of *C. pareira*. Scientific reports demonstrating pharmacological properties of this plant include; anti-nociceptive and anti-arthritic activity, anti-inflammatory activity, antioxidant activity, chemo-preventive effects [Amresh et al., 2001; 2007a; 2007b; 2007c; 2007d], anti-fertility activity [Ganguly et al., 2007], anti-haemorrhagic effects [Badilla et al., 2008] etc.

❖ PHYTOCHEMISTRY:

Chemical profiling of *C. pereira* have shown the occurrence of biologically important classes of compounds such as alkaloids, tannins, flavonoids, terpenoids and others. These classes of compounds have demonstrated significant biological properties thereby validating the health promoting properties of *C. pereira*. Cissampelosine was reported from *C. pareira* which was later on shortened as pelosine [Wiggers, 1838]. A comparative analysis of *C. pareira* demonstrated presence of starch, gum, tannin, phlobaphene and an alkaloid [Ringer and Brooke, 1982]. The aerial parts of *C. pareira* contain a group of plant chemicals like isoquinoline alkaloids Cissampareine, (++)-4" -O-methylcurine, pareirubrine A and B, novel tropoloisoquinoline alkaloids; pareitropone, a novel azafluoranthene alkaloid; norimeluteine, norruffscine [Haynes et al., 1966; Kupchan, 1964; Morita et al., 1993a; 1993b; 2002; Roy et al., 1952]. An anti-protozoal chalcone-flavone dimer, cissampeloflavone, D-qurecitol and grandirubrine has been isolated from the aerial parts of *C. pareira*. Roots of *C. pereira* has been found to be store house of major bioactive constituents. The chemical investigation on the roots reported for presence of 0.33% of alkaloids, mainly hayatine, bebeerines, d-iso-chondrodendrine, hayatidine, hayatinine, cissamine, cycleanine, l-curine, menismine, pareirine, hayatinine tetrandrine, dicentrine, dihydrodicentrine, cycleanine, insularine and isochondrodendrine 0.2% essential oils, 3.4% fixed oils and a sterol [Anwer et al., 1968; Bhatnagar and Popli, 1967; Bhatnagar et al., 1967; Bhattacharji et al., 1952; Combes et al., 1965; Dwuma-Badu et al., 1975; Kirtikar and Basu, 2005; Kupchan et al., 1966; Rojanasonthorn, 1970].

1.8.4. *COCCULUS HIRSUTUS* (L.) DIELS:

❖ **TAXONOMIC HIERARCHY:**

- Kingdom: Plantae- Plants
- Division: Magnoliophyta
- Class: Magnoliopsida – Dicotyledons
- Order: Ranunculales
- Family: Menispermaceae
- Genus: *Cocculus*
- Species: *C. hirsutus L.*
- English name: Jaljamini

❖ **BOTANICAL DESCRIPTION:**

Cocculus hirsutus (L.) Diels (Synonym; *Cocculus villosus*) is a climbing scandent shrub with green flowers that blooms in February-March and bears fruits in the months of May-June. Leaves are simple, alternate, ovate, sub deltoid or three lobed, obtuse and mucronate. The fruit is a drupe which is size of small pea with dark purple endocarp (Fig. 1.9D). The flowers are very small, unisexual and green [Kirtikar and Basu, 2005].

Fig. 1.9 (D): *Cocculus hirsutus* (Menispermaceae). Its roots and leaves is patronized for its unique property of curing various diseases and healing all types of cuts, wounds and boils.

❖ HABITAT AND GEOGRAPHICAL DISTRIBUTION:

C. hirsutus is widely distributed in the tropical and sub-tropical regions of India, China, Africa, Arabia and Ceylon [Chatterjee et al., 2000]. The plant grows all over India, especially in dry regions.

❖ TRADITIONAL MEDICINAL REPORTS:

Traditionally, *C. hirsutus* was patronized for its unique property of curing various diseases and healing all types of cuts, wounds and boils. The roots and leaves of *C. hirsutus* have great medicinal value and are used both, internally as well as externally for medicinal purpose. According to Ayurveda, *C. hirsutus* is known as "Patalagarudi" in Sanskrit and describes the uses of roots to "Kapha" and "Vata", lessen bile and burning sensation. It enriches the blood and is useful in urethral discharges. It is also used as refrigerant, laxative, in chronic rheumatism, venereal diseases, fever and syphilitic cachexia [Nadkarni et al., 1999]. Roots are bitter in taste and are also used as alterative, laxative, demulcent, tonic, diuretic, anti-periodic fever, in malaria, joint pains, in treatment of skin diseases constipation and kidney problems [Caius, 1986; Chopra et al., 1996]. According to Unani system of medicine, it is anti-pyretic, tonic, diminishes thirst, helpful in fractures, and useful in tubercular glands related problems. It is well known herb which is commonly used as first aid remedy in minor injuries. This plant has a special potency as a detoxifier. It is an aphrodisiac and tonic in properties [Anonymous, 2004]. Juice of leaves coagulates in water and forms mucilage which is used externally as cooling medicine in eye problems and soothing application in prurigo, eczema, impetigo and dyspepsia and acute gonorrhoea [Chadha et al., 1950; Maasilamani and Shokat, 1981; Nandkarni et al., 1976]. The water soluble fraction of ammonical extract has sedative, hypotensive, bradycardiac, cardiotonic, spasmolytic and slight anti-convulsant actions [Maasilamani and Shokat, 1981].

❖ BIOLOGICAL ACTIVITIES:

The *C. hirsutus* plant has been evaluated for different medicinal properties over the years. There are several scientific reports on pharmacological properties of *C. hirsutus*, such as acute toxicity [Ganapaty and Dash, 2002], anti-diabetic [Badole et al., 2006; Sangameswaran and Jayakar, 2007; Satyanarayana et al., 1994], anti-inflammatory and analgesic [Nayak et al., 1993], anti-microbial [Nayak and Singhai, 2003], cardiotonic

[Satyanarayana et al., 1994], diuretic and laxative [Badole et al., 2009], immune- stimulant [Rastogi et al., 2008], spermatogenic [Sangameswaran et al., 2007] etc.

❖ **PHYTOCHEMISTRY:**

The research work in the field of phytochemical investigation of the plant has been carried out by many research groups over the years to identify and isolate different biologically active constituents. *C. hirsutus* has been reported to contain essential oil, β-sitosterol, (+) ginnol, glycosides, sterols and alkaloids [Das et al., 1964; Merchant et al., 1962]. The preliminary phytochemical analysis of leaves showed presence of alkaloids, phenolic compounds, flavonoids, glycosides, and carbohydrates. The phytochemical studies showed the presence of bis-benzyl isoquinoline alkaloids; viz. Cohirsinine [Viquaruddin et al., 1991], Hirsutine, Shaheenine [Rasheed et al, 1991a; 1991b], Jamtinine [Viqaruddin and Iqbal, 1993], Jamitine- N –oxide [Viqaruddin et al., 1987a], Cohirsine [Viqaruddin et al., 1987b], Cohirsitine [Viquaruddin and Iqbal, 1992] and Haiderine [Viqaruddin and Iqbal, 1993] which are isolated from stem and roots of *C. hirsutus*. The alkaloids present in the leaves of *C. hirsutus* are D-trilobine and DL-coclaurine, isotrilobine, (+)syringaresinol and protoquericitol [Jagannadha and Ramachandra, 1961]. Roots are reported for the presence of D-trilobine and coclaurine, sterols and resins [Viqaruddin and Tahir, 1986].

1.9. OBJECTIVES OF THE PRESENT STUDY:

Based on an exploration of published literature of selected Indian traditional medicinal plants with significant medicinal value, the following objectives were designed to evaluate their antioxidant and anti-cancer efficacies. The specific objectives of the study were:

1) Screening and determination of phytochemicals constituents with respect to total phenolic and flavonoid contents.

2) Evaluation of antioxidant and free radical scavenging activities of plant extracts in a cell free system by different *in vitro* methods.

3) Bioassay guided fractionation of crude plant extracts using column chromatography.

4) Determination of growth inhibition of different cancer cells through induction of apoptosis.

5) Identification of active constituents from active fractions using LC-MS/MS analysis and accurate mass database searching.

Chapter II:

Materials and Methods

2.1. CHEMICALS AND REAGENTS:

❖ KITS, ENZYMES, ANTI-BODIES, CHEMICALS, SOLVENTS AND REAGENTS:

Cell culture media, Molecular biology kits and standard chemicals were procured from Invitrogen Life technologies (Germany), Gibco (Germany), Cell Signalling technology (USA), Sigma-Aldrich (USA) and Clontech Laboratories (Japan), Thermo Scientific (USA), Fermentas (USA). Primary and secondary anti-bodies were obtained from Cell Signalling Technologies (USA). The solvents, chemicals and reagents were of analytical grade and obtained from Merck (India), Sigma-Aldrich (USA) and Himedia (India), SRL (India), Qualigens fine chemicals (India). Precautions were taken as per the manufacturer's instructions.

2.2. COLLECTION OF PLANT MATERIALS AND IDENTIFICATION:

All the plants were easily accessible and readily available for collection. The fresh plants of *Abrus precatorius* and *Artemisia nilagirica* were kindly provided by the Central Research Institute of Unani Medicine (CRIUM), Hyderabad; *Cissampelos pareira* and *Cocculus hirsutus* were collected from University of Hyderabad campus in the months of July-August during the period of study. The accessibility of the plants allowed immediate processing of fresh plant parts prior to extraction. The plants were identified and authenticated by Dr. V.C. Gupta, Taxonomist, (CRIUM, Hyderabad). Voucher specimens (UoH/VS/AP-2; UoH/VS/AN-3; UoH/VS/CP-4; UoH/VS/CH-5) were maintained at Department of Ethano-pharmacology, Central Research Institute of Unani Medicine (CRIUM), Hyderabad.

2.3. ETHICS STATEMENT:

The necessary permissions for collection of materials was obtained for the described studies and the party(s) involved were duly acknowledged. The species are not endangered and the habitat is not threatened and it is also not listed in the appendices of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). For animal studies, the experimental protocols were undertaken in accordance with National Institutes of Health (NIH)- guidelines and the necessary approvals were obtained from the University of Hyderabad, School of Life Sciences' Animal Ethics Committee (LS/IAEC/IAG/11/10; dated 15/4/2011).

2.4. PREPARATION OF PLANT MATERIAL AND EXTRACTION:

The fresh leaves of *A. precatorius*, *C. pereira* and *C. hirsutus* and aerial parts of *A. nilagirica* were collected and immediately sprayed with alcohol to cease the enzymatic degradation of secondary metabolites and shade dried at room temperature for 5-10 days. After shade drying, plant materials were coarsely powdered separately using a mechanical grinder. All the dried materials were stored at room temperature until required. Soxhlet extraction is a well-established technique, widely used for extraction of phyto-constituents because of its continuous process, less time and solvent-consumption than other procedures involving maceration and percolation. The powdered plant material(s) (30.0 g each) was placed in a soxhlet apparatus (Fig 2.1A), which is on top of a collecting flask beneath a reflux condenser. The hot extraction was carried out successively with organic solvents from non-polar to polar, which were added to flask and refluxed. The steam of the solvent, which comes in contact with the material dissolves metabolites and brings them back to flask. In the present work, four solvents of different polarities were used for the extraction purpose (*n*-hexane, ethyl acetate, ethanol and water) successively in order of increasing polarity. Continuous extraction was carried out for a period of 8-10 h at a temperature not exceeding the boiling point of the solvent with about 20 refluxes or till the solvent in siphon tube of soxhlet apparatus becomes colourless [Lin et al., 1999]. Each time before extracting with the next successive solvent, it was ensured that the plant material(s) being extracted was free from previous organic solvent. The solvents in the round-bottomed flask were collected, filtered using Whatman® filter paper (No. 1) and concentrated to dryness in a flash evaporator (Buchii®, USA) under reduced pressure and controlled temperature (35-40^{o}C). Water extracts were prepared by soaking 10 g powder of respective plant material in 100 mL of distilled water overnight at room temperature. The extracts were centrifuged at 10,000 rpm for 10-20 min at room temperature. The supernatant volumes were collected and subsequently concentrated by using rotary vacuum evaporator and the water was removed by freeze drying [Tilak et al., 2004]. A schematic representation of the extraction procedure illustrates the different steps performed to obtain the plant extracts (Fig 2.1B). On concentration, it yielded respective solvent extracts and weight of the different extracts obtained were recorded and used for percentage yield calculation in terms of dried weight of the plant material. The extracts designated as APH, APE, APA, APW for *A. precatorius n*-

hexane, ethyl acetate, ethanol and water extracts respectively; ANH, ANE, ANA, ANW for *A. nilagirica n*-hexane, ethyl acetate, ethanol and water extracts respectively; CPH, CPE, CPE and CPW for *C. pareira n*-hexane, ethyl acetate, ethanol and water extracts respectively. Similarly, CHH, CHE, CHA were designated for *n*-hexane, ethyl acetate and ethanol extracts

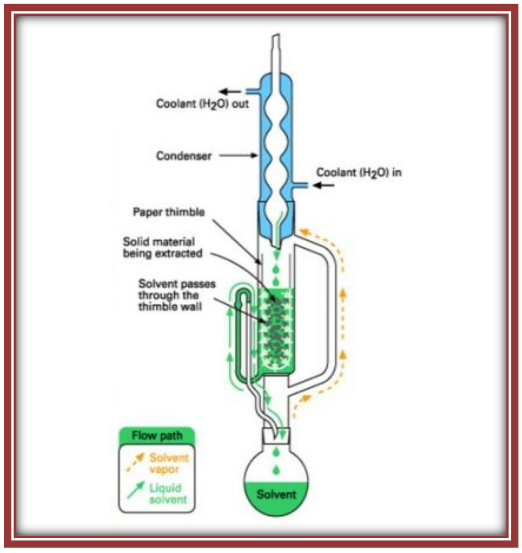


Fig. 2.1 (A): Soxhlet apparatus used in this study

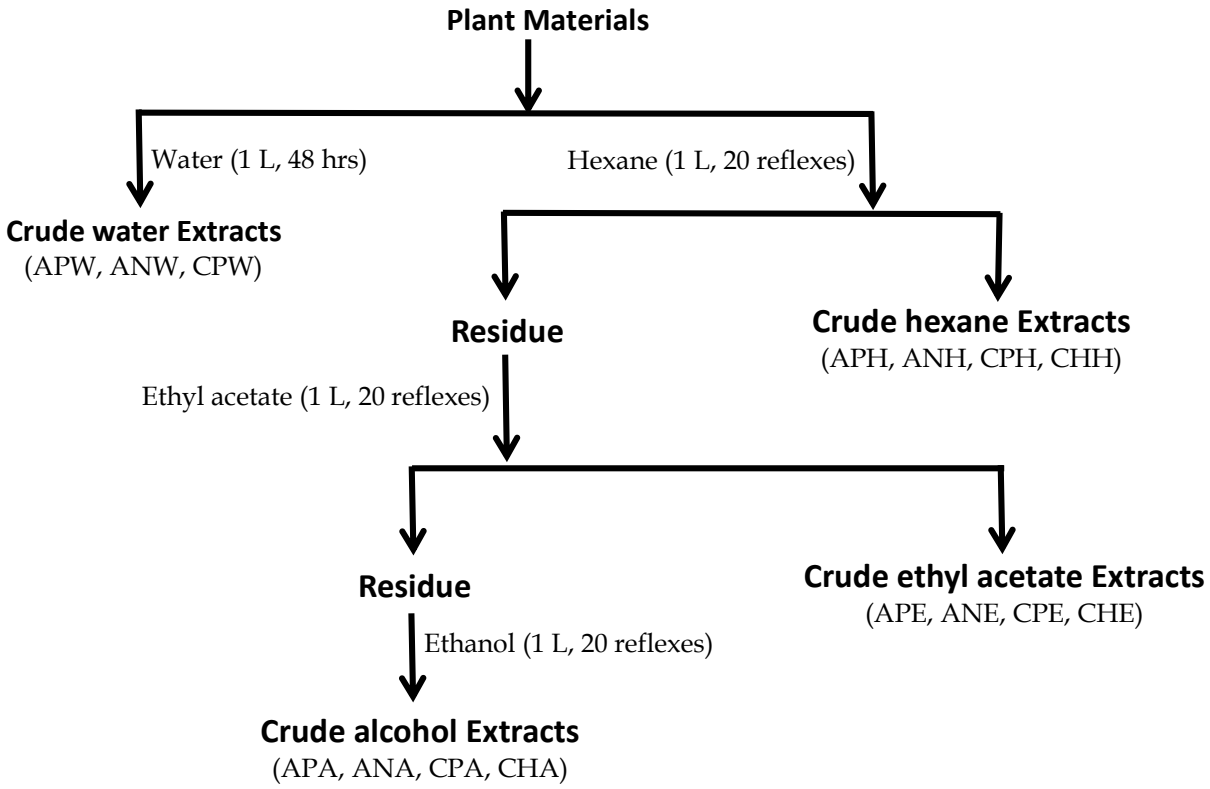


Fig. 2.1 (B): Systematic representation of sequential solvent extraction method for selected medicinal plants.

for *C. hirsutus*. It is worth to mention that water extract of leaves of *C. hirsutus* could not be prepared as it gets solidified on soaking in water. The extraction process was repeated three times at different time periods to check the variability in yield of extracts. No significant difference was observed in the percentage yield and contents of phyto-constituents that are believed to play an important role in biological activities. Plant extracts were stored at -20^{o}C and later used for phytochemical and biological studies. It was assumed that dried material remained stable as it has been showed that material stored for 80 years retains its activity [Katerere and Eloff, 2004].

❖ **DETERMINATION OF EXTRACTION YIELD (% YIELD):**

The yield (%, w/w) from all the plant extracts was calculated as:

$$\textbf{Yield (\%)} = (W_1 \times W_2) \times 100$$

where W_1 is the weight of the extract after removal of organic solvent under reduced pressure/lyophilisation, and W_2 is the weight of the plant powder.

2.5. PHYTOCHEMICAL ANALYSIS OF PLANT EXTRACTS:

Preliminary phytochemical screening was carried out to quantitate total amounts of polyphenols and flavonoids in the extracts.

2.5.1. DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC):

For the quantification of the total contents of phenolic compounds, a Folin- Ciocalteau (FC) method described by Yang et al., [2007] with minor modifications was used. Folin-Ciocalteu reagent is molybdotungsto phosphoric heteropolyanion reagent that can reduce phenols specifically. A blue colour is developed which can be measured at λ 765 nm by using spectrophotometer. Intensity of blue colour depends upon concentration of phenolic compounds in the test substance. Working solutions of Gallic acid with concentrations of 0, 50, 100, 150, 250, and 500 µg/mL were prepared from the stock of 50 mg/mL. An aliquots of 10 µL from each of the working solutions (20 mg/mL) of plant extracts into separate tubes were added to 0.2 mL of distilled water (dH_2O), followed by addition of 0.1 mL of FC reagent. The contents were shaken vigorously and incubated at room temperature for 10 min. Further, 0.3 mL of 20% sodium carbonate (Na_2CO_3) solution was added and vortexed thoroughly. Final volume was made up to 1 mL with dH_2O. The mixtures were allowed to stand for 2 h and 30 min at room temperature for complete reaction. The total phenolic content was determined spectrophotometrically by measuring the absorbance at λ 725 nm

(UV-160A spectrophotometer, Shimadzu, Japan) against a reagent blank. The amount of total polyphenols was calculated as Gallic acid equivalent (GAE)/g dry weight (dwt) plant material from the calibration curve of Gallic acid standard solutions ($R^2 \geq 0.99$) and expressed as mg GAE/g of dwt of plant material.

2.5.2. DETERMINATION OF TOTAL FLAVONOID CONTENT (TFC):

The total flavonoid content was determined using the $NaNO_2$-$AlCl_3$ -NaOH system described by Barreira et al., [2008]. Briefly, 10 µL from each of the working solutions (20 mg/mL) of plant extracts into separate tubes were diluted with 0.5 mL of dH_2O. To this mixture, 30 µL of 5% sodium nitrite ($NaNO_2$) and 60 µL of 10% aluminium chloride were added and incubated at room temperature for 10 min. After incubation, 350 µL of 1 M sodium hydroxide (NaOH) was added and final volume was made up to 1 mL with dH_2O. Finally, absorbance was measured against the prepared blank at λ 510 nm. A yellow colour indicated the presence of flavonoids. Quercetin was used as a reference standard. The amounts of total flavonoids were expressed in mg quercetin equivalents (mg QE)/g of dwt of plant extracts. Standard curve was prepared with 0.1 mg/mL to 1.2 mg/mL concentrations of quercetin ($R^2 \geq 0.99$).

2.6. ANTIOXIDANT ABILITY ASSAYS:

2.6.1. PHOSPHOMOLYBDENUM ASSAY:

Total antioxidant activity of plant extracts were determined by green phosphomolybdenum complex method described by Prieto et al., [1999]. The assay is based on the reduction of Mo(VI)–Mo(V) by the antioxidant compound(s) and subsequent formation of a green phosphate/Mo(V) complex at acidic pH. Different aliquots of standard solutions of ascorbic acid (5-50 µg/mL) and plant extracts (10 µL from each extract) were mixed with 1 mL of chemical reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in 1.5 mL eppendrof tubes. Tubes containing reaction mixture were capped and incubated in a dry thermal bath at 95°C for 1 h and 30 min. The tubes were cooled down to room temperature and subsequently, the absorbance was measured at λ 695 nm against a blank. Ascorbic acid have been used ($R^2 \geq 0.99$) for reference and the reducing capacities of the analysed extracts were expressed as mg of ascorbic acid equivalents/g (mg ASE/g) of dwt.

2.6.2. FERRIC-REDUCING ABILITY /ANTIOXIDANT POWER [FRAP] ASSAY:

Reducing power of different plant extracts was determined by the method of Oyaizu [1986] with a slight modifications. According to this method, the reduction of Fe^{3+} to Fe^{2+} was determined by measuring absorbance of the Perl's Prussian blue complex. This method is based on a redox reaction in which an easily reduced oxidant (Fe^{3+}) is used in stoichiometric excess and antioxidants acts as reductants. Briefly, different plant extracts/ascorbic acid (standard reference) in 1 mL of appropriate solvents were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v) and then mixture was incubated at 50°C for 30 min. Later, 2.5 mL of trichloro-acetic acid (10% w/v) was added to the mixture, which was then centrifuged at 5000 rpm for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1% w/v). The absorbance was measured at λ 700 nm and the reducing power of the extracts was presented as mg ASE/g of dwt.

2.7. RADICAL SCAVENGING ACTIVITIES:

2.7.1. DPPH FREE RADICAL-SCAVENGING ACTIVITY:

2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical-scavenging activity of the plant extracts/standard compounds was measured spectrophotometrically according to the method described by Braca et al., [2002] with minor modifications, wherein the bleaching rate of a stable free radical, $DPPH^{\bullet}$ is observed at a characteristic wavelength in presence of the plant extracts/reference compound. $DPPH^{\bullet}$ in its radical form absorbs at λ 517 nm, but upon reduction by an antioxidant, its absorption decreases. Briefly, 0.004% solution of $DPPH^{\bullet}$ was prepared in ethanol and 0.90 mL of this solution 900 µL of this solution was mixed with 100 µL of extract solution of different concentrations (40-400 µg/mL) of dried extracts. These solutions were vortexed thoroughly and incubated in dark. After the incubation of 30 mins, the absorbance was measured at λ 517 nm against blank samples. Lower absorbance of the reaction mixture compared to blank indicates higher $DPPH^{\bullet}$ free radical scavenging activity. Ascorbic acid and Quercetin were used as positive controls. The DPPH free radical-scavenging activity was calculated according to the following equation:

$$\textit{DPPH radical-scavenging activity (\%)} = [(A_o - A_t)/A_o] \times 100$$

where A_o is the absorbance without samples and A_t the absorbance in the presence of plant extracts. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity.

2.7.2. HYDROXYL RADICAL SCAVENGING ACTIVITY:

The ability of the plant extracts to impede site-specific hydroxyl radical-mediated peroxidation was performed according to the method described by Kunchandy and Rao, [1990] with minor changes by studying the competition between deoxyribose and extracts for hydroxyl radicals generated by Fenton's reaction. Briefly, the mixture containing $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), deoxyribose (28 mM) and different concentrations of plant extracts (40-400 µg/mL) in 500 µL of phosphate buffered saline (PBS, 20 mM, pH 7.4) was incubated for 30 min at 37°C. After adding 1 mL of trichloro-acetic acid (TCA; 10%, w/v) and 1 mL thiobarbituric acid (TBA; 2.8% w/v) in 25 mM NaOH, the reaction mixture was heated for 15 min. The extent of oxidation was estimated at λ 532 nm. Ascorbic acid was used as the positive control. The scavenging activity of samples were expressed as the percentage inhibition of the deoxyribose degradation to malondialdehyde. The percentage of scavenging of hydroxyl radical by plant extracts or reference compound was calculated using the formulae:

$$\textit{Percentage (\%) inhibition} = [(A_o - A_t)/A_o] \times 100$$

where A_o is the absorbance without samples and A_t the absorbance in the presence of plant extracts.

2.7.3. HYDROGEN PEROXIDE SCAVENGING ACTIVITY:

The hydrogen peroxide scavenging activity of the plant extracts/reference compound was performed according to the method described by Long et al., [1999]. Phosphate buffer of 0.1 M (pH 7.4), 40 mM hydrogen peroxide (H_2O_2) in PBS and xylenol orange reagent containing 256 mM ammonium iron (II) sulphate, 25 mM H_2SO_4, 4.4 mM BHT and 1 mM xylenol orange were prepared and stored at 4°C in an amber coloured bottle. Different concentrations of plant extracts (20-200 µg/mL) were pre-incubated with 10 µL of 40 mM hydrogen peroxide for 15 min in dark and 400 µL of xylenol orange reagent was added. The reaction mixture was vortexed and subsequently incubated at room temperature for 30 min. After the incubation, the absorbance of the reaction mixture was read for the intensity of chromophore (ferric-xylenol orange complex) at λ 560 nm. The percentage scavenging of

hydrogen peroxide radical by plant extracts or reference compound was calculated using the formulae:

$$\textit{Percentage inhibition (\%)} = [(A_o - A_t)/A_o] \times 100$$

where A_o is the absorbance without samples and A_t the absorbance in the presence of plant extracts.

2.7.4. NITRIC OXIDE SCAVENGING ACTIVITY:

The free radical scavenging potential of plant extracts was further substantiated by scavenging of nitric oxide radical assayed by sodium nitroprusside method described by Sreejayan and Rao, [1997]. The reaction solution (50 µL) containing 10 mM sodium nitroprusside in PBS (pH 7.0) was mixed with different concentration (40-400 µg/mL) of plant extracts, followed by incubation at 37°C for 20 min under light conditions. After incubation, the mixture was diluted with 300 µL of Griess reagent (1% sulfanilamide in 2% H_3PO_4). The reaction mixture was further incubated for 45 min under light conditions followed by addition of 10 µL of 0.1% *N*-(1-Naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4. The absorbance was recorded at λ 546 nm. Ascorbic acid was used as a positive control. The results were expressed as percent of scavenged nitric oxide with respect to the negative control without addition of any antioxidant. Percentage inhibition was calculated as per the following formulae:

$$\textit{Percentage inhibition (\%)} = [(A_o - A_t)/A_o] \times 100$$

where A_o is the absorbance without samples and A_t the absorbance in the presence of plant extracts.

2.7.5. SUPEROXIDE ANION RADICAL SCAVENGING ACTIVITY:

The scavenging activity of the plant extracts and reference compound towards superoxide anion radicals was measured by nitro-blue tetrazolium (NBT) reduction method as defined by Nishikimi et al., [1972] with minor modifications. Superoxide anion radicals were generated in a non-enzymatic phenazine methosulphate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of NBT. In the experiment, the superoxide anion was generated in 2 mL of phosphate buffer (100 mM, pH 7.4) containing 500 µL of 156 µM NBT solution, 500 µL of 468 µM NADH solution and 300 µL of different concentrations (40-400 µg/mL) of extracts. Dimethyl sulfoxide (DMSO) and ascorbic acid were used as solvent and positive

control respectively. The reaction was initiated by adding 100 µL of 60 µM PMS to the mixture. The reaction was incubated at room temperature for 5-20 min and then absorbance was measured at λ 560 nm against blank. Ascorbic acid was used as the reference. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition was calculated by the equation:

$$\textit{Percentage inhibition (\%)} = [(A_o - A_t)/A_o] \times 100$$

where A_o is the absorbance without samples and A_t the absorbance in the presence of plant extracts.

2.7.6. IN VITRO Fe(II)-INDUCED LIPID PEROXIDATION IN RAT LIVER HOMOGENATE:

Fe^{2+} induced lipid peroxidation is one of the established system for assessing anti-oxidant action of different plant extracts. Lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate by the method of Bishayee and Balasubramaniyam, [1971] was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al., [1979]. The assay is based on the extent of formation of TBARS from the peroxides formed by lipid by oxidizing agents. Polyunsaturated fatty acids with three or more double bonds are particularly more susceptible to peroxidation. Once the reaction is initiated, the process proceeds as a free radical chain reaction. The secondary end product of polyunsaturated fatty acid -Malondialdehyde, reacts with two molecules of TBA yielding a pinkish red chromogen.

❖ EXPERIMENTAL ANIMALS:

Healthy rats of the Wister strain weighing 200-250 g and with age from 12-16 weeks were used. The animals were housed in a poly acrylic cages and maintained under standard laboratory conditions (temperature 22±3°C; relative humidity 60-70% and 12 h light/dark cycles). They were fed commercial rat feed and distilled water *ad libitum.* The animals were sacrificed following the administration of anaesthesia (Thiopentone barbitone sodium).

❖ RAT LIVER TISSUE HOMOGENATE:

Rat liver was excised after decapitation, weighed and rinsed with ice-cold 0.9% NaCl to get rid of blood. Liver tissue homogenate was prepared in 10% (w/v) of 0.15 M KCl using a teflon homogenizer followed by centrifugation at 5,000 rpm for 15 min at 4°C, and clear cell-free supernatant was used for thiobarbutaric acid assay.

Different extracts of plants at different concentrations (40-400 µg/mL) were mixed with the liver microsome preparation and incubated at room temperature for 10 min. Then, 50 µL Fenton's reagent (10 mM $FeCl_3$; 10 µL of 2.5 mM H_2O_2; 0.1 M ascorbic acid) in PBS (200 mM, pH 7.4) were added, and the volume was made to 1 mL. The sample mixture were then incubated for 30-45 min at 37°C to induce lipid peroxidation. Thereafter, the reaction was terminated by adding 2 mL of ice-cold HCl (0.25 N) containing 15% (v/v) TCA 0.5% (w/v), TBA and 0.5% (w/v) butylated hydroxytoluene (BHT). Subsequently, the reaction mixtures were heated at 80-90°C for 10-15 min. Then the reaction mixtures were cooled down in an ice bath for 10-15 min and subsequently centrifuged at 1000 rpm for 10 min. The extent of lipid peroxidation was then observed by means of formation of TBARS as pink chromogen in presence/absence of extracts and ascorbic acid. The absorbance of the supernatant was measured spectrophotometrically at λ 532 nm and decrease in formation of pink chromogen in pre-treated reactions was considered as inhibition of lipid peroxidation. The effect of different extracts or test/standard compound against lipid peroxidation were calculated as follows:

$$\textit{Percentage inhibition (\%)} = [(A_o - A_t)/A_o] \times 100$$

where A_o is the absorbance without samples and A_t the absorbance in the presence of plant extracts.

2.8. BIOASSAY-GUIDED FRACTIONATION OF ACTIVE CRUDE EXTRACTS OF *A. PRECATORIUS* AND *A. NILAGIRICA* USING COLUMN CHROMATOGRAPHY:

2.8.1. PREPARATION OF CRUDE EXTRACTS FOR BIOASSAY GUIDED FRACTIONATION:

The bulk quantity of dried plant material of *A. precatorius* and *A. nilagirica* (200 g each) were grounded and extracted with ethyl acetate (EA) and methanol (MeOH) at room temperature by successive extraction using soxhlet apparatus (as described in section 2.4). The EA extract of *A. precatorius* leaves filtrate was collected, filtered through Whatman® filter paper (No. 1) and excess solvent was evaporated under reduced pressure using a rotary evaporator at 40°C to dryness producing 25.4 g of dark-greenish EA extract (APE) and the MeOH filtrate of *A. precatorius* yielded 28.2 g of MeOH extract (APA). Similarly, the ethyl acetate and methanol filtrates of aerial parts of *A. nilagirica* yielded 22.7 g and 24.2 g of EA (ANE) and methanol (ANA) extracts, respectively.

2.8.2. COLUMN CHROMATOGRAPHY:

Column chromatography was used as a fractionation step for crude extract(s), which provided a partial separation of chemical compounds based on polarities. A sintered glass column of internal diameter 40 mm and length 1000 mm was packed with 500 g of silica gel (60-120 mesh) as stationary phase prepared as slurry in *n*-hexane. The column was allowed to stabilise for 24 h, after which, the solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The active crude extracts were adsorbed on silica gel by preparing the slurry in methanol and the solvent was recovered under reduced pressure. This slurry was loaded on the stationary phase of the column. Further, varying solvent combinations of increasing polarity were used as the mobile phase for the separation of the fractions from the active crude extracts.

2.8.3. COLUMN CHROMATOGRAPHY SEPARATIONS OF ETHYL ACETATE AND METHANOL EXTRACTS OF *A. PRECATORIUS* LEAF EXTRACT:

The APE crude extract (20 g) was subjected to column chromatography to separate the extract into its component fractions. The elution was carried out with systems of gradually increasing polarity using *n*-hexane, ethyl acetate (EA) and methanol (MeOH). The following ratios of solvent combinations were sequentially used in the elution process; Hexane:Ethyl acetate: 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70 and 20:80 (v/v). A measured volume (1 L) of each solvent combination was collected gradually with a wash bottle and sprayed uniformly by the sides of the glass into the column each time. Eluents collected in portions of 50 mL volume were collected in number labelled conical flasks. Finally, the column was eluted with 100% MeOH. A total of 142 fractions of 50 mL each were collected and all the eluents were pooled according to the similarity of the chemical composition detected on pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} plates (as described in section 2.8.5). The eluents were pooled according to the similarity of the chemical composition detected on TLC and the excess solvent was evaporated under reduced pressure using a rotary evaporator to yield a total of 11 fractions, designated as APH-1, APH-2, APH-3, APH-4, APH-5, APH-6, APH-7, APH-8, APH-9, APH-10 and APH-11. Each fraction was weighed and stored at -20^{o}C for further use.

Similarly, the whole methanol extract (APA) weighing 20 g was separated by silica gel (60-120 mesh) column chromatography. The extract was eluted using a combination of *n*-

hexane, ethyl acetate and methanol with an initial ratio of Hexane-MeOH, 99:1 followed by 98:2, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100 (v/v). A total of 127 fractions of 50 mL each were collected and all the eluted fractions were than monitored individually by TLC and the fractions with same TLC profile were pooled; thereby 8 major fractions were obtained that were designated as APM-1, APM-2, APM-3, APM-4, APM-5, APM-6, APM-7 and APM-8. The fractions were then concentrated to dryness under reduced pressure, weighed and stored at -20^{o}C.

2.8.4. COLUMN CHROMATOGRAPHY SEPARATIONS OF ETHYL ACETATE AND METHANOL EXTRACTS OF *A. NILAGIRICA* CRUDE EXTRACTS:

The 20 g of active ethyl acetate crude extract was chromatographed on a glass column packed silica gel (60-120 mesh). The column was developed with a solvent gradient of Hexane-EA in order of increasing polarity (Hexane:EA; 100:0, 90:10, 80:20, 70:30, 60:40 and 40:60 v/v) and volume of the solvent combination used in each gradient step was 1000 mL. Eluents were collected in portions of 50 mL. Finally the column was flushed with MeOH. A total of 65 fractions of 50 mL each were collected and all the eluents were pooled according to the similarity of the chemical composition detected on pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} plates. The excess solvent was evaporated under reduced pressure using a rotary evaporator to yield a total of 8 fractions designated as ANE-A, ANE-B, ANE-C, ANE-D, ANE-E, ANE-F, ANE-G, and ANE-H. Each fraction was weighed and stored at -20^{o}C for further use.

Similarly, the methanol extract (ANA) weighing 20 g was fractionated with column chromatography over silica gel (60-120 mesh). The column was eluted sequentially with following ratios of solvent combinations; Hexane: EA; 100:0, 80:20, 60:40, 40: 60 and 20: 80 and 100% (v/v) followed by chloroform ($CHCl_3$): MeOH combination with following ratios of 98:2, 95:5, 90:10, 80:20, 70:30, 60:40, 40:60. At the end, the column was flushed with 100% MeOH. Eluents were collected in numbered conical flasks and total of 132 fractions of 50 mL each were collected and grouped in 10 sub-fractions according to their chemical profiles analysed by thin layer chromatography and were designated as ANM-1, ANM-2, ANM-3, ANP-4, ANM-5, ANM-6, ANM-7, ANM-8, ANM-9 and ANM-10**.** Each fraction was then concentrated to dryness under reduced pressure on rotary evaporator, weighed and stored at -20^{o}C for further analysis.

2.8.5. ANALYTICAL THIN LAYER CHROMATOGRAPHY (TLC):

Thin layer chromatography (TLC) is widely used in natural product analysis. TLC fingerprints of medicinal plant extracts/fractions are used for identification of separated components. The identification of separated components can be attained on the basis of retention factor (R_f) values and colour spots. The phytochemical profiles of each of the aforesaid eluents/fractions was monitored on pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} aluminium strips supplied by Merck, Germany by spotting 10 µL. The TLC strips was dipped into a small chromatographic jars containing the different developing solvent system. The plates were developed by employing varying solvent polarities such as ethyl acetate in *n*-hexane; ethyl acetate in chloroform; methanol in chloroform and methanol in water with different ratios to create developing solvent. The combinations within the parenthesis were used:

a) Hexane:Ethyl acetate = (95:5; 90:10; 85:15;80:20; 75:25 v/v)

b) Choloroform:Ethyl acetate= (95:5; 90:10; 85:15;80:20; 75:25 v/v)

c) Choloroform:Methanol = (95:5; 90:10; 85:15;80:20; 75:25 v/v)

d) Ethyl acetate:Methanol = (95:5; 90:10; 85:15;80:20; 75:25 v/v

e) Water:Methanol = (95:5; 90:10; 85:15;80:20; 75:25 v/v)

All the jars were covered with a glass lid to prevent the evaporation of developing solvents. The solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dryer. The developed spots were visualized under UV light at λ 254 nm and λ 365 nm, visible, or by means of spraying the plates with a mixture of ethanol solution of vanillin (1:1 v/v), Iodine, Ninhydrin and $KMNO_4$ The developed TLC plates were further sprayed with 10% v/v sulphuric acid in methanol solution and heated at 100^{o}C for 5-10 min to allow colour development [Herborne, 1973; Stahl, 1969]. The eluents were pooled according to the similarity of the chemical composition detected on TLC and the excess solvent was evaporated under reduced pressure using a rotary evaporator to yield sub-fractions. All the major sub-fractions obtained were further subjected to MTT cytotoxic activity assay using different cancer cell lines for bioactivity and fractions APH-11 & APM-3 from APE and APA and ANE-B & ANM-9 from ANE and ANA, respectively were revealed as the most active fractions in cytotoxic activity and therefore selected and further evaluated for phytochemical analysis and studied

for their apoptotic effects towards the selected cancer cell lines. A concentrated stock solution of active sub fractions was prepared in DMSO and stored at -20°C until required.

2.9. CELL LINES AND CELL CULTURE:

A panel of five human cancer cell lines namely; (a): Human acute monocytic leukaemia cell line - THP-1, (b): Human T-cell lymphoblastic lymphoma- SupT1, (c): Human B-cell lymphoma leukaemia - JM1, (d): Human hepatocellular carcinoma cells - HepG2, (e): Human cervix adenocarcinoma- HeLa. They are all included in the 60 human tumour cell lines panel, which is a tool developed by the "Developmental Therapeutics Program" of National Cancer Institute for early stage of drug discovery screening [Shoemaker, 2006]. The murine peritoneal macrophages and Human Embryonic Kidney 293 (HEK-293) cells were used as a model of normal cells. The cell lines; THP-1, SupT1, JM-1, HepG2, HeLa and HEK-293 were obtained from National Centre for Cell Sciences (NCCS), Pune, India whereas, the peritoneal macrophages were harvested from female BALB/C mice (procedure described in section 2.9.2). The cell lines HepG2, HeLa and HEK were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4.5g/L D-glucose, L- glutamine; THP-1 and SupT1 in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with L- glutamine. JM1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-glutamine and 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2 mercaptoethanol. In addition, all the three media were supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, antibiotics. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C during growth and treatments and seeded onto standard tissue culture 75 cm^2 flasks. All cells were sub-cultured at 4-6 days interval upon reaching 70-80% confluence and cells were regularly examined. The cell lines were used until 20 passages. Only cells growing at the logarithmic growth phase were used to perform all the experiments. They were seeded at a density of 1×10^4 cells/mL, then washing of the monolayers was performed with PBS, pH 7.4. The washed cells were isolated by centrifugation and re-suspended in culture medium for plating or counting. Adherent cell lines were detached with 0.025 % trypsin-EDTA. Cell viability was assessed by trypan blue exclusion test. This test is based on the principle that live cells hold intact cell membranes that exclude certain dyes, such as trypan blue whereas, dead cells do not. In this test, a cell suspension of 20 µL was mixed with 10 µL of (0.004%) dye and then

examined under microscope. All viable cells had the clear cytoplasm whereas; non-viable/dead cells were appeared in blue colour [Strober, 2001]. The cell numbers were determined by haemocytometer.

2.9.1. EVALUATION OF CYTOTOXIC EFFECTS OF THE EXTRACTS/FRACTIONS:

❖ CELL PROLIFERATION BY MTT REDUCTION ASSAY:

In vitro response to extracts/sub-fractions and reference compound was evaluated by means of a growth inhibition using the (3- (4, 5-dimethyl-thiazol-2-yl) -2, 5-diphenyltetrazolium bromide - MTT assay [Mosmann, 1983; Polydoro et al., 2004]. The MTT assay is based on metabolic reduction of colourless tetrazolium salt by mitochondrial enzyme activity in viable cells, to blue formazan salt/crystals, which can be quantified spectrophotometrically. It is particularly useful for assaying cell suspensions because of its specificity for living cells [Mosmann 1983].

The crude extracts/fractions were dissolved in DMSO at 50 mg/mL as stock solutions which were further diluted with Milli-Q water to desired concentrations and sterilized using 0.2 µm membrane filters. The final concentration of DMSO in each sample did not exceed 0.01% v/v, to keep the cytotoxicity of DMSO at less than 10%. Cells were seeded in 96-well micro-plates at the density of 1×10^4 cells/mL (in 200 µL of growth medium). The plates were pre-incubated for 24 h at 37°C to allow adaptation of cells prior to addition of sample tests. The concentration-dependent effects of the extracts/fractions on the viability was carried out on selected five different tumour cells. Control groups received the same amount of DMSO (0.01% v/v). Untreated cells were used as a negative control, while cells treated with Doxorubicin were used as a positive control. After treatment with various concentrations of each extract/sub fraction/Doxorubicin, the cells were incubated for 48 h at 37°C. After incubation, the medium was removed and a fresh medium was added followed by addition of 20 µL of MTT solution (5 mg/mL in PBS) to each well and further incubated for 4 h at 37°C. After incubation, the supplement was carefully removed and 100 µL of DMSO were added to solubilize the insoluble formazan crystals. The plates were shaken for 5 min before optical density was measured at λ 570 nm with background subtraction at λ 690 nm using a micro plate reader (Tecan, Japan). Media without cells with respective concentrations of extracts or standard compounds were taken as respective blanks. Data were obtained from triplicate wells. The effect of extracts/sub fractions on growth inhibition was assessed as percent cell

viability, where control-treated cells were taken as 100% viable. Experiments were repeated three times (n=3). The resulting growth data represents the net outcome of cell proliferation and cell death. The IC_{50} value (in µg/mL), which represents the concentration of test samples that lowers cell viability by 50% compared to the untreated control, was calculated for each extract/fraction from the concentration-response curve. Data were calculated as the percentage of inhibition by using following formula:

$$\textit{Percentage Inhibition (\%)} = [1-(A_0 / A_t)] \times 100\%$$

A_0 and A_t designated the optical density of cell lines incubated with sample and vehicle control, respectively.

The MTT assay was undertaken at three stages. In the 1st stage, different crude extracts of each plant were tested against the different cancer cell lines. Extracts that caused more than 50% inhibition of proliferation were selected for further investigations in stage 2nd. In the second stage, each fraction of active plant extracts was tested against the three cell lines. Fractions that showed more than 50% inhibition were selected for IC_{50} determination in stage II. In the 3rd stage, five concentrations (100, 75, 50, 25, 10 µg/mL) were prepared from each active fraction and tested against the three cell lines. The IC_{50} value (in µg/ml), which represents the concentration of test samples that lowers cell viability of cells by 50% compared to the untreated control, was calculated for each extract/fraction/Doxorubicin from the concentration–response curve.

2.9.2. EFFECT OF CRUDE EXTRACT/ACTIVE FRACTIONS ON NORMAL CELLS (CELL TOXICITY):

To assess the selectivity of crude plant extracts/active fractions towards selected cancer cells, murine peritoneal macrophages and human embryonic kidney - 293 (HEK-293) were used.

❖ EXPERIMENTAL ANIMALS:

Female BALB/C mice (6-8 weeks old, weighing 18-25 g) were used for isolation of murine peritoneal macrophage cells. The animals were maintained in a poly acrylic cages (at 24±4°C, 60±10% relative humidity and 12 h light/dark cycle), with water and food available *ad libitum*.

❖ ISOLATION OF PERITONEAL MURINE MACROPHAGES:

Thioglycollate-elicited mouse peritoneal exudate cells were obtained from mice following intraperitoneal injection of 3 mL thioglycollate medium [3.0 g/100 mL] and lavage

of the peritoneal cavity with 5 mL of ice cold PBS (10 mM; pH 7.2) 3-4 days later [Bhattacharjee et al., 2009; Bonacorsi et al., 2004]. Cells were washed, re-suspended in HEPES-buffered RPMI-1640 medium (supplemented with 10% heat inactivated FBS and antibiotics: 100 U/mL penicillin G and 100 mg/mL streptomycin and 50 mM 2-mercaptoethanol. These cells were seeded ($5x10^4$ cells/mL) in sterile disposal plates (60 mm) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The non-adherent cells were detached by centrifugation washing with RPMI-1640 and fresh culture medium was added to the adherent cells to grow at 80-90% confluency for cytotoxicity assay.

❖ **CYTOTOXICITY ASSAY:**

Macrophages cell suspension (1 x 10^4 cells/mL; 200 µL) was added to each well of a 96-well plate and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 prior to the experiment. After 2 h, the wells were washed and adhering cells exposed to different concentrations of plant extracts/sub fractions for 48 h. The test was accompanied by a viability positive control [medium + cells]. Similarly, HEK-293 cells were seeded in separate 96-well plates (200 µL/well at a density of $1×10^4$ cells/mL) and treated with various concentrations of plant extracts/sub fractions for 48 h. After incubation, cells were washed twice with PBS and the supernatant was discarded. Finally, MTT assay was performed (as described in section 2.9.1). The cytotoxicity was obtained by comparing the absorbance between the samples and the control and results were expressed as percentage cellular viability of the extracts/sub fractions.

2.10. APOPTOSIS STUDIES:

2.10.1. DNA FRAGMENTATION ANALYSIS BY AGAROSE GEL ELECTROPHORESIS:

DNA fragmentation was used to determine the induction of apoptosis by observing the biochemical change [Elmore, 2007; Kalinina et al., 2002]. Cells were grown to 80% confluence and seeded in 6-well plates at the density of $1×10^6$ cells/mL (in 1 mL of growth medium). Cells were treated with active fractions *A. precatorius* and *A. nilagirica* at a concentration of 50 µg/mL and incubated for 48 h. After incubation, the cells were washed twice with ice cold PBS (pH 7.4) and the DNA in the cell pellets was extracted by lysing the cells using 500 µL ice cold lysis buffer (5 mM of Tris-Cl, pH 7.4; 20 mM of EDTA; 1% (w/v); Triton X-100) for 20-30 min [Gao et al., 2002]. The cell lysates were incubated at 37°C for 1 h after the addition of RNase A (0.1 mg/mL). Then, 10 µL of proteinase K (100 µg/mL) was

added to each sample and were incubated at 56°C overnight. Next day, the samples were centrifuged at 14000 rpm at 4°C. DNA was extracted from the aqueous phase with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). DNA was precipitated with 10 M of NH_4COONa and 90% (v/v) ice cold ethanol for 2 h at -20°C. The DNA precipitate was centrifuged at 14,000 rpm at 4°C for 15 min. The pellet was air-dried and dissolved in 20 µL of sterile Tris-EDTA buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0). Concentrations of the samples were determined using a nanodrop UV spectrophotometer. Simultaneously, the equivalent amount of DNA samples diluted with the 4X DNA loading dye were loaded onto to 0.8% agarose gel containing 0.05% ethidium bromide. Electrophoresis was performed in 1X TAE buffer in company with DNA ladder marker (Fermentas, Life Sciences, 1 kb) at 50 V for 90 min. Finally the fragmented DNA bands were visualized by UV transilluminator and photographed. Apoptosis induction is indicated by the appearance of DNA ladder fragments of approximately 200 bp multiples on the agarose gel [El-Shemy et al., 2007].

2.10.2. TUNEL ASSAY (APOALERT® DNA FRAGMENTATION ASSAY):

The nuclear DNA fragmentation was further assessed by a commercial kit (ApoAlert® DNA Fragmentation Assay, Clontech Laboratories, Inc.) in accordance with the manufacturer's instructions. The ApoAlert® DNA Fragmentation Assay Kit detects apoptosis-induced nuclear DNA fragmentation via a fluorescence assay. The assay is based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL). TdT catalyses incorporation of fluorescein-dUTP at the free 3'-hydroxyl ends of fragmented DNA. Briefly, the cells 1×10^6 cells/mL were seeded into 60 mm sterile dishes and incubated with 1 mL of complete respective culture medium for 24 h. Then, the cells were treated with the active fractions of *A. precatorius* and *A. nilagirica* at the concentration of 50 µg/mL, complete media (as negative control) and Doxorubicin 10 µg/mL (as positive control) and re-incubated for 48 h. After the incubation period, the culture medium was aspirated off, and cells were attached on poly-L-lysine-coated slides and washed thrice with PBS (pH 7.4) in coupling jars. The cells were fixed with 4% (v/v) methanol-free formaldehyde/PBS solution at 4°C for 25 min and rinsed again with PBS. Subsequently, the cells were permeabilised by immersion in 0.2% Triton X-100 in PBS (pH 7.4) for 5 min, rinsed in PBS and equilibrated with the equilibration buffer for 10 min. Cells were labelled by incubating at 37°C with the TdT (terminal deoxynucleotidyl transferase) incubation buffer for 60 min. The reaction was

stopped by immersing the slides in saline sodium citrate for 15 min. Finally, slides were washed with PBS and treated with propidium iodide (10 µg/mL in PBS) for 15 min in dark conditions. Observations were made by using confocal fluorescence scanning microscope (Carl-Zeiss, Germany). At least 10 microscopic fields were observed for each sample. The green fluorescent cells and red fluorescent cells is considered as an apoptotic hallmark.

2.10.3. FLOW CYTOMETRY FOR CELL CYCLE ANALYSIS AND DETECTION OF APOPTOTIC CELLS:

The effect of sub fractions treatments on cell cycle distribution was determined by flow cytometry following staining with propidium iodide as described previously [Ait-Mohamed et al., 2011; Tang et al., 2010]. Briefly cells (1×10^6 cells/mL) treated with 50 µg/mL of active fractions of *A. precatorius* and *A. nilagirica* and Doxorubicin 10 µg/mL (positive control) for 24 and 48 h were fixed in 1 mL of 70% (v/v) ethanol and kept at 4°C for overnight. Next day, the cell pellets were collected by centrifugation (3000 rpm for 5 min) and washed twice with PBS. The cells were then resuspended in 1 mL hypotonic fluorochrome solution (50 µg/mL propidium iodide (PI), 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 10 µg/mL of RNase A). After 30 min incubation in dark conditions at 37°C, cells were analysed with a flow cytometer (FACScalibur, BD Biosciences, USA). Fluorescence emitted from the propidium iodide-DNA complex was estimated on a minimum of 10,000 cells per sample and analysed using CellQuest Pro software (BD Biosciences, USA). The percentage of hypodiploid cells (Sub-G1) over total cells was calculated and expressed as percentage of apoptotic cells.

2.10.4. ELECTROPHORESIS AND IMMUNOBLOTTING:

To investigate production of proteins involved in apoptosis, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting experiments were performed. Electrophoresis and related applications have contributed significantly towards the understanding of the molecular bases of cell structure and function. To enhance resolution and discrimination of proteins on the basis of molecular size rather than charge or shape, the proteins are denatured by SDS, prior to electrophoresis. The western blotting is extensively acknowledged analytical technique to detect the specific proteins in the given sample. Proteins separated by electrophoretic technique are electrophoretically transferred (“electroblotted”) onto a membrane. The membrane, which acts as a model of polyacrylamide gel, is then probed with anti-bodies to specific proteins. The primary anti-

bodies can be revealed by an additional incubation with HRP conjugated, followed by enhanced electrochemiluminescent (ECL) detection [Krizek and Rick, 2002].

❖ PROTEIN EXTRACTION:

Different cancer cells were plated at the density of 1×10^6 cells/mL in 100 mm dishes and incubated for 24 h prior to the treatment. Twenty four hours later, the medium was removed and replaced with fresh medium containing 50 µg/mL of active fractions of *A. precatorius* and *A. nilagirica.* The wells with no fraction was taken as negative controls and for positive control, cells were treated with Doxorubicin (10 µg/mL). After the incubation period of 24 h, the cells were harvested, pelleted down at 2500 rpm for 10 min and washed twice with ice cold PBS (pH 7.4). The pellets were lysed with 50 µL of lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, and protease inhibitor cocktail] and left for constant agitation on the rocker for 30 min at 4°C. The cell suspension was then subjected to three rounds of sonication at 20 mm for 5 sec each. Thereafter, the lysates were cleared by centrifugation at 14,000 rpm for 20-30 min, and supernatant were aliquoted and stored at -80°C as whole cell lysate till further use. The protein concentrations in the cell lysates was determined using Pierce® BCA protein assay kit (Thermo Scientific, USA) according to the manual provided by the manufacturer. A microplate reader (Tecan, Japan) was used to measure the absorbance at λ 595 nm and the concentration of the protein was calculated based on a bovine serum albumin (BSA) standard curve with a range of 0 to 1 mg/mL.

❖ SDS-PAGE AND IMMUNOBLOTTING:

To investigate production of proteins involved in apoptosis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting experiments were performed as described by Ravi et al., [2011]. The aliquots of whole cell lysates containing equal amount of proteins (100 µg) were heat denatured at 95-100°C for 5 min and resolved by electrophoresis on (10% for poly-ADP ribose polymerase (PARP); 12% for caspase -3, -7 and β-actin) SDS-polyacrylamide gel electrophoresis at 3 V/cm^2 for 45-60 min on SDS-PAGE and immobilized onto the NC membrane (Pall, USA). After gel electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane (0.45 mm) at 15 V for overnight. Membranes were then blocked in blocking solution (5% non-fat dry milk in TBST [10 mM Tris-HCl (pH 8.0), 137 mM NaCl, 0.05% Tween-20 (v/v)] for 3 h at 70 rpm, room temperature,

followed by washing for 3 times for 10 min each at 110 rpm. Thereafter, the membranes were probed with corresponding primary anti-bodies (1:1000 dilution; e.g. PARP, caspase-3, caspase-7, for proteins of interest overnight at 4°C; 70 rpm. After overnight incubation, the membranes were washed 3 times for 10 min each at 110 rpm before probing with corresponding secondary anti-bodies (anti-rabbit IgG conjugated with horse-radish peroxidase) diluted 1:5000 in blocking buffer away from direct light for 1 h and 30 min at room temperature and followed by washing 3 times for 10 min each. Subsequently, the immunoblots were examined with the enhanced chemiluminescence kit (ECL western blotting detection kit, GE Healthcare, USA). β-actin was used in each blot as a loading control to ensure equal loading of proteins for each sample. The membranes were then imaged with the help of Kodak Image Station 4000MM Pro.

2.11. METABOLITE PROFILING OF ACTIVE PLANT FRACTIONS BY LC-MS/MS:

High performance liquid chromatography coupled with mass spectrometry (LC/MS) is a key enabling technology for the detection and characterization of metabolites of biological samples, providing the researchers with one of the most powerful analytical tools of modern times. Mass spectrometry has had an instant and intense effect as a result of high sensitivity and the wealth of structural information for the analysis of organic compounds ranging from low molecular weight drug molecules to large molecular weight bio-polymers of assorted polarities in complex biological sample [Ho et al., 2003].

❖ SAMPLE PREPARATION:

The powdered material of APH-11, APM-3, ANE-B and ANM-9 (5 mg each) were dissolved in HPLC-grade methanol, sonicated, centrifuged (10,000 rpm) and filtered through 0.2 µm membrane filters. Separately obtained solutions were used for LC–DAD and LC–MS/MS analysis.

❖ INSTRUMENT:

LC-MS/MS analysis was performed at the Metabolic facility of School of Life Sciences, University of Hyderabad, Hyderabad on mass spectrometer coupled with DAD-UV detector of Agilent 1200 series (Agilent, Santa Clara, CA) that was equipped with Agilent Technologies 6520 with Accurate Mass Q-TOF mode. Instrument control, data acquisition and processing were performed using Mass Hunter workstation software (Qualitative Analysis, version B.03.01, Agilent). Zorbax XDB- C18 column with rapid resolution of 1.8 µm, 4.6 × 50 mm was

used. Detection of peaks were performed by HPLC-PDA (photo decode array detector) with UV absorbance at λ 250, 260, 272, 280, 300 nm in addition to the detection by MS.

❖ LC–MS/MS AND LC–DAD CONDITIONS AND PARAMETERS:

The determination of active compounds was performed by positive mode ESI-MS/MS since higher ion intensities were observed using positive as compared to negative mode. To characterize the fragment ions of the investigated compounds, an electrospray interface with good sensitivity, fragmentation and linearity was optimized. The analyses were performed using binary gradients of Milli-Q water: mobile phase-(A) 5mM ammonium formate in 0.1% (v/v) formic acid and (B) 100% (v/v) HPLC grade acetonitrile with the following elution profile; from 0 min: 35% (B) in (A); 1 min: 35% (B) in (A); 25 min: 90% (B) in (A); 29 min: 98% (B) in (A); 40 min: 35% (B) in (A). Separation was carried out at a flow rate of 0.2 mL/min and the injection volume 2 µL/sample. Mass spectra of the column eluate were recorded in the range m/z 100-1700. The instrument was operated with a capillary voltage of 3500 V and a nozzle voltage of 4 KV with scan rate 1.4. Helium was used as a nebulizer gas at 35 psi, a carrier gas of 8 L/min at 325^{o}C. MS data were acquired in both positive and negative modes for the accurate determination of the m/z of the parent ions. MS/MS data were acquired in negative ionization mode to obtain the m/z of the fragmented ion. The chromatographic data were processed using Dictionary of Natural Products on DVD software (CRC Press, Taylor and Francis Group, www.netbeans.org) Mass hunter software (Agilent, Santa Clara, USA) and Mass Bank. The MS signals were used only for qualitative analysis based on specific mass spectra of each compound. The MS spectra obtained from each fraction were integrated in a mass spectra library. Later the MS traces/spectra of the analysed samples were compared to the spectra from library which allowed positive identification of the compounds, based on the spectra match [Horai et al., 2010].

2.12. CALCULATIONS AND STATISTICAL ANALYSIS:

The results were expressed as mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in triplicate. IC_{50} value (the concentration of the extracts required to scavenge/inhibit 50% of radicals/cell growth) was calculated for different extracts/fractions of selected plants. The statistical calculations were performed using GraphPad Prism Version 6. Statistical differences between the samples were evaluated using appropriate statistical tests (one-way ANOVA, repeated measures ANOVA, Turkey's

and Dunnett's tests). A *p*-values of ≤ 0.05, ≤ 0.01 and ≤ 0.001 were considered significant where probability values were found to be equal to or less than 0.05. The graphical representation of the results were performed using GraphPad Prism Version 6.03 (San Diego, USA) and Sigmaplot version 11.0 (USA) software's.

Chapter III:

Results and Discussion

3.1. YIELD OF PLANT EXTRACTIONS (GRAM %):

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals for the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical and cosmetic products. The extractive yield obtained from four different plants using solvents of various polarities (*n*-hexane, ethyl acetate, methanol and water) is presented in Table 3.1. A wide range of the yields among extracts was observed showing considerable variations ($p<0.001$) depending on the extraction solvent and the plant material used. All extracts of selected plants were yellow to yellowish brown in colour. The extractive yield in methanol was an extremely efficient extractant in all the four plants followed by ethyl acetate. Results for the total extract yields reported as gram percentage. *A. precatorius* on dry basis indicated that the methanol gave the highest total extract yield (16.62±1.01%), followed by ethyl acetate (9.22±0.59%), *n*-hexane (4.74±0.04%) and water (2.83±0.18%). In case of *C. periera*, similar trend was observed where the maximum yield was obtained for the methanol solvent (17.58±0.40%) followed by ethyl acetate (13.85±0.92%), while water extract yielded the least yield of 2.71±0.49%. Similarly, in *C. hirsutus*, the plant material extracted with methanol also gave maximum yield of extract (18.16±2.12%) followed by ethyl acetate (13.75±0.49%). The *n*-hexane extract was found to be least efficient in the extraction (6.42±0.59. The extraction yields of *A. nilagirica* was found to be ranging between 4.95±0.30% to 21.07±1.04%. Water extract gave maximum yield of 21.07±1.04% followed by methanol (13.63±0.18%). The *n*-hexane and ethyl acetate solvents gave lower extraction yields; 6.97±0.52% and 4.95±0.30% respectively. Overall, the extraction yields with different solvents varied significantly and can be ranked from high yield to low yield in the following order: methanol extract > ethyl acetate extract > *n*- hexane extract > water extract for three plants; *A. precatorius, C. pereira* and *C. hirsutus*. However, in case of *A. nilagirica*, the extraction yield was in the order of water extract > methanol extract > hexane extract > ethyl acetate extract.

Extraction method plays an important role in separation and characterization of different phytochemicals from herbs, and screening of plant extracts for novel leads. Solvent extraction is the most frequently used procedure for preparation of extracts from plant materials due to their ease of use, efficiency, and wide applicability. It is generally known that the extract yield and resulting biological activities of the plant materials are strongly

Table 3.1: Percentage yield of successive extracts of selected medicinal plants

Plant	*Extract*	Method of extraction	*Colour*	*Physical nature*	*Yield (%)*
Abrus precatorius	*n*-Hexane (APH)	Successive solvent extraction in Soxhlet apparatus	*Green*	*Semisolid*	4.74±0.04^{ab}
	Ethyl acetate (APE)		*Greenish brown*	*Semisolid*	9.22±0.59^{ab}
	Alcohol (APA)		*Yellowish green*	*Solid*	16.62±1.01^{a}
	Water (APW)		*Brownish green*	*Solid*	2.83±0.18^{b}
Artemisia nilagirica	*n*-Hexane (APH)		*Green*	*Semisolid*	6.97±0.52^{ab}
	Ethyl acetate (APE)		*Greenish brown*	*Semisolid*	4.95± 0.30^{b}
	Alcohol (APA)		*Yellowish green*	*Solid*	13.63±0.18^{ab}
	Water (APW)		*Brownish green*	*Solid*	21.07±1.04^{a}
Cissampelos pereira	*n*-Hexane (APH)		*Green*	*Semisolid*	4.14±0.97^{ab}
	Ethyl acetate (APE)		*Greenish brown*	*Semisolid*	13.85±0.92^{ab}
	Alcohol (APA)		*Yellowish green*	*Solid*	17.58±0.40^{a}
	Water (APW)		*Brownish green*	*Solid*	2.71±0.49^{b}
Cocculus hirsutus	*n*-Hexane (APH)		*Green*	*Semisolid*	6.42±0.59 ^{ab}
	Ethyl acetate (APE)		*Greenish brown*	*Semisolid*	13.75±0.49 ^{ab}
	Alcohol (APA)		*Yellowish green*	*Solid*	18.16±2.12^{a}

Results are means ± SD of triplicate measurements. ***a****-Highest yield and significantly different (p <0.001) from other yields (ab & b) of each row.* ***ab****- significantly different (p <0.001) from b only. Extract yields with identical alphabets show no significant difference (p <0.05).*

dependent on the nature of extracting solvents (polarity), extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples [Shabir et al. 2011; Sultana et al. 2009]. The solubility of phyto-constituents is governed by the chemical nature of the plant sample, as well as the polarity of the solvents used [Dai and Mumper, 2010]. Plant crude extracts usually contain large amounts of carbohydrates and/or lipoidal material and the concentration of the phenolics in the crude extract may be low. To overcome this problem and obtain a concentrated, polyphenol-rich extract/fraction before analysis, strategies including sequential extraction based on polarity have been commonly used. Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. In the sequential extraction, solvents, such as methanol, ethanol, ethyl acetate, acetone, chloroform and their combinations are suitable solvents and have been used for the extraction of therapeutically desired constituents from plant materials, often with different proportions of water to get better extraction efficiency. Selecting the right solvent affects the amount and rate of extraction of polyphenols [Peschel et al., 2006; Xu and Chang, 2007]. Earlier studies have demonstrated that methanol/ethanol and ethyl acetate have been generally found to be more efficient in extraction of lower molecular weight polyphenols due to their significant selectivity in respect of natural products [Abdille et al., 2005; Li et al., 2006; Prior et al., 2001; Rehman, 2006; Shabir et al. 2011; Shi et al., 2005; Sultana et al., 2009; Wang et al., 2011]. In our study, extraction yield using four different solvents showed the following order: methanol > ethyl acetate > *n*-hexane > water and significant differences ($p<0.001$) were found among them. The largest yield obtained by methanol/ethanol and ethyl acetate solvents with intermediate polarity may be probably due to high temperature and solvent recycle in soxhlet method, which contribute to increase solubilisation of components from raw material. *n*-hexane and water showed lower extraction yield in case of *A. precatorius*, *C. pereira* and *C. hirsutus* plants because water has affinity with polar compounds, the –OH group turns water in to a bad solvent for organic compounds. Although water presents higher polarity index than all other solvents used in soxhlet but the yield was lower, because the polarity is not the only factor affecting the extraction efficiency, and it is important to understand the different interactions between solute and solvent [Almeida et al., 2012]. The differences in the extract yields from the selected plant materials in the present analysis might be ascribed to the different availability of extractable components, resulting from the varied chemical

composition of plants [Hsu et al., 2006]. Our findings were in agreement with previous investigations where it was shown that maximum extract yield were obtained with methanol and ethyl acetate [Abdille et al., 2005; Li et al., 2006; Peschel et al., 2006; Rehman, 2006].

3.2. PHYTOCHEMICAL ANALYSIS OF PLANT EXTRACTS:

3.2.1. DETERMINATION OF TOTAL PHENOLIC CONTENT:

The phytochemical active constituents (TPC and TFC) of four selected medicinal plants were quantitatively analysed for different extracts based on the absorbance values after reaction with Folin-Ciocalteu reagent. The results are presented in Table 3.2 and Fig. 3.1A. Total polyphenolic content in the different extracts of plants expressed as mg GAE/g dw varied significantly with the solvent used. The results of total phenolic content of different *A. precatorius* extracts indicated that APW possessed an abundance of phenolics amounting to 25.48±0.62 mg GAE/g dw, while GAE value of APE extract was 23.57±0.31 mg/g dw. APA and APH showed less amount of GAE at 7.44±0.10 mg/g dw and 1.65±0.22 mg/g dw respectively. In *A. nilagirica*, the total phenol content was found in the range of 0.44±0.04 to 12.13±0.20 mg of GAE/g dw. ANA had the highest content as 12.13±0.20 mg GAE/g dw, whereas ANH contained least amount of phenolics as 0.44±0.04 mg GAE/g dw. The ANW and ANE showed moderate amount of phenolic content (4.00±0.03 and 3.52±0.09 mg GAE/g dw), respectively. Total phenolic contents of *C. pereira* extracts varied from 2.44±0.05 mg GAE/g dw to 10.14±0.14 mg GAE/g dw. Among the extracts, the highest amount of total phenolics was observed in CPE (10.14±0.14 mg GAE/g dw), whereas CPH showed least (2.44±0.05 mg GAE/g dw) values for phenolics. The other two extracts, CPW and CPE possessed intermediate contents of total phenolics (7.00±0.18 and 6.09±0.15 mg GAE/g dw respectively). The total phenolics content of the *C. hirsutus* extracts was found in the order of CHA>CHE>CHH. A higher quantity was found in CHA (20.67±0.27 mg GAE/g dw), whereas, CHH contained least polyphenols (1.73±0.22 mg GAE/g dw) among the other extracts of this plant.

The total flavonoid content of various extracts of selected plants obtained with different solvent polarities expressed in terms of mg quercetin equivalents/g dw is presented in Table 3.2 and Fig 3.1B. In *A. precatorius*, the flavonoid contents of extracts were between 6.20±0.11 and 17.16±1.04 mg QE/g dw. It was noticed that APE showed higher total flavonoid content than other three extracts (17.16±1.04 mg QE/g dw) followed by APW

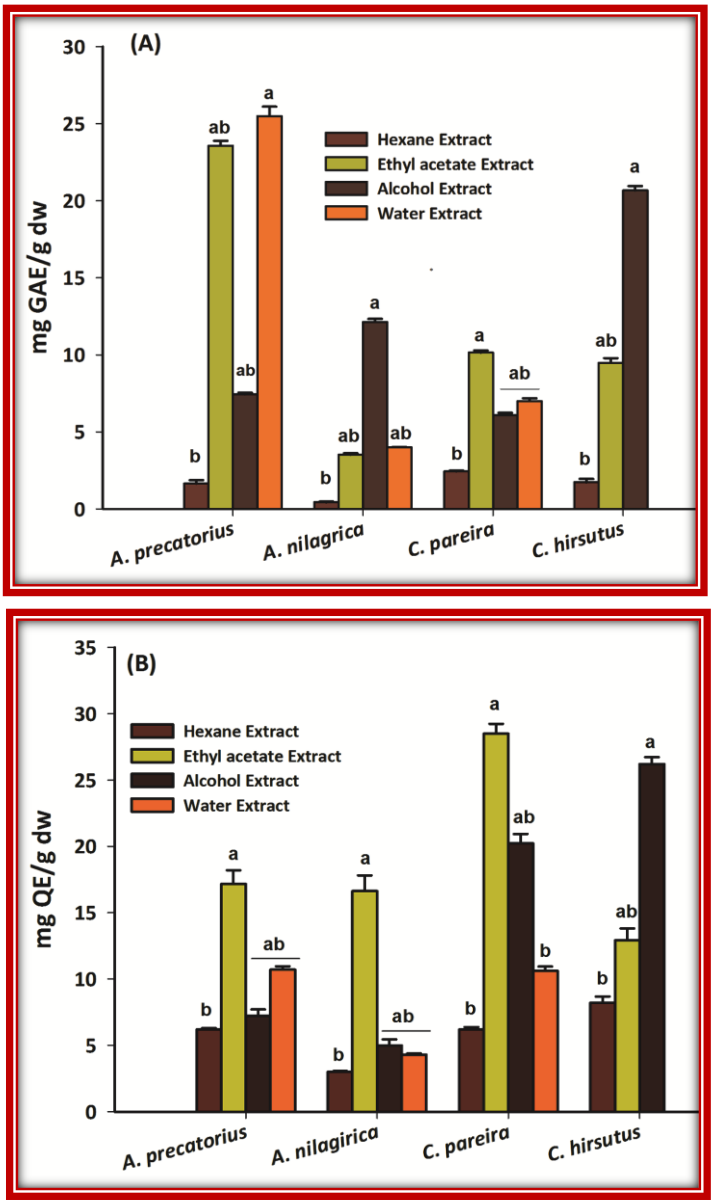


Fig. 3.1: Quantitative estimation of phytochemical constituents from different extracts of selected plants. **(A)** Total phenolics **(B)** Total flavonoid content. Each value is expressed as a mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in triplicate. **a**-Highest value and significantly different ($p \leq 0.05$) from other values (**ab** and **b**) of each row; **ab**- significantly different ($p \leq 0.05$) from **b** only. Values with identical alphabets show no significant difference ($p < 0.05$).

and APA extracts. The least amount was recorded in APH as 6.20±0.11 mg QE/g dw. For *A. nilagirica* extracts, the flavonoid content varied between 3.02±0.05 and 16.63±1.18 mg QE/g dw, highest being in ANE (16.63±1.18 mg QE/g dw) and lowest in ANH (3.02±0.05 mg QE/g dw). The other two extracts, ANA and ANW showed moderate values for total flavonoid content (5.0±0.46 and 4.30±0.10 mg QE/g dw). The extracts of *C. pereira* possessed varied

amounts of total flavonoid content in terms of mg QE/g dw. The CPE showed highest flavonoid content (28.49±0.75 mg QE/g dw) followed by CPA (20.24±0.69 mg QE/g dw), CPW (10.62±0.32 mg QE/g dw) and CPH (6.92±0.19 mg QE/g dw). Among the different extracts of *C. hirsutus;* CHA possessed significant amount of flavonoid content (26.20±0.52 mg QE/g dw) followed by CHE (12.92±0.90mg QE/g dw). The least quantity was observed in the extract of CPH with the value of 8.21±0.47 mg QE/g dw.

Phenolic compounds are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins [Baxter et al., 1999]. They have been considered the most important group of compounds in the plant kingdom. They are generally involved in defence against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as contributing to plants' colours. They are ubiquitous constituents of plant foods (fruits, vegetables, cereals, legumes, etc.) and beverages (tea, coffee, beer, wine, etc.) [Naczk and Shahidi, 2004]. The growing interest in these substances is mainly because of their antioxidant potential and the association between their consumption and the prevention of some diseases. Over the past decade or so, numerous experimental and epidemiological studies have shown that these compounds are able to prevent or slow down oxidative stress induced damage leading to carcinogenesis by upsetting the molecular events in the initiation, promotion or progression conditions [Arts and Hollman, 2005; Cole et al., 2005; Hertog et al., 1994; Rasmussen et al., 2005]. In the last few years, great attention has been paid to the plant phenolic compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases like cancer and diabetes, reduction in risk factors of cardiovascular diseases, antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory, and anti-microbial effects among others [Martins et al., 2011]. Due to these countless beneficial characteristics for human health, researches have been intensified aiming to find fruits, vegetables and plants, agricultural and agro-industrial residues as sources of bioactive phenolic compounds. Therefore, it was worthwhile to evaluate the different extracts of selected medicinal plants for their phytochemical (TPC and TFC) contents to understand the healing property of these medicinal herbs. Our results have shown the presence of differential amount of phyto-constituents in different extracts of selected plants. The phenolic content (TPC and TFC) in

different extracts indicated significant variation among the selected plants and was found to be solvent dependent. The results showed that ethyl acetate, alcohol and water extracts possessed significantly high ($p<0.001$) phyto-constituent content than the non-polar extracts (*n*-hexane) in all the four plants under study. Variation in phenolic content (TPC and TFC) in the extracts may be attributed to the polarities of different solvents. This suggested that polar solvents are efficient solvents for the extraction and have better solubility for polyphenol and flavonoid type of constituents in the selected plants.

3.3. ANTIOXIDANT ABILITY ASSAYS:

3.3.1. PHOSPHOMOLYBDENUM ASSAY:

Total antioxidant activity of different plant extracts were determined by phosphomolybednum method which is based on the reduction of Mo (VI) -Mo (V) by the antioxidant compounds and subsequent formation of green phosphate/Mo (V). The results were expressed as mg of ascorbic acid equivalents per g (mg AAE/g) of the dried extract. All the extracts from selected plants exhibited varied degrees of activity as shown in Table 3.3 and Fig. 3.2A. In *A. precatorius*, the antioxidant activity was in the range of 7.16±0.43 to 24.73±0.72 mg AAE/g dw. APA had higher antioxidant capacity (24.73±0.72 mg AAE/g dw) than other three extracts which showed antioxidant capacity in the order of: APE (17.92±0.38 mg AAE/g dw) > APW (16.66±0.68 mg AAE/g dw) > APH (7.16±0.43 mg AAE/g dw). The results showed that the extracts of *A. nilagirica* possess antioxidant potential but discrepancies were noticed in the extracts of different polarity. The results obtained exhibited that ANE had higher antioxidant capacity (15.92±0.44 mg AAE/g dw) among the four extracts. The ANA and ANW displayed moderate activities in this experiment, whereas, ANH displayed least antioxidant activity (0.49±0.97 mg AAE/g dw). The extracts derived from *C. pereira* showed various degrees of antioxidant activity in the experimental conditions and the activity was in the range of 8.72±0.13 to 30.52±1.07 mg AAE/g dw. Among the four extracts from this plant, CPE revealed maximum antioxidant capacity (30.52±1.07 mg AAE/g dw). CPA also showed strong antioxidant activity close to CPE with the value of 27.43±0.61 mg AAE/g dw. The CPW showed moderate potential (18.06±0.14 mg AAE/g dw) compared to CPE and CPA, whereas least activity was observed in CPH extract (8.72±0.13 mg AAE/g dw). In *C. hirsutus;* CHA and CHE exhibited higher antioxidant capacity 46.35±0.69 and

37.24±1.14 mg AAE/g dw, respectively. The CHH showed least antioxidant activity (12.37±0.11 mg AAE/g dw).

3.3.2. FERRIC REDUCING ABILITY/ANTIOXIDANT POWER (FRAP) ASSAY:

The ability of plant extracts obtained from investigated plants to reduce ferric ions was determined by FRAP assay. Table 3.3 and Fig. 3.2B shows differences in total antioxidant capacity measured by the FRAP method between the examined plants. In *A. precatorius*, all the four extracts showed some degree of electron donating capacity and reduced Fe^{3+} to Fe^{2+}. The reducing ability of the extracts was in range of 2.67± 0.40 to 13.34±0.35 mg AAE/g dw. The FRAP values for APW (13.34±0.35 mg AAE/g dw) was found to be higher than the other three extracts followed by APA and APE (10.28±0.47 and 8.45±0.65 mg AAE/g dw respectively) while the least values were observed for APH (2.67±0.40 mg AAE /g dw). Different extracts of varied polarities from *A. nilagirica* also exhibited varied degree of reducing power potential. Among the extracts, ANA possessed the highest values of FRAP (4.43±0.11 mg AAE/g dw) followed by the APE and APW (3.69±0.13 and 1.94±0.03 mg AAE/g dw respectively). The least value was observed for ANH with the measurement of 1.27±0.02 mg AAE/g dw. The reducing power of *C. pereira* extracts ranged from 3.40±0.12 to 16.81±0.55 mg AAE/g dw. The CPA displayed highest reducing activity (16.81± 0.55 mg AAE/g dw), followed by CPE and CPW with the values of 14.65±0.32 and 11.41±0.40 mg AAE/g dw, respectively. In case of *C. hirsutus*, the reducing power of CHA was the highest amongst extracts, with 13.75±0.32 mg AAE/g dw, followed by CHE (10.46±0.41 mg AAE/g dw), whereas that of CHH showed least reducing power of 3.21±0.16 mg AAE/g dw.

The different extracts of selected medicinal plants were screened to unravel their antioxidant potential. The antioxidant capacity of the plant extracts largely depends on both the composition of the extract and the test system. It can be influenced by a large number of factors and cannot be fully evaluated by one single method. It is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action [Frankel and Meyer, 2000]. There are many methods to determine antioxidant capacity. These methods differ in terms of their assay principles and experimental conditions; consequently, in different methods particular antioxidants have varying contributions to total antioxidant potential [Cao and Prior, 1998]. In this study, the antioxidant capacities of selected medicinal plant extracts were evaluated by employing two

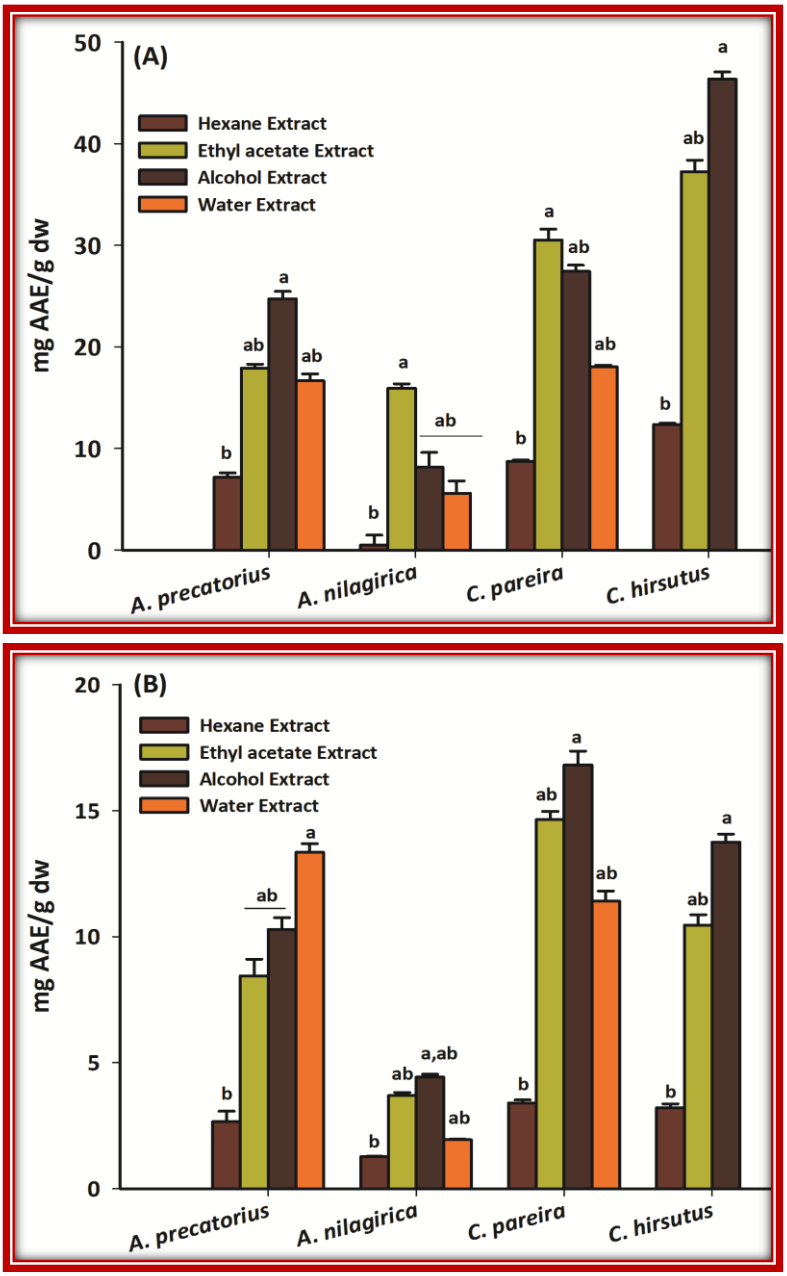


Fig. 3.2: Evaluation of antioxidant potential **(A)** Phosphomolybednum assay **(B)** Ferric reducing power (FRAP) of different extracts of selected medicinal plants. Each value is expressed as a mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in triplicate. a-Highest value and significantly different ($p \leq 0.05$) from other values (ab and b) of each row; ab- significantly different ($p \leq 0.05$) from b only. Values with identical alphabets show no significant difference ($p < 0.05$).

widely used methods; phosphomolybednum and FRAP assays. These methods are sensitive and appropriate to measure the total antioxidant capacity and state of medicinal herbs if they can be used in phytotherapy [Prieto et al., 1999; Szollosi and Varga, 2002]. In phosphomolybdenum assay which is a quantitative method (since the total antioxidant

Table 3.2: Quantitative estimation of phytochemical constituents from different extracts of selected medicinal plants

Plant Name	Total Phenolic Content (mg of GAE/g dw)				Total Flavonoid Content (mg of QE/g dw)			
	Type of extracts							
	Hexane	Ethyl acetate	Alcohol	Water	Hexane	Ethyl acetate	Alcohol	Water
Abrus precatorius	1.65±0.22 [b]	23.57±0.31 [ab]	7.44±0.10 [ab]	25.48±0.62 [a]	6.20±0.11 [b]	17.16±1.04 [a]	7.23±0.48 [b]	10.70±0.26 [ab]
Artemisia nilagirica	0.44±0.04 [b]	3.52 ±0.09 [ab]	12.13±0.20 [a]	4.00±0.03 [ab]	3.02±0.05 [b]	16.63±1.18 [a]	5.0±0.46 [ab]	4.30±0.10 [b]
Cissampelos pareira	2.44±0.05 [b]	10.14±0.14 [a]	6.09±0.15 [ab]	7.00±0.18 [ab]	6.92±0.19 [b]	28.49±0.75 [a]	20.24±0.69 [ab]	10.62±0.32 [ab]
Cocculus hirsutus	1.73±0.22 [b]	9.48±0.30 [ab]	20.67±0.27 [a]	NA	8.21±0.47 [b]	12.92± 0.90 [ab]	26.20±0.52 [a]	NA

Each value is expressed as a mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in triplicate. **a**-Highest value and significantly different (p≤0.05) from other values (**ab** and **b**) of each row; **ab**- significantly different (p≤0.05) from **b** only. Values with identical alphabets showed no significant difference (p<0.05).

Table 3.3: Quantitative estimation of antioxidant potential of different extracts of selected medicinal plants

Plant Name	Total Antioxidant Capacity (mg of AAE/g dw)				Ferric Reducing Power (mg of AAE/g dw)			
	Type of extracts							
	Hexane	Ethyl acetate	Alcohol	Water	Hexane	Ethyl acetate	Alcohol	Water
Abrus precatorius	7.16± 0.43 [b]	17.92±0.38 [ab]	24.73±0.72 [a]	16.66±0.68 [ab]	2.67± 0.40 [b]	8.45±0.65 [ab]	10.28±0.47 [ab]	13.34±0.35 [a]
Artemisia nilagirica	0.49±0.97 [b]	15.92±0.44 [a]	8.16±1.45 [ab]	5.58±1.23 [ab]	1.27±0.02 [b]	3.69±0.13 [b]	4.43±0.11 [b]	1.94±0.03 [b]
Cissampelos pareira	8.72±0.13 [b]	30.52±1.07 [a]	27.43±0.61 [ab]	18.06±0.14 [ab]	3.40±0.12 [b]	14.65±0.32 [ab]	16.81±0.55 [a]	11.41±0.40 [ab]
Cocculus hirsutus	12.37±0.11 [b]	37.24±1.14 [ab]	46.35±0.69 [a]	NA	3.21±0.16 [b]	10.46±0.41 [ab]	13.75±0.32 [a]	NA

Each value is expressed as a mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in triplicate. **a**-Highest value and significantly different (p≤0.05) from other values (**ab** and **b**) of each row; **ab**- significantly different (p≤0.05) from b only. Values with identical alphabets showed no significant difference (p<0.05).

capacity, antioxidants reduce molybdenum (VI) to green coloured molybdenum (V) complex. The molybdenum (V) complex shows absorption maxima at λ 695 nm [Prieto et al., 1999]. The reducing ability of a compound generally depends on the presence of reductants which have exhibited anti-oxidative potential by breaking the free-radical chain, donating a hydrogen atom [Duh et al., 1999; Gordon, 1990]. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Perl's Prussian blue at λ 700 nm, it is possible to determine the Fe^{2+} concentration. In the present study, most of the extracts obtained from selected medicinal plants showed appreciable antioxidant activity. Our results showed that ethyl acetate, alcohol and water extracts had substantial ability to convert Fe^{3+} into Fe^{2+} ($p<0.05$) that is, capability to donate hydrogen atom, a property of antioxidants and that could be attributable to the presence of phenolic compounds. However, the difference in antioxidant capacity of different extracts may be attributed to differences in their chemical composition. The antioxidant activities of the individual phenolic compounds may depend on structural factors, such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups, and other structural features [Patt and Hudson, 1990]. Recent reports indicated that several bioactive compounds present in plants have strong antioxidant activity [Miguel, 2010]. Earlier reports have also shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging and reducing ability of medicinal plants [Benzie and Szeto, 1999; Khan et al., 2012; Sharififar et al., 2009]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential [Rice-Evans et al., 1995].

3.4. RADICAL SCAVENGING ACTIVITIES:

Based on significant amount of phyto-constituents and total antioxidant potential of extracts, we expected that the extracts would present radical scavenging activities against the several biological reactive species. Keeping in view the effectiveness of antioxidant study, it has been recommended to employ at least three *in vitro* methods due to the variability of values in free radical-scavenging assay systems [Aruoma, 1998; Schlesier et al., 2002]. Thus, the extracts were analysed for radical scavenging activities by a series of

different analytical methods to assess the antioxidant potential of these medicinal plants. The DPPH values for investigated extracts were expressed as percentage inhibition and IC_{50}. A higher DPPH radical scavenging activity was associated with a lower IC_{50} value.

3.4.1. DPPH FREE RADICAL SCAVENGING ACTIVITY:

DPPH (1, 1-diphenyl-2-picrylhydrazyl) analysis is one of the best-known, sensitive, accurate and frequently employed methods to study the antioxidant activity of a specific compound or plant extracts [Zhou and Yu, 2004]. It is a stable free radical because of its spare electron delocalization over the whole molecule. In presence of an active radical scavenger, the absorption vanishes and the resulting decolourization is stoichiometric at a selected range with respect to the degree of reduction. The degree of discoloration of violet colour of DPPH, as it gets reduced, indicates the radical scavenging potential of the antioxidant [Kulisic et al., 2004]. The DPPH scavenging activities of different extracts from four medicinal plants are presented in Table 3.4; Fig. 3.3A - 3.3D. The experimental data revealed that DPPH radical-scavenging activities of different solvent extracts of all selected plants and the positive control increased with increasing concentration, that is, the higher the concentration, the highest scavenging potential.

In *A. precatorius*, DPPH activity values for APE, APA and APW were found to be nearer to each other. The APE and APA were able to inhibit the formation of DPPH• radicals with a percentage inhibition of 96.35±2.98 and 95.14±3.44, respectively at the highest concentration of 400 µg/mL with the IC_{50} values of 57.66±1.32 and 79.97±1.84 µg/mL, respectively. APW also exhibited potent DPPH scavenging activity (92.63±4.63%) and its IC_{50} (60.67±1.03 µg/mL) was closer to that of APE. APH showed less inhibitory action as compared to other extracts with the percentage inhibition of 50.84±5.68 at same concentration with the IC_{50} value of 196.70±2.06 µg/mL (Table 3.4; Fig. 3.3A).

A. nilagirica crude extracts exhibited significant DPPH radical scavenging abilities in a concentration dependent manner (Fig. 3.3B). The ability to scavenge DPPH radicals of various solvent extracts was in the order of ANH (32.55±1.18%) < ANW (63.48±3.35%) < ANE (70.95±1.58%) < ANA (77.11±0.80%) at the concentration of 400 µg/mL. The IC_{50} values showed wide variability, ranging between 266.13±1.21 µg/mL to 713.50±6.23 µg/mL. It was found that ANA was efficient in quenching the DPPH radical expressed as with the lowest IC_{50} value of 266.13±1.21 µg/mL. ANA was followed in this activity by ANE and ANW extracts

Table 3.4: IC_{50} values obtained for different crude extracts of selected plants and reference compounds in various antioxidant assays *in vitro*

Plant Extract/Chemical	IC_{50} values (µg/mL)					
	Name of the radical/anion					
	DPPH$^{\bullet}$	$^{\bullet}OH$	H_2O_2	NO$^{\bullet}$	$O_2^{\bullet-}$	LPO
APH	196.70±2.06^{b}	464.25±4.43^{b}	112.59±3.24^{ab}	192.45±3.76^{ab}	427.26±5.72^{b}	377.07±5.23^{b}
APE	57.66±1.32^{a}	205.51±3.08^{ab}	121.02±3.14^{ab}	107.58±2.12^{a}	143.44±3.28^{a}	45.46±3.71^{a}
APA	79.97±1.84^{ab}	209.33±4.13^{a}	92.83±3.23^{a}	145.96±2.17^{ab}	157.07±2.56^{ab}	285.22±4.63^{ab}
APW	60.67±1.03^{ab}	309.90±5.21^{ab}	152.35±1.06^{b}	264.95±4.24^{b}	201.45±6.23^{ab}	302.02±4.11^{b}
ANH	713.50±6.23^{b}	345.06±9.11^{b}	193.23±2.34^{b}	209.20±7.33^{b}	609.99±8.12^{b}	297.35±5.78^{ab}
ANE	305.25±1.32^{ab}	247.52±8.71^{ab}	115.12±3.67^{ab}	257.30±6.78^{b}	330.32±7.12^{a}	229.64±7.89^{ab}
ANA	266.13±1.21^{a}	335.51±3.91^{b}	88.23±5.11^{a}	264.60±1.71^{b}	509.94±1.71^{ab}	451.06±4.95^{b}
ANW	337.95±4.21^{ab}	232.02±7.41^{ab}	187.67±2.15^{b}	258.09±5.43^{b}	536.12±4.33^{ab}	236.21±9.00^{ab}
CPH	425.53±0.33^{b}	374.11±0.94^{b}	100.18±1.32^{ab}	175.25±0.95^{ab}	580.88±0.35^{b}	178.77±0.28^{a}
CPE	113.65±0.52^{a}	241.50±2.65^{ab}	75.79±1.63^{a}	142.27±1.23^{ab}	150.85±0.52^{a}	209.20±0.81^{ab}
CPA	149.03±0.44^{ab}	255.05±1.08^{ab}	102.82±1.65^{ab}	85.98±0.56^{a}	142.92±0.38^{a}	230.55±0.76^{ab}
CPW	161.32±0.48^{ab}	182.36±1.13^{a}	314.07±0.77^{b}	284.40±1.10^{b}	444.84±0.46^{ab}	288.36±3.23^{ab}
CHH	174.04±1.75^{b}	429.18±1.55^{b}	100.68±1.32^{a}	168.31±2.38^{b}	387.37±1.23^{b}	433.84±1.30^{b}
CHE	56.33±0.88^{ab}	312.87±1.22^{ab}	99.21±1.20^{a}	101.16±0.37^{ab}	163.43±1.65^{b}	264.50±1.30^{ab}
CHA	35.21±0.37^{a}	205.13±1.69^{a}	60.61±0.90^{a}	109.58±2.0^{ab}	115.85±1.76^{a}	227.19±1.40^{ab}
Ascorbic acid	6.86±0.92	62.40±3.72	245.30±4.60	19.90±2.30	32.86±3.78	48.72±3.20
Quercetin	14.34±1.64	--	--	21.09±2.16	--	--

Each value is expressed as a mean ± standard deviation (n = 3). Values in the same column followed by a different letter ($^{a, ab}$) are significantly different ($p< 0.05$).' --' not determined. **a**-Highest value and significantly different ($p\leq0.05$) from other values (**ab** and **b**) of each row; **ab**- significantly different ($p\leq0.05$) from b only. Values with identical alphabets showed no significant difference ($p<0.05$).

(305.25±1.32 and 337.95±4.21 µg/ml respectively). The ANH was a weak scavenger of the DPPH radical with highest IC_{50} value among the extracts (713.5±6.23 µg/mL) (Table 3.4).

According to the results established for *C. pereira*, it is found that all the extracts exhibited potent or moderate scavenging activities against DPPH model (Fig. 3.3C). At a concentration of 400 µg/mL, the DPPH radical-scavenging activity of CPE was the highest, with the percentage inhibition of 96.02±1.45 and an IC_{50} value of 113.65±0.52 µg/mL and proved to be more effective scavenger of DPPH radicals. At the same concentration, the CPA (94.35±1.20%) and CPW (95.26±0.19%) also presented similar activity but their IC_{50} values 149.03±0.44 and 161.32±0.48 µg/mL respectively) were on little higher side than CPE. CPH showed the lowest activity (47.00±0.33%) among the extracts, as it required much higher concentrations to reduce 50% of free-radical concentrations (IC_{50}=425.53±0.33 µg/mL) (Table 3.4).

C. hirsutus extracts also showed varied degrees of scavenging activity in a concentration dependent manner. The IC_{50} values for extracts ranged between 35.21±0.37 to 174.04±1.75 µg/mL. The results revealed CHA was most active free radical scavenger and exhibited 99.16±0.16% decrease at a concentration of 400 µg/mL with IC_{50} of 35.21±0.37 µg/mL, followed by CHE with the decrease of 97.35±0.48% at the same concentration thereby providing an IC_{50} value of 56.33±0.88 µg/mL. The CHH exhibited relatively less activity with the decrease of DPPH radical by 52.81±0.88% and presenting IC_{50} value of 174.04±1.75 µg/mL compared to other two extracts (Table 3.4; Fig. 3.3D).

The positive control, ascorbic acid and quercetin showed maximum scavenging effects offering lowest IC_{50} values of 6.86±0.92 and 14.34±1.64 µg/mL respectively. The differences in the $DPPH^{\bullet}$ radical scavenging activity among the different extracts of different plants were statistically significant ($p<0.05$). The results of DPPH scavenging activity revealed that ethyl acetate, alcohol and water which are polar solvent extracts, were more effective antioxidants compared to non-polar hexane extracts. This was observed by comparing the IC_{50} values. In fact, the IC_{50} values of the ethyl acetate, alcohol extracts were significantly lesser than that of water and hexane extracts. This behaviour can be explained by the different composition of each extract, since there are compounds that react quickly with the DPPH and others that have a slower reaction mechanism [Tsimogiannis and Oreopoulou, 2004]. Furthermore, this could be explained on the basis of the similarity between compounds with high antioxidant activity extracted by these organic solvents. Many other

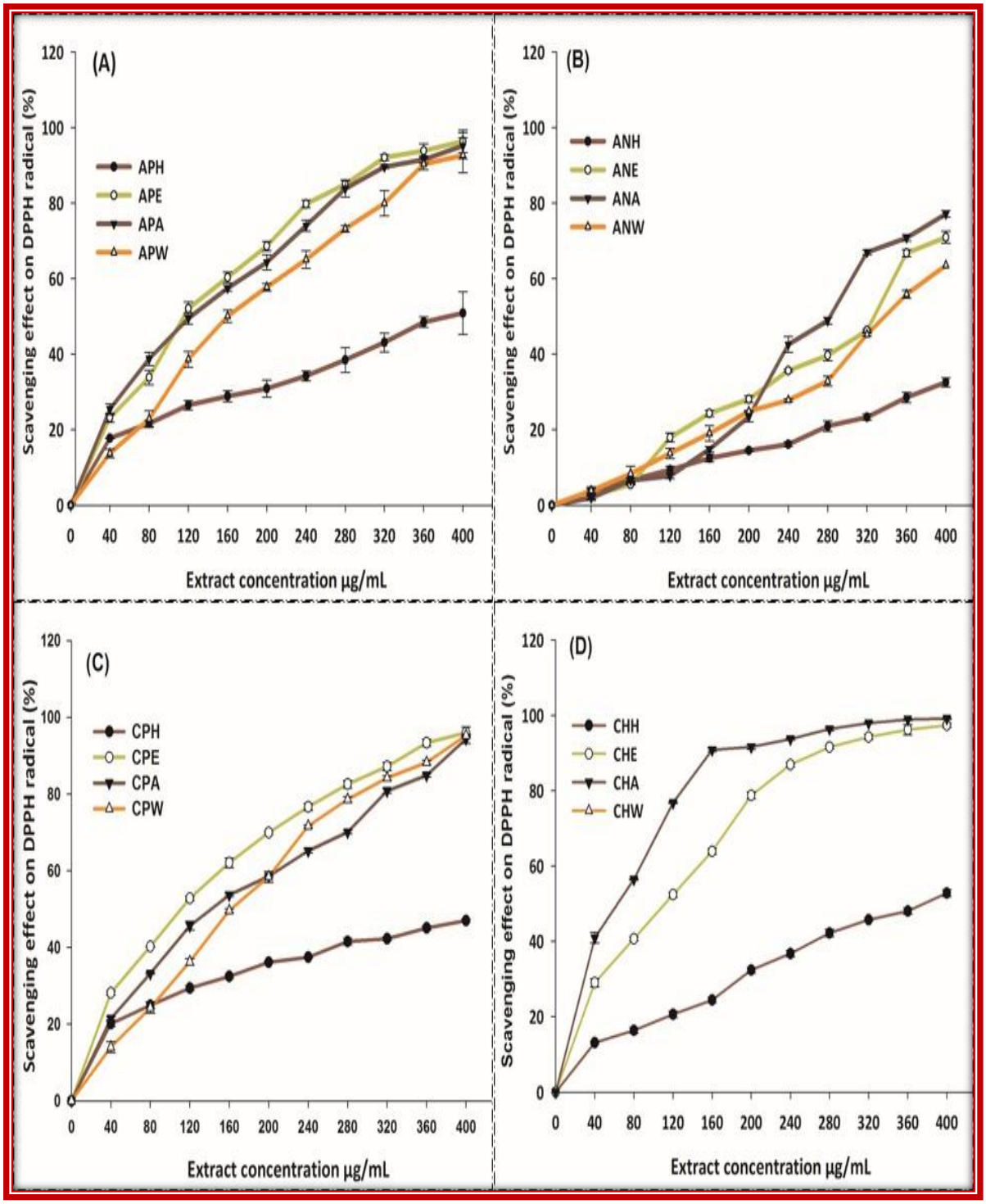


Fig. 3.3: DPPH radical scavenging activities of different extracts from 4 selected medicinal plants at various concentrations. Each value represents a mean ± SD (n = 3).
(A): *Abrus precatorius*; **(B)**: *Artemisia nilagirica*; **(C):** *Cissampelos pereira* and **(D):** *Cocculus hirsutus*.

studies have demonstrated that ethyl acetate and alcohol extracts/fractions are good sources of antioxidant compounds [Irshad et al., 2012; Schubert et al., 2007; Tung et al., 2009; Turkmen et al., 2006]. The ethyl acetate and alcohol extracts exhibited higher phenolic contents in this study, and these extracts showed potent free radical scavenging activity on DPPH. This indicated that DPPH radical-scavenging activities of extracts from selected plants were related to the amount of antioxidant phyto-constituents extracted by various solvents. Several authors have described a positive correlation between phenolic content and antioxidant activity using similar assay systems [Adedapo et al., 2008; Chandra and Mejia, 2004; Shyamala et al., 2005; Tung et al., 2007].

3.4.2. HYDROXYL RADICAL SCAVENGING ACTIVITY:

The effect of different extracts on the inhibition of free radical-mediated deoxyribose damage was assessed by means of hydroxyl radical scavenging assay. Table 3.4; Fig. 3.4A - 3.4D presents the results of the effects of examined various solvent extracts of selected plants as well as known antioxidant (ascorbic acid) on $^{\bullet}OH$ radical production.

A significant decrease in concentration of $^{\bullet}OH$ was observed due to *A. precatorius* extracts. All the four extracts exhibited significant activity, above 40% in a concentration - dependent manner with maximal inhibition of 79.52±2.57% at 400 µg/mL by APE with an IC_{50} value of 205.51±3.08 µg/mL. APA (78.97±1.60%; IC_{50} = 209.33±4.13 µg/mL) and APW (68.18±3.14%; IC_{50}=309.90±5.21 µg/mL) extracts were also found to be significant quenchers of $^{\bullet}OH$ radicals. APH was weak scavenger of $^{\bullet}OH$ radicals by inhibiting the concentration up to 43.08±1.66% with the IC_{50} value of 464.25±4.43 µg/mL (Table 3.4; Fig. 3.4A)

The extracts of *A. nilagirica* were found to possess concentration dependent scavenging activity on $^{\bullet}OH$. The inhibition percentage ranged between 53.74±0.95% to 85.55±1.14%. Concentrations of ANW, ANE, ANA and ANH required for 50% inhibition were 232.02±7.41; 247.52±8.71; 335.51±3.91 and 345.06±9.11 µg/mL respectively. ANW was found to be more potent with the percentage inhibition of 85.55±1.14% compared to other extracts. ANE also quenched the $^{\bullet}OH$ in a significant manner with percentage inhibition reached close to ANW (84.60±0.98%) thereby presenting the IC_{50} value of 247.52±8.71. However, ANA and ANH were weak scavengers compared to ANW and ANE whose IC_{50} values were found to be higher (Table 3.4; Fig. 3.4B).

The *C. pereira* extracts exhibited appreciable scavenging properties against hydroxyl radicals, and the inhibition percentage was proportional to the concentration of each extract. The IC_{50} values were found in the range between 182.36±1.13 and 374.11±0.94 µg/mL. CPW showed the highest activity with the percentage inhibition of 80.75±1.94 at the concentration of 400 µg/mL whose concentration of 182.36±1.13 µg/mL was enough to inhibit 50% of hydroxyl radical-mediated deoxyribose degradation followed by CPE (75.67±1.40%) and CPA (73.67±0.86% at the same concentration presenting IC_{50} values of 241.50±2.65 and 255.05±1.08 µg/mL respectively. The CPH extract showed minimal percentage inhibition of 53.46±1.86 with higher IC_{50} value of 374.11±0.94 µg/mL than other extracts of this plant (Table 3.4; Fig. 3.4C).

The $^{\bullet}OH$ scavenging activity of *C. hirsutus* extracts was also found to be concentration dependent and the percentage inhibition ranged from 46.60±0.76% to 88.20±1.50%. Among the extracts, CHA was found to be most effective $^{\bullet}OH$ scavenger with highest inhibition percentage (88.20±1.50) and lowest IC_{50} value of 205.13±1.69 µg/mL. The other two extracts; CHE and CHH showed moderate scavenging activity with percentage inhibition of 61.56±1.27 and 46.60±0.76, respectively with IC_{50} values of 312.87±1.22 and 429.18±1.55 µg/mL respectively. The scavenging activity obtained for ascorbic acid (standard compound) was found to be significant with an IC_{50} value of 62.40±3.72 µg/mL (Table 3.4; Fig. 2.4D).

Hydroxyl radical is the neutral form of hydroxyl ion and the most reactive free radical in biological systems generated by the Fenton reaction in the presence of reduced transition metals such as Fe^{2+} and H_2O_2. It is known to be extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells. These radical combines with nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis and cytotoxicity [Duan et al., 2007; Halliwell and Gutteridge, 1981; 1984; McCord and Day 1987; Spencer et al., 1994]. Scavenging of $^{\bullet}OH$ is an important antioxidant activity, because of very high reactivity that enabling it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids, and nucleotides [Wang et al., 2008]. Thus, the removal of $^{\bullet}OH$ is, therefore, probably one of the most effective defences of a living body against various diseases. The results of our study showed the abilities of various solvent extracts of selected plants to inhibit $^{\bullet}OH$ mediated deoxyribose degradation in a concentration dependent manner. A significant decrease in concentration of $^{\bullet}OH$ was observed in plant extracts with varied IC_{50}

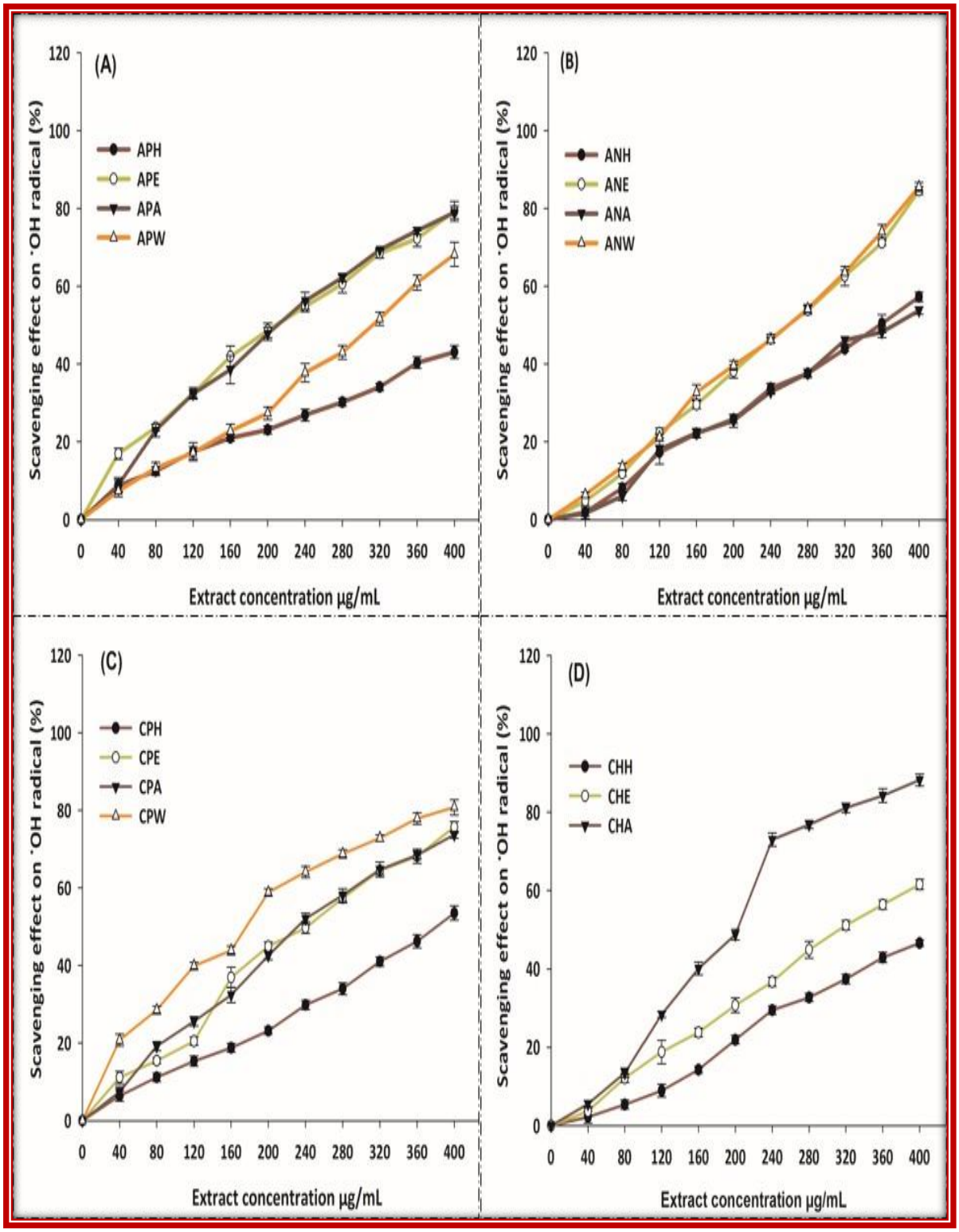


Fig. 3.4: Hydroxyl (•OH) radical scavenging activities of different extracts from 4 selected medicinal plants at various concentrations. Each value represents a mean ± SD (n = 3).
(A): *Abrus precatorius*; **(B)**: *Artemisia nilagirica*; **(C):** *Cissampelos pereira* and **(D):** *Cocculus hirsutus*.

values ($p<0.05$). Ethyl acetate, alcohol and water extracts were capable of scavenging the $^{\bullet}OH$ radicals more effectively than the *n*-hexane extracts of selected plants. The efficient inhibition of $^{\bullet}OH$ by these extracts can be supported by their similarity as potent scavengers of DPPH stable radicals. Our results further suggested that $^{\bullet}OH$ scavenging ability of extracts are comparable to or even higher than earlier published reports [Gul et al., 2011; Gülçin et al., 2007; Sacan et al., 2010]. The reducing properties of plant extracts are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain thereby donating a hydrogen atom [Gordon, 1990].

3.4.3. HYDROGEN PEROXIDE (H_2O_2) SCAVENGING ACTIVITY:

The ability of the different extracts of plants to scavenge hydrogen peroxide was determined and results showed that extracts triggered a significant concentration dependent inhibition of H_2O_2 (Table 3.4; Fig. 3.5A - 3.5D).

The *A. precatorius* extracts showed significant ability of scavenging H_2O_2 in a concentration dependent manner (Fig. 3.5A). Among the different extracts, APA was found to be efficient scavenger of H_2O_2 as the percentage inhibition reached up to 98.94±5.98 at a concentration of 200 µg/mL and presented minimal IC_{50} value of 92.83±3.23 µg/mL. The APH and APE extracts also exhibited comparable efficiency by inhibiting the H_2O_2 to 90.58±1.59% and 87.69±2.27% with an IC_{50} values of 112.59±3.24 and 121.02±3.14 µg/mL respectively. On the other hand, APW was least efficient, as it inhibited the H_2O_2 concentration up to 32.82±.21% at the same concentration, thereby, giving the IC_{50} value of 152.35±1.06 µg/mL) (Table 3.4).

The scavenging effect of extracts of *A. nilagirica* on H_2O_2 was concentration-dependent (20-200 µg/mL). ANA displayed stronger H_2O_2 scavenging activity (IC_{50}=88.23±5.11 µg/mL). The other extracts, ANE also showed similar activity by presenting IC_{50} values close to ANA (115.12±3.67 µg/mL). The ANW demonstrated moderate activity with the IC_{50} value of 187.67±2.15 µg/mL whereas, ANH has shown minimal scavenging ability with the IC_{50} value of 193.23±2.34 µg/mL (Fig. 3.5B).

The *C. pereira* extracts were also capable of scavenging H_2O_2 in an concentration dependent manner (Fig. 3.5C). Results illustrated a significant decrease in the concentration of H_2O_2 due to the scavenging activities of the extracts. The scavenging efficiency increased as follows: CPW<CPA<CPH<CPE. At a concentration of 200 µg/mL, CPE markedly scavenged

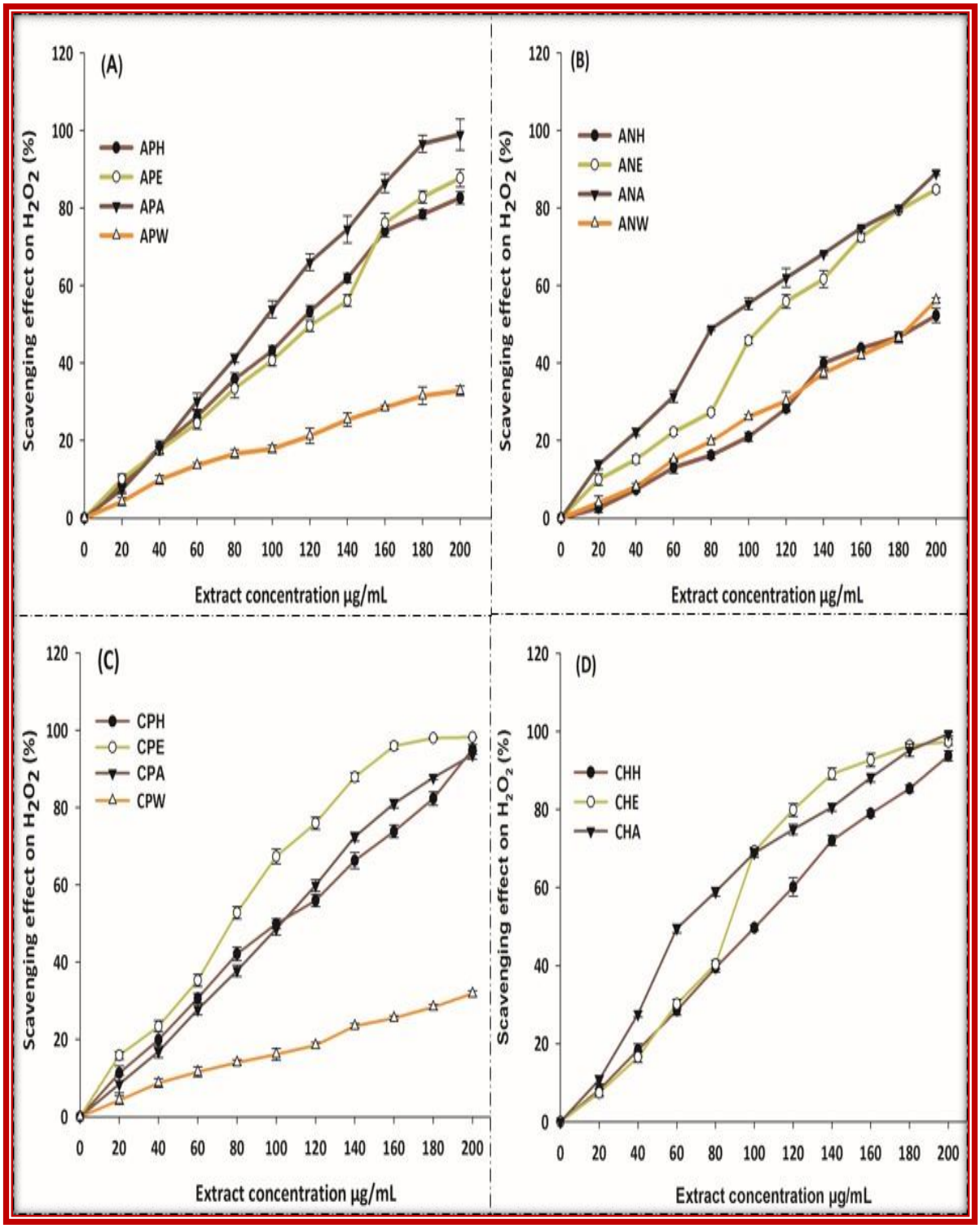


Fig. 3.5: Hydrogen peroxide (H_2O_2) scavenging activities of different extracts from 4 selected medicinal plants at various concentrations. Each value represents a mean ± SD (n = 3).
(A): *Abrus precatorius*; **(B)**: *Artemisia nilagirica*; **(C):** *Cissampelos pereira* and **(D):** *Cocculus hirsutus*.

the H_2O_2 up to 98.19±0.14% with lowest IC_{50} value of 75.79±1.63 µg/mL. On the other hand, at the same concentration, CPH and CPA exhibited 95.06±1.26% and 93.63±1.15% H_2O_2 scavenging activity with the IC_{50} value of 100.18±1.32 and 102.82±1.65 µg/mL respectively. The CPW was found to be weak scavenger of H_2O_2 among the extracts with highest IC_{50} value of 314.07±0.77 µg/mL (Table 3.4; Fig. 3.5C).

The scavenging activity for H_2O_2 of extracts from *C. hirsutus* was in the order of CHA>CHE>CHH respectively. It was observed that scavenging effect increased as the concentration of the solvent extract increased. Among the extracts, CHA at a concentration of 200 µg/mL exhibited substantial H_2O_2 scavenging activity (99.32±0.48%) with lesser IC_{50} value of 60.61±0.90 µg/mL. The CHE and CHH had similar inhibitory effects on H_2O_2 by constraining the concentration up to 97.34±0.88% and 93.73±1.33 respectively, thereby presenting the IC_{50} values of 99.21±1.20 µg/mL and 100.68±1.32 µg/mL (Table 3.4; Fig. 3.5D).

Hydrogen peroxide (H_2O_2) is a biologically relevant, non-radical reactive oxygen species and is inevitably generated as a by-product of normal aerobic metabolism. It itself is not very reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals that can initiate lipid peroxidation and cause DNA damage [Halliwell and Gutteridge, 2007]. High concentration of H_2O_2 is lethal to the cells, and its build-up causes oxidation of cellular targets such as DNA, proteins, and lipids, eventually leading to cell death [Yen and Duh, 1994]. Moreover, $^{\bullet}OH$ radicals are known to be capable of abstracting hydrogen atoms from membrane lipids and brings about peroxidation reaction of lipids [Van-wijk et al., 2008]. It can cross membranes and may slowly oxidize a number of compounds. In consequence, removing H_2O_2 is very important for antioxidant defence of living systems. The measurement of H_2O_2 -scavenging activity is one of the useful methods of determining the ability of antioxidants to decrease the level of prooxidants such as H_2O_2. In our study, the H_2O_2 - scavenging activity of different extract and the standard ascorbic acid increased in a concentration-dependent manner. Results showed that H_2O_2 scavenging capacity of all extracts was significantly better than that of ascorbic acid (245.30±4.60 µg/mL) tested in the same conditions, thereby presented higher H_2O_2 scavenging ability. IC_{50} values of the extracts in scavenging hydrogen peroxide were significantly different ($p<0.05$). Alcohol and ethyl acetate extracts efficiently scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralizing it into water. The

decomposition of H_2O_2 into H_2O may occur according to the antioxidant compounds as the antioxidant component present in the extracts are good electron donors as they may accelerate the conversion of H_2O_2 to H_2O. The results of this study strongly suggested that these extracts contained the necessary compounds for H_2O_2 elimination. Many reports have already proven that nutritive phenols play a significant role in protecting mammalian and bacterial cells from cytotoxicity induced by H_2O_2 [Bourgou et al., 2008; Nakayama, 1994; Ksouri et al., 2009], indicating that the observed activity of plants extracts could be due to the presence of phenols.

3.4.4. NITRIC OXIDE SCAVENGING ACTIVITY:

In the present study, the extracts of selected plants were further analysed for their inhibitory effects on nitric oxide production. It was observed that extracts of selected plants exerted inhibition activity against the production of nitric oxide (NO) radical from its artificial source sodium nitroprusside (Table 3.4; Fig. 3.6A - 3.6D).

A. precatorius extracts showed significant decrease in $NO^{\bullet}$ due to the scavenging ability of extracts. All the four extracts of this plant exhibited significant $NO^{\bullet}$ scavenging activity in a concentration dependent manner. The results clearly identified APE as better $NO^{\bullet}$ scavenger, where percentage inhibition reached to 97.58±3.12% with an IC_{50} value of 107.58±2.12 µg/mL followed by APA whose inhibition of generation of $NO^{\bullet}$ reached up to 92.70±2.13% (IC_{50}=145.96±2.17 µg/mL) in a concentration-dependent manner and a gradual decline thereafter at higher concentrations. The APH and APW were also efficient scavengers of $NO^{\bullet}$ (83.11±0.89% and 80.62±2.11%) with slightly higher IC_{50} values; 192.45±3.76 and 264.95±4.24 µg/mL respectively (Fig. 3.6A).

The scavenging of $NO^{\bullet}$ by *A. nilagirica* extracts was concentration-dependent, exhibiting significant decrease in the $NO^{\bullet}$. $NO^{\bullet}$ scavenging was most efficient with ANH with a maximum of 76.47±0.41% achieved at the concentration of 400 µg/mL presenting the IC_{50} value of 209.2±7.33 µg/mL. Lower efficiency, yet still considerable, was exhibited by ANE, ANW and ANA extracts with maximum inhibition of 73.93±1.19%, 72.11±0.57% and 71.75±1.23%, respectively at the same concentration of 400 µg/mL. The effective concentrations at which 50% of the $NO^{\bullet}$ radical were inhibited by these extracts (257.30±6.78, 258.09±5.43, 264.60±1.71 µg/mL, respectively) were not statistically different

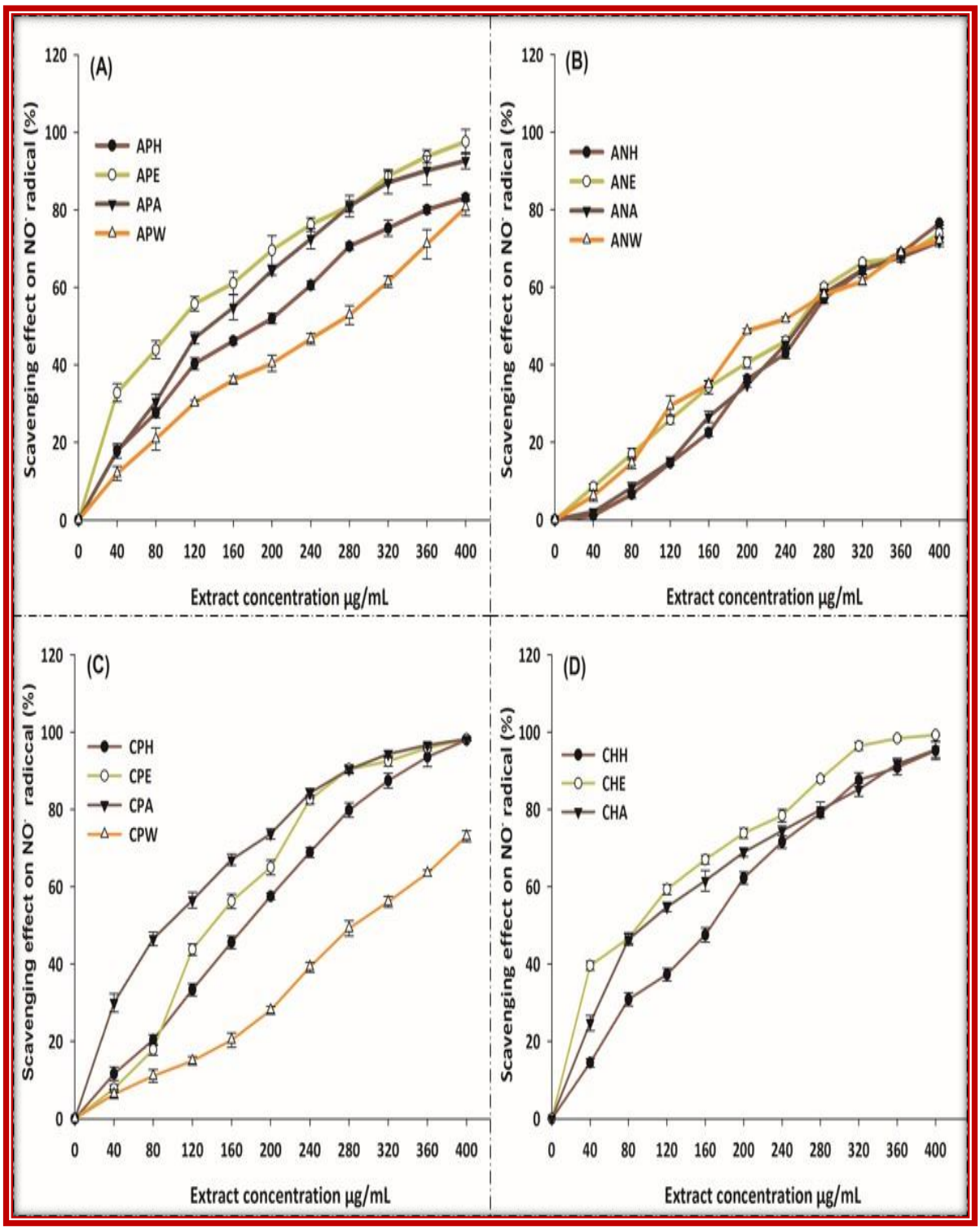


Fig. 3.6: Nitric oxide (NO•) radical scavenging activities of different extracts from 4 selected medicinal plants at various concentrations. Each value represents a mean ± SD (n = 3). **(A):** *Abrus precatorius*; **(B)**: *Artemisia nilagirica*; **(C):** *Cissampelos pereira* and **(D):** *Cocculus hirsutus*.

from each other, hence showed similar inhibiting activity towards the NO• production (Fig. 3.6B).

C. pereira extracts exhibited significant protective effect against NO•. The scavenging of NO• by the extracts was increased in concentration dependent manner. As shown in Fig. 3.6C, all the four extracts reduced the absorbance and the concentration of extracts was directly proportional to the reduction. The results clearly recognised CPA as better NO• scavenger as it had the highest efficiency (98.18±0.27%) to quench the NO• radical with minimal IC_{50} value of 85.98±0.56 µg/mL). The CPE and CPH had almost similar scavenging activities and also possessed significant effectiveness to scavenge the NO• as the percentage inhibition reached up to 98.22±0.44 and 97.96±0.37 with IC_{50} values of 142.27±1.23 µg/mL and 175.25±0.95 µg/mL), respectively. The CPW showed the lowest activity among the extracts with the percentage inhibition of 73.06±1.43 and IC_{50} value of 284.40±1.10 µg/mL.

The extracts of *C. hirsutus* also established strong NO• scavenging activity *in vitro* and inhibited the generation of NO• in a concentration dependent manner (Fig. 3.6D). Among the extracts of this plant, CHE and CHA showed a strong scavenging ability that reached a peak of 99.27±0.37% and 95.45±2.0% at the concentration of 400 µg/mL with IC_{50} values of 101.16±0.37 and 109.58±2.0 µg/mL, respectively. The CHH also demonstrated a concentration dependent elevation in NO• scavenging activity with the percentage inhibition level of 95.23±2.38 but required slightly higher concentration for inhibiting 50% of NO• production (168.31±2.38 µg/mL).

Nitric oxide radical (NO•) is an important bio-regulatory mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes [Lata and Ahuja, 2003]. Initially NO• was regarded to have only beneficial effects like cell signalling, pathological defence, but it has been found that over production of NO• is closely related with different pathological diseases, such as chronic inflammation, autoimmune diseases and cancer [Szekanecz and Koch, 2007]. Nitric oxide is known to be a ubiquitous free-radical moiety, which is distributed in tissues or organ systems and play an important role in inducing inflammatory responses and their toxicity multiplies only when they react with $O_2^{\cdot-}$radicals to form peroxynitrite which damages the biomolecules such as proteins, lipids and nucleic acids, and therefore injures the host tissue [Gülçin et al., 2007]. The measure of NO• radical scavenging activity was based on the principle that sodium nitroprusside (SNP) in an aqueous solution at physiological pH

spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using a Griess reagent. It is well recognised that the mechanism of inhibition of the production of short-lived $NO^{\bullet}$ radical from SNP involves either direct scavenging of $NO^{\bullet}$ by anti-radical or through the scavenging of the oxide (oxygen), which reacts with the formed $NO^{\bullet}$ leading to the formation of nitrite ions [Janzen et al., 1993]. Scavengers of $NO^{\bullet}$ competes with oxygen, leading to reduced production of nitrite ions which can be monitored at λ 546 nm suggesting their antioxidant potential [Sreejayan and Rao 1997]. The results of this study clearly identified the different extracts of selected medicinal plants as potent $NO^{\bullet}$ scavengers. The IC_{50} values were significantly different ($p<0.05$) amongst each other. This can be explained that various polyphenolic phytochemicals may react with free radicals in different ways, depending of their chemical structure, and thus, lead to different scavenging activities [Hu et al., 2003]. On the other hand, different plant extracts could present different behaviour according to the antioxidant evaluation methodologies. And also in the extracts, a synergies or antagonisms among the antioxidants in the mixture could occur. It appeared that these extracts have the scavenging character in accordance with the standards and they contained active compounds that are highly reactive against $NO^{\bullet}$. At the same time, the tested extracts exhibited low scavenging activity compared to that of positive controls, ascorbic acid and quercetin, which exhibited minimal IC_{50} values of 19.90±2.30 µg/mL and 21.09±2.16 µg/mL, respectively. This may be explained because of pure nature of these compounds. Over the years, it has been shown that the plant/plant products may have the property to counteract the effect of $NO^{\bullet}$ formation and in turn may be of considerable interest in preventing the ill effects of excessive $NO^{\bullet}$ generation *in vivo*. Inhibition of $NO^{\bullet}$ production by plant polyphenols is well-documented and potency of $NO^{\bullet}$ radical inhibition has been correlated with the number of hydroxyl residues of polyphenolics, including flavonoids [Janzen et al., 1993; Klotz and Sies, 2003; Van-Acker et al., 1995].

3.4.5. SUPEROXIDE ANION SCAVENGING ACTIVITY:

The superoxide anion ($O_2^{\cdot-}$) scavenging activity of extracts were assayed by the PMS-NBT-NADH system by measuring their ability to quench $O_2^{\cdot-}$ radical and the results are presented in Table 3.4; Fig. 3.7A - 3.7D.

The extracts of *A. precatorius* were found to be proficient in scavenging of $O_2^{\cdot-}$ among which the APE and APA displayed the maximum activity. At 400 µg/mL, the quenching ability of $O_2^{\cdot-}$ were found to be 95.01±4.29% with IC_{50} value of 143.44±3.28 µg/mL for APE and 93.15±4.36% with IC_{50} value of 157.07±2.56 µg/mL for APA. The APW and APH also possessed the moderate scavenging effect of 73.25±4.50% and 46.81±2.87%, however with higher IC_{50} values of 201.45±6.23 µg/mL and 427.26±5.72 µg/mL, respectively (Fig. 3.7A).

The abilities of *A. nilagirica* extracts to quench $O_2^{\cdot-}$ from reaction mixture was found to be concentration dependent and a lower IC_{50} value reflects better ability to reduce the radical generation. The results revealed that ANE had the highest $O_2^{\bullet-}$ scavenging ability as the scavenging percentage achieved 61.39±0.44 at the concentration of 400 µg/mL (IC_{50} value=330.32±7.12 µg/mL). The ANA and ANW also showed better scavenging action, where IC_{50} was achieved at 509.94±1.71 and 536.12±4.33 µg/mL concentration. The ANH showed weak antioxidant potential of 34.68±0.50% at the highest concentration of 400 µg/mL with higher IC_{50} value (609.99±8.12 µg/mL) compared to other extracts (Fig. 3.7B).

C. pereira leaf extracts potently inhibited $O_2^{\bullet-}$ dependent NBT reduction in this biochemical assay in a concentration dependent manner thus indicating their abilities to quench $O_2^{\bullet-}$ in the reaction mixture. The percentage inhibition values of CPH, CPE, CPA and CPW were found to be in the order of CPA (93.69±0.27%) > CPE (93.27±0.55%) > CPW (44.96±0.46%) > CPH (34.43±0.35%) at the highest concentration of 400 µg/mL (Fig. 3.7C). The IC_{50} values of ascorbic acid and the extracts (CPH, CPE, CPA and CPW) are presented in Table 3.4.

The effect of leaf extracts obtained from *C. hirsutus* on the superoxide anions showed antioxidant activities in terms of $O_2^{\bullet-}$ quenching ability in a concentration dependent manner (Fig. 3.7D). At the concentration of 400 µg/mL), $O_2^{\bullet-}$ scavenging activity of CHE and CHA accounted for an inhibition of 97.81±0.18% and 86.02±0.36%. However, the IC_{50} value of CHE (163.43±1.65 µg/mL) was slightly higher than that of CHA (115.85±1.76 µg/mL). On the other hand, at the same concentration, CHH weakly exhibited 43.46±0.34% $O_2^{\bullet-}$ scavenging activity with the IC_{50} value on higher side (387.37±1.23 µg/mL) (Table 3.4).

Superoxide anion ($O_2^{\bullet-}$) the one-electron reduced form of molecular oxygen, is one of the strongest reactive oxygen species among the free radicals that are generated continuously by auto-oxidation processes or by several cellular processes, including the microsomal and mitochondrial electron transport systems. Although, it is a weak oxidant,

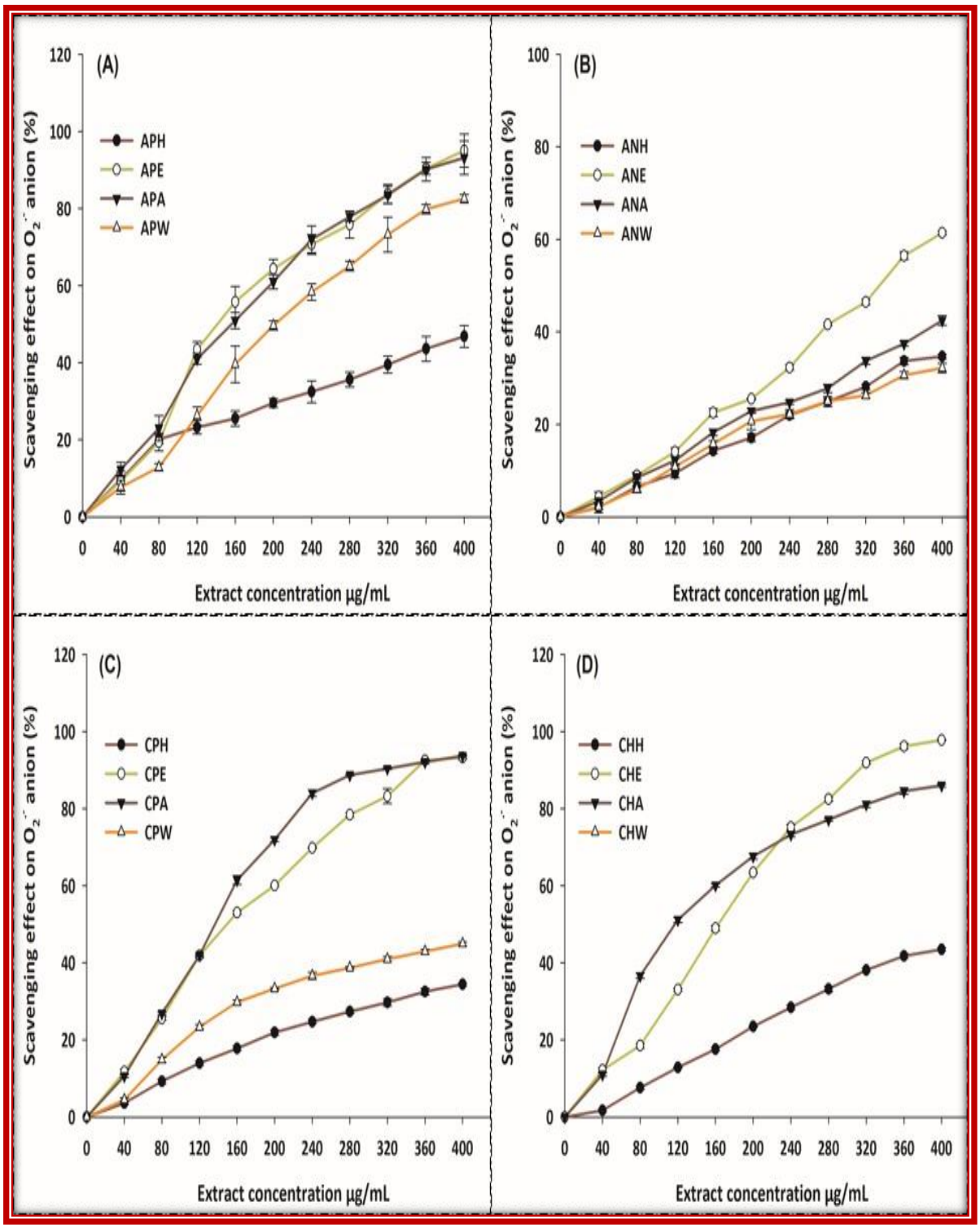


Fig. 3.7: Superoxide ($O_2^{-\bullet}$) anion scavenging activities of different extracts from 4 selected medicinal plants at various concentrations. Each value represents a mean ± SD (n = 3). **(A):** *Abrus precatorius*; **(B)**: *Artemisia nilagirica*; **(C):** *Cissampelos pereira* and **(D):** *Cocculus hirsutus*.

superoxide anion plays an important role in the formation of other ROS such as H_2O_2, $^{\bullet}OH$ and $^1O_2$ [Liu et al., 1997; Pietta, 2000]. It has been implicated in several pathophysiological processes, due to its transformation into more reactive species such as $^{\bullet}OH$ that initiate oxidative damage in lipids, proteins and DNA [Halliwell and Gutteridge, 1984; Wickens, 2001]. Therefore, it is of great importance to scavenge superoxide anion radical. In the present work, we verified the capacity of different extracts to scavenge the $O_2^{\bullet-}$ by employing the PMS-NADH-NBT system, a non-enzymatic universal method. The extracts were found to be an efficient scavenger of $O_2^{\bullet-}$ and were able to diminish the reduction of NBT in a concentration dependent manner (40-400 µg/mL in the reaction mixture). Of all tested extracts, the ethyl acetate of three plants, (*A. precatorius, A. nilagirica*, and *C. pereira*) and alcohol extract of *C. hirsutus* were found to be an efficient scavenger of $O_2^{\bullet-}$ generated in PMS/NADH/NBT assay system and percentage of inhibition increases markedly with the increase in concentration of extracts. Significant differences ($p<0.05$) were observed for IC_{50} values of these extracts than other counterparts. It can be inferred that ethyl acetate and alcohol extracts contained highly potent $O_2^{\bullet-}$ scavengers soluble in both solvents used for extraction. These result clearly indicated that the tested extracts have a noticeable effect on scavenging $O_2^{\bullet-}$ and antioxidant activity of these extracts may be arising mainly from the presence of well-known antioxidant compounds (i.e. polyphenols). It is acknowledged that different antioxidants help to scavenge $O_2^{\bullet-}$ by inhibiting initiation and breaking of chain reaction, suppressing formation of free radicals by binding to the metal ions, reducing H_2O_2, and quenching $O_2^{\bullet-}$ and $^1O_2$. The hydroxyl group of the phenolics contributes to $O_2^{\bullet-}$ scavenging activity by their electron donation. The hydroxyl moieties have the ability to terminate propagation of chain carrying radicals by acting as H-atom donor [Bravo, 1998; Chao et al., 2010; Yan et al., 2006]. Nonetheless, when compared to ascorbic acid, the $O_2^{\bullet-}$ scavenging activity of the extracts was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract.

3.4.6. IN VITRO Fe(II)-INDUCED LIPID PEROXIDATION IN RAT LIVER HOMOGENATE:

In addition to free radical scavenging activities, the plant extracts were evaluated for their ability to protect biomembrane from oxidative damage. Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or

through •OH radical by Fenton's reaction. Oxidative stress can lead to peroxidation of cellular lipids and can be measured by determining the levels of TBARS. The inhibition of lipid peroxidation induced by Fenton reagent in liver homogenate was assayed by measuring the amount of lipid peroxidation product malondialdehyde (MDA). Extracts from selected medicinal plants inhibited Fe(II)/ascorbate induced lipid peroxidation in rat liver homogenate in a concentration dependent manner indicated lipoprotective response as summarised in Table 3.4; Fig. 3.8A - 3.8D.

Different extracts of *A. precatorius* inhibited the Fe^{2+} induced lipid peroxidation and considerably reduced MDA content in a concentration-dependent manner (Fig. 3.8A). APE had the greatest inhibiting activity (98.70±2.56%); with the lowest IC_{50} value 45.46±3.71 µg/mL. When compared to the activity of standard, (ascorbic acid, IC_{50}=48.72±3.20 µg/mL), inhibiting activity against lipid peroxidation of APE was significant considering that the extract was a mixture of number of components against pure compound used as standard. The other extracts (APA, APW and APH) proved to be much weaker inhibitors of lipid peroxidation than APE with the percentage inhibition of 73.75±1.60%, 68.09±3.26% and 56.73±3.81%, respectively with much higher IC_{50} values than that of APE. The IC_{50} values of these extracts are presented in Table 3.4.

The extract of *A. nilagirica* showed concentration dependent anti-lipid peroxidation activity *in vitro* and exhibited capability of protecting tissues from peroxidative damage. Among the extracts, ANE, ANW and ANH in presence of highest concentrations of extracts (400 µg/mL) showed highest LPO inhibition potential with the percentage inhibitions of 88.15±0.99%, 75.32±1.80% and 69.90±0.94%. The IC_{50} value of these extracts were found to be 229.64±7.89, 236.21±9.00 and 297.35±5.78 µg/mL respectively. The ANA accounted for least activity at the same concentration with of 46.88±0.75% thereby presenting highest IC_{50} value of 451.06±4.95 µg/mL (Fig. 3.8B).

Similarly, the extracts of *C. pereira* exhibited concentration-dependent and significant inhibition of Fe^{2+}/ascorbate-induced lipid peroxidation *in vitro*. CPH possessed highest inhibitory activity against lipid peroxidation by 78.62±0.88% with an IC_{50} value of 178.77±0.28 µg/mL. Other extracts; CPE, CPA and CPW also showed significant levels of inhibition of lipid peroxidation but their IC_{50} values were recorded to be on higher side than that of CPH (209.20±0.81, 230.55±0.76, 288.36±3.23 µg/mL, respectively) (Table 3.4; Fig. 3.8C).

The lipid peroxidation results of *C. hirsutus* revealed that the extracts had significant anti-lipid peroxidation potential. Percentage inhibition of lipid peroxide in liver homogenate by the extracts was found to be in the order of CHA > CHE > CHH at the highest concentration of 400 µg/mL. The CHA produced higher inhibition by 90.40±1.84% with an IC_{50} value of 227.19±1.40 µg/mL. The CHE also demonstrated significant percentage inhibition (83.35±1.86) with an IC_{50} value of 264.50±1.30 µg/mL. The CHH was found to be least effective among the extracts as 46.10±1.30% inhibition was achieved at the highest concentration of 400 µg/mL suggesting that its chemical components are extremely weak hydrogen or electron donors (Table 3.4; Fig. 3.8D).

Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated lipids. In biological systems, lipid peroxidation generates many aldehydes products, among which MDA is considered to be the most important derivative and a hallmark of this process. The increase in MDA content that results from lipid peroxidation in presence of iron could be attributed to the fact that iron (Fe^{2+}) can analyse one electron transfer reaction that generate reactive oxygen species such as the reactive $^{\bullet}OH$ which is formed from H_2O_2 through the Fenton reaction. Iron also decomposes lipid peroxides, thus generating peroxyl and alkoxyl radical that ultimately propagates the chain reaction in lipids, disrupts cell membrane and finally results in cell damage [Barreira et al, 2008; Halliwell and Gutteridge, 1981; Zago et al., 2000]. Moreover, MDA can react with DNA bases to form adducts, which were seen elevated in different pathological conditions especially in cancer [Marnett, 1999; Wang and Rossman, 1996]. Due to the effective sequestration of iron by the various metal-binding proteins, the cells contain only the negligible amounts of free catalytic iron. To avoid the harmful effects of free iron, its proper chelation is of key importance [Kell, 2009; Kumar and Pandey, 2012; Kumar et al., 2013]. Thus, antioxidants capable of scavenging peroxy ($LOO^{\bullet}$) and alkoxyl (R-O) radicals could prevent lipid peroxidation. In the present study, we examined the plant extracts for their ability to inhibit lipid peroxidation in rat liver microsomes, induced by the Fe^{2+}/ascorbate system. The results of our study established that the different extracts had potential to inhibit lipid peroxidation *in vitro* by reducing the formation of lipid peroxidation end product MDA. However, the ethyl acetate extracts of *A. precatorius, A. nilagirica* and *C. hirsutus* and hexane extract of *C. pereira* exhibited good antioxidant capacity as these extracts presented significantly lower IC_{50} values for abrogation of MDA in the reaction mixture than other extracts ($p<0.05$). Metal ion

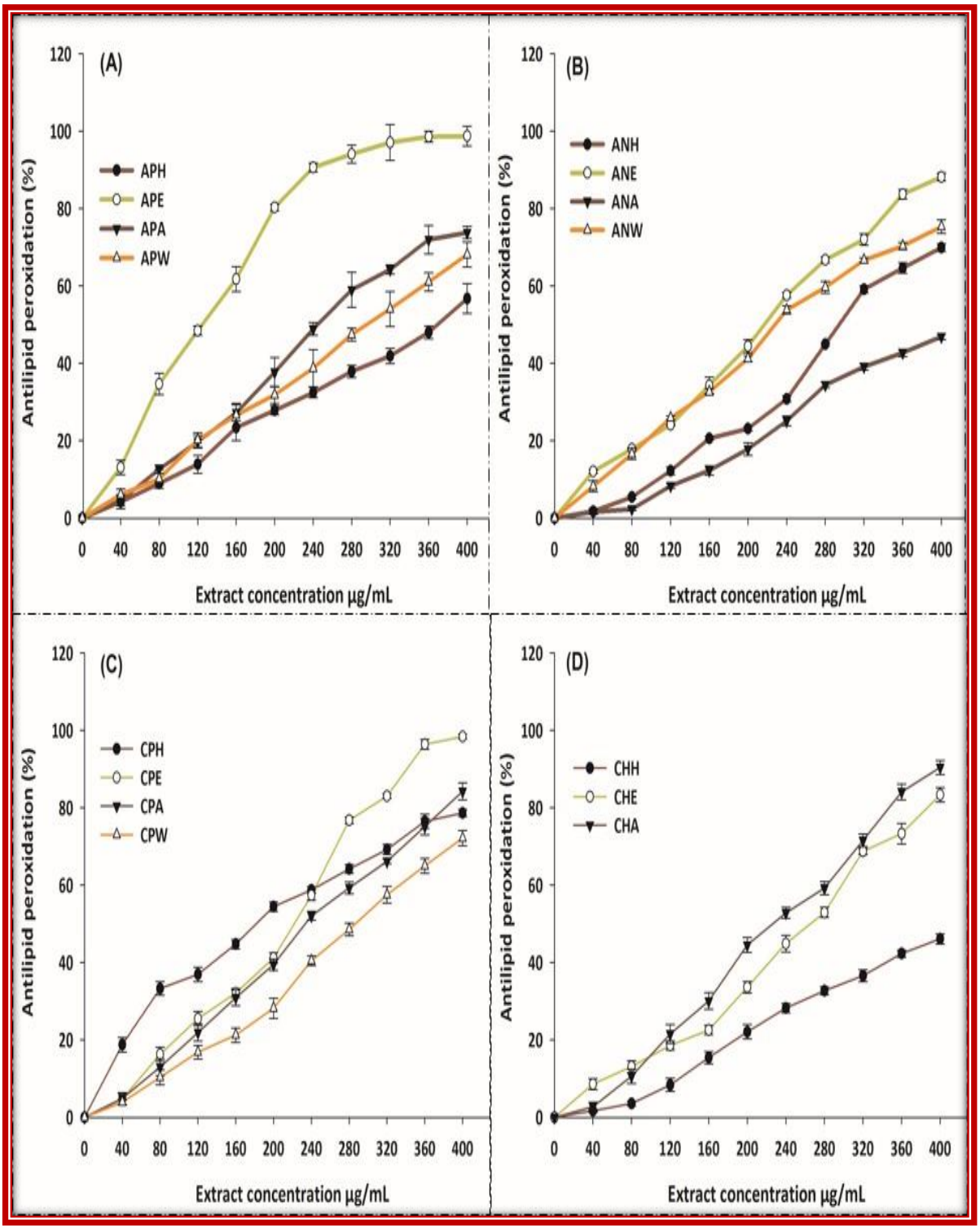


Fig. 3.8: Antilipid peroxidation (LPO) activities of different extracts from 4 selected medicinal plants at various concentrations. Each value represents a mean ± SD (n = 3).
(A): *Abrus precatorius*; **(B)**: *Artemisia nilagirica*; **(C):** *Cissampelos pereira* and **(D):** *Cocculus hirsutus*.

chelating capacity plays a significant role in the antioxidant mechanism since it reduces the concentration of the catalysing transition metal in lipid peroxidation. The natural compounds which exhibit high lipid peroxidation inhibition potential can replace the synthetic antioxidants which are known to be toxic. It is generally assumed that ability of the plant phenolic compounds to chelate iron in lipid peroxidation system is very important for their antioxidant property. The protective ability exhibited by tested plant extracts on lipid peroxidation may be attributed to the presence of a particular polyphenolic class of compounds (reductones/antioxidants) that may be dominant in these extracts, as they are known to exist in different classes [Pietta, 2000; Shimada et al., 1992]. The antioxidant activity of these reductones is believed to be due to the breakage of free radical chain reaction by donating a hydrogen atom from the phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate or propagate further oxidation of the lipid and hence there might be the primary and secondary antioxidants [Doss et al., 2010; Gordon, 1990; Sharma and Gujral, 2011]. The data obtained from the present study revealed that the plant extracts may be offering protection against Fe^{2+}/ascorbate induced lipid peroxidation by either metal chelation or absence of ferryl perferyl complex, which is essential for inhibition of lipid peroxidation. Therefore, these extracts can act as effective antioxidants and are therefore valuable in maintaining the membrane integrity of liver organ.

3.5. SCREENING OF EXTRACTS FROM MEDICINAL PLANTS FOR ANTI-PROLIFERATIVE ACTIVITY:

The contribution of new and novel products from potential bioactive plants or their extracts for cancer treatment and prevention is still vast, despite the overshadowing by recent synthetic chemistry as a method of drug discoveries and drug productions. The persistence in search for new anti-cancer compound(s) from medicinal plants and traditional foods is a realistic and promising strategy for its prevention [Ullah and Khan, 2008]. The evaluation and the discovery of new anti-cancer agents is long-term process that encompasses many steps. The step broaches with the screening for anti-cancer properties, followed by the isolation and identification of bioactive compounds obliged to anti-cancer properties, toxicity estimation of the isolated compounds and finally *in vivo* anti-cancer activity testing to verify the aptitude of the compounds. Extracts of selected medicinal plants were believed to contain a wide array of polyphenolic compounds which might possess cancer preventive and/or therapeutic properties [Mantle and Wilkins, 2005]. To screen for

Table 3.5: Cytotoxic effects of the extracts/fractions in MTT assay and their IC_{50} values

Sample	Type of Cell Line									
	THP-1		SupT1		JM-1		HepG2		HeLa	
	%age Inhibition	IC_{50} Value	%age Inhibition	IC_{50} Value	%age Inhibition	IC_{50} Value	%age Inhibition	IC_{50} Value	%age Inhibition	IC_{50} Value
APE (200 µg/mL)	58.30±6.16	116.84±7.09	61.34±1.32	37.00±2.38	65.43±7.22	54.82±5.63	53.33±1.20	44.31±3.07	76.50±7.33	75.80±5.24
APA (200 µg/mL)	78.11±0.66	298.68±11.17	94.12±3.34	26.89±3.24	63.67±1.92	44.24±5.17	88.52±3.04	27.03±1.03	70.72±4.75	80.98±4.75
ANE (200 µg/mL)	89.25±1.22	38.21±7.37	76.28±4.01	59.02±5.90	77.33±1.63	35.87±1.68	73.96±3.15	101.01±2.46	67.09±13.52	51.67±9.84
ANA (200 µg/mL)	61.21±12.00	132.41±7.19	78.18±6.00	112.24±3.66	68.80±4.45	40.91±9.78	39.72±3.09	164.36±5.36	56.88±4.72	92.25±5.55
APH-11 (100 µg/mL)	87.32±0.17	14.64±1.84	86.37±3.37	11.56±4.69	83.77±4.10	8.49±0.85	--	--	--	--
APM-3 (100 µg/mL)	85.84±7.52	20.90±3.58	78.51±2.13	13.49±2.12	67.87±1.44	16.76±0.15	--	--	--	--
ANE-B (100 µg/mL)	91.06±1.31	27.04±2.54	89.33±2.03	9.56±3.06	85.77±1.56	6.15±1.95	--	--	--	--
ANM-9 (100 µg/mL)	84.85±0.69	12.70±4.79	91.13±8.49	6.21±6.96	90.93±2.16	9.23±2.83	--	--	--	--
Doxorubicin (10 µg/mL)	91.80±0.58	1.63±0.58	92.93±0.52	1.89±0.52	92.93±0.52	1.79±1.66	85.84±3.04	0.47±0.08	74.73±1.52	6.69±1.52
DMSO (0.1%)	6.66±2.60	--	4.33±0.78	--	3.067±2.86	--	3.13±1.01	--	5.190±2.70	--

Values were the means of four replicates ± standard deviation (SD).Doxorubicin (10 µg/mL) was used as positive control and DMSO as vehicle control.

anti-cancer drug candidates, the cell proliferation inhibition activity is obviously a choice as it validates the target for anti-cancer drug discovery. On the whole, our aim was to determine whether the extracts of these plants can exert an inhibitory effect on cancer cell proliferation and cause cell death. Therefore, the anti-proliferative effects of all the extracts from four plants were measured *in vitro* by employing MTT assay using different human cancer cell lines. This was done to test the hypothesis that extracts from selected plants would contain potent anti-cancer drug candidates that could be discovered by monitoring their cytotoxicity potential.

Out of the fifteen extracts from 4 different plants, screened in the cell proliferation assay, only 4 extracts (two extracts from *A. precatorius*, APE & APA and two extracts from *A. nilagirica*, ANE & ANA) had marked cell growth inhibitory action on tested cancer cells in a concentration dependent manner. None of the extracts from *C. pereira* and *C. hirsutus,* presented any significant anti-proliferative effect in the evaluated range (25-200 µg/mL) for any cell line, thus indicating their non-inhibitory properties against cancer cell growth. The active extracts demonstrated significantly effective anti-proliferative activities against the cancer cell panel. The extracts of *A. precatorius* (APE & APA) and *A. nilagirica* (ANE & ANA) showed different patterns of IC_{50} values. Considerable differences in the sensitivity of cell lines towards these extracts were observed. The results of this screening are summarized in Table 3.5 and Fig. 3.9A - 3.9E. At the highest concentration of 200 µg/mL, APA resulted in 78.11±0.66% inhibition of cell proliferation with an IC_{50} value of 298.68±11.17 µg/mL, whereas APE extract provided 58.30±6.16% inhibitions presenting the IC_{50} value of 116.84±7.09 µg/mL for THP-1 cell line. The ANE and ANA too showed significant anti-proliferative ability on THP-1 cells and appeared in a concentration-dependent manner with percentage inhibition of 89.25±1.22 and 61.21±12.00 exhibiting the IC_{50} value of 38.21±7.37 µg/mL and 132.41±7.19 µg/mL, respectively (Table 3.5; Fig. 3.9A).

The extracts also displayed high potentiality to inhibit the growth of SupT1 cancer cells. The APA and APE exerted paramount anti-proliferative ability (94.12±3.34 and 61.34±1.32% inhibition at 200 µg/mL) with IC_{50} values of 26.89±3.24 and 37.00±2.38 µg/mL, respectively. ANA and ANE were also found to be sensitive towards the growth of SupT1 cells and inhibited the growth up to 78.18±6.00 and 76.28±4.01% at the same concentration. These extracts presented the IC_{50} values of 112.24±3.66 and 59.02±5.90 µg/mL (Table 3.5; Fig. 3.9B). Lowest IC_{50} values of active extracts for JM1 cell line also illustrated that these extracts

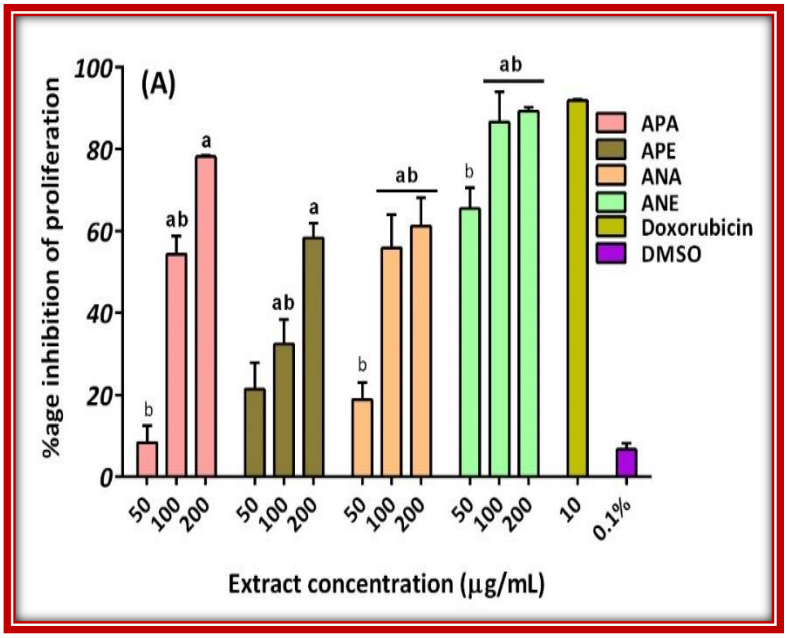


Fig. 3.9 (A): Antiproliferative activity of different crude extracts of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on THP-1 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

had significant potentiality to exterminate the growth of these cancer cells as well. The APE inhibited the growth up to 65.43±7.22% with an IC_{50} value of 54.82±5.63 µg/mL whereas APA demonstrated percentage inhibition of 63.67±1.92 and required 44.24±5.17 µg/mL to inhibit 50% of cells in the experimental conditions. Similarly, ANE and ANA confirmed their anti-proliferative property against JM1 cells where the percentage inhibition reached to 77.33±1.63 and 68.80±4.45, respectively at the highest concentration of 200 µg/mL. The IC_{50} values of these extracts against JM1 cells were found to be 35.87±1.68 and 40.91±9.78 µg/mL respectively (Table 3.5; Fig. 3.9C).

Furthermore, these active extracts treatments also significantly suppressed the proliferation of other two human cancer cell lines, HepG2 and HeLa. APE resulted in 53.33±1.20% and 76.50±7.33% inhibition for Hep2G and HeLa cells, respectively at a concentration of 200 µg/mL with IC_{50} value of 44.31±3.07 and 75.80±5.24 µg/mL, respectively. At the same concentration, APA supressed the proliferation of HepG2 cells up to 88.52±3.04% and HeLa cells up to 70.72±4.75% and presented the IC_{50} value of 27.03±1.03 and 80.98±4.75 µg/mL respectively. The ANE inhibited growth up to 73.96±3.15% and

67.09±13.52% against HepG2 and HeLa cells respectively at the same concentration of 200 µg/mL with IC_{50} values of 101.01±2.46 and 51.67±9.84 µg/mL respectively.

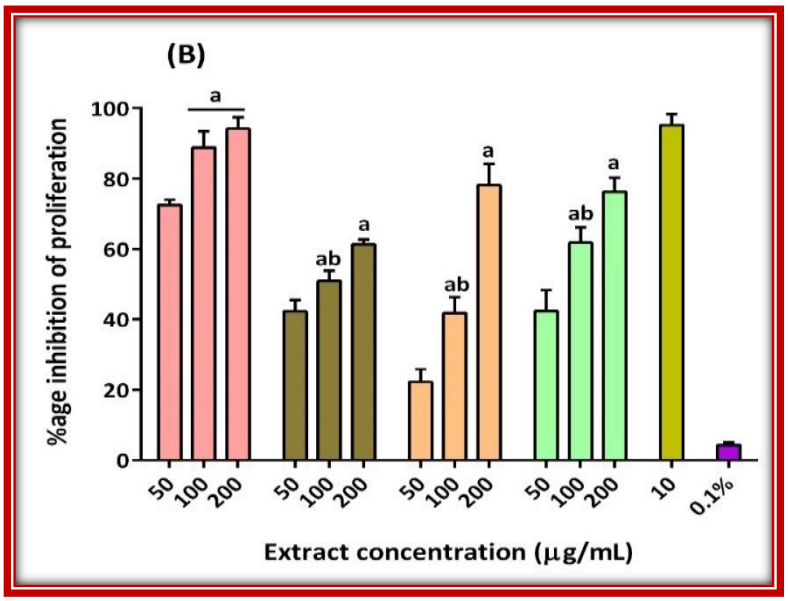


Fig. 3.9 (B): Antiproliferative activity of different crude extracts of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on SupT-1 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

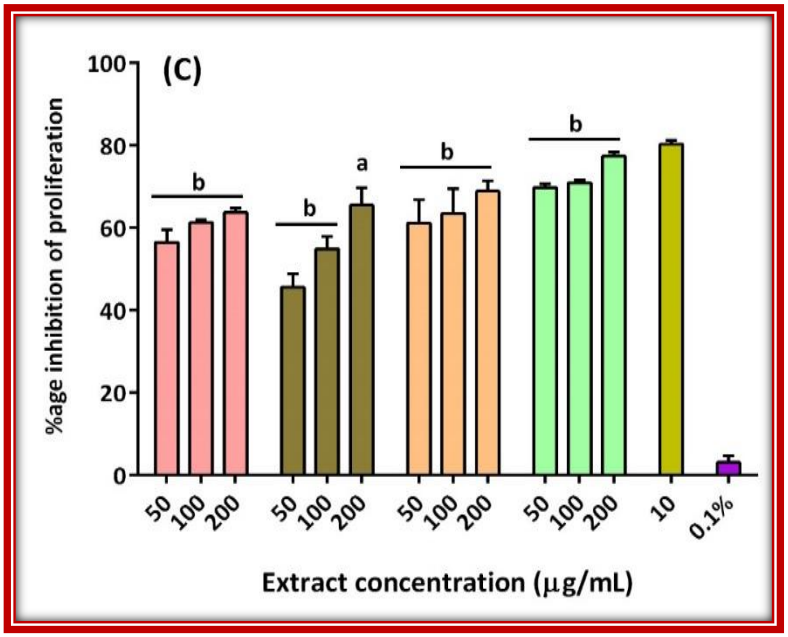


Fig. 3.9 (C): Antiproliferative activity of different crude extracts of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on JM-1 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

ANA also restricted the proliferation of HepG2 and HeLa cells (39.72±3.09% and 56.88±4.72% of inhibition, respectively, at 200 µg/mL), however the IC_{50} values were found to be on the higher side (164.36±5.357 and 92.25±5.55 µg/mL respectively) (Table 3.5; Fig. 3.9D; 3.9E). Doxorubicin was used as the positive control in the present study because it is a

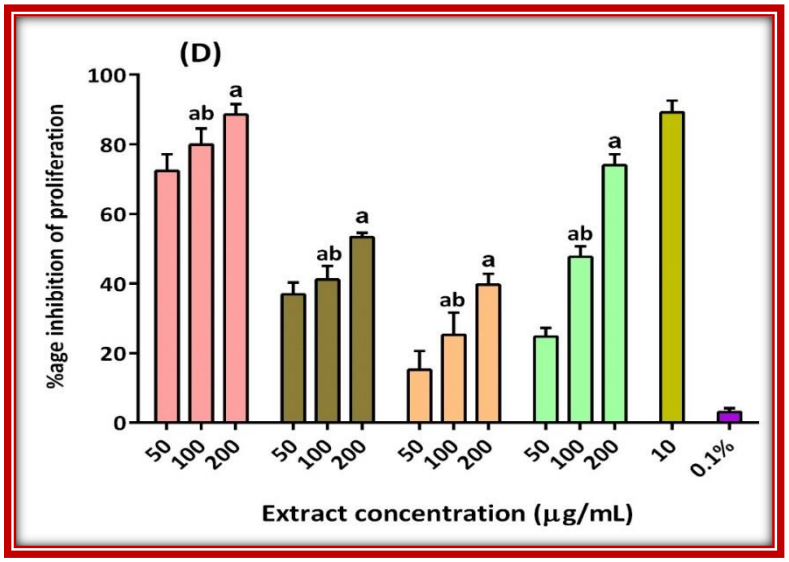


Fig. 3.9 (D): Antiproliferative activity of different crude extracts of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on HepG2 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

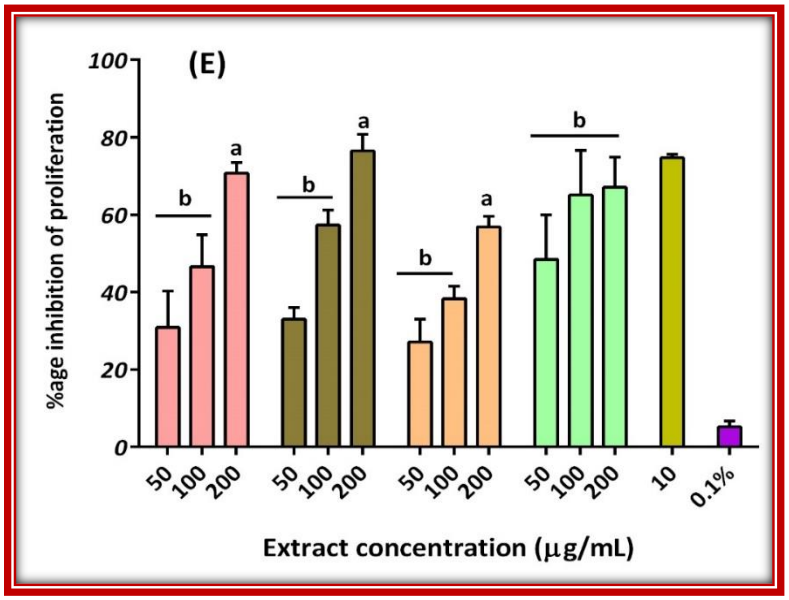


Fig. 3.9 (E): Antiproliferative activity of different crude extracts of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on HepG2 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

commonly used chemotherapeutic drug for the treatment of acute leukaemia, lymphomas and different types of solid tumours such as breast, liver and lung cancers [Tokarska-Schlattner et al., 2006]. Doxorubicin, in our study showed excellent indication of cancer cell inhibition on THP-1, SupT1, JM-1, HepG2 and HeLa cancer cells lines with IC_{50} values of 1.63±0.58, 1.89±0.52, 1.79±1.66, 0.47±0.08 and 6.69±1.52 µg/mL, respectively. It was found that Doxorubicin was needed in much lesser concentrations than the active crude extracts to produce significant cytotoxic effects on the cells. Obviously, the standard drugs are highly purified as compared to assayed crude extracts.

The results of present study suggested that extracts of *A. precatorius* (APE & APA) and *A. nilagirica* (ANE & ANA) caused marked cell growth inhibition on all the five human cancer cell lines in a concentration-dependent manner. However, there were diverse inhibition levels on different cells shown by active extracts and the inhibition levels of each extract was significantly different ($p<0.001$). It is known that different cell lines exhibit different sensitivities towards an anti-cancer compound(s), so the use of more than one cell line was therefore considered necessary in the evaluation of anti-cancer effects. This specificity of plant extracts is likely to be due to the presence of different classes of compounds in the extracts, as it has been documented in the case of known classes of compounds [Conforti et al., 2008; Cragg et al., 1994]. Moreover, it was reported that types of solvent used in the extract preparation greatly influenced the bioactive compound extraction [Pinelo et al., 2005]. Therefore, we found the different effects with different extraction solvents. However, the high IC_{50} values were likely due to very low concentrations of compounds of interest, which would be considerably enriched upon bioactivity-guided fractionation.

3.6. ASSESSMENT OF CYTOTOXICITY OF PLANT EXTRACTS ON NORMAL CELLS:

An important criterion in the search of anti-cancer compound(s) with therapeutic potential, is to determine whether they show toxic effects on normal cells. Undesirable side effects might limit the use of some extracts. Therefore, in order to define whether the strong inhibitory effects of active crude extracts *A. precatorius* (APE & APA) and *A. nilagirica* (ANE & ANA) were specific to cancer cells, the effects of these extracts on the proliferation of normal cells were subsequently monitored. For this purpose, a test of cytotoxicity to HEK and peritoneal macrophages was performed in order to determine the selectivity to

biological activity. The results of this study clearly indicated that assayed extracts were virtually non-toxic and had negligible inhibitory effects on cell proliferation against HEK cells and peritoneal macrophages and there was minimal reduction in cell survivability (Fig. 3.10A - 3.10B).

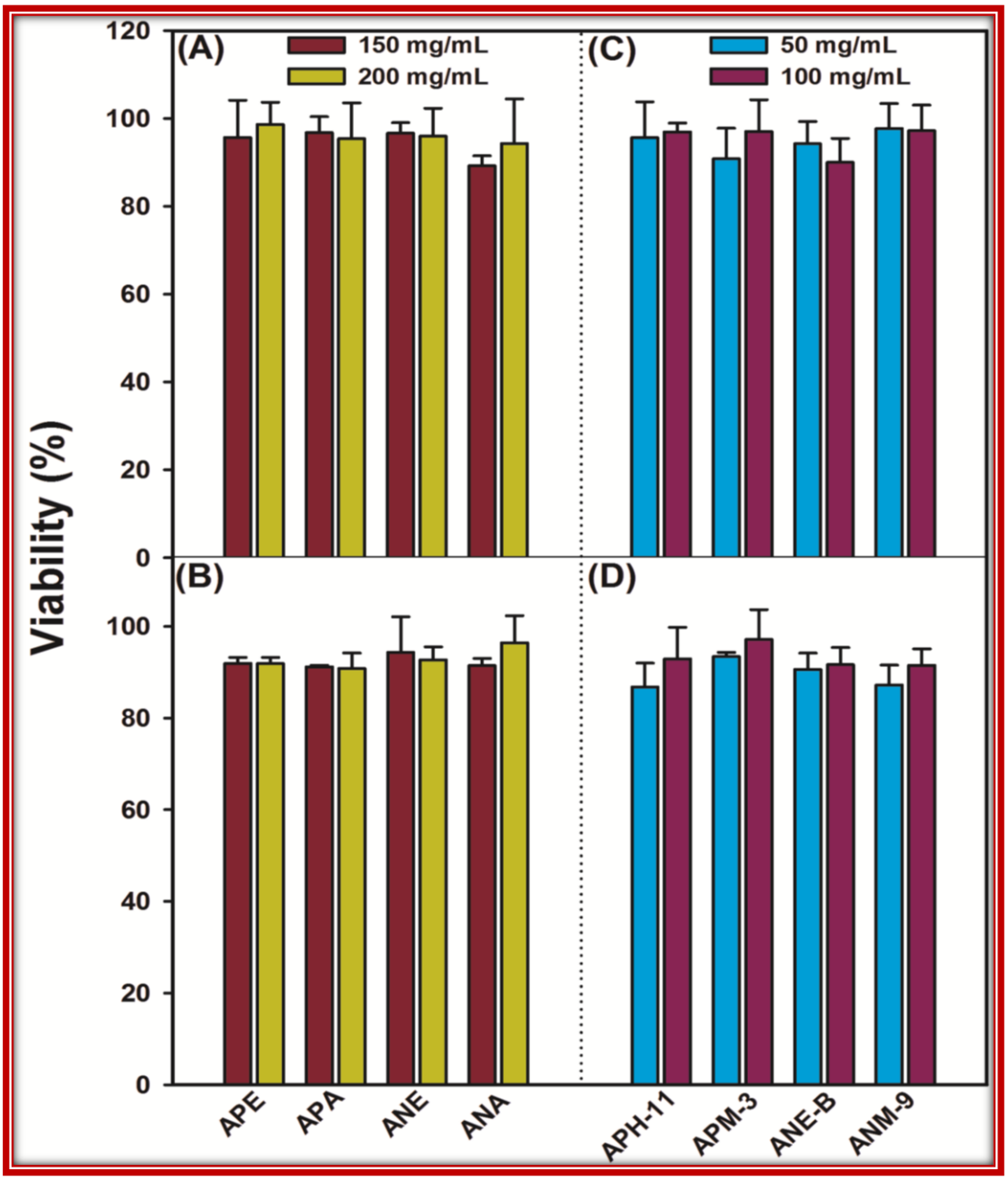


Fig. 3.10: Growth inhibitory effects of crude/active fractions of *Abrus precatorius* and *Artemisia nilagirica.* The values were means ± SD of 4 independent experiments. **(A):** Effect of crude extracts on murine peritoneal macrophages; **(B):** Effect of crude extracts on HEK-293 cells; **(C):** Effect of active fractions on murine peritoneal macrophages; **(D):** Effect of active fractions on HEK-293 cells.

The percentage viability was found to be above 90% at the highest concentration of 200 µg/mL. These results suggested that HEK-293 cells and murine peritoneal macrophages were much less susceptible to the cytotoxic effects of active extracts. Therefore, the anti-proliferative properties of these extracts were specific to cells of tumour origin and the extracts were found to be selective against the cancer cell lines used and displays application potential in cancer chemoprevention and chemotherapy.

3.7. BIOASSAY GUIDED FRACTIONATION OF ACTIVE EXTRACTS OF *A. PRECATORIUS* (APE & APA) AND *A. NILAGIRICA* (ANE & ANA):

The discovery of cytotoxic agents from natural sources by following up the results of previous anti-proliferative screening has had a rich and fruitful past, with the identification of novel agents such as taxoids, campthotecine, podophyllotoxin derivatives and Vinca alkaloids [Valeriote et al., 1992]. Different methods have been employed in the search for active constituents from plants. They include bioassay guided isolation, and biochemical combinatorial chemistry approaches [Rimando et al., 2001]. The bioassay-guided isolation integrates the processes of separation of compounds in a mixture, using various analytical methods, with results obtained from biological testing. The process begins with the testing of an extract to confirm its activity, and further fractionation is carried out on the fractions that are determined to be active. The crude extracts of *A. precatorius* (APE & APA) and *A. nilagirica* (ANE & ANA) exhibited pronounced and concentration-dependent anti-proliferative activities in the tested cell lines and were worthy of further investigation to explore active compounds responsible for the anti-proliferative activity. Given the wide therapeutic window, we next aimed to enhance the cytotoxic potential of these crude extracts and subsequently identify the responsible bioactive constituent(s). Thus, we first fractionated these active crude extracts separately using classical column chromatography by employing a gradient method to ensure absolute separation with a reduced analysis time. A series of solvent systems with a gradual increase in polarity were used to elute these extracts along the silica gel column.

3.7.1. BIOASSAY-GUIDED FRACTIONATION OF THE CRUDE EXTRACTS OF *A. PRECATORIUS* (APE & APA):

APE extract was fractionated with n-hexane, ethyl acetate and methanol successively. The fractionation disseminated the chemicals on polarity basis and fashioned varying heft of crude fractions. A total of 142 fractions of 50 mL each were collected from the column and

the eluents were pooled according to the similarity of the chemical composition detected on pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} plates. This reduced the number of fractions from 142 to 11 and the nomenclature of fractions were given as APH-1, APH-2, APH-3, APH-4, APH-5, APH-6, APH-7, APH-8, APH-9, APH-10 and APH-11.

Similarly, APA extract was fractionated with n-hexane, ethyl acetate and methanol. A total of 127 fractions of 50 mL each were collected and all the eluted fractions were then monitored individually by TLC and the fractions with same TLC profile were pooled; thereby 8 major fractions were obtained and designated as APM-1, APM-2, APM-3, APM-4, APM-5, APM-6, APM-7 and APM-8. All the major fractions obtained from the fractionation of crude extracts were examined for anti-proliferative activity on different tested cells therefore, APH-11 and APM-3 were found to be most promising fractions to inhibit the cancer cells proliferation and hence were studied further to explore their mechanisms of action.

3.7.2. BIOASSAY-GUIDED FRACTIONATION OF THE CRUDE EXTRACTS OF *A. NILAGIRICA* (ANE & ANA)*:*

The ethyl acetate extract of *A. nilagirica* (APE) which gave the promising anti-proliferative activity was also fractionated sequentially with *n*-hexane, ethyl acetate and methanol. A total of 65 fractions of 50 mL each were collected and all the eluents were pooled according to the similarity of the chemical composition detected on pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} plates. Eight fractions were obtained from the fractionation based on the similarity of their chemical composition and the fractions were designated as ANE-A, ANE-B, ANE-C, ANE-D, ANE-E, ANE-F, ANE-G, and ANE-H. In the same way, alcoholic extract (ANA) was subjected for sequential fractionation with *n*-hexane, ethyl acetate, chloroform and methanol. A total of 132 (50 mL) aliquots were collected, evaporated and run on silica gel thin layer chromatography (TLC) plates. Aliquots with similar TLC profiles were combined to yield 10 fractions in total, labelled as ANM-1, ANM-2, ANM-3, ANM-4, ANM-5, ANM-6, ANM-7, ANM-8, ANM-9 and ANM-10.

Each of the fractions obtained as detailed above, were evaluated for their anti-proliferative activity against selected cancer cell lines. The fractions ANE-B and ANM-9 provided the best selective anti-proliferative activity, which was the highest activity on the cancer cell lines, were selected for further studies to find out their mechanisms of action.

3.8. ANTI-PROLIFERATIVE ACTIVITY OF ACTIVE FRACTIONS AGAINST HUMAN CANCER CELL LINES:

The potential effects of the active fractions of *A. precatorius* and *A. nilagirica* obtained through fractionation were investigated on inhibition of cell growth against three human leukemic cell lines; THP-1, SupT1 and JM1 over a concentration range of 10-100 µg/mL by MTT assay, with Doxorubicin being used as the positive control and culture medium containing 0.1% DMSO used as the negative control. These active fractions presented superior cytotoxic effects and exhibited an increased rate of cell death at a lower concentrations than those of parent crude extracts. The results are summarized in Table 3.5; Fig. 3.11A- 3.11C.

Proliferation of THP-1 cells was significantly inhibited by the active fractions in a concentration-related manner with more than 80% suppression with lower IC_{50} values ($p<0.001$). APH-11 displayed strong inhibition against THP-1 cell proliferation with the percentage inhibition of 87.32±0.17 at the highest concentration of 100 µg/mL, giving the IC_{50} value of 14.64±1.84 µg/mL. The methanol fraction, APM-3 also demonstrated the similar anti-proliferative potential by suppressing the growth of THP-1 cells up to 85.84±7.52% with an IC_{50} value of 20.90±3.58 µg/mL. The inhibitory percentage of ANE-B at 48 h treatment were found to be 91.06±1.31% at the highest concentration of 100 µg/mL with IC_{50} value of 27.04±2.54 µg/mL. Similarly, ANM-9 also presented enhanced cytotoxic effects compared to ANA presenting the IC_{50} value of 12.70±4.79 µg/ml with 84.85±0.69% suppression at the same concentration (Table 3.5; Fig. 3.11A).

Active fractions of both the plants exhibited a concentration dependent decline in viability of SupT1 cells after 48 h treatment. APH-11 exerted significant anti-proliferative potential with cell growth inhibition of 86.37±3.37% at the highest concentration of 100 µg/mL and 50% inhibition of cell growth was obtained at concentration of 11.56±4.69 µg/mL. APM-3 inhibited the growth of SupT1 cells up to 78.51±2.13% at the same concentration of 100 µg/mL with the IC_{50} value of 13.49±2.12 µg/mL. The ANE-B and ANM-9 also supressed the growth of SupT1 cells significantly. The inhibitory percentage of ANE-B at 48 h after treatment were found to be 89.33±2.03% with an IC_{50} value of 9.56±3.06 µg/mL. A similar cytotoxic effect was also observed for ANM-9, where the proliferation of 91.13±8.49% was observed at the same concentration of 100 µg/mL with the IC_{50} value of 6.21±6.96 µg/mL (Table 3.5; Fig. 3.11B).

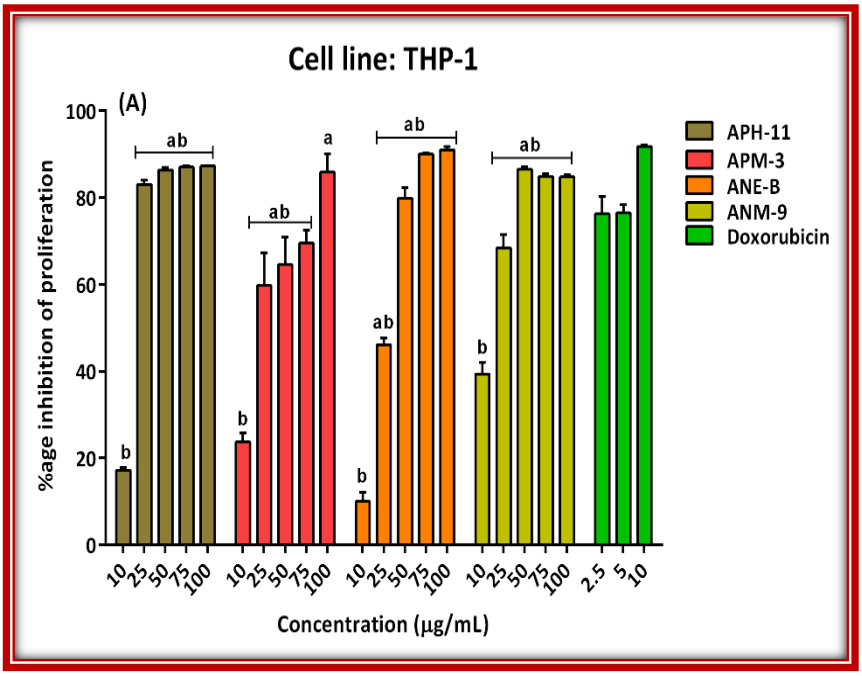


Fig. 3.11 (A): Antiproliferative activity of active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on THP-1 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

The active fractions further revealed efficient anti-proliferative effects towards JM-1 cell line in concentration dependent manner. APH-11 exhibited significant activity and inhibited the growth up to 83.77±4.10% at the concentration of 100 µg/mL and achieved an IC_{50} value of 8.49±0.85 µg/mL. APM-3 resulted in 67.87±1.44% inhibition of cell proliferation and presented IC_{50} value of 16.76±0.15 µg/mL. The ANE-B and ANM-9 also exhibited significant inhibitory effects towards the JM-1 cell line. At 48 h, a marked reduction in viability was observed with the concentration of 100 µg/mL. ANE-B suppressed the growth of cells up to 85.77±1.56% presenting with the IC_{50} value of 6.15±1.96 µg/mL. The ANM-9 was also found to be sensitive towards JM-1 cells and presented better anti-proliferative with the IC_{50} value of 9.23±2.83 µg/mL with the percentage inhibition of 90.93±2.15% (Table 3.5; Fig. 3.11C). The Doxorubicin (chemotherapeutic agent) was used as drug control which was able to inhibit cancer, cell proliferation. It had an IC_{50} of 1.63±0.58, 1.89±0.52 and 1.79±1.66 µg/mL for THP-1, SupT1 and JM-1 cells, respectively.

According to the results obtained, it is clear that anti-proliferative activity of 4 active fractions derived from their respective parent crude extract were more sensitive and

displayed distinct suppression of growth of all the three leukemic cell lines. The fractionation procedure potentiated the anti-proliferative activities of the crude extracts. The active fractions showed an increased anti-proliferative ability with lowest IC_{50} values when compared to that of their parent crude extracts suggested that activity was more specific towards cancer cells and more effective. The IC_{50} values clearly indicated that the semi-purified fractions had better ability to supress the growth and presented remarkable anti-proliferative activity towards the three human cancer cell lines at minimum concentrations. The criteria of cytotoxicity for the crude extracts/ semi-purified fractions, as established by the U.S. National Cancer Institute (NCI), is an IC_{50} <30 µg/mL in the preliminary assays [Suffness and Pezzuto, 1990]. This standard was used as an indicator of significant cytotoxic activity in several other studies focusing on the induction of cell death in cancer cell lines through MTT cytotoxicity assay [Chang et al., 2010; Jokhadze et al., 2007]. The crude extracts when pursued for further fractionation and bioassays demonstrated potent activity in the MTT assay according to this standard value. This cytotoxic activity could be associated to the presence of active chemical constituents that could probably have highly anti-growth effects.

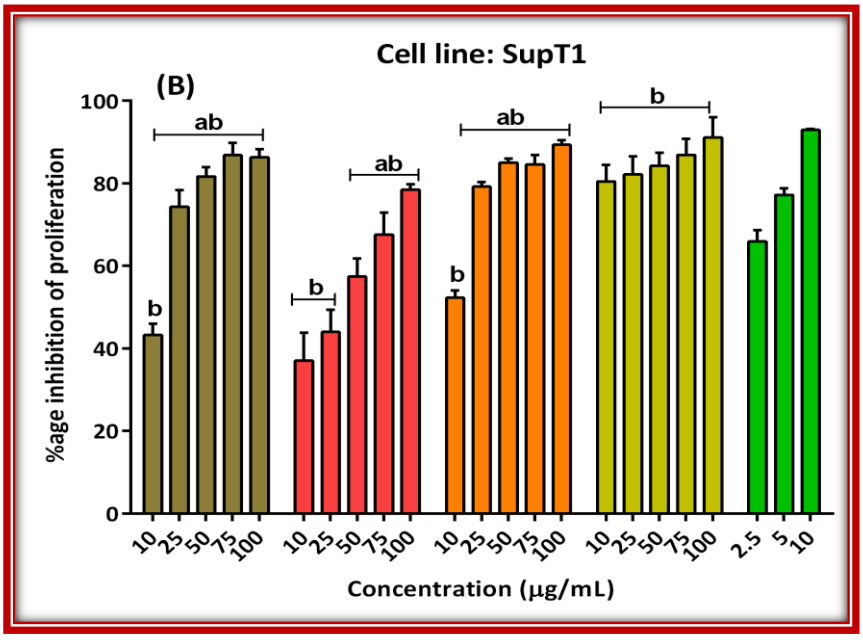


Fig. 3.11 (B): Antiproliferative activity of active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on SupT-1 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

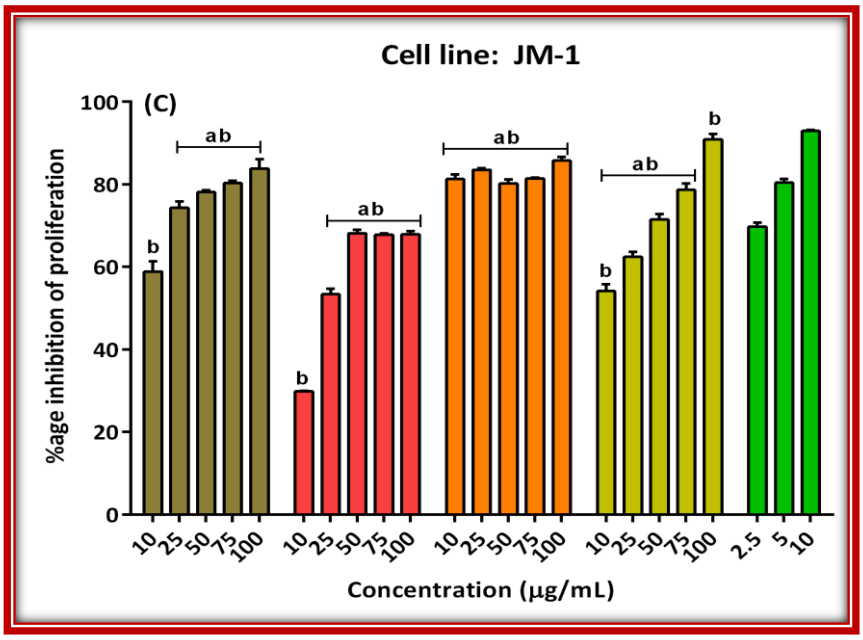


Fig. 3.11 (C): Antiproliferative activity of active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin on JM-1 cells. The values were means ± SD of 4 independent experiments. Letters (a, b, ab) are significantly different by the analysis of variance.

In order to understand the characteristic of the cytotoxicity effects of active fractions on cancer cells, the above three cells lines (THP-1, SupT1 and JM-1) were selected for further investigation in this study.

3.9. ASSESSMENT OF CYTOTOXICITY OF ACTIVE FRACTIONS ON NORMAL CELLS:

In order to consider an extract/fraction or a compound as cytotoxic agent in treatment of cancers, it is best to have no cytotoxicity on normal cell lines. To assess whether the extracts demonstrate toxicity to normal cells, the cytotoxic effect was also studied in normal human embryonic kidney cells (HEK) and mice peritoneal macrophages. The assayed fractions were found to be devoid of any activity against any of the tested non-cancer cells. As shown in Fig. 3.10C and 3.10D, a minimal reduction in cell survivability was observed when they were treated with active fractions in the evaluated range of 150-200 µg/mL. To achieve the IC_{50} values on HEK and peritoneal macrophages significantly higher concentrations of all the four fractions were needed (IC_{50} >300 µg/mL). Active fractions demonstrated prominent inhibitory activity towards cancer cells with no or little adverse effect on non-cancer cell lines. The selective toxicity demonstrated by the extracts/fractions is an important requirement of cancer chemotherapy. A good anti-cancer compound(s) must selectively exterminate cancerous cells whilst sparing normal fast growing cells in the body.

Many synthetic anti-cancer drugs however, failed in this respect, hence their numerous side effects are reported [Chan and Gaccia, 2011]. Our results suggested that the anti-proliferative properties are specific to cells of tumour origin and the active fractions could be a potential source of anticancer compound(s) with little side effects. However, these findings suggested the need for further investigations to clarify the features underlying the anti-proliferative potential of these fractions.

3.10. APOPTOSIS STUDIES:

3.10.1. DNA FRAGMENTATION ANALYSIS BY AGAROSE GEL ELECTROPHORESIS:

Extensive investigations into the molecular mechanisms underlying apoptotic cell death pointed to the presence of biochemical markers of apoptosis, that is, the fragmentation of nuclear DNA into oligonucleosome-sized DNA fragments by an apoptotic nuclease. To investigate if the decrease in viability was due to a specific death type, nuclear morphology of active fraction treated cells was analysed using DNA fragmentation analysis by agarose gel electrophoresis. The cells treated with active fractions of *A. precatorius* (APH-11 & APM-3) and *A. nilagirica* (ANE-B & ANM-9) presented with well-defined cleavage of genomic DNA in an oligonucleosomal laddering pattern upon agarose gel electrophoresis. The results depicted in Fig. 3.12A - 3.12C illustrated that there was clear disintegration of genomic DNA in THP-1, SupT1 and JM-1 cells, at the level of treated concentration of the fractions for 48 h. DNA disintegration can also be seen clearly in the THP-1, SupT1 and JM-1 cells treated with doxorubicin (positive control) whereas DNA from untreated cells did not show any fragmentation or smearing. However, in the cells treated with cytotoxic active fractions, the formation of typical DNA ladder due to the release of oligonucleosome associated DNA fragments was observed less clearly. The interspersing smears were witnessed in the lanes. Nevertheless, these changes are suggestive of apoptosis, as described elsewhere [Matassov et al., 2004; Ramasamy et al., 2012].

Apoptotic cells are characterised by a number of morphological features such as cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies [Orienius, 2004; Zimmerman et al., 2001;]. The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In apoptosis, cells are lysed gradually and systematically to produce membrane-bound apoptotic bodies, which was suggested to play a major role in suppressing inflammatory

responses to other neighbouring cells. Apoptotic bodies or cells which underwent apoptosis produce a specific pattern of DNA fragments with the multiples of 200 bp due to specific action of activated nucleases [Bortner et al., 1995]. The morphological changes associated with apoptosis occur as a result of the activation of endogenous endonucleolytic and proteolytic (caspases) enzymes that, in turn, mediate the cleavage of DNA into fragments as well as protein substrates, which usually determine the integrity and shape of the cytoplasm and organelles [Denault and Salvesen, 2002; Kasibhatla and Tseng, 2003; Seraste and Pulkki, 2000].

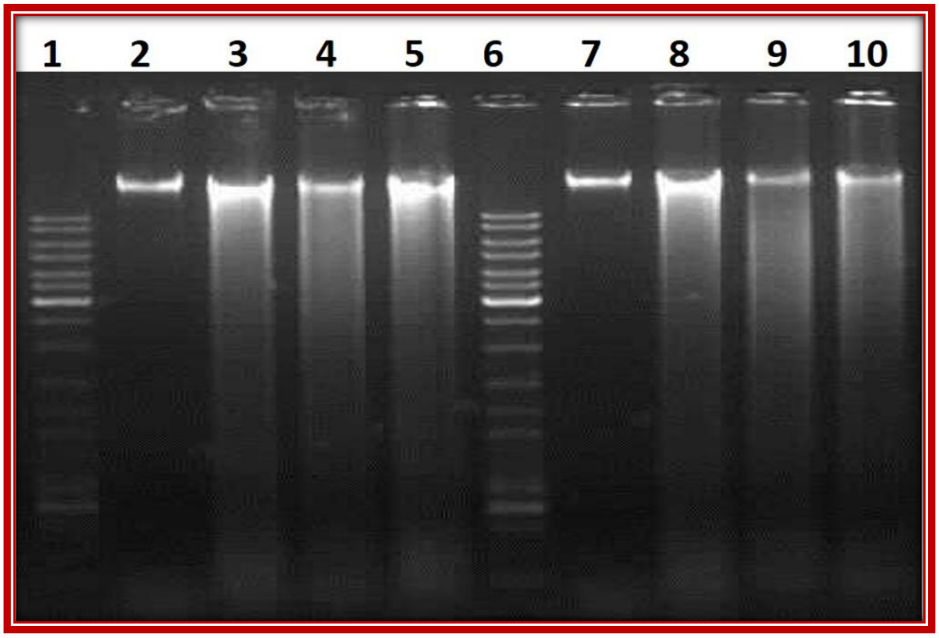


Fig. 3.12 (A): Analysis of DNA fragmentation in THP-1 cells using agarose gel electrophoresis. 1 kb marker **(Lane 1)**, Untreated cells- negative control **(Lane 2)**, Doxorubicin- positive control **(Lane 3)**, ANE-B fraction **(Lane 4)**, ANM-9 fraction **(Lane 5)**, 1000 bp marker **(Lane 6)**, Untreated cells- negative control **(Lane 7)**, Doxorubicin- positive control **(Lane 8)**, APH-11 fraction **(Lane 9)**, APM-3 fraction **(Lane 10)**.

In our results, we observed that DNA extracted from untreated cells showed no fragmentation, while DNA from active fraction treated cells at a concentration of 50 µg/mL showed disintegration as a result of endonuclease action at sites between nucleosomes. The patterns were consistent with nuclear fragmentation and condensation that occurs during apoptotic cell death. However, the smearing could be due to some post-apoptotic necrosis cells [Ramasamy et al., 2012]. Besides, the large band present at the top of the gels as observed in treated cells may represent large semi-fragmented pieces of DNA and indicates

incomplete apoptotic fragmentation in the sample material [Matassov et al., 2004]. Breakdown of DNA molecule is one of the sign of inhibition of DNA replication due to inter-nucleosomal cleavage associated with apoptosis and continuous smears may also indicate DNA fragmentation due to apoptosis. Most anti-cancer drugs of plant origin or chemically synthesised have been known to cause DNA damage or suppress its replication, not necessarily killing the cells directly but inducing apoptosis. During apoptosis, a specific nuclease (now known as caspase-activated DNase or CAD and pre-existed in living cells as an inactive complex) cuts the genomic DNA between nucleosomes and generates DNA fragments [Brown et al., 2005; Hanahan et al., 2000; Nagata, 2000; Wyllie 1980]. Our finding established that all the four fractions (APH-11, APM-3, ANE-B and ANM-9) possessed selective apoptosis induction effect in selected cancer cell lines. The constituents present in these active fractions might alkylate the DNA and cause DNA strand breakage and damage, leading to death of cancer cells.

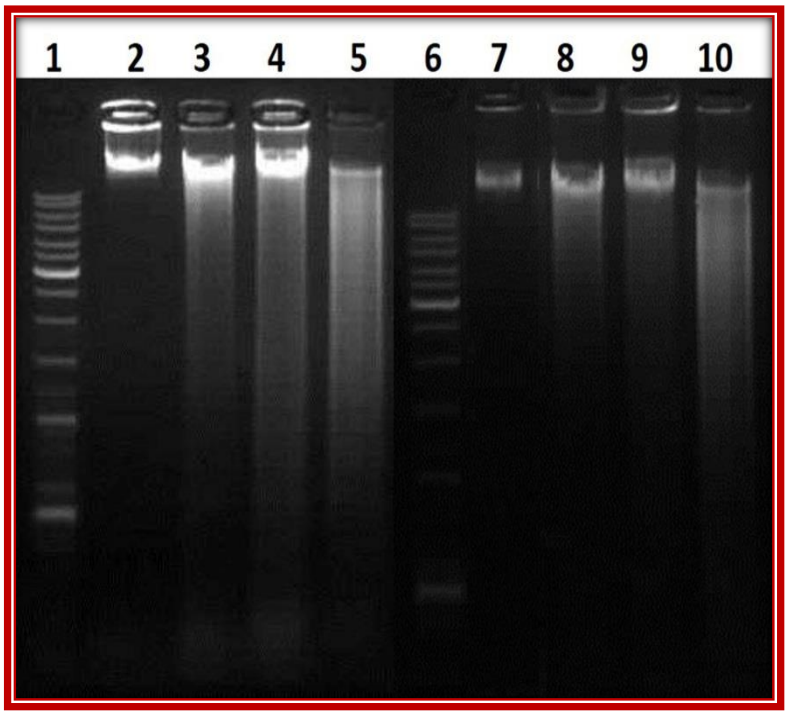


Fig. 3.12 B: Analysis of DNA fragmentation in SupT-1 cells using agarose gel electrophoresis. 1 kb marker **(Lane 1)**, Untreated cells- negative control **(Lane 2)**, Doxorubicin- positive control **(Lane 3)**, ANE-B fraction **(Lane 4)**, ANM-9 fraction **(Lane 5)**, 1000 bp marker **(Lane 6)**, Untreated cells- negative control **(Lane 7)**, Doxorubicin- positive control **(Lane 8)**, APH-11 fraction **(Lane 9)**, APM-3 fraction **(Lane 10)**.

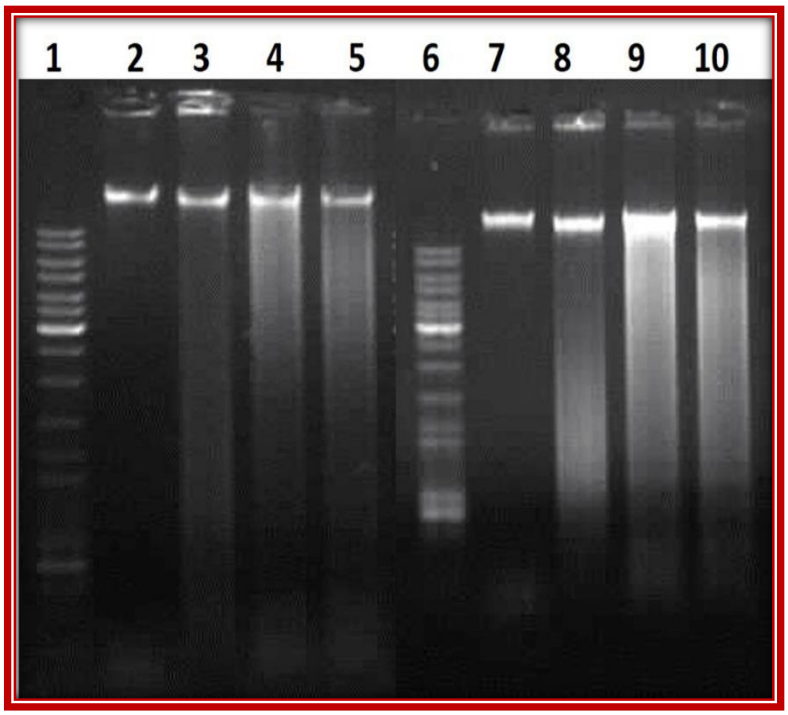


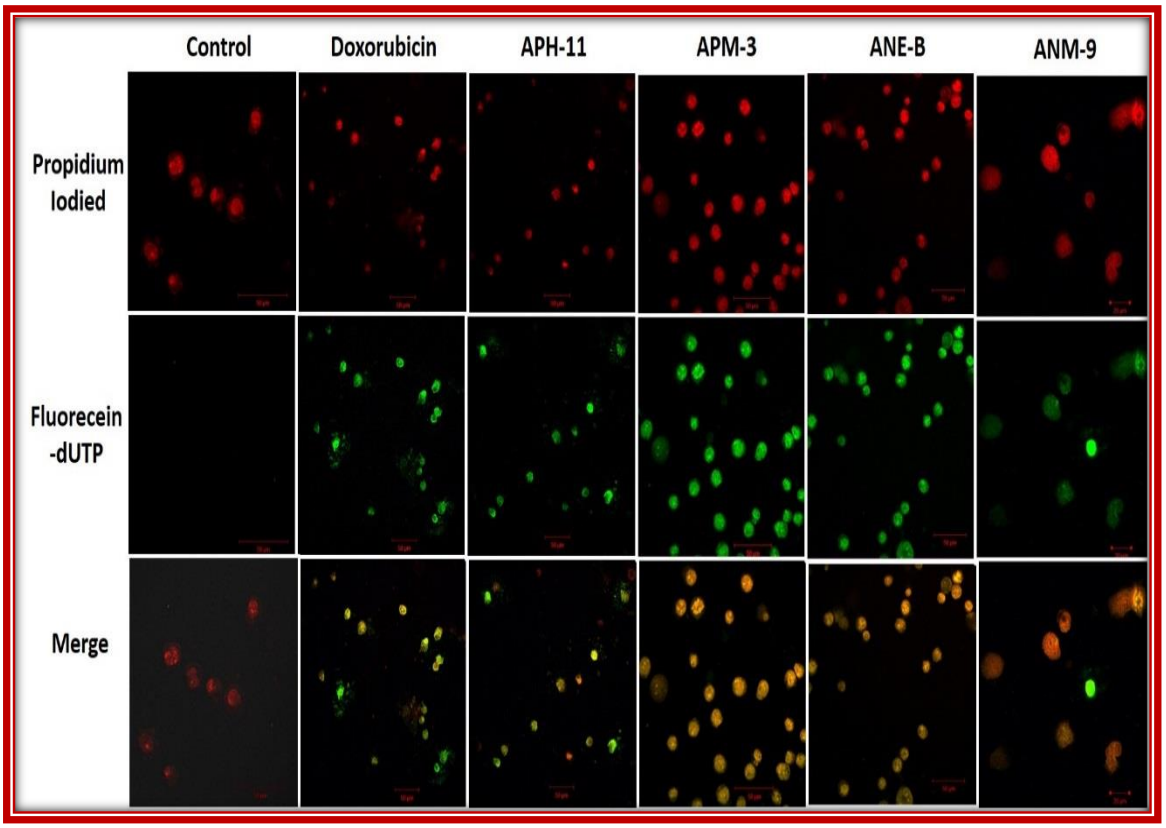
Fig. 3.12 (C): Analysis of DNA fragmentation in JM-1-1 cells using agarose gel electrophoresis. 1 kb marker **(Lane 1)**, Untreated cells- negative control **(Lane 2)**, Doxorubicin- positive control **(Lane 3)**, ANE-B fraction **(Lane 4)**, ANM-9 fraction **(Lane 5)**, 1000 bp marker **(Lane 6)**, Untreated cells- negative control **(Lane 7)**, Doxorubicin- positive control **(Lane 8)**, APH-11 fraction **(Lane 9)**, APM-3 fraction **(Lane 10)**.

3.10.2. DETECTION OF APOPTOSIS THROUGH TUNEL ASSAY:

The pro-apoptotic efficacy of active fractions were further verified and substantiated on cancer cell lines by TUNEL assay. Fragmented DNA has a free 3' hydroxyl group which is a substrate for binding of fluorescein – dUTP using TUNEL assay and provide a better evaluation of DNA damage in the cell. Apoptosis in cells usually leads to introduction of nicks in the genome and these nicks can be visualized as orange to yellowish in colour using the TUNEL assay by a fluorescent microscope. At the concentration of 50 µg/mL of active fractions (APH-11, APM-3, ANE-B and ANM-9), the significant increase in the number of apoptotic cells (positively TUNEL stained cells) with fragmented DNA was observed after the treatment of 48 h, thereby indicating DNA damage (Fig. 3.13A-3.13C). The pattern of TUNEL staining in treated cells confirmed that DNA fragmentation is initiated at the nuclear periphery and progresses towards the centre. Doxorubicin was used as positive control

which also showed a significant DNA damage in the cells whereas little or no significant DNA damage was observed in untreated cells.

The cleavage of nuclear DNA into nucleosome-sized fragments is a signature of apoptosis. Because such cleavage degrades the genetic materials that are crucial for producing cellular proteins, DNA fragmentation arguably represents the most severe damage to the cell [Zhang et al., 2002]. Once the morphological observation confirmed that fractions have ability to induce apoptosis, we switched on our study for TUNEL assay for quantification of apoptosis inducing ability of active fractions. Until today, the microscopic examination has been the gold standard for the most precise detection of apoptosis [Xiao et al., 2007; Yasuhara et al., 2003]. With this technique the whole process of apoptosis can be observed and evaluated based on the original morphological criteria [Wyllie et al., 1980].



ig. 3.13 (A): TUNEL nuclear staining on THP-1 cells. Cells were treated with 50 µg/mL of active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin for 24 h. Cells were collected, washed in phosphate-buffer saline, fixed and permeablised and subjected to TUNEL nuclear staining, mounted and viewed by fluorescence microscopy.

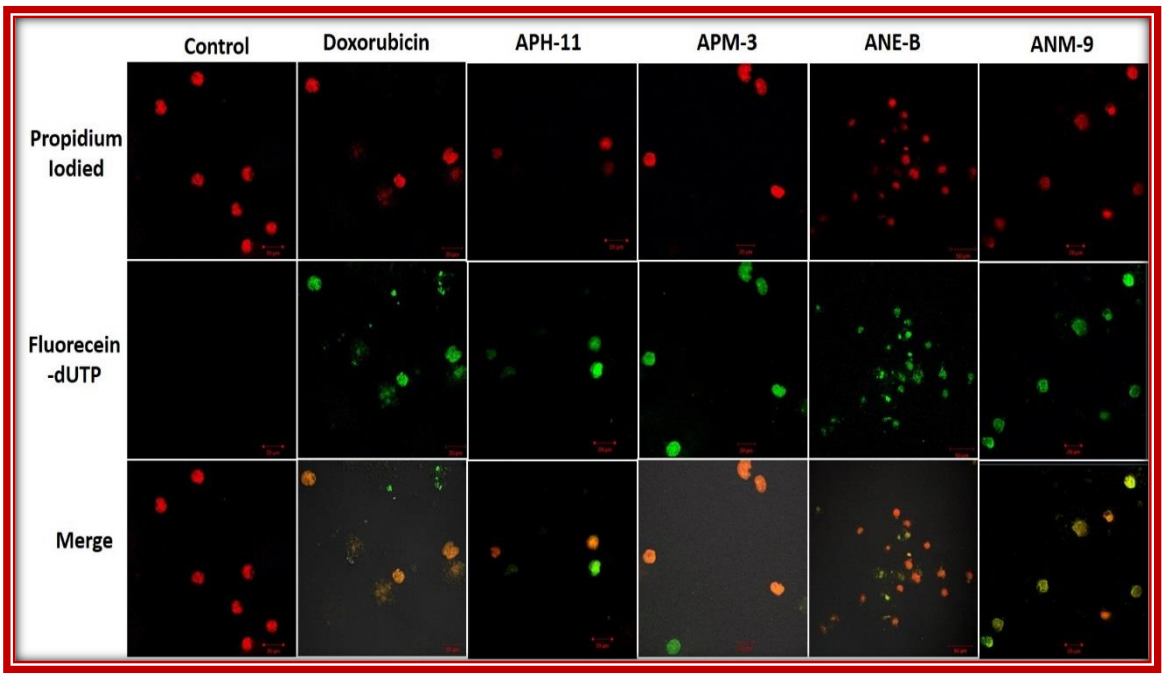


Fig. 3.13 (B): TUNEL nuclear staining on SupT-1 cells. Cells were treated with 50 µg/mL of active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin for 24 h. Cells were collected, washed in phosphate-buffer saline, fixed and permeablised and subjected to TUNEL nuclear staining, mounted and viewed by fluorescence microscopy.

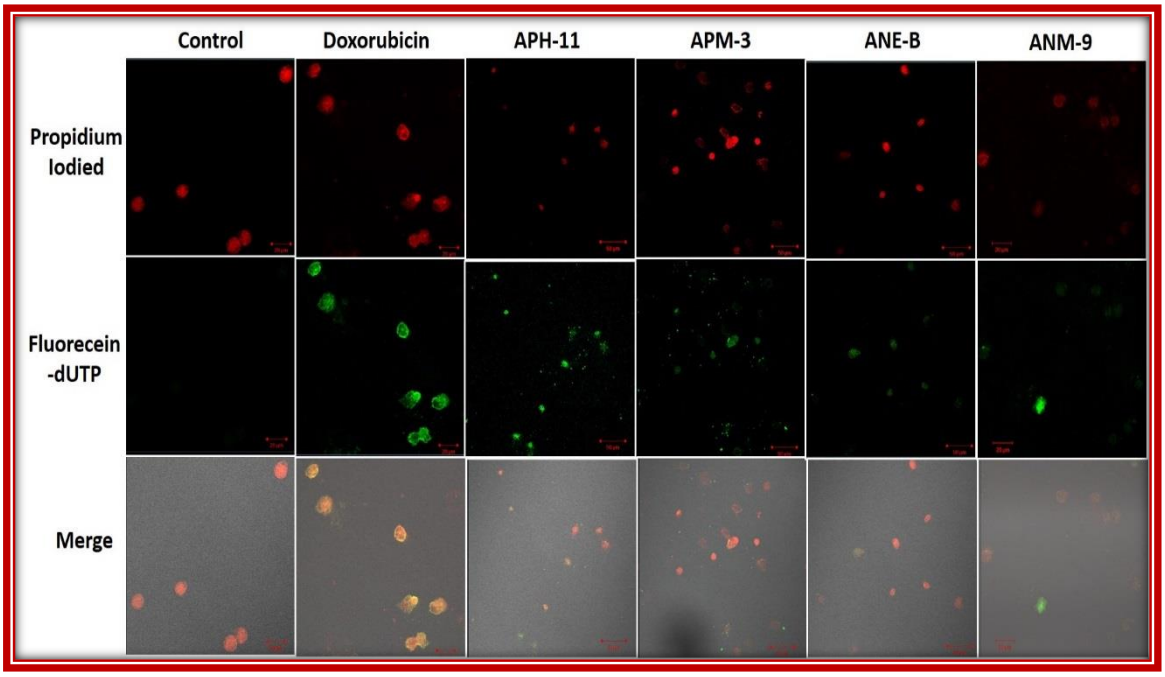


Fig. 3.13 (C): TUNEL nuclear staining on JM-1 cells. Cells were treated with 50 µg/mL of active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin for 24 h. Cells were collected, washed in phosphate-buffer saline, fixed and permeablised and subjected to TUNEL nuclear staining, mounted and viewed by fluorescence microscopy.

Apoptosis was originally defined by structural alterations in cells which can be observed by various microscopic techniques [Taatjes et al., 2008]. TUNEL assay is a common and an efficient combination of molecular biology and morphological observation method used to detect the intact single apoptotic nucleus or apoptotic bodies *in situ* accurately [Goh and Kadir, 2011; Hasan et al., 2011; Mazahery et al., 2009; Tan et al., 2013; Wu et al., 2013; Zakaria et al., 2009]. Extensive DNA degradation is a characteristic event which often occurs in the early stages of apoptosis. During apoptosis, the chromatin condenses against the nuclear envelope and the DNA inside the nucleus gets fragmented. Terminal deoxynucleotidyl transferase (TdT) when used with a fluorescent marker allows detection of cells with apoptotic DNA fragmentation. TdT is an enzyme that catalyzes the repetitive addition of dUTPs (Alexa 488) to the 3'-OH ends of a DNA fragment. In TUNEL analysis, the fluorescently conjugated dUTP are added to the 3'-OH groups of the DNA fragments making the apoptotic cells visible by fluorescent microscopy [Darzynkiewicz et al., 2008; Meenaksh et al., 2003; Xu et al., 2000]. This approach is also helpful to differentiate cellular apoptosis and necrosis [Gold et al., 1994]. Therefore, we further confirmed our results by TUNEL assay and the results showed that TUNEL positive were rarely observed in cells of the control group and in contrast, a number of apoptotic cells were observed in the treated cells. The morphological observation revealed that both extra and intracellular structures were intensely affected following the fraction treatment. The reduction of number of viable cells in the treatment was in accordance with their cytotoxicity property showed by the fractions. These cells had typical morphological changes associated with apoptosis such as DNA fragmentation and apoptotic body formation as observed under fluorescence microscopy on staining. It is well known that, in apoptosis, the earliest recognized morphological changes are compaction and segregation of the nuclear chromatin, with the result of chromatin margination and condensation of the cytoplasm [Kerr et al., 1972]. Therefore, our results indicated that active fractions can inhibit the proliferation of cancer cells via apoptosis induction.

3.10.3. FLOW CYTOMETRY FOR CELL CYCLE ANALYSIS AND DETECTION OF APOPTOTIC CELLS:

Since cell cycle progression is an essential event for cellular growth, we examined whether the cell growth inhibitory effect by active fractions by monitoring the alteration in a specific phase of the cell cycle. In this experiment, flow cytometric analysis of the three

different treated leukemic cancer cells was conducted after staining cells with propidium iodide. The results obtained in the present study provide convincing evidence that the fractions exerted their effects on cell cycle progression. The relative DNA content in treated and untreated cancer cells was represented as three distinct stages within the interphase of the cell cycle, called sub G0, G1, S and G2/M phases. In treated THP-1 cells, the cell cycle profile was altered after the treatment. Table 3.6; Fig. 3.14A showed that treatment with *A. precatorius* fractions resulted in a significant increase in sub-G0 population (apoptotic cells) with time compared to control group (APH-11; 70.8% for 24 h, 67.9% for 48 h; APM-3; 41.7% for 24 h, 52.5% for 48 h). APH-11 treatment exhibited growth arrest in S- phase as there was no change in G1 and G2/M percentage cells at 24 and 48 h, respectively but APM-3 exhibited growth arrest at G1 phase due to which there is decrease in S and G2/M cells at the time point of 24 h and 48 h. Due to the growth arrest in cell cycle the cells were not able to recover from the arrest so cells enter the sub G0 phase which ultimately leads to apoptosis. In case of *A. nilagirica* treated THP-1 cells, it was also found that its active fractions can effectively alter cell cycle distribution of THP-1 cells. After 24 h incubation with both fractions, stability was generally noticed in all cell cycle sub-populations of the THP-1 cells, with a drastic increase in sub-G0 population in treated cells. We have also observed decrease in the S-phase sub-population in treated cells compared to control. After 24 h of treatment, the sub-G0 population sharply increased to 49.5% in ANE-B treated cells and 32.0% in ANM-9 treated cells compared to control cells. Concomitantly, there is a decrease in G2/M and S-phase populations, in both ANE-B and ANM-9 treatment compared to the control. At 48 h, there is a significant increase in sub G0 population up to 64.4% in ANE-B and 71.8% in ANM-9 in comparison to control. We have observed that a maximum of sub-G0 cell population was reached at 48 h post treatment. The positive control doxorubicin treatment also showed increase in sub-G0 population of 58.8% at 24 h and 64.6% at 48 h, respectively (Table 3.6; Fig. 3. 14A).

In SupT1 cells, the active fractions induced time-dependent alteration of cell cycle, associated with a significant increase in sub G0 population of the cells. Results showed that there were no changes in the population of the untreated cells. Treatment with *A. precatorius* fractions resulted in a significant increase in sub G0 population (apoptotic cells) with time compared to control group (APH-11: 25.3% for 24 h, 33.5% for 48 h; APM-3; 21.3% for 24 h, 14.8% for 48 h). The fractions of *A. nilagirica* at the concentration of 50 µg/mL can

also effectively alter the cell cycle distribution of SupT-1 cells in a time-dependent manner. After 24 h incubation with the both fractions there was an increase in sub G0 population in treated cells. We have also observed decrease in the S phase population in treated cells compared to control. After 24 h of treatment, the sub G0 population sharply increased to 13.9% in ANE-B treated cells and 23.3% in ANM-9 treated cells compared to control cells. Concomitantly, there is a decrease in G2/M and S-phase populations, in both ANE-B and ANM-9 treatment compared to the control. Similarly at the 48 h of treatment, we found further increase in sub G0 sub-population up to 43.2% and 51.9% after ANE-B and ANM-9 treatment respectively in comparison to control cells. We have observed that a maximum of sub G0 cell population was achieved 48 h post treatment. The positive control doxorubicin treatment also showed increase in sub G0 population of 7.7% at 24 h and 18.4% at 48 h, respectively (Table 3.6; Fig. 3.14B).

The DNA content and cell cycle distribution was also analysed in treated and untreated JM-1 cells using flow cytometry. A typical time dependent alteration of cell cycle distribution in JM-1 cells was observed with 50 µg/mL (Table 3.6; Fig. 3.14C). On the treatment of APH-11, the sub G0 population was found to be 28.1% at 24 h and 44.5% at 48 h respectively. But, in APM-3 treated cells, this pattern was not observed which may be due to its ineffectiveness in inducing the apoptosis. After 24 h treatment, the population of cells in sub G0 phase was 14.9%, while after 48 h of treatment, the population of cells in sub G0 phase slightly decreased to 11.4%. Likewise, the fractions of *A. nilagirica* displayed alteration of cell cycle distribution of JM-1 cells where the sub G0 populations increased from 56.2% to 92.7%, for two time points (24 h and 48 h) when cells were treated with ANE-B. Similar results were observed when JM-1 cells were treated with ANM-9 at the same time points. The sub G0 populations increased from 39.3% to 85.9% from 24 h to 48 hr post treatment. The positive control doxorubicin treatment showed increase in Sub G0 population from 82.1% at 24 h and 94.0% at 48 h respectively. Taken together, the results indicated that the active fractions of *A. precatorius* and *A. nilagirica* mediated growth inhibition of three different cancer; THP-1, SupT-1 and JM-1 was associated with alteration of different phases of cell cycle ultimately leading to apoptosis.

Uncontrolled cellular proliferation is a hallmark of all cancer cells, and the blockade of the cell cycle is regarded as an effective strategy for eliminating cancer cells [Adhami et al., 2004]. The quantitative analysis of cell cycle is very important in the study of molecular

Table 3.6: Cell cycle phase distribution of three different leukemic cells treated active fractions of *Abrus precatorius* and *Artemisia nilagirica* and Doxorubicin.

Sample	Cell Line: THP-1							
	Time points							
	24 h				48 h			
	Cell cycle phases							
	SubG0	G1	S	G2/M	SubG0	G1	S	G2/M
Neg. control	20.9	44.5	12.3	22.3	23.2	43.5	12.3	21.0
Doxorubicin	58.8	19.5	8.4	13.4	64.6	13.5	9.3	12.6
APH-11	70.8	14.1	9.3	5.8	67.9	14.6	10.8	6.7
APM-3	41.7	27.7	12.9	17.6	52.5	28.1	15.3	14.1
ANE-B	49.5	25.8	9.1	15.6	64.4	17.3	7.9	10.4
ANM-9	32.0	44.6	8.9	14.5	71.8	17.2	6.8	4.1
Sample	Cell Line: SupT-1							
	Time points							
	24 h				48 h			
	Cell cycle phases							
	SubG0	G1	S	G2/M	SubG0	G1	S	G2/M
Neg. control	5.1	53.4	28.1	8.0	9.4	53.1	20.5	4.6
Doxorubicin	7.7	54.3	32.9	2.2	18.4	46.0	28.7	2.3
APH-11	25.3	36.2	28.9	5.8	33.5	28.8	21.7	9.4
APM-3	21.3	47.4	18.9	4.9	14.8	33.4	31.3	6.1
ANE-B	13.9	48.4	26.0	5.4	43.2	33.8	15.2	5.2
ANM-9	23.3	24.1	37.5	10.4	51.9	18.5	20.4	5.4
Sample	Cell Line: JM-1							
	Time point							
	24 h				48 h			
	Cell cycle phases							
	SubG0	G1	S	G2/M	SubG0	G1	S	G2/M
Neg. control	10.0	56	18.8	15.2	15.1	58.0	14.9	12.1
Doxorubicin	82.1	7.8	7.3	2.7	94.0	3.0	2.1	0.8
APH-11	28.1	47.3	10.2	14.4	44.5	39.2	8.2	8.1
APM-3	14.9	59.3	11.0	14.8	11.4	68.0	8.4	12.2
ANE-B	56.2	25.5	7.9	10.4	92.7	4.8	1.7	0.8
ANM-9	39.3	34.6	10.2	15.8	85.9	9.3	2.5	2.2

The data is from one of the two independent experiments with similar results

mechanism of cell death and cell cycle progression [Tao et al., 2004]. Cell cycle is required to monitor and maintain the genomic integrity of cells by means of a complex network of DNA repair pathways or cell cycle checkpoints. In recent years, many chemotherapeutic agents have been shown to impart anti-proliferative effects via arrest of cell division at certain checkpoints in the cell cycle [Adhami et al., 2004; Kögel et al., 2010]. Cell cycle checkpoints detect various types of structural defects in DNA, or in chromosome function and induce a multifaceted cellular response that activates DNA repair and delays cell cycle progression [Bartek and Lukas, 2001]. DNA damage is one of the major mechanisms behind anti-cancer drug-induced cell cycle arrest and apoptosis [Cai et al., 2007; Zhu et al., 2007]. Under normal conditions, genomic integrity in DNA damaged-cells is usually restored through DNA repair. If DNA repair cannot be achieved properly or cells are overwhelmed by sustained damage, apoptosis ensues in order to remove genetically aberrant cells [Ljungman 2010; Norbury and Zhivotovsk, 2004]. Apoptosis induction and/or cell proliferation inhibition is extremely correlated with the activation of a variety of intracellular signalling pathways to arrest the cell cycle in different phases. The process of replication and division of chromosomes within the nucleus prior to cell division can be described by the cell cycle. It is found that cancer cells develop when the normal mechanisms for regulating cell cycle gets disrupted, therefore, it is essential to identify the genetic basis for this disruption [Ravi et al., 2011]. Cell cycle kinetics is one of the ways of detecting the changes in cell cycle pattern due to effect of a therapeutic drug or effect of specific genes [Kessel and Luo, et al., 2000; Tian et al., 2006]. Flow cytometric analysis of cell cycle measures the apoptotic changes in cells by staining them with DNA dyes [Telford et al., 1994]. This method is useful for quantitative estimates of the fractions of cells in the different phases of the cell cycle [Ali et al., 2011]. Cell cycle modulators of natural and synthetic origin are gaining widespread attention in recent years. Given that disruption of cell cycle plays a crucial role in cancer progression, its modulation by phytochemicals seems to be a logical approach in control of carcinogenesis [Singh et al., 2002]. There are a number of herbs that have shown the ability to induce cell cycle arrest and to play an important role in cancer prevention and therapy [Huang et al., 2009]. Our findings on cell cycle analysis indicated that the used concentration of fractions was capable of triggering cell death of cancerous cell lines in a time dependent manner and maximum of sub-G0 cell population was reached 48 h post treatment. However, a slight increase was observed at Sub G0 phase of untreated cells over time. The observed

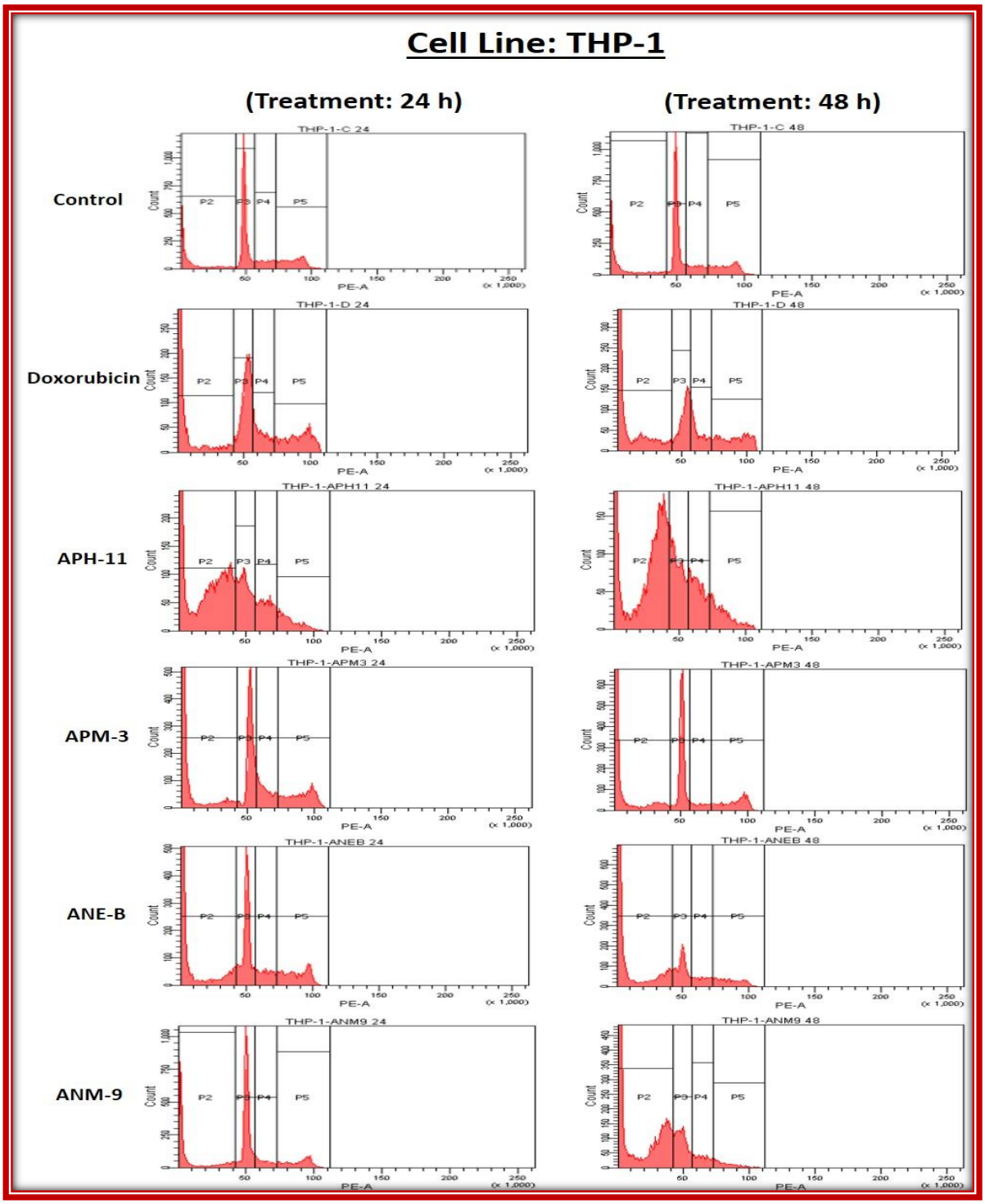


Fig. 3.14 (A): Effect of active fractions of *Abrus precatorius* and *Artemisia nilagirica* on cell cycle distribution. Histogram of cell cycle distribution in active fractions of *Abrus precatorius* and *Artemisia nilagirica* treated THP-1 cells. After exposure to 50 µg/mL of the active fractions for 24 h and 48 h, cells were harvested, stained with propidium iodide, and analysed by flow cytometry. The results are representative of three separate experiments.

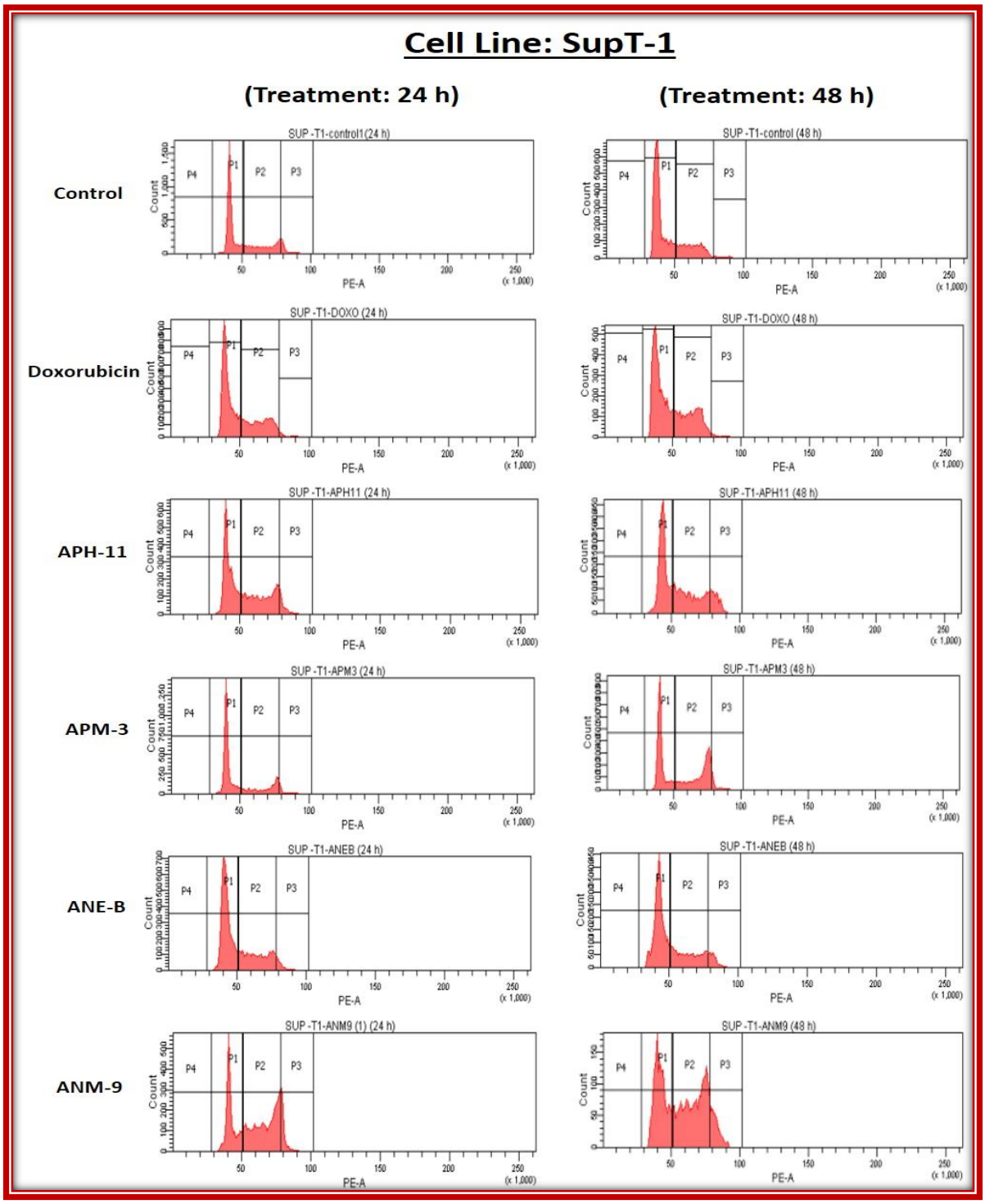


Fig. 3.14 (B): Effect of active fractions of *Abrus precatorius* and *Artemisia nilagirica* on cell cycle distribution. Histogram of cell cycle distribution in active fractions of *Abrus precatorius* and *Artemisia nilagirica* treated SupT1 cells. After exposure to 50 µg/mL of the active fractions for 24 h and 48 h, cells were harvested, stained with propidium iodide, and analysed by flow cytometry. The results are representative of three separate experiments.

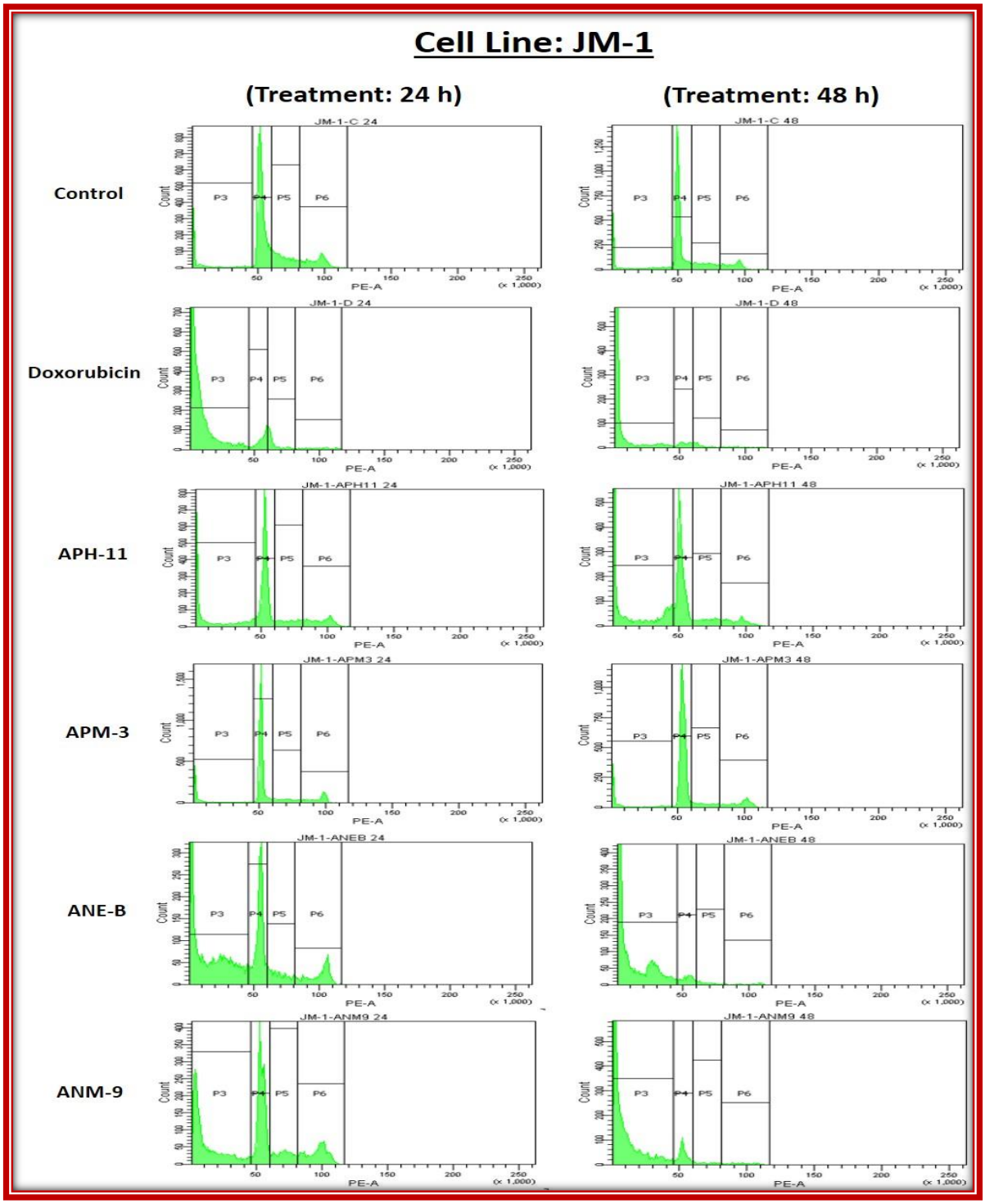


Fig. 3.14 (B): Effect of active fractions of *Abrus precatorius* and *Artemisia nilagirica* on cell cycle distribution. Histogram of cell cycle distribution in active fractions of *Abrus precatorius* and *Artemisia nilagirica* treated SupT1 cells. After exposure to 50 µg/mL of the active fractions for 24 h and 48 h, cells were harvested, stained with propidium iodide, and analysed by flow cytometry. The results are representative of three separate experiments.

effects could be mediated by two sub-classes of cytotoxic molecules, a first class could act fast and require high concentration to induce cell death, and a second class plays a role in cell cycle arrest by preventing the G1-to-S transition. The checkpoint arrest at G1/S of the cell cycle is pivotal to let the DNA to be repaired and prevents the replication of damaged cells [Vermeulen et al., 2003]. Alternatively, all these effects could be attributed to a single molecule. This conclusion arises from previous similar results described in the literature with Resveratrol [Opipari et al., 2004]. Although the majority of cells were still accumulated in the S phase at 48 h, some were arrested at the G2/M phase. This difference in response may be due to the possible effect of several differently active components in the sub-fractions. It is indeed established that the content of DNA remaining in apoptotic cells for cytometric analysis varies markedly depending on the extent of DNA degradation and cell washing steps [Vermes et al., 1995].

10.3.4. MECHANISM OF CELL DEATH THROUGH IMMUNOBLOTTING:

The induction of apoptosis in tumour cells is considered very useful in the management and therapy of cancer. Activation of aspartate-specific cysteine proteases also known as caspases is a crucial biochemical event during apoptosis. Different caspases are activated during the initiation and execution phases of apoptosis. Procaspases-7, 8 and 9 as initiator are activated by self-processing and cleave downstream procaspase-3 to active dimeric form of caspase-3 as executioner of apoptosis. PARP (poly-ADP ribose polymerase) cleavage is triggered by the Caspase-3 which is the effector caspase and it is one of the hallmarks of apoptosis. To delineate the possible signalling pathways by which active fractions induced apoptosis in three cell lines, the changes in the expression levels of various apoptosis-regulating proteins effector caspases (caspase-3 and -7), and PARP were studied by western blotting. Doxorubicin (10 µg/mL), a conventional drug inducing caspase-dependent apoptosis was used as positive control. After exposure to active fractions of both the plants at the concentration of 50 µg/mL for 24 h, the active fractions induced activation of procaspase-7 (38 kDa) into cleaved caspase-7 (20 kDa) and cleavage of procaspase-3 (32 kDa) into caspase-3 (20 kDa) in THP-1 cells (Fig. 3.15A-3.15C). Surprisingly, active caspase-7 and active caspase-3 were absent after treatment with the fractions in SupT1 and JM-1 cells, even with alteration of concentrations and incubation times. The cleavage of PARP from the native 116 kDa to a truncated 85 kDa product, which is one of the substrates for caspase-3,

is also a characteristic of apoptosis which was evident in all the three human cancer cells and the fractions cleaved PARP into 85 kDa fragment. The cleavage of PARP suggesting further that PARP might answer, at least in part, for the ultimately apoptotic death of leukemic cell lines induced by the active fractions. When compared with the results of the DNA-fragmentation and TUNEL assays, activation of caspase and PARP cleavage happened very fast and was an early event during active fraction induced apoptosis in all the three cell lines. Therefore, our results indicated that APH-11, APM-3, ANE-B and ANM-9 can induce caspase dependent apoptosis in THP-1 cells, which is parallel to apoptosis detected by the DNA-fragmentation and TUNEL assays. While in SupT1 and JM-1 cells the results suggested that some other pathways may be involved in fraction-induced cell death in these cell lines. Apoptosis is a multi-step, multi-pathway program for cell death that is inherent in every cell of the body. It plays a crucial role in eliminating the mutated or proliferating neoplastic cells from the biological system and it is characterised by a sequential cascade of cellular events, resulting from chromatin condensation, DNA fragmentation, cytoplasmic membrane blabbing and cell shrinkage [Bøe et al., 1991; Hickman, 1992]. Loss of apoptosis in cancer cells is the key event for the process of cancer development. Successful treatment with chemotherapeutic drugs largely depends on their ability to trigger cell death in cancer cells, which at least partially involves activating apoptosis [Barry et al., 1990; Bose et al., 1995; Fisher, 1994; Hannun, 1997]. Apoptosis can be triggered in a cell through two fundamental pathways: the mitochondrial or intrinsic pathway and the death receptor or extrinsic pathway [Bröker et al., 2005; Hsu et al., 2009]. A family of cytosolic proteases, the caspases, can be divided into two major subgroups, initiator (caspases-2, -8, -9, and -10), which activate the effector caspases (caspases-3, -6 and -7). Initiator caspases are activated by apoptotic signals, resulting in the activation of the effector caspases [Devarajan et al., 2002; McGee et al., 2002; Thornberry and Lazebnik, 1998]. Caspase-3, the most commonly activated caspase that plays a critical role in apoptotic pathway by cleaving some proteins, like PARP and exhibits some of the typical morphological and biochemical features such as membrane blabbing, induction of phagocytes migration to the site of apoptotic cells and DNA fragmentation [Jänicke, 2009; Thornberry and Lazebnik, 1998; Wolf and Green, 1999].

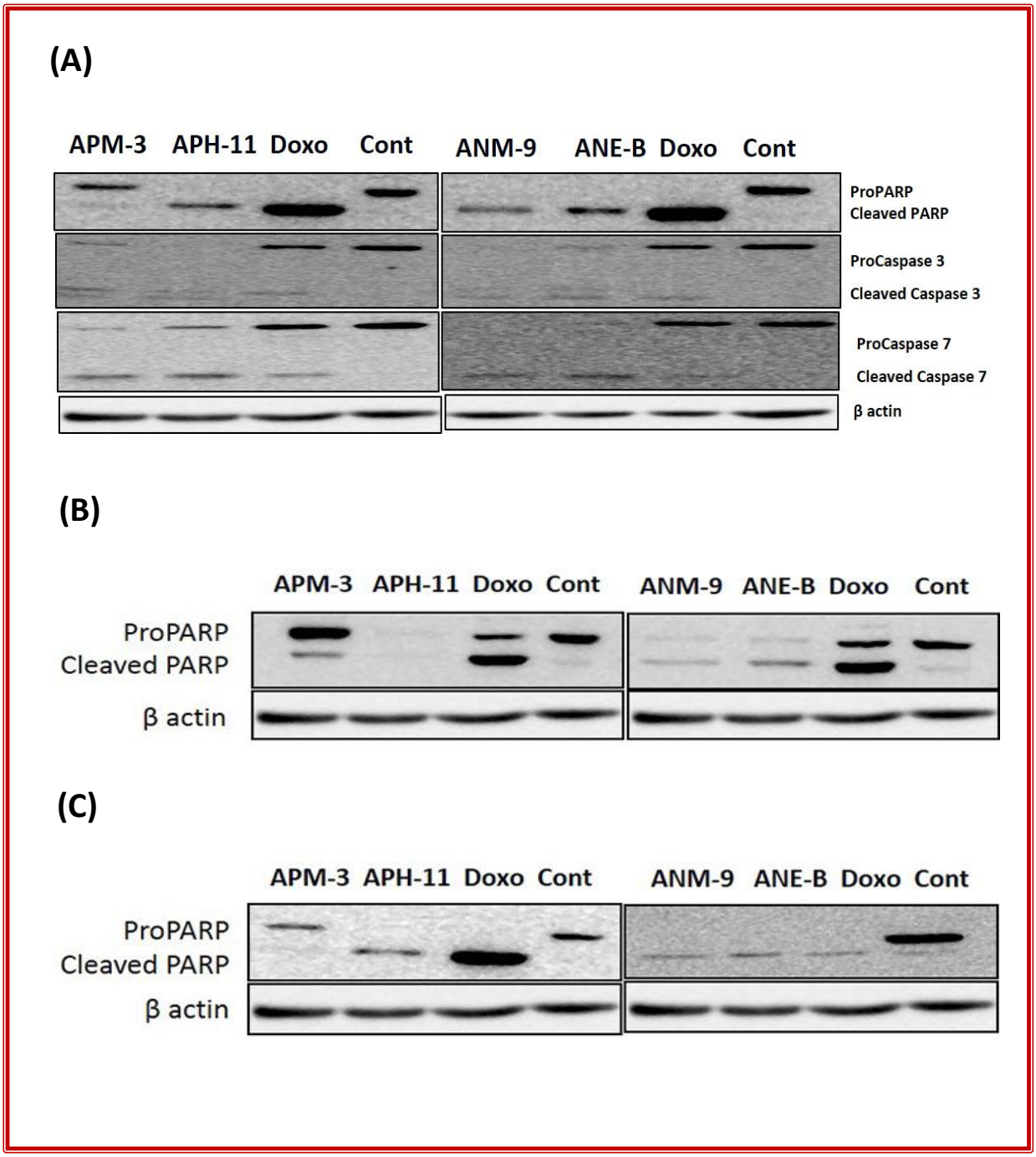


Fig. 3.15: Effects of active fractions of *Abrus precatorius* and *Artemisia nilagirica* on activation of caspases. **(A)**: Immunoblots of total cell lysates from THP-1 cells, revealing the cleavage of PARP, Caspase 3 and 7 demonstrating the occurrence of apoptosis with caspase 3 activation. Immunoblots of total cell lysates from **(B):** SupT1 and **(C):** JM-1 cells showing the presence of the cleaved form of PARP, hall marks of apoptosis, at the same time, the blot reveals the absence of active caspase 3, demonstrating the occurrence of apoptosis without caspase 3 activation.

In the present work, western blotting revealed that the active fractions (APH-11, APM-3, ANE-B and ANM-9) activated the caspase 7, caspase 3 cascade and cleaved PARP, in THP-1 cells. Cleaved fragment of PARP supports the finding that the processing of procaspase-7 and procaspase-3 resulted in its catalytic activation. PARP binds to DNA breaks and generates polymers of ADP-ribose bound to chromatin-associated proteins, including itself, thus making it accessible to DNA repair enzyme. The possible mechanism by which these fractions induced apoptosis in THP-1 cells was caspase dependent pathway in which sequential activation of caspase-7 and caspase-3 and ultimately, PARP cleavage. These observations were in agreement with earlier reports in which plant extracts/compounds were shown to induce apoptosis through the activation of caspases in different human cancer cell lines [Elkady et al., 2012; Hsieh et al., 2013; Kottke et al., 2002; Looi et al., 2013; McGee et al., 2002; Valiyari et al., 2013; Wong et al., 2013]. However, more investigation on initiator caspase 8 and 9 would be required to distinguish between the extrinsic and intrinsic apoptosis pathways [Elmore, 2007; Nicholson, 1999]. Furthermore, to our surprise, the active fractions, on the other hand, were unable to augment the assembly of the SupT1 and JM-1 death-inducing signalling complex responsible for the activation of cleaved PARP in the experimental conditions. This signified the caspase-independent mechanism might be involved in the cell death by these fractions in these two cell lines. Although caspases may be a necessary factor in the execution of programmed cell death, the process of caspase activation is not the sole factor in determining the triggering of apoptosis. It can be inferred that death of SupT1 and JM-1 cells might be related to autophagy, triggered by metabolic stress created by damaged mitochondria that caused an energy-deprivation state, or the autophagy is coupled to an apoptosis cell death independent of caspase 3 activation, since we noticed occurrence of DNA damages related apoptosis (DNA fragmentation and presence of cleaved PARP). Some studies have reported the model of caspase-independent cell death in different cell types, such as Jurkat, MCF-7 and Hela cells [Chen and Wong; 2009; Wang et al., 2012; Xiang et al., 1996]. Taken together, our present investigation indicated that all the three human leukaemia cell lines treated with *A. precatorius* and *A. nilagirica* fractions underwent phytochemical-specific programmed cell death, and did not induce non-specific necrotic death in the cells. These findings suggested that the activation of caspases involved in the apoptotic pathway is one of the major mechanisms by which these fractions affected THP-1 cells, whereas it can be hypothesized that anti-proliferative effectiveness of fractions

may invoke other possible pathways in SupT1 and JM-1 cell lines and further studies are necessary to investigate the precise mechanisms responsible.

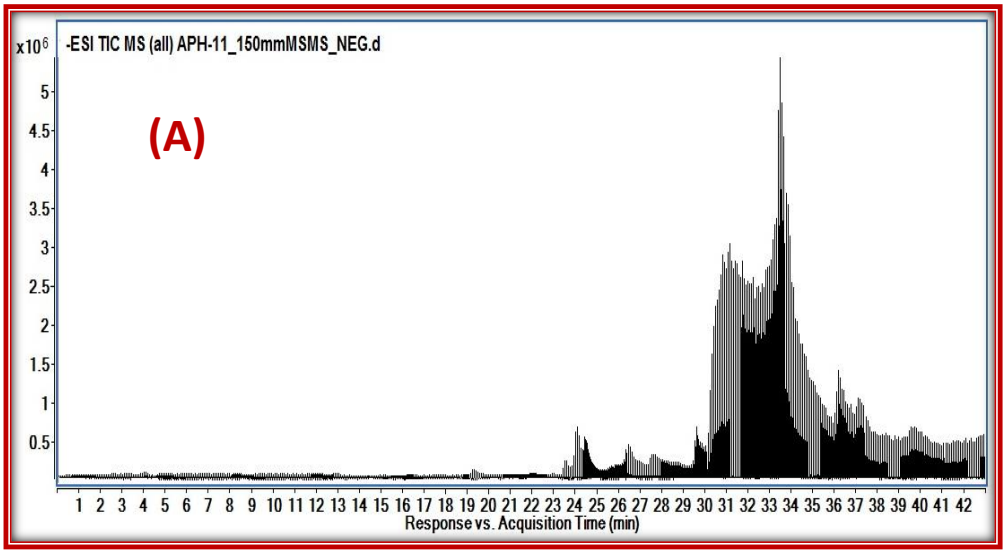
10.4. METABOLITE PROFILING OF ACTIVE FRACTIONS BY LC-MS-MS ANALYSIS:

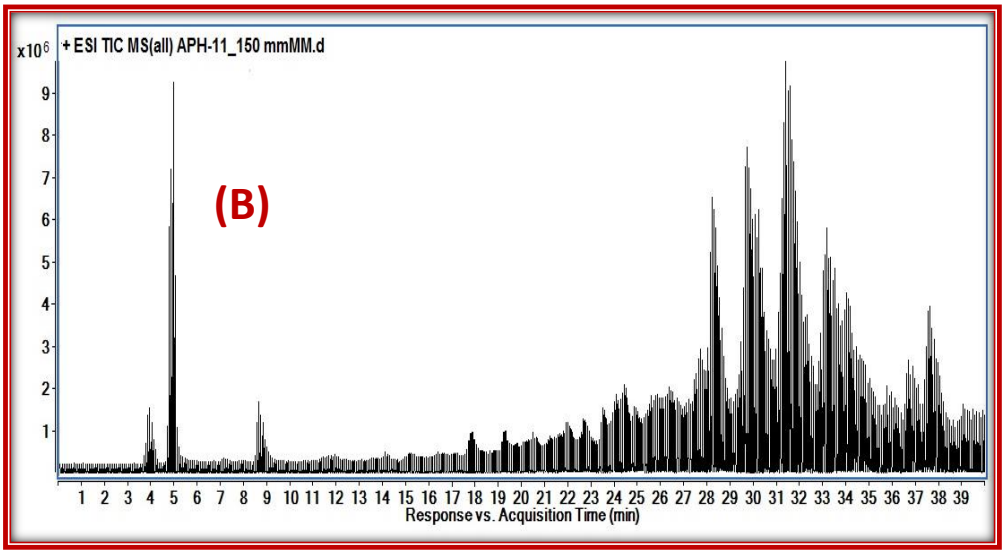
Isolation and identification of bioactive compounds present in a crude extract/semi-purified samples, has emerged as the major path of drug development from natural products particularly medicinal plants besides their biological activities. Isolation and identification of bioactive compounds found in crude extracts/semi-purified fractions as building blocks of new/novel drug provides unique opportunities for the development of new drug with new mechanisms of action to treat various human ailments. There is an enormous bioactive compound diversity of a plants extracts/semi- purified fractions to be covered. This presents a considerable challenge for the isolation and identification of bioactive compounds. During the last decade, Liquid chromatography (LC) combined with mass spectrometry (MS) techniques were developed employing soft ionization methods like electro-spray (ESI) or photoionization (APPI) and, simultaneously, mass spectrometers have become both more sophisticated and more robust modern day research. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful tool in inter alia, pharmaceutical and plant metabolism analytics, and has been widely used to analyse complex mixtures, such as biological samples [Chen et al., 2009; Cuyckens and Claeys, 2004; Korfmacher, 2005; Spacil et al., 2010]. This technique has gained attention as a convenient method for identification, structural determination and quantitative analysis of various bioactive compounds in complex extracts. LC-MS is considered to be a major breakthrough in the analysis of natural products possessing significant biological activities. Of the various LC-MS techniques, high performance liquid chromatography coupled to electro spray ionization-mass spectrometry (HPLC-ESI-MS) is an ideal technique for the analysis of a wide variety of analytes [Niessen et al., 1998]. This soft ionization technique coupled to a single quadrupole mass spectrometer provides the molecular weight of compounds, and further structural details can be obtained with HPLC-MS-MS systems (those with triple quadrupole or ion trap mass analysers), which provide information on the characteristic fragmentation pattern typical of a compound. In contrast to other ionization techniques, multiple charged analytes can easily be formed with ESI, making it possible to analyse larger molecules. Small polar to medium polar analytes are also readily ionized with this technique, making HPLC-ESI-MS useful in phyto-chemical

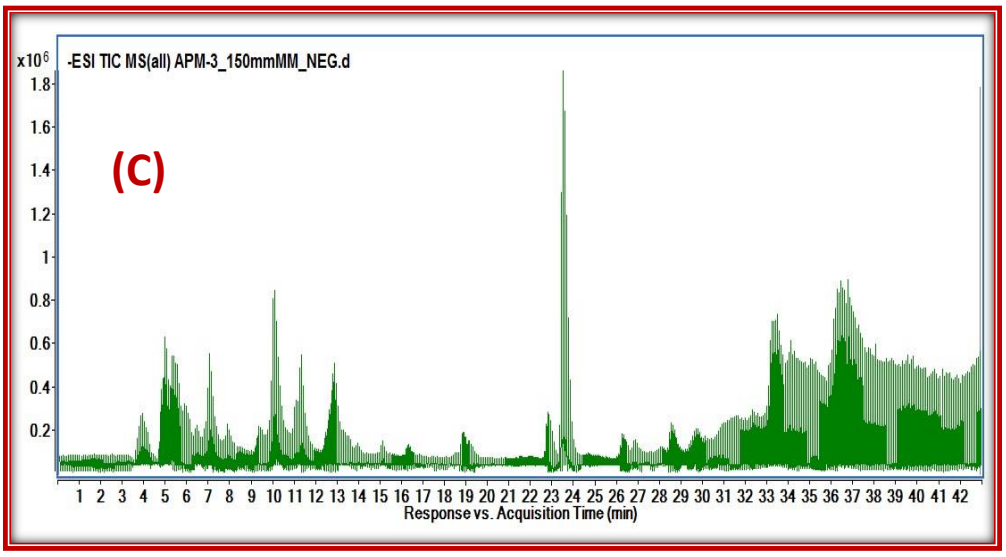
investigation [Moberg, 2006]. In most quantitative HPLC-ESI-MS methods, low detection limits and reproducible results are the desirable features. Hence, in this study, LC-MS/MS technique was utilized to identify the bioactive compounds with anti-cancer activity from active fractions of *A. precatorius* and *A. nilagirica*.

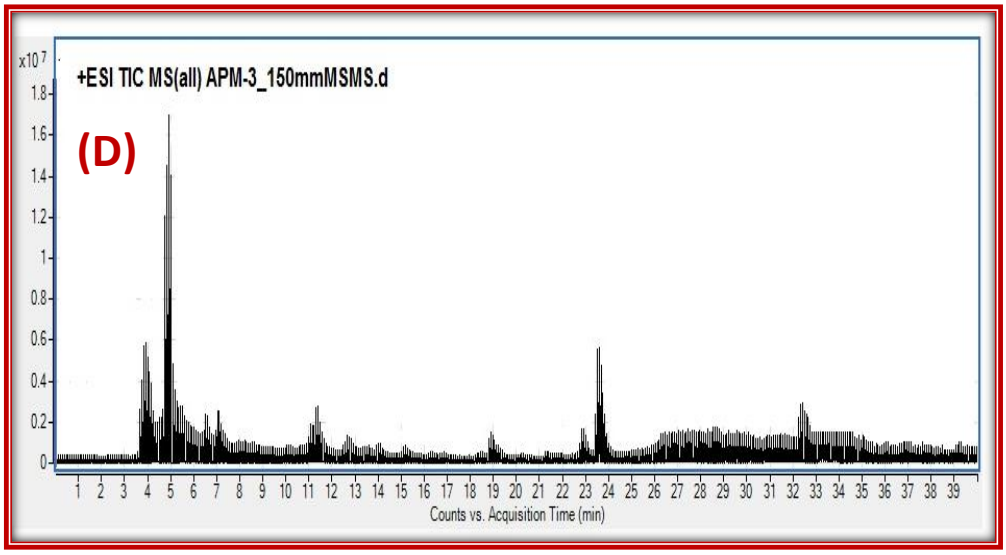
Phenolic compounds constitute one of the most numerous and ubiquitous groups of plant metabolites, and are an integral part of the human diet. In recent past, many epidemiological studies have shown that plant-derived polyphenols are promising nutraceuticals associated with a reduced risk of a number of chronic diseases, including cardiovascular disease (CVD) neurodegenerative disorders and neoplastic diseases [Ullah and Khan, 2008; Vauzour et al., 2010]. The phenolic compounds from a large variety of plants have been shown to inhibit or attenuate the initiation, progression and spread of cancers in cells *in vitro* and in animals *in vivo* [Dai and Mumper, 2010]. Furthermore, plant-derived polyphenolic compounds have shown anti-cancer properties against different types of cancers [Bracke et al., 2008; deKok et al., 2008; Fresco et al., 2006; Lamoral-Theys et al., 2010; Rodríguez et al., 2012; Wilken et al., 2011], which explains the high interest and initiation of many studies to evaluate the biological activities as well as bio availabilities of polyphenolic compounds. Consequently, the identification and development of cancer chemo-preventive phenolic agents has become, most relevant issue in public health-related research in the last few years. In this direction, we made an attempt to identify the bioactive constituents in the active fractions.

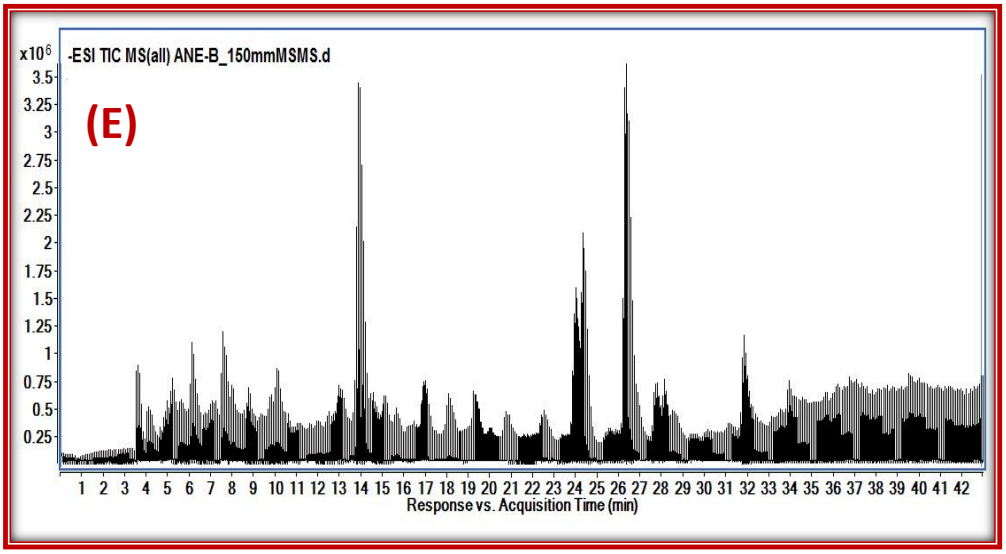
In order to identify putative bioactive compounds responsible for above described anti-proliferative effects, all the four active fractions; APH-11, APM-3, ANE-B and ANM-9 were subjected to LC-MS/MS analysis using hyphenated technique LC-DAD-ESI-MS. DAD-UV is absorption maxima λ of 250, 260, 272, 280, 300 nm(s) were employed which are characteristic for polyphenolic class of compounds particularly flavonoids and coumarins [Markham, 1982]. Analysis of samples using high-resolution mass spectrometry by electrospray ionization (HRMS-ESI) with time-of-flight (TOF) in both positive and negative modes was performed. All samples showed more sensitive and selective mass patterns in negative ESI mode than positive mode, the total ion chromatograms are represented in Fig. 3.16 A-H.

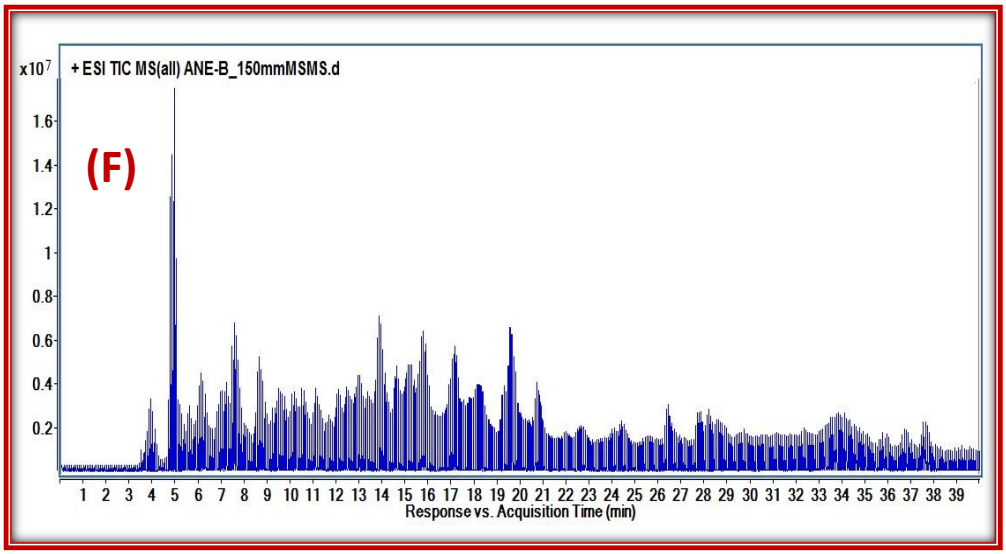

x10^6
-ESI TIC MS (all) APH-11_150mmMSMS_NEG.d
(A)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Response vs. Acquisition Time (min)


x10^6
+ ESI TIC MS(all) APH-11_150 mmMM.d
(B)
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
Response vs. Acquisition Time (min)


x10^6
-ESI TIC MS(all) APM-3_150mmMM_NEG.d
(C)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
Response vs. Acquisition Time (min)


+ESI TIC MS(all) APM-3_150mmMSMS.d
(D)
x10 7
Counts vs. Acquisition Time (min)


-ESI TIC MS(all) ANE-B_150mmMSMS.d
(E)
x10 6
Response vs. Acquisition Time (min)


+ESI TIC MS(all) ANE-B_150mmMSMS.d
(F)
x10 7
Response vs. Acquisition Time (min)

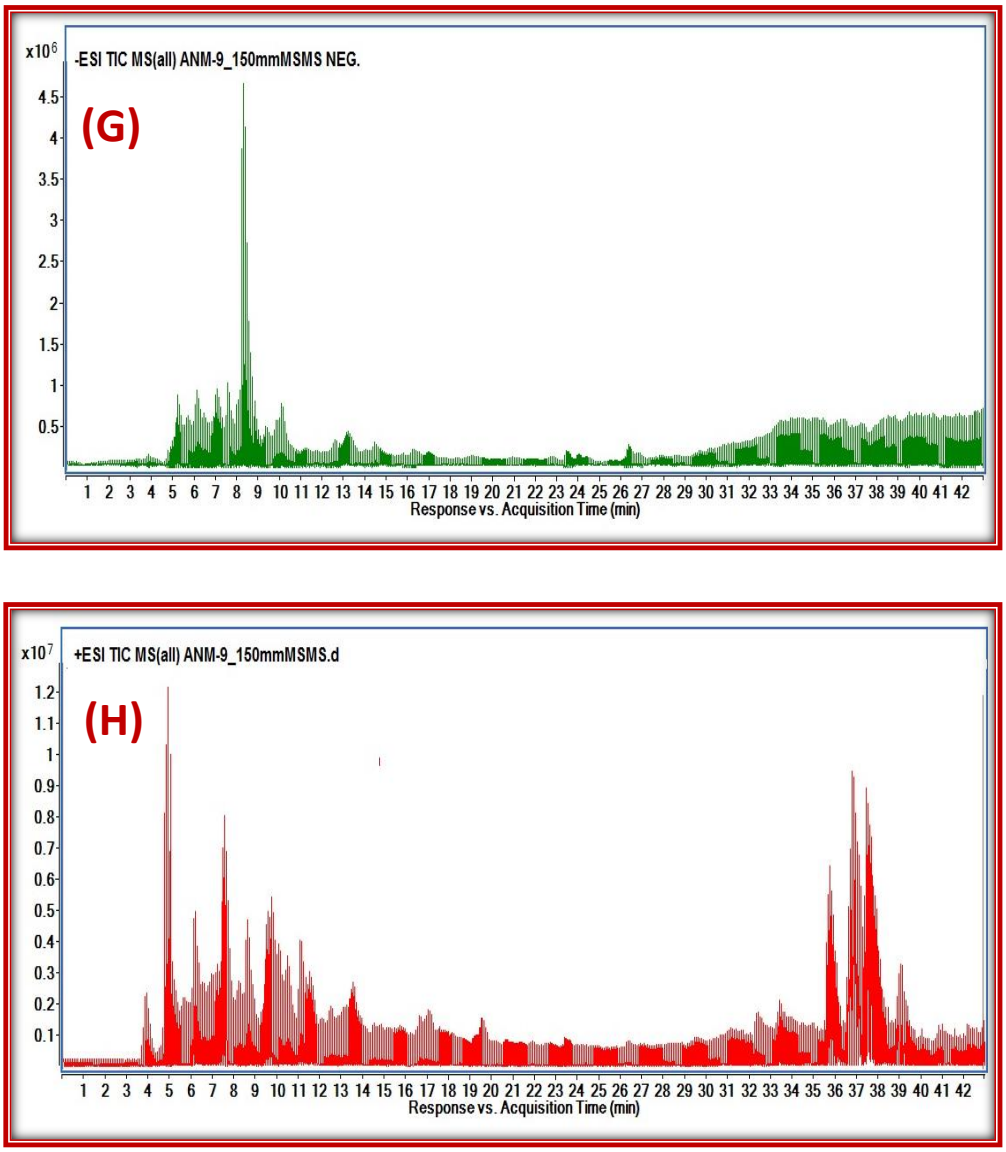


Fig. 3.16: HPLC-DAD-ESI-MS chromatograms of active fractions of *Abrus precatorius* and *A. nilagirica*. **(A):** APH-11, Positive (+ve) mode **(B)** APH-11, Negative (-ve) mode **(C)** APM-3, Positive (+ve) mode **(D)** APM-3, Negative (-ve) mode **(E)** ANE-B, Positive (+ve) mode **(F)** ANE-B, Negative (-ve) mode **(G)** ANM-9, Positive (+ve) mode **(H)** ANM-3, Negative (-ve) mode.

Table 3.7: Characterisation of 17 chromatographic peaks in *Abrus precatorius* and *Artemisia nilagirica* active fractions with HPLC-ESI-QTOF-MS data recorded in the positive and negative ion modes.

S. No.	Plant	Fraction	Compound	Mol. Formula	RT (min)	λ max (min)	Mol. Weight	Precursor ion (m/z)	Product ions (m/z)	Reference
1	AP	APH-11	**Abrectorin****	$C_{19}H_{20}O_7$	16.305	260, 272, 280, 300	314.2895	$[M+H]^+$ 315.0853	136,149,152,254,282	Alessandro et al., 1979
2	AP	APM-3	**Abruquinone A***	$C_{17}H_{14}O_6$	4.892	260, 272, 280, 300	360.3579	$[M-H]^-$ 359.1015	135,149,151,209	Bhardwaj, et al., 1980
3	AP	APM-3	**Luteolin****	$C_{15}H_9O_6$	9.685	272, 280, 300	286.2363	$[M-H]^-$ 285.0426	107,133,151,175	Mass Bank
4	AN	ANE-B	**Scopoletin***	$C_{10}H_8O_4$	7.404	250, 260, 272, 280, 300	192.1681	$[M+H]^+$ 193.0504	122, 133, 137, 150, 165, 178	Mass Bank
5	AN	ANE-B	**Isoferulic acid****	$C_{10}H_{10}O_4$	20.135	-NA-	194.1840	$[M+H]^+$ 195.3523	149,177	Martens et al., 2012
6	AN	ANE-B	**3,4-Dicaffeoylquinic acid****	$C_{25}H_{24}O_{12}$	3.222	250	516.4509	$[M+H]^+$ 515.1122	173,335,353	Clifford et al.2003
7	AN	ANE-B	**Euoniside**	$C_{17}H_{20}O_{10}$	16.053	280, 300	384.3619	$[M-H]^-$ 383.1287	163,221,339,355	He et al., 2009
8	AN	ANE-B	**Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy*****	$C_{18}H_{16}O_8$	29.2936	260, 272, 280	360.3148	$[M-H]^-$ 359.0713	151,163,195,329,343	Kupchan et al., 1969
9	AN	ANE-B	**Penduletin****	$C_{18}H_{16}O_7$	30.9818	260, 272, 280	344.3154	$[M-H]^-$ 343.0768	147,151,163,195,313,343	Turi et al., 2014
10	AN	ANE-B	**Casticin***	$C_{19}H_{18}O_8$	31.7655	272, 280	374.3414	$[M-H]^-$ 373.0868	151,195,284,328,343, 358	Mass Bank
11	AN	ANM-9	**Quercetin 3,7-dimethyl ether****	$C_{17}H_{14}O_7$	13.391	250, 260, 272, 280, 300	330.2889	$[M+H]^+$ 331.0792	137,151,273,301,316	Turi et al., 2014
12	AN	ANM-9	**3,5-Dihydroxy-6,7,8-trimethoxyflavone*****	$C_{18}H_{16}O_7$	17.330	250, 260, 272, 280, 300	344.3154	$[M+H]^+$ 345.0946	106,119,135,159,183,199 ,211,259,283,315,330	Romo et al., 1970
13	AN	ANM-9	**Kaempferol***	$C_{15}H_{10}O_6$	9.785	250, 260, 272, 280, 300	286.2363	$[M-H]^-$ 285.0363	107,133,151,175	Mass Bank
14	AN	ANM-9	**Pedilatin****	$C_{16}H_{12}O_7$	10.217	250, 260, 272, 280, 300	316.2623	$[M-H]^-$ 315.0466	136, 201, 243, 300	Moharram et al., 2012).
15	AN	ANM-9	**Axillarin****	$C_{17}H_{14}O_8$	10.891	250, 260, 272, 280, 300	346.2883	$[M-H]^-$ 345.0571	136,287,330,315	Turi et al., 2014
16	AN	ANM-9	**Apigenin***	$C_{15}H_{10}O_5$	12.406	250, 260, 272, 280, 300	270.2369	$[M-H]^-$ 269.0415	107,117,149,151,225,241	Mass Bank.
17	AN	ANM-9	**Diosmetin***	$C_{16}H_{12}O_6$	13.163	250, 260, 272, 280, 300	300.2629	$[M-H]^-$ 299.0518	107,151,227,256,284	Mass Bank

AP=*Abrus precatorius*; AN= *Artemisia nilagirica*; *Mass Bank; **MS/MS fragmentation of reported compound; *** Tentatively assigned. For the sake clarity, MS/MS derived ions are presented in unit numbers.

To characterize the fragment ions of the investigated compounds, an electrospray interface with good sensitivity, fragmentation and linearity was optimized and, therefore, resulted in the detection of a series of masses. Three compounds were identified from *Abrus precatorius* fractions (APH-11 & APM-3) and fourteen compounds from *Artemisia nilagirica* fractions (ANE-B & ANM-9), thus in total 17 compounds were identified using LC–DAD–ESI-MS. Individual components were identified by comparison of their m/z values in the Total Ion Count (TIC) profile with those of the selected compounds from each empirical formula from its mass as described in literature. The compounds were determined by comparing spectral data with previous work or by matching their MS/MS spectra with those reported in a public repository of mass spectral data called Mass Bank, MetLin database and the Dictionary of Natural Products software (CRC Press, Taylor and Francis Group) [Horai et al. 2010]. LC-MS/MS analysis of the samples has indicated the presence of coumarins, cinnamic acids and flavonoids class of phytochemicals. The major compounds identified includes; - **(1)**: Abrectorin, **(2)**: Abruquinone A, **(3)**: Luteolin, **(4)**: Scopoletin, **(5)**: Isoferulic acid, **(6)**: 3,4-Dicaffeoylquinic acid, **(7)**: Euoniside, **(8)**: Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy (Eupatin), **(9)**: Penduletin, **(10)**: Casticin, **(11)**: Quercetin 3,7-dimethyl ether, **(12)**: 3,5-Dihydroxy-6,7,8-trimethoxyflavone, **(13)**: Kaempferol, **(14)**: Pedilatin, **(15)**: Axillarin, **(16)**: Apigenin, **(17)**: Diosmetin. The chemical structures of these compounds are presented in Fig. 3.17A-Q. Details of the compounds including retention time, mass, formula and fragmentation pattern type are given in Table 3.7.

In APH-11 only one peak was identified in both positive and negative ionisation mode. According to the LC-ESI-MS/MS spectrum of compound **1** assigned it as **Abrectorin**, the positive ESI-MS (Rt= 16.305 min; MW=314.2895) showed ion $[M+H]^+$ with *m/z* 315.0853. MS/MS analysis found loss of a H_2O and a methyl groups with 33 a.m.u., with *m/z* at ~282 and followed by loss of CO with 28 a.m.u. with *m/z* at ~254. Ions peaks at ~152, ~149, ~136 correspond to cleavage of central (C) ring by $^{0,2}B^+$, $^{1,3}B^+$ and $^{1,2}B^+$ respectively. Thus, $[M+H]^+$ along with its fragments suggested compound **1** (Fig. 3.17A) as **Abrectorin** [Alessandro et al., 1979].

In APM-3, two peaks were identified in negative ionisation mode. Compound **2** (Rt = 4.892 min; MW=360.3579) had $[M-H]^-$ at *m/z* 359.1015 and was identified as **Abruquinone A** with loss of one methyl group and loss of ring B benzoquinone with 107 a.m.u. at *m/z* ~151. Ions peaks at ~209, ~149 and ~135 correspond to cleavage of central (C) ring by $^{0,3}B^-$,

$^{0,3}A^-$, and $^{0,4}A^-$ respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **2** (Fig. 3.17 B) as **Abruquinone A** [Bhardwaj et al., 1980].

According to the LC-ESI-MS-MS spectrum of compound **3** from APM-3 was assigned it as **Luteolin**, the negative ESI-MS (Rt= 9.685 min; MW=286.2363) showed ion $[M-H]^-$ with *m/z* 285.0426. MS/MS analysis found loss of a B ring with 110 a.m.u., with *m/z* at ~175. Ions peaks at ~151, ~133, ~107 correspond to cleavage of central (C) ring by $^{1,3}A^-$, $^{1,3}B^-$ and $^{0,4}A^-$, respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **3** (Fig. 3.17C) as **Luteolin** [Mass Bank].

In fraction ANE-B, compounds **4-10** were identified; compound **4** and **5** in positive mode and **6-10** in negative mode. According to the LC-ESI-MS/MS spectrum of compound **4** assigned as **Scopoletin**, the positive ESI-MS (Rt=7.404 min; MW=192.1681) showed a $[M+H]^+$ ion peak at *m/z* 193.054 and the analysis of the MS/MS spectrum of the protonated ion of compound **4** found a peak by loss of 15 a.m.u. of methyl group with *m/z* ~178 A peak at m/z ~165 formed readily by elimination of a CO group from the molecular ion which is characteristic of coumarins to give the corresponding benzofuran which highlighted the presence of product as coumarin. This benzofuran further fragments with loss of methyl group with 15 a.m.u., loss of CO group with 28 a.m.u., loss of hydroxyl and methoxy groups with 32 a.m.u. and loss of $C(OCH_3)$ group with m/z ~150, ~137, ~133, ~122 respectively suggested the presence of a coumarin, **Scopoletin** as compound **4** (Fig. 3.17D). Identification of compound 4 as Scopoletin was done by matching its tandem mass spectra with that of Mass Bank.

The LC-ESI-MS/MS data of compound **5** designated as **Isoferulic acid**, the positive ESI-MS (Rt=20.135 min; MW=194.1840), a $[M+H]^+$ ion peak at *m/z* ~195.3523. Analysis of MS/MS further through proton transfer and endothermic loss of water, protonated molecular ion forms a $[(M+H^+)+(-H_2O)]$ ion with m/z ~177, which can be considered as resonance hybrid between acylium $(-C\equiv O^+)$ and a ketene $(-C^+=C=O)$ structural moieties, subsequently loss of carbon monoxide forms ion at m/z ~149. Identification of compound **5** (Fig. 3.17E) as **Isoferulic acid** was done by matching its tandem mass spectra with that of Mass Bank and earlier report of Martens et al., [2012].

According to the LC-ESI-MS/MS spectrum of compound **6** designated as **3, 4-Dicaffeoylquinic acid**, the negative ESI-MS (Rt=3.222 min; MW=516.4509) showed ion $[M-H]^-$ with *m/z* 515.1122 and further MS/MS analysis ion peak at *m/z* ~353 due to the loss of

one cinnamoyl unit 162 a.m.u., peak at *m/z* ~335 due to the loss of a one H_2O molecule 18 a.m.u. Ion m/z ~173 is characteristic for isomer substituted at position 4 must be due to loss of the caffeoyl moiety at position 3 (Fig. 3.17F). Interestingly, the MS/MS spectrum of the $[M-H]^-$ ion showed a fragmentation pattern very similar to what was proposed by Clifford et al., 2003.

The analysis of the LC-ESI-MS/MS spectrum of compound **7** assigned it as **Euoniside**, the negative ESI-MS (Rt=16.053 min; MW=384.3619) showed ion $[M-H]^-$ with *m/z* 383.1287. Further analysis of the MS/MS spectrum of the deprotonated ion of compound **7** found a peak at *m/z* ~355 with loss of 28 a.m.u. formed readily by elimination of a carbon monoxide from the molecular ion which is characteristic of coumarins to give the corresponding benzofuran with loss of 44 a.m.u a peak at *m/z* ~339 corresponding to substituted 1H-indene which highlighted the presence of product as coumarin. The tandem mass experiment on the $[M-H]^-$ ion allowed to observe a product ion at *m/z* ~221 formed due to neutral loss of one deoxy-hexose unit 163 a.m.u. and corresponding to **Euoniside** (Fig. 3.17G) [He et al., 2009].

The analysis of LC-ESI-MS-MS spectrum of compound **8** assigned it as **Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy**, the negative ESI-MS (Rt=29.2936 min; MW=360.3148) showed ion $[M-H]^-$ with *m/z* 359.0713. In MS/MS analysis found loss of oxygen atom with 16 a.m.u., loss of 2 methyl groups with 30 a.m.u., *m/z* at ~343, ~329 respectively. Ions peaks at ~195, ~163, ~151 correspond to cleavage of central (C) ring by $^{1,3}A^-$, $^{1,3}B^-$ and $^{0,4}A^-$ respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **8** (Fig. 3.17H) as **Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy** [Kupchan et al., 1969].

According to the LC-ESI-MS-MS spectrum of compound **9** assigned it as **Penduletin**, the negative ESI-MS (Rt= 30.9818 min; MW=344.3154) showed ion $[M-H]^-$ with *m/z* 343.0768. MS/MS analysis found loss of 2 methyl groups with 30 a.m.u., with *m/z* at ~313 respectively. Ions peaks at ~195, ~163, ~151, ~147 correspond to cleavage of central (C) ring by $^{1,3}A^-$, $^{0,3}B^-$, $^{0,4}A^-$ and $^{1,3}B^-$ respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **9** (Fig. 3.17I) as **Penduletin** [Sy and Brown, 1998].

The negative mode LC-ESI-MS/MS profile of compound **10** assigned it as **Casticin**, the ESI-MS (Rt= 31.7655 min; MW=374.3414) showed ion $[M-H]^-$ with *m/z* 373.0868. Further MS/MS analysis found loss of a methyl group with 15 a.m.u., loss of two methyl groups with 30 a.m.u., with *m/z* at ~358, ~343 respectively. From the ion with *m/z* at ~358 found with

further loss of two methyl groups with 30 a.m.u. with *m/z* at ~328 followed by loss of CO_2 44 a.m.u with *m/z* at ~284. Ions peaks at ~195, ~151 correspond to cleavage of central (C) ring by $^{1,3}A^-$ and $^{0,4}A^-$ respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **10** (Fig. 3.17J) as **Casticin** and identification done by matching its tandem mass spectra with that of Mass Bank.

In fraction ANM-9, compounds **11-17** were identified; compound **11** and **12** in positive mode and **13-17** in negative mode. The LC-ESI-MS/MS spectrum of compound **11** assigned it as **Quercetin 3,7-dimethyl ether**, the positive ESI-MS (Rt= 13.391 min; MW=330.2889) showed ion $[M+H]^+$ at *m/z* 331.0792. In MS/MS analysis found loss of a methyl group with 15 a.m.u., loss of 2 methyl group with 30 a.m.u, with loss of $C_2O_2H_2$ (2, hydroxyl groups ortho to each other) 58 a.m.u., m/z at ~316, ~301, ~273 respectively. Ions peaks at ~151, ~137 corresponds to cleavage of central (C) ring by $^{0,3}B^+$ and $^{1,4}A^+$ respectively. Thus, $[M+H]^+$ along with its fragments suggested compound **11** (Fig. 3.17K) as **Quercetin 3,7-dimethyl ether** [Bergendorff and Sterner, 1995].

The analysis of the LC-ESI-MS/MS spectrum of compound **12** assigned it as **3,5-Dihydroxy-6,7,8-trimethoxy flavone**, the positive ESI-MS (Rt= 17.330 min; MW=344.3154) showed ion $[M+H]^+$ at *m/z* 345.0946. Further MS/MS analysis loss of a methyl group with 15 a.m.u., loss of 2 methyl group with 30 a.m.u., loss of 2 methoxy group with 30 a.m.u., loss of $C(OCH_3)$-$C(OCH_3)$ with 62 a.m.u., loss of $C(OCH_3)=C(OCH_3)$-$C(OCH_3)=C(OH)$ with 186 a.m.u with *m/z* ~330, ~315, ~283, ~259, ~159 respectively. Ions peaks at ~211, ~199, ~183, ~135, ~119, ~106 corresponds to cleavage of central (C) ring by $^{0,3}A^+$, $^{1,4}A^+$, $^{0,4}A^+$, $^{0,3}B^+$, $^{1,3}B^+$ and $^{0,2}B^+$ respectively. Thus, $[M+H]^+$ along with its fragments suggested compound **12** (Fig. 3.17L) as **3,5-Dihydroxy-6,7,8-tri methoxy flavone** [Romo et al., 1970].

According to the LC-ESI-MS-MS spectrum of compound **13** assigned it as **Kaempferol**, the negative ESI-MS (Rt= 9.785 min; MW=286.2363) showed ion $[M-H]^-$ with *m/z* 285.0363. The further MS/MS analysis found cleavage of its central (C) ring resulted in the formation of ions at *m/z* ~151, ~133, ~107 corresponding to $^{1,3}A^-$, $^{1,3}B^-$, $^{0,4}A$- respectively and ~175 corresponds to loss of B ring along with a hydroxyl group. Thus, $[M-H]^-$ along with its fragments suggests compound **13** (Fig. 3.17M) as **Kaempferol** and identification done by matching its tandem mass spectra with that of Mass Bank.

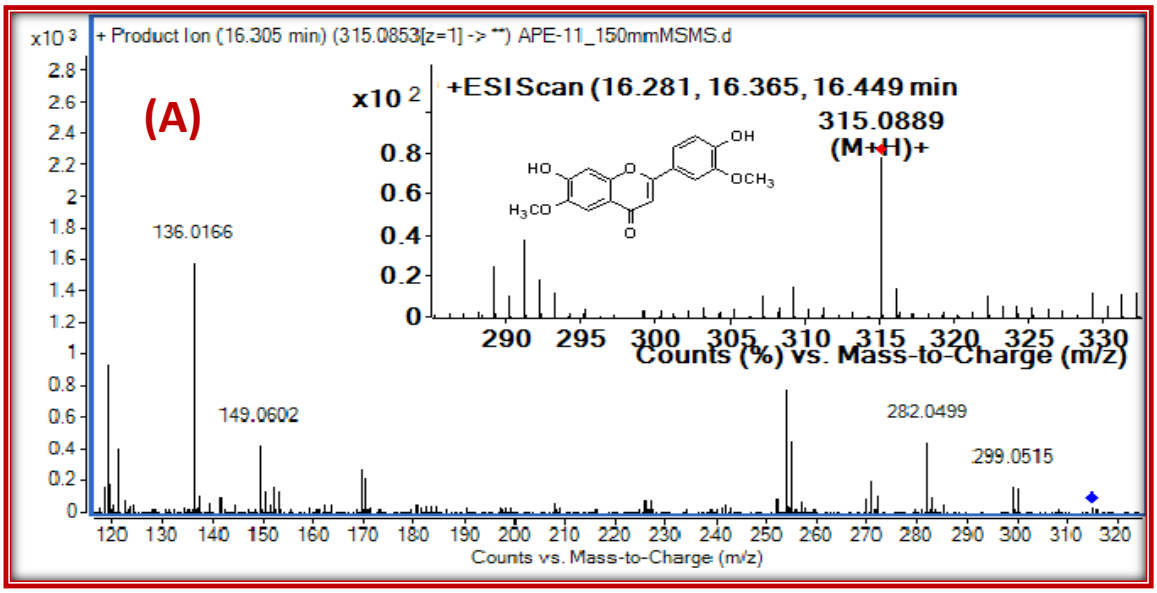
The positive LC-ESI-MS-MS spectrum of compound **14** assigned it as **Pedalitin (6-hydroxy luteolin 7 methyl ether)**, the negative ESI-MS (Rt= 10.217 min, MW=316.2623)

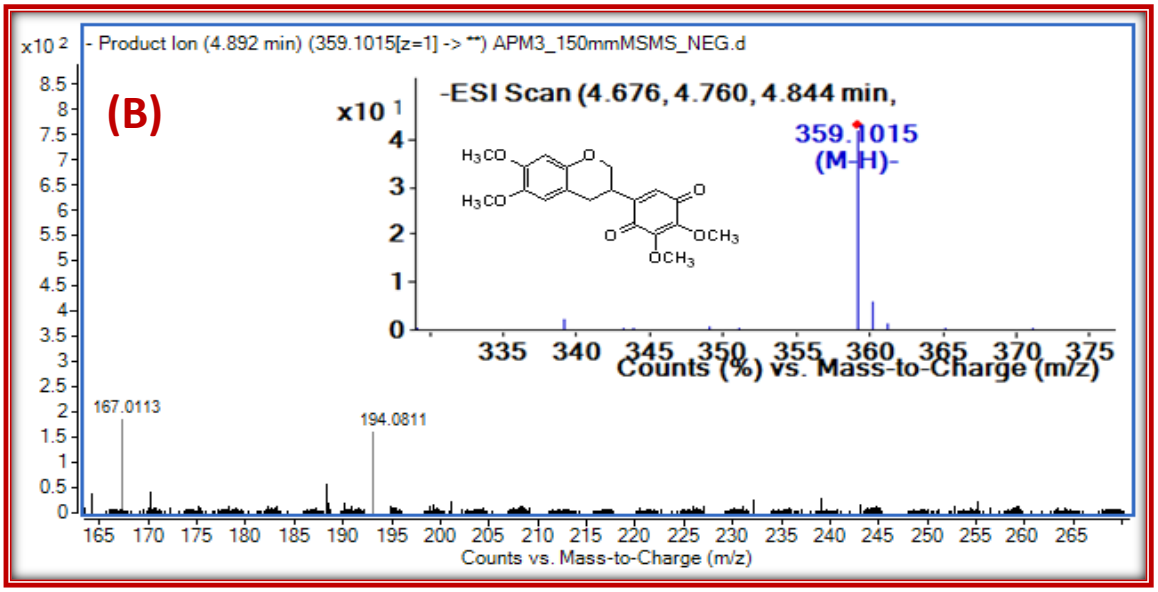
showed ion $[M-H]^-$ with *m/z* 315.0466. In further MS/MS analysis ions peaks with *m/z* ~300, ~ 243, ~201 which corresponds to loss of methyl group, loss of $C(OCH_3)$-C(OH) and loss of -$CH=C(OCH_3)$-C(OH)=C(OH)- with 15 a.m.u., 72 a.m.u. and 114 a.m.u. of ring A respectively and ~136 ion peak corresponds to cleavage of central (C) ring by $^{0,2}B^-$. Thus, $[M-H]^-$ along with its fragments suggested compound **14** (Fig. 3.17N) as **Pedalitin** [Moharram et al., 2012].

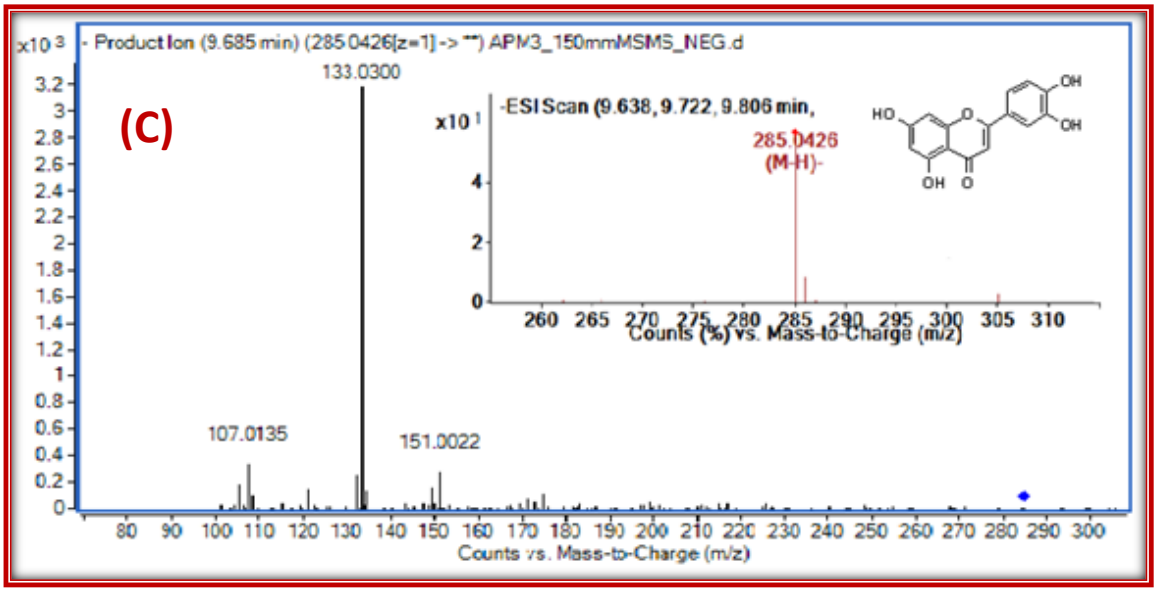
The LC-ESI-MS/MS spectrum of compound **15** assigned it as **Axillarin**, the negative ESI-MS (Rt= 10.891 min; MW=346.2883) showed ion $[M-H]^-$ with *m/z* 345.0571. In MS/MS analysis found loss of a methyl group with 15 a.m.u., loss of 2 methyl group with 30 a.m.u., loss of $C_2O_2H_2$ (2 hydroxyl groups ortho to each other) 58 a.m.u., with *m/z* at ~330, ~315, ~287 respectively and ~136 ion peak corresponds to cleavage of central (C) ring by $^{0,2}B^-$. Thus $[M+H]^+$ along with its fragments suggested compound **15** (Fig. 3.17O) as **Axillarin** [Forgo et al., 2012].

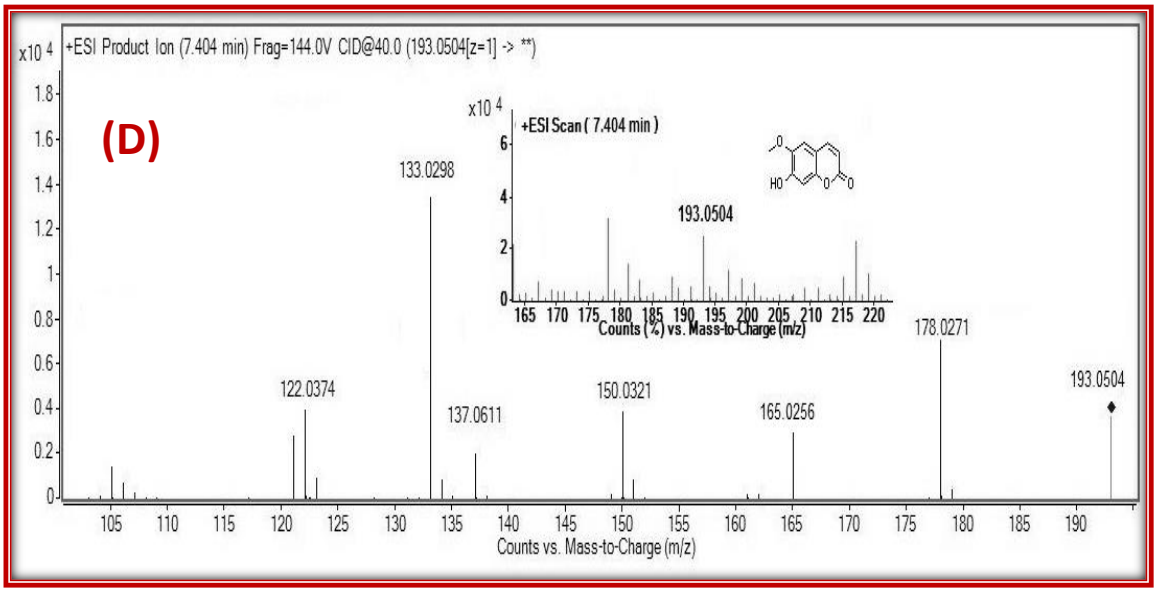
According to the LC-ESI-MS-MS spectrum of compound **16** assigned it as **Apigenin**, the negative ESI-MS (Rt=12.406 min; MW=270.2369) showed ion $[M-H]^-$ with *m/z* 269.0415. Further MS/MS analysis ion peaks with *m/z* ~241, ~225 which corresponds loss of CO and CO_2 with 28 a.m.u. and 44 a.m.u., respectively. Cleavage of its central (C) ring resulted in the formation of ions at m/z ~151, ~149, ~107 and ~117 corresponding to $^{1,3}A^-$, $^{1,4}B^-$+2H, $^{1,3}A$-CO_2, $^{1,3}B^-$ respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **16** (Fig. 3.17P) as **Apigenin** and identification done by matching its tandem mass spectra with that of Mass Bank.

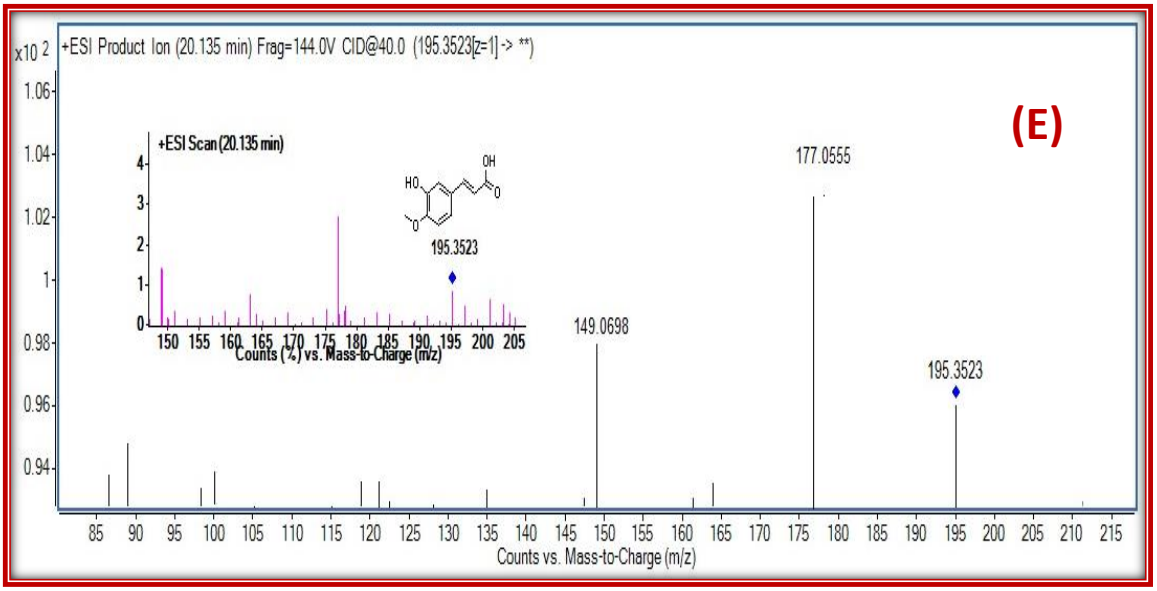
According to the LC-ESI-MS-MS spectrum of compound **17** assigned it as **Diosmetin**, the negative ESI-MS (Rt= 13.163 min; MW=300.262905) showed ion $[M-H]^-$ with *m/z* 299.0518. In MS/MS analysis an ion peaks with *m/z* ~284, ~256 which corresponds loss of methyl group with 15 a.m.u., loss of –C-OCH_3 with 43 a.m.u. Ion peaks with *m/z* ~227 corresponds to cleavage of –C(OH)=C(OCH_3) group with 72 a.m.u. Cleavage of its central (C) ring resulted in the formation of ions at m/z ~107 and ~151, corresponding to $^{0,4}A^-$ and $^{1,3}A^-$, respectively. Thus, $[M-H]^-$ along with its fragments suggested compound **17** (Fig. 3.17Q) as **Diosmetin** and identification done by matching its tandem mass spectra with that of Mass Bank.

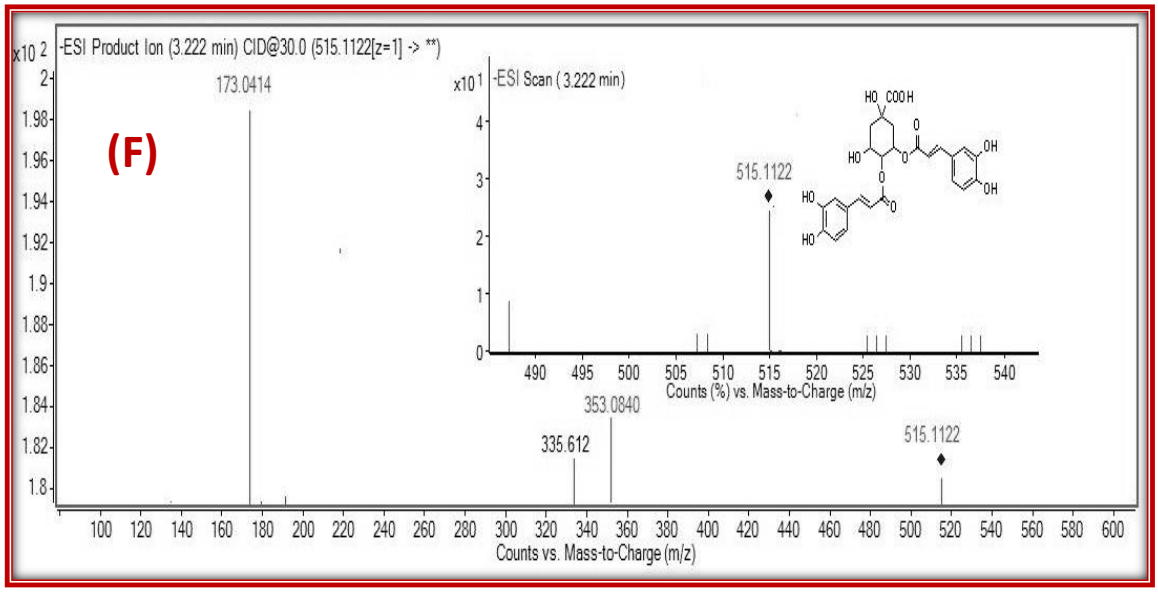

(A)
+ Product Ion (16.305 min) (315.0853[z=1] -> **) APE-11_150mmMSMS.d
+ESI Scan (16.281, 16.365, 16.449 min
315.0889
(M+H)+
136.0166
149.0602
282.0499
299.0515
Counts (%) vs. Mass-to-Charge (m/z)
Counts vs. Mass-to-Charge (m/z)

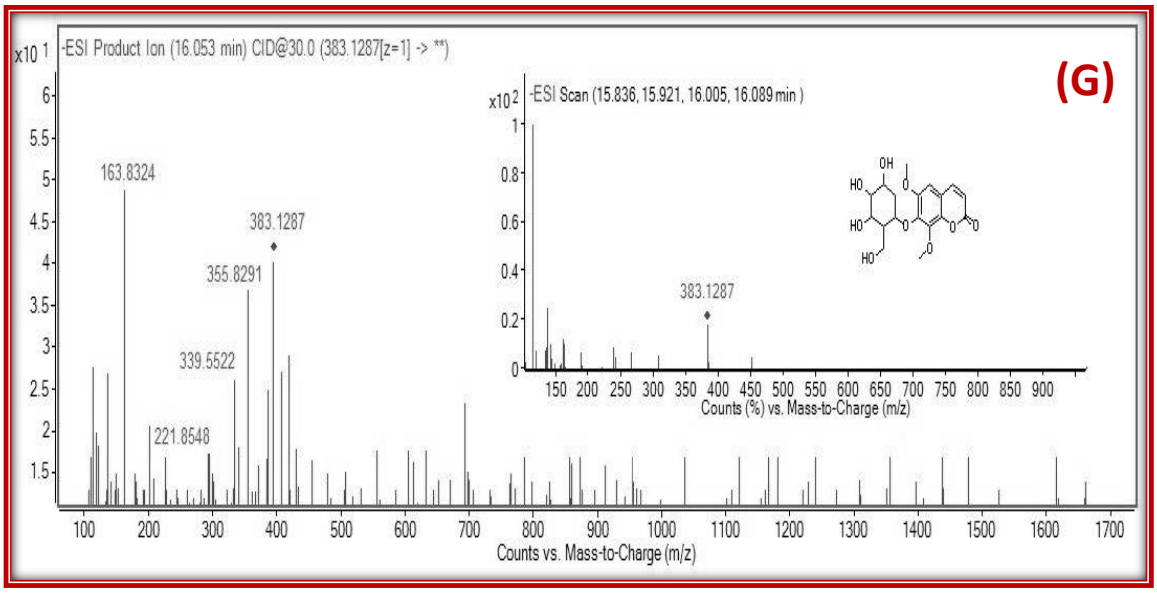

(B)
- Product Ion (4.892 min) (359.1015[z=1] -> **) APM3_150mmMSMS_NEG.d
-ESI Scan (4.676, 4.760, 4.844 min,
359.1015
(M-H)-
167.0113
194.0811
Counts (%) vs. Mass-to-Charge (m/z)
Counts vs. Mass-to-Charge (m/z)

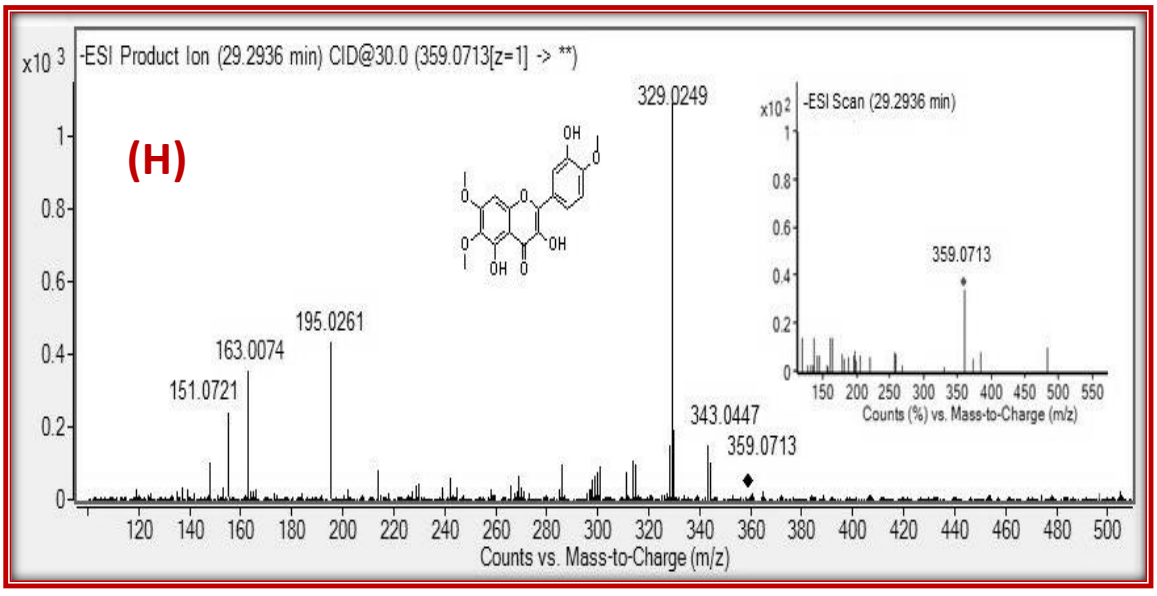

(C)
- Product Ion (9.685 min) (285.0426[z=1] -> **) APM3_150mmMSMS_NEG.d
133.0300
-ESI Scan (9.638, 9.722, 9.806 min,
285.0426
(M-H)-
107.0135
151.0022
Counts (%) vs. Mass-to-Charge (m/z)
Counts vs. Mass-to-Charge (m/z)

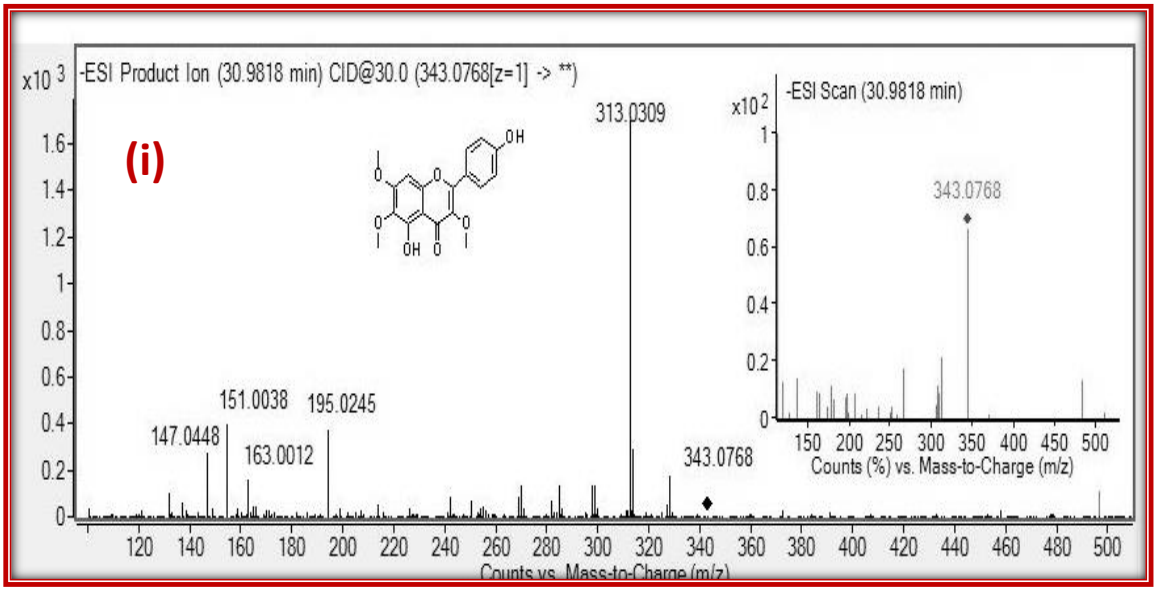

(D)
+ESI Product Ion (7.404 min) Frag=144.0V CID@40.0 (193.0504[z=1] -> **)
+ESI Scan (7.404 min)
193.0504
133.0298
122.0374
137.0611
150.0321
165.0256
178.0271
193.0504
Counts vs. Mass-to-Charge (m/z)

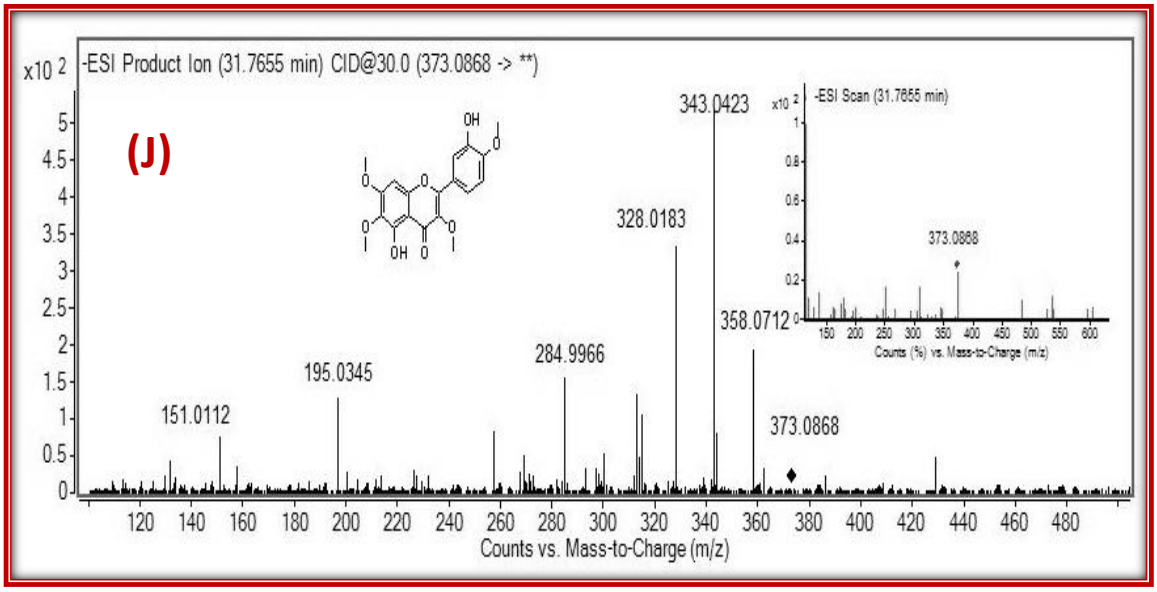

(E)
+ESI Product Ion (20.135 min) Frag=144.0V CID@40.0 (195.3523[z=1] -> **)
+ESI Scan (20.135 min)
195.3523
177.0555
149.0698
195.3523
Counts vs. Mass-to-Charge (m/z)

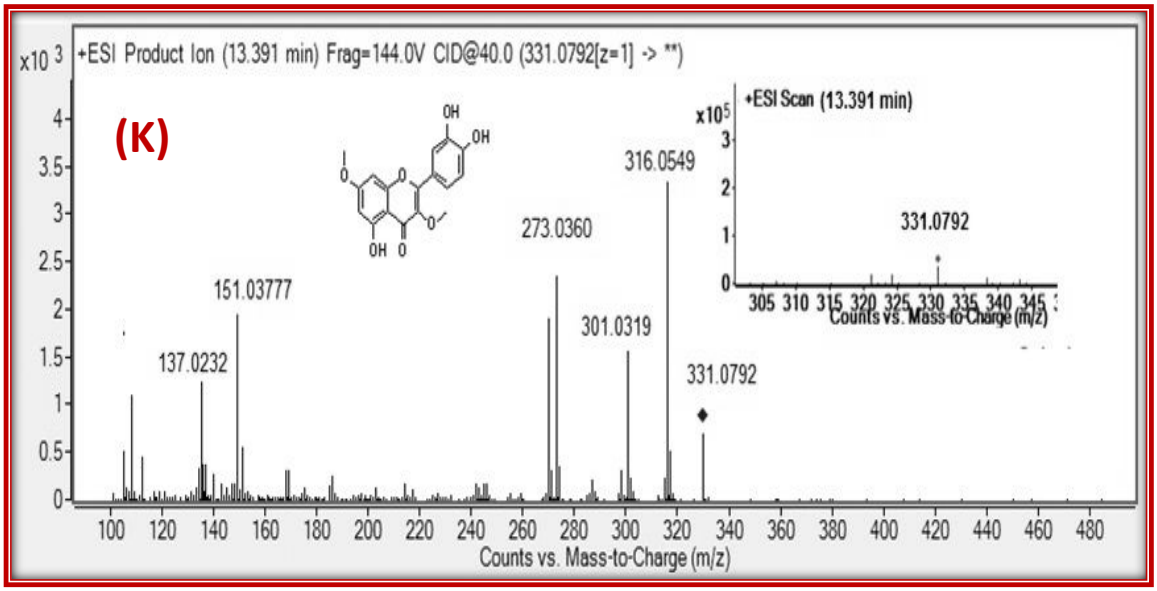

(F)
-ESI Product Ion (3.222 min) CID@30.0 (515.1122[z=1] -> **)
-ESI Scan (3.222 min)
173.0414
515.1122
353.0840
335.612
515.1122
Counts vs. Mass-to-Charge (m/z)

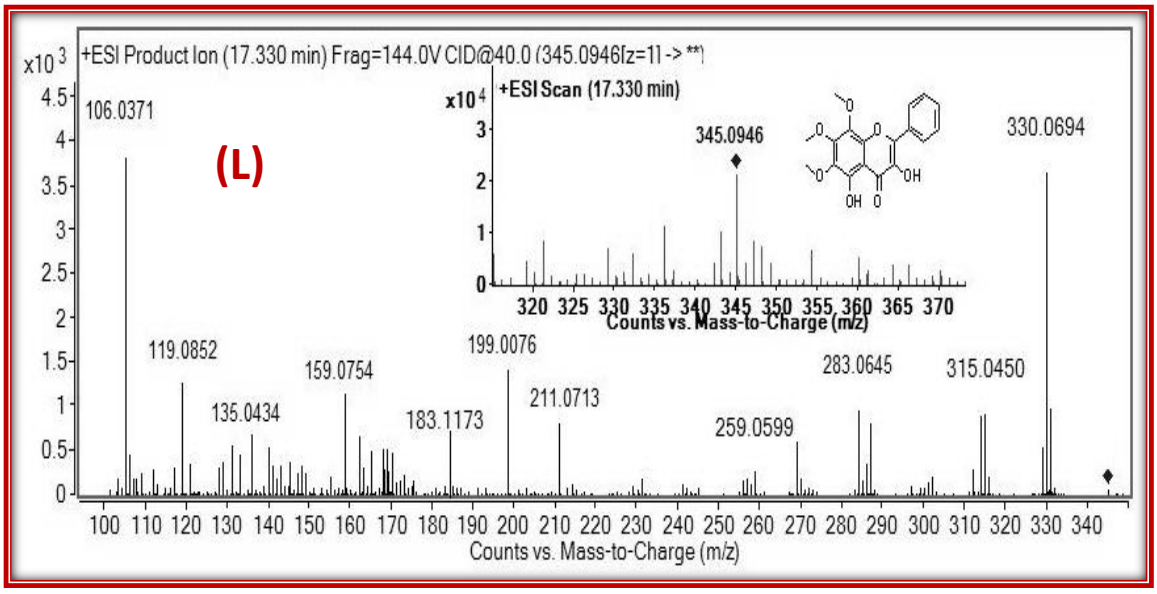

(G)
-ESI Product Ion (16.053 min) CID@30.0 (383.1287[z=1] -> **)
-ESI Scan (15.836, 15.921, 16.005, 16.089 min)
163.8324
383.1287
355.8291
339.5522
221.8548
Counts vs. Mass-to-Charge (m/z)
Counts (%) vs. Mass-to-Charge (m/z)

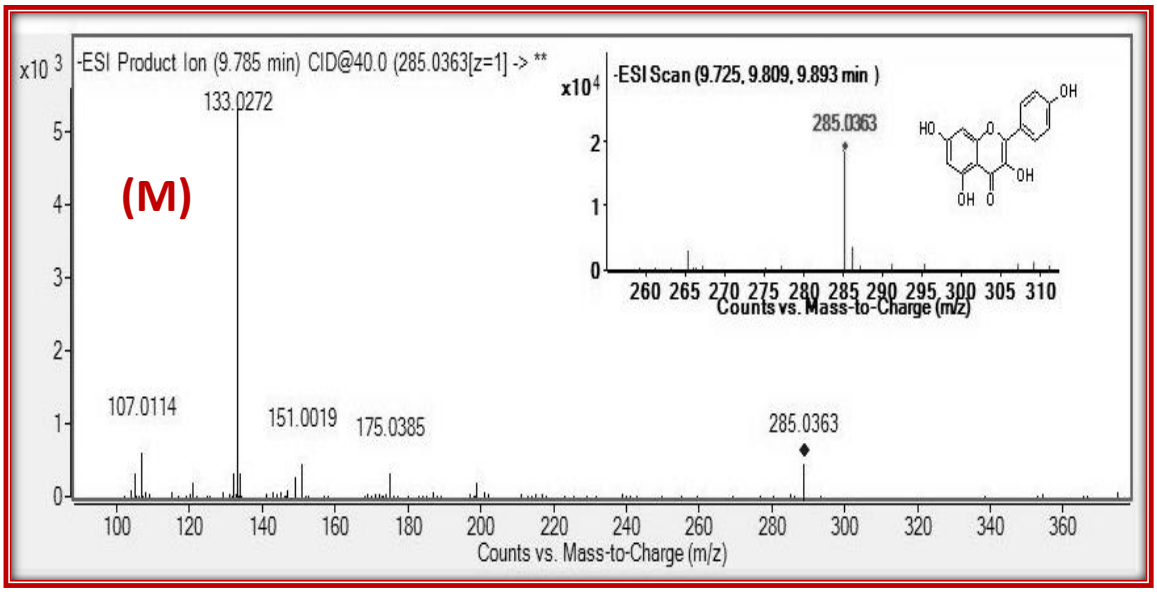

(H)
-ESI Product Ion (29.2936 min) CID@30.0 (359.0713[z=1] -> **)
-ESI Scan (29.2936 min)
329.0249
195.0261
163.0074
151.0721
343.0447
359.0713
Counts vs. Mass-to-Charge (m/z)
Counts (%) vs. Mass-to-Charge (m/z)

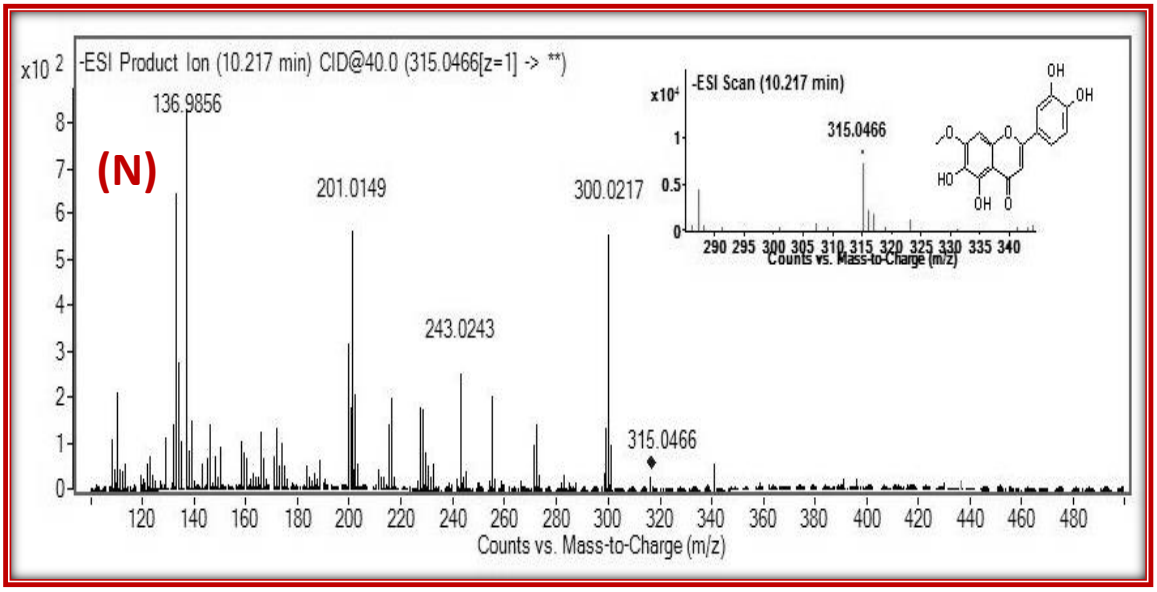

(i)
-ESI Product Ion (30.9818 min) CID@30.0 (343.0768[z=1] -> **)
-ESI Scan (30.9818 min)
313.0309
151.0038
195.0245
147.0448
163.0012
343.0768
Counts vs. Mass-to-Charge (m/z)
Counts (%) vs. Mass-to-Charge (m/z)

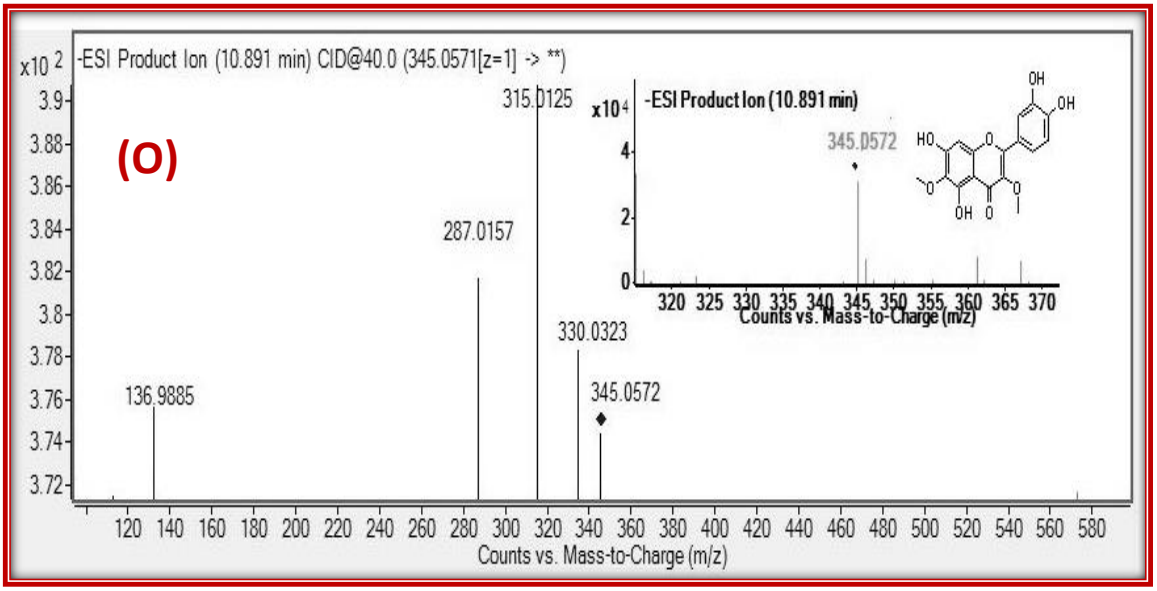

(J)
-ESI Product Ion (31.7655 min) CID@30.0 (373.0868 -> **)
343.0423
328.0183
358.0712
284.9966
195.0345
151.0112
373.0868
-ESI Scan (31.7655 min)
373.0868
Counts vs. Mass-to-Charge (m/z)


(K)
+ESI Product Ion (13.391 min) Frag=144.0V CID@40.0 (331.0792[z=1] -> **)
316.0549
273.0360
151.03777
301.0319
137.0232
331.0792
+ESI Scan (13.391 min)
331.0792
Counts vs. Mass-to-Charge (m/z)


(L)
+ESI Product Ion (17.330 min) Frag=144.0V CID@40.0 (345.0946[z=1] -> **)
106.0371
345.0946
330.0694
119.0852
159.0754
199.0076
135.0434
183.1173
211.0713
283.0645
315.0450
259.0599
+ESI Scan (17.330 min)
Counts vs. Mass-to-Charge (m/z)


(M)
-ESI Product Ion (9.785 min) CID@40.0 (285.0363[z=1] -> **
133.0272
107.0114
151.0019
175.0385
285.0363
Counts vs. Mass-to-Charge (m/z)
-ESI Scan (9.725, 9.809, 9.893 min)
285.0363
Counts vs. Mass-to-Charge (m/z)


(N)
-ESI Product Ion (10.217 min) CID@40.0 (315.0466[z=1] -> **)
136.9856
201.0149
300.0217
243.0243
315.0466
Counts vs. Mass-to-Charge (m/z)
-ESI Scan (10.217 min)
315.0466
Counts vs. Mass-to-Charge (m/z)


(O)
-ESI Product Ion (10.891 min) CID@40.0 (345.0571[z=1] -> **)
315.0125
287.0157
330.0323
345.0572
136.9885
Counts vs. Mass-to-Charge (m/z)
-ESI Product Ion (10.891 min)
345.0572
Counts vs. Mass-to-Charge (m/z)

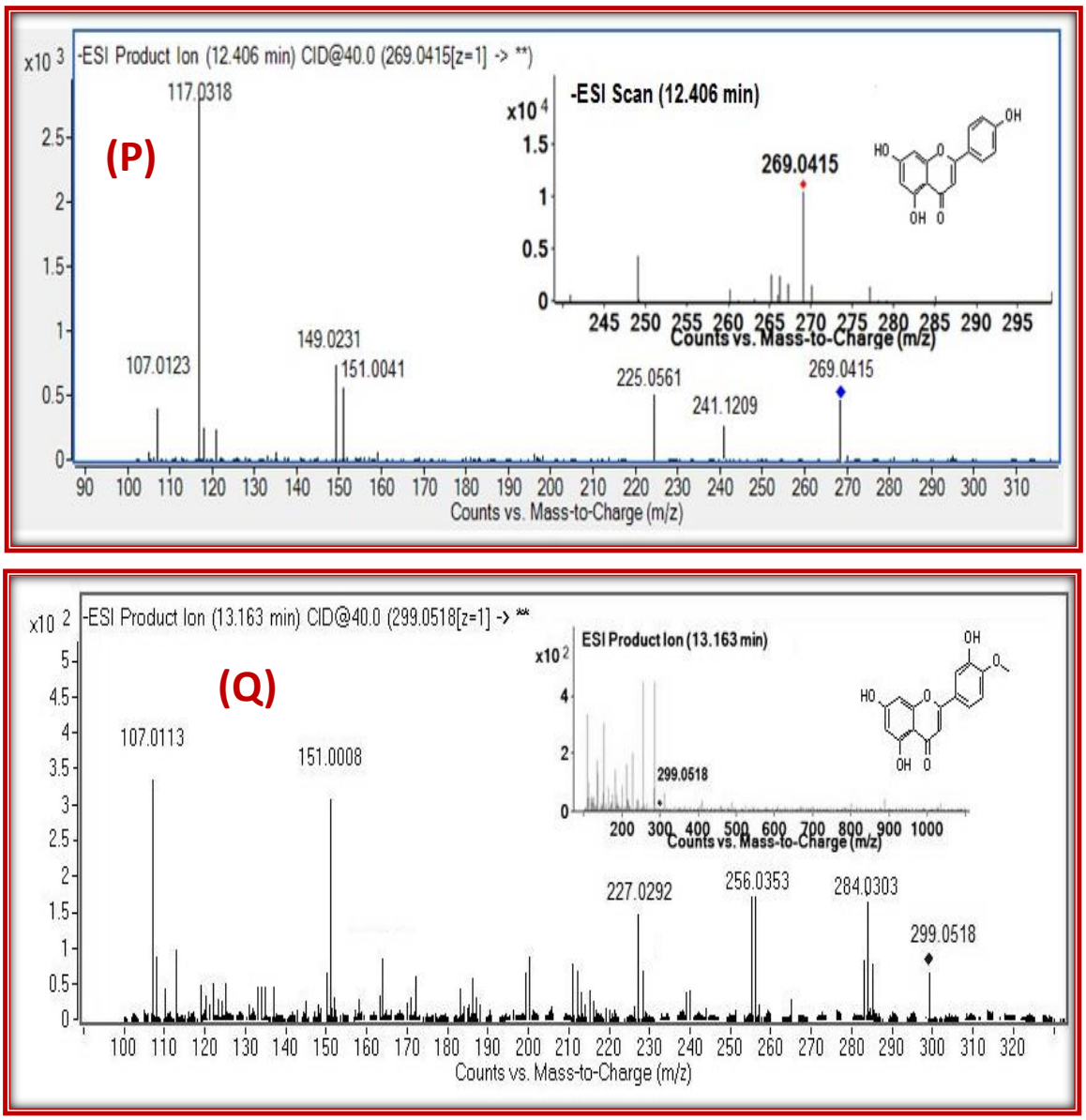


Fig. 3.17: HPLC-ESI-QTOF and MS chromatograms of the active fractions of *Abrus precatorius* (APH-11 & APM-3) and *Artemisia nilagirica* (ANEB & ANM-9). **A)**: Abrectorin, **(B)**: Abruquinone A, **(C)**: Luteolin, **(D)**: Scopoletin, **(E)**: Isoferulic acid, **(F)**: 3,4-Dicaffeoylquinic, **(G)**: Euoniside, **(H)**: Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy, **(I)**: Penduletin, **(J)**: Casticin, **(K)**: Quercetin 3,7-dimethyl ether, **(L)**: 3,5-Dihydroxy-6,7,8-trimethoxyflavone, **(M)**: Kaempferol, **(N)**: Pedilatin, **(O)**: Axillarin, **(P)**: Apigenin, **(Q)**: Diosmetin. Names and details of the compounds to corresponding peak numbers are given in Table 3.7.

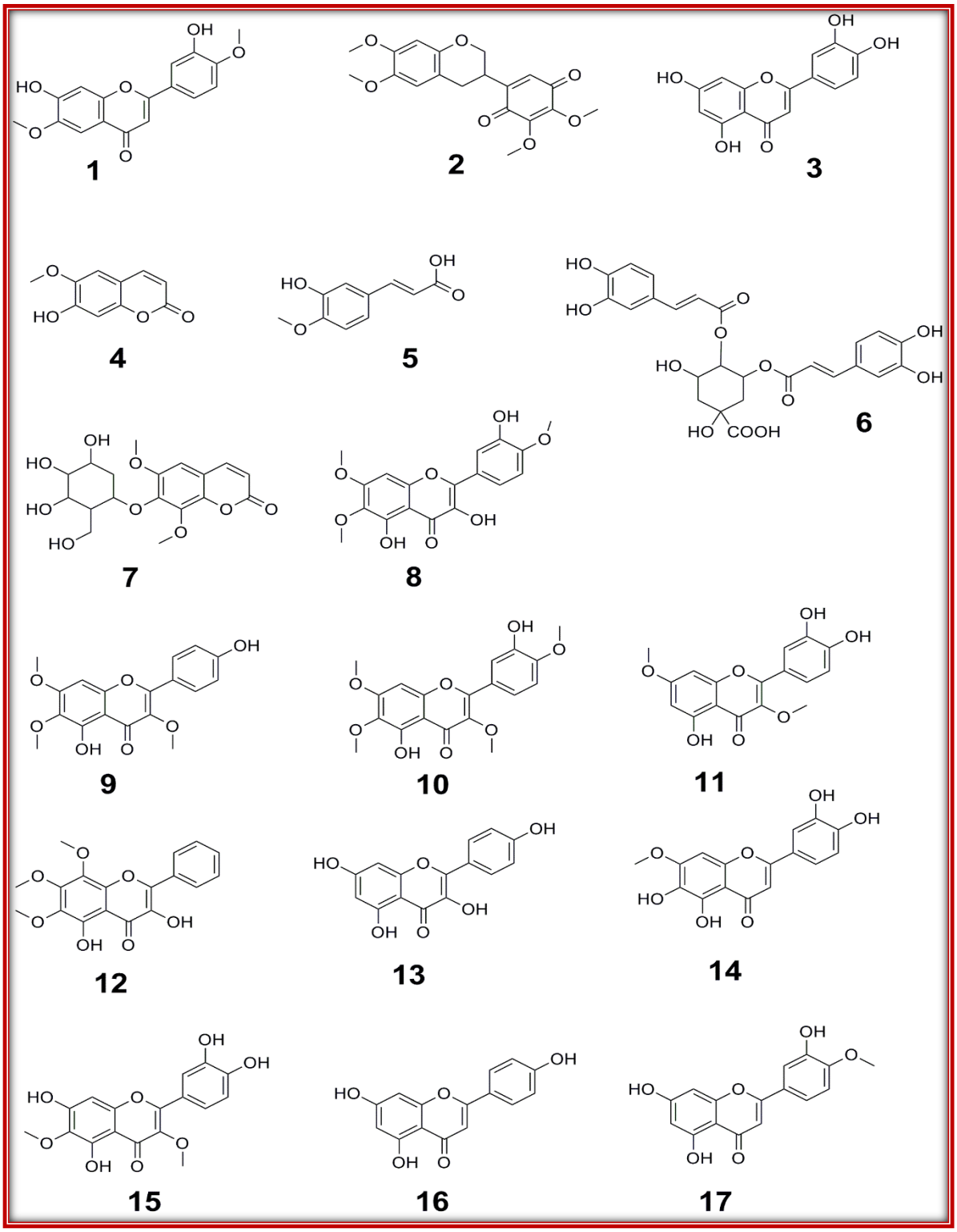


Fig. 3.18: Chemical structures of characterised compounds from active fractions of *Abrus precatorius* (APH-11 & APM-3) and *Artemisia nilagirica* (ANEB & ANM-9).
(1): Abrectorin **(2):** Abruquinone A **(3):** Luteolin **(4):** Scopoletin **(5):** Isoferulic acid **(6):** 3,4-Dicaffeoylquinic **(7):** Euoniside **(8):** Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy **(9):** Penduletin **(10):** Casticin **(11):** Quercetin 3,7-dimethyl ether **(12):** 3,5-Dihydroxy-6,7,8-trimethoxyflavone **(13):** Kaempferol **(14):** Pedilatin **(15):** Axillarin **(16):** Apigenin **(17):** Diosmetin.

LC-MS/MS technologies are extremely important for characterization and quantitation of herbal medicines. It provides important information that may be of relevance in helping to elucidate which components are the possible active ingredients within herbal medicines because full characterization of these products is a desirable goal [Wang et al., 2000]. Moreover, MS/MS pattern give further structural information on the target compounds and increases the probability of their presence in the samples [Cuyckens and Claeys, 2000; Sánchez-Rabaneda et al., 2003]. ESI was preferred for the study of compound identification because it has proven effective in generating molecular ions of a variety of compounds without causing much fragmentation [D'Acquarica et al., 2010]. Because it is a rapid heating technique, thermal degradation is also minimized. Therefore, in order to analyse the metabolite profiling and major bioactive constituents of *A. precatorius* (APH-11 & APM-3) and *A. nilagirica* (ANE-B & ANM-9) that may be playing the role in their cytotoxicity properties, LC-MS/MS analysis were employed as it provides several advantages in terms of sensitivity, selectivity and peak purity considerations. Based on the UV and MS/MS results, 17 different chemical compounds were identified in four different fractions (Table 3.7; Fig. 3.18). The Identified compounds were from coumarins, cinnamic acids and flavonoids class of phytochemicals.

The identified compounds from *A. precatorius* fractions (Abruquinone A, Luteolin and Abrectorin) in APH-11 and APM-3 were reported to have numerous biological activities. Abruquinone A, which is classified as a flavonoid, has been isolated previously from *A. precatorius* roots and proven to possess anti-inflammatory, anti-allergic and cardiovascular effects in several studies [Hsu et al., 1997; Kuo et al., 1995; Lupi et al., 1979]. Luteolin belongs to the flavonoid group of compounds that exhibits a widespread pharmacological benefits including antioxidant, hypertension, inflammatory disorders, cancer and other chemopreventive effects [Lin et al., 2008]. Abrectorin has been confirmed in *A. precatorius* and other plant species with medicinal properties [Bhardwaj et al., 1980; Harborne and Williams, 2000].

A total of 14 compounds were identified from ANE-B and ANM-9 fractions of *A. nilagirica.* As per the available information, compounds such as Scopoletin, 3, 4-Dicaffeoylquinic acid, Apigenin and Diosmetin were previously reported from *A. nilagirica* species [Haggag et al., 2003; Lee et al., 1998; Okuda and Hideyuki 2011]. However, Quercetin 3, 7-dimethyl ether, Kaempferol, Pedilatin have been reported previously as their derivatives

or as glycosides [Bora and Sharma, 2011]. Although the presence of Isoferulic acid, Euoniside, Flavone-3-3'-5-trihydroxy-4'-6-7-trimethoxy (eupatin), Penduletin, Casticin, Quercetin 3,7-dimethyl ether, Kaempferol, Pedilatin, Axillarin are already reported in *Artemisia* genus [Bora and Sharma, 2011; Turi et al., 2014] but not in *A. nilagirica*. Therefore, this is the first time that these compounds are being reported in this particular species. Some of these identified compounds from *A. nilagirica* fractions are reported to exhibit multifunctional biological activities and have great pharmacological importance, while some compounds are yet to be studied for their biological activities particularly cancer. Scopoletin and Euoniside which belongs to the class of coumarins have exhibited significant pharmacological activities like an antioxidant, anti-inflammatory, anti-arthritic, anti-angiogenetic agent and also displays anti-tumor activity by inducing cell cycle arrest and increase apoptosis in different human cancer cells in earlier studies [He et al., 2009; Hsieh et al., 2013; Liu et al., 2012; 2013]. Isoferulic acid, 3,4-Dicaffeoylquinic acid belongs to the cinnamic acid group of compounds that exhibits a widespread pharmacological benefits including antioxidant, hypertension, inflammatory disorders, cancer and other chemopreventive effects [Farah et al., 2008; Rocha et al., 2012; Urushisaki et al., 2011; Wang et al., 2005]. Penduletin, Casticin, Flavone-3-3'-5-Trihydroxy-4'-6-7-Trimethoxy, 3,5-Dihydroxy-6,7,8-trimethoxyflavone, Quercetin 3,7-dimethyl ether, Kaempferol, Diosmetin, Apigenin, Axillarin, 3,5-Dihydroxy-6,7,8-trimethoxy flavone, Quercetin 3,7-dimethyl ether are from flavonoids group of natural substances. These compounds have exhibited a broad spectrum of beneficial biological properties including cancer [Androutsopoulos et al., 2009; Caltagirone et al., 2000; Chen and Chen 2013; HaÏdara et al., 2006; Hsieh et al., 2013; Jiang et al., 2013; Kadifkova et al., 2005; Li et al., 2005; Liu et al., 2013; Meirinhos et al., 2005; Ruela-de-Sousa et al., 2010; Wang et al., 2000; Zeng et al., 2012]. The presence of rich bioactive compounds can serve as candidate indexing compounds in the active fractions of *A. precatorius* and *A. nilagirica* plants and makes these plants a potential source of bioactive compounds. In addition, our observations support the notion that the combination of several bioactive compounds in these fractions exerts their anti-cancer activity, and hence, pave the way for further research efforts aimed at elucidating if any of the identified compounds could be mainly responsible for these effects.

Chapter IV:

Summary and Conclusion

Keeping in view the demand for developing natural antioxidants and effective anti-proliferative drugs, the present study was aimed to scientifically explore the bio-efficacy with respect to *in vitro* antioxidant and anti-proliferative activities of four potent medicinal plants widely used in Indian traditional system of medicine, namely, *Abrus precatorius, Artemisia nilagirica, Cissampelos periera* and *Cocculus hirsutus.* The selection of these plants for evaluation was based on their traditional usage due to their potential health promoting and pharmacological attributes. Even though these plants are widely used against several disorders, no systematic study on the nature of antioxidant and anti-proliferative action has been reported so far. The objectives of the present study were, therefore, to evaluate these selected plants for their antioxidant and anti-cancer activities. It was proposed to screen their successive extracts for their *in vitro* antioxidant and anti-cancer activity using standard procedures and to carry out the bioactivity-guided investigation in order to identify the compounds responsible for their anti-proliferative action.

The results of the sequential extraction of selected plant powders with n-hexane, ethyl acetate, ethanol and water suggested that methanol was a better solvent for the extraction of *A. precatorius, C. pereira* and *C. hirsutus* powders, while water was better solvent for the extraction of *A. nilagirica* plant powder material. Overall, the yield percentage was high in polar solvents than non- polar or low polar solvents.

Preliminary phytochemical analysis revealed significant aggregate of phenolic and flavonoid contents in the selected medicinal plants. The enrichment of phenolic constituents in the extracts was found to be dependent on the extracting solvents. Methanolic extracts of *A. nilagarica* and *C. hirsutus* had high total phenolic content while water extract and ethyl acetate extracts of *A. precatorius* and *C. pareira* respectively showed highest total phenolic content suggested that these solvents could function as appropriate for the extraction of phenolic compounds. This study also showed ethyl acetate extracts of *A. precatorius, A. nilagirica* and *C. pareira* respectively had a significantly high ($P<0.05$) flavonoid content than other extracts of each plant. This indicated that ethyl acetate was the best solvent for the extraction of flavonoid in *A. precatorius, A. nilagirica* and *C. pareira.* The methanol solvent was found to be efficient solvent of extraction for flavonoids in *C. hirsutus*.

In vitro antioxidant potential of extracts of selected plants using phosphomolybednum and FRAP methods, showed significant activity with ascorbic acid as a standard reducing agent. Ethyl acetate extracts of *A. nilagirica* and *C. pareira* whereas

alcohol extracts of *A. precatorius, C. hirsutus* showed pronounced antioxidant activity than water and hexane extracts. This suggested that these were better solvents for the extraction of plant antioxidants in the studied plants.

The extracts obtained by different solvents were further subjected to screening for their possible antioxidant activity. Six complementary test systems, namely, $DPPH^{\bullet}$, hydroxyl radical, hydrogen peroxide, nitric oxide radical, superoxide anion and lipid peroxidation scavenging assays were used for this purpose. It was evident from the results that the different solvent extracts of selected plants exhibited efficient antioxidant capacity and strong free radical scavenging effects on a series of free radicals and oxidants ($DPPH^{\bullet}$, $^{\bullet}OH$, H_2O_2, $NO^{\bullet}$, $O_2^{\bullet-}$ lipid peroxidation). Results of this study demonstrated that different extracts exhibited varying degrees of inhibition towards the tested radicals and oxidants, therefore, showing that antioxidant activity of extracts was strongly dependents on the extraction solvent. These results suggested that extracts of selected medicinal plants have reducing capacities, thus neutralizing the free radical, and may have potential health benefits to reduce ROS, hence the oxidative stress in the body. This diverse antioxidative effects observation can be attributed to different mechanisms exercised by phenolic compound(s) and to the synergistic effects of different compounds. However, the components responsible for the antioxidative activities of the selected medicinal plants are still unclear, if properly and extensively studied, could provide many chemically interesting and biologically active drug candidates.

In the present study, we further studied the cytotoxic effects of crude extracts of selected plants against five human cancer cell lines, namely; human acute monocytic leukaemia cell line (THP-1), human T-cell lymphoblastic lymphoma cell line (Sup-T1), human B cell lymphoma cell line (JM-1), human hepatocellular carcinoma cell line (HepG2), human cervix adenocarcinoma cell line (HeLa) using an *in vitro* MTT cytotoxicity assay. The murine peritoneal macrophages and Human embryonic kidney cell line (HEK-293) were used as a model for normal cells. In the course of our preliminary screening of all the crude extracts of selected plants, only four crude extracts, two from *A. precatorius* (APE and APE) and two from *A. nilagirica* (ANE and ANA) exerted marked inhibitory potency against all selected human cancer cell lines in a concentration dependent manner and presented different patterns of IC_{50} (µg/mL) among the cell lines tested. These extracts demonstrated to be more

active in the cancer cell lines without affecting the normal cells (HEK-293 and murine peritoneal macrophages).

The active crude extracts of both the plants (*A. precatorius* and *A. nilagirica)* were subsequently submitted to bioassay guided fractionation using column chromatography with solvents of increasing polarity and re-assayed under same conditions. Bioassay-guided fractionation of active crude extracts from *A. precatorius* and *A. nilagirica* produced successively four more cytotoxic fractions, APH-11 & APM-3 and ANE-B & ANM-9 respectively. According to cytotoxic results, the fractionation procedure potentiated the cytotoxic activity of the crude extracts and it was found that the active fractions from crude extracts were more effective in inhibiting proliferation of panel of cell lines. Furthermore, these fractions not only exhibited selectivity, but also did not show any significant cytotoxic effects in non-cancerous cell lines; HEK-293 and murine peritoneal macrophages. It proved that these active fractions of *A. precatorius* and *A. nilagirica* had potential cytotoxicity against panel of human cancer cells, but they were virtually non-toxic to the normal cells.

Apoptosis is the pharmacodynamic end point of anti-cancer drug remedy as this phenomenon safeguards that no cancer resistance to chemotherapy will occur. Moreover, apoptosis is an autonomous dismantled progression to eliminate individual components of cells and avoid inflammatory effects which are generally concomitant with necrosis. Therefore, no toxicity to the normal nearby cells would occur when cells undergo apoptosis. To investigate whether apoptosis was involved in the cell death caused by these active fractions on three leukemic cell lines, DNA laddering on agarose gel electrophoresis, TUNEL assay and cell cycle analysis were carried out. In our results, the changes were observed in all the three tested cell lines by degradation of chromosomal DNA which is indicative of the late stage of apoptosis. TUNEL assay based on labelling of DNA strand breaks generated during apoptosis revealed that fractions of *A. precatorius* and *A. nilagirica* induced apoptosis in cells. Apoptotic effects of fractions on these cancer cell lines was further investigated by examining the cell cycle distribution using PI staining. The quantitative data indicated that on exposure to fractions, the cells underwent apoptosis accompanied by an alteration in the cell cycle in a time dependent manner.

Further experimental insight into the mechanism of action towards leukemic cells has been acquired to confirm early stage apoptotic cell death through activation of pro-apoptogenic proteins by western blotting. Studies have identified caspases as important

mediators of apoptosis induced by various apoptotic stimuli. We investigated the activation of caspase-3/7 and PARP in cells treated with active fractions for 24 h. Both procaspases-3/7 and PARP were significantly activated when THP-1 cells were exposed to 50 µg/mL of fractions. In contrast, no active form of caspase-3/7 was observed in SupT1 and JM-1 cells, but PARP was activated in both these cell lines. The probable mechanism by which these fractions induced apoptosis in THP-1 cells was caspase dependent pathway in which sequential activation of caspase-3/7 and ultimately, PARP cleavage was observed. However, the active fractions, on the other hand, were unable to trigger the caspase cascade in SupT-1 and JM-1 cells. This indicted the caspase-independent mechanism might be involved in the cell death in SupT1 and JM-1 cells. The results suggested that the activation of caspases involved in the apoptotic pathway is one of the major mechanisms by which these fractions affected THP-1 cells, whereas it can be hypothesized that anti-proliferative effectiveness of fractions may invoke other possible pathways in SupT1 and JM-1 cell lines.

We also made an attempt to identify the components in the active fractions of both the plants by LC-DAD-ESI-MS analysis. The chemical investigation led to the identification of **17** compounds in which 3 from *A. precatorius* fractions and 14 from *A. nilagirica* fractions. The Identified compounds were from coumarins, cinnamic acids and flavonoids class of phytochemicals. Some of the identified compounds have been reported to exhibit multifunctional biological activities and possess great pharmacological importance, while some compounds are yet to be studied for their biological activities particularly cancer. Based on the results for the chemical composition of fractions, we can conclude that these compounds may contribute to the anti-proliferative activities. The presence of rich bioactive compounds can serve as candidate indexing compounds in the active fractions of *A. precatorius* and *A. nilagirica* plants and makes these plants a potential source of bioactive compounds. In addition, our observations support the idea that the combination of several bioactive compounds in these fractions exerts their anti-cancer activity, and henceforth, paves the way for further research efforts designed at elucidating if any of the identified compounds could be principally responsible for these anti-cancer effects.

In conclusion, the overall findings of the study provide substantial evidence that selected medicinal plants used in Indian traditional system of medicine have significant antioxidant properties and making them promising source of natural antioxidants. These plants species may have great relevance in the prevention and therapies of diseases in which

oxidants or free radicals are implicated after further *in vitro* and *in vivo* research for understanding their mechanism of action as antioxidant.

Furthermore, the results of the present study suggested that *A. precatorius* and *A. nilagirica* may be useful for integrative and complementary medicine by promoting cell death in three leukemic cell lines via proliferation, alteration of cell cycle and induction of apoptosis without inducing substantial damage to non-cancerous cells; murine peritoneal macrophages and HEK-293 cells. Although the clear molecular mechanism by which apoptosis is induced by these fractions remains unclear, our findings increased the present level of understanding of the mechanisms of action of fractions of A. *precatorius* and *A. nilagirica*, explaining their promising biological properties.

However, further studies are required to i) understand the complete molecular basis of their anti-leukemic selectivity, ii) standardizing the active fractions and evaluating the latter with *in vivo* models, iii) elucidating the compounds responsible for the observed cytotoxic activities and their exact mechanism of apoptosis determine which of the identified bioactive compounds are responsible for these effects and iv) perform preclinical developmental research aimed at ensuring their safety and efficacy as therapeutic agents. The positive outcomes of such studies could form a strong basis for the development of *A. precatorius* and *A. nilagirica* as a source for the prevention and/or intervention of leukemia.

Chapter V:

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Annexure:

Publications and Conferences attended

❖ LIST OF RESEARCH PAPERS PUBLISHED PEER REVIEWED JOURNALS:

1. **Gul MZ,** Bhakshu LM, Ahmad F, Kondapi AK, Qureshi IA, Ghazi IA: Evaluation of *Abelmoschus moschatus* extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using in vitro assays. *BMC Complement Altern Med*., 2011; 11:64.
2. Kumar A, **Gul MZ**, John MM, Waikhom B, Ghazi IA: Differential Responses of Non-enzymatic Antioxidative System under Water Deficit Condition in Rice (*Oryza sativa* L.). IPCBEE, 2011; 9, *IACSIT Press*, Singapore.
3. **Gul MZ,** Vidya A, Qureshi IA, Ghazi IA: Antioxidant and α-glucosidase inhibitory activities of *Murraya koenigii* leaf extracts. *Phcog J*., 2012; 4(32):65-72.
4. **Gul MZ,** Ahmad F, Kondapi AK, Qureshi IA, Ghazi IA: Antioxidant and antiproliferative activities of *Abrus precatorius* leaf extracts-an *in vitro* study. *BMC Complement Altern Med*., 2013; 13:53.
5. Kumar A, **Gul MZ*,** Zeeshan A, Waikhom B, Qureshi IA, Ghazi IA: Differential antioxidative responses of three different rice genotypes during bacterial blight infection. *AJCS*, 2013; 7(12):1893-1900. **(* Equal Contribution)**

❖ MANUSCRIPT UNDER PREPARATION:

6. **Gul MZ,** Sambamurthy C, Manjulatha K, Radheshyam M, Qureshi IA, Ghazi IA: Evaluation of the antiproliferative activity of Leaves from *Abrus precatorius* by Bioassay-guided fractionation.
7. **Gul MZ,** Sambamurthy C, Manjulatha K, Radheshyam M, Qureshi IA, Ghazi IA: Bioassay-guided fractionation and *in vitro* antiprliferative effects of fractions of *A. nilagirica*.
8. **Gul MZ,** Sambamurthy C, Radheshyam M, Qureshi IA, Ghazi IA. Antioxidant and enzyme inhibitory activities of *Cissampelos pereira* leaf extracts.
9. **Gul MZ,** Bhat MY, Sambamurthy C, Radheshyam M, Qureshi IA, Ghazi IA: Antiproliferative and antioxidant properties of extracts from *Artemisia nilagirica*.

❖ PRESENTATIONS IN CONFERENCES/SEMINARS:

- Poster presentation at 82nd Annual meeting of The Society of Biological Chemists (India) and International Conference on Genomes: Mechanism and Function, at School of Life Sciences, University of Hyderabad on 2-5 December, 2013.
- Poster presentation at International Workshop on "Frontiers in Biological Sciences" organized by University of Hyderabad and Academica Sinica Taiwan on 8-9, April, 2013
- Poster presentation at International symposium on “Chemistry and chemical biology of natural products”, August 2-4, 2012 CSIR-IICT, Hyderabad, India.
- Oral Presentation in Plant Science Colloquim-2012 organized by Department of Plant Sciences, University of Hyderabad, 10th January, 2012.

Reprints of Published articles

BMC Complementary & Alternative Medicine

RESEARCH ARTICLE Open Access

Evaluation of *Abelmoschus moschatus* extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using *in vitro* assays

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Abstract

Background: *Abelmoschus moschatus* Medik. leaves and seeds are considered as valuable traditional medicine. The aromatic seeds of this plant are aphrodisiac, ophthalmic, cardio tonic, antispasmodic and used in the treatment of intestinal complaints and check queasiness. To give a scientific basis for traditional usage of this medicinal plant, the seed and leaf extracts were evaluated for their antioxidant, free radical scavenging, antimicrobial and antiproliferative activities.

Methods: In this study, antioxidant, antimicrobial and antiproliferative activities of *A. moschatus* extracts were evaluated in a series of *in vitro* assay involving free radicals, reactive oxygen species and their IC_{50} values were also determined. The antioxidant activities of the seed and leaf extracts of *A. moschatus* were determined by total antioxidant, DPPH, and ferrous reducing antioxidant property (FRAP) methods. In addition, the antiproliferative activity was also evaluated using colorectal adenocarcinoma and retinoblastoma human cancer cell lines. Moreover, six bacterial reference strains, two gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), four gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris* and *Salmonella enterica paratyphi*) and one fungal strain (*Candida albicans*) were used to evaluate its antimicrobial activity.

Results: The results from this study showed that the antioxidant activities of *A. moschatus* as determined by the total phenol, flavonoids, total antioxidant and FRAP methods were higher in leaf than that of the seed extracts. On the other hand, the aqueous overnight seed extract (AMS-I) has shown significant radical scavenging activity as in 1, 1- Diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide, hydroxyl radical, superoxide and lipid peroxidation as compared to other seed and leaf extracts. The AMS-I and AML-IV have shown activity against six and seven microorganisms respectively. Simulteneously, AMS-IV and AML-IV have demonstrated potential antiproliferative activity against two human cell lines - Colorectal adenocarcinoma (COLO-205) and retinoblastoma (Y79).

Conclusion: The seed and leaf extracts of *A. moschatus* possess significant antioxidant activity and could serve as free radical inhibitors or scavenger, or substitute, probably as primary antioxidants. The plant possesses moderate antibacterial activity against bacterial strains used in this study. Hydroalcoholic seed and leaf extracts also exhibited antiproliferative activity against two human cancer cell lines. *A. moschatus* may therefore, be a good candidate for functional foods as well as pharmaceutics.

Background

The free radicals (FR) and reactive oxygen species (ROS) are produced through frequent physiological and biochemical processes in the human body as byproduct [1,2]. ROS includes a number of chemically reactive molecules derived from oxygen, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (OH^-) etc. Over production of such free radicals might leads to oxidative damage of biomolecules in the body (e.g. lipids, proteins, DNA) that can initiate number of diseases like atherosclerosis, diabetes mellitus, cancer, heart and neurodegenerative diseases etc. [3,4]. The harmful effect of the free radicals can however, be blocked by antioxidant substances. Plants produce wide

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array of secondary metabolites such as phenolic compounds (phenolic acids, flavonoids, quinines and coumarins), nitrogen compounds (alkaloids and amines), vitamins, terpenoids and other secondary metabolites that have been proved for antioxidant activities [5,6]. Current research has confirmed that antioxidants are the most effective tools to eliminate free radicals which cause oxidative stress and are possible protective agents that protect the cells from ROS and retard the progress of many diseases as well as lipid peroxidation [7-9]. Moreover, in recent past, the polyphenols have found to be beneficial as strong antioxidants [10]. In this context, evaluation of the polyphenols and antioxidant activity in herbs has become important tool to understand the healing property of medicinal plants.

Natural products from the medicinal plants provide unlimited opportunities for new drugs because of the unparalleled accessibility of diverse chemical compounds [11]. Cancer is a leading cause of death worldwide and it accounted for 7.9 million deaths (around 13% of all deaths) in 2007. It is also reported that more than 70% of all deaths of cancer occurred in middle and low income countries. Anticancer drugs from natural sources such as plants, marine organisms and microorganisms account approximately 60% of all anticancer drugs [12]. For thousands of years, human beings have used natural substances especially plants to relieve pain, heal wound and maintain health. Most of the bioactive components in medicinal plants probably evolved as chemical defence agents against infections or predators. Many plants were proved to be very important source of anticancer agents due to reducing risk factors of the cancer by consuming vegetables and fruits which are rich in naturally occurring phytochemicals including phenols and flavonoids [13]. Several investigations were carried out to evaluate anticancer properties of herbs and are being used as potent anticancer drugs [14]. In addition to antioxidant activity, the valuable health benefits of different medicinal plants were claimed as antibiotic agents against pathogenic microorganisms. There is also an urgent necessity to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants [15]. The antimicrobial compounds generated by these medicinal plants are active against plant and human pathogenic microorganisms [16]. They are efficient in the treatment of infectious diseases and at the same time mitigate many of the side effects namely, hypersensitivity, immune-suppression and allergic reactions that are often associated with synthetic antimicrobials [17]. Moreover, there is an alarming prevalence of antibiotic resistance in bacteria of medical importance [18].

Keeping in view of the demand for developing natural antioxidants, effective antiproliferative and antimicrobial drugs, the present study was aimed to investigate the antioxidant, anti free radical, antimicrobial and antiproliferative activities of different extracts of *A. moschatus*, which belongs to family, malvaceae and popularly known as Mushkdana/Kasturi bhendi. The selection of this plant for evaluation was based on its traditional usage. A survey of the literature revealed that the seeds of this plant (powdered form in lukewarm milk) have been recommended for use in various traditional systems of medicine for the treatment of intestinal complaints, constipation, dyspepsia and gonorrhea. The seeds are used as stimulant, relaxant and also for casting out the poison of snakes. The seeds also serve as cardiac tonic, aphrodisiac, diuretic, antispasmodic. Moreover, the leaf decoction has been effective against intestinal complaints and checks vomiting. The tincture of leaf powder is applied for skin diseases [19]. Thus, our main objective of the present investigation was to evaluate the antioxidant, antiproliferative and antibacterial potential of this plant, in addition to quantifying the polyphenols of extracts, which might be responsible for biological activities.

Methods

Chemicals

All the chemicals were purchased from Hi-Media and Merck, India. Standard drugs were purchased from Sigma-Aldrich chemicals co. (St. Louis O., USA); and RPMI-1640, DMEM & serum from Gibco, (Invitrogen, USA). Analytical grade solvents were used in this study.

Plant material

Seeds and healthy leaves of *A. moschatus* were collected from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Regional Centre Hyderabad, India during the month of September-October, 2009. The seeds and leaves were cleaned, dried under shade, ground to a coarse powder and stored in an air-tight container at 25°C for further use.

Microbial cultures

Bacterial reference strains *Bacillus subtilis* ATCC 5740, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 6380, *Salmonella enterica paratyphi (Salmonella paratyphi*) ATCC 9150 and *Candida albicans* ATCC 10231 were obtained from Central Research Institute of Unani Medicine, Hyderabad, India. The pure cultures were maintained on nutrient agar slants for the entire study. All the isolates were sub-cultured at regular time period and stored at 4°C as well as at -80°C by making their suspension in 10% glycerol.

Preparation of plant extracts

The seed and leaf powder of *A. moschatus* were subjected to different modes of extraction using ethanol

and water in order to find out the suitable extract with maximum biological activities. Aqueous extracts of *A. moschatus* seed (AMS-I) and leaf (AML-I) were prepared by soaking 1 g of dried powder in 4 mL of distilled water for 24 h at room temperature. Other aqueous extracts (AMS-II and AML-II) were prepared using dried powder of seed and leaf (1 g each) through slow evaporation at 30-40°C. Also decoctions were prepared from the seed and leaf powder (1 g each) by mixing with 20 mL distilled water for 3-4 h at 80-90°C and the extracts were designated as AMS-III and AML-III respectively.

Hydroalcoholic extracts of seed (AMS-IV) and leaf (AML-IV) were prepared by dissolving the dried powder of seed and leaf (1 g each) in 20 mL of 80% (v/v) ethanol for 3-4 h at 40-50°C and were evaporated to 4 mL. The suspensions prepared in all above cases were centrifuged at 10,000 rpm for 15 min. The supernatant were collected into separate tubes and concentrated to the dry mass using vacuum evaporator. The residues were stored in amber glass bottles at -20°C for further analysis. The dried extracts of 20 mg/mL stock solution were prepared and different concentrations were used in the experiments.

Determination of total phenolic content

The amount of total soluble phenolic content in different seed and leaf extracts was determined according to Folin-Ciocalteu method [20] with slight modifications. Briefly, 10 μL of extract solution from the stock solution was mixed with 100 μL of Folin-Ciocalteu reagent. After 10 min of incubation, 300 μL of 20% Na_2CO_3 solution was added and the volume was adjusted to 1 mL using distilled water. The mixture was incubated in dark for 2 h and the absorbance was measured at 765 nm using a UV-Vis spectrophotometer against blank sample. The total phenolic content was measured as gallic acid equivalents (mg GAE)/gram of dry weight (dw) and the values were presented as means of triplicate analysis.

Determination of total flavonoid content

Total flavonoid content was estimated by a colorimetric method [21] by taking 20 μL of each extract and mixed with 500 μL Milli-Q water and 30 μL of 5% $NaNO_2$ solution. After 5 min of incubation at room temperature, 60 μL of 10% $AlCl_3$ solution was added. Subsequently, 350 μL of 1 M NaOH and 40 μL of Milli-Q water were added to make the final volume 1 mL. Samples were further incubated for 15 min at room temperature and the absorbance of the samples was measured at 510 nm. The total flavonoids were determined as qurecetin equivalents (mg QE)/g of dw and the values were expressed as means of triplicate analysis.

Evaluation of antioxidant capacity

Determination of total antioxidant activity (TAA)

The total antioxidant activity of both seed and leaf extracts of *A. moschatus* were evaluated by phosphomolybdenum method [22]. The assay is based on the reduction of Mo (VI) - Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. Different extracts of 10 μL each from the stock solution were dissolved in 90 μL distilled water and 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in 1.5 mL tubes. The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the solution of each reaction was measured at 695 nm against blank samples. Ascorbic acid (AA) was used as standard and the total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (mg AAE/g) of dw.

Determination of reducing antioxidant power (FRAP)

The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method [23] was used to measure the reducing capacity of seed and leaf extracts with a slight modification which involves the presence of extracts to reduce the ferricyanide complex to the ferrous form. The FRAP method is based on a redox reaction in which an easily reduced oxidant (Fe^{3+}) is used in stoichiometric excess and antioxidants acts as reductants. Various concentrations of extracts (seed and leaf) of *A. moschatus* from the stock solutions and the standard (ascorbic acid) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 min. Then 2.5 mL of trichloroacetic acid (10% w/v) was added to the reaction mixture, which was then centrifuged at 1000 g for 10 min. The upper layer of the solution (2.5 mL) was mixed with deionised water (2.5 mL) and ferric chloride (0.5 mL, 0.1% w/v). The absorbance was measured at 700 nm at the reaction time of 30 min. The reducing power of the extracts was represented as mg AAE/g of dw.

DPPH radical scavenging activity

The antioxidant activity of the plant extracts was assessed on the basis of the radical scavenging effect using stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) [24]. DPPH solution (0.004% w/v) was prepared in 95% methanol and serial dilutions were carried out with the stock solutions (20 mg/mL) of the extracts. Various concentrations of extracts were mixed with DPPH solution (900 μL), incubated in dark for 30 min and then absorbance was measured at 517 nm. Methanol (95%), DPPH solution and ascorbic acid (AA) were used as blank, control and reference standard respectively.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to a ferrous ion oxidation - xylenol orange (FOX) assay [25] with minor changes. FOX reagent was prepared by adding nine volumes of reagent 1 to one volume of reagent 2, where reagent 1 was 4.4 mM butylated hydroxytoluene (BHT) in methanol and reagent 2 was 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 250 μM H_2SO_4. Plant extracts of different concentrations were incubated with 10 μL of 40 mM H_2O_2 for 10 min at room temperature in dark and 0.2 mL of FOX reagent was added and the volume was made upto 1 mL with distilled water. The reaction mixture was then vortexed and incubated at room temperature for 30 min. Development of violet colour indicates control reaction and discoloration was considered as scavenging activity after the addition of plant extracts or standard (ascorbic acid). The FOX reagent without extracts/H_2O_2 served as blank and with H_2O_2 served as control. The absorbance of the ferric-xylenol orange complex was measured at 560 nm.

Superoxide radical scavenging activity

The superoxide radical scavenging activity of seed and leaf extracts of *A. moschatus* was performed according to the method given by Kakkar *et al.* [26] with minor modifications. Briefly, solutions containing 156 μM nitroblue tetrazolium (NBT) dissolved in 50 mM phosphate buffer (pH 7.4), 468 μM nicotinamide adenine dinucleotide (NADH) and various concentrations of extracts were mixed. The reaction was started by addition of 100 μL of 60 μM phenazine methosulfate (PMS) solution and the final volume of the reaction was 3 mL. The reaction mixture was incubated at 25°C for 5 min and absorbance at 560 nm was observed against control samples (with NADH).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured as per the protocol of Kunchandy and Rao [27] with minor changes by studying the competition between deoxyribose and test extracts for hydroxyl radicals generated by Fenton's reaction. Briefly, solution of Fenton's reagent [Fe (III) chloride, ascorbic acid and H_2O_2] was prepared in distilled water just prior to use. To 0.1 mL Fenton's reagent, thiobarbituric acid (1% w/v) in 25 mM NaOH (1 mL) and tricholoroacetic acid (1 mL, 2.8% w/v) were added and volume was made to 3 mL with distilled water. The mixture was heated for 90 min on water bath at 80°C and the amount of pink chromogen produced was considered as control. Finally it was measured spectrophotometrically at 532 nm. The protection of oxidation of D-ribose has been conducted by pre-incubation with the *A. moschatus* extracts in different concentrations and decrease in the formation of pink colour was considered as antioxidant property which was compared to the standard ascorbic acid.

Inhibition of Fenton's reagent-induced strand breaks in plasmid DNA

The ability of different extracts to protect super coiled pBR322 DNA from devastating effects of hydroxyl radicals generated by Fenton's reagent was assessed by DNA nicking assay [28] with minor modifications. The reaction mixture contained 2.5 μL of plasmid DNA (0.25 μg) and 10 μL Fenton's reagent (30 mM H_2O_2, 500 μM ascorbic acid and 800 μM $FeCl_3$) followed by the addition of 5 μL of extracts and the final volume of the mixture was brought upto 20 μL with distilled water. The reaction mixture was then incubated for 45 min at 37°C and analyzed on 0.9% agarose gel electrophoresis by staining with ethidium bromide.

Determination of inhibition of Lipid peroxidation

Lipid peroxidation inhibitory activity of *A. moschatus* extracts and the standard (ascorbic acid) were carried out according to the standard protocol [29]. The rat liver homogenate was used for induction of lipid peroxidation, mediated by $FeCl_3$ as pro-oxidant. Healthy albino rats of the wister strain (250 g) were sacrificed and perfused the liver with 0.15 M KCl and homogenate was centrifuged at 800 g for 15 min at 4°C and the supernatant was used for the thiobarbutaric acid assay. The extracts of *A. moschatus* at different concentrations were mixed with the liver microsome preparation and the mixtures were incubated in the presence and absence of Fenton's reagent (50 μL of 10 mM $FeCl_3$; 10 μL of 2.5 mM H_2O_2) in phosphate buffer (0.2 M, pH 7.4) and the final volume was made to 1 mL. The reaction mixtures were incubated at 37°C for 30 min. After incubation, 2 mL of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid, 0.5% thiobarbutaric acid, and 0.5% butylated hydroxytoluene (BHT) was added to the reaction mixture, followed by heating at 100°C for 60 min. The reaction mixture was put in an ice bath for 10 min. The mixture was centrifuged at 1000 g for 10 min and the extent of lipid peroxidation was subsequently monitored by the formation of thiobarbutaric acid reactive substances (TBARS) as pink chromogen in the presence or absence of extracts and standard (ascorbic acid). The absorbance of the supernatant was measured spectrophotometrically at 532 nm. The decline in the formation of pink chromogen in the pretreated reactions was considered as inhibition of lipid peroxidation.

Antiproliferative activity

For the assessment of the antiproliferative activity of plant extracts, two human tumor cells, colorectal adenocarcinoma (COLO-205) and retinoblastoma (Y79) cells were used. The cell lines were purchased from National Centre for Cell sciences (NCCS), Pune, India. The antiproliferative activity of the selected cell lines was performed and the reduction of 3- (4, 5- dimethylthiozol-2-yl) - 2, 5- diphenyltetrazolium bromide was chosen as an optimal

end point of cell viability measurement. COLO-205 and Y79 cells (0.2×10^6 cells per well) were grown in DMEM and RPMI 1640 respectively, alongwith 10% Fetal bovine serum (FBS) in 96-well plates. Increasing concentrations (25, 50, 100, 200 μg) of seed and leaf extracts of *A. moschatus* dissolved in 10% Dimethyl sulfoxide (DMSO) were added to the cells (final concentration of DMSO was 2%) and incubated at 37°C under 5% CO_2 in a humidified incubator for 14 h. The cell suspension was centrifuged at 1000 g for 10 min and the medium was aspirated. Subsequently, the fresh growth medium containing 20 μL of MTT solution of 5 mg/mL was added to each well [30]. After incubation for 4 h in a humidified atmosphere, the medium was removed by centrifugation at 1000 g for 10 min and 200 μL of DMSO was added to the wells to dissolve the MTT-formazan crystals. The plates were shaken and absorbance was determined by ELISA reader (TECAN) at 570 nm. The conventional anticancer drug, ifosfamide was used as a positive control and 2% DMSO as solvent control. Controls and samples were assayed in triplicates for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing the absorbance between samples and controls.

Antimicrobial activity

The seed and leaf extracts of *A. moschatus* were tested against the reference strains for antimicrobial activity using micro dilution method in 96 well microtiter plates [31] with minor modifications and recommended by the National Committee for Clinical Laboratory Standard [32]. The antimicrobial activity of the extracts was evaluated against two gram positive (*B. subtilis* ATCC 5740, *S. aureus* ATCC 25923), four gram negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *P. vulgaris* ATCC 6380, *S. enterica paratyphi* ATCC 9150) bacterial strains and one fungal strain (*C. albicans* ATCC 1023). Briefly, antimicrobial activity was carried out in 96 well microtiter plate containing different concentrations of extracts. The culture suspension (100 μL) was added to each well having 10^5 CFU/mL and final volume was made to 200 μL by adding LB broth. Plates were incubated at 37 ± 1°C for 18 h and then 10 μL of MTT (5 mg/mL) was added to each well. The plates were examined with ELISA reader (TECAN) at 530 nm and the lowest concentration of each extract which showed complete inhibition was taken as its minimum inhibitory concentration (MIC). In control experiments, sterile distilled water and ethanol were added in place of plant extracts; whereas, antibiotics such as ampicillin, kanamycin and nystatin (1 mg/mL) were used as positive controls. For blank reaction, the sterile broth was used in place of suspension cultures (without inoculums).

Calculations and Statistical analysis

The percentage inhibition of free radicals, lipid peroxidation and cytotoxic activities of the extracts were calculated using the formula:

$$\%\,\text{Inhibition} = \left[(\text{A control} - \text{A sample})/\text{A control}\right] \times 100$$

All analyses were performed in triplicates. The experimental results were expressed as mean ± standard deviation of mean (SEM) of three replicates. The concentration of the extract that was required to scavenge 50% of radicals (IC_{50}) was calculated for different seed and leaf extracts of *A. moschatus*. The graphical representation of the results was done using Sigma -11 software.

Results

Total phenolic and flavonoid content

The results of total phenolic content of different seed and leaf extracts of *A. moschatus* were significant and shown in Table 1. The total phenol content in the seed extracts (AMS-I, II, III and IV) expressed as gallic acid equivalent (GAE) were in the range of 1.56 to 3.74 mg GAE/g dw. AMS-I had the highest content as 3.74 mg GAE/g dw, whereas AMS-IV contained a much smaller amount as 1.56 mg GAE/g dw. In leaf extracts, the total phenol content was significantly higher compared to seed extracts and varied from 9.49 to 13.84 mg GAE/g dw, AML-IV extract showed higher level of total polyphenol content (13.84 mg GAE/g dw), whereas the lowest content of total polyphenol was found in AML-I (9.49 mg of GAE/g dw).

Total flavonoid content of the seed and leaf extracts was recorded in least quantities in quercetin equivalents (QE) and in comparison to the total phenolics (Table 1). All the four extracts of seeds (AMS-I, AMS-II, AMS-III

Table 1 Total polyphenol, flavonoid, antioxidants and ferric reducing antioxidant power of *A. moschatus* extracts

Extract	Polyphenols[a]	Flavonoids[b]	Antioxidants[c]	Ferric reducing antioxidant power[d]
AMS-I	3.74 ± 0.13	0.10 ± 0.02	10.78 ± 0.16	0.54 ± 0.05
AMS-II	2.35 ± 0.08	0.13 ± 0.08	8.89 ± 0.04	0.46 ± 0.03
AMS-III	1.73 ± 0.02	0.22 ± 0.03	9.12 ± 0.06	0.38 ± 0.04
AMS-IV	1.56 ± 0.02	0.26 ± 0.02	8.08 ± 0.08	0.42 ± 0.04
AML-I	9.49 ± 0.17	5.60 ± 0.02	13.30 ± 0.33	3.02 ± 0.05
AML-II	11.86 ± 0.11	2.00 ± 0.08	15.30 ± 0.15	4.51 ± 0.04
AML-III	13.38 ± 0.26	3.12 ± 0.03	19.85 ± 0.07	6.07 ± 0.02
AML-IV	13.84 ± 0.10	6.00 ± 0.02	21.52 ± 0.07	6.28 ± 0.01

a: gallic acid; b: quercetin; c & d: ascrobic acid equivalents mg/g dw plant material respectively; Results represented in means ± standard deviation (n = 3).

and AMS-IV) contained total flavonoids in minimum amount, highest being in AMS-IV (0.26 mg QE/g dw). The leaf extracts also contained some flavonoid content with the highest value observed in AML-IV (6.0 mg QE/g dw). The overall levels of total polyphenol and flavonoid content in the plant extracts were found significantly lower when compared to the standard compounds used in this study.

Total antioxidant activity (TAA) and ferric reducing antioxidant power (FRAP)

The extracts of seed and leaf exhibited significant antioxidant activity, thus establishing the extracts as an antioxidant. The results of the antioxidant measurements are summarized in Table 1. The antioxidant activity was in the range of 8.08 to 10.78 mg AAE/g dw in the seed extracts. The highest value of 10.78 mg AAE/g dw was observed in AMS-I whereas the lowest value (8.08 mg AAE/g dw) was found in AMS-IV. The leaf extracts of *A. moschatus* showed reasonably higher antioxidant activity in comparison to the seed extracts. The activity was in the range of 13.30-21.52 mg AAE/g dw whereas AML-IV exhibited highest activity with value of 21.52 mg AAE/g dw and AMS-I with least activity 13.30 mg AAE/g dw.

The extracts of *A. moschatus* expressed electron donating activity, but their power was inferior to ascorbic acid, which is known to be a strong reducing agent (Table 1). Leaf extracts exhibited considerably higher reducing power for Fe^{3+} than the seed extracts. The reducing ability of the leaf extracts was in range of 3.02-6.28 mg AAE/g dw. The highest value was observed in AML-IV (6.28 mg AAE/g dw), whereas the lowest value was recorded in AML-I (3.02 mg AAE/g dw). The FRAP values for the seed extracts were in the range of 0.38-0.54 mg AAE/g dw. AMS-I showed highest value of 0.54 mg AAE/g dw whereas AMS-III depicted least value (0.38 mg AAE/g dw).

DPPH radical scavenging activity

In this study, all the extracts showed tendency to quench the DPPH free radicals, as indicated by the concentration dependent increase in percentage inhibition. The results revealed that the leaf extracts had the higher DPPH radical scavenging ability than those of the seed extracts. The IC_{50} values (concentration of the extract that was able to scavenge half of the DPPH radical) are presented in Table 2. Among the seed extracts, AMS-IV exhibited stronger radical scavenging ability and its percentage inhibition reached to 91.6% with the lowest IC_{50} value of 38.1 μg GAE/mL, which indicates its good antioxidant potential. The other seed extracts showed moderate DPPH radical scavenging effects (Figure 1a; Table 2). On the other hand, leaf extracts showed significantly stronger activities and quenched DPPH radicals to different degrees at higher concentrations. The scavenging activity reached to 91.7% with IC_{50} value of 42.8 μg GAE/mL in AML-IV, followed by AML-III. The lowest percentage of inhibition was observed in AML-I (28.4% with IC_{50} value of 176.1 μg GAE/mL) (Figure 1b; Table 2).

Hydrogen peroxide scavenging activity

Among the seed and leaf extracts of *A. moschatus*, only three seed extracts (AMS-I, AMS-II and AMS-III) were capable of scavenging H_2O_2 in a concentration dependent manner and IC_{50} values for scavenging of H_2O_2 were 22.6, 26.3 and 24.6 μg GAE/mL respectively (Table 2; Figure 2). The IC_{50} for ascorbic acid was 44.8 μg GAE/mL. Since any of the leaf extracts did not show inhibition of the peroxide radical generation, therefore, no figure or IC_{50} values were provided.

Superoxide radical scavenging activity

The superoxide radical scavenging activity of *A. moschatus* extracts assayed by the PMS-NBT-NADH system was shown in Figure 3; Table 1. Three extracts of seed (AMS-I, AMS-II and AMS-III) and one leaf extract (AML-I) were found to be an efficient scavenger of superoxide radical generation. The maximum inhibition of 87.4% with IC_{50} value of 22.3 μg GAE/mL was observed in AMS-I, whereas AMS-II showed inhibition value of 69.9% with the IC_{50} value of 26.3 μg GAE/mL. The leaf extracts, AML-I inhibited superoxide radical upto 66.6% with IC_{50} value of 30.6 μg GAE/mL (Table 2; Figure 3). This result clearly indicated that the tested extracts had a noticeable effect on scavenging superoxide radical.

Hydroxyl radical scavenging activity

This assay showed the abilities of the extracts and standard (ascorbic acid) to inhibit hydroxyl radical-mediated deoxy-ribose degradation. The *A. moschatus* seed and leaf extracts showed significant inhibition of hydroxyl radicals generated by Fenton's reagent in a concentration dependent manner. The OH^- radical scavenging data (Table 2) indicated that extracts of *A. moschatus* does possess the ability to scavenge this reactive oxygen species (ROS). Among the seed extracts, it was found that AMS-I was efficient in quenching the hydroxyl radical formation and expressed as an IC_{50} value of 16.3 μg GAE/mL, followed by AMS-II (IC_{50} = 18.5 μg GAE/mL). The other two extracts AMS-III and AMS-IV also showed significant hydroxyl radical scavenging effect (Table 2; Figure 4a). On the other hand, the leaf extracts were also found to be potent scavenger of OH^-. The extract AML-I was the most efficient inhibitor and hence, inhibited the formation of hydroxyl radical to 98.5% followed by AML-II and AML-IV respectively. It is worth to mention that ascorbic acid was shown to be weak inhibitor than the extracts tested (Table 2; Figure 4b).

Table 2 IC_{50} values of *A. moschatus* extracts on tested radicals

Name of the Assay	Seed*				Leaf*				Standard†
	AMS -I	AMS -II	AMS -III	AMS -IV	AML -I	AML-II	AML-III	AML-IV	
DPPH	93.6 ± 3.0	70.7 ± 6.0	56.3 ± 15.0	38.1 ± 8.0	176.1 ± 14.0	58.5 ± 1.2	47.5 ± 1.0	42.8 ± 1.0	3.5 ± 0.2
Hydrogen peroxide	22.6 ± 5.0	26.3 ± 4.0	24.6 ± 10.0	138. ± 12.0	NA	NA	NA	NA	44.8 ± 0.4
Super oxide radical	22.3 ± 2.0	26.3 ± 3.0	28.4 ± 14.0	NA	30.6 ± 3.0	NA	NA	NA	25.5 ± 0.6
Hydroxyl radical	16.3 ± 2.0	18.5 ± 4.0	20.1 ± 12.0	22.8 ± 7.0	10.7 ± 3.0	18.7 ± 3.0	22.7 ± 4.0	22.4 ± 2.0	55.3 ± 0.8
Lipid peroxidation	76.2 ± 2.0	136.3 ± 8.0	146.3 ± 4.0	148.3 ± 6.0	60.5 ± 4.0	65.4 ± 3.0	85.4 ± 4.0	88.9 ± 4.0	45.2 ± 0.3

(*Values expressed in μg of GAEs/mL; †: Ascorbic acid in μg/mL; Results represented in means ± standard deviation (n = 3); NA: No activity.

Inhibition of Fenton's reagent-induced strand breaks in plasmid DNA

Two seed (AMS-I and AMS-IV) and two leaf (AML-I and AML- IV) extracts have shown significant antioxidant activity in the *in vitro* studies compared to all other extracts. Hence, they were selected for oxidative damage protective activity against a model DNA (pBR322). Hydroxyl radicals generated by Fenton's reaction are known to cause oxidative DNA damage leading to DNA strand breaks and open circular or relaxed DNA forms. This study has revealed that three extracts (AMS-I, AMS-IV and AML-IV) showed effective protection of DNA from damage (nicking) caused by the hydroxyl radicals (Figure 5).

Inhibition of lipid peroxidation

The percentage inhibition of lipid peroxidation by *A. moschatus* extracts were presented in Figure 6a & Figure 6b and the IC_{50} values were given in Table 2. Compared with the control, AML-I showed significant level of inhibition of lipid peroxidation by 96.2% at 60.5 μg GAE/mL. Other extracts of the leaf (AML-II, AML-III and AML-IV) as well as seed extract (AMS-I) performed poorly in this assay. The seed extracts (AMS-II, AMS-III and AMS-IV) did not exhibit minimal inhibition of lipid peroxidation at the same concentrations.

Antiproliferative activity

The antiproliferative activities of seed (AMS-IV) and leaf (AML-IV) extracts of *A. moschatus* and ifosfamide on the growth of cell lines *in vitro* were presented in Figure 7a and 7b. At the concentration of 200 μg/mL, AMS-IV showed significant antiproliferative activity against the both cell lines COLO-205 and Y79 with the corresponding percentage inhibitory activities of 73.33 ± 1.6 and 74.40 ± 1.6 respectively, under the experimental conditions. Similarly, at the same concentration (200 μg/mL), the leaf extract (AML-IV) showed the 78.25 ± 1.6 and 78.8 ± 0.65 percent inhibitory activity in COLO-205 and Y79 cancer cell lines respectively. Other extracts of seed (AMS-I, II and III) and leaf (AML-I, II and III) did not show any antiproliferative activity on these cell lines.

Antimicrobial activity

In the present study, the seed and leaf extracts of *A. moschatus* were tested for its antimicrobial activity at various concentrations and evaluated for minimum inhibitory concentration (MIC) values which are presented in Table 3. The extracts showed varying degrees of antimicrobial activity against tested microorganisms. AMS-I and AML-IV extracts exhibited higher degrees of antimicrobial activity than the other extracts. On the contrary, the seed extracts (AMS-II, AMS-III and AMS-IV) and leaf extracts (AML-I, AML-II and AML-III) showed least inhibition of growth of microorganisms. *B. subtilis,*

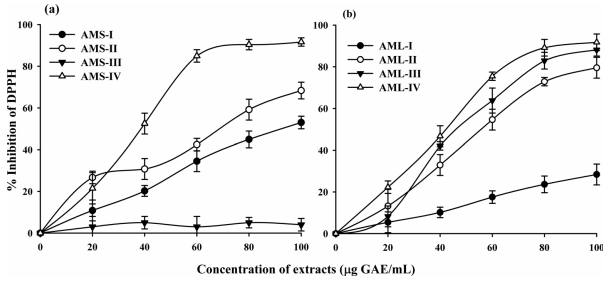

Figure 1 DPPH scavenging activity of the *A. moschatus* seed (a) and leaf (b) extracts.

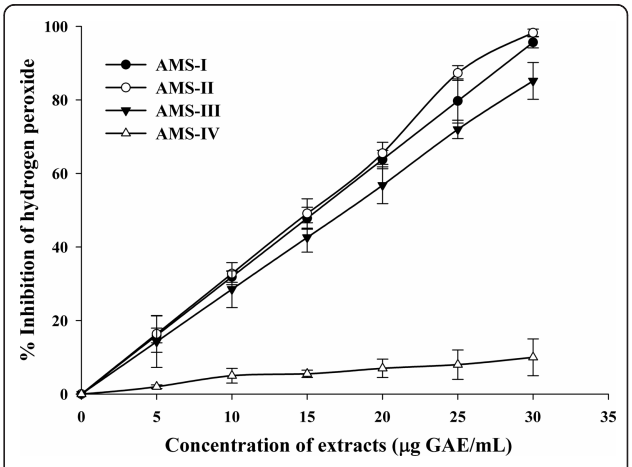

Figure 2 Hydrogen peroxide scavenging activities of seed extracts of *A. moschatus*.

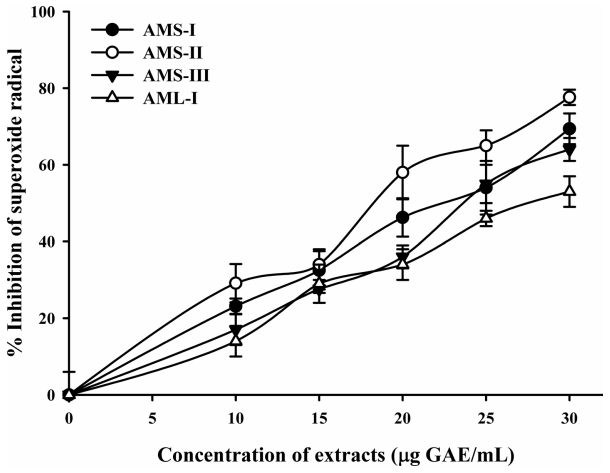

Figure 3 Superoxide scavenging activities of seed extracts of *A. moschatus*.

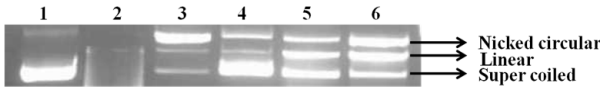

Figure 5 Effect of seed and leaf extracts of *A. moschatus* on the integrity of pBR322 plasmid DNA in the presence of Fenton's reagents. (Lane 1: pBR322 DNA + H_2O; Lane 2: pBR322 DNA + FR; Lane 3: standard antioxidant compound (quercetin) in the presence of FR; Lane 4: pBR322 DNA + FR + AMS-IV; Lane 5: pBR322 DNA + FR + AML-IV; Lane 6: pBR322 DNA + FR + AML-I).

S. aureus and *P. aeruginosa* were most susceptible to AMS-I with MICs value of 101.6 ± 10.2, 109.6 ± 8.3 and 109.6 ± 10.2 µg GAE/mL respectively. Whereas AML-IV has shown strong inhibition with MIC value of 91.7 ± 2.0, 92.0 ± 4.1, 92.5 ± 1.8, 102.6 ± 2.0 µg GAE/mL on *B. subtilis, S. aureus, P. aeruginosa* and *P. vulgaris* respectively. AML- IV also manifested very strong inhibition on growth of *C. albicans* with the MIC value of 45.5 ± 2.2 µg GAE/mL. The inhibitory effects of the extracts were compared with the standard antibiotics such as ampicillin for gram positive bacteria, kanamycin for gram negative bacteria and nystatin for fungal strain. There was no inhibitory effect of ethanol on all tested microorganisms at given concentrations of the solvent.

Discussion

Oxidation processes are intrinsic in the energy management of all living organisms and are therefore, kept under strict control by several cellular mechanisms [33]. However, the aberrant production and unbalanced mechanisms of antioxidant protection leads to several human diseases and conditions such as cancer, diabetes, inflammatory disorders, as well as aging processes etc. [34,35]. Natural antioxidants, which are ubiquitous in fruits, vegetables and medicinal plants, have received great attention and have been studied extensively, since they are effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants [36]. The present study is a step towards the exploration of natural antioxidants from seed and leaf extracts of *A. moschatus* employing free radical scavenging assays in addition to anti-proliferative and antimicrobial activities.

Among the various natural antioxidants, phenolics are very important constituents because of their multiple biological effects and direct contribution to antioxidative activity [28]. The results of our study reveal that there is a strong coincidence between antioxidant activity and phenolic content. Several studies on total phenolic content had been published over the years demonstrating its importance in the medicinal field [37-39].

In the present study, antioxidant activity in AMS-I and AML-IV suggests that polyphenols are largely contributing to the total antioxidant activity of these extracts. It is found that the highest antioxidant activity, measured as total antioxidant activity (TAA) values depends on quantities of total polyphenols. Similar results have been published earlier also suggested a causative relationship between total polyphenol content and antioxidant activity [40,41]. Our study indicates that polyphenol present in the extracts of *A. moschatus* might be responsible for the antioxidant properties.

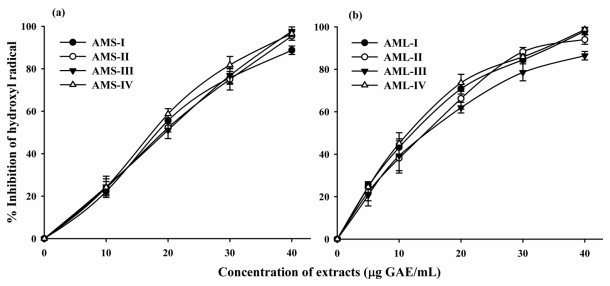

Figure 4 Hydroxyl radical scavenging activities of the extracts of seed (a) and leaf (b) of *A. moschatus*.

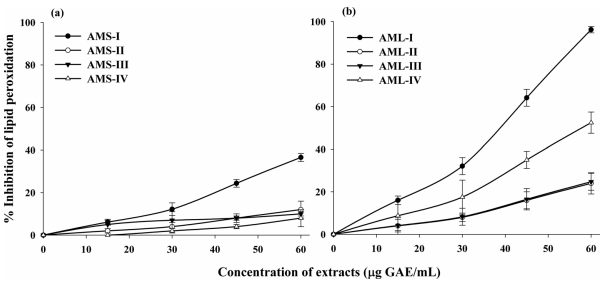

Figure 6 Inhibition of Lipid peroxidation of seed (a) and leaf (b) extracts of *A. moschatus*.

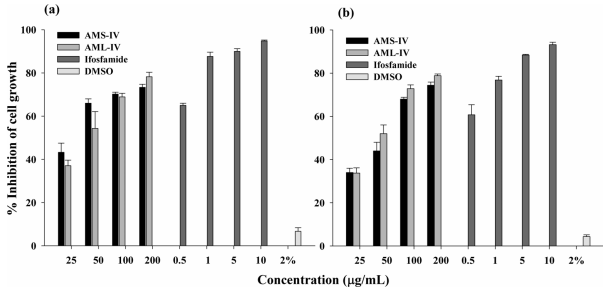

Figure 7 Antiproliferative activity of *A. moschatus* seed and leaf extracts against COLO-205 (a) and Y79 (b) cell lines.

Since the antioxidant activity of a substance is usually correlated directly to its reducing capacity, the FRAP assay provides a reliable method to study the antioxidant activity of various compounds [42]. This method has been frequently used for a rapid evaluation of the total antioxidant capacity of different plant extracts containing flavonoids [43]. As shown in Table 1, the ferric reducing power is higher in AMS-I and AML-IV than the rest of the extracts and show a similar trend for total antioxidant activity and this could be attributed to the presence of antioxidant phytomolecules.

The DPPH radical has been used widely as a model system to investigate the scavenging activities of several natural compounds including phenolic compounds, flavonoids or crude mixtures of plants. The effect of antioxidants on DPPH was thought to be due to their hydrogen donating ability [44]. The DPPH radical scavenging abilities of the *A. moschatus* extracts are observed in all the extracts under study in a concentration dependent manner. They are significantly comparable to that of ascorbic acid (100%) showing that the extracts have proton-donating ability and could serve as free radical inhibitors or scavengers, possibly acting as primary antioxidants. It is clear that the antioxidant activity of *A. moschatus* extracts in DPPH assay increased proportionally to the polyphenol content and same trend was observed in earlier reports where increased antioxidant activities showed linear relationship between DPPH values and total polyphenols [45,46].

Hydrogen peroxide is an oxidant that is being continuously generated in living tissues as a result of several metabolic processes. The detoxification of H_2O_2 is vital for preventing it from reacting in damaging Fenton-type reactions, which generate extremely reactive oxygen species including hydroxyl free radical [47]. As shown in Figure 2 and Table 2, *A. moschatus* extracts have an effective radical scavenging activity for H_2O_2 in a concentration dependent manner and results reveal that these extracts have significant scavenging character in accordance with the standard, ascorbic acid. Similar results have shown that scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2 and neutralize it to water [48,49].

Several biological reactions generate superoxide radical which is a relatively weak oxidant and exhibits only limited chemical reactivity. It can also generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids, thus study of scavenging of this radical is important [50]. In the present study, the seed extracts of *A. moschatus* are found to be

Table 3 Minimum inhibitory concentrations (MIC) of *A. moschatus* extracts against the microorganisms by micro-dilution broth assay

Microorganisms	Seed extracts*				Leaf extracts*				Standard
	AMS -I	AMS-II	AMS -III	AMS - IV	AML - I	AML-II	AML-III	AML-IV	
Gram-positive Bacteria									
B. subtilis ATCC 5740	101.6 ± 10.2	NA	NA	243.5 ± 7.2	NA	NA	NA	91.7 ± 2.0	75.3 ± 2.3[a]
S. aureus ATCC 25923	109.6 ± 8.3	323.4 ± 7.1	497.1 ± 6.2	352.5 ± 12.1	NA	195.6 ± 3.3	197.3 ± 4.6	92.0 ± 4.1	65.3 ± 3.7[a]
Gram-negative Bacteria									
E. coli ATCC 25922	406.41 ± 11.3	948.9 ± 5.4	1543.3 ± 6.4	935.8 ± 2.4	244.4 ± 3.8	195.6 ± 3.9	174.8 ± 9.2	184.2 ± 5.0	102.4 ± 4.7[b]
P. aeruginosa ATCC-27853	109.6 ± 10.2	378.7 ± 7.2	485.5 ± 9.3	487.1 ± 3.2	240.2 ± 5.6	197.4 ± 5.0	197.3 ± 4.0	92.5 ± 1.8	67.67 ± 3.8[b]
P. vulgaris ATCC 6380	401.7 ± 7.1	948.9 ± 8.4	1375.7 ± 11.0	974.3 ± 4.2	170.7 ± 4.1	195.2 ± 2.0	173.9 ± 2.6	102.6 ± 2.0	58.7 ± 2.9[b]
S. enterica paratyphi ATCC 9150	296.7 ± 12.0	714.8 ± 8.8	1063.5 ± 12.4	944.3 ± 3.1	139.9 ± 2.2	194.9 ± 5.5	179.3 ± 4.2	128.6 ± 2.0	35.7 ± 5.6[b]
Fungi									
C. albicans ATCC 10231	NA	NA	NA	487.1 ± 3.5	NA	NA	NA	45.5 ± 2.2	10.7 ± 0.3[c]

*Values are expressed in µg of GAE/ml; a: ampicillin; b: kanamycin; c: nystatin (µg/ml); Results represented as means ± standard deviation (n = 3); NA: No activity.

an efficient scavenger of superoxide radical generated in PMS/NADH/NBT assay system and percentage of inhibition increases markedly with the increase in concentrations. It suggests that the extracts are potential scavengers of superoxide anion and possibly renders them as promising antioxidants (Table 2; Figure 4). It has also been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical [51].

The hydroxyl radical is said to be detrimental and initiates auto-oxidation, polymerization and fragmentation of biological molecules [47,52]. The identification of compounds that have excellent hydroxyl scavenging activity would be significant for some diseases caused by oxidative stress. It has been demonstrated that plants contain many natural antioxidants compounds which have been identified as hydroxyl radical scavengers [53]. Therefore, OH^- scavenging effects of *A. moschatus* extracts are assessed in the present study. The result shows that the scavenging activity of both seed and leaf extracts are significantly higher than those of ascorbic acid (Table 2). Hence, *A. moschatus* extracts can be used to minimize the adverse effects from the hydroxyl radicals.

Hydroxyl radicals are also known to be the most reactive species, causes damage to DNA, protein and other life essential biological molecules, leading to mutagenesis, carcinogenesis, and aging [28]. DNA guanosine residues are attacked by hydroxyl radicals generated from Fenton reactants, resulting in strand breakage and transformation from native circular DNA to nicked open circular or relaxed forms. Polyphenols are potential protecting agents against the lethal effects of oxidative stress and offer protection of DNA by chelating redox-active transition metal ions [54]. AMS-I, AMS-IV and AML-IV show effective reduction in the formation of nicked DNA and increased super coiling of DNA (Figure 5). Similar studies have been performed and reported on the protection of DNA by different medicinal plants, thereby confirming antioxidant properties [55,56].

Oxidative damage to cellular components such as cell membrane by free radicals is believed to be associated with pathology of many diseases and conditions including diabetes, cancer, ageing, cardiovascular diseases and inflammatory conditions [57]. One of the degradation products of lipid peroxidation is malondialdehyde (MDA) which causes cell damage and form a pink colour chromogen with thiobarbituric acid. Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation through many other mechanisms and thus prevent diseases [58,59]. Therefore, the inhibition of lipid peroxidation is considered to be important index of antioxidant activity. In our study, the leaf extract (AML-I) shows very strong inhibition of MDA formation (96.2% at 16.5 μg GAE/mL), compared to the other extracts of leaf as well as seed, proving that this extract offers a good degree of protection against the biological end point of oxidative damage.

There has been a 22% increase in cancer incidence and mortality, with over 10 million new cases and over 6 million deaths worldwide in the year 2000 and cases could further increase by 50% to 15 million new cases in the year 2020 [60]. Colon cancer is rapidly rising and is strongly related to age, with 90% of the cases arising in people who are 50 years or above [61]. It is now the third most common malignant disease in both men and women in Asia [62]. Similarly, retinoblastoma is the most common intraocular tumor of childhood and lead to metastatic disease and death in 50% of children worldwide [63]. The use of medicinal plant and fruit extracts for cancer therapy is rapidly evolving as they are affordable, with limited or no side effects. The active components present in such extracts have been shown to efficiently inhibit the process of multi-stage carcinogenesis in a synergistic manner. The identification and characterization of components with potential anti-cancer activity derived from herbal or medicinal plant extracts has been gaining attention. Earlier reports revealed that the antioxidant activity prevents development of cancers [64-67]. So in this context, we have also examined the antiproliferative ability of *A. moschatus* extracts using two human cancer cell lines, COLO-205 and Y79. We found that the proliferation was inhibited in a concentration dependent manner after the exposure to AMS-IV and AML-IV extracts to these cell lines (Figure 7a & Figure 7b). The cytotoxicity was slightly higher in leaf extract than seed extract in both the cell lines tested. Although, the activity is low in comparison to the standard drug, this may be due to the crude nature of the extracts, which can be further enhanced by the purification. It can be inferred that the hydroalcoholic extracts of seed and leaf of *A. moschatus* might be useful as an antiproliferative agent due to the presence of potent bioactive principles [68].

Furthermore, medicinal herbs had been used in ayuverdic traditional medicine for their effectiveness against wide range of diseases due to the advantage of diverse secondary metabolites such as phenolic compounds including flavonoids, alkaloids and tannins [55-57]. Therefore, we also examined the antimicrobial activity of *A. moschatus* extracts against a panel of seven pathogenic microorganisms. Our results indicate that the different extracts of *A. moschatus* exhibit antimicrobial activity and among them, AMS-I and AML-IV are more effective which signifies the antibiotic nature of these extracts (Table 3). Moreover, our observation suggests that organic solvent extract of leaf (AML-IV) is more efficient than other aqueous extracts. Literature also reveals that organic solvent extraction has been proved to be suitable for antimicrobial activities of medicinal

plants [69-71]. It is known that the gram negative bacteria are more resistant than the gram-positive ones [72,73] and our results also demonstrate that all the extracts except AML-IV are less effective to these microorganisms even at higher concentrations. The non-activity of the aqueous extracts against most bacterial strains investigated in this study was also in agreement with previous studies which showed that aqueous extracts of plant generally show little or no antibacterial activities [74].

Conclusions

The present study indicated that *A. moschatus* contains considerable amount of total polyphenols and flavanoids and exhibited good antioxidant activity by effectively scavenging various free radicals. In addition, it has been demonstrated that *A. moschatus* is a potential antiproliferative and antimicrobial agent. The antioxidant and biological activities might be due to the synergistic actions of bioactive compounds present in them. However, it is still unclear which components are playing vital roles for these activities. Therefore, further studies are still needed to elucidate mechanistic way how the plant contributes to these properties. Phytochemical investigation is also proposed to isolate the active fraction and eventually the pure compound(s) from this plant.

Acknowledgements

The authors would like to thank Central Institute of Medicinal and Aromatic Plants (CIMAP), Regional centre, Hyderabad and Central Research Institute of Unani Medicine, Hyderabad Andhra Pradesh, for providing the plant material and bacterial strains respectively. The authors (MZG, LMB and FA) were thankful to UGC (RFSMS, DSKPDF scheme and JRF respectively) for the fellowship and financial support to the department from the DST, UoH DBT-CREBB, DST-FIST, UGC-SAP-CAS, UGC-XI plan seed money. The authors also thank Prof. A.S.Raghavendra for allowing to use his lab facilities.

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Authors' contributions

MZG conceived the study, carried out all the experimentation, acquisition and analysis of data and drafting of the manuscript. LMB assisted with the concept and analysis of data. FA was involved in cell culturing, MTT assay. AKK provided technical support and advice in cytotoxic studies. IAQ helped in nystatin study and revision of the manuscript. IAG conceived, designed and supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 27 April 2011 Accepted: 17 August 2011
Published: 17 August 2011

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1472-6882/11/64/prepub

doi:10.1186/1472-6882-11-64
Cite this article as: Gul *et al.*: Evaluation of *Abelmoschus moschatus* extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using *in vitro* assays. *BMC Complementary and Alternative Medicine* 2011 **11**:64.

Antioxidant and α-glucosidase inhibitory activities of *Murraya koenigii* leaf extracts

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Submission Date: 1-6-2012; Review Completed: 19-7-2012

ABSTRACT

Medicinal plants are source for a wide variety of natural antioxidants. Dietary antioxidant consumption may be an important approach for inhibiting or delaying the oxidation of susceptible cellular substrates and thus is relevant to disease prevention in many paradigms. *Murraya koenigii* is one of the important medicinal herb which is used as a food ingredient across India. In this study, we demonstrated the reducing power, total antioxidant potential, radical scavenging capabilities and α-glycosidase inhibitory property of different crude extracts of curry leaves (*Murraya koenigii* L.). The extracts were evaluated for their radical scavenging activities by means of DPPH, NO, •OH, $O2^-$ and anti-lipid peroxidation assays. In addition, extract of *M. koenigii* were tested for α-glycosidase inhibitory property. The extracts of *M. koenigii* scavenged radicals effectively in varied degree. Similarly, the total reducing power of alcohol extract was found higher in both phosphomolybednum and FRAP methods. *In vitro* assay of α-glucosidase activity of MKA and MKW showed an IC_{50} of 174.74 and 287.00 µg/ml respectively, while other two extracts did not show any significant effect. Simultaneously, total phenolic and total flavonoid contents of extracts were studied, where values of MKW were found to be higher than that of other extracts. In present study, we found that MKA and MKW extracts contain effective antioxidant and radical scavenging activities as compared to other extracts. Our study provides a proof for the ethno-medical claims and reported biological activities that curry leaves have significant therapeutic potential.

Keywords: *Murraya koenigii*, antioxidant, free radical, α-glucosidase.

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl (•OH) radical are often generated as byproducts of biological reactions or from exogenous. These ROS create homeostatic imbalance which generate oxidative stress and cause cell death and tissue injury.[1] Free radicals and ROS are well known inducers of cellular and pathological processes including diabetes, cell proliferation, inflammatory conditions and many neurodegenerative disorders apart from aging processes.[2,3,4] A potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases.[5] It has been established that antioxidants provide protection to living organisms from damage triggered by uncontrolled production of ROS and associated lipid peroxidation, protein damage and DNA strand breaking.[6] The health promoting effect of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species. Plant-derived antioxidants could function as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors or synergists.[7]

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DOI: 10.5530/pj.2012.32.12

Most plant species possess tremendous medicinal properties because they are used both to sustain health and to cure illness.[8] Dietary antioxidant intake may be an important approach for inhibiting or delaying the oxidation of susceptible cellular substrates and is thus relevant to disease prevention in many models. Phenolic compounds such as flavonoids, phenolic acids, diterpenes etc. have received attention for their high antioxidative activity.[9,10,11] *In vitro* bioassay systems have been extensively used to monitor biological activities of medicinal plant extracts used in traditional medicines.

Numerous plants used in traditional medicine are effective in treating various ailments caused by oxidative stress, bacterial and/or viral infections. Since ancient times, spices and herbs have been added to different types of food to improve flavours as well as for their antioxidant capacity. Converging evidence from both experimental and epidemiological studies have demonstrated that medicinal plants in particular contain a myriad of phenolic compounds and still present a large source of natural antioxidants that might serve as leads for the development of novel drugs.[12] Therefore, the exploration for natural antioxidants and other preparations of plant origin to achieve this objective has been gained importance over the years.

Murraya koenigii L. Spreng (Rutaceae) is a small and strong smelling perennial shrub or small tree commonly found in forests as undergrowth, cultivated in India for its characteristic flavour and aroma. Leaves of this plant are used as a condiment in the preparation of curry powder, pickle, chutney, sausages and seasonings.[13,14] The flavour and fragrance of leaves is retained even after drying.[15] Leaves relieve nausea, indigestion, vomiting and used as a cure for diarrhoea and dysentery.[13,16]

In this study, we have demonstrated the antioxidant efficiency of different *M. koenigii* using series of *in vitro* assays. In addition, we also evaluated its α-glycosidase inhibitory activity apart from determination of phytochemical constituents.

MATERIALS AND METHODS

Materials

All solvents used in this study were of analytical grade. Methanol, ethyl acetate, hexane, and Folin- Ciocalteau reagent obtained from Merck (Merck, India) while other chemicals were procured from HiMedia chemicals, India.

Plant Material

Curry leaves (*M. koenigii* L.) were purchased from local market in Hyderabad, India in the month of July, 2011. The identity of the plant was confirmed by Dr. VC Gupta, Taxonomist, Central Research Institute of Unani Medicine, Hyderabad. The voucher specimen of the plant was kept for future reference. One hundred grams of curry leaves were dried at ambient temperature for 10–15 days. After drying completely, leaves were grounded to a coarse powder using domestic electric grinder.

Preparation of Extracts

The coarse powder was subjected to successive extraction in a soxhlet apparatus using different solvents such as hexane, ethyl acetate, ethanol and water. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. Furthermore, extracts were filtered through Whatman No.1 paper filter and concentrated to the dry mass with the aid of rotary evaporator. The yield of each extract was measured and residues were stored in dark for further analysis. Different extracts were designated as MKH (for hexane extract), MKE (for ethyl acetate extract), MKA (for ethanol extract) and MKW (for water extract). Dried extracts of 20 mg/ml stock solution were prepared and different concentrations were used in various experiments.

DETERMINATION OF PHYTOCHEMICAL CONSTITUENTS

Total Phenolic Content

Total phenolic content of extracts were determined by Folin-Ciocalteu method[17] with little modification. Briefly, 10 µl of the extracts were taken to which 500 µl of double distilled water was added, followed by 100 µl of Folin Ciocalteu's reagent. After incubating the mixture for 10 min at room temperature, 300 µl of 20% Na_2CO_3 was added, thoroughly vortexed and the volume of the reaction mixture was adjusted to one ml with double distilled water. The mixture was then incubated for 2 hrs in dark and the absorbance was measured at 765 nm against blank. Results were expressed as mg of gallic acid equivalents (GAE)/g dry weight (dw).

Total Flavanoid Content

Total flavonoid content was quantified by following the method of Barreira[18] with minor modifications. Briefly, 20 µl of each extract were mixed with 500 µl double

distilled water and 30 µl of 5% $NaNO_2$ solution. After 5 min of incubation at room temperature, 60 µl of 10% $AlCl_3$ solution was added. Subsequently, 350 µl of 1 M NaOH and 40 µl of double distilled water were added to make the final volume to one ml. Samples were further incubated for 15 min at room temperature and the absorbance of samples was measured at 510 nm. The total flavonoids were determined as qurecetin equivalents (mg QE)/g of dw and the values were expressed as means of triplicate analysis.

Determination of Total Antioxidant Activity

Total antioxidant activities of extracts were evaluated by phosphomolybdenum method.[19] The assay is based on the reduction of Mo^{+6}–Mo^{+5} by the antioxidant compounds and subsequent formation of a green phosphate/Mo^{+5} complex at acidic pH. The reagent solution contains ammonium molybdate (4 mM), disodium hydrogen phosphate (28 mM) and sulfuric acid (0.6 M) mixed with the extracts. Samples were incubated for 60 min at 90°C and the absorbance of the green phosphomolybedenum complex was recorded at 695 nm. Ascorbic acid was used as reference and reducing capacity of the extracts was expressed as the mg ascorbic acid equivalents per gram dry weight.

Determination of Reducing Antioxidant Power (FRAP)

The reducing antioxidant power of the plant extracts was determined according to the method described by Oyaizu[20] with slight modifications. Briefly, 10 µl of each extract was taken and the volumes were made to 250 µl with double distilled water. Further, 250 µl of potassium ferricyanide (1%) was added to the tubes and incubated for 20 min at 50°C. Then 250 µl of trichloroacetic acid (10%) was added to the incubated mixture. Upper part of the mixtures (500 µl) were taken and mixed with 400 µl of double distilled water and 100 µl of ferric chloride (0.1%). The absorbance of the mixture was measured at 700 nm and reducing power of extracts was expressed as mg ascorbic acid equivalents (AAE) per gram (g) of dry weight (dw).

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the plant extracts was determined according to the method described by Braca.[21] The activity was assessed using stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH). DPPH solution (0.004% w/v) was prepared in 95% methanol, mixed with different dilutions of plant extracts and thoroughly vortexed. The reaction mixture was then incubated in dark at room temperature for 45 min and absorbance solution was measured at 517 nm against the blank. Methanol (95%) and ascorbic acid were used as blank and reference compound respectively.

Nitric Oxide Radical Scavenging Activity

The activity was measured according to the modified method of Sreejayan and Rao.[22] To 100 µl of the extract having different concentrations (40–400 µg/ml), 20 µl of sodium nitroprusside (SNP) solution (10 mM) was added and incubated for 15 min under light conditions. After incubation, the mixture was diluted with 300 µl of Griess reagent (1% sulfanilamide in 2% H_3PO_4). The reaction mixture was further incubated for 45 min under light conditions at 30°C followed by addition of 10 µl of 0.1% naphthylethylene diamine dihydrochloride in 2.5% H_3PO_4. Final volume was made to 1 ml with double distilled water. The absorbance of the chromophore was taken immediately at 546 nm and compared to the standard (ascorbic acid).

Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was measured as per the protocol of Kunchandy and Rao[23] with minor changes by studying the competition between deoxyribose and extracts for hydroxyl radicals generated by Fenton's reaction. Briefly, solution of Fenton's reagent [Fe (III) chloride, ascorbic acid and H_2O_2] was prepared in distilled water just prior to use. To 0.1 mL of Fenton's reagent, thiobarbituric acid (1% w/v) in 25 mM NaOH (1 ml) and tricholoroacetic acid (1 ml, 2.8% w/v) were added and volume was made to 3 ml with distilled water. The mixture was then incubated at 80°C for 90 min and amount of pink chromogen produced was considered as control and was measured spectrophotometrically at 532 nm. The protection of oxidation of D-ribose has been conducted by pre-incubation with extracts in different concentrations and decrease in the formation of pink colour was considered as antioxidant property which was compared to standard (ascorbic acid).

Superoxide Radical Scavenging Activity

The activity was evaluated using nitro blue tetrazolium (NBT) reduction method.[24] The reaction mixture consisted of 0.5 ml of NBT solution (156 µM, 0.5 ml nicotinamide adenine dinucleotide (468 µM, NADH), and extracts of different concentrations (40–400 µg/ml). The reaction was initiated by adding 50 µl of phenazine methosulfate solution (60 µM, PMS) in phosphate buffer

(pH 7.4). The reaction was incubated at 25°C for 20 min and then absorbance was measured at 560 nm against blank. Ascorbic acid was used as the reference.

Determination of Inhibition of Lipid Peroxidation

Lipid peroxidation inhibitory activity of *M. koenigii* extracts and standard (ascorbic acid) were performed as per the protocol given by Halliwell and Gutteridge.[25] The rat liver homogenate was used for induction of lipid peroxidation, mediated by $FeCl_3$ as pro-oxidant. Healthy albino rats of the wister strain (250 g) were sacrificed and perfused the liver with 0.15 M KCl followed by centrifugation of homogenate at 800 g for 15 min at 4°C and supernatant was used for thiobarbutaric acid assay. The extracts at different concentrations (40–400 µg/ml) were mixed with the liver microsome preparation and the mixtures were incubated in presence and absence of fenton's reagent (50 µl of 10 mM $FeCl_3$; 10 µl of 2.5 mM H_2O_2) in phosphate buffer (0.2 M, pH 7.4) and the final volume was made to 1 ml. The reaction mixtures were incubated at 37°C for 30 min. After incubation, 2 ml of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid, 0.5% thiobarbutaric acid, and 0.5% butylated hydroxytoluene (BHT) was added to the reaction mixture followed by heating at 100°C for 10 min. The reaction mixture was put in an ice bath for 5 min for cooling. After that the mixture was centrifuged at 1000 g for 10 min and the extent of lipid peroxidation was subsequently monitored by the formation of thiobarbutaric acid reactive substances (TBARS) as pink chromogen in the presence or absence of extracts and standard (ascorbic acid). The absorbance of supernatant was measured spectrophotometrically at 532 nm and decline in the formation of pink chromogen in the pre-treated reactions was considered as inhibition of lipid peroxidation.

Effects of Extracts on α-glucosidase Activity *In Vitro*

The α-glucosidase inhibitory activity was determined according to the method given by Matsui[26] with slight modifications by measuring the release of 4-nitrophenol from 4-nitrophenyl α-D-glucopyranoside (4-NPGP). Briefly, the enzyme reaction was performed using p-nitrophenyl-α-D-glucoside (PNP-glycoside) as a substrate in 0.1 M phosphate buffer (pH 6.8). PNP-glycoside (10 mM) and 10 µl of GSH (3 mM) was pre-mixed with samples at various concentrations. Each mixture was added to an enzyme solution (0.01 units) to make 1 ml of final volume. The reaction was terminated by adding 5 µl of 100 mM sodium carbonate solution. Enzymatic activity was quantified by measuring the p-nitrophenol released from PNP-glycoside at 400 nm. All reactions were carried out at 37°C for 30 min with three replications. Acarbose was used as a positive control and IC_{50} values were calculated by the graphic method.

Calculations and statistical analysis

The percentage inhibitions of radicals, lipid peroxidation and α-glucosidase inhibitory activities of the extracts were calculated using the formula:

$$\text{Percentage inhibition} = (A_{control} - A_{sample})/A_{control} \times 100.$$

All the experiments were performed in triplicates and experimental results were expressed as mean ± standard deviation of mean (SEM) of three replicates. IC_{50} value (the concentration of the extracts required to scavenge 50% of radicals) was calculated for different extracts of *M. koenigii*. Graphpad prism 5 software was used for statistical analysis and to prepare the graphical representation of results.

RESULTS AND DISCUSSION

Determination of Phytochemical Constituents

The results of total phenolic content of different leaf extracts of *M. koenigii* were significant and found in the range of 2.37 to 28.84 mg GAE/g dw (Table 1). Among the tested extracts, the highest amount of total phenolics was observed in MKW (28.84 mg GAE/g dw) whereas MKH showed least (2.37 mg GAE/g dw) content of phenolics. The flavonoid contents of the extracts in terms of quercetin equivalent were between 8.28 and 39.90 mg QE/g dw (Table 1), highest being in MKW (39.90 mg QE/g dw) and lowest in MKH (8.28 mg QE/g dw). It is well known that plant polyphenols are widely distributed in the plant kingdom and are very important plant constituents.[27] It has been recognised that phenolic compounds such as flavonoids, phenolic acid and tannins possess diverse biological activities like anti-diabetic anti-

Table 1. Total Polyphenol, Flavonoid, Antioxidants and Ferric Reducing Antioxidant Power of *M. koenigii* Extracts.

Extract	Total polyphenol content[a]	Total flavonoid content[b]	Total antioxidant activity[c]	Ferric reducing power[c]
MKH	2.37±0.36	8.28±0.18	6.04±0.25	3.31±0.012
MKE	12.41±1.60	14.93±1.19	21.86±1.04	9.91±0.02
MKA	21.18±1.47	23.52±0.81	23.46±0.52	17.12±0.47
MKW	28.84±0.49	39.90±1.23	25.54±0.72	2.69±0.08

a: gallic acid; b: quercetin; c: ascrobic acid equivalents mg/g dw plant material respectively; Results represented in means ± standard deviation (n = 3).

inflammatory, anti-carcinogenic and anti-atherosclerotic activities and their effects on human nutrition and health are considerable.[28] It is interesting to note that both the polyphenol and flavonoid contents of MKW are higher than other extracts. This may be due to the better solubility of the polyphenol and flavonoid type of constituents in the aqueous solvent than the other medium. According to our study, the high contents of these phytochemicals in *M. koenigii* can explain its high radical scavenging activity.

Determination of Total Antioxidant Activity

Total antioxidant activity of plant extracts was determined by phosphomolybednum method which is based on the reduction of Mo (VI)–Mo (V) by the antioxidant compounds and subsequent formation of green phosphate/Mo (V), is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid (AA) per gram dry weight of extract. All the four extracts possess antioxidant potential (Table 1), but discrepancies were noticed in the extracts of different polarity. The antioxidant activity was in the range of 6.04 to 25.54 mg AAE/g dw in the leaf extracts. MKW showed the greatest value of 25.54 mg AAE/g dw, as was the case of total phenols and flavonoids, whereas the lowest value of 6.04 mg AAE/g dw was found in MKH. In our study, total polyphenol, flavonoid content and antioxidant activity of the plant extracts correlated significantly and could also contribute to the overall antioxidant potential.

Determination of Reducing Antioxidant Power (FRAP)

The reducing ability to convert Fe^{3+} to Fe^{2+} is also an indirect evidence for the antioxidant activity of an extract or a compound.[29] In this assay system, the antioxidants present in the extract causes the reduction of the Fe^{3+}/ferricyanide complex to form Fe^{2+} ions, which was monitored spectrophotometrically by recording the absorbance of the reaction mixture at 700 nm.[30] All the tested samples showed some degree of electron donation capacity (Table 1). The reducing power of MKA was the highest amongst all the tested samples, with 17.12±0.47 mg AAE/g dw, followed by MKE (9.91±0.02 mg AAE/g dw), whereas those of the others were much lower, with a varied range from 2.69±0.08 to 3.31±0.012 mg AAE/g dw. The data presented here indicate that the marked reducing power of *M. koenigii* extracts seem to be attributed to their antioxidant activity.

DPPH Radical Scavenging Activity

Substances that are capable of donating hydrogen or an electron to DPPH$^{\bullet}$, nitrogen centered free-radical are considered as antioxidants and therefore, radical scavengers. The degree of discoloration of violet colour of DPPH, as it gets reduced, indicates the radical scavenging potential of the antioxidant.[31] In this assay, all extracts showed significant dose-dependent DPPH radical scavenging capacity (Figure 1). The IC_{50} values ranged from 22.12±2.97 to 32.55±1.42 and the DPPH radical-scavenging efficiency increased as follows: MKH < MKA < MKW < MKE. MKE was most efficient, with the lowest IC_{50} value, 22.12 µg/ml. The antioxidant(s) in crude extracts neutralized the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH[32], thereby changing the colour from purple to the yellow-coloured diphenyl picrylhydrazine. This experiment suggests that the plant extracts could contain more bioactive compounds that may attribute the antioxidant properties of *M. koenigii*.

Nitric Oxide Radical Scavenging Activity

Abnormally high level of NO has been linked with chronic inflammation and may be associated with the etiology and pathology of a number of chronic diseases.[33] Besides its own toxicity, this radical can further react with other species instigating even more toxic radicals, such as peroxynitrite, which results from its reaction with superoxide. We tested *M. koenigii* extracts for their inhibitory effect on nitric oxide production andnitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by the extracts. The extracts at varied concentrations (40–400 µg/ml) showed significant inhibitory effect of nitric oxide radical scavenging activity. The percentage scavenging activity increased with increasing concentration. Among the different extracts, the lower IC_{50} value was observed for MKE (162.27±2.26 µg/ml) followed by MKW (163.06±1.86 µg/ml) and highest IC_{50} was observed for MKA (Table 2; Figure 2).

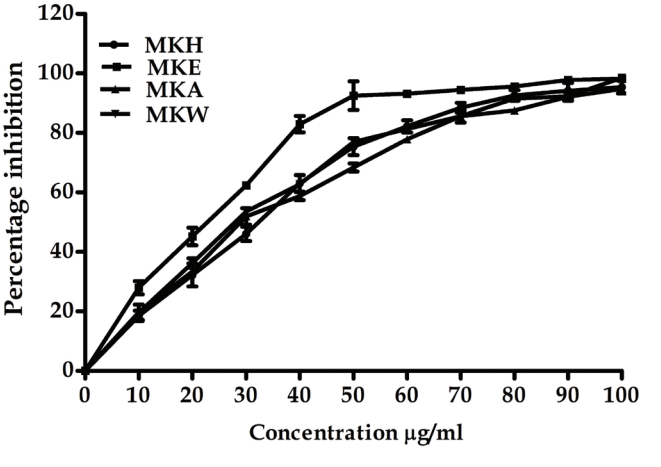

Figure 1. DPPH scavenging activity of different *M. koenigii* leaf extracts.

Table 2. IC_{50} Values of *M. koenigii* Extracts on Tested Radicals.

Extract	DPPH	Nitric oxide	Superoxide	Hydroxyl radical	Lipid peroxidation	-glucosidase inhibition
MKH	32.55±1.42	188.82±0.78	90.38±0.64	380.44±0.38	281.12±0.47	ND
MKE	22.12±2.97	162.27±2.26	129.73±0.42	535.90±0.36	31.67±1.65	ND
MKA	28.88±2.76	267.20±2.035	141.76±0.53	177.52±0.28	72.25±1.9	287.00±0.49
MKW	28.00±0.72	163.06±1.86	203.56±1.13	82.23±0.33	260.41±0.53	174.74±0.72

(Values expressed in µg/ml; Results represented in means ± standard deviation (n = 3); NA: No activity.

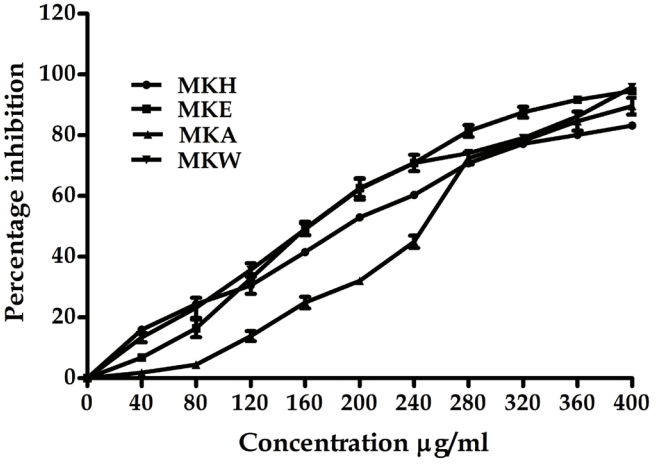

Figure 2. Nitric oxide scavenging activity of different *M. koenigii* leaf extracts.

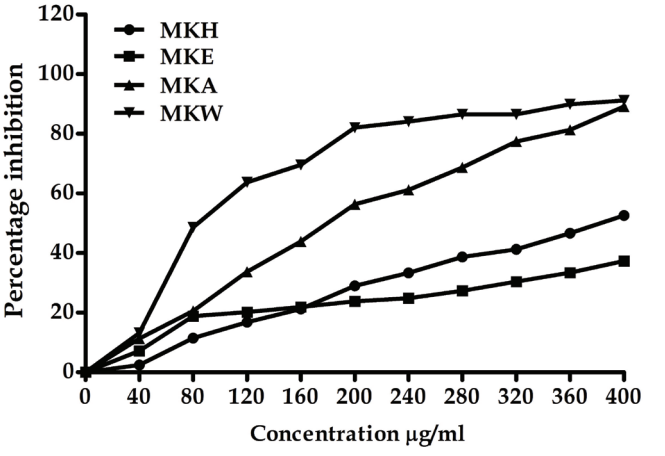

Figure 3. Superoxide scavenging activity of different *M. koenigii* leaf extracts.

Superoxide Radical Scavenging Activity

Superoxide anion is a free radical generated from the normal energy process of energy generation in the human body. It is toxic to cells and tissues and can act as precursors to other reactive oxygen species.[34] It was found that the superoxide-scavenging activities of different extracts of *M. koenigii* were increased markedly with increasing concentrations (Figure 3). The inhibitory activity of MKH was significantly higher than that of other extracts (Figure 3). The IC_{50} values of extracts was found to be in the order of MKW > MKA > MKE > MKH (Table 2). These results imply that water extract is better superoxide scavenger and its capacity to scavenge superoxide may contribute to its antioxidant activity.

Hydroxyl Radical Scavenging Activity

Hydroxyl radicals are the major reactive oxygen species that are responsible for oxidation of lipids and massive biological damage.[35,36] The evaluation of radical scavenging activity was based on the generation of •OH by Fenton reaction. The percentage inhibitions against the hydroxyl radical of different extracts of *M. koenigii* are presented in Figure 4. MKW was found to be efficient scavengers of hydroxyl radicals with least IC_{50} value (82.23±0.33 µg/ml), while other extracts were found to be less efficient scavengers. By comparing the IC_{50} values of all extracts, we can point out that the aqueous extracts of this plant (MKW) was more efficient hydroxyl radical scavengers than its other counterparts.

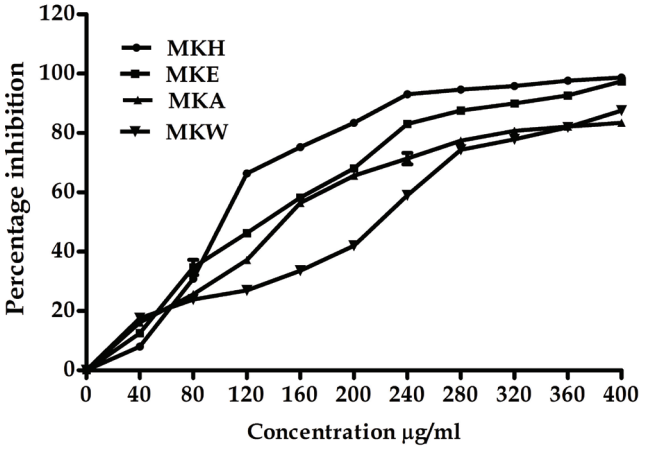

Figure 4. Hydroxyl radical scavenging activity of different *M. koenigii* leaf extracts.

Determination of Inhibition of Lipid Peroxidation

In biological systems, lipid peroxidation generates a number of degradation products such as malondialdehyde which is one of the causes of cell membrane destruction

and cell damage. In order to determine if the *M. koenigii* extracts were capable of reducing *in vitro* oxidative stress, lipid peroxidation was assessed by means of an assay that determines the production of malondialdehyde and related compounds in rat liver homogenates. TBARS are produced as by-products of lipid peroxidation that occurs in the hydrophobic core of biomembranes. Our results clearly indicate that extracts in rat liver homogenate were capable of quenching the extent of lipid peroxidation caused by a Fe^{2+}/ascorbate system (Figure 5). The IC_{50} value was found to be least for MKE (31.67±1.65 µg/ml) followed by MKA (72.25±1.9 µg/ml). The IC_{50} of other two extracts MKH and MKW were much higher (Table 2). This inhibition of lipid peroxidation may have been either due to chelation of Fe^{2+} ions or by trapping of free radicals produced by Fe^{2+}/ascorbate in the reaction system.

Effects of the Extracts on α-glucosidase Activity *In Vitro*

Natural products are still the most readily available source of α-glucosidase inhibitors.[37] In recent reports from other traditional plants, polyphenols were observed to contribute to strong α-glucosidase inhibition.[38] Therefore, we investigated the activity in different extracts. It was found that out of four extracts tested for α-glucosidase inhibitory activity, only MKA and MKW showed significant inhibition property (Figure 6), whereas other extracts (MKH and MKE) did not inhibit α-glucosidase at all. The percentage inhibition of α-glucosidase by MKA and MKW exhibited significant inhibitory activity at dose-dependent acceleration suggesting a competitive type of inhibition. MKW (IC_{50}=174.74 µg/ml) exerted the most powerful

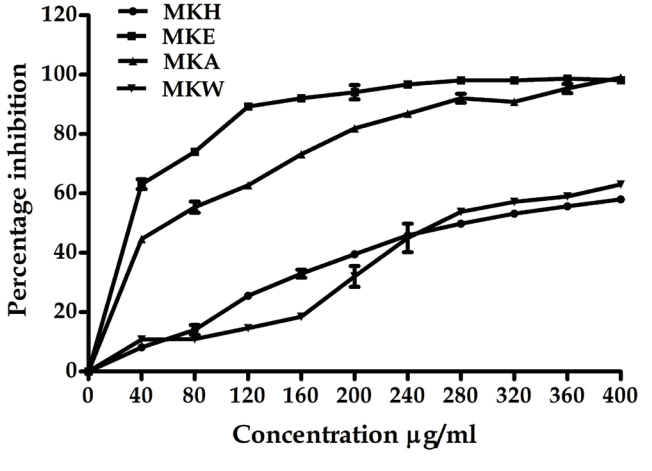

Figure 5. Inhibition of lipid peroxidation of different *M. koenigii* leaf extracts.

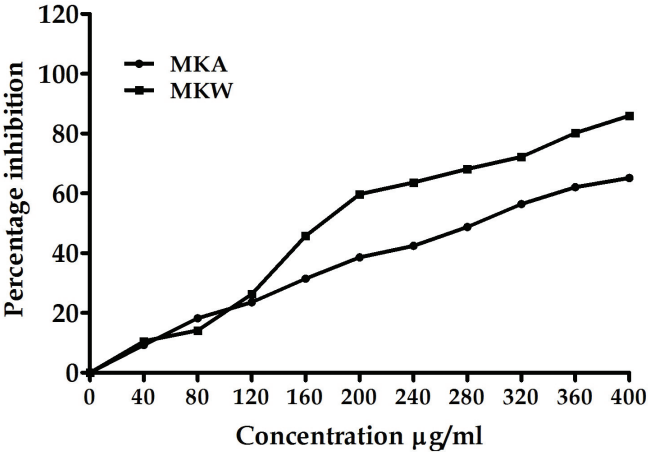

Figure 6. α-glycosidase inhibitory activity of different *M. koenigii* leaf extracts.

inhibitory activity. Acarabose, an antidiabetic drug exerts almost similar effects (IC_{50}=128 µg/ml) under our assay conditions. The IC_{50} value for MKA was found to be 287.00 µg/ml. Based on our results presented here, we can say that *M. koenigii* exert inhibitory effect on α-glucosidases, with MKW being the most effective. With these results, we can further support the traditional use of the plants for its wide medicinal applications.

CONCLUSION

In conclusion, the results of this study clearly indicate that *M. Koenigii* has powerful antioxidant activity against various oxidative systems *in vitro*. Various antioxidant properties of this potent medicinal plant may be attributed to its components effectiveness as scavengers of free radicals, reductive capacity, and metal chelating ability, as well as lipid peroxidation inhibition. The free radical-scavenging property may be one of the mechanisms by which this plant is attributed as useful for foodstuff as well as traditional medicine. Thus, our results support *M. koenigii* as an accessible source of natural antioxidants and a food supplement.

ACKNOWLEDGMENTS

The author MZG acknowledges the financial support from University Grants Commission, New Delhi, India in the form of Research fellowship (RFSMS). We are thankful to DBT-CREBB, DBT-FIST and UGC-SAP-CAS, UGC-XI plan seed money for supporting infra structural facilities of Department of Plant Sciences and School of Life Sciences.

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2011 International Conference on Food Engineering and Biotechnology
IPCBEE vol.9 (2011) © (2011)IACSIT Press, Singapore

Differential Responses of Non-enzymatic Antioxidative System under Water Deficit Condition in Rice (Oryza sativa L.)

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Abstract. Drought stress is one of the foremost causes for crop yield reduction in many agricultural regions of the world. Present study gives insight into the relationship between oxidative damages caused by drought stress and variations in the level of antioxidants as part of the plants' protective mechanism in three rice genotypes IRBB21, Pusa Basmati 1 (PB1) and *O. longistaminata*. Augmentation in antioxidants, predominantly flavanoids, phenolics and total antioxidants were many folds higher in IRBB21 over PB1 and *O. longistaminata* during drought stress. The drought tolerant genotype *O. longistaminata* might have accumulated smaller amounts of free radicals and hence it produced lesser amount of antioxidant. The novelty of present work is that IRBB21 (resistant to biotic stress i.e., Bacterial blight (BB)) has produced more antioxidant, phenolics and flavanoids compared to Pusa Basmati1 (susceptible to BB) and *O. longistaminata* (tolerant to drought) to dehydration stress. Such observation suggests that increased water deficit induces a severe oxidative stress in rice plants, which apparently lead to enhance the antioxidant defense.

Keywords: Drought, antioxidant, free radicals, Oryza sativa, susceptible, flavanoids

1. Introduction

Rice is a major staple food on which more than half of the world's population depends on. In Asia, rice supplies 30 – 80% of the daily calories consumed (Narciso and Hossain 2002). It is a semi-aquatic tropical C_3 crop and hence it requires high level of soil moisture (Mckersie and Lesham 1994). Drought stress is one of the major constraints reducing crop yield in various water limited regions of the world. There are some stress specific generic responses of plants particularly production of excess reactive oxygen species (ROS). Reactive oxygen species play a major role in energy production, phagocytosis, regulation of cell growth, intercellular signalling and synthesis of biologically important compounds. Its production and removal is balanced under tight control which is disturbed by a number of adverse abiotic stress factors such as high light, drought, low temperature, high temperature and mechanical stress. However, its overproduction causes lipid peroxidation, protein denaturation and membrane deterioration in plants (Yu and Rengel 1999; Prochazkova et al. 2001). It also inhibits the photochemical performance and decreases the activities of enzymes in the Calvin cycle (Monakhova and Chernyadev 2002). A common effect of drought stress is an imbalance between the generations and quenching of ROS resulting in oxidative stress (Smirnoff 1998). Therefore, survivals of plants under stress depend on the ability of recognition, generation and transmission of the stimulus, and initiation of various physiological as well as biological changes accordingly (Nguyen et al. 2004). Plants have developed its own machinery to overcome drought stress through production of enzymatic (catalase, peroxidise, superoxide dismutase) and non enzymatic (flavanoids, anthocyanins, carotenoids) free radical scavengers.

This study was conducted to assess the extent of the changes in the activities of non-enzymatic antioxidants and its concentration in rice genotypes under drought stress. A detailed study of the behaviour of antioxidant activities induced under drought tolerance was essential in order to identify genes encoding

them, which may give new leads to produce transgenic drought tolerant rice crops employing biotechnological approaches.

2. Material and methods

Seeds of *Oryza longistaminata,* Pusa Basmati 1 and IRBB21 were germinated for 21 days and the healthy seedlings were transplanted in 20 litres earthen pots (three plants in each pot) filled with a mixture of clay and peat. The experiment was carried out at the greenhouse complex of University of Hyderabad, Hyderabad, India. The plants were subjected to drought stress after 20 days of transplantation by withholding water. In growth room, the plants were maintained at 25±2^{o}C (day/ night) temperature in natural photosynthetically active radiation. The control plants were kept at 100% pot water holding capacity (PWC) by supplying water regularly (Zero stress), while the test plants were subjected to mild (4 days), moderate (5 days) and severe (6 days) drought stress.

Ethanol extracts (80%) were prepared from 500mg leaf sample for each rice genotypes. Different antioxidant assays were carried out to determine the antioxidant nature of the extracts. The flavanoids content was determined by aluminium chloride method using quercetin standard solution (Zhishen et al. 1999). Flavanoids form internal complex (chelate) with the aluminium ion (Al^{3+}) and give yellow colour whose intensity determine the content of flavanoids spectrophotometrically. The total phenolic content of the extract was estimated by well known Folin–Ciocalteau method (Osawa and Namiki 1981; Singleton and Rossi 1965) and it was expressed as gallic acid equivalents. We estimated total non-enzymatic antioxidants as described by Preito et al. (1999) taking ascorbic acid as standard. Statistical analysis was performed using one way analysis of variance (ANOVA) and data presented are mean ± standard errors (SE).

3. Results and discussion

In the present study, there was significant increase in polyphenol content in all the three genotypes. IRBB21 treated (IRBB21T) showed the maximum polyphenol content (4.84 mg GAE/g) followed by Pusa Basmati 1 (4.29 mg GAE /g) and *O. longistaminata* (3.85mg GAE / g) at 6 d of drought stress. Almost same pattern was seen in the case of flavanoids content as well, where it was induced maximally in IRBB21T (16.35 mg QE / g) at 6 d followed by Pusa basmati 1 and *O. longistaminata* (15.30 mg QE / g and 11.20 mg QE / g; 5 d, respectively). Plant extracts and plant-derived antioxidants can elicit a number of *in vivo* effects which amplifies the synthesis of endogenous antioxidants or act directly as antioxidants. It is also reported that composition of antioxidant varies with duration and nature of stress. Antioxidant enzymes and metabolites increases under various environmental stresses, with their comparatively higher activity in stress tolerant genotypes, suggesting that higher antioxidant activity impart tolerance (Sairam and Saxena 2000). The concentrated action of low molecular weight antioxidants like polyphenol (Sgherri et al. 2004) and flavanoids (Hernandez et al. 2004) can effectively scavenge harmful radicals and stabilize lipid oxidation.

IRBB21 is a near isogenic line (NILs) developed by Khush et al. (1990) which contain *Xa21* gene, confer resistance to bacterial blight (BB), is also responding to drought stress by increasing significant amount of antioxidants during water deficit. Higher production of non-enzymatic antioxidants such as phenolics and flavanoids in susceptible genotype (Pusa Basmati 1) compared to tolerant genotype (*O. longistaminata*) is in contrast to the previous reports (Jagtap and Bhargava 1995; Sairam et al. 1998). IRBB21T has appreciably more amount of antioxidant content in its leaves facing severe drought stress at 6 d (8.28 mg ASE /g). With increase in stress degree there was no significant changes in the antioxidant content of *O. longistaminata* (6.04, 6.36 and 6.99 mg ASE / g at 4, 5 and 6 d respectively). There was also less variation of antioxidant content between control and treated Pusa Basmati 1 at 5 d and 6 d of drought stress whereas, there was enhancement in antioxidant content on 4 d and 5 d. (Table 1). *O. longistaminata* was showing much lesser production of phenolics, flavanoids as well as total antioxidants, may be due to appreciably higher repair mechanism to free radical damage, which is a prominent feature of drought tolerant genotype. Alternatively, other antioxidants might be compensating the need of low molecular weight antioxidants in drought tolerant genotype (*O. longistaminata*).

4. Conclusion

The information about the comparative role of plant antioxidant systems in relation to drought tolerance in rice is limited. Therefore, to gain more insight on drought response of PB1, IRBB21 and *O. longistaminata* in terms of oxidative damages, its relation to antioxidant capacity with drought stress was compared. PB1 behaved as drought sensitive which support earlier study whereas the corresponding parameters were few in *O. longistaminata*, which therefore showed appreciable repair to free radical damages and hence prominent drought tolerance. The above mentioned antioxidants might be limited up to the defence mechanism of the susceptible genotypes under drought. Although laboratory conditions may not always reflect the behaviour of the plants exposed to water stress under field conditions, but such finding may help to understand the mechanism of drought stress management and selection or development of rice genotypes resistant to drought stress.

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Table 1: Effect of drought treatment on phenolics (mg GAE /g), flavanoids (mg QE /g) and total antioxidant (mg AAE /g) fresh weight.

Variety	Phenolics						Flavonoids						Total antioxidants					
	4th Day		5th Day		6th Day		4th Day		5th Day		6th Day		4th Day		5th Day		6th Day	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
Pusa basmati 1	0.84 ± 0.12	0.96 ± 0.14	2.14 ± 0.17	2.69 ± 0.14	3.24 ± 0.14	4.29 ± 0.12	5.87 ± 0.13	6.64 ± 0.09	7.95 ± 0.14	12.28 ± 0.13	8.21 ± 0.15	15.30 ± 0.13	3.65 ± 0.11	4.98 ± 0.12	7.73 ± 0.17	6.16 ± 0.15	6.54 ± 0.13	7.55 ± 0.14
IRBB 21	3.04 ± 0.13	3.23 ± 0.11	3.19 ± 0.10	3.55 ± 0.17	4.54 ± 0.12	4.84 ± 0.17	5.21 ± 0.13	7.20 ± 0.08	6.37 ± 0.13	12.80 ± 0.17	8.12 ± 0.18	16.35 ± 0.13	3.48 ± 0.13	4.45 ± 0.14	6.81 ± 0.16	6.85 ± 0.15	7.55 ± 0.15	8.28 ± 0.14
Oryza longistaminata	0.46 ± 0.15	1.13 ± 0.14	2.30 ± 0.16	3.13 ± 0.13	3.06 ± 0.11	3.85 ± 0.13	4.11 ± 0.14	7.47 ± 0.13	5.25 ± 0.17	11.20 ± 0.18	6.29 ± 0.14	13.34 ± 0.11	3.75 ± 0.13	6.04 ± 0.15	4.78 ± 0.13	6.36 ± 0.14	6.45 ± 0.14	6.99 ± 0.13

Gul et al. BMC Complementary and Alternative Medicine 2013, 13:53
http://www.biomedcentral.com/1472-6882/13/53

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RESEARCH ARTICLE Open Access

Antioxidant and antiproliferative activities of *Abrus precatorius* leaf extracts - an *in vitro* study

Mir Z Gul[1], Farhan Ahmad[2], Anand K Kondapi[2], Insaf A Qureshi[2] and Irfan A Ghazi[1*]

Abstract

Background: The use of traditional medicine at the primary health care level is widespread and plant-based treatments are being recommended for curing various diseases by traditional medical practitioners all over the world. The phytochemicals present in the fruits, vegetables and medicinal plants are getting attention day-by-day for their active role in the prevention of several human diseases. *Abrus precatorius* is a widely distributed tropical medicinal plant with several therapeutic properties. Therefore in the present study, *A. precatorius* leaf extracts were examined for their antioxidant and cytotoxic properties *in vitro* in order to discover resources for new lead structures or to improve the traditional medicine.

Methods: In this study, antioxidant and antiproliferative properties of the different leaf extracts (hexane, ethyl acetate, ethanol and water) from *A. precatorius* were investigated along with the quantification of the polyphenol and flavonoid contents. The ability of deactivating free radicals was extensively investigated with *in vitro* biochemical methods like DPPH$^{\bullet}$, $^{\bullet}$OH, NO, SO_{2-} scavenging assays and inhibition capability of Fe(II)-induced lipid peroxidation. Furthermore, antiproliferative activities using different human cancer cell lines and primary cell line was carried out by MTT method.

Results: Total phenolic content and total flavonoid content of the extracts were found in the range of 1.65 ± 0.22 to 25.48 ± 0.62 GAE mg/g dw and 6.20 ± 0.41 to 17.16 ± 1.04 QE mg/g dw respectively. The experimental results further revealed that *A. precatorius* extracts showed strong antiradical properties, capable to chelate Fe^{2+} and possess good inhibition ability of lipid peroxidation. In addition, as a first step towards the identification of phytoconstituents endowed with potent chemopreventive activities, we evaluated the inhibitory effects of *A. precatorius* extracts on the proliferation of four different human tumour cell lines such as human colon adenocarcinoma cells (Colo-205), human retinoblastoma cancer cells (Y79), human hepatocellular carcinoma cells (HepG2) and Leukemia cells (SupT1). Ethanol extract (APA) and ethyl acetate extract (APE) of *A. precatorius* had apparent capabilities of inhibiting the survival of tested human cancer cell lines. Moreover, it was observed that the *A. precatorius* extracts did not inhibit the growth of mice peritoneal macrophages, thus confirming that plants extracts are selective against the cancer cell lines.

Conclusion: This work provides a scientific support for the high antioxidant and antiproliferative activity of this plant and thus it may find potential applications in the treatment of the diseases caused by ROS. Further studies are needed to confirm *in vivo* anti-tumorgenicity and subsequent chemical characterization of the active molecule(s).

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Background

The human body possesses numerous antioxidant defences and repair mechanisms against oxidative stress. However, these mechanisms are insufficient to prevent the damage entirely as production of reactive oxygen species (ROS) is certain to play multiple important roles in tissue damage and loss of function in a number of tissues and organs [1]. Free radicals and ROS have been implicated as endogenous initiators in the etiology of cancer and several other degenerative or pathologic processes of various serious diseases, as well as in aging processes [2]. Oxidative damage to DNA is considered a critical step in cancer development [3]. Over the past decade or so, numerous experimental and epidemiological studies have shown that a wide variety of phytochemicals such as phenolics, flavonoids, isoflavone, flavones, anthocyanins, catechin, isocatechin and carotenoids are able to prevent or slow down oxidative stress-induced damage leading to carcinogenesis by upsetting the molecular events in the initiation, promotion or progression conditions. Recent studies demonstrated that the high dietary intake of fruits and vegetables could be associated with lower cancer prevalence in humans [4-7]. Natural products mainly from plant kingdom offer a wide range of biologically active compounds that act as natural antioxidants with recognized potential in drug discovery and development [8]. Great interest is currently being paid to natural products for their interesting anticancer activities. High percentages (~ 60%) of all the drugs applied in the treatment and/or prevention of cancer are from natural products and their derivatives, of which higher plants contribute around 25%. Approximately 60% of drugs approved for cancer treatment are of natural origin [9,10]. This has elicited the pursuit of effective antioxidant and anticancer agents from various sources particularly medicinal and edible plants [11]. Investigations on natural products have regained prominence in the recent past with increasing understanding of their biological significance such as antioxidant, radical scavenging, antiproliferative activities and increasing recognition of the origin and function of their structural diversity [12-15].

Abrus precatorius L. (Fabaceae) is a vine originally native to India that is now commonly found throughout the tropical and subtropical parts of the world [16]. It grows best in fairly dry regions at low elevations. Leaves, roots and seeds are used as a medicament in traditional system of Indian medicine for antihelminthic, antidiarrhoeal, antiemetic and inhibits intestinal motility. Researchers have reported that seeds are used for the treatment of diabetes and chronic nephritis [17]. The leaves of *A. precatorius* are sweeter [18,19] and as equivalent in sweetness potency to sucrose [20]. In West Tropical Africa, *A. precatorius* leaves have been employed to sweeten foods and certain medicines used for stomach complaints, to treat fevers, cough and cold (used as decoction). The leaves are casually chewed and the vine sometimes sold as a masticatory in Curacao [21,22]. The plant is also traditionally used to treat tetanus, and to prevent rabies. Though considerable work has been done on the seeds for different activities, however, scientific information on antioxidant and antiproliferative properties of leaves of this plant is still not available or rather scarce. Thus, we evaluated the abilities of leaf extracts of *A. precatorius* to function as an antioxidant agent using *in vitro* assays. Additionally, the ability of the leaf extracts to inhibit proliferation of various cancer cell lines was investigated. Since elimination of cancer in early stages is an integral part of chemoprevention, measuring antiproliferative properties against cancer cells provide useful insight on the chemoprotective potential of natural extracts. Thus, the objective of this study was to examine the efficacy of *A. precatorius* as an antioxidant as well as its inhibitory effect on human cancer cell proliferation.

Methods

Chemicals

The analytical grade chemicals were purchased from Hi-Media and Merck, India. Standard drugs were purchased from Sigma-Aldrich chemicals co. (Germany); RPMI-1640, DMEM and foetal bovine serum (FBS) from Gibco (USA).

Plant material

Abrus precatorius leaves were kindly provided by Central Research Institute of Unani Medicine, Hyderabad. A voucher specimen (UoH/VS/AP-2) has been preserved in our laboratory for future reference.

Preparation of extracts

The air-dried leaves of the plant were powdered with a mechanical grinder to obtain a coarse powder, which was then subjected to successive extraction in a soxhlet apparatus using hexane, ethyl acetate, ethanol and water. Each time before extracting with the next solvent, the material was dried in hot air oven at 40°C. Extracts were then filtered through a Whatman No.1 paper filter and concentrated to the dry mass with the aid of rotary evaporator. The extraction process was repeated three times at different time periods. It was observed that there was no significant difference in the percentage yield and content of phyto-constituents that are believed to play an important role in biological activities. The yield of each extract was measured and residues were stored in dark glass tubes for further analysis. The different extracts were designated as APH (for hexane extract), APE (for ethyl acetate extract), APA (for ethanol

extract) and APW (for water extract). The dried extracts were dissolved in dimethyl sulfoxide (DMSO) as 20 mg/mL and diluted with phosphate-buffered saline (PBS, pH 7.4) to give final concentrations.

Determination of phytoconstituents

Determination of total phenols

Total phenolics were determined using Folin-Ciocalteu reagent as described by Yang *et al.* [23] with minor modifications. Total phenolic assay was conducted by mixing 2.7 mL of deionised water, 0.01 mL of extracts, 0.3 mL 20% Na_2CO_3 and 0.10 mL Folin-Ciocalteu reagent. Absorbance of mixture was measured at 725 nm. A standard curve was prepared with gallic acid ($r^2 = 0.9454$) and final results were given as mg gallic acid equivalents (GAE) g dw.

Determination of total flavonoids

The total flavonoids was measured by addition of aluminium chloride reagent to the solution containing the extract using the method of Barrera et al. [24]. Briefly, 10 μL of plant extracts of known concentrations (20 mg/mL) were diluted with 0.5 mL of double distilled water. To this mixture, 30 μL of 5% sodium nitrite ($NaNO_2$) and 60 μL of 10% aluminium chloride were added and incubated at room temperature for 10 min. After incubation, 350 μL of 1 M NaOH was added and total volume was made up to 1 mL with distilled water. Finally, absorbance was measured against the prepared blank at 510 nm and results were given as quercetin equivalents (mg QE)/g of dw. Standard curve was prepared with known concentrations of quercetin ($r^2 = 0.955$).

Antioxidant ability assays

Phosphomolybdenum assay

The total antioxidant activity of extracts was evaluated by green phosphomolybdenum complex according to the method of Prieto *et al.* [25]. An aliquot of 10 μL of sample solution was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in micro centrifuge tube. Tubes were incubated in a dry thermal bath at 95°C for 90 min. After cooling, the absorbance of the mixture was measured at 695 nm against a blank. Ascorbic acid have been used ($r^2 = 0.964$) for reference and the reducing capacities of the analyzed extracts were expressed as mg of ascorbic acid equivalents (mg AAE)/g of dw.

Ferric-reducing/antioxidant power (FRAP) assay

The Fe^{3+} reducing power of the extracts was determined by the method of Oyaizu [26] with slight modifications. Briefly, extracts and standard (ascorbic acid) in 1 mL of appropriate solvents were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%), and then mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 5000 rpm for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%). The absorbance was measured at 700 nm and the reducing power of the extracts was presented as mg AAE/g of dw.

DPPH˙ radical scavenging activity

The DPPH free radical scavenging activity of leaf extracts of *A. precatorius* was measured in term of hydrogen donating or radical scavenging ability using the stable radical DPPH [27]. Briefly, 0.004% w/v of DPPH radical solution was prepared in methanol and then 900 μL of this solution was mixed with 100 μL of extract solution containing 40–400 μg/mL of dried extract. The absorbance was measured at 517 nm after 30 min of incubation. Methanol (95%), DPPH solution and ascorbic acid were used as blank, control and reference respectively.

Hydroxyl radical scavenging activity

The ability of the extracts to inhibit site-specific hydroxyl radical-mediated peroxidation was carried out according to the method given by Hinneburg *et al.* [28] with some modifications. Briefly, the mixture containing $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), deoxyribose (28 mM) and different concentrations of test samples (40–400 μg/mL) in 500 μL phosphate buffered saline (PBS, 20 mM, pH 7.4) was incubated for 30 min at 37°C. After adding 1 mL of trichloroacetic acid (10%, w/v) and 1 mL thiobarbituric acid (2.8% w/v; in 25 mM NaOH), the reaction mixture was boiled for 15 min. The extent of oxidation was estimated at 532 nm and the scavenging activity of test sample was expressed as the percentage inhibition of the deoxyribose degradation to malondialdehyde. Ascorbic acid was used as the positive control.

Hydrogen peroxide scavenging assay

The ability of plant extracts to scavenge hydrogen peroxide was determined according to Long *et al.* [29]. A 40 mM of H_2O_2 solution was mixed with different concentrations of plant extracts (20–200 μg/mL) and incubated for 3.5 h at room temperature. After incubation, 90 μL of the H_2O_2-sample solution was mixed with 10 μL of HPLC-grade methanol and 0.9 mL of FOX reagent was added (prepared by mixing 9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was vortexed and then incubated at room temperature for 30 min.

The absorbance of ferric-xylenol orange complex was measured at 560 nm. Ascorbic acid was used as the reference compound.

Nitric oxide scavenging activity

The free radical scavenging potential of *A. precatorius* was further substantiated by scavenging of nitric oxide radical assayed by sodium nitroprusside method [30]. The reaction solution (50 μL) containing 10 mM sodium nitroprusside in PBS (pH 7.0) was mixed with different concentration (40–400 μg/mL) of sample extracts, followed by incubation at 37°C for 20 min under light. After incubation, the samples were mixed with 300 μL of Griess reagent (1% sulfanilamide, 2% H_3PO_4). The samples were again incubated for 30 min at room temperature under light conditions followed by the addition of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride. The absorbance was recorded at 546 nm and the results were expressed as per cent of scavenged nitric oxide with respect to the negative control without addition of any antioxidant. Ascorbic acid was used as a positive control.

Superoxide radicals scavenging activity

The scavenging activity of the plant extracts towards superoxide anion radicals was measured by the nitro-blue tetrazolium (NBT) reduction method [31] with minor modifications. Superoxide anions were generated in a non-enzymatic phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium. In the experiment, the superoxide anion was generated in 2 mL of phosphate buffer (100 mM, pH 7.4) containing 500 μL of 156 μM nitroblue tetrazolium (NBT solution), 500 μL of 468 μM nicotinamide adenine dinucleotide (NADH) solution and 300 μL of different concentrations (40–400 μg/mL) of extracts. DMSO and L-ascorbic acid were used as solvent and positive control respectively. The reaction was initiated by adding 100 μL of 60 μM phenazine methosulfate (PMS) to the mixture. After 5 min of incubation at room temperature, the absorbance was measured at 560 nm against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Inhibition of lipid peroxidation assay

Fe^{2+} induced lipid peroxidation is one of the established system for assessing antioxidant action of different plant extracts. A modified thiobarbituric acid-reactive species (TBARS) assay [32] was used to measure the lipid peroxide formed using rat liver homogenate as lipid rich media. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen. Healthy albino rats of the wister strain (250 grams) were sacrificed (procedure described was reviewed and approved by the University of Hyderabad, School of Life Sciences' animal ethics committee) and liver was perfused with 0.15 M KCl, homogenate was centrifuged at 800 g for 15 min at 4°C and the supernatant was used for thiobarbituric acid assay. The extracts of *A. precatorius* at different concentrations (40–400 μg/mL) were mixed with the liver microsome preparation and incubated at room temperature for 10 min. Then, 50 μL Fenton's reagent (10 mM $FeCl_3$; 10 μL of 2.5 mM H_2O_2; 0.1 M L-ascorbic acid) in phosphate buffer (0.2 M, pH 7.4) were added, and the volume was made to 1 mL. The tubes were then incubated for 30–45 min at 37°C to induce lipid peroxidation. Thereafter, 2 mL of ice-cold HCl (0.25 N) containing 15% trichloroacetic acid, 0.5% thiobarbituric acid and 0.5% butylated hydroxytoluene (BHT) were added in each sample followed by heating at 100°C for 15 min. The reaction mixture was put in an ice bath for 10 min. The mixture was centrifuged at 1000 rpm for 10 min and the extent of lipid peroxidation was subsequently monitored by formation of thiobarbituric acid reactive substances (TBARS) as pink chromogen in presence or absence of extracts and standard (L-ascorbic acid). The absorbance of the supernatant was measured spectrophotometrically at 532 nm and decline in formation of pink chromogen in pre-treated reactions was considered as inhibition of lipid peroxidation.

Anti-proliferative activity

A panel of four human cell lines namely, (a): human colon adenocarcinoma cells - Colo-205, (b): human retinoblastoma cancer cells - Y79, (c): human hepatocellular carcinoma cells - HepG2 and (d): Leukaemia cells - SupT1 were used to study antiproliferative activity. The cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cell lines HepG2 & Colo-205 were cultured in Dulbecco's modified Eagle's medium (DMEM) and Y79 & SupT1 in RPMI 1640 containing 10% (v/v) FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator with 5% CO_2 for 24 h at 37°C and seeded onto 75 cm^2 culture flasks. *In vitro* response to extracts and standard drug was evaluated by means of a growth inhibition using the MTT assay [33]. The cells were seeded at a density of $\sim 5 \times 10^3$ per well using a brief trypsinization. Furthermore, Doxorubicin and sample extracts (25–200 μg/mL) dissolved in dimethylsulfoxide (DMSO; not exceeding the concentration of 2%), and further diluted in cell culture medium were added into a 96-well plate. After 48 h of incubation, 20 μL of MTT reagent (5 mg/mL) were added and mixtures were reincubated for 4 h. Finally the

absorbance of formazan was measured at 550 nm. Doxorubicin was also assayed as a positive control at the concentration of 0.5–10 μg/mL. The resulting growth data represents the net outcome of cell proliferation and cell death. The cell viability (%) was obtained by comparing the absorbance between the samples and a negative control.

Assessment of extract toxicity in normal cells

To assess the toxicity of the plant extracts on primary cells (peritoneal murine macrophages), the MTT toxicity assay was used. Thioglycollate-elicited mouse peritoneal macrophages were harvested from female BALB/C mice [34]. Experimental protocol was again undertaken in accordance with the ethical guidelines and the permission of the University of Hyderabad, School of Life Sciences' animal ethics committee was obtained. Toxicity toward mouse peritoneal macrophages was assessed with cells plated in 96-well plates at a density of 2×10^6 cells per well (in 200 μL volume). After adherence, the medium was removed and replaced by one of the media containing the plant extracts. The plates were incubated for 72 hrs at 37°C in a humidified 5% CO_2 incubator. Control cells were incubated with culture medium alone. Cell viability was determined by a colorimetric assay with the tetrazolium salt MTT [33]. Absorbance of the formed formazan product was measured at a test wavelength of 540 nm. Results were expressed as percentage cellular viability of the extracts.

Calculations and statistical analysis

The percentage inhibitions of radicals, lipid peroxidation and cytotoxic activities of the extracts were calculated using the formula:

$$\text{Percentage inhibition} = \left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}} \times 100$$

All results are expressed as mean ± standard deviation (SD) values average from 3 to 4 independent experiments performed in duplicate. IC_{50} value (the concentration of the extracts required to scavenge 50% of radicals) was calculated for different extracts of *A. precatorius*.

Statistical differences between correlated samples were evaluated using Student's *t*-test and composite treatments were compared using one-way analysis of variances (ANOVA) and considered significantly different where probability values were found to be equal to or less than 0.05. Statistical tests as well as mean and SD calculations and graphical representation of the results were performed using GraphPad Prism v5 and Sigmaplot v11.0 software's.

Results and discussion

Determination of phyto-constituents

The results of total phenolic content in leaf extracts of *A. precatorius* evaluated using Folin-Ciocalteu method are presented in Table 1. Total polyphenolic content in different extracts varied with the solvent used in this study. The results indicated that water extract (APW) possessed an abundance of phenolics amounting to 25.48 ± 0.62 mg GAE/g dw, while GAE value of ethyl acetate (APE) extract was 23.57 ± 0.31 mg/g dw. Ethanol extract (APA) and hexane extract (APH) showed less amount of GAE at 7.44 ± 0.10 mg/g dw and 1.65 ± 0.22 mg/g dw respectively. It suggested that major phenolics of *A. precatorius* were mainly located in water extract. The extracts of *A. precatorius* also contained significant amount of flavonoids (Table 1). The flavonoid contents of extracts in terms of quercetin equivalents were between 6.20 ± 0.41 and 17.16 ± 1.04 mg/g dw. The flavonoid contents in APE (17.16 ± 1.04 mg/g dw) was higher compared to other three extracts. The plant derived antioxidants especially polyphenols and flavonoids have recently attracted medicinal attention as bioactive agents with anticancer, antidiabetic, antimicrobial, hepatoprotective, neuroprotective and cardioprotective properties [35-37]. They have been ascribed to have resilient antioxidant activity and help in protecting cells against oxidative damage caused by free radicals due to their redox properties, which enable them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [38-41].

Antioxidant ability assays

Phosphomolybdenum assay

In phosphomolybdenum assay, which is a quantitative method to evaluate the antioxidant capacity, all the extracts exhibited different degrees of activity as shown in Table 1. The reducing power of a compound is associated with electron donating capacity and serves as an indicator of antioxidant activity [42,43]. Results indicated that APA has higher antioxidant capacity (24.73 ± 0.72 mg ascorbic acid equivalent/g dw) than other three extracts which showed antioxidant capacity in the order: APE (17.92 ± 0.38 mg AAE/g dw) > APW (16.66 ± 0.68 mg AAE/g dw) > APH (7.16 ± 0.43 mg AAE/g dw).

Ferric-reducing/antioxidant power (FRAP) assay

In reducing power assay, the presence of reductants (antioxidants) in samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron which serves as a significant reflection of antioxidant activity [44]. The amount of Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability [45]. Earlier reports suggest that some phenolic

Table 1 Quantitative estimation of phytochemicals and antioxidant activities of different extracts of *A. precatorius*

Type of Extract	Total Phenols[a]	Total Flavonoid[b]	Total antioxidant capacity[c]	Ferric reducing antioxidant power[c]
APH	1.65 ± 0.22	6.20 ± 0.41	7.16 ± 0.43	2.67 ± 0.40
APE	23.57 ± 0.31	17.16 ± 1.04	17.92 ± 0.38	8.45 ± 0.65
APA	7.44 ± 0.10	7.23 ± 0.68	24.73 ± 0.72	10.28 ± 0.47
APW	25.48 ± 0.62	10.70 ± 0.56	16.66 ± 0.68	13.34 ± 0.35

a: Gallic acid; b: Quercetin; c: Ascrobic acid equivalents mg/g dw plant material respectively. Each value is expressed as a mean ± standard deviation (n = 3).

compounds such as flavonoids and phenolic acids exhibited antioxidant activity through their reductive capacity in a Fe^{3+}- Fe^{2+} system [46]. All the four extracts showed some degree of electron donating capacity and reduced Fe^{3+} to Fe^{2+}. The reducing ability of the extracts was in range of 13.34 ± 0.35 to 2.67 ± 0.40 AAE mg/g dw (Table 1). The FRAP values for APW was found to be higher than other three extracts. This suggests that APW has a significant ability to react with free radicals to alter them into more stable non-reactive species and to terminate radical chain reaction.

DPPH• radical scavenging activity

DPPH assay provides basic information on antiradical activity of extracts and its results can indicate the presence of phenolic and flavonoid compounds in plant extracts [47]. Very significant antioxidant activities were found in all the four extracts and positive control, which increased with increasing concentration (Figure 1). DPPH activity values for APE, APA and APW were found to be nearer to each other. The APE and APW were able to inhibit the formation of DPPH• radicals with a percentage inhibition of 96.35 ± 2.98 and 92.63 ± 4.63% respectively at the highest concentration of 400 μg/ml with the IC_{50} values of 57.66 ± 1.32 and 79.97 ± 1.84 μg/mL respectively (Table 1). Previous reports have demonstrated that ethyl acetate fractions are good sources of antioxidant compounds [48,49]. APA also exhibited potent DPPH scavenging activity (95.14 ± 3.44%) in this study and its IC_{50} (60.67 ± 1.03 μg/mL) was near to that of APE, in which phenolic levels were approximately 3.5 times higher. Several groups have pronounced a positive correlation between phenolic content and antioxidant activity [50-52] using similar assay systems, but our study could not establish correlation in similar manner. It could be due to the presence of other reducing compounds that probably interfere with the Folin-Ciocalteu assay and/or the presence of other non-phenolic compounds with antioxidant effects. APH showed less inhibitory action as compared to other extracts with the percentage inhibition of 50.84 ± 5.68 at same concentration with the IC_{50} value 196.70 ± 1.84 μg/mL. The IC_{50} values of ascorbic acid and quercetin (positive controls) were found to be 3.80 ± 0.43 and 9.84 ± 0.6 μg/mL respectively. This behaviour can be explained by different composition of each extracts as there are compounds that react quickly with DPPH and others that have a slower reaction mechanism and required extremely high concentration to have a significant effect [53].

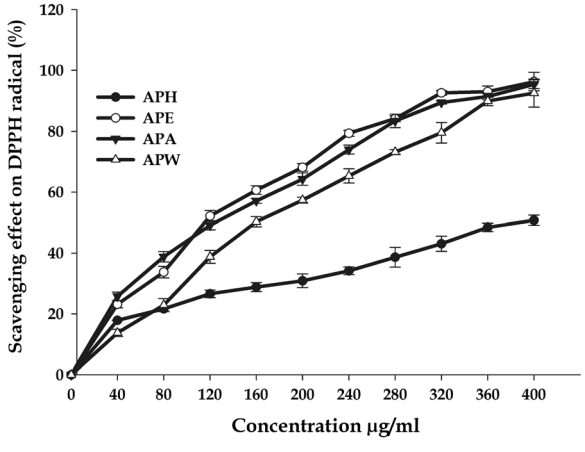

Figure 1 DPPH radical-scavenging activities of *A. precatorius* leaf extracts at different concentrations.

Hydroxyl radical scavenging activity

The hydroxyl radical is known to be the most reactive oxygen radical and it severely damages neighbouring bio-molecules in the body, such as protein and DNA, resulting into mutagenesis, carcinogenesis and cytotoxicity [54,55]. Therefore, removal of hydroxyl radical is possibly one of the most effective defences of a living body against various diseases. A significant decrease in concentration of hydroxyl radical was observed due to *A. precatorius* extracts (Figure 2; Table 2). All the extracts exhibited significant activity, above 40% in a concentration - dependent manner with maximal inhibition of 79.52 ± 2.57% at 400 μg/mL by APE with IC_{50} value of 205.51 ± 3.08 μg/mL. APA (78.97 ± 1.60%; IC_{50} = 209.33 ± 4.13 μg/mL) and APW (68.18 ± 3.14%; IC_{50} = 309.90 ± 5.21 μg/mL) extracts were also found to be significant powerful quenchers of •OH radical as compared to ascorbic acid (IC_{50} = 62.40 ± 3.72). APH was found to be weak scavenger of •OH with the IC_{50} value of 464.25 ± 4.43 μg/mL. Our results suggested that hydroxyl radical scavenging ability of extracts of *A. precatorius* are comparable to or even higher than

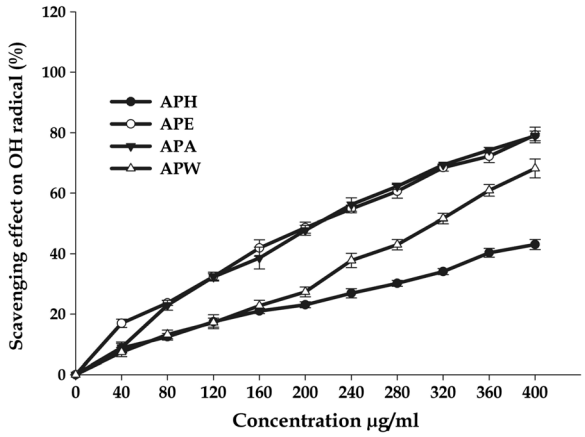

Figure 2 Hydroxyl radical scavenging activities of *A. precatorius* leaf extracts at different concentrations.

earlier published reports [12,56,57] and could help in preventing oxidative damage in the human body.

Hydrogen peroxide scavenging assay

Hydrogen peroxide (H_2O_2) is a biologically relevant, non-radical reactive oxygen species and is inevitably generated as a by-product of normal aerobic metabolism. However, when concentration increases under stress conditions, H_2O_2 could be detrimental for cells [58] and, furthermore, could be converted into other ROS such as hydroxyl radicals. Thus, H_2O_2 scavenging activity becomes a crucial characteristic of total antioxidant activity. In this study, APA extract (IC_{50} = 92.83 ± 3.23 µg/ml) was found to be efficient scavenger of hydrogen peroxide radical, while APW extract (IC_{50} = 152.35 ± 1.06 µg/mL) was least efficient. The APH and APE extracts also exhibited comparable efficiency with IC_{50} = 112.59 ± 3.24 and 121.02 ± 3.14 µg/mL respectively (Figure 3; Table 2). The H_2O_2 scavenging capacity of all extracts was also better than that of quercetin tested in the same conditions. The results strongly suggest that these extracts contain the necessary compounds for radical elimination. Many reports have already proven that nutritive phenols play a significant role in protecting mammalian and bacterial cells from cytotoxicity induced by H_2O_2 [59-61], indicating that the observed activity of plants extracts could be due to the presence of phenols.

Nitric oxide scavenging activity

Initially NO was regarded to have only beneficial effects, but it has been found that over production of NO is closely associated with different pathological diseases, such as chronic inflammation, autoimmune diseases and cancer [62]. The NO radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with O_2^- radicals to form peroxynitrite which damages the biomolecules such as proteins, lipids and nucleic acids, and therefore injures the host tissue [56]. The measure of NO radical scavenging activity was based on the principle that sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using a Griess reagent. Scavengers of nitric oxide act against oxygen, leading to reduced production of nitrite ions which can be monitored at 546 nm. *A. precatorius* extracts showed significant decrease in NO radical due to the scavenging ability of extracts. All the extracts exhibited significant NO scavenging activity in a concentration dependent manner (Figure 4). The results clearly identify APE as better NO scavenger where percentage inhibition reached to 97.58 ± 3.12% with an IC_{50} value of 107.58 ± 2.12 µg/mL followed by APA whose inhibition of generation of NO reached up to 92.70 ± 2.13 (IC_{50} = 145.96 ± 2.17 µg/mL) in a concentration - dependent manner and a gradual decline thereafter at the higher concentrations. The APH and APW extracts were also efficient scavengers of NO (83.11 ± 0.89% and 80.62 ± 2.11%) with slightly higher IC_{50} Values, 192.45 ± 3.76 and 264.95 ± 4.24 µg/mL respectively.

Superoxide radicals scavenging activity

Superoxide anion (O_2^-) is one of the most important representatives of free radicals. It acts as a precursor of more reactive oxidative species such as single oxygen and hydroxyl radicals that have the potential of reacting

Table 2 IC_{50} values obtained in the antioxidant activity assays

Sample	IC_{50} µg/mL					
	DPPH	OH	H_2O_2	NO	O_2^-	Lipid peroxidation
APH	196.70 ± 2.06	464.25 ± 4.43	112.59 ± 3.24	192.45 ± 3.76	427.26 ± 5.72	377.07 ± 5.23
APE	57.66 ± 1.32	205.51 ± 3.08	121.02 ± 3.14	107.58 ± 2.12	143.44 ± 3.28	45.46 ± 3.71
APA	60.67 ± 1.03	209.33 ± 4.13	92.83 ± 3.23	145.96 ± 2.17	157.07 ± 2.56	285.22 ± 4.63
APW	79.97 ± 1.84	309.90 ± 5.21	152.35 ± 1.06	264.95 ± 4.24	201.45 ± 6.23	302.02 ± 4.11
Ascorbic acid	6.86 ± 0.92	62.40 ± 3.72	–	19.90 ± 2.30	32.86 ± 3.78	48.72 ± 3.20
Quercetin	14.34 ± 1.64	–	245.30 ± 4.60	21.09 ± 2.16	–	–

Each value is expressed as a mean ± standard deviation (n = 3).

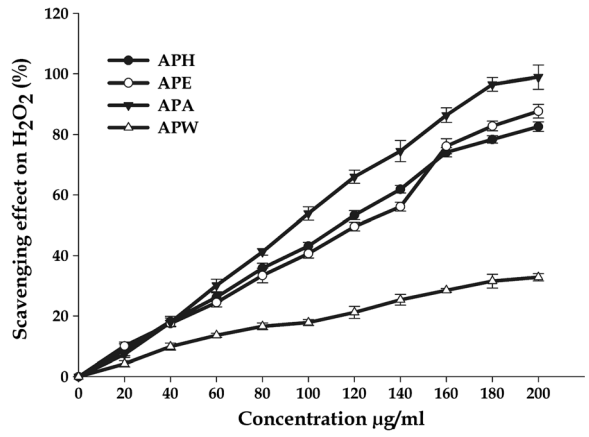

Figure 3 Hydrogen peroxide scavenging activities of *A. precatorius* leaf extracts at different concentrations.

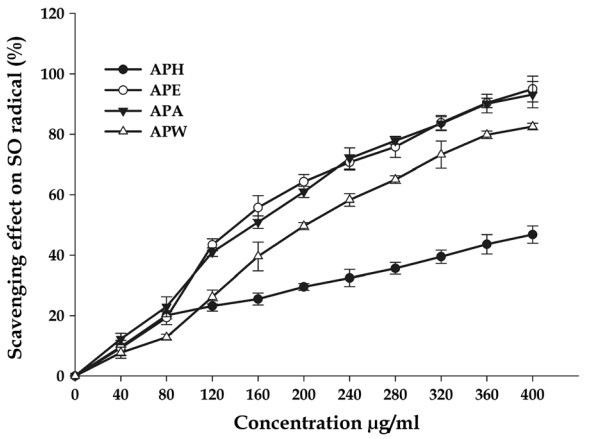

Figure 5 Superoxide scavenging activities of different extracts of *A. precatorius* at different concentrations.

with biological macromolecules and thereby inducing tissue damage, and plays a vital role in peroxidation of lipids [63-65]. In the present study, the inhibitory effect of *A. precatorius* extracts on superoxide radicals was in a concentration dependent manner (Figure 5). High inhibitions were observed at very low extract concentrations. At 400 µg/mL of tested extract, the scavenging effects on superoxide radical were found to be 95.01 ± 4.29%; IC_{50} value = 143.44 ± 3.28 µg/mL for APE and 93.15 ± 4.36%; IC_{50} value = 157.07 ± 2.56 µg/mL for APA. Moreover, APW and APH also possess the significant scavenging effect 73.25 ± 4.50%; IC_{50} value = 201.45 ± 6.23 µg/mL and 46.81 ± 2.87%; IC_{50} value = 427.26 ± 5.72 µg/mL respectively, however with higher IC_{50} values.

Inhibition of lipid peroxidation assay

Lipid peroxidation involves the formation and propagation of lipid radicals with numerous deleterious effects, including destruction of membrane lipids, metabolic disorders and inflammation. Production of malondialdehyde (MDA) is a hallmark of this process. This process is initiated by hydroxyl and superoxide radicals leading to the formation of peroxy radicals ($LOO^{\cdot}$) that ultimately propagates chain reaction in lipids. Thus, antioxidants which are capable of scavenging peroxy radicals could prevent lipid peroxidation. In this study, we measured the potential of *A. precatorius* extracts to inhibit lipid peroxidation in rat liver microsomes, induced by the Fe^{2+}/ascorbate system (Figure 6). Different extracts protected against lipid peroxidation induced by Fe^{2+}, considerably reduced MDA content in a concentration-dependent manner. APE had the greatest inhibiting activity (98.70 ± 2.56%); with the lowest IC_{50} value 45.46 ± 3.71 µg/mL. When compared to the activity of standard, (ascorbic acid, IC_{50} = 48.72 ± 4.23 µg/mL), inhibiting activity against lipid peroxidation

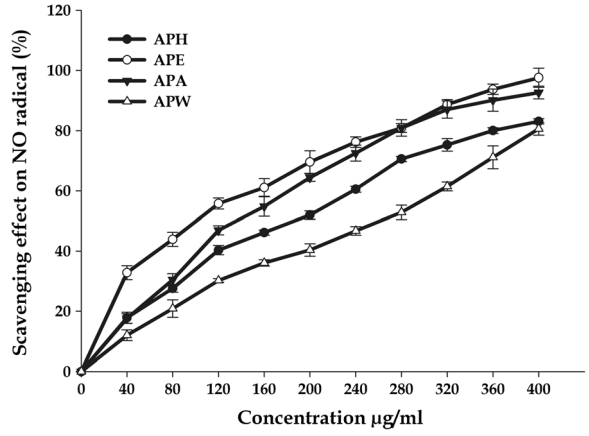

Figure 4 Nitric oxide scavenging activities of *A. precatorius* leaf extracts at different concentrations.

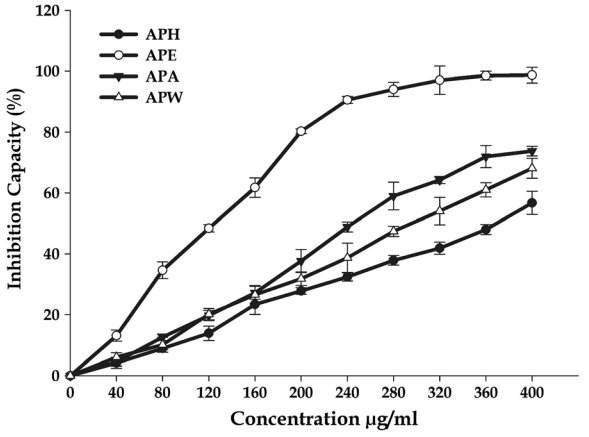

Figure 6 Effects of different concentrations of crude extracts from the leaf of *A. precatorius* on Fe (II) induced TBARS production in liver.

of APE was very high considering that the extract was a mixture of a great number of components against pure compound used as standards. The other extracts (APA, APW and APH) proved to be much weaker inhibitors of lipid peroxidation than APE with the percentage inhibition of 73.75 ± 1.60%, 68.09 ± 3.26% and 56.73 ± 3.81% respectively with higher IC_{50} values than that of APE (Table 2).

Antiproliferative activity

Deregulation of cell proliferation, together with suppressed apoptosis, is a minimal, common platform for all cancer evolution and progression [66]. Uncontrolled cell division is the primary key in the progression of cancer tumors. In order to evaluate *A. precatorius* as a potential therapy for cancer, different extracts were assayed against a panel of four human cancer cell lines: Colo-205, Y79, HepG2 and SupT1. The antiproliferative effects were quantified in terms of cytotoxicity (percentage inhibition) and IC_{50} values were also determined with lower IC_{50} values indicating a higher antiproliferative activity. Out of four extracts tested, only APA and APE demonstrated significantly effective antiproliferative activities in a concentration dependent manner, whereas APH and APW extracts did not inhibit the proliferation of tumor cells, thus indicating their non - cytotoxic properties. In fact, APA was by far the strongest inhibitor of tumor cell proliferation with above 85% growth inhibition of all tested cell lines, while as APE was slightly weaker inhibitor of growth of cell lines than APA (Figure 7a-7d; Table 3). APA exerted the highest cytotoxicity at a concentration of 200 μg/mL against Colo-205 (92.25 ± 2.05%) and Y79 (92.80 ± 6.34%) cells with an IC_{50} value of 18.91 ± 1.06 and 26.74 ± 1.34 μg/mL respectively. Whereas APE inhibits growth up to 68.33 ± 1.41% and 66.40 ± 5.44% against Colo-205 and Y79 cells respectively at the same concentration of 200 μg/mL with higher IC_{50} values of 29.57 ± 2.02 and 35.94 ± 2.10 μg/mL respectively. In addition, APA also showed significant inhibition activity on other two human cancer cell lines HepG2 (88.52 ± 3.04%) and SupT1 (94.12 ± 3.34%) at 200 μg/mL with the

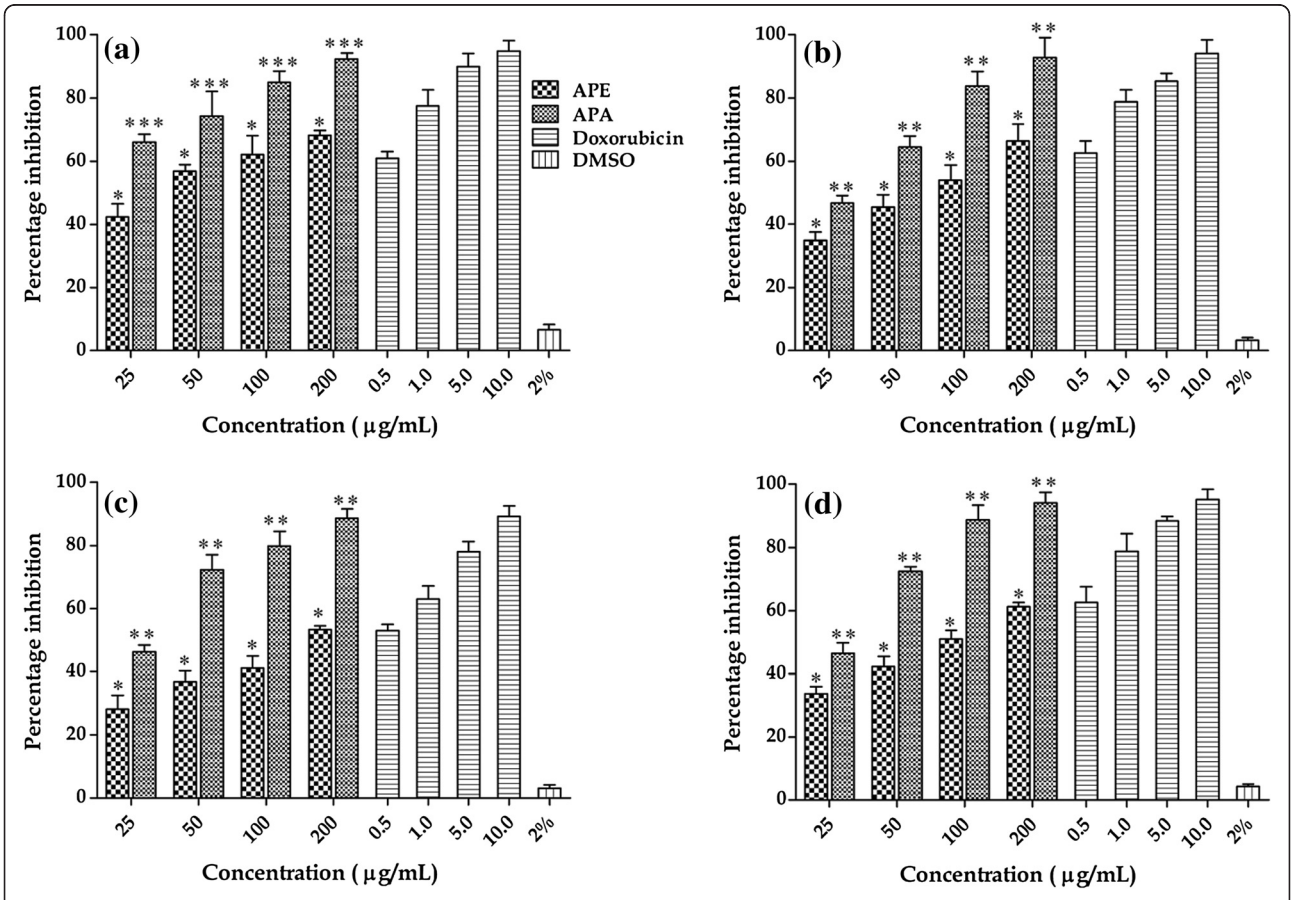

Figure 7 Antiproliferative activity of *A. precatorius* leaf extracts (APA and APE) against: (a) COLO-205 (b) Y79 (c) HepG2 and (d) SupT1 cell lines. Significant p value (*** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ were obtained by Student's *t* test analysis. Composite treatments were compared using one-way analysis of variances (ANOVA) and probability values were found to be equal to or less than 0.05 for all the four cell lines.

Table 3 Percentage inhibition of cancer cell proliferation and IC_{50} values

Sample	Type of cell line							
	Colo – 205		Y79		HepG2		SupT1	
	%age Inhibition	IC_{50} Value	% age Inhibition	IC_{50} Value	% age Inhibition	IC_{50} Value	% age Inhibition	IC_{50} Value
APE (200 μg/mL)	68.33 ± 1.41*	29.57 ± 2.02	66.40 ± 5.44*	35.94 ± 2.10	53.33 ± 1.21*	44.31 ± 3.07	61.34 ± 1.32*	37.00 ± 2.38
APA (200 μg/mL)	92.25 ± 2.05***	18.91 ± 1.06	92.80 ± 6.34*	26.74 ± 1.34	88.52 ± 3.04**	27.03 ± 1.03	94.12 ± 3.34**	26.89 ± 3.24
Doxorubicin (Standard) 10 μg/mL	94.81 ± 3.42	0.41 ± 0.08	94.20 ± 4.11	0.39 ± 0.10	89.18 ± 3.42	0.47 ± 0.08	95.20 ± 3.11	0.39 ± 0.11
DMSO 2% (Solvent control)	6.67 ± 1.67	–	3.33 ± 0.78	–	3.13 ± 1.01	–	4.33 ± 0.78	–

Values were the means of four replicates ± standard deviation (SD). Significant p value (*** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ were obtained by Student's *t* test analysis. Composite treatments were compared using one-way analysis of variances (ANOVA) and probability values were found to be equal to or less than 0.05 for all the four cell lines.

IC_{50} value 27.03 ± 1.03 and 26.89 ± 3.24 μg/mL respectively. The APE showed moderate ability to inhibit cancer cell growth in a concentration-dependent manner with IC_{50} value of 44.31 ± 3.07 μg/ml for HepG2 and 37.00 ± 2.38 μg/ml for SupT1.

The criterion for cytotoxicity for the crude extracts, as established by the National Cancer Institute (NCI), is an IC_{50} value lower than 30 μg/mL [67]. In this study, the APA crude extract displays an IC_{50} values less than 30 μg/mL in all the four tested cell lines, which established APA as more active extract than APE. Comparatively, Doxorubicin, an anticancer drug used in this study as a positive control, demonstrated IC_{50} values in the tumor cell lines ranging from 0.39-0.47 μg/mL. Although, the activity of APA and APE are weak in comparison to the standard drug, which could be due to the crude nature of the extracts and can be further enhanced by the purification.

Assessment of drug toxicity

The cytotoxic effect of APA and APE was studied in murine peritoneal macrophages using the MTT method. The results clearly indicated that plant extracts were virtually nontoxic and had no inhibitory effect on cell proliferation in peritoneal macrophages and there was minimal reduction in cell survivability (Figure 8). The percentage viability was above 90% at the highest concentration of 200 μg/mL. This suggests that APA and APE extracts did not show any kind of toxic effect on the normal cells. Therefore, the cytotoxicity of the active extracts was found to be highly selective against the cancer cell lines used.

During the past decade, both *in vivo* and *in vitro* studies have suggested that natural antioxidants such as phenolics, carotenoids, tocotrienols exhibit antitumor activities by inhibiting the growth and proliferation of many cancer cells such as breast, lung and liver cancer cells [68-71]. These observations and reports (with regard to the cytotoxicity of the plant extracts) indicate that there are great differences among the antiproliferative activity of the same plant species, depending on plant parts and extraction solvents used. Furthermore, the different cell lines vary in their sensitivity to the same plant extract.

Conclusion

This work has gathered experimental evidence that *A. precatorius* leaf extracts contained substantial amount of polyphenols and flavonoids and exhibited significant antioxidant activity by effectively scavenging various free radicals. Additionally, it has been demonstrated that the *A. precatorius* leaf extracts (APA and APE) are potential antiproliferative agents without any toxic effect on normal cells. The antioxidant and antiproliferative activities might be due to the synergistic actions of bioactive compounds present in them. Therefore, the plant has promising compounds to be tested as potential antioxidant drugs for treatment of diseases resulting from oxidative stress. However, these findings warrant extensive studies on chemical profiles and mechanistic action of antiproliferative and antioxidant activities. The study will be helpful to understand this important herbal medicine and further studies are underway in our laboratory.

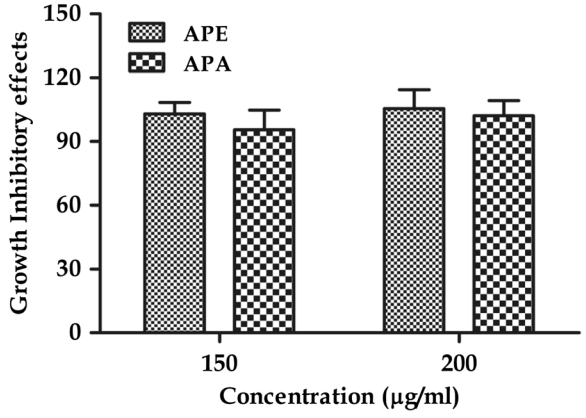

Figure 8 Growth inhibitory effects of *A. precatorius* leaf extract (APA and APE) on peritoneal macrophages. Cell viability was determined by MTT assay as described in material and methods section.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MZG conceived the study, carried out all the experimentation, acquisition and analysis of data and drafting of the manuscript. FA was involved in cell culturing, MTT assay. AKK provided technical support and advice in cytotoxic studies. IAQ helped in drafting and revision of manuscript. IAG conceived, designed, supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgement
The authors would like to thank Central Research Institute of Unani Medicine, Hyderabad Andhra Pradesh for providing the plant material. Authors gratefully acknowledge the help of S. Chandrasekaran, Research Scholar, Dept. of Animal Sciences, UoH in animal studies. The authors; MZG and FA acknowledge the financial support in the form of Senior Research Fellowship (SRF) from Council of Scientific and Industrial Research (CSIR) and University Grants Commission (UGC), New Delhi, India respectively. Authors are also thankful to DBT-CREBB, DBT-FIST and UGC-SAP - CAS, UGC- XI plan seed money for supporting infra structural facilities of Department of Plant Sciences and School of Life Sciences.

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Received: 2 November 2012 Accepted: 21 February 2013
Published: 2 March 2013

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doi:10.1186/1472-6882-13-53
Cite this article as: Gul *et al.*: Antioxidant and antiproliferative activities of *Abrus precatorius* leaf extracts - an *in vitro* study. *BMC Complementary and Alternative Medicine* 2013 **13**:53.

Australian Journal of Crop Science AJCS
AJCS 7(12):1893-1900 (2013) ISSN:1835-2707

Differential antioxidative responses of three different rice genotypes during bacterial blight infection

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Abstract

Using three rice genotypes exhibiting different disease symptoms towards bacterial blight (BB) disease, *O. longistaminata* (BB-highly resistant), IRBB21 (BB-resistant) and Pusa Basmati 1 (BB-sensitive), we investigated the variable antioxidant profile and oxidative damages resulting from bacterial blight infection to elucidate the antioxidative protective mechanism governing differential BB resistance. Rice genotypes were grown in growth chamber and after 45 days of transplantation in pots, they were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) isolate DX133 following the leaf clipping method. Different biochemical assays were performed with leaf samples collected on 5th day after infection (5th DAI), 10th DAI and 15th DAI. The results showed that *O. longistaminata* exhibited higher level of total chlorophyll (~4.42±0.09 mg/g fw) and carotenoid (~2.74±0.04 mg/g fw) pigments throughout the studied periods of infection when compared to other genotypes. At 5th DAI, the higher production of non-enzymatic antioxidants including total phenolic content (10.82±0.01 mg GAE/g fw) and flavonoid content (18.60±0.03 mg QE/G fw) was observed in highly resistant genotype *O. longistaminata*, compared to Pusa Basmati1 and IRBB21. The activity of catalase (CAT) was increased in all the three genotypes under BB infection. However, *O. longistaminata* showed highest CAT activity on later stages of infection (15th DAI). The level of total antioxidant and ferric reducing power (FRAP) increased in the infected plants compared to controls on the onset of infection.The present study clearly demonstrates higher level of antioxidative protection in the highly resistant wild genotype *O. longistaminata* and can significantly contribute to understand the physiological mechanisms in rice conferring BB resistance.

Keywords: Antioxidant enzymes, non-enzymatic antioxidants, Radical scavenging, *Oryza sativa*.
Abbreviations: BB-bacterial blight; DPPH-1, 1-diphenyl-2-picrylhydrazyl; FRAP-ferric reducing antioxidant potential; FW-fresh weight; GAE-gallic acid equivalents; GPX-Glutathione peroxidase; PWC-pot water holding capacity; ROS-reactive oxygen species; SWC- Soil water content; SOD-superoxide dismutase.

Introduction

Rice (*Oryza sativa* L.) is one of the most extensively cultivated food crop of the world, whose production is remain constrained by diseases of fungal, bacterial, viral and nematode origin. Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most noted and oldest recognized diseases of rice. BB is known to occur in epidemic proportions almost across the world and hampered productivity up to 80% (Singh et al., 1977). Plants encounter both biotic as well as abiotic stresses which adversely affect the growth and productivity. It also initiates a chain of anatomical, morphological, physiological, biochemical and molecular changes in plants. The chlorotic zone formed on the leaves of rice by bacterial blight lead to reduction in photosynthetic rate which inturn the yield loss in rice (Rajarajeswari and Muralidharan, 2006; Kumar et al., 2013). Plants produces singlet oxygen, perhydroxyl radical, hydroxyl radicals, hydrogen peroxide and alkoxy radical like reactive oxygen species upon encounter with biotic stresses. (Jaleel et al., 2006). The ROS generated following stresses reacts with proteins, lipids and DNA, causing oxidative damage and impairing the normal functions of cells (Wu et al., 2004). This effect of ROS is minimized in the plants by enzymatic and non-enzymatic detoxification systems and thereby protect the cells from oxidative damage. This protection is shown by a variety of antioxidant enzymes and lipid-soluble or scavenging molecules produced during different stresses (Walker et al., 1993). The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) function in detoxification of superoxide and hydrogen peroxide (H_2O_2) (Asada et al., 1994). The primary scavenger is SOD, which converts O_2 to H_2O_2. The APX, dehydro ascorbate reductase (DHAR) and GR remove the toxic product of SOD reaction, (Manivannan et al., 2008). H_2O_2 is also scavenged by CAT though the enzyme which is less efficient than APOX-GR system (Zhao

et al., 2008). Cystein, reduced glutathione and ascorbic acid are components of non–enzymatic system (Jaleel et al., 2008a). In biotic stress tolerance, such as bacterial blight, high activities of antioxidant enzymes and high content of non-enzymatic constituents are important. Superoxide radicals and H_2O_2 are also scavenge by various POX and peroxiredoxins apart from CAT through ascorbate glutathione cycle. The production of enzymatic and non-enzymatic antioxidants during stress together causes the oxidative damage to plant tissue. Among them the most important include β-carotenes, ascorbic acid, α-tocopherol, GSH and enzymes including SOD, POX, APX, CAT, PPO and GR (Blokhina et al., 2003). Free radical-induced peroxidation of lipid membranes is reported to be a reflection of stress-induced impairment at the cellular level (Sankar et al., 2007). Different rice species exhibit variation in their sensitivity towards *Xoo* infection. Based on the phenotypic response of disease symptoms after *Xoo* infection, we had classified *Oryza longistaminata* (OL), IRBB21 and Pusa Basmati-1 (PB-1) into highly resistant (HR), resistant (R) and susceptible (S), respectively. Till date, not much is known about the BB resistance of these genotypes and their association with antioxidant profiles. There are very few reports regarding the difference in the biochemical responses of these cultivars to BB stress. At present, our ability to improve BB resistance is limited due to poor understanding of the stress physiology and their adaptation processes. Therefore, the aim of the present study was, to study various antioxidant constituents actively involved during BB disease and compare the antioxidant profile in three phenotypically variable rice genotypes towards *Xoo* infection and also to examine the relationship between imposition of BB stress and induction of oxidative stress in susceptible, resistant and highly resistant genotypes of rice.

Results

Effect of BB stress on photosynthetic pigments in three rice genotypes

Bacterial infection induces several physiological changes in rice leaves at different stages of infection. Bacterial blight disease led to decrease in Chl (a + b) content in all three genotypes due to lesion formation (Fig 1). The total chlorophyll content was significantly reduced in infected plants (Table 1). The change in total chlorophyll content of PB-1 was minimal at first two stages of infection, however, this genotype showed drastic reduction on 15^{th} DAI (1.53±0.10 mg/g fw). Almost similar trend was observed in IRRB21 wherein chlorophyll content depleted progressively, whereas in OL, we found that the cholorphyll content was maintained at the same level even at 15^{th} DAI. Similar trend was observed with that of carotenoid content in all the three genotypes.

Gradual decrease in total polyphenol and flavanoid content in infected rice

Leaf phenolics are important protective components of the plant system. In the present study, the phenolic contents were also found to be enhanced significantly due to BB disease. At the initial stages of infection, the higher level of non-enzymatic antioxidants such as phenolics and flavanoids were produced in highly resistant genotype OL, compared to PB-1 and IRBB21. Infected OL substantially produced double the amount of phenolics and flavonoids in comparison to control at 5^{th} DAI. During the progression of disease, the inhibitory effect of biotic stress on phenolic content was observed in all the three genotypes (Table 2). The phenolic content reduced dramatically at the later stage of infection in all the three genotypes. The highest rate of phenolic content reduced after infection being observed in OL (from 10.82±0.01 to 4.81±0.06 mg/g fw), followed by PB-1 (from 12.23±0.06 to 4.96±0.05 mg/g fw) and IRBB21 (from 11.28±0.05 to 4.31±0.04 mg/g fw). The flavonoid accumulations in infected plants were increased during the earlier stages of infection and subsequently declined at the later stages, thus flavonoids might play a protective role under stress conditions. Like phenolics, flavonoid content in OL showed highest production at the 5^{th} DAI (18.6±0.03 mg QE/g fw) in camparison to control plants (8.7±0.05 mg QE/g fw) and started to decrease and reached to (7.0±0.06 mg QE/g fw) at 15^{th} DAI. The other two genotypes, PB-1 and IRBB21 showed similar pattern (Table 2). The change in flavonoid content in stressed PB-1 was found 22.2±0.02 to 8.73±0.04 mg QE/g fw and in IRBB21, the reduction was from 19.1 ± 0.05 to 8.98±0.05 mg QE/g fw.

Total antioxidant activity shoot up during infection

Earlier studies have shown that stress in plants induce the oxidative burst followed by the activation of antioxidative system (Saleh et al., 2009). We conducted our studies on the antioxidative responses of rice genotypes to assess the redox potentials under BB stress condition. Our results showed that the total antioxidative capacity of three rice genotypes sharply increased during initial stage of stress period. The effect of BB stress on the activities of total antioxidants participating in the scavenging of ROS which showed gradual decrease in all the three genotypes is shown in Table 2. The level of total antioxidant and ferric reducing power increased in the infected plants in comparison to the control plants on the onset of infection. The maximum antioxidant level was observed in treated OL (16.19±0.02 mg AAE/g fw) compared to control (9.48 ±0.02 mg AAE/g fw). Similar patterns of ferric reducing power (increased activity during first stage followed by decreased activity at the later stages) were also observed for all the three genotypes (Table 2). OL was found to be more active as compared to other genotypes with maximum level of ferric reducing power value on 5^{th} DAI (10.09±0.02 mg AAE/g fw), while there was less significant change in the FRAP activity in other two genotypes, PB-1 and IRBB21, 9.23 ±0.02 and 10.48±0.01 mg AAE/g fw, respectively on the same day in comparison to control plants (8.53±0.06 and 9.16±0.01 mg AAE/g fw for PB-1 and IRBB21, respectively).

BB stress enhanced the accumulation of total free amino acid

The free amino acid pool did not change very much in control samples during the entire period of investigation,

Table 1. Bacterial blight stress induced changes in chlorophyll and carotenoid contents (mg/g fw) of three rice genotypes.

Sample	Chl (a+b)		Carotenoid	
	Untreated	Treated	Untreated	Treated
5th Day after Infection (5th DAI)				
OL	6.06±0.02	5.24±0.07*	3.09±0.07	3.03±0.03
PB-1	6.47±0.03	4.99±0.07*	3.08±0.05	2.99±0.04*
IRBB21	6.43±0.02	4.93±0.10*	3.08±0.02	3.00±0.04
10th Day after Infection (10th DAI)				
OL	5.43±0.08	5.00±0.09*	3.06±0.06	2.98±0.05
PB-1	5.66±0.08	4.81±0.08*	3.07±0.06	2.98±0.04
IRBB21	4.99±0.05	4.02±0.07*	3.03±0.08	2.65±0.03*
15th Day after Infection (15th DAI)				
OL	5.37±0.08	3.07±0.13*	3.04±0.04	2.22±0.06*
PB-1	5.62±0.08	1.53±0.10*	3.05±0.04	1.07±0.06*
IRBB21	5.83±0.08	2.21±0.07*	3.01±0.05	1.38±0.07*

Values are presented as mean of triplicate determinations ± standard deviation. (* = p≤0.001; ANOVA one way variance, Student's t test).

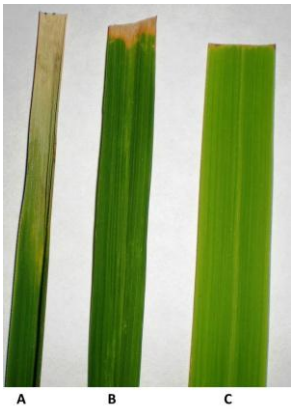

Fig 1. The effect of *Xoo* inoculation on the leaf blades of three rice genotypes (A) PB1; (B) IRBB21 and (C) *O. longistaminata* on 10th day after inoculation. Rice seedlings were inoculated with *Xoo* isolate DX133 by leaf clipping method.

while in stress induced plants, total free amino acid content increased with increasing time period of stress in all three genotypes particularly at 15th day of infection **(**Table 2). Mean TFA (total free amino acid) levels ranged from 21.82 to 29.21 µg/g fw at 15th DAI under stress conditions. The highest accumulation of the amino acid was recorded in PB-1 (29.21±0.25µg/g fw), followed by OL (26.89±0.78µg/g fw) and IRBB21 (21.82±0.48µg/g fw). During the early stages of stress, all the three genotypes evidenced the minimal accumulation of total free amino acids.

Higher DPPH free radical scavenging activityis related to resistance

Antioxidant potential of three genotypes was determined by employing 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity of leaf tissues of plants grown under different days of treatments. The leaf tissues of OL under bacterial stress condition at the 5th DAI showed a significantly higher capacity to detoxify oxygen radicals (51.2±0.01%) over the antioxidant potential of the leaves of non-treated (18.06±0.01%). In other two genotypes, PB-1 and IRBB21, the activity was similar throughout the experimental period which shows that only OL had evident tendency to detoxify oxygen radicals to counter act the stress conditions (Fig 2).

Comparative scavenging activity of CAT and GPX

Catalase activity (CAT) was measured in three rice genotypes which steadily increased during infection period and highest activity was observed at later stage, *i.e*; 15th DAI (Fig 3). CAT activity increased significantly upon exposure to BB infection in all three genotypes. The highest activity was observed in IRBB21 at all time points followed by OL and PB-1 respectively. The peroxidase activity showed significant increase with regard to biotic stress in comparison to control plants (Fig 2). Peroxidase activity significantly increased with increasing stress period in OL at 10th DAI, however, slight decrease in activity was observed upon increasing exposure to bacterial stress. The resistant IRBB21 also showed an increased peroxidase activity with increasing stress. Similar results were observed in PB-21 genotype where activity started to increase from initial phase of the infection.

Discussion

BB is one of the most important biotic stresses limiting crop productivity. Oxidative stress is regarded as a major detrimental factor in plants exposed to large range of biotic and abiotic stresses. In order to improve stress tolerance in crops and specially BB tolerance, it is essential to identify salient components of antioxidative defence system which are induced under BB stress and may have role in conferring BB tolerance. In our experiments, a substantive increase in phenolics and flavonoids was observed in the leaves of highly resistant; OL compared to resistant IRBB21 and suceptible PB1. The induced accumulation of flavonoids and phenolics during infection might have occurred to protect the plant from further invasion and growth of the pathogens population. Increase in production of flavonoids after invasion by pathogens or pests is a well known phenomenon

Table 2. Effect of bacterial blight on total polyphenol content (TPC), total flavonoid content (TFC), total antioxidant capacity (TAC), ferric reducing power (FRAP) and free amino acid content (FAA) on three rice genotypes.

Sample	TPC[a]		TFC[b]		TAC[c]		FRAP[c]		FAA[d]	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
5th Day after Infection (5th DAI)										
OL	5.52±0.03	10.82±0.01†	8.70±0.05	18.60±0.03†	9.48±0.02	16.19±0.02†	6.47±0.05	10.09±0.02†	6.43±0.05	8.30±0.42†
PB-1	10.14±0.01	12.23±0.06†	19.32±0.07	22.20±0.02†	13.13±0.02	17.05±0.03†	8.53±0.06	9.22±0.02†	2.82±0.04	4.14±0.52†
IRBB21	9.75±0.03	11.28±0.05†	16.40±0.01	19.10±0.05	15.59±0.03	15.25±0.05	9.16±0.01	10.48±0.01†	7.92±0.02	9.54±0.34†
10th Day after Infection (5th DAI)										
OL	7.68±0.03	10.47±0.03†	11.90±0.09	18.03±0.03†	11.90±0.02	17.15±0.01†	8.32±0.05	9.75±0.06†	2.31±0.02	6.54±0.11†
PB-1	9.40±0.02	11.11±0.06†	14.28±0.03	17.73±0.05†	15.27±0.05	16.03±0.04	7.74±0.04	10.38±0.01†	4.42±0.01	11.34±0.98†
IRBB21	10.07±0.05	12.80±0.04†	16.28±0.02	19.85±0.06	14.85±0.01	15.18±0.02	8.45±0.05	9.83±0.06†	6.34±0.08	13.10±0.36
15th Day after Infection (15th DAI)										
OL	2.77±0.02	4.81±0.06†	5.45±0.03	7.0±0.06†	5.08±0.02	10.28±0.04†	5.04±0.01	6.02±0.02†	4.12±0.02	26.89±0.78†
PB-1	4.30±0.07	4.96±0.05†	7.40±0.08	8.73±0.04†	6.53±0.05	10.35±0.02†	5.64±0.03	5.93±0.05†	4.35±0.02	29.21±0.25†
IRBB21	4.94±0.04	4.31±0.02†	7.78±0.09	8.98±0.05†	8.91±0.04	9.03±0.02†	5.83±0.06	6.10±0.04†	8.65±0.06	21.82±0.48†

a: gallic acid; b: quercetin; c & d: ascrobic acid equivalents mg/g fw plant material respectively; Values are presented as mean of triplicate determinations ± standard deviation. († = p≤0.01; ANOVA one way variance, Bartlett's test).

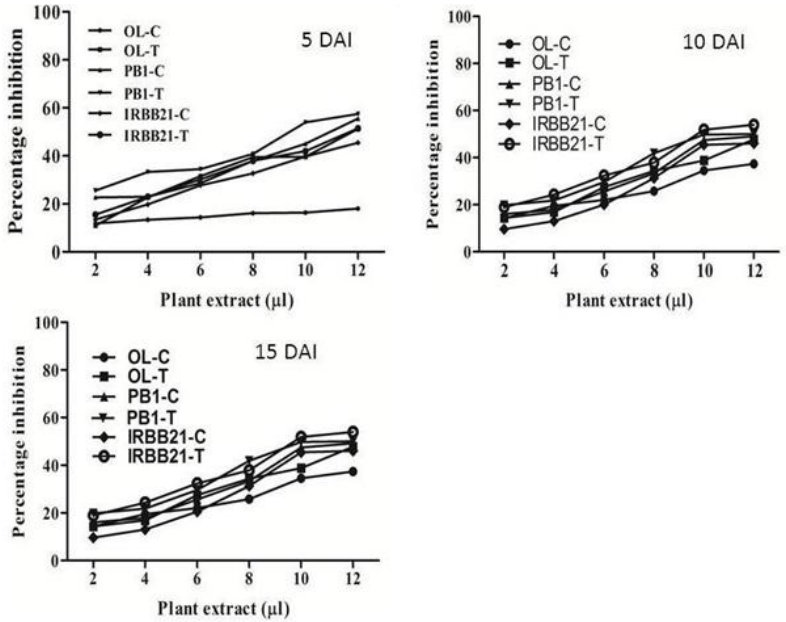

Fig 2. DPPH scavenging activity of extracts of different genotypes of *Oryza.* The leaf tissues of OL under bacterial stress condition at the 5th DAI showed a significantly higher capacity to detoxify oxygen radicals'when compared to IRBB21 and PB1.

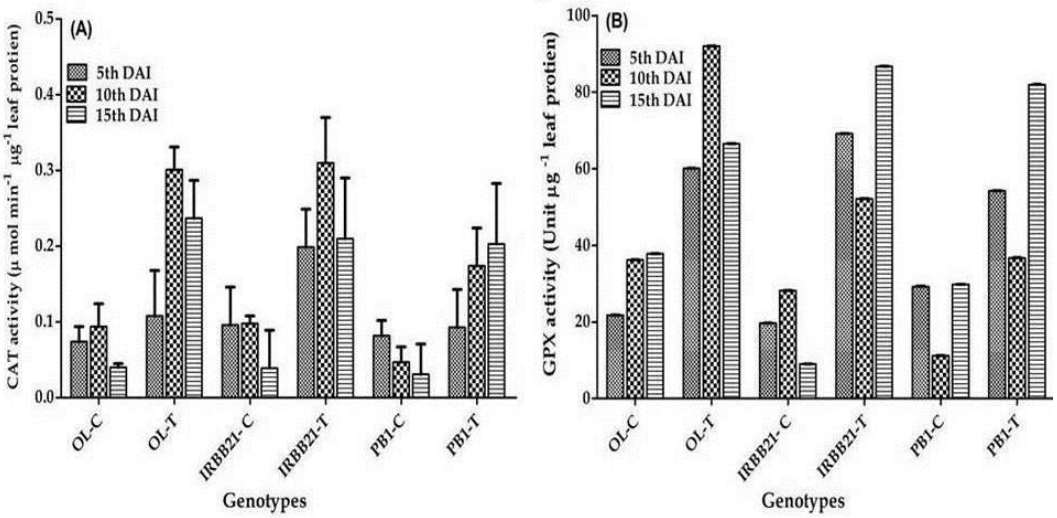

Fig 3. Activity of antioxidative enzymes, CAT (A) expressed as Units μmol min^{-1} $μg^{-1}$ protein and GPX (B) as Units $μg^{-1}$ protein. Values are mean ± SD for three observations (n=3). The highest activity was observed in IRBB21 at all time points followed by OL and PB-1 respectively.

(Gallet et al., 2004). In cotton leaves, epidermal anthocyanin production is an indicator of resistance to bacterial blight *Xanthomonas campestris* pv. *malvacearum* (Kangatharalingam et al., 2002). Higher levels of H_2O_2 have been detected under various stress conditions (Smirnoff, 1993; Menconi et al., 1995). H_2O_2 also predisposes the chloroplasts to photodisintegration of thylakoid membrane causing the observed loss of chlorophyll as in pea (Angelopoulos et al., 1996; Moran et al., 1994) and *Arabidopsis* (Jung et al., 2004). Therefore, resistant genotype OL might be able to hold the integrity of thylakoid membrane and hence, have maintained its level of chlorophyll pigments during the progression of the disease. Low molecular weight antioxidants like carotenoids effectively scavenge the harmful radicals and stabilize lipid oxidation (Strzalka et al., 2003). The carotenoid level was found to be higher in highly resistant OL during disease progression at all the three time periods. There was severe reduction of carotenoid content in susceptible PB-1 and resistant IRBB21 during the same time periods. Antioxidants and metabolites increase under various stresses, with their comparatively higher activity in stress-resistant cultivars, suggesting that higher antioxidant activity imparts tolerance (Polle, 1997; Sairam et al., 2000). The higher activities of antioxidants were observed in highly resistant OL which is in support of earlier studies, whereas the susceptible genotype maintained a lower protection from oxidants. Moreover, BB sensitive genotype has a lower antioxidant capacity than do the resistant genotypes. The reducing power ability of the antioxidants enable them to scavenge the DPPH radical by donating hydrogen and forming reduced DPPH**(** Duh, 1998; Sun et al., 1999). In our study, resistant OL has shown maximum scavenging activity during infection compared to rest of the two genotypes. The activation and increment of DPPH-radical scavenging activity also appeared to be correlated with BB induced tolerance in rice genotypes. The ability to donate a hydrogen atom to free anionic radicals so as to terminate the free radical reactions is measured by the reducing power of a sample. Increased oxidative damages might have led to the induction of reducing power ability, maximum being in OL, thus enabling their sustenance under BB stress conditions. The induced reducing power, due to BB stress, donating free H^+ ions, was a means to neutralize the excess free radicals, which are mostly negative ions, thus trying to restore overall homeostasis and overcome metabolic imbalance under BB infection. The total free amino acid content increased in all genotypes during disease progression. The highest free amino acid accumulation was found in the case of PB-1 (sensitive to BB), followed by OL and IRBB21. Significant differences was found between highly resistant OL and susceptible PB-1. The up-regulation of free amino acids during stress helps in renaturing or removal of denatured proteins which increased under stress conditions. H_2O_2 also functions as an intercellular signal to either stimulate or deactivate certain antioxidative enzymes like SOD, CAT and GPX (Lee et al., 2001). The induction of CAT activity was far greater in IRBB21 and OL compared to PB1. The observed less increase in CAT activity in BB susceptible PB1 could diminish the ability to scavenge harmful radicals favouring accumulation of oxygen radical species, which could cause membrane damages. The GPX activity was higher in OL and IRBB21 compared to PB1. The higher induction of GPX in highly resistant and resistant genotypes may enable these genotypes to limit the accumulation of harmful radicals.

Materials and methods

Plant material, growth conditions and stress treatments

Three rice genotypes; *Orzya longistaminata* (OL), near isogenic line (IRBB21) and Pusa Basmati 1 (PB-1) were grown in a growth chamber under 28ºC/22ºC, relative humidity of 85%, and photoperiod of 12 hrs. The germinated seeds were uniformly watered and fertilized with a half-strength Hoagland nutrient solution. After 30 days, healthy seedlings were transplanted to 20 Ltrs earthen pots. Six pots (3 control; 3 treated) were kept for each genotype. The pots were filled with a mixture of clay and peat (1:1; v/v). After 45 days of transplantation, all plants were challenged with DX133 (Indian virulent *Xoo* isolate) strain which was obtained from Department of Plant Pathology, Directorate of Rice Research, Hyderabad, India and cultured in modified Wakimoto's medium. The bacteria were then scraped and suspended in sterile distilled water and the concentration was adjusted to 0.1 - 0.2 OD ($1x10^8$-$1x10^9$ CFU/ml). Using this bacterial suspension, 5-6 uppermost leaves of plants at the

booting stage were inoculated following the leaf clipping method (Kauffman et al., 1973). The control plants were mock inoculated by clipping with scissors dipped in sterile water. PB-1 was used as a susceptible check and OL was included as a resistant check for the *Xoo*. Soil water content (SWC) was kept at 100% pot water holding capacity (PWC) and periodically measured (gravimetrically) in different points of the pot to check the homogeneity of moisture content in soil.

Sample collection and preparation

Leaf sample were collected on different time points such as 5^{th} day after infection (5^{th} DAI), 10^{th} DAI and 15^{th} DAI. The leaf samples were ground with 80% ethanol to fine pulp. The homogenates were centrifuged at 12000 g for 20 min and supernatants were collected and used for assays. For enzymatic assays, fresh leaves were homogenized (w/v) in 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone at 4ºC and centrifuged at 15000 g for 15 min at 4ºC. Supernatants were used to determine different enzymatic activities.

Extraction and estimation of chlorophyll content

On three different days of experiment, leaf discs were taken from five fully expanded leaves of comparable physiological age. Leaf sections were ground in 80% acetone and the total chlorophyll concentration was determined (Arnon et al., 1949).

Determination of non-enzymatic antioxidants

Estimation of total phenolic content

The total phenolic content of the rice leaf extracts were estimated by the Folin- Ciocalteu method (Gul et al., 2011). In brief, 20 µl of the extracts were mixed with 180 µl of distilled water and after 5 min, 100 µl of Folin- Ciocalteu reagent was added. After 10 min of incubation, 300 µl of 20% sodium carbonate was added, thoroughly vortexed and the final volume was made to 1 ml. The reaction mixture was further incubated for 90 min in dark and thereafter the absorbance was measured at 765 nm using UV-Visible Spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of fresh weight calculated from standard graph of gallic acid.

Estimation of total flavonoid content

A slightly modified version of the spectrophotometric method (Barreira et al., 2008) was used to determine the flavonoid content of samples. Plant extracts (20 µl each) were taken and diluted with 480 µl of double distilled water, followed by addition of 30 µl of 5% sodium nitrite. The solution was mixed well and kept at room temperature for 10 min. To this solution, 60 µl of 10% aluminium chloride was added. After 5 min, 350 µl of 1 M sodium hydroxide was added. The final volume was made to 1 ml with distilled water Samples were further incubated for 30 min at room temperature and subsequently, the absorbance was recorded at 510 nm. Flavonoid content was determined as mg quercetin equivalents (mg QE/g) of fresh weight using a standard curve of quercetin and the values were expressed as means of triplicate analysis.

Determination of total antioxidant potential

The antioxidant activity of leaf extracts were evaluated as per the protocol based on the reduction of Mo (VI) – Mo (V) by the extract, following formation of a green phosphate/Mo (V) complex at acidic pH (Prieto et al., 1999). Aliquots of the leaf samples were mixed properly with 1 ml of the reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4 and 4 mM $(NH_4)6Mo_7O_{24}$). The tubes were incubated in dry thermal blocks at 90ºC for 90 min. Further, absorbance of sample solutions were measured at 695 nm against methanol (blank). Total antioxidant activity was expressed as mg of ascorbic acid equivalents (mg AAE/g) of fw.

Determination of reducing activity by FRAP assay

The capability to reduce ferric ions was measured using the method reported by Oyaizu et al., 1986. About 10 µl of leaf sample were mixed with 0.25 ml of distilled water and 0.25 ml of 1% potassium ferric cyanide and reaction mixture was incubated in a water bath at 50ºC for 30 min. 0.25 ml of 10% tricholoro acetic acid was added and centrifuged at 8000 g for 10 min. 0.5 ml of supernatant were taken to which 100 µl of ferric chloride was added and volume was made to 1 ml. The increase in absorbance at 700 nm was measured after 30 min. The antioxidant capacity of the extract was expressed as mg ascorbic equivalents per gram of leaf material based on fresh weight. Ascorbic acid was used for standardization.

Measurement of the DPPH radical scavenging activity

The DPPH free radical scavenging activity was carried out by employing the standard protocol given by Braca et al., 2002. Different dilutions of the extract were incubated with 1 ml of DPPH solution (0.004% w/v) in dark for 45 min. The decrease in absorbance due to scavenging DPPH radicals by extract was determined at 517 nm using a spectrophotometer.

Estimation of total free amino acids

The total amino acids were quantified by the ninhydrin method (Moore et al., 1968). In 1 ml of ninhydrin solution, 0.1 ml of leaf extracts were mixed and volumes were made upto 2 ml with distilled water. The tubes were incubated in boiling water bath for 20 min followed by addition of 5 ml diluents solvents (1:1 ratio of 1-propanol and water). After 15 min, the intensity of the colour formed was read at 570 nm against a blank. Leucine was used for standardization.

Evaluation of enzymatic antioxidants

Determination of Catalase Activity

CAT activity was determined by monitoring the disappearance of H_2O_2 at 240 nm ($\varepsilon = 40 mM^{-}1\ cm^{-}1$) (Aebi et al., 1984). The reaction mixture comprised 50 mM sodium phosphate buffer (pH 7.0), 20 mM H_2O_2 and 50 µg of plant extract. Absorbance was taken at 470 nm. The molar extinction coefficient of hydrogen peroxide at 240 nm was taken as 0.04 sq. cm/µ mole. Enzyme activity was expressed as µmoles of hydrogen peroxide degraded/min g fw.

Determination of Guaiacol peroxidase activity

Glutathione peroxidase (GPX) activity was determined as per the standard protocol (Hemeda et al., 1990).

A reaction mixture containing 1% Guaiacol (v/v) 0.3% hydrogen peroxide and 80 ml of 50 mM phosphate buffer (pH 6.6) to which enzyme extracts (50 µl each) were added to a final volume of 3 ml. The extinction coefficient of guaiacol was considered 26.6/mM cm. Absorbance was taken at 470 nm. Enzyme activity was expressed as µmoles of guaiacol oxidized/min g fw.

Statistical analyses

The percentage inhibitions of free radicals were calculated using the formula:
Percentage Inhibition = $A_{control}$ - A_{sample} / $A_{control}$ × 100.
All the experimental data values were expressed as means of three measurements ± standard deviation (SD). Statistical differences between the samples were evaluated using ANOVA one way variance; treatments were compared using Bartlett's test and considered significantly different where *p* values were found to be equal to or less than 0.05. Statistical tests and graphical representation of the results was done using graphpad prism 5 software.

Conclusions

BB resistant genotype accumulates lesser amounts of peroxide and free radicals. Apparently OL seems to have potentially more tolerance to BB than the other two genotypes. The higher induction of major antioxidants, especially flavonoids and phenolics, and no-decrease in the activity of CAT in OL, probably enabled this genotype to limit H_2O_2 accumulation, thus warding off the irreversible damaging effects at the cellular and subcellular levels. IRBB21 behaved some what similarly to PB1 with respect to antioxidative nature suggesting that some other mechanism govern the resistance conferred by *Xa21* gene present in IRBB21. Although a short-term effect of BB stress on seedlings in the green house conditions may not always reflect exactly the behaviour of the plants exposed to a long term BB stress under field conditions, findings of such experiments could help to assess the plant genotypes for survival under BB stress conditions in the early stage of the life cycle. Comparison of biochemical responses, as undertaken in this study, might be useful to understand the mechanism of BB stress management, selection and development of rice genotypes resistant to BB stress.

Acknowledgements

This work has been supported by funding from UGC project F No. 41-559/2012 (SR). The authors are thankful to DBT-CREBB, DBT-FIST and UGC-SAP-CAS, UGC- XI plan seed money for supporting infra structural facilities. The authors (MZG and WB) acknowledge the financial support from CSIR, New Delhi, in the form of Senior Research fellowship.

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