Bioactivities of *Polyalthia korintii* (Dunal) Benth. & Hook.F. with special emphasis towards its antiproliferative potential against leukemia and colon cancer cell lines

Thesis submitted to
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In
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By

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DECLARATION

I hereby declare that the work presented in this Thesis entitled '**Bioactivities of *Polyalthia korintii* (Dunal) Benth. & Hook. F. with special emphasis towards its antiproliferative potential against leukemia and colon cancer cell lines**' submitted to the University of Calicut, as partial fulfillment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Biotechnology, is original and carried out by me under the supervision of Prof. (Dr.) K. K. Elyas, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any University.

C.U Campus Tancia Rosalin
Date:

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Tancia Rosalin

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ABBREVIATIONS

µg	: Microgram
µm	: Micometer
µM	: Micomolar
•OH	: Hydroxyl radical
AAE	: Ascorbic acid equivalent
ABL Kinase	: Abelson Kinase
ABTS	: 2, 2-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid)
ACTN	: Acetone
ADME	: Absorption, distribution, metabolism, excretion
amu	: atomic mass unit
ANOVA	: Analysis of variance
AO – EB	: Acridine orange- ethidium bromide
AQ Sox	: Aqueous soxhlated extract
ARG	: Arginine
ASP	: Aspartic Acid
ATCC	: American Type Culture Collection
AU	: Absorbance unit
BHT	: Butylated hydroxytoluene
bp	:base pair
B-RAF	: B-rapidly accelerated fibrosarcoma
BSA	: Bovine serum albumin
C	: Celsius
CAA	: Cellular Antioxidant Activity

CDK2 : Cyclin-dependent kinase 2

CHL Sox : Chloroform soxhlated extract

DAB : 3, 3' -Diaminobenzidine

DCF : 2', 7'-dichlorofluorescein

DCFH-DA : 2′, 7′ dichlorofluorescin diacetate

DE : Diosgenin equivalents

DMEM : Dulbecco's Modified Eagle's Medium

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

DNA–MG : Deoxyribonucleic acid- Methyl Green

DPPH : 2, 2-diphenyl-1-picrylhydrazyl

EA Sox : Ethyl acetate soxhlated extract

EB : Ethidium bromide

ECM : Extracellular matrix

ED 50 : Median effective dose

EDTA : Ethylene di amine tetra acetic acid

EGFR : Epidermal growth factor receptor

FACS : Fluorescence activated cell sorting

FBS : Fetal bovine serum

FITC : Fluorescein isothiocyanate

Fluo-3AM : Fluo-3 Acetoxymethyl ester

FRAP : Ferric Reducing Antioxidant Power

FTIR : Fourier Transform Infrared Spectroscopy

g : gram

GAE : Gallic Acid Equivalent

GC-MS : Gas chromatography-Mass Spectrometery

GLN : Glutamine

GLU : Glutamic Acid

h : hour

HBSS : Hank's Balanced Salt Solution

HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hPBLs : Human peripheral blood lymphocytes

HPLC : High performance liquid chromatography

HPTLC : High performance thin layer chromatography

HRP : Horse radish peroxidase

IC : Inhibitory Concentration

ILE : Isoleucine

kDa : kilo Dalton

Kg : kilogram

LC-HR-MS : Liquid Chromatography-High Resolution-Mass Spectrometry

LSM : Lymphocyte Separation Medium

LYS : Lysine

M : Molar

m/z : Mass/charge

MeOH : Methanol

MET : Methionine

MET Sox : Methanol soxhlated extract

mg : milligram

min : minutes

MMP : mitochondrial membrane potential

ml : millilitre

mM : millimolar

MTCC : Microbial Type Culture Collection

MTT : 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

MW : molecular weight

N : Normal

NBT : Nitro Blue Tetrazolium

NCCS : National Centre for Cell Science

NIST : National Institute of Standards and Technology

nm : nanometer

nM : nanomolar

NO : Nitric oxide

OD : optical density

Pa : Probable activity

PARP : Poly (ADP-ribose) polymerase

PASS : Prediction of Activity Spectra for Substances

PBS : Phosphate buffered saline

PDB : Protein data bank

PE Sox : Petroleum ether soxhlated extract

PHE : Phenylalanine

Pi : Probable inactivity

PI : Propidium iodide

PI3K : Phosphoinositide 3-kinase

PVDF : Polyvinylidene fluoride

PVPP : Polyvinylpolypyrrolidone

QE : Quercetin Equivalent

RBC : Red Blood Cells

Rf : Retardation factor

RI : Retention Index

RNA : Ribonucleic acid

RNase : Ribonuclease

ROS : Reactive Oxygen Species

RPMI : Roswell Park Memorial Institute medium

RT : Retention Time

SD : Standard Deviation

SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SER : Serine

SF : Sub-Fraction

SMILES : Simplified Molecular-Input Line-Entry System

SPSS : Statistical Package For The Social Sciences

TAE : Tannic Acid Equivalent

TBARS : Thiobarbituric Acid-Reactive Species

TBST : Tris Buffered Saline With Tween 20

TCA : Tri-Chloro Acetic Acid

TFC : Total Flavonoid Content

THR : Threonine

TLC : Thin Layer Chromatography

TPC : Total Phenolic Content

TPTZ : 2, 4, 6- tripyridyl-s-triazine

TRP : Tryptophan

TYR : Tyrosine

UV : Ultra Violet

v/v : volume/volume

VAL : Valine

VEGFR : Vascular Endothelial Growth Factor Receptor

w/v : weight/volume

WHO : World Health Organization

µl : microliter

INTRODUCTION

The knowledge, skills, and practices to use plants to cure human diseases have been passed down from generation to generation. To date, a majority of the world depends on their own traditional medicinal plants for their medical purposes. These medications involve the use of herbs, herbal materials, herbal preparations, and finished herbal products (Facchinetti, F. et al., 2015).

The herbal material may contain various phytoconstituents, such as alkaloids, phenolics, terpenoids, flavonoids, *etc* which function alone or in combination with one another to deliver the desired therapeutic effect (Parasuraman, S. et al., 2014). Notably, many valuable drugs derived from plants in clinical medicine today were discovered through their application in traditional medicines and from their recorded ethnomedicinal uses (Li-Weber M., 2009). This is evident from WHOs investigation of the pharmacopeias and the scientific literature which revealed that the traditional knowledge of medicinal plants indeed leads to the development of new effective drugs. The study established that of the 122 compounds studied, 80% were found to be compounds originating from 94 plant species already recorded in traditional practices in relation to their pharmaceutical effects (Fabricant, D. S., & Farnsworth, N. R., 2001)

The tropical regions of the world are bulk suppliers of natural medicine to meet the global demands. India, being a major center for the diversity of medicinal plants possesses 20,000 species of higher plants of which one-third are endemic and 500 species have medicinal value. The Western Ghats of India- a biodiversity hotspot is the major repository of medicinal plants known to harbor 4,000 species of plants including the 450 threatened species (Krishnan, P. N. et al., 2011). About 50 species of Western Ghats are known to hold a very high value in the folk and herbal health forms for the therapy of different ailments (Suja 2005; Joy et al. 2001). Various phytochemicals used in modern medicine such as berberine, camptothecin, forskolin, L-Dopa, and reserpine are from *Coscinium fenestratum*, *Nothapodytes foetida*, *Coleus forskohlii*, *Mucuna pruriens*, and *Rauvolfia serpentine;* the

medicinal plants of Western Ghats (Gilani, A. H., 2005). Literature reports suggest that only 3–5% of terrestrial plants have been investigated and only a few species from the Western Ghats have been fully documented and prospected (Krishnan, P. N. et al., 2011). In general, the necessity of extensive and intensive research on medicinal plants is thus noteworthy.

Annonaceae is a diverse family of angiosperms with about 200 genera and 2500 species around the world in the tropics. *Polyalthia* is one of the notable and largest genera in the *Annonaceae* family possessing anti-inflammatory, antimalarial, antioxidant, analgesic, antifiliarial, antimicrobial, anti-AIDS, cytotoxic, hypotensive, and hepatoprotective phytochemicals (Paarakh and Khosa, 2009).

Polyalthia korintii is an Indo - Sri Lankan ethnomedicinal species reported from Western Ghats, India. It grows as a shrub or small tree, 3 - 5 m tall (Ashton, M. S. et al., 1997) and is mainly confined to peninsular India. It is commonly called "Karuvalli" and "Korintipanel" in Malayalam, "Uluvintai" in Tamil, and "Ulkenda" in Sinhala. Several researchers have reported the presence of these species in the Eastern Ghats and the southern Western Ghats (Singh Richard, P. S., & Muthukumar, S. A., 2012) of India. *P. korintii* has ethnomedicinal claims and is mostly used by tribes as these plants are highly confined and available to those areas. For instance, the Tribes of Kambakam Hills, Eastern Ghats, India uses the root bark to cure stomach ache. The village folk at Kadapa district of Andhra Pradesh, India use oral administration of root powder decoction as a good antidote for Russell Viper bite (Bijauliya, R. K. et al., 2017). The number of mature plants and the area of its distribution are too low and this species is placed under the vulnerable critically endangered category.

Despite the ethno pharmacological significance of this plant, the literature review shows that this species is scientifically unexplored and its availability possesses a limit to its exploitation. So there is an indispensable need to first set up parameters to identify and authenticate the plant specimen before any study is carried out. In this regard, the present work first aimed at standardization of the leafy

parts of this pharmacologically important endemic species from peninsular India, in reference to its pharmacognostic and physicochemical characteristics.

The study was also undertaken to decide on the best extraction method and an ideal solvent to extract the phytoconstituents from *P. korintii* leaves using different extraction methods involving various solvents, incorporating the use of high temperatures, acid, and alkali. A set of preliminary experiments to gain basic information on the nature of the various extracts that is, phytochemical composition, and investigations to assess the therapeutic potentials like antioxidant, antimicrobial, and antiproliferative abilities of the extracts were carried out.

The study mainly focused on the antiproliferative and apoptogenic potentials of the leaves of *P. korintii.* The ability of extract for selective targeting of cancer cells without significant deleterious effects on normal human cells was also studied. Bioactivity-guided fractionation, GC-MS and *in silico* prediction was employed to isolate and characterize the putative anti-cancer compounds. Additionally, essential oils, isolated from fresh leaves of *P. korintii*, were also characterized to identify the anticancer volatile constituents and evaluated for antiproliferative activity. The outline of the doctoral research work is presented in Fig.1.1.

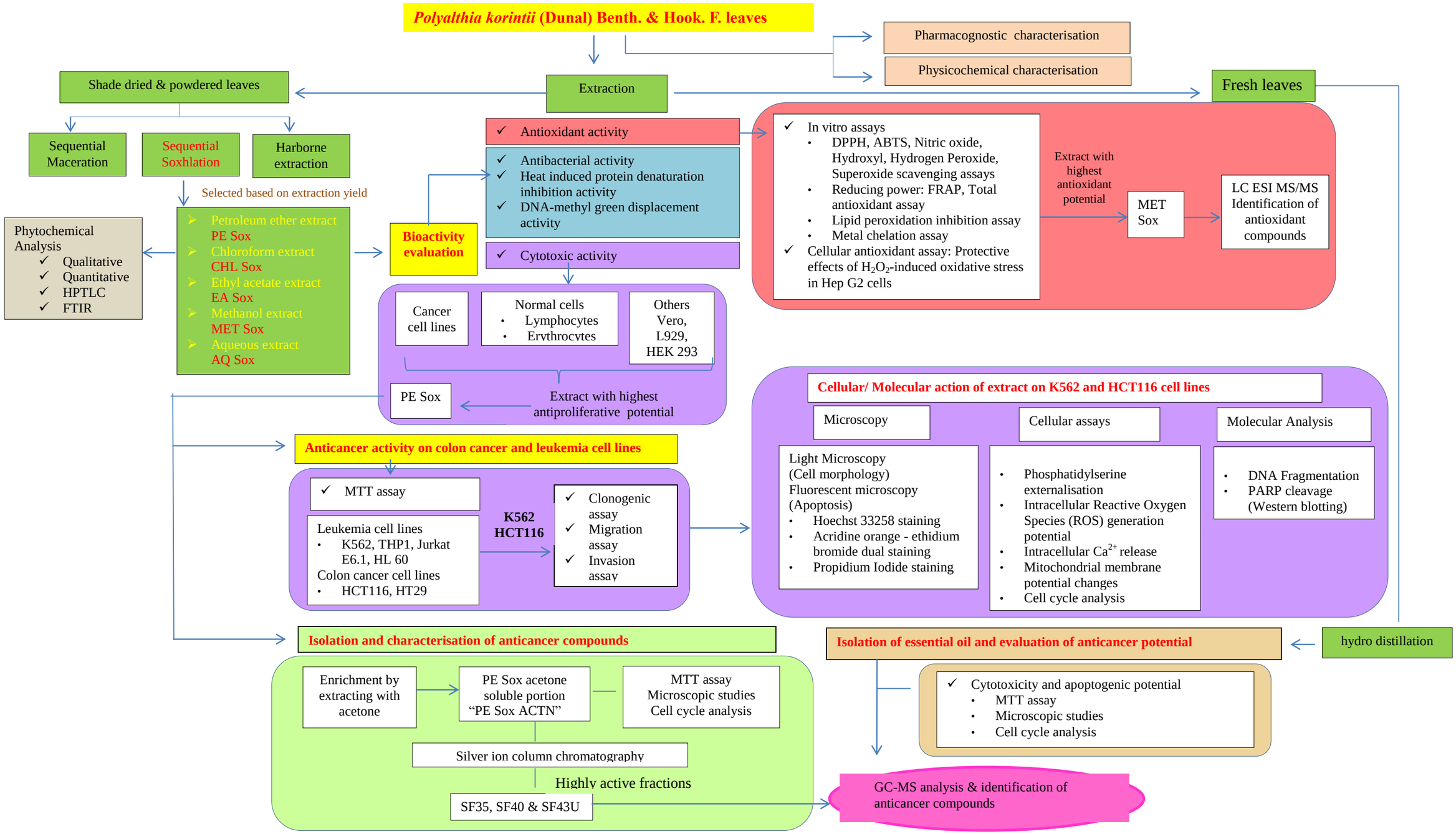

Fig 1.1 Flowchart of the doctoral research work presented in this Thesis

OBJECTIVES

1. Pharmacognostic and physicochemical characterisation of *Polyalthia korintii* leaves.
2. Screening of phytochemical constituents present in solvent extracts of *P. korintii* leaves.
3. Evaluation of the antioxidant, antimicrobial, DNA interacting ability, and cytotoxic potentials of solvent extracts of *P. korintii* leaves..
4. Assessment of the effect of the extract on cell viability, migration, invasion and colony forming capacity of K562, a chronic myelogenous leukemia cell line and HCT116, a colon cancer cell line.
5. Elucidation of the mode of action of the leaf extract against K562 and HCT116 cell lines.
6. Bioassay guided isolation and characterisation of anticancer compounds of leaves extract.
7. Evaluation of the antiproliferative potential of essential oils from *P. korintii* leaves.

REVIEW OF LITERATURE

There are several written records of the existence of sophisticated medicinal systems involving the applications of plants such as Mesopotamian (2600 BC), Egyptian medicine (2900 BC) (Borchardt, 2002; Cragg and Newman, 2013; Sneader, 2005), Traditional Chinese medicine (TCM) (Unschuld, 1986), and the Indian Ayurveda system (Patwardhan, 2005). During all that time, medicinal plants were used without any knowledge of their active constituents.

2.1 Plants in drug discovery

Drug discovery from plants was started when Friedrich Sertürner succeeded in isolating morphine, an analgesic and sleep-inducing agent from opium (Sertürner, 1817). This set off the investigations on other medicinal plants. By the 19th century, many bioactive natural products were isolated, for instance, caffeine, quinine, nicotine, atropine, codeine, cocaine, colchicine, and capsaicin (Felter and Lloyd, 1898; Corson and Crews, 2007; Kaiser, 2008; Kruse, 2007; Hosztafi, 1997; Zenk and Juenger, 2007; Sneader, 2005).

Despite the advent of newer techniques during the last decades, the usage of natural products for drug discovery is still in demand. Among the 1073 new chemical entities approved between 1981 and 2010, only 36% were purely synthetic, and more than half were derived or inspired from nature (Newman and Cragg, 2012). Many such compounds were discovered in higher plants (Kinghorn et al., 2011). Prominent examples of plant-derived drugs that have become indispensable for modern pharmacotherapy are the anti-cancer agents, e.g., paclitaxel and its derivatives (from *Taxus* species), vincristine and vinblastine (from *Catharanthus roseus*), and camptothecin and its analogs (from *Camptotheca acuminate*) (Cragg and Newman, 2013; Kinghorn et al., 2011). Other leads with notable pharmacologic activities are the cholinesterase inhibitor galanthamine (from *Galanthus nivalis* L.) (Mashkovsky and Kruglikova-Lvova, 1951), antimalarial and the anti-cancer agent artemisinin (from *Artemisia annua* L.) (Klayman et al., 1984).

The modern techniques for screening, separation, structure elucidation, and combinatorial synthesis have led to the resurrection of plant products and lead compounds as sources of new drugs. Although some natural compounds have unique therapeutic effects, their clinical efficacy is limited due to their physicochemical properties and their toxicity. But plant occurring secondary metabolites are excellent leads for drug development, modifying the chemical structure of these more promising compounds is one strategic way to increase their pharmacologic activity and selectivity (Seca, A. M., & Pinto, D. C. 2018). The list of recent plant-derived constituents and their modified derivatives already in clinical use and undergoing clinical trials as therapeutic agents are summarised in Table 2.1.

Table 2.1 Plant-derived compounds in clinical use and clinical trials

Lead/drug	Disease	Reference
Physostigmine	Dementia–Alzheimer's disease	Saklani, A., & Kutty, S. K.,2008
Artemisinin	Antimalarial	Southgate, J., 2001
Galanthamine	Alzheimer's disease	Southgate, J., 2001
Retinoic acid derivatives	Cutaneous T cell lymphoma	Southgate, J., 2001
L-Dopa	Parkinson's diseases	Southgate, J., 2001
Quinine	Antimalarial	Saklani, A., & Kutty, S. K., 2008
Tubocurarine	Neuromuscular blocking agent/anesthesia	Southgate, J., 2001
Galanthamine	Dementia–Alzheimer's	Southgate, J., 2001
Leptospermone	Antityrosinaemia	Lloyd, I., 2003
Tiotropium	Chronic obstructive pulmonary disease	Lloyd, I., 2003
Morphine	Pain	Saklani, A., & Kutty, S. K., 2008
1-Deoxynojirimycin	Type1 Gaucher disease	Saklani, A., & Kutty, S. K., 2008
Tiotropium	Chronic obstructive pulmonary disease	Saklani, A., & Kutty, S. K., 2008
Apomorphine	Parkinson's diseases	Saklani, A., & Kutty, S. K., 2008
Morphine	Post-surgical pain relief	Saklani, A., & Kutty, S. K., 2008
Camptothecin	Ovarian & small lung cancer	Saklani, A., & Kutty, S. K., 2008
Retinoic acid derivatives	Acute myelogenous leukemia	Saklani, A., & Kutty, S. K., 2008
Paclitaxel	Breast cancer	Saklani, A., & Kutty, S. K., 2008
Docetaxel	Antineoplastic (head and neck cancer) and stomach cancer	Saklani, A., & Kutty, S. K., 2008
Atropine	Antidote (Exposure to organophosphorus nerve	Saklani, A., & Kutty, S. K., 2008

Continued…

	agents)	
Physostigmine	Dementia–Parkinson's	Saklani, A., & Kutty, S. K., 2008
Camptothecin	Cervical cancer	Saklani, A., & Kutty, S. K., 2008
Delta-9-THC	For treatment of chemotherapy nausea and vomiting	Saklani, A., & Kutty, S. K., 2008
Green tea polyphenol (catechin) extract	Genital and perianal warts	Saklani, A., & Kutty, S. K., 2008
Artemisone/Artemifon	antimalarial	Robert, A. et al., 2001
Calanolides calanolide B (costatolide), dihydrocalanolide B and oxocalanolide	anti-HIV coumarin	Yu, D. et al., 2003 ; Kashman, Y.et al., 1992
Crofelemer	to treat different types of diarrhea.	Saklani, A., & Kutty, S. K., 2008
DCK 3-hydroxymethyl-4-methyl Khel lactone coumarin derivative	anti-HIV	Yu, D. et al., 2003
3,5-di-O-caffeoylquinic acid	anti- HIV and anti-Hepatitis C	Saklani, A., & Kutty, S. K., 2008
6-O-butanoyl castanospermine	Against Chronic Hepatitis C	Saklani, A., & Kutty, S. K., 2008
4-Methylumbelliferone	Against Hepatitis C and B	Saklani, A., & Kutty, S. K., 2008
Milk Thistle	for diseases of the liver and biliary tract	Saklani, A., & Kutty, S. K., 2008
3-O-(30,30 –dimethyl succinyl) betulinic acid	antiviral drug	Butler, M. S., 2005; Li, F. et al., 2003
PYN6	antibacterial	Saklani, A., & Kutty, S. K., 2008
Sho-Saiko-to (H09)	Anti-Hepatitis C	Schuppan, D., et al., 1999
Sutherlandia frutescens	anti-HIV activity	Mills, E., et al., 2005
DA-5018 (synthetic capsaicin analog)	non-narcotic analgesic	Butler, M.S., 2005
Dexanabinol	neuroprotective product	Saklani, A., & Kutty, S. K., 2008
Ganstigmine	AChE inhibitor	Saklani, A., & Kutty, S. K., 2008
IP-751 (Ajulemic acid, CT-3)	treatment of neuropathic pain	Butler, M.S., 2005; Whelan, J., 2002
LLL-2011 (Amigra)	as a nasal spray for prophylaxis of migraine	Saklani, A., & Kutty, S. K., 2008
Lobeline	to treat attention deficit hyperactivity disorder	Butler, M.S., 2005
M6G morphine 6-glucuronide	for postoperative pain	Kilpatrick, G. J., & Smith, T. W., 2005
NGX-4010 synthetic trans-capsaicin	in post-herpetic neuralgia (PHN) and neuropathic pain related to HIV-associated neuropathy	Saklani, A., & Kutty, S. K., 2008
P58 (PYM-50028, CoganeTM)	for the treatment of Parkinson's disease and AD type dementia.	Saklani, A., & Kutty, S. K., 2008
Phenserine (Phenserine tartrate, PosiphenTM) derivative of	to treat mild-to-moderate Alzheimer's disease	Butler, M.S., 2005

Continued…

phytostigmine		
RU 47213 is a pro-drug based on arecoline	for treatment of AD	Camps, P., & Munoz-Torrero, D., 2002
THC-CBD (Dronabinol/cannabidiol, GW-1000-02, Sativex1)	delta 9-tetrahydrocannabinol (THC) and cannabidiol (CBD) as an adjunctive treatment for neuropathic pain and cancer pain with multiple sclerosis	Saklani, A., & Kutty, S. K., 2008
ZT-1 a pro-drug of huperzine	Anti-Alzheimer's product	Butler, M.S., 2005
P-57 (P-57AS3) a steroid glycoside	For oral treatment of obesity	Saklani, A., & Kutty, S. K., 2008
PMI-5011 botanical extract	anti-diabetic effects	Saklani, A., & Kutty, S. K., 2008
Flavocoxid An extract	to inhibit cyclooxygenase (COX)-1, COX-2 and 5-lipooxygenase	Saklani, A., & Kutty, S. K., 2008
Grazax standardized extract of the protein allergens	for use in the treatment of grass pollen-induced rhinoconjunctivitis with or without asthma	Saklani, A., & Kutty, S. K., 2008
Paxceed (micella Paclitaxel)	An intravenous chemotherapy agent	Saklani, A., & Kutty, S. K., 2008
PMI-001	for auto-immune disease	Saklani, A., & Kutty, S. K., 2008
PMI-005	for rheumatoid arthritis	Saklani, A., & Kutty, S. K., 2008
PYN17	treatment for the symptoms of Chronic Hepatitis C	Saklani, A., & Kutty, S. K., 2008
QS-21A and QS-21B The saponins	adjuvants are an integral part of experimental vaccines	Butler, M.S., 2005
Bruceatin	anticancer	Da Rocha et al., 2001
Dimethyl xanthene-9-one-4-acetic acid	anticancer	Shah, U. et al., 2013
Flavopiridol	anticancer	Newcomb, E. W., 2004
Homoharringtone	anticancer	Da Rocha et al., 2001
Ingenyl 3-angelate	anticancer	Saklani, A., & Kutty, S. K., 2008
4-Ipomeanol	anticancer	Da Rocha et al., 2001
Kahalalide F	anticancer	Hamann, M. T.et al., 1996
Kanglaite	anticancer	Camps, P., & Munoz-Torrero, D., 2002
Meisoindigo	anticancer	Bradbury, J., 2005

2.2 Cancer

Cancer is the most feared diagnosis among patients and remains the leading cause of mortality globally. Cancer is characterized by uncontrolled cell growth and metastasis. Activation of oncogenes, the inactivation of tumour-suppressing genes, mutagenesis due to external factors, and epigenetic modifications are notable causes.

2.2.1 Cancer statistics

The pronounced nature of the disease is evident from the global cancer statistics which is predicted to be 28.4 million cases in 2040 accounting for a 47% rise from 2020. The on-going coronavirus disease pandemic has delayed progress in cancer care, screening, diagnosis, and treatment. A survey by World Health Organisation reveals that disruption of services for cancer prevention and treatment was found in 42% of countries (Ranganathan, P.et al., 2021). Missed cancer diagnoses may lead to repercussions later indicating a rise in mortality over the years to come.

There were an estimated 10 million cancer deaths and 19.3 million new cases globally in 2020. In Asia, which contributes 59.5% of the global population, an estimate of 58.3% of deaths due to cancer was expected. Europe accounts for 19.6% of the cancer mortality and 22.8% of the total cancer cases and in the Americas' 20.9% of incidence and 14.2% of cancer deaths. Globally, female breast cancer is the most commonly diagnosed cancer followed by lung, colorectal, prostate, and stomach cancers contributing to 11.7%, 11.4%, 10.0%, 7.3%, and 5.6% of total cases respectively. Whereas the leading cause of cancer deaths are lung, colorectal, liver, stomach, and female breast cancers accounting for 18.0%, 9.4%, 8.3%, 7.7%, and 6.9% of the total cancer deaths respectively. Among males, lung cancer is the most diagnosed and the leading cause of cancer, followed by prostate and colorectal cancer for incidence and liver and colorectal cancer for mortality. Among females, the most frequently occurring and the leading cause of death is breast cancer, followed by colorectal and lung cancer for incidence (Sung, H et al., 2021).

Noncommunicable diseases in India accounted for 63% of all deaths, among which 9 % were due to cancer. Population-Based Cancer Registries showed the highest incidence rate of cancer in both sexes in the North East. In men, lung, mouth, oesophagus, and stomach were the usual cancer sites whereas, in women, the breast, cervix uteri, and ovary were the most common sites. In Kerala, Population-Based Cancer Registries of Thiruvananthapuram and Kollam reported thyroid cancer as the second most common cancer (Mathur, P. et al., 2020).

The projected incidence of patients with cancer in India for the year 2020 was 679,421 (94.1 per 100,000) and 712,758 (103.6 per 100,000) respectively among males and females. It was speculated that one in 68 males (lung cancer), 1 in 29 females (breast cancer), and 1 in 9 Indians will develop cancer during their lifetime. Lung, mouth, oesophagus, stomach, and nasopharynx cancers were the most common cancers in men. In Indian metropolitan cities and the southern region, lung cancer was the leading site whereas, in the West and Central regions, mouth cancer was the leading site (Sharma, J. D et al., 2014). According to Bray F. et al. (2018), lung cancer and oral/mouth cancer were the most common cancers among males in the Indian subcontinent. Cancer of the breast and cervix uteri was the most common cancer in women. Tobacco is a contributing risk factor and almost one-third of the cancers are associated with the use of tobacco in India. Gandhi, A. K. et al., 2017 state that approximately 70% of cancers in India were potentially preventable through modifiable risk factors.

Colorectal cancer is a common cancer globally but very low incidence is reported in India. The literature reports that there are geographical diversities in incidence rates and most cases are reported from developed countries. Leukemia was once a rare disease, and its incidence and prevalence is increasing in India. Among several types of leukemia, chronic myeloid leukemias are most common followed by acute lymphoblastic leukemia, acute myeloblastic leukemia and chronic lymphocytic leukemia (Ahirwar, D. R. et al., 2018).

Estimates of 28.4 million new cancer cases are projected to occur in 2040 which will likely be paralleled by increases in mortality rates (Sung, H et al., 2021). Currently available modalities for cancer treatment involve surgical removal of tumors, radiation treatment, and chemotherapy. The efficacy of the treatments remains low owing to the high toxicity, drug resistance, and side effects of chemotherapeutics. This has vitalized researchers to hunt for more beneficial approaches and to find novel anticancer drugs.

2.2.2 Cancer treatment

Cancer therapy has been characterized by variability depending on the type and stages of cancer. Early stage diagnosis of cancer generally provides the best chance for a cure. Surgery, radiation therapy and chemotherapy alone and in combinations are the major treatments modes. Apart from treatments, delaying or blocking the process of carcinogenesis known as cancer chemoprevention by the intake of active agents has gained much attention.

Surgery is most effective only at an early stage of disease progression and surgical resections of the tumors are followed by radiotherapy and/or chemotherapy. The chemotherapeutic treatment inhibits cell proliferation and tumor multiplication, thus evading invasion and metastasis. They are classified according to their mechanism of action and include alkylating agents like Bifunctional alkylators (Cyclophosphamide, Mechlorethamine, Chlorambucil, Melphalan) and Monofunctional alkylators (Dacarbazine, Nitrosoureas, Temozolomide, Anthracyclines, Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Mitoxantrone, Valrubicin); Cytoskeletal disruptors (taxanes like Paclitaxel, Docetaxel, Abraxane, Taxotere, Epothilones); Histone deacetylase inhibitors (Vorinostat, Romidepsin); Inhibitors of topoisomerase I (Irinotecan, Topotecan); Inhibitors of topoisomerase II (Etoposide, Teniposide, Tafluposide); Kinase inhibitors (Bortezomib, Erlotinib, Gefitinib, Imatinib, Vemurafenib, Vismodegib); Nucleotide analogs and precursor analogs (Azacitidine, Azathioprine, Capecitabine, Cytarabine, Doxifluridine, Fluorouracil, Gemcitabine, Hydroxyurea, Mercaptopurine, Methotrexate); Peptide antibiotics (Bleomycin, Actinomycin); Platinum-based agents (Carboplatin, Cisplatin, Oxaliplatin); Retinoids (Tretinoin, Alitretinoin, Bexarotene); and Vinca alkaloids and derivatives (Vinblastine, Vincristine, Vindesine, Vinorelbine)

Though the combined therapy reduces the morbidity and mortality, radiation therapy and most of the chemotherapeutic agents results in damage to healthy cells. Drug resistance is another major problem with chemotherapy wherein the cancer cells that initially were susceptible to an anti-cancer drug develops resistance to the

same owing to reduced drug uptake and increased drug efflux. So there are some limitations for the traditional cancer chemotherapeutics (Amjad, M. T et al., 2021).

Investigations on cancer cells have led to significant insight into mechanisms of cell survival and cell death including apoptosis, metastasis, angiogenesis, cell signal transduction, growth factor modulation and differentiation. Researchers have designed molecular targeted therapy employing these pathways. Targeted therapy works by targeting specific genes, proteins, or the tissue environment that contributes to cancer growth and survival. Most targeted therapies are either small-molecule drugs or monoclonal antibodies.

Monoclonal antibodies are proteins designed to attach to specific targets found on cancer cells such that they are better recognised and destroyed by the immune system. Some monoclonal antibodies directly stop cancer cells from growing or cause them to self-destruct by binding to ligands or receptors to interrupt essential cancer cell processes. Whereas others carry lethal loads like toxins and radioisotope to target cancer cells (Adams, G. P., & Weiner, L. M. (2005). For example, Gemtuzumab is a CD33+ specific monoclonal antibody which in conjugation with calicheamicin is currently in use for acute myeloid leukemia treatment (Sorokin, P., 2000) and Ibritumomab tiuxetan is an anti-CD20, a 90Y metal isotope-based used in the treatment of non-Hodgkin's lymphoma (Jacobs, S. A., 2007).

Small molecule inhibitors ($\leqslant$500 Da) translocate through plasma membranes and interfere with the intracellular tyrosine kinase signalling (Tanner, J. E., 2005). For example, gefitinib and erlotinib inhibits epidermal growth factor receptor (EGFR) kinase and EGFR in non-small cell lung cancer patients, respectively. Lapatinib and sorafenib inhibits EGFR/Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2) for ERBB2-positive breast cancer and VEGFR kinase, in renal cancer (Yap, T. A., & Workman, P., 2012).

Treatment of cancers whose growth is dependent on hormonal stimulation-breast, prostate and endometrial cancers is often treated with hormone therapy

(Archumpong and Sweetland, 2015). For treatment of breast cancers, aromatase inhibitors (anastrozole, exemestane, letrozole), selective estrogen receptor modulators (tamoxifen and raloxifene), Estrogen receptor antagonists (fulvestrant and toremifene), Luteinizing hormone-releasing hormone agonists (goserelin, leuprolide, and triptorelin) are made use.

Therapeutic stem cell is also an option for cancer treatment and is yet in the experimental clinical trial. Mesenchymal stem cells are currently being used in trials. Blood and marrow stem cell transplantations are the most effective for blood cancers like leukemia, lymphoma and myeloma (Naji, A. et al., 2019).

Gene therapy strategies involves the expression of genes such as pro apoptotic genes, chemosensitizing genes, wild-type tumor suppressor genes, genes able to elicit anti-tumor immune responses, and targeted silencing of oncogenes (Pucci, C.et al., 2019). For instance, a recombinant adenovirus carrying wild-type p53 (Gendicine) induced regression in head and neck squamous cell cancer (Räty, J. K.et al., 2008).

Liposomes (Kim, H. S.et al., 2006) , nanoparticles conjugated with chemotherapeutics (Wagner, M. J. et al., 2017), immunotherapy, gene therapy strategies like pro-drug activating suicide-gene therapy, oncolytic virotherapy, anti-angiogenic gene therapy, gene therapy-based immune modulation, correction/compensation of gene defects, genetic manipulation of apoptotic and tumor invasion pathways, antisense, and RNAi strategies (Meng, Z., & Lu, M. ,2017; Subhan, M. A., & Torchilin, V. P., 2020) are other strategies being experimented for cancer treatment.

Apart from all the above mentioned strategies, natural antioxidants like vitamins, polyphenols, and plant-derived bioactive compounds are used as preventive and therapeutic drugs after recognising their anti-proliferative and proapoptotic properties. Compounds like curcumin, berberine, quercetin, and others, are examples of natural antioxidants screened in vitro and in vivo. Curcumin exerts cytotoxic effects while sparing normal cells at effective therapeutic doses. Similarly,

Berberine and quercetin, effective against different cancers is also under clinical study (Debela, D. T. et al., 2021). Thus the hunt for novel therapeutic agents from plants is still kept going.

2.3 Polyalthia

The taxonomic position of the Genus *Polyalthia* is as follows:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Magnoliidae
Order : Mognoliids
Family : Annonaceae
Tribe : Annoneae
Genus : Polyalthia

The genus *Polyalthia* belongs to the Family Annonaceae. The Greek word poly means much or many and àltheo means to cure. The genus *Polyalthia* consists of about 120 species distributed in Africa, South and South-Eastern Asia, Australia, and New Zealand. Geographically, India has 14 *Polyalthia* species (Katkar et al., 2010), China, has 17 species (Lu et al., 2009), Peninsular Malaysia has 30 species (Johnson and Murray, 1999), Australia has 4 native species (Williams, 2013), Singapore has 32 species, 27 species from Vietnam, 7 species in Sri Lanka and 8 species in Burma (Huber, 1985; Ban, 2000; Sinclair, 1955).

Being highly polyphyletic, resulting in a morphologically heterogeneous assemblage of species, and due to the absence of conspicuous synapomorphies, *Polyalthia* is known as a "default" genus and has never been monographed (Mols J B. et al., 2004; Xue B et al., 2012; Johnson, D. M., & Murray, N. A.1999). The genus *Polyalthia* is widely distributed especially in Southern India and Sri Lanka (Bakker M. E., 2000; Sinclair J., 1955).

The major *Polyalthia* species in India are *Polyalthia cerasoides* Bedd., distributed throughout India; *Polyalthia fragrans* Benth. and Hk., found in Western Ghats and *P. longifolia* (Sonn.) Thw., under cultivation. *P. longifolia* cv. *pendula*, is

commonly used in indigenous medicine (Katkar, K. V.et al., 2010). In India, *Polyalthia longifolia* is also called Ashoka or Indian mast tree. In Taiwan, it is commonly cultivated as landscape trees to avoid noise pollution (Chen, Y. C et al., 2021). The Polyalthia species found in India are summarised in Table 2.2 (Viswanathan, M. B., & Manikandan, U., 2001; Kundu, S. R., 2006).

Table 2.2 *Polyalthia* of the Indian subcontinent

Sl No.	*Polyalthia* species	Place of occurrence
1	*Polyalthia cauliflora* Hook. F. & Thoms. var. desmantha (Hook. f. & Thoms.).	India, Thailand, Singapore
2	*P. cerasoides* (Roxb.) Beddome	India, Myanmar, Thailand, Kampuchea, Vietnam, Laos
3	*P. coffeoides* (Hook. f. & Thoms.) Thw	India, Sri Lanka
4	*P. crassa* R. Parker	India
5	*P. fragrans* (Dalzell.) Beddome	India
6	*P. jenkinsii* (Hook.f. & Thoms.) Hook.f. & Thoms.	India, Malaysia, Philippines, Indo-China
7	*P. korintii* (Dunal) Thw.	India, Sri Lanka
8	*P. lateriflora* (Blume) Kurz.	India, Myanmar
9	*P. longiflora* (Sonner.) Thw.	India, Bhutan, Sri Lanka
10	*P. meghalayensis* Prakash & Mehrotra	India
11	*P. parkinsonii* Hutch.	India
12	*P. rufescens* Hook.f. & Thoms	India
13	*P. simiarum* (Hook.f. & Thoms.) Hook.f. & Thoms	India, Bhutan Bangladesh, Myanmar, SriLanka, Philippines, Singapore, Thailand, Kampuchea, Vietnam, Laos
14	*P. suberosa* (Roxb.) Thw	India, Myanmar, Sri Lanka, Indonesia, Thailand, Malaysia, Philippines, Singapore, Laos, Vietnam
15	*P. tirunelveliensis*	India

2.4 Pharmacologic activities of *Polyalthia* plants

Several literature reports on laboratory experiments and data have demonstrated that *Polyalthia* species have significant pharmacologic potential due to the presence of active compounds and the same is illustrated with a strong correlation to many of their biological activities against a variety of diseases. Polyalthia plants are made used in the Traditional Indigenous System of medicines and have great ethno pharmacological relevance.

In Indian Medicine, Polyalthia plants are used as an abortifacient, a respiratory stimulant, bitter tonic, febrifuge, a cure for scorpion stings, and high blood pressure (Paarakh, P. M., & Khosa, R. L., 2009). In south India, *Polyalthia cerasoides* are used for their edible fruits and seeds. Medicinal practitioners use the stem bark as a tonic to combat stress and pain (Ravikumar, Y. S. et al., 2008). The fruits of *Polyalthia suberosa* are used to prevent diarrhea and to treat lung issues. Leaves are used in the treatment of coughs, colds, and diarrhea. The bark is also used as a febrifuge to prevent diarrhea and dysentery; it is an analgesic and laxative. Seeds have a diuretic, sedative, and soporific action. Latex is utilized as inexpensive filler for dental cavities in the tropics (Amin, R. et al., 2022).

In Thai medicine, *Polyalthia evecta* Finet & Gagnep is used as a carminative and as a galactagogue for inducing milk secretion in breastfeeding mothers (Machana, S. et al., 2012). *Polyalthia obliqua* mostly grown on Hainan Island, China is known for its folk medicine usage against several ailments such as stomach ache, dysmenorrhea, and pharynx neurosis. P. longifolia is traditionally been used in the treatment of fever and other clinical symptoms such as skin diseases, diabetes, hypertension, and helminthiasis (Jothy, S. L.et al., 2013).

Apart from the cited ethnomedicinal uses, the scientific literature provides explicit evidence of various biologic potentials of extracts from Polyalthia species. The details of the literature reported pharmacological activity of Polyalthia plants is listed in Table 2.3.

Table 2.3 Pharmacological activity of Polyalthia plants

Plant	Parts used	Bioactivity	References
Polyalthia longifolia	Leaf	Inhibits the replication of paramyxoviruses at viral entry and budding level	Yadav P. et al., 2020
P. longifolia	Leaf	ameliorates the cadmium-induced hepatotoxicity and oxidative stress in rats	Oyeyemi, A. O.et al., 2020
P. longifolia	Twigs, Leaf	Antifungal Activity against Plant Pathogens	Nguyen, M. V.et al., 2021
P. longifolia	Leaf	inhibits fructose-mediated protein glycation and oxidation	Rai, A. K.et al., 2021
P. longifolia	Leaf	Triggers ER Stress in Prostate Cancer and induces apoptosis	Afolabi, S. O.et al., 2019
P. longifolia	Leaf	Antioxidant, radical scavenging properties, hepatoprotective	Jothy, S. L et al., 2012
P. longifolia var. angustifolia	Leaf	Hepatoprotective against paracetamol-	Jothy, S. L et al., 2012;

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		induced oxidative damage	Ghosh, G.et al., 2010
P.longifolia var. pendula	Leaf	Anti-inflammatory, antimicrobial, and antitumor activities	Faizi, S.et al., 2008;Tanna, A., Nair, R., & Chanda, S., 2009
P. longifolia	stem bark	Antitumor, antioxidant	Manjula, S. N et al., 2010
P. longifolia	Leaf	apoptosis, cell cycle arrest, mitochondrial potential depolarization	Vijayarathna, S.et al., 2017
P. longifolia	Leaf	antiplasmodial activity cytotoxicity	Kwansa-Bentum, B et al., 2019
P. longifolia	Leaf	Radioprotective activity	Jothy, S. L. et al., 2016
P. longifolia (Sonn) Thw. var. pendula	stem bark	antimalarial	Gbedema, S. Y.et al., 2015
P. longifolia var. pendula	stem	antimicrobial	Faizi, S.et al., 2003
P. longifolia	Leaf	mitigates sulfate-reducing bacteria influenced microbial corrosion	Vaithiyanathan, S et al., 2018
P. cerasoides (Roxb.)	Stem bark, seed	Antiproliferative, apoptotic, and antimutagenic	Ravikumar, Y. S et al., 2021
P. cerasoides	Stem bark	Against oxidative stress, anti-nociceptive activity	Goudarshivananavar, B. C. et al., 2015
P. cerasoides	Seed	Antiproliferative, apoptotic, and antimutagenic activity	Ravikumar, Y. S.et al., 2010
P. cerasoides	Root	antimalarial antimycobacterial activity	Kanokmedhakul, S. et al., 2007
P. cerasoides	Stem bark	Antioxidant, cytotoxic	Ravikumar, Y. S. et al., 2008
P. cerasoides	Stem bark	hepatoprotective	Padma, P. et al., 1999
P. cerasoides	Stem bark	Anti-inflammatory	Vila, L. et al., 2022
P. suberosa	Leaf	Antidepressant, analgesic, antidiarrheal	Amin, R et al., 2022
P. suberosa	Leaf	Analgesic and anti-inflammatory	Yasmen, N. et al., 2018
P. suberosa	Leaf	Anti-HIV	Li, H. Y. et al., 1993
P. suberosa	Leaf	Antioxidant	Thangnipon, W et al., 2012
P. longifolia (Sonn.) Thwaites	Unripe fruit	Reduced NO production in LPS induced cells	Wu et al., 2014
P. longifolia (Sonn.) Thwaites	Fruit	Antioxidant, hepatoprotective	Rajangam and Christina, 2013
P. longifolia (Sonn.) Thwaites	Seed	Anti-inflammatory, antioxidant	Thonangi and Akula 2018
P. longifolia (Sonn.) Thwaites	Root, leaf, stem bark	Antipyretic activity	Annan et al., 2013
P. parviflora	Leaf	Antioxidant	Liou et al., 2014
P. debilis Finet & Gagnep	Root	Antioxidant	Boonpangrak et al., 2015
P. debilis Finet & Gagnep	Root	Antiproliferative activity	Prachayasittikul et al., 2009
P. simiarum (Buch.-Ham. ex Hook. f. & Thomson)	Stem bark	Radical scavenging activity	Kabir et al., 2013
P. consanguinea Merr.	Root	Radical scavenging activity	Wang, J., et al., 2012
P. rumphii (Blume ex Hensch.) Merr	Stem	Antiproliferative activity	Wang, T., et al., 2012
P. nemoralis Aug. DC.	Leaf	Apoptosis induction	Wu. C. et al., 2017
P. bullata	Leaf	Antioxidant activity	Kamarul Zaman, M. A.et al., 2020
P. simiarum	Leaf	Cytotoxic activity	Duan, X. Y.et al.,2020

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P. petelotii	Stem, Leaf	Cytotoxic activity	Yang, C. et al., 2016
P. longifolia (Sonn.) Thwaites	Leaf	Anti-ulcer activity	Malairajan, P. et al., 2008
P. laui	Roots	Cytotoxic, antibacterial activity	Yu, Z. X. et al., 2017
P. cinnamomea	Twig	NO production inhibitory activity; α-Glucosidase inhibitory activity.	Suthiphasilp, V et al., 2020
P. evecta (Pierre) Finet & Gagnep	Leaf	Anticancer	Machana, S., Weerapreeyakul, N., & Barusrux, S., 2012
P. evecta (Pierre) Finet & Gagnep	Root	Anti Plasmodial, anti-mycobacterial	Kanokmedhakul, S et al., 1998
P. evecta (Pierre) Finet & Gagnep	Leaf	*Antioxidant, antimutagenicity*	Sripanidkulchai, B. et al., 2008
P. debilis	Root	antimycobacterial activity	Panthama, N. et al., 2010
P. oblique	Branch, Leaf	Antibacterial activities	Wang, L. K. et al., 2014
P. oliveri	Stem bark	antiplasmodial activity, cytotoxicity	Kouam, S. F. et al., 2014
P. plagioneura	stem	Antiproliferative	Liu, B. et al., 2010
P. obliqua Hook.f. & Thomson	Branch, Leaf	Cytotoxic activities	Wu, L. J. et al., 2016
P. evecta	roots	antiplasmodial activity, antiviral activity, cytotoxicity	Kanokmedhakul, S. et al., 2006
P. parviflora	Leaf	Anti-inflammatory, cytotoxic activity	Liou, J. R. et al., 2014

2.5 Phytochemicals from *Polyalthia* species

Scientific reports on various parts (leaves, bark, stem bark, root, twigs, and seeds) of *Polyalthia* have revealed the presence of plenty of phytoconstituents with numerous biological and pharmacological potentials such as anti-bacterial (Faizi, S. et al., 2003; Kanokmedhakul, S.et al., 2007; Murthy, M. M et al., 2005), anti-viral (Kanokmedhakul, S.et al., 2006; Li, H. Y. et al., 1993), anti-fungal (Bhattacharya, A. K.et al., 2015), anti-plasmodial (Misra, P et al., 2010; Gbedema, S. Y et al., 2015; Ngantchou, I.et al., 2010), anti-ulcer (Olate, V. R et al.,2012), anti-cancer (Thiyagarajan, V. et al., 2016; Afolabi, S. O et al., 2019; Cheng, M. Fet al., 2017), anti-inflammatory (Chang, F. R et al.,2006; Bermejo, A. et al., 2019; Nguyen, H. T et al., 2020), anti-tumor (Wu, Y. C et al.,1990; Duan, X. Y et al., 2020; Ma, X et al.,1994), and chemopreventive potentials. The most abundant phytochemicals in *Polyalthia* plants are alkaloids and terpenes (Paarakh, P. M., & Khosa, R. L., 2009). The chemical compounds isolated from the *Polyalthia* species are presented in Table 2.4.

Table 2.4 Chemical constituents isolated from *Polyalthia* species

Sl No.	Plant	Plant part	Compound	References
1	*P. acuminata*	bark, leaves	Anonaine, N-methyl coclaurine, Anolobine, Cavarine, Noroliveroline,Norushinsunine, O-methyl moschatoline, Stepharine, Reticuline , Coclaurine , Stepholidine	Zarga, M. H. A. & Shamma, M.; 1982
		leaves	Asimilobine, 3-hydroxy-nornuciferine, Isoboldine, Isopiline, 3-hydroxy nuciferine, O-methyl isopiline, Norannuradhapurini	
			Norliridinine, Nornuciferine, Tuduranine	
		bark	Liriodenine, Kikemanine	
2	*P. barnesii*	stem bark	16ahydroxyl cleroda,3,13 Z dien,16,15,olide ; 3b,16 a-dihydroxy cleroda 4C(18)- 13(14) Z dien-15,16 olide	Ma, X. et al., 1994
3	*P. bullata*	stem bark	7,7-bisdehydro-O-methyl isopiline ; 7dehydronornuciferine-7'dehydro-O-methyl isopiline; Urabaine	Ma, X. et al., 1994
4	*P. cauliflora*	bark	Norushinsunine, Ushinsunine	Jossang, A. et al.,1984
5	*P. cauliflora var beccari*	bark	Boldine, Predicentrine, Atherospermidine, Thailandine, Beccapoline, Beccapolinium, Polybeccarine, Beccapolydione, Sebiferine	Jossang, A. et al.,1984
		leaves	Dehydropredicentrine	
		stem bark	O-methyl moschatoline, Oxostephanine, Liriodenine, Lysicamine	
6	*P. cerasoides*	stem bark	Polycerasoidin methyl ester, Polyalthidin, 6 E 10E isopolycerasoidol, Trans asarone, Polycerasoidin, Polyacerasoidol	González, M. C. et al., 1996 ; Zafra-Polo, M. C. et al., 1996
		root	Bidebiline E, Reticuline, Laudanosine, Codamine, laudanidine, a–humulene, Caryophyllene oxide, a –cadinol	Kanokmedhakul, S. et al., 2007
7	*P. chelienesis*	stem bark	16ahydroxyl cleroda,3,13 Z dien,16,15,olide; 3b,16 a-dihydroxy cleroda 4C(18)- 13(14) Z dien-15,16 olide ; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); Cleroda 6 (18) 13 E diene 15 oic acid	Hao, X. J. et al., 1995
8	*P. crassa*	leaves	Altholactone, 3-acetyl altholactone, Crassalactones A-D, Howiicin A	Tuchinda, P. et al., 2006
9	*P. debilis*	root	Bidebilines A-D; Bis 7,7'dehydroanonaine; 7 dehydro anonaine 7' dehydro -8' methoxy anonaine; Bis 7,7' dehydro 8,8' dimethoxy anonaine; Bis 7,7' dehydro 10,10' dimethoxy anonaine	Kanokmedhakul, S et al., 2003
10	*P.emarginata*	trunk bark	Lanuginosine	Paarakh, P. M., & Khosa, R. L., 2009
		leaves	Liriodenine, Anonaine	
11	*P. evecta*	root	19(2-furyl)nonadeca 5,7 diynoic acid; 19(2-furyl)nonadeca 5ynoic acid; 1(2-furyl) pentacosa 7,9 diyne 21 (2furyl) heneicosa 14,16 diyne; 19 (2-furyl)nonadeca 5,7 diynoate	Kanokmedhakul, S et al., 2006

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12	*P. fragrans*	stem bark	Polyalthic acid	Paarakh, P. M., & Khosa, R. L., 2009
13	*P. insignis*	bark	Asimilobine, Liriodenine, O-methyl moschatoline, Oxostephanine	Lee, K. H.et al., 1997
14	*P. longifolia*	leaves	O-methyl bulbo-capnine ß-N-oxide ; Hyperoside; Anonaine; O-methyl bulbo-capnine a-N-oxide ; N-methyl nandigerine ß-N-oxide ; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); 3,13E kolavadien-15-oic acid 16 al; 16 hydroxy cleroda 3, 13(14)- E dien 15 oic acid; Rutin; Quercetin; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); 16ahydroxyl cleroda,3,13 Z dien,16,15,olide	Wu, Y. C. (1989); Phadnis, A. P.et al., 1988; Seetharaman, T. R., 1986; Zhao, G. et al., 1991;
		stem	Noroliveroline, 16ahydroxyl cleroda,3,13 Z dien,16,15,olide; Oliveroline N-oxide; Liriodenine; Darienine; Polyfothine; Isoncodine; Onychine; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); Polyfothine; Darienine	Wu, Y. C., 1989; Zhao, G. et al., 1991; Phadnis, A. P. et al., 1988; Chakrabarty, M., & Nath, A. C., 1992;
		stem bark	2 mercapto pyridine N oxide Zinc salt; Altholactone; Ent halima 1 (10) 13 E dien 15 oic acid; Ent halima 5 (10) 13 E dien 15 oic acid; 16 hydroxy ent halima 5(10) 13-dien 15,16 olide; Cleroda 6 (18) 13 E diene 15 oic acid; 16 hydroxy cleroda 4 (18) 13-dien 15,16 olide; Kolavenic acid; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); Isoncodine; 3b,16 a-dihydroxy cleroda 4C(18)- 13(14) Z dien-15,16 olide; Polyfothine; Oliveridine; Oliverine; Norlirioferine; Stepholidine; Darienine; 16ahydroxyl cleroda,3,13 Z dien,16,15,olide	Loder, J. W., & Nearn, R. H., 1977; Hara, N. et al., 1995; Zhao, G. et al., 1991; Phadnis, A. P.et al., 1988; Chakrabarty, M., & Nath, A. C., 1992; Paarakh, P. M., & Khosa, R. L., 2009
15	*P. longifolia var pendula*	root	Isoursuline, Pendulamine A, Pendulamine B, Penduline	Jossang, A. et al,1984; Faizi, S.et al., 2003
		leaves	kolavadien 15 oic acid 3 al; 3b,16 a-dihydroxy cleroda 4C(18)- 13(14) Z dien-15,16 olide; 17 (R & S) 3,13 Z kolavadien - 15,16 olide 2-one; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); Kolavenic acid; 2-oxo-kolavenic acid; 16 (R &S) hydroxyl cleroda 3,13-(14) Z dien 15,16 olide; Methyl 16 oxo cleroda 3,13-(14)E dien 15 oate; Solidagonal acid	Faizi, S. et al., 2008; Ma, X. et al., 1994; Hasan, C. M. et al., 1994; Faizi, S. et al., 2008
		Stem bark	Lanuginosine; Liriodenine; 16 oxocleroda 3,13 (14) E dien - 15 oic acid (Polyalthialdoic acid); Kolavenic acid; 16 ß hydroxy cleroda 3,13(14)Z - dien 15,16 olide; 16 hydroxy cleroda 13 ene - 15,16 olide 3 one; 3b 5b 16 a trihydroxyhalima-13,14 en 15,16 olide	Paarakh, P. M., Khosa, R. L., 2009; Hasan, C. M. et al., 1994; Faizi, S. et al., 2008; Chen, C. Y. et al., 2020

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16	*P. macropoda*	stem bark	Oliverine N-oxide; Oliveroline; Liriodenine; Coclaurine; Thaipetaline; (4S,9R,10R) methyl 18-carboxy labda 8,13 E dien 15 oate	Lavault, M. et al., 1990; Richomme, P. et al., 1996; Richomme, P. et al., 1990
17	*P. nemoralis*	stem, leaves	Polynemoralines A; Polynemoralines B; Polynemoralines C; Polynemoralines D	Lu, Z. M et al., 2008
		stem bark	Zinc polyanemine; 2 mercapto pyridine N oxide Zinc salt	Paarakh, P. M., & Khosa, R. L., 2009
18	*P. nittidissima*	bark	Norushinsunine, Ushinsunine; Daurisoline; Stepholidine; Lindoldhamine; Liriodenine; Protosinomenine; O-methyl-7-lindoldhamine; N-N'-dimethyl lindoldhamine; Isodaurisoline; Dauricine	Jossang, A et al., 1983;Johns, S. R., & Lamberton, J. A., 1970;
19	*P. oligosperma*	bark	Polygospermine, Kikemanine, Norconovine,Xylopinine	Paarakh, P. M., & Khosa, R. L., 2009
20	*P. oliveri*	stem bark	N-methyl pachypodanthine N-oxide, Anonaine, N-methyl cordine, Noroliveridine, Noroliverine, Oliveridine, Oliverine, Oliveroline, Oliveroline N-oxide, Pachypodanthine, Polyalthine, Polysuavine, Lanuginosine, Liriodenine, Uvariopsamine, Polyalthenol, Polycarpol	Paarakh, P. M., & Khosa, R. L., 2009; Achenbach, H., & Löwel, M.,1993; Leboeuf, M. et al., 1976
		leaves	Anonaine, N-methyl pachypodanthine N-oxide, N-methyl cordine, Noroliveridine, Noroliverine, Oliveridine, Oliverine, Oliveroline, Oliveroline N-oxide, Pachypodanthine, Polyalthine, Polysuavine, Lanuginosine, Liriodenine,	Paarakh, P. M., & Khosa, R. L., 2009
21	*P. plagioneura*	stem	Plagionicin A, Howiicin A	Paarakh, P. M., & Khosa, R. L., 2009
22	*P. purpurea*	whole plant	Anonaine, Atherospermidine, Liriodenine	Paarakh, P. M., & Khosa, R. L., 2009
23	*P. sclerophylla*	stem bark	Polycerasoidin, Polyacerasoidol, 6 E 10E isopolycerasoidol, Trans asarone	González, M. C. et al., 1996
24	*P. sericea*	bark	O-methyl moschatoline	Paarakh, P. M., & Khosa, R. L., 2009
25	*P. stenopetala*	stem bark	Asimilobine, Liriodenine, Oxostephanine, Thaipetaline, Discretamine, Isoursuline	Lavault, M. et al., 1990
26	*P. suaveolens*	bark	Oliveridine N-oxide, Noroliverine, Polycarpol, Guatterine,Oliveridine, Oliverine, Oliverine N-oxide, Oliveroline, Pachypodanthine, Polyalthine, Polysuavine, Lysicamine, Oxostephanine	Hasan, C. M et al., 1982; Cave, A.et al., 1978; Kunesch, N. et al., 1985; Paarakh, P. M., & Khosa, R. L., 2009
		stem	Polyalthenol, Isopolyalthenol, Neopolyalthenol, Polyveoline, Polyavolensine, Polyavolensinol, Polyavolensione, Polyavolinamide, Greenwayodendrine, Greenwayodendrine-3-one, Greenwayodendrine-3ß-yl acetate, Greenwayodendrine 3ß-ol, Greenwayodendrine 3-ol	Kunesch, N. et al., 1985; Cave, A.et al., 1978; Hocquemiller, R. et al., 1981; Okorie, D. A.,1980; Okorie, D. A., 1981; Hasan, C. M et al., 1982
27	*P. suberosa*	stem bark	Asimilobine, Lanuginosine, Liriodenine, Oxostephanine, Tetrahydropalmatine	Reichling, J., 1999

Continued…

		stem	Kalasinamide, Suberosol, 1(2-furyl)pentacosa 16,18 diyne, 23 (2-furyl) tricosa 5,7 diynoic acid	Tuchinda, P.et al., 2000
		leaves	Suberosol	Li, H. Y et al., 1993
28	*P. viridis*	bark	Methyl 2E 6E farnesoate- 3,13 E kolavadien 15 oic acid; 16 (R & S) 3,13 Z kolavadien - 15,16 olide 2-one; 16ahydroxyl cleroda,3,13 Z dien,16,15,olide; 16 a methoxycleroda 3,13 Z-dien 15,16 olide; 16 (R) 3,13 Z kolavadien 15,16 olide ; 2-oxo-kolavenic acid; 3,13E kolavadien 15 oic acid 16 al ; 14,15 bisnor 3,11 E kolavadien 13 one (4*2) abeo 16 (R&S)2,13 Z-; kolavadien 15,16 olide 3 ol (4*2) abeo 16 (R&S)2,13 Z-; 3,12 E kolavadien 15 oic acid 16 al; Bisclerodaneimide	Kijjoa, A. et al., 1990; Hasan, C. M. et al., 1982; Lopes, L. M et al., 1987

Even though different *Polyalthia* plant extracts and isolated compounds have shown strong pharmacological activities as mentioned in Table 2.3 and section 2.5 of this review, a large number of *Polyalthia* species are yet to be analysed for realizing their therapeutic potential.

2.6 *Polyalthia korintii (Dunal) Benth. & Hook. F.*

Fig. 2.1 Leaf, flower and fruit of *Polyalthia korintii* (Research specimen)

Polyalthia korintii is an Indo - Sri Lankan ethnomedicinal species. It grows as a shrub or small tree, 3 - 5 m tall, found spreading its young branches, with edible fruits (Ashton, M. S. et al., 1997) and is mainly confined to peninsular India. It is commonly called “Karuvalli” and “Korintipanel” in Malayalam, “Uluvintai” in Tamil, and “Ulkenda” in Sinhala. Several researchers have reported the presence of

these species in the Eastern Ghats and the southern Western Ghats (Singh Richard, P. S., & Muthukumar, S. A., 2012) of India. The number of mature plants and the area of its distribution are too low and this species is placed under the vulnerable critically endangered category.

The presence of this endangered species is reported from Sree puthiya bhagavathi sacred grove, Kalloori at Kannur district, Kerala (Poovathur, P., & Joseph, S., 2016), Iriveri Sree Pulideva Temple associated sacred groove, Kannur district, Kerala (Swathi, B. R., & Joseph, S., 2017), Mundanthurai Range in the Kalakad-Mundanthurai Tiger Reserve of the southern Western Ghats, India (Richard, P. S. S., & Muthukumar, S. A., 2012), Cheruvathur Grama Panchayath of Kasargod district, Kerala (Sharma, J. K. et al., 2003), Kadapa district of Andhra Pradesh (Sekhar, J. et al., 2011), and Kambakam hills, Andhra Pradesh (Basha, S.et al., 2014).

P. korintii has ethnomedicinal claims and is mostly used by tribes as these plants are highly confined and available to those areas. For instance, the Tribes of Kambakam Hills, Eastern Ghats, India uses the root bark for the cure of stomach ache. The village folk at Kadapa district of Andhra Pradesh, India use oral administration of root powder decoction as a good antidote for Russell Viper bite (Bijauliya, R. K. et al., 2017).

Even though the plant possesses medicinal properties, it is not scientifically studied. So this plant was selected to know more about its biological properties such as antioxidant, antimicrobial, and anti-proliferative potentials and for the isolation of anticancer compounds.

MATERIALS AND METHODS

3.1 Collection and authentication of plant material

Disease-free fresh *Polyalthia korintii* leaves were collected from the Calicut University Botanical Garden (CUBG), University Campus, Kerala, India. The plant was authenticated by Dr. A.K. Pradeep, Department of Botany, University of Calicut, and a voucher specimen (Accession No: 6917) was deposited at the University herbarium.

3.2 Pharmacognostic analysis

3.2.1 Macroscopic evaluation: Fresh leaves were used for the evaluation of macroscopic characteristics such as size, shape, and color. The magnifying lens was used for the observation of plant material.

3.2.2 Microscopic evaluation: The studies were done following standard procedures recommended by Ayurvedic Pharmacopoeia of India. The leaves were transverse sectioned through the midrib and used for microscopic examination (Singh M et al., 2017).

3.3 Physicochemical evaluation

The determination of ash values (total ash, water-soluble ash, acid insoluble ash, and sulfated ash) was carried out as per the methods of Indian Pharmacopoeia (1996). Assessment of foreign organic matter (Pawaskar SM and Sasangan K.C, 2017) moisture content (Geetha DH et al., 2017) , and crude fiber content (Singh et al., 2017) were done following the methods of Ayurvedic Pharmacopoeia of India and Indian Standards.

3.3.1 Sample preparation

The leaves were cleaned with distilled water, dried in shade, and crushed into a coarse powder using the blender. These were stored in air-tight containers for further use.

3.3.2 Determination of ash values

3.3.2.1 Total Ash value: Accurately, two grams of leaf powder were taken in a pre-weighed silica crucible. The crucible was incinerated at a higher temperature (450^0C) and then cooled and reweighed. The percentage of total ash with reference to the initially used air-dried powder was calculated.

3.3.2.2 Acid Insoluble Ash value: The ash obtained in the above procedure was boiled for 5 minutes with 25 ml of dilute Hydrochloric acid. Using ashless filter paper, the insoluble matter was collected and washed with hot water. The insoluble ash was transferred into a pre-weighed silica crucible, ignited, cooled, and weighed. The percentage of acid-insoluble ash was then calculated with reference to the air-dried powder.

3.3.2.3 Water Soluble Ash value: The total ash obtained was boiled for 5 minutes with 25 ml of water and the insoluble matter was collected on ash-less filter paper. In a pre-weighed silica crucible, the insoluble ash was ignited for 15 minutes at 450^0 C. The weight of the insoluble matter was subtracted from the weight of the total ash to obtain the weight of water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried powder.

3.3.2.4 Sulphated Ash value: Accurately weighed two grams of the powder, moistened with Sulphuric acid, and gently ignited. The procedure was repeated, cooled, and weighed. The value thus obtained was used to calculate the percentage of sulphated ash with reference to the air-dried powder.

3.3.3 Determination of Foreign Organic Matter

Examine in daylight with the unaided eye and using a magnifying glass and separate the foreign matter. Weigh the sorted foreign matter and calculate the foreign matter content in per cent with reference to the dried powder.

3.3.4 Determination of Moisture Content

Accurately weighed two grams of the powdered sample and dried at 105°C for 5h and allowed to cool in desiccators and weighed. The process was repeated

until the weight of the sample became constant. Then the percentage of loss on drying was calculated.

3.3.5 Determination of Crude Fibre Content

Accurately weighed one gram of plant material and boiled in 200 ml of 1.25% sulphuric acid for 30 minutes. The filtered content was then neutralized and boiled in 200 ml of 1.25% sodium hydroxide for 30 minutes. A crucible was weighed and the sample was ignited in a furnace at 550-600 ^{0}C for 2- 3 hours. It was then cooled, and weighed, and the percent fiber content was determined.

3.3.6 Fluorescence Analysis

The coarsely powdered dried leaves were studied initially under daylight and also under ultraviolet radiation. 0.5 g of the powder were taken into each test tube and treated with 5 ml of various solvents like distilled water, acetone, benzene, chloroform, methanol, petroleum ether, and hexane; alkaline solutions like aqueous and alcoholic sodium hydroxide (1N) and potassium hydroxide (5%); acidic reagents *viz*. 1N hydrochloric acid, 50% sulphuric acid, 50% nitric acid, glacial acetic acid, and picric acid; ferrous chloride solution, iodine solution, and ammonia. All the tubes were properly mixed and allowed to stand for about 30 min. The solutions thus obtained were observed under visible light and UV light. The characteristic colours that appeared were recorded (Mukhi S. et al., 2016) (Mamillapalli V et al., 2016).

3.4 Preparation of solvent extracts

The leaves were collected and washed under running tap water to remove the dust and any surface pollutants. They were air-dried under shade. The dried sample was coarsely powdered and stored in air-tight containers for use. Different extraction methods were followed to prepare crude extracts from dried leaves of *P. korintii.*

3.4.1 Sequential Soxhlet Extraction

Soxhlet apparatus was used in the preparation of leaves extracts. The pre-weighed leaf powder was filled into the thimble and extracted with different organic

solvents in the order of increasing polarity as petroleum ether, chloroform, ethyl acetate, methanol, and water (Fig. 3.1).

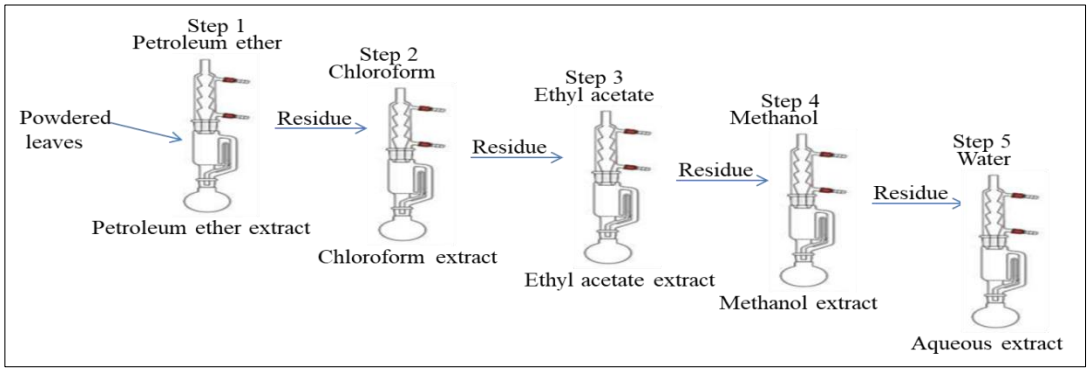


Fig. 3.1 Diagrammatic representation of extraction of *P. korintii* leaves using soxhlet apparatus.

The extraction went on until the siphon tube is colourless, almost 48 hours. The boiling point of each solvent was set as the temperature for extraction. After the extraction with the first solvent, the filtrate was collected, cooled, passed on through layers of filter papers, and then evaporated to get the crude extract. The weight of the extract thus obtained was noted for calculating the extraction yield as per the following equation.

$$\text{Extraction yield} = \frac{\text{Weight of the dried extract (g)}}{\text{Weight of the leaf powder used (g)}} * 100$$

The powder or residue remaining after the extraction was left under air to evaporate the solvent completely and was fed into the thimble for the extraction with the next solvent. The process was repeated to obtain crude extracts of different solvents. Yield values (%) were calculated and the extract characteristics were noted.

3.4.2 Sequential Maceration

Accurately weighed leaf powder was extracted with different organic solvents one after the other, in the order of their increasing polarity (petroleum ether, chloroform, ethyl acetate, methanol, and water). This mixture taken in the conical flask was constantly agitated in an orbital shaker for 48 hours at room temperature.

Extraction was done twice with a single solvent and then the filtrate was collected and the residue was air-dried. The filtrate was evaporated to obtain the crude extract. The scheme of extraction was completed as shown in Fig. 3.2.

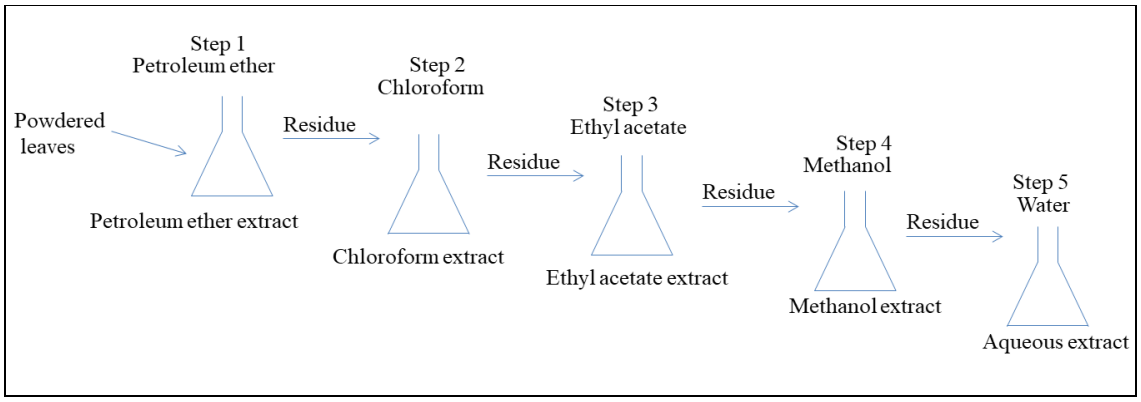


Fig. 3.2 Representation of sequential shaker extraction of *P. korintii* leaves.

3.4.3 Harborne Extraction / Acid-base extraction

The extractions were performed according to the scheme given in the figure below (Harborne, A. J., 1998). This method is followed in order to separate the main classes of phytoconstituent from each other based on varying polarity.

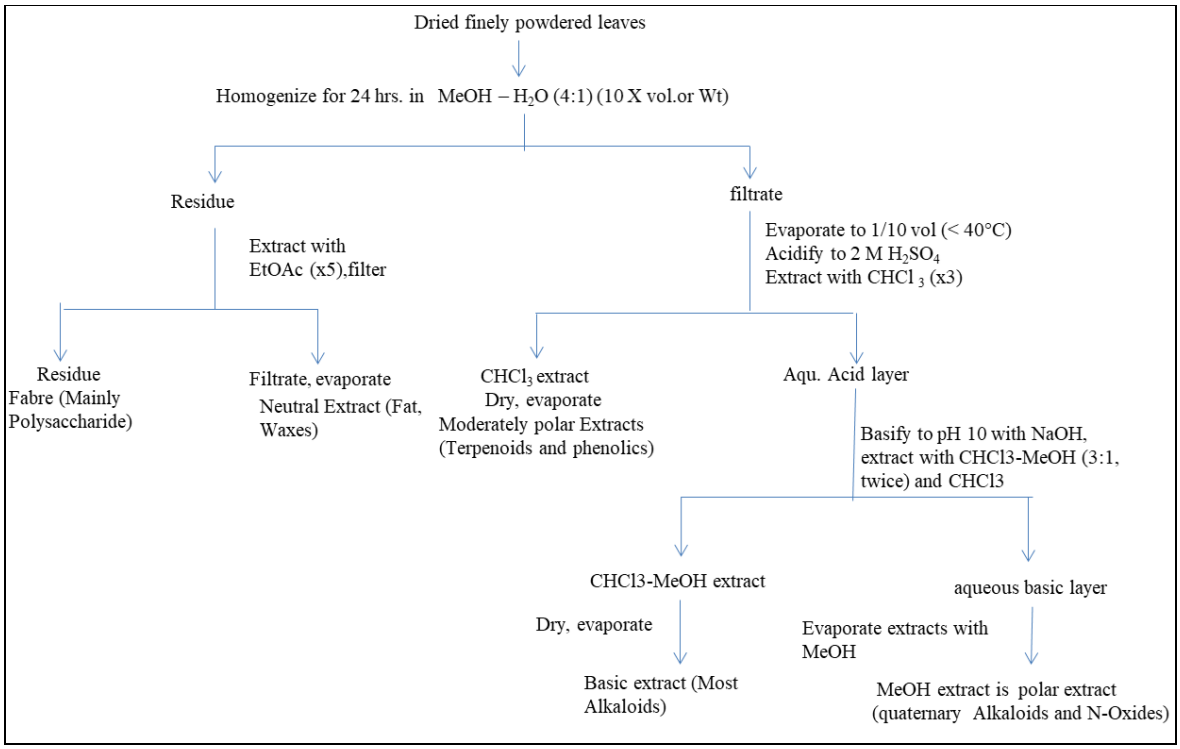


Fig. 3.3 Flowchart of Harborne method extraction of *P. korintii* leaves.

3.5 Phytochemical analyses of extracts

3.5.1 Qualitative analyses

A preliminary phytochemical screening of the plant extracts to detect the presence of various secondary metabolite classes was carried out (Tyagi and Agarwal, 2017; Saxena Mamta et al., 2012).

(i) Alkaloids (Dragendroff's test): The extract was warmed with 2% H_2SO_4 and the filtrate was collected. A few drops of Dragendroff's reagent were added to the filtrate. Orange-red precipitate indicated the presence of alkaloids.

(ii) Flavonoids (Shinoda test): Two drops of conc. HCl and magnesium turnings were added to the extract. The appearance of pink color indicated the presence of flavonoids.

(iii) Phenolics (Ferric chloride test): Extracts were treated with 10% aqueous ferric chloride solution. The formation of bluish-black color indicated the presence of phenolics.

(iv) Tannins (Lead acetate test): To the extract, 10% solution of lead acetate was added. The formation of precipitate indicated the presence of tannins.

(v) Carbohydrates (Benedict's test): To the extract, a few drops of Benedict's solution were added and boiled in the mixture in a water bath. A sequential change in the color (blue-green-orange) indicated the presence of carbohydrates.

(vi) Proteins (Biuret test): To the extract, 2 ml of 20% KOH solution was added and mixed thoroughly. Then, 1 ml of 0.5% $CuSO_4$ solution was slowly added. The development of pale purple color indicated the presence of proteins.

(vii) Steroids (Lieberman Burchard test): The extract was dissolved in acetic anhydride, followed by the addition of concentrated sulphuric acid along the sides of the test tube. The formation of green color indicated the presence of steroids.

(viii) Terpenoids (Lieberman Burchard test): The extract was treated with chloroform, acetic anhydride, and drops of H_2SO_4. The formation of dark green color indicated the presence of terpenoids.

(ix) Glycosides (Keller Killani test): The extract was mixed with a few drops of glacial acetic acid and ferric chloride solution. In addition to H_2SO_4, the development of reddish-brown coloration at the junction of two layers and a bluish-green color in the upper layer indicated the presence of glycosides.

(x) Amino acids (Ninhydrin test): The extract was treated with a ninhydrin reagent. The formation of blue-violet color indicated the presence of amino acids.

(xi) Saponins (Foam test): The extract was vigorously shaken with distilled water and allowed to stand for 10 min. The appearance of stable froth indicated the presence of saponins.

3.5.2 Quantitative analyses

3.5.2.1 Determination of Total Phenolic Content

Total phenolic content in the extracts was determined with the Folin-Ciocalteu reagent as described by Singleton and Rossi (1965). About 500 µl of the sample (1mg/ml) was reacted with 2.5 ml of 0.2 mol/l Folin-Ciocalteu reagent for 4 min, followed by 2 ml saturated sodium carbonate solution (about 75 g/l). After incubation at room temperature for 2 hours, the absorbance readings were taken at 760 nm. Gallic acid was used as the reference standard to plot the calibration curve and the results were expressed as milligram Gallic Acid Equivalent (mg GAE)/g dry weight.

3.5.2.2 Determination of Total Flavonoid Content

Total Flavonoid Content was tested using Aluminum Chloride Colorimetric Method as described by Chang et al., 2002. Quercetin was used to make the standard calibration curve. Stock quercetin solution was prepared by dissolving 5.0 mg quercetin in 1.0 ml methanol, then the standard solutions of quercetin were prepared by serial dilutions using methanol (0–100 µg/ml). 0.5 ml of the diluted standard

solutions were mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the mixture was filtered through Whatman filter paper and the filtrate was collected. The absorbance of the filtrate was measured at 415 nm. The blank was set up with the same amount of water instead of 10% aluminum chloride. Similarly, 0.5 ml of each extract at a specific concentration was treated with aluminum chloride. The corresponding absorbance was plotted on a standard calibration curve and the results were expressed as milligram quercetin equivalent (mg QE)/g dry weight of the extract.

3.5.2.3 Determination of Total Tannins

Analysis of total tannins was conducted following the colorimetric method as described by Makkar et al., 1993. Total phenols in each extract were determined using Folin Ciocalteu Reagent. These extracts were then treated with PVPP (a tannin-binding agent). PVPP formed precipitate with tannins and was removed on centrifugation. The filtrate thus obtained has only simple phenols. Total phenolic content in the filtrate was then determined. Tannic acid was used as the reference standard to obtain the calibration curve. The difference between total phenolic content values obtained before and after treatment with PVPP represents the Total Tannin Content and is expressed as Tannic Acid Equivalents.

3.5.2.4 Determination of Total Saponin Content

Total Saponins Content in the extracts was estimated as described by Hiai *et al*., 1976 using colorimetric methods. Extracts at a specific concentration were treated with 0.25 ml of vanillin reagent. Test tubes were placed in an ice-cold water bath and 2.5 ml of 72% (v/v) sulphuric acid was added slowly along the sides. After thorough mixing and 3 minutes of incubation, the tubes were warmed to 60^0C for 10 min in a water bath. On cooling, the absorbance was measured at 544 nm using a spectrophotometer against the reagent blank. Diosgenin was used as a reference to determine the standard calibration curve. The results were expressed as Diosgenin equivalents (DE μg/mg extract).

3.5.3 High-Performance Thin Layer Chromatography (HPTLC)

HPTLC was done on silica gel 60 $F_{254,}$ 20 x 10 cm HPTLC plates (Merck), with toluene: ethyl acetate [93:7 (v/v)] as a mobile phase for petroleum ether, chloroform, and ethyl acetate extracts. Ethyl acetate: toluene: methanol (7:3:1) and ethyl acetate: methanol: water: formic acid: acetic acid (9:1:1:1:1) was used for methanol and aqueous extracts respectively. 5.0 µl of each extract were applied to the plates as bands, sample application with CAMAG-Linomat IV automated spray on band applicator equipped with a 100 µl syringe and operated with following settings: band length 10 mm, application rate 10 sec/ µl, the distance between 4 mm, distance from the plate side edge1.5 cm and distance from the bottom of the plate 2 cm. CAMAG TLC Scanner 3 was used densitometrically to quantify the bands using WIN CATS software (Version 4 X). The scanner operating parameters were mode: absorption / reflection; Slit dimension: 5 x 0.1 mm; scanning rate: 20 mm/s and monochromator bandwidth: 20 nm at an optimized wavelength 254, 366 nm and in visible range.

3.5.4 Spectroscopic analysis of the extracts

3.5.4.1 Fourier-Transform Infrared Spectroscopy (FTIR) analysis

Dried extracts of *Polyalthia korintii* were used for FTIR analysis. 1 mg of the dried extract was ground with 10 mg of KBr to prepare translucent discs. These encapsulated discs were loaded in an FTIR spectroscope (Shimadzu, Japan) and scanned from 400 to 4000 cm-1 with a resolution of 2 cm^{-1}.

3.5.4.2 GC-MS analysis

The extracts except aqueous extract were dissolved in their respective solvents and filtered. The GC/MS analysis of these extracts was performed by using the Shimadzu GC-MS instrument (QP2010S). The column used was Rxi-5Sil MS of 30m X 0.25mm X 0.25µm size. The carrier gas used is Helium with a flow of 1.0 ml/min. For MS detection, the electron ionization mode with ionization energy of 70 eV was used, with a mass range of m/z 50–550. Analysis was performed by injecting 1 µl of the sample with a split ratio of 50. The column oven temperature

was set at 80°C and held for 2 min, raised at a rate of 5°C per min to 200°C and held for 6 min, and finally held at 260°C for 2 min. The identification of the compounds was achieved by comparing obtained mass spectra of unknown peaks with those stored in the NIST 11(National Institute of Standards and Technology) and Wiley 8.0 mass spectral electronic libraries.

3.5.4.3 HR-LC MS Analysis

The extract was prepared in water and subjected to HR- LC-ESI/MS analysis on Agilent 6550 iFunnel Q-TOFs using the ZORBX Eclipse Plus C18, Narrow Bore 2.1 x 150mm, 5-micron column. The HR-LCMS of the sample was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Pawai, Mumbai. The acquisition method was set to be MS- minimum range 120 (m/z) and maximum 1200 (m/z) with a scanning rate of one per second. The solvent system used was 0.1% formic acid in water and 90% Acetonitrile with 10% water containing 0.1% formic acid at a constant flow rate of 0.3 ml/min.

3.6 Bioactivity evaluation of crude extracts

3.6.1 Antioxidant activity assays

3.6.1.1 DPPH radical scavenging activity

The antioxidant activity of the extracts was measured according to the method described by Brand-Williams et al., 1995. The procedure relies on the basis of the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. DPPH solution (0.1mM) in methanol was mixed with 1ml of plant extract solution of varying concentrations. The reaction mixture was incubated at room temperature in the dark for 30 minutes. Absorbance was recorded at 517 nm. Ascorbic acid served as the reference standard. The inhibition % was calculated using the following formula.

$$\text{Inhibition \%} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}} \times 100}{\text{Absorbance}_{\text{control}}}$$

3.6.1.2 ABTS scavenging activity

ABTS radical cation was produced by reacting ABTS stock solution (7mM) in water with potassium persulfate (final concentration of 2.45 mM). The mixture was allowed to stand in the dark at room temperature for 12–16 h before use. For the study, the solution was diluted with methanol to an absorbance of 0.70 at 734 nm. 5 µl of plant extract was added to 3.995 ml of diluted $ABTS^{\cdot+}$ solution, mixed well, incubated for 30 minutes and absorbance was measured at 734 nm. Ascorbic acid was used as a standard substance (R. Re et al., 1999). Percent inhibition was calculated using the formula,

$$ABTS^{\cdot+} \text{ scavenging effect (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}}}{\text{Absorbance}_{\text{control}}} \times 100$$

3.6.1.3 Nitric oxide scavenging activity

Nitric oxide radical inhibition was estimated using Griess reagent. In a 3 ml reaction mixture, 0.5 ml of extracts or reference compounds at different concentrations was mixed with 2 ml of 10 mM sodium nitroprusside and made up to volume with saline phosphate buffer. After incubation at 25^0C for 150 minutes, 0.5 ml of the reaction mixture was mixed with 1 ml of the Greiss reagent and then allowed to stand for another 30 minutes at 25^oC. The concentration of nitrite was assayed at 546 nm and was calculated with the control absorbance of the standard nitrite solution. Buffers and ascorbic acid were used as blank and standard respectively (Zeidan and Oran, 2014). The scavenging activity (%) was calculated as follows:

$$\text{Nitric oxide scavenged (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}}}{\text{Absorbance}_{\text{control}}} \times 100$$

3.6.1.4 Reducing power assay

The reducing power of extracts was determined by the method of Oyaizu., 1986. An aliquot of 0.75 ml of extract at varying concentrations was mixed with phosphate buffer (0.75 ml, 0.2 M, pH 6.6) and potassium hexacyanoferrate ($K_3Fe(CN)_6$) (0.75 ml, 1%, w/v).After incubation at 50^oC in a water bath for 20 min,

the reaction was stopped by adding trichloroacetic acid (TCA) solution (0.75 ml,10%). The mixture was centrifuged at 800 g for 10 min and 1.5 ml of the supernatant was mixed with distilled water (1.5 ml) and ferric chloride ($FeCl_3$) solution (0.1 ml, 0.1%, w/v). On incubation for 10 minutes, the absorbance of the reaction mixture was read at 700 nm. A higher absorbance value of the reaction mixture indicated greater reducing power.

3.6.1.5 FRAP assay

The Ferric Reducing Antioxidant Power (FRAP) assay was carried out according to Benzie IFF and Strain JJ (1996). Freshly prepared FRAP solution (25 ml of 300 mM acetate buffer pH 3.6, 2.5 ml of 10mM 2,4,6-tripyridyls- triazine (TPTZ) in 40mM HCl, and 2.5 ml of 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$)solution) was used. 1.5 ml of this reagent was added to 1ml of the extracts at different concentrations. After incubation at 37^oC for 30 min, the increase in absorbance at 593 nm was measured. $FeSO_4$ was used for the calibration curve and ascorbic acid served as the positive control. FRAP values for the extracts are extrapolated from the standard curve and expressed as mg Fe (II)/g of the extract.

3.6.1.6 Metal chelating activity

The Ferrous ion chelating ability of the extracts was determined in 96 well plates as per the protocol of Khatua et al., (2017). Samples at different concentrations were dispersed in each well of the plate. 5 µl of aqueous ferrous chloride (3 nM) solution was added and mixed well. Then, 10 µl of ferrozine (0.12 nM) was added and made up the volume to 200 µl with water. The reaction mixture was incubated at room temperature for 10 min, and absorbance was measured at 595 nm. EDTA was used as a reference standard. The degree of scavenging was calculated by the following equation:

$$\text{Scavenging effect (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) \times 100}{\text{Absorbance}_{\text{control}}}$$

3.6.1.7 Phosphomolybdenum assay

According to Pietro et al. 1999, the total antioxidant capacity was evaluated by phosphomolybdenum assay and expressed as Ascorbic acid equivalents. 0.1 ml of sample solution at varying concentrations was combined with 1 ml of reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 90˚C for 90 min. The absorbance was measured at 695 nm using reagent and solvent as blank.

3.6.1.8 Superoxide scavenging assay

The reaction mixture constitutes 50 mM phosphate buffer, pH 7.6, 20 µg riboflavin, 12 mM EDTA, and 35µg/ml NBT. Different concentrations of the extracts were added and followed by illuminating the reaction mixture with a fluorescent lamp for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. Identical reaction mixture kept in the dark served as blanks (Kumaran, A., 2006). The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{extract}}) \times 100}{\text{Absorbance}_{\text{control}}}$$

3.6.1.9 Hydroxyl radical scavenging activity

The reaction mixture contained 0.8 mL of 50 mmol/l, pH 7.4 phosphate buffer solution, 0.2 ml of extracts and standard at different concentrations, 0.2 mL 1.04 mmol/l of EDTA, 0.2 ml of 1 mmol/l $FeCl_3$, and 0.2 ml of 28 mmol/l 2-deoxy-D-ribose. The mixtures were maintained at 37 °C and added 0.2 ml of 2 mmol/l of ascorbic acid and 0.2 ml of 10 mmol/l H_2O_2. 1.5 ml 10 g/l of cold thiobarbituric acid was added to the reaction mixture after 1 hour followed by 1.5 ml of HCl. The mixture was heated at 100 °C for 15 min. On cooling, the absorbance was measured at 532 nm. The percentage of hydroxyl radical scavenging activity was calculated as

$\%$ hydroxyl radical scavenging activity $= [A_0 - (A_1 - A_2)] \times 100/A_0$

where A_0 is the absorbance of the control without extract.

A_1 is the absorbance after adding the extract and 2-deoxy-D-ribose.

A_2 is the absorbance of the sample without 2-deoxy-D-ribose.

The percentage of inhibition was plotted against concentration to obtain the IC_{50} value from the graph (Rahman, M. M. et al., 2015).

3.6.1.10 Hydrogen peroxide scavenging ability

The extracts at different concentrations (1-10 mg/ml) were treated with 2 mM H_2O_2 for 5 minutes at room temperature in dark. The excess H_2O_2 remaining in the reaction mixture was treated with dichromate in acetic acid to produce a green color which can be measured at 620 nm. The amount of H_2O_2 scavenged by the extracts was calculated. Percentage hydrogen peroxide scavenging ability was plotted against each concentration and IC_{50} values were noted. Ascorbic acid served as a reference compound (Beevi, S. S.et al., 2010).

3.6.1.11 Lipid peroxidation inhibition assay

Egg-yolk homogenates were employed as lipid-rich media (10% in distilled water, v/v) and different concentrations of the extracts were tested. $FeSO_4$ (0.07 M) was used to induce lipid peroxidation. Then, 20% acetic acid (pH 3.5), 0.8% TBA (w/v), and 20% TCA were added sequentially, mixed well, and heated in a boiling water bath for 1 hour. On cooling, 1-butanol was added and centrifuged at 3000 rpm for 10 min. The upper organic layer containing the pinkish-red chromogen was collected and absorbance was measured at 532 nm (Upadhyay R. et al., 2014).

3.6.2. Protective effects of *P. korintii* extracts against H_2O_2-induced oxidative stress in Hep G2 cells - Cellular antioxidant assay

The cellular antioxidant activity (CAA) assay was carried out according to Wolfe and Liu with minor modifications. Hep G2 was seeded at a density of 10^4 cells/well on a 96-well black microplate in 100 µl of growth medium. The cells were then incubated for 24 h, after which the growth medium was removed and the wells were washed with Phosphate Buffered Saline (PBS). Triplicate wells were treated with 100 µl of various concentrations of extracts (0–1000 µg/ml) containing 25 µM DCFH-DA dissolved in the treatment medium. After 1 h of incubation, the media

was removed and the cells were once again washed with 100 µL of PBS. Then, H_2O_2 (100 mol/l) was applied to the cells in 100 µL of Hank's Balanced Salt Solution (HBSS) and the fluorescence intensity was measured with the microplate reader. Emission at 538 nm was measured with excitation at 485 nm every 5 min for 1 h. Each plate included a triplicate control and blank. After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the CAA value. The median effective dose (EC50) was determined.

3.6.3 Anti-microbial activity by Microwell dilution method

A total of nine bacterial strains were used for the study: *Proteus mirabilis* (clinical isolate), *Chromobacterium violaceum* (ATCC 22019), *Pseudomonas aeruginosa* (clinical isolate), *Klebsiella pneumoniae* (MTCC 3384), *Escherichia coli* (MTCC 41), *Streptococcus pyogenes* (ATCC 19615), *Enterococcus feacalis* (ATCC 29212), *Listeria monocytogenes* (clinical isolate), and *Staphylococcus aureus* (MTCC 87). Serial dilutions of extracts (0-25 mg/ml) and the standard antibiotic chloramphenicol (0- 10 µg/ml) were made and diluted bacterial cultures from 0.5 McFarland standards were added. Sterility control without culture and growth control without drug was also employed. The assays were performed in sterile, 96-well microtitre plates with Mueller-Hinton broth. To control for the color of different extracts, absorbance at 600 nm (OD_{600}) measurements were taken before incubation at 37°C and after 12 hours. The values presented are the mean ± standard error of three independent experiments. The IC_{50} values were calculated from the average OD_{600} value (Mogana, R.et al., 2020; Khan, M. et al., 2018).

3.6.4 Heat-induced protein denaturation inhibition potential

BSA (0.5 % w/v) (pH 6.0) was prepared and added to extracts at different concentrations (100-500 µg/ml). The reaction mixtures were incubated at 25°C for 20 min followed by heating to 70°C for 5 minutes to allow denaturation of proteins. On cooling the samples, the turbidity was measured at 660 nm. Diclofenac sodium was used as a standard drug. Percentage inhibition of protein denaturation was

calculated relative to control, which represents 100% of protein denaturation (Grabowska, K et al., 2016).

3.6.5 DNA intercalating activity by DNA-MG displacement assay

DNA–MG (20 mg) was suspended in assay buffer (0.05 M Tris–HCl pH 7.5, 7.5 mM $MgSO_4$) (100 mL) with continuous stirring at 37°C for 24 hours. The extracts at different concentrations (0-500 µg/ml) were added to 200 µl of the assay buffer containing DNA-MG. Ethidium bromide was used as the positive control. DNA–MG complex absorbs maximally at 645 nm and agents that interact with DNA and displace methyl green from the complex result in the decrease of absorbance which can be detected spectrophotometrically (Rajan, I. et al., 2014).

3.6.6 Hemolysis assay

Fresh human blood (5.0 mL, heparinized) was centrifuged at 1500 rpm for 10 min to obtain an erythrocyte pellet and to remove the plasma and the buffy coat. The pellet was then washed thrice with sterile phosphate-buffered saline (PBS) and suspended in PBS to get approximately 10^8 cells/ml. 0.5 mL of cell suspension was incubated with different concentrations of extracts for 1 h at 37°C and centrifuged at 1500x g for 10 min. The absorbance of the supernatant was measured at 540 nm. Triton X-100 (1% v/v) and PBS served as positive and negative control respectively. The percentage of haemolysis was quantified (Rajan et al. 2014).

3.6.7 Antiproliferative and cytotoxic activity evaluation

3.6.7.1 Cells and culture conditions

Human and murine cell lines were used in the studies to assess the therapeutic efficacy of the extracts, their purified fractions, and essential oil. The cell lines used in the present study were leukemia - K562, HL60, THP1, Jurkat E6.1; colorectal carcinoma - HCT116, HT-29; hepatocellular carcinoma - Hep G2, Hep 3B; non-small lung alveolar carcinoma - A549, prostate cancer - PC3, DU145, LNCaP; breast cancers - MCF7 and MDA-MB-231, Cervical carcinoma- HeLa,

SiHa; Fibrosarcoma- HT-1080; Ovarian carcinoma- PA-1; Pancreatic carcinoma- PANC-1; Monocyte/Macrophage cell line (BALB/c Mice)- RAW 264.7; human embryonic kidney - HEK293; Mouse fibroblast- L929; and VERO- kidney epithelial cells from African green monkey were purchased from the National Centre for Cell Science (NCCS), Pune. Leukemia cell lines were maintained and propagated in RPMI 1640 medium (HiMedia, Mumbai), and PC3 cells were grown in Ham's F12 medium (HiMedia, Mumbai). All other cell lines were grown in DMEM/ MEM (HiMedia, Mumbai), as recommended by NCCS. All media were supplemented with 10% (v/v) fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). Cell cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2. All cells were cultured in a 25 cm^2 culture flask. The leukemic cell lines were split following centrifugation with subsequent resuspension in a fresh medium. All the other adherent cell lines were trypsinized for subculturing. Trypsinzation was done with 0.25% (w/v) Trypsin-0.53 mM EDTA solution (1.0 ml) for 5 to 15 min to detach the cells. Cells were collected and resuspended in fresh growth media.

Human peripheral blood lymphocytes (hPBLs) were isolated from freshly drawn human blood. The blood was diluted with an equal volume of phosphate-buffered saline (PBS) and carefully overlaid on a Hi-SepTM Lymphocyte separation medium (LSM) in a centrifuge tube. After centrifugation at 400 x g for 30 min at room temperature, the buffy coat obtained at the fluid interface was aspirated using a sterile Pasteur pipette and washed twice with PBS. The cells were then cultured in HiKaryo XLTM RPMI medium by incubating at 37 °C.

3.6.7.2 Cytotoxicity assay (MTT)

Cytotoxicity / antiproliferative activity was determined using the yellow-colored, water-soluble dye, MTT (3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide) which can be reduced by mitochondrial dehydrogenases to purple-colored formazan crystals. Briefly, in 96-well microtitre plates cells were seeded at a density of 15x 10^3 cells/well followed by overnight incubation. The cell was then treated with varying concentrations of various extract(s) / its purified fraction /essential oils solubilized in DMSO and incubated

further for an appropriate time (24, 48, and 72 h) in a CO_2 incubator. DMSO served as vehicle control and appropriate amounts were added to cells to get control readings. After treatment, the spent media was removed and MTT (5 mg/ml) was added to each well and further incubated at 37°C in the dark for 3 - 4 hours. The formazan formed was then solubilized in DMSO and the absorbance was measured at 570 nm on a microplate reader. The difference in absorbance of untreated control and treated samples were noted and the percentage cell viability was calculated (Mosmann, 1983) using the formula:

$$\text{Percentage cell viability} = \frac{(\text{Absorbance}_{\text{treated}} - \text{Absorbance}_{\text{blank}}) \times 100}{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{blank}})}$$

3.6.7.3 Trypan blue dye exclusion method

The viability of cells treated with the samples was also evaluated using the trypan blue dye exclusion method. Live cells with intact cell membranes exclude trypan blue dye and dead cells take up the stain and appear blue. 10^5cells/ml were seeded into 24-well plates and treated with the samples for an appropriate time. The cells were collected, stained with 0.4% trypan blue solution, and counted using a hemocytometer.

3.6.7.4 Microscopy

Cytomorphological changes induced by the extract and its purified fractions were observed and photographed using light (phase-contrast) and fluorescence microscopic techniques. The treated and untreated HCT116 and K562 cells (1×10^5 cells/ml) were harvested and washed with ice-cold PBS. The cells thus collected were stained with appropriate dyes like Hoechst 33258 (1.0 mg/ml) incubated for 10 min at room temperature in the dark, AO - EB solution added to the cell suspension at a final concentration of 100 µg/ml, and propidium iodide (20 µg/ml). The cells were then transferred to glass slides, covered with coverslips, and visualized under the fluorescence microscope.

3.6.7.5 Assessment of phosphatidylserine externalization

The externalization of phosphatidylserine in the treated and untreated cells was evaluated by staining with Alexa Fluor® 488 - annexin V (Apoptosis kit, Invitrogen, USA) in accordance with the manufacturer‘s instructions. The cells were collected and washed with ice-cold PBS, resuspended in 1x annexin V- binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2.2H_2O$; pH 7.4), stained with 5 µL of annexin V and 10 µL of propidium iodide solution and incubated for 10 min at room temperature in the dark. The cells were then mounted on slides, and the images were photographed. Flow cytometric measurement of fluorescence on BD FACS ARIA II machine and the results were analyzed using BD FACS Diva software 6.1.3.

3.6.7.6 Assessment of intracellular reactive oxygen species (ROS)

2′, 7′ dichlorofluorescin diacetate (DCFH-DA) was used to study the variations in cellular ROS levels due to treatment with the extract. In the presence of ROS, non-fluorescent DCFH-DA is converted to the highly fluorescent 2', 7'-dichlorofluorescein (DCF). The cells were stained with 10 µM DCFH-DA for 30 min at 37°C, washed twice with PBS, mounted on a slide, and observed under a fluorescence microscope and quantification of ROS was carried out using fluorometry.

3.6.7.7. Assessment of intracellular Ca^{2+} concentration

Since intracellular Ca^{2+} homeostasis is involved in the regulation of cell death, any variations in the intracellular Ca^{2+} levels were determined by using a fluorescent dye, Fluo-3 AM. The treated and untreated cells were incubated with 5 µM Fluo-3AM for 30 min at 37°C and subjected to fluorescence microscopy and fluorometry.

3.6.7.8. Assessment of mitochondrial membrane potential

Loss of mitochondrial membrane potential is considered an early event in apoptosis induction in some systems. Mitochondrial membrane potential was

measured employing the lipophilic cationic dye, rhodamine 123. The cells were treated with rhodamine 123 (5 µg/ml) for 30 min at 37°C. The collected cells were then washed with PBS and mounted on slides for fluorescence microscopy. Quantitative analysis was performed with fluorometry.

3.6.7.9 Cell cycle analysis

Cell cycle distribution was studied by staining the treated and untreated cells with DNA intercalating dye, propidium iodide. Using the BD FACS-ARIA II cytometer, the DNA content and the percentage of cells distributed in each phase-sub-G0, G0/G1, S, and G2/M were quantified. Cells were harvested and fixed in 70% ice-cold ethanol and preserved at -20°C until use. PI solution (20 µg/ml) containing DNase-free RNase A (200 µg/ml) was added and incubated for 30 minutes before data acquisition and analysis.

3.6.7.10 DNA fragmentation assay

DNA fragmentation is the hallmark of apoptosis. The treated and control cells were lysed in buffer containing 20 mM Tris; pH 8.0, 10 mM EDTA, and 0.2% Triton X-100. The lysates were treated with proteinase K (200.0 µg/ml), incubated for 90 min at 37 °C, and added RNase A (0.5 mg/ml) followed by incubation for 2 h at 37°C. Using phenol: chloroform: isoamyl alcohol (25:24:1), DNA was extracted and the DNA pellet was dissolved in Tris-EDTA buffer (pH 8.0). Electrophoresis was performed on 1.8% agarose gel at 50 V incorporating ethidium bromide dye.

3.6.7.11 Western blot analysis

The control and treated cells were pelleted and lysed in RIPA buffer containing freshly added protease inhibitors to isolate the protein under cold conditions. Protein estimation from the lysates was done by Bradford‘s method and equivalent amounts of proteins were subjected to SDS-PAGE on a 12 % gel. The gel was blotted onto a polyvinylidene fluoride (PVDF) membrane followed by blocking using 5% (w/v) skimmed milk prepared in TBST (TBS with 0.1% Tween 20) for 1 h at room temperature. Primary antibodies (rabbit) were added against β-actin and PARP and incubated overnight at 4 °C. The membranes were then washed in TBST

and probed with anti-rabbit HRP-conjugated secondary antibodies. Following washes with TBST, the blots were exposed to DAB and H_2O_2 to visualize the polypeptides.

3.6.7.12 Clonogenic assay

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay to assess the single cell's ability to grow into a colony. Treated and untreated cells were counted and seeded out to form colonies in a complete medium and incubated for 14 days. Colonies were fixed with glutaraldehyde, stained with crystal violet, and counted.

3.6.7.13 Cell migration assay

The serum-starved HCT116 cells were grown to a confluent monolayer and a straight scratch was made using a sterile micropipette tip to create a wound. The floating cells were removed by a gentle wash with the growth media. Cells were then exposed to different concentrations of the extract and the cell migration potential was measured in terms of wound closure due to moving cells by monitoring and capturing images at time intervals.

3.6.7.14 Invasion assay

Tumor cells invade nearby tissues by degrading the basement membrane and extracellular matrix, by the virtue of a property called metastasis. The invasion ability of HCT116 cells was studied using the Geltrex® matrix coated transwell chambers (8 μm pore size). Geltrex® matrix contains extracellular matrix (ECM) proteins such as laminin, collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. Serum starved cells at 2.5 x 10^5 cells/ml were seeded in serum-free medium to the upper chamber along with varying concentrations of the extract. To induce invasion, the bottom chamber was supplied with a medium containing 10 % FBS which serves as a chemo-attractant. After incubation at 37°C, cells that had not migrated were removed from the upper face of the filters using cotton swabs. The lower surface of the transwell was fixed in methanol, stained with crystal violet, and

counted to measure the population of cells that invaded the matrix into the lower surface.

3.7 Bioactivity-guided sub-fractionation of active extract by silver ion silica gel column chromatography

Silver ions (Ag^+) interact and form complexes with compounds containing olefinic double bonds. The chromatographic technique that utilizes these interactions to achieve the separation process is termed silver ion (argentation) chromatography. Separation and isolation of Lipids (Fatty acid mixtures, Isomeric mixtures, Oxygenated unsaturated acids, Triacylglycerol mixtures, Phospholipids, Sterol esters), Terpenes, Hydrocarbons, Steroids, Sterols, Derivatized unsaturated aldehydes and ketones, Prostaglandins, Hydroxyprogesterones, Estrogens, Mineral oils, etc. are literature reported uses of silver ion chromatography (Nikolova-Damyanova B., 2000).

In the study, silica gel 60-120 mesh was impregnated with a 10% aqueous solution of silver nitrate. The impregnated silica gel was washed several times with methanol. The mixture was dried and stored away from light. A predefined quantity of gel was mixed with petroleum ether to degas, packed into a glass column, and left for equilibration. The column was wrapped in aluminium foil for the whole process. The extract was mixed with a small amount of silica gel and applied on top of the packed column without any disturbances to the underlying packed silica gel. Applying different solvents and their combinations, based on the increasing polarity, fractions were collected. The sub-fractions obtained as aliquots were evaporated, weighed, and used for the assessment of their anticancer activity and characterization of phytoconstituents present in them.

3.8 Molecular docking of anticancer compounds to cancer related protein targets

Molecular docking studies were done using Schrodinger software package (Schrodinger, LLC, New York). The ligands used in this study were prepared using LigPrep module with MMFF force field and the protein structures were retrieved from PDB and pre-processed using the protein preparation wizard of the

Schrodinger. The structures were then subjected to energy minimisation. The proteins and their PDB IDs used for the present study are ABL Kinase (3CS9), AKT (3MVH), B-RAF (30G7), VEGFR2 (3WZE), EGFR (3POZ), CDK2 (4BCP), and PI3K (4JPS). Best poses of intermolecular interaction between the compounds (ligand) and the active site of the protein (target) were analysed and the Glide ligand docking scores were calculated. It represented the total of all interactions between the amino acid residues at the binding site and the ligand molecule, thus indicating the potentiality of binding. Docking studies were performed in the extra precision (XP) mode (Vysyan, S. M.et. al, 2021).

3.9 Prediction using PASS (Prediction of Activity Spectra for Substances)

Computer-aided structure activity-based studies are a boon in identifying compounds with pharmacological potentials. PASS predictions are also based on the structure-activity relations and physicochemical characteristics of a compound. The anticancer activity spectra of the phytoconstituents were obtained by using the canonical SMILES (Table 3.1) entered into the PASS online server available from www.pharmaexpert.ru/passonline/predict.php/. The PASS prediction results provide Pa (Probable activity) and Pi (Probable inactivity) values to interpret the potential of a compound. Compounds with Pa > Pi is considered active. Pa values higher than 0.7 indicate the probability to obtain high activity experimentally and also there is a chance that the compound is an analog of an existing active agent. If $0.5 < Pa < 0.7$, usually less probable to obtain an activity experimentally, and the substance is likely to be different from the existing active agents. Finally, Pa values less than 0.5 imply the least probability to find the activity experimentally but the probability of finding a novel chemical entity is high (Basanagouda, M. et al., 2011).

Table 3.1 Canonical SMILES used for PASS prediction

No.	Compounds	Canonical SMILES
1	15-Methylhexadecanoate	CC(C)CCCCCCCCCCCCCC(=O)[O-]
2	1-Iodo-2-Methylnonane	CCCCCCCC(C)CI
3	2,6,10.14-Tetramethyl Hexadecane	CCC(C)CCCC(C)CCCC(C)CCCC(C)C
4	2,6-Di-Tert-Butyl-P-Benzoquinone	CC(C)(C)C1=CC(=O)C=C(C1=O)C(C)(C)C
5	3,3-Dimethyl Hexane	CCCC(C)(C)CC
6	3,7- Dimethyldecane	CCCC(C)CCCC(C)CC
7	3,7,11,15- Tetramethyl-2-Hexadecen-1-Ol	CC(C)CCCC(C)CCCC(C)CCC/C(=C/CO)/C
8	3-Methylene-7,11,15-Trimethylhexadec-1-Ene	CC(C)CCCC(C)CCCC(C)CCCC(=C)C=C
9	6,10.14-Trimethylpentadecan-2-One	CC(C)CCCC(C)CCCC(C)CCCC(=O)C
10	7,9-Di-Tert-Butyl-1-Oxaspiro(4,5)Deca-6,9-Diene-2,8-Dione	CC(C)(C)C1=CC2(CCC(=O)O2)C=C(C1=O)C(C)(C)C
11	9-Octadecen-1-Ol, (Z)-	CCCCCCCC/C=C\CCCCCCCCO
12	Dibutyl Phthalate	CCCCOC(=O)C1=CC=CC=C1C(=O)OCCCC
13	Di-Iso-Butyl Phthalate	CC(C)COC(=O)C1=CC=CC=C1C(=O)OCC(C)C
14	Heptadecane	CCCCCCCCCCCCCCCCC
15	Hexadecane	CCCCCCCCCCCCCCCC
16	Iodododecane	CCCCCCCCCCCCI
17	Isopropyl Tetradecanoate	CCCCCCCCCCCCCC(=O)OC(C)C
18	Kauran-13-Ol	CC1CC23CCC4C(CCCC4(C2CCC1(C3)O)C)(C)C
19	Methyl Hexadecanoate	CCCCCCCCCCCCCCCC(=O)OC
20	Methyl Octadecanoate	CCCCCCCCCCCCCCCCCC(=O)OC
21	N-Heptadecanol-1	CCCCCCCCCCCCCCCCCO
22	Nonacosane	CCCCCCCCCCCCCCCCCCCCCCCCCCCCC
23	Nonadecane	CCCCCCCCCCCCCCCCCCC
24	Octadecane	CCCCCCCCCCCCCCCCCC
25	Octadecanol	CCCCCCCCCCCCCCCCCCO
26	Octadecene	CCCCCCCCCCCCCCCCC=C
27	Pentacosane	CCCCCCCCCCCCCCCCCCCCCCCCC
28	Squalene	CC(=CCC/C(=C/CC/C(=C/CC/C=C(/CC/C=C(/CCC=C(C)C)\C)\C)/C)/C)C
29	Tetratetracontane	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC

RESULTS

4.PHARMACOGNOSTIC, PHYSICOCHEMICAL AND PHYTOCHEMICAL CHARACTERISTICS OF *Polyalthia korintii* (Dunal) Benth. & Hook. F LEAVES

4.1 Pharmacognostic and Physicochemical Characteristics of *Polyalthia korintii* leaves

According to the world health organization's terms on establishing quality standards and specifications for herbal preparations, the samples should be pharmacognostically and physicochemically validated. It is an indispensable need to first identify and authenticate the plant specimen before any study is carried out. In this regard, the present work is aimed at standardization of the leafy parts of this pharmacologically important endemic species from peninsular India, in reference to its pharmacognostic and physicochemical characteristics.

4.1.1 Pharmacognostic study

4.1.1.1 Macroscopic characters

The leaf is oval in shape, entire, apiculate with rounded leaf base and short petiole. The adaxial surface is green and abaxial is dull green. The surface is smooth and shiny. Mature leaf is 7.5 - 9 cm long (Fig. 4.1). No characteristic odour and taste was noted. The observations are summarised in Table 4.1.

Fig.4.1 Morphology of *P. korintii*

Table 4.1. Macroscopic characters of *Polyalthia korintii* leaf

Parameters	Leaf
Color	Green
Shape	Oval
Length	7.5-9 cm
Breadth	3.5-5 cm
Base	Rounded
Margin	Entire
Apex	Apiculate
Texture	Shiny
Touch	Smooth

4.1.1.2 Microscopic characters

Microscopic analysis revealed the following characteristics. The transverse section of the leaf through the midrib showed the presence of upper and lower epidermis with trichomes. Epidermis was covered with cuticle. Collenchyma was seen below the epidermis. Stele is located at the center position. Stele consists of xylem and phloem and the vascular region is surrounded by lignified fibers- the bundle sheath. Leaf lamina is narrow, dorsiventral with upper and lower epidermis covered with cuticle and hairs. Stomata were present. Mesophyll cells with palisade and spongy parenchyma were observed (Fig. 4.2). The leaf powder microscopy revealed the presence of fragments of trichomes, spiral vessels, lignified fibers, collenchyma cells etc. It also showed fragments of mesophyll in both surface and transverse view (Fig. 4.3).

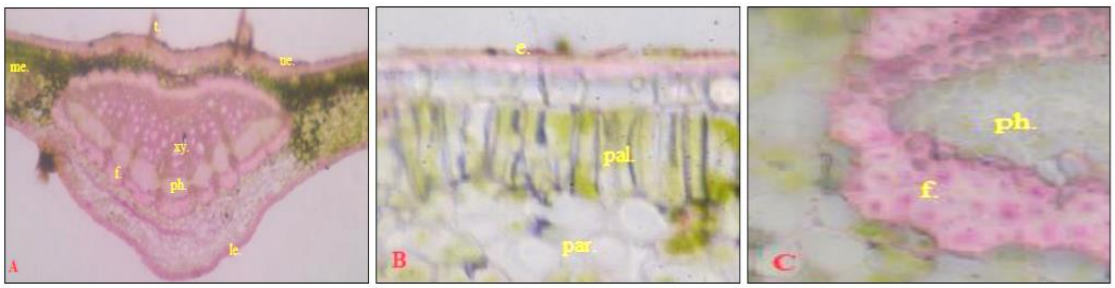


Fig. 4.2 Transverse section of *P. korintii* leaf showing t.,trichome; e.,epidermis; ue.,upper epidermis; le.,lower epidermis; ph.,phloem; xy.,xylem; me.,mesophyll cells; f., fibers.

Fig. 4.3 Powder microscopy of leaf powder showing t.,Trichome; f.,Fibers. v.,Vessel; me.,Mesophyll Cells

Pharmacognostic study thus details the structures and is one among the simplest and cheapest methods to initiate the study on unexplored species. The present investigation on *P. korintii* leaf might be worthy to add on information in respect to its identification parameters.

4.1.2 Physicochemical study

Physicochemical parameters aids in assessing the purity and quality of a preparation and can be employed to rule out the presence of any adulterants and other impurities which may be present due to improper handling of the samples. The physicochemical parameters of *P. korintii* leaves are represented in Table 4.2.

In the present study, the absence of foreign organic matter in the leaves powder clearly states that the plant was healthy and the specimen collection and processing was followed with cleanliness. The moisture content of dry powder was 11.16%, which is pretty less, and hence it would discourage the infection with microorganisms, insects, *etc.* Low moisture content will even discourage deterioration following hydrolysis and help in increasing the shelf life.

The total ash value is primarily important in the evaluation of the purity of drugs. The residue remaining after the incineration of plant material represents the inorganic salts naturally found in it or deliberately added to it as adulterants. The ash

usually consists of carbonates, phosphates, and silicates of sodium, potassium, calcium, and magnesium. Thus it indirectly determines the presence or absence of foreign matter such as metallic salts and silica. Acid-insoluble ash is a part of total ash, soluble in dilute hydrochloric acid, and measures the amount of silica present, especially as sand and siliceous earth. Water-soluble ash is the water-soluble portion of the total ash. The total ash of powdered leaves of *P. korintii* was 4.34%, acid insoluble ash was 0.008%, water-soluble ash was 2.67% and sulphated ash was 6.22%.

Table 4.2. Physicochemical parameters of *P. korintii* leaf

Parameters	Yield (w/w) (%)
Moisture content	11.16 ± 0.8
Total ash	4.34 ± 0.2
Acid-insoluble ash	0.008 ± 0.45
Water-soluble ash	2.67 ± 1.03
Sulphated ash	6.22 ± 0.65
Crude fiber	19.63 ± 1.7
Foreign organic matter	absent

The value of a plant as an herbal formulation is dictated by the phytochemicals present in it and the exploitability of these phytoconstituents, in turn, depends on their extractability from the source into an easily used form. The extractive values and properties of the extracts obtained by successive extraction using a rotary shaker are noted in Table 4.3. In the study, methanol showed higher extractive values (12%) followed by petroleum ether and chloroform with 4% each, and finally ethyl acetate with the least. These variations in the extractable matter suggest that the plant leaves contain a high amount of alcohol-soluble polar substances like phenols, tannins, and glycosides.

Table 4.3. Extractive values and properties of the successive extracts from *P. korintii* leaves

Extract	Extractive values (% w/w)	Consistency	Colour under visible light
Petroleum ether	4.0 ± 1.8	Sticky solid	Pale green
Chloroform	4.06 ± 2.3	Sticky solid	Light green
Ethyl acetate	2.0 ± 1.5	Sticky semi-solid	Dark green
Methanol	14.0 ± 1.3	Syrupy	Dark brown

Fluorescence analysis, yet another important pharmacognostic parameter is used to qualitatively assess the powdered herbal preparations. Every leaf powder has its specific fluorescent colors produced when treated with different reagents. Any deviation from these colors indicates the presence of contamination with adulterants, microorganisms, and other impurities. The results of the fluorescence study of the *P. korintii* powdered leaves on treatment with various solvents and observations under ultraviolet and visible light are tabulated in Table 4.4. Further in the study, reaction with $FeCl_3$ showed the presence of phenolic compounds, ammonia, and NaOH reagent detected the presence of flavonoids. These observations also indicate that the powder contains active phytochemicals which can attribute to the pharmacological quality of the plant material.

Table 4.4. Fluorescence analysis of powder of *P. korintii* leaves

No.	Powder Treatment	Color observed	
		Visible light	UV light (365 nm)
1.	Powder as such	Dark green	Green and red-tinted
2.	Powder + distilled water	Yellowish green	Dark olive green
3.	Powder + acetone	Greenish-yellow	Orange
4.	Powder + benzene	Yellowish green	Deep pink
5.	Powder + chloroform	Green	Orange
6.	Powder + methanol	Green	Sandy brown
7.	Powder + petroleum ether	Pale yellow	Dark red
8.	Powder + hexane	Very pale green	Bright red
9.	Powder + glacial acetic acid	Yellow	Dark red
10.	Powder + 50 % H_2SO_4	Brownish-yellow	Sky blue
11.	Powder + 50 % HNO_3	Yellow	Yellow
12.	Powder + 1N HCl	Pale yellow	Pale yellow

Continued…

13.	Powder + 5 % $FeCl_3$	Black	Black
14.	Powder + 5 % I_2	Yellow	Pale yellow
15.	Powder + picric acid	Bright yellow	Black
16.	Powder + 1N aqueous NaOH	Bright yellow	Dark yellow
17.	Powder + 1N alcoholic NaOH	Light green	Light pink
18.	Powder + ammonia	Dark yellow	Pale green
19.	Powder + 5% alcoholic KOH	Light green	Light pink
20.	Powder + 5 % aqueous KOH	Dark yellow	Greenish-yellow

The pharmacognostic and physicochemical properties of the *P. korintii* leaf have been reported for the first time. This could serve as an aid in the identification, quality control, and preparation of a monograph of the plant.

4.2 C**omparative study of the extractive yield from *Polyalthia korintii* leaves using different extraction methods and solvents**

Different pharmacological activities of plants are attributed to the presence of specific phytoconstituents in them. So extracting them is an important step involved in the identification of bio-actives from medicinal plants. The extraction techniques, solvents, and other parameters adopted for the present study are summarised in Table 4.5.

Table 4.5. Comparison of extraction parameters

Extraction conditions	**Extraction methods**		
	Sequential maceration	**Sequential soxhlation**	**Harborne method**
Solvents	Petroleum ether, Chloroform, Ethyl acetate, Methanol, Distilled water	Petroleum ether, Chloroform, Ethyl acetate, Methanol, Distilled water	Methanol: Water (4:1), Ethyl acetate, Chloroform, Chloroform: Methanol (3:1)
Other reagents	-	-	H_2SO_4, NaOH
Sample to solvent ratio (w/v)	1:10	1:10	1:10
Temperature	Room temperature	Boiling points of corresponding solvents	27 ^oC - 40 ^oC
Time for the entire process	15 days	3 days	6 days

The sequential maceration of leaves of *P. korintii* using different solvents resulted in semi-solid residues. The percentage yield, characteristics like color, and consistency of the extracts are noted in Table 4.6. The highest yield was obtained for methanol extract followed by chloroform, petroleum ether, aqueous, and ethyl acetate. Petroleum ether, chloroform, and ethyl acetate extracts were sticky, the aqueous extract was lyophilized and methanol extract on concentration provided extract with a syrupy consistency.

Table 4.6. Extraction yield of *P. korintii* leaves by sequential maceration

Extract	% Yield	Colour	Consistency
Petroleum ether	4.0 ± 1.8	Dark green	Sticky
Chloroform	4.06 ± 2.3	Dark green	Sticky
Ethyl acetate	2.0 ± 1.5	Dark green	Sticky
Methanol	14.0 ± 1.3	Dark brown	Syrupy
Aqueous	3.9 ± 2.1	Dark brown	Powdery

Sequential soxhlation resulted in high extractive yield (Table 4.7) with aqueous extract showing 19% followed by methanol (10.7 %), chloroform (6.94 %), petroleum ether (6.3 %), and ethyl acetate (3.2 %). The aqueous extract was dried and appeared powdery. The methanol extract was syrupy and the other three extracts had sticky consistency.

Table 4.7. Extraction yield of *P. korintii* leaves by sequential soxhlation

Extract	% Yield	Colour	Consistency
Petroleum ether	6.3 ± 1.4	Dark green	Sticky
Chloroform	6.94 ± 2.0	Dark green	Sticky
Ethyl acetate	3.2 ± 0.5	Dark green	Sticky
Methanol	10.7 ± 2.2	Dark brown	Syrupy
Aqueous	19.0 ± 1.7	Dark brown	Powdery

Compared to above-mentioned methods, Harborne extraction resulted in very low yields. The percentage yields were in the order methanol (8%), chloroform: methanol (5 %), ethyl acetate (3.1%), and chloroform (2.5%). Ethyl acetate,

chloroform, and chloroform: methanol extract were sticky and methanol extract was syrupy (Table 4.8).

Table 4.8. Extract characteristics and extraction yield of *P. korintii* leaves by Harborne method

Extract	% Yield	Colour	Consistency
ethyl acetate	3.1 ± 0.8	Dark green	Sticky
chloroform	2.5 ± 2.2	Green	Sticky
chloroform: methanol	5.0 ± 1.5	Yellow	Sticky
methanol	8.0 ± 1.2	Dark brown	Syrupy

From the results noted, it is evident that though the sample to solvent ratio was kept a constant and same solvents were used for extraction, the time required for extraction and the extractive yield varied considerably (Fig.4.4). The sequential soxhlation method furnished higher extractive yield for most of the solvents used and process were less time consuming.

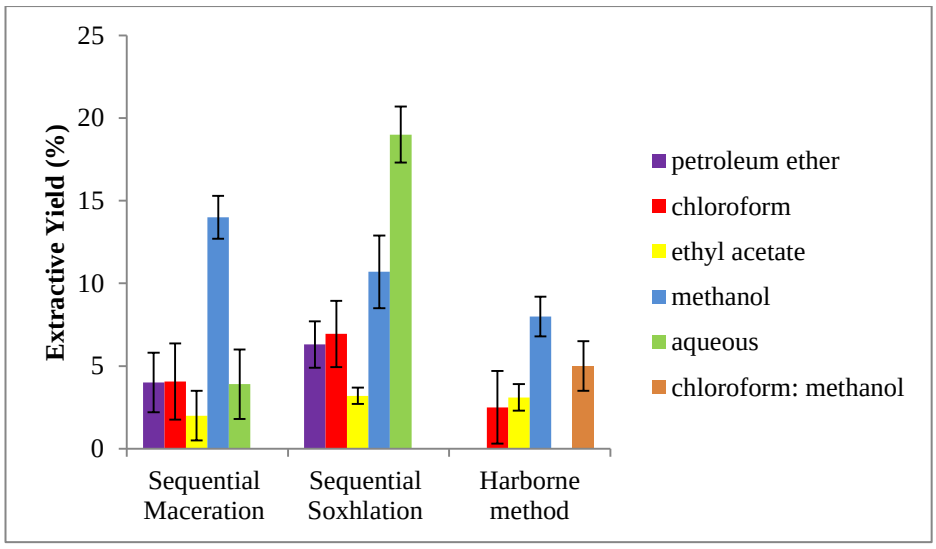


Fig. 4.4. Comparison of percentage extractive yield from *Polyalthia korintii* leaves. The figure shows the comparison of yield obtained using the mentioned solvents following three different extraction methods. In all the three methods, distilled water and methanol was efficient in extracting more and can be ascribed to the presence increased amount of polar constituents in the leaves. Values represent mean± S.D. of three experiments.

These results suggest that the majority of extractable phytochemicals are high in polarity since the methanol and aqueous extracts showed high percentage yield in all the methods tested. Also on increasing the extraction temperature there is increase in the yield. Considering the extraction yield and the time required for extraction, it is evident that the sequential soxhlation method is more efficient and less time consuming in extracting phytoconstituents from *Polyalthia korintii* leaves. Thus, the extracts obtained from sequential soxhlation method were used for all the further studies. These extracts are designated as PE Sox (Petroleum ether extract), CHL Sox (chloroform extract), EA Sox (Ethyl acetate extract), MET Sox (Methanol extract) and AQ Sox (aqueous extract).

4.3 Phytochemical Screening, HPTLC Profiling and Spectroscopic Analysis of *Polyalthia korintii* Leaves Extracts

Plants are an immense source of therapeutic potential owing to the presence of their metabolites especially the secondary metabolites or the plant phytochemicals. Thus it is mandatory to have knowledge of the classes of compounds present in the crude extracts before its biological activities are explored. The present investigation details the presence of different classes of secondary metabolites from the crude extracts of *Polyalthia korintii* leaves.

4.3.1 Preliminary qualitative phytochemical analysis of extracts

The qualitative phytochemical screening of the *P. korintii* leaves extract revealed the presence of plant metabolites like alkaloids, flavonoids, phenolics, tannins, carbohydrates, steroids and saponins (Table 4.9). All the extracts tested showed the presence of alkaloids, flavonoids, phenolics, tannins, carbohydrates, and saponins. Steroids were detected only in methanol and aqueous extracts. In the extracts, terpenoids, proteins, amino acids and glycosides were not detected by the reagents used.

Table 4.9. Preliminary qualitative analysis of *Polyalthia korintii* leaves extracts

Phyto constituents	Test Method	Extracts				
		PE Sox	CHL Sox	EA Sox	MET Sox	AQ Sox
Alkaloids	Dragendroff's test	+	+	+	+	+
Flavonoids	Shinoda test	+	+	+	+	+
Phenolics	Folin ciocalteau reagent test	+	+	+	+	+
Tannins	Ferric chloride test	+	+	+	+	+
Carbohydrates	Benedict's reagent test	+	+	+	+	+
Proteins	Biurette reagent test	-	-	-	-	-
Steroids	Lieberman Burchard test	-	-	-	+	+
Terpenoids	Lieberman Burchard test	-	-	-	-	-
Glycosides	Picric acid solution test	-	-	-	-	-
Amino acids	Ninhydrin test	-	-	-	-	-
Saponins	Foam test	+	+	+	+	+

+ Presence, - absence. PE Sox (Petroleum ether extract), CHL Sox (chloroform extract), EA Sox (Ethyl acetate extract), MET Sox (Methanol extract) and AQ Sox (aqueous extract)

The qualitative phytochemical screening helped to understand the variety of secondary metabolites present in the extracts. Alkaloids, flavonoids, phenolics, tannins, saponins and carbohydrates were detected in all the extracts. The phytochemical classes noted in the extracts have shown to possess numerous therapeutic values.

Alkaloids are known to have antitumor, antipsychotic, hypoglycemic, antiviral, antibacterial, insecticidal and anti-inflammatory properties (shi et al., 2014). Phenolics are characterised by their prominent antioxidant activity which confers protection from oxidative stress. Significant anti-inflammatory, anti-carcinogenic, bactericidal, antiseptic, anthelmintic, cardioprotective and immune system promoting properties are also attributed to phenolic compounds (Tungmunnithum et al., 2018).

Flavonoids are known for their anti-cancer, anti-allergic, anti-inflammatory, anti-oxidant, and anti-viral potentials (Guardia et al., 2001; Hertog et al., 1995). Tannins have anticaries, bactericidal, antitumor, anti-oncogenic, anti-viral and antioxidant properties (Pizzi A., 2019). Plant steroids exhibits pharmaceutical activities like anti-inflammatory, immunosuppressive, anti-tumor, antibacterial, cytotoxic, hepatoprotective, antihelminthic, and cardiotonic activity (Patel and Savjani, 2015). Literature illustrates that saponins are hemolytic factors and possess antiviral, antibacterial, antifungal, anti-inflammatory, insecticidal, cytotoxic, molluscicidal and anticancer properties. They also exhibit cholesterol-lowering action (El Aziz et al., 2019).

4.3.2 Quantitative phytochemical analysis of extracts

The quantification of the secondary metabolites will aid in extraction, purification for surmising the biological activities of the extracts. So the quantitative estimation of the phytochemicals in the leaf extracts of *Polyalthia korintii* was carried out and the data are tabulated in Table 4.10.

4.3.2.1 Total phenolic content

The *P. korintii* leaves extracts showed significant total phenolic content. Higher phenolic content was observed in AQ Sox (11.072 ± 0.01 mg/g GAE) followed by MET Sox (5.303 ± 0.003 mg/g GAE), EA Sox (3.213 ± 0.001 mg/g GAE), CHL Sox (2.963 ± 0.003 mg/g GAE), and PE Sox (0.918 ± 0.001 mg/g GAE). The results show that phenolic compounds were extracted in to the solvents depending on their polarity. For instance, Oreopoulou, V. and Tzia, C. (2007) explained that certain phenolic terpenes were easily extracted with non-polar solvents like hexane and petroleum ether, some low molecular weight compounds (flavonoid aglycons) and phenolic acids were recovered with diethyl ether and ethyl acetate. Whereas flavonoid glycosides and higher molecular weight phenols was extracted with polar solvents.

The results obtained indicated that as the polarity of extraction solvent was increased during the sequential extraction process, the yield of phenolics also showed an increase. This observation is in accordance with the fact that the highly polar solvents are best at extracting most of the phenolic compounds. In other way, the results also show that the plant contains more of higher polarity phenolic compounds. Thus, the solubility behaviours of phenolic compounds in *P. korintii* reveal the presence of least polar to highly polar phenolic compounds.

4.3.2.2 Total Flavonoid content

The *P. korintii* leaves extracts showed significant total flavonoid content. Higher flavonoid content was observed in AQ Sox (139.291 ± 11.403 mg/g QE) and MET Sox (133.617 ± 4.958 mg/g QE) followed by EA Sox (36.312 ± 1.977 mg/g QE), CHL Sox (29.362 ± 2.815 mg/g QE), and PE Sox (11.773 ± 4.341 mg/g QE). As stated for phenolics, the flavonoids were also extracted in to the solvents according to their polarity. MET Sox and AQ Sox extracts had higher flavonoid content indicating the presence of more amount of polar flavonoids compared to other types of flavonoids.

Findings of Chávez-González et al., 2020 substantiate the results obtained. Their study on the methodologies for extraction of flavonoids found that the nature of the extracting solvent affected the type of flavonoid extracted; ethanol and methanol were the highest yielding etc. They also specified that apolar solvents (diethyl ether, ethyl acetate, dichloromethane, chloroform) are usually used in the extraction of methylated flavones, flavones and isoflavones; and polar solvents (ethanol and methanol) are employed to extract flavonoid glycosides and aglycones (polar flavonoids).

4.3.2.3 Total saponin content

Significant saponin content was observed in *P. korintii* leaves extracts. Higher saponin content was observed in PE Sox (309.7 ± 3.7 mg/g DE) and CHL Sox (309.7 ± 1.2 mg/g DE) followed by AQ Sox (241 ± 4.9 mg/g DE), EA Sox (196.2 ± 8.0 mg/g DE), MET Sox (195.9 ± 3.7 mg/g DE).

4.3.2.4 Total Tannin Content

Tannin content was observed in all the leaves extracts of *P. korintii*. Higher tannin content was observed in MET Sox (48.233 ± 0.002 mg/g TAE) followed by CHL Sox (28.061 ± 0.001mg/g TAE), EA Sox (24.447 ± 0.005 mg/g TAE), PE Sox (11.044 ± 0.001mg/g TAE) and comparatively negligible amount in AQ Sox (3.769 ± 0.001mg/g TAE).

Though the saponins contain glycone and sapogenin components which are polar and non-polar respectively, their extraction uses single solvent as well as mixture of solvents. The methanol extract had higher saponin content which is in agreement with the reports that water, methanol and ethanol are the mostly used solvents for tannin extraction (Yuliana et al. 2014, Case et al., 2014; Romero et al., 2020, Naima et al., 2015; Rhazi et al., 2019).

Table 4.10 Quantitative phytochemical analysis of *Polyalthia korintii* leaves extracts

Extracts	Total Phenolic Content (TPC) expressed as mg Gallic Acid Equivalents (GAE)/ g dry weight of extract	Total Flavonoid Content (TFC) expressed as mg Quercetin Equivalents (QE) / g dry weight of extract	Total Saponin Content expressed as mg Diosgenin Equivalents (DE) / g dry weight of extract	Total Tannin Content expressed as mg Tannic Acid Equivalents (TAE) / g dry weight of extract
PE Sox	0.918 ± 0.001	11.773 ± 4.341	309.7 ± 3.7	11.044 ± 0.001
CHL Sox	2.963 ± 0.003	29.362 ± 2.815	309.7 ± 1.2	28.061 ± 0.001
EA Sox	3.213 ± 0.001	36.312 ± 1.977	196.2 ± 8.0	24.447 ± 0.005
MET Sox	5.303 ± 0.003	133.617 ± 4.958	195.9 ± 3.7	48.233 ± 0.002
AQ Sox	11.072 ± 0.01	139.291 ± 11.403	241 ± 4.9	3.769 ± 0.001

All values are Mean ± SD

4.3.3 High Performance Thin Layer Chromatography analysis of the *Polyalthia korintii* leaves extract

High performance thin layer chromatography (HPTLC) is a common method to analyse the phytoconstituents owing to its efficiency in terms of sample and solvent usage, time, separation efficiency by the use of finer sorbent particles and thinner layers, band resolution, automation and reproducibility of results (Salmon, C. N et al., 2012). It is highly recommended for the standardization and quality control of herbal extracts.

Various solvent compositions of the mobile phase were examined for *Polyalthia korintii* leaves extract HPTLC analysis. PE Sox, CHL Sox and EA Sox extracts were developed under chamber saturation conditions using toluene: ethyl acetate (7:3) as the mobile phase. Ethyl acetate: Toluene: methanol (7:3:1) gave good separation of MET Sox extract components and for AQ Sox, Ethyl acetate: methanol: water: formic acid: acetic acid (9:1:1:1:1) was used.

Exposure of the developed HPTLC plate under UV 254 nm detected the presence of many organic compounds as dark and light bands in the green background. On UV 366 nm exposure, multi-colored bands of varying intensities were observed. The individual chromatograms were subjected to densitometric scanning using winCATS software and the percentage area units (AU) under individual peaks of the fractionated bands are included in the tabulated data.

Each of the extract showed different HPTLC pattern. The HPTLC analysis of PE Sox extract revealed 5 peaks at 254 nm and 4 peaks at 366nm (Fig.4.5 (a), (d) & (g)). Post-chromatographic detection done with anisaldehyde sulphuric acid reagent detected the blue spots representing monoterpenes (Gerlach et al. 2018). HPTLC chromatogram of CHL Sox extract exhibited 10 bands in longer UV and 7 bands in shorter UV. Many bands were iodine reactive and showed positive reaction towards anisaldehyde reagent.

Six bands under 366 nm and 3 bands under 254 nm were observed for EA Sox. Some bands were iodine reactive (yellow colored) and blue coloured spots (monoterpenes) were detected with anisaldehyde reagent (Fig.4.5 (b), (e) & (h)). On detection with iodine reagent, two spots were visible at Rf 0.09 and 0.13. Spots at Rf 0.09, 0.13 and 1.00 showed the presence of monoterpenes appearing blue colored with the use of Anisaldehyde sulphuric acid reagent (Fig.4.10 (c), (f) &(i)). Spots at Rf 0.10 (PE Sox), 0.09 (CHL Sox & EA Sox) and 0.91 (PE Sox), 0.92 (CHL Sox & EA Sox) were common in all the three extracts.

HPTLC analysis of MET Sox extract and AQ Sox extract are presented in Fig.4.6 and Fig.4.7 respectively. Detection with iodine reagent did not show specific separated bands in MET Sox chromatogram; instead the spot at 0.01 was yellow brown coloured and showed the presence of iodine reactive compounds. In AQ Sox chromatogram, spots at Rf 0.01 and 1.78 were iodine reactive. Usage of Anisaldehyde sulphuric acid reagent showed gray spot at Rf 0.01 of MET Sox; 0.01 and 1.78 of AQ Sox chromatogram.

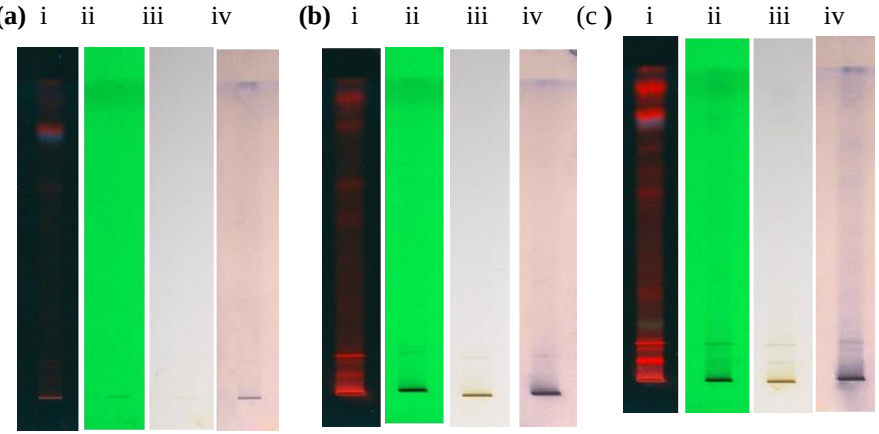
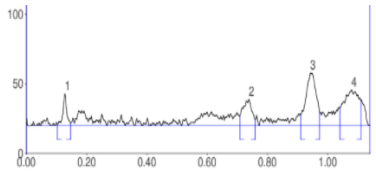


Fig. 4.5. HPTLC fingerprint of (a)PE Sox (b)CHL Sox and (c)EA Sox extract of *P. korintii* leaf. The extracts were developed under chamber saturation conditions using toluene: ethyl acetate (7:3) as the mobile phase. The lanes shows image taken under (i) UV 366 nm, (ii) 254 nm, (iii) detected with iodine and (iv) detected with anisaldehyde sulphuric acid reagent respectively. The densitometric scan from the chromatogram of extracts under UV 365 nm is represented in (d) PE Sox (e) CHL Sox and (f) EA Sox. The densitometric scan from the chromatogram of extracts under UV 254 nm is represented in (g) PE Sox (h) CHL Sox and (i) EA Sox. Observations under visible light for (iii) & (iv) are presented in the text.

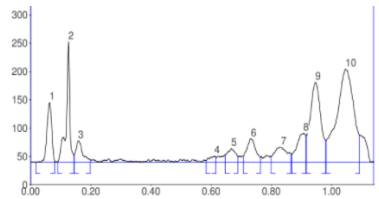
AU

(d)



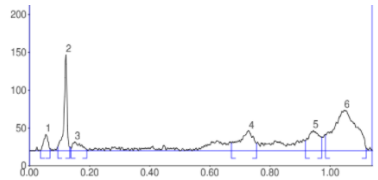
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.10	1.2	0.13	22.7	21.56	0.15	1.9	243.1	8.70
2	0.71	7.4	0.74	19.0	17.98	0.76	4.4	467.9	16.74
3	0.91	7.9	0.94	38.0	36.06	0.98	9.6	1055.5	37.76
4	1.04	13.0	1.08	25.8	24.41	1.11	18.3	1028.8	36.80

(e)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.02	0.0	0.06	105.2	12.87	0.08	2.4	1352.4	6.88
2	0.09	0.2	0.13	213.1	26.06	0.14	12.4	2082.5	10.60
3	0.15	13.4	0.16	38.2	4.67	0.20	4.9	689.0	3.51
4	0.58	3.2	0.61	11.1	1.35	0.62	9.6	192.4	0.98
5	0.65	13.3	0.67	24.4	2.99	0.69	11.4	541.7	2.76
6	0.71	10.8	0.73	41.3	5.06	0.77	8.7	1010.7	5.14
7	0.80	10.8	0.83	27.1	3.31	0.87	14.8	935.0	4.76
8	0.87	14.8	0.91	51.7	6.32	0.92	46.6	1227.1	6.25
9	0.92	47.2	0.95	140.9	17.23	0.98	38.2	4065.6	20.69
10	0.98	38.6	1.05	164.7	20.14	1.10	47.0	7550.0	38.43

(f)

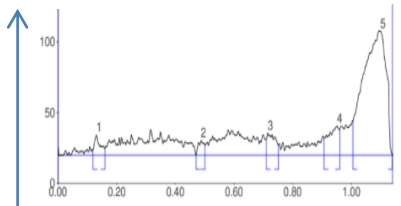


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.04	0.3	0.05	21.0	7.91	0.07	2.6	251.9	3.83
2	0.09	5.5	0.12	126.7	47.68	0.13	5.3	1032.6	15.71
3	0.14	3.3	0.15	11.6	4.35	0.19	1.7	261.8	3.98
4	0.67	10.0	0.73	26.2	9.86	0.76	9.7	969.7	14.76
5	0.92	13.5	0.95	26.7	10.05	0.97	17.9	813.9	12.39
6	0.99	21.0	1.05	53.6	20.15	1.12	3.0	3241.6	49.33

Rf

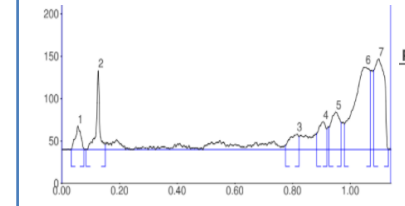
AU

(g)



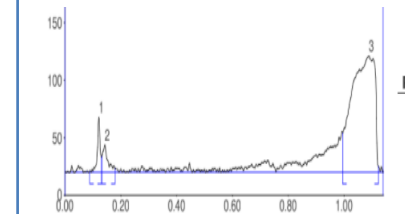
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.12	3.9	0.13	14.0	9.50	0.16	4.6	230.1	3.34
2	0.47	0.0	0.49	10.2	6.93	0.50	7.2	160.0	2.33
3	0.71	11.2	0.72	15.7	10.61	0.75	7.4	366.2	5.32
4	0.91	12.2	0.95	20.3	13.76	0.96	17.9	612.8	8.91
5	1.01	23.9	1.10	87.5	59.21	1.14	0.6	5509.8	80.10

(h)



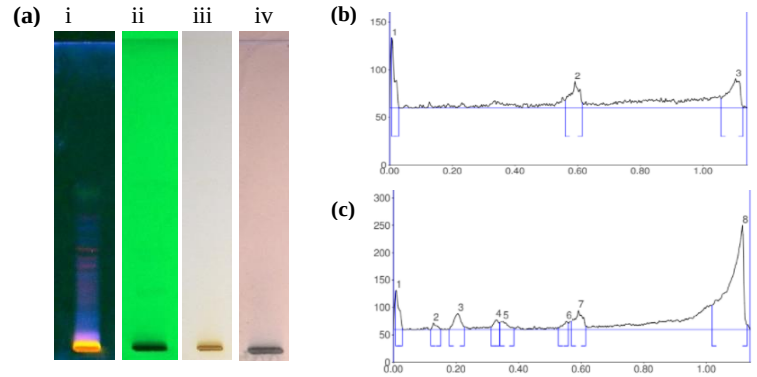
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	0.3	0.06	28.0	6.66	0.08	0.6	419.9	3.84
2	0.08	0.5	0.13	93.3	22.16	0.15	5.9	885.9	8.11
3	0.78	5.0	0.82	17.9	4.25	0.82	14.9	464.7	4.25
4	0.89	19.5	0.91	32.8	7.79	0.92	24.6	712.5	6.52
5	0.93	26.6	0.95	44.4	10.56	0.97	32.3	1123.1	10.28
6	0.98	30.3	1.06	97.5	23.16	1.07	92.9	4379.5	40.09
7	1.08	94.1	1.10	107.0	25.42	1.13	0.4	2938.8	26.90

(i)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.09	0.9	0.12	48.1	27.72	0.13	13.0	395.6	5.20
2	0.13	14.0	0.14	24.0	13.84	0.18	3.6	376.5	4.95
3	1.00	34.4	1.09	101.4	58.44	1.13	5.4	6832.1	89.85

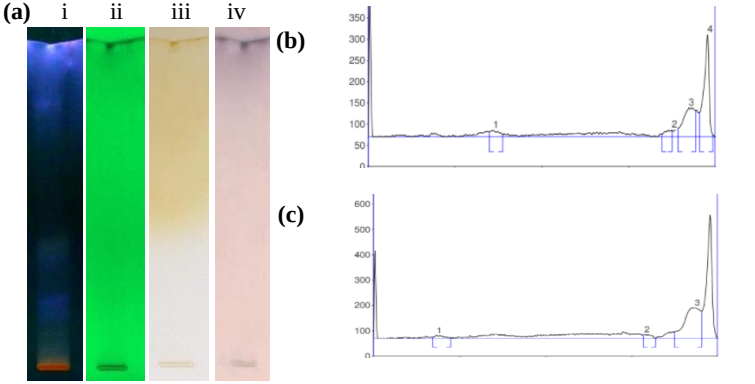
Rf



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.01	73.8	0.01	73.8	55.83	0.03	1.5	540.0	26.11
2	0.56	7.6	0.59	27.8	21.02	0.62	5.7	614.8	29.73
3	1.06	9.9	1.11	30.6	23.15	1.13	0.9	913.1	44.16

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.01	70.3	0.01	71.6	18.58	0.03	3.1	633.1	6.71
2	0.12	0.6	0.13	11.9	3.09	0.15	0.5	129.7	1.37
3	0.18	0.2	0.21	29.1	7.55	0.23	5.2	533.5	5.65
4	0.31	4.6	0.33	18.6	4.83	0.34	11.9	248.5	2.63
5	0.34	13.0	0.35	14.3	3.72	0.39	2.5	287.3	3.04
6	0.53	3.4	0.55	15.0	3.89	0.56	12.2	208.4	2.21
7	0.57	15.5	0.59	34.8	9.02	0.62	6.1	702.1	7.44
8	1.02	44.5	1.12	190.1	49.32	1.13	8.6	6694.6	70.94

Fig. 4.6. HPTLC fingerprint of MET Sox extract of *P. korintii* leaf. The extracts were developed under chamber saturation conditions using Ethyl acetate: Toluene: methanol (7:3:1) as the mobile phase. The lanes i-iv shows image taken under UV 366 nm, 254 nm, detected with iodine and detected with anisaldehyde sulphuric acid reagent respectively. (b) The densitometric scan from the chromatogram of extract under UV 366 nm. (c) The densitometric scan from the chromatogram of extract under UV 254 nm. Observations under visible light for (iii) & (iv) are presented in the text.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.35	12.1	0.36	15.0	4.41	0.78	5.7	370.0	5.98
2	0.845	5.7	0.875	15.8	4.64	1.75	15.1	308.1	4.98
3	0.89	19.5	0.925	68.2	20.09	1.89	61.9	2134.9	34.48
4	0.955	57.8	0.98	240.6	70.86	1.99	3.7	3378.0	54.6

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.17	4.6	0.185	13.3	8.76	0.45	1.6	309.9	5.22
2	0.785	14.2	0.79	16.4	10.82	1.64	1.7	335.7	5.66
3	0.875	26.7	0.935	121.6	80.42	1.91	107.4	5288.1	89.12

Fig. 4.7. HPTLC fingerprint of AQ Sox extract of *P. korintii* leaf. The extracts were developed under chamber saturation conditions using Ethyl acetate: methanol: water: formic acid: acetic acid (9:1:1:1:1) as the mobile phase. The lanes i-iv shows image taken under UV 366 nm, 254 nm, detected with iodine and detected with anisaldehyde sulphuric acid reagent respectively. (b) The densitometric scan from the chromatogram of extract under UV 366 nm. (c) The densitometric scan from the chromatogram of extract under UV 254 nm. Observations under visible light for (iii) & (iv) are presented in the text.

4.3.4 Spectroscopic analysis of extracts

The analysis of phytoconstituents will help in determining the various biological activities of the plant extracts. The spectroscopic techniques being simple and cost-effective are widely used analytical tool for the analysis of pharmaceutical materials.

4.3.4.1 Fourier-Transform Infrared Spectroscopy (FTIR) analysis

FTIR analysis of plant extracts aids in identifying the characteristic functional groups present in them. The peak values obtained as a spectrum in the infra-red region corresponds to the specific molecular fingerprint of the compounds contained in the extract and thus provides structural insights (Karpagasundari and Kulothungan, 2014).

The absorption spectrum frequency recorded from the extracts was compared with those reported from the literature and the results are tabulated. The results of the FTIR spectroscopy confirmed the presence of various chemical constituents such as alcohols, carboxylic acids, carbohydrates, glycosides, amines, flavonoids, polyphenolics, aromatic compounds etc. in PE Sox extract (Table 4.11).

Presence of phenolics, alcohols, aromatics, nitro compounds, alkyl halides, polysaccharides etc. were detected in CHL Sox (Table 4.12). Alcohols, aldehydes, alkane, keto, ether, alkyne, and amine were identified in EA Sox extract (Table 4.13).MET Sox extract showed the presence of alcohols, alkanes, aromatic esters, amines, aliphatic amines, and aromatics (Table 4.14). Alcohols, phenols, alkyl, alkenes, alkanes, ethers, aromatics, aliphatic amines, and alkenes were noticed in AQ Sox extract (Table 4.15). The FTIR spectrum of extracts is provided in Appendix.

Table 4.11. FTIR spectral peak values of PE Sox extract of *P. korintii*

Frequency (cm^{-1})	Component peaks	References
3462.56	-NH vibration	Krstić et al., 2015
3019.01	alcohol or amino groups	Solíz-Guerrero, J. B., et al.,2002
2971.77	CH- stretching vibrations alkane type C−H stretching (C−H stretching vibration in CH_3)	Sultana, J., & Ahmed, F. R. S. (2013).
2934.16	asymmetric stretching vibration of the C-H bond of alkane	Nayem, S. M et al 2020
1714.41	carbonyl groups C=O stretching	Ashokkumar, R., & Ramaswamy, M. (2014)
1455.03	CH_2 deformation stretching; CH deformation asymmetric in plane; HCH and OCH in plane bending vibration.	Brangule, A., Šukele, R., & Bandere, D. (2020).
1377.89	C-H bending in alkane, especially for methyl group	Ashokkumar, R., & Ramaswamy, M. (2014)
1214.93	C-O stretching of Alcohols, carboxylic acids, esters, ethers	Omar, S et al., 2020
1045.23	–CH_2OH groups and to the C−O stretching and bending of the C–OH moiety of carbohydrates	Balan, V et al., 2019
929.521	vibration of β-glycoside	Fu, Z et al., 2020
744.388	N-H stretching vibration presence of primary and secondary amines	Kabra, A et al., 2019
667.25	presence of flavonoids and polyphenols	Sarkar, S., & Kotteeswaran, V. (2018
624.823	aromatic class. acetylenic C–H bending vibrations	Prabu, H. J., & Johnson, I. (2015) Kaur, P., Thakur, R., & Chaudhury, A. (2016)

Table 4.12. FTIR spectra analysis of CHL Sox extract of *P. korintii*

Frequency (cm^{-1})	Component peaks	References
3419.17	O-H stretching alcohols	Coates J, 2006
	O-H stretching vibration of alcohol or phenolic compounds	Choudhury S.R. et al. ,2013 Nayem, S. M et al 2020
2917.77	asymmetric stretching vibrations of the methylene groups	Heredia-Guerrero, J. A et al., 2014
	asymmetric CH stretching in aromatic methoxyl groups and in methyl and methylene groups of side chains	Turki, A et al., 2018
2849.31	symmetric stretching in CH_2 ,	Topală, C. M., & Rusea, I. (2018).
	C–H stretching in methyl of aromatic and methylene groups of side chains and aromatic methoxyl groups.	Salim, R. M., Asik, J., & Sarjadi, M. S. (2021)

Continued…

1714.41	carbonyl/ carboxyl groups	Heredia-Guerrero, J. A et al., 2014
	carbonyl groups C=O stretching	Wahyono, T et al., 2019
	due to carboxylic acid	Lionetto, F et al.,2012
1461.78	-CH_3 and –CH_2 bending	Zhang, Z. P., Rong, M. Z., & Zhang, M. Q. (2013
	the bending vibration peak of methylene	Shi, L., et al., 2013
1381.75	Nitro compounds (N–O asymmetrical stretching	Janakiraman, N., & Johnson, M. (2015).
1165.76	Alkyl halides (C–F stretch)	Janakiraman, N., & Johnson, M. (2015).
1048.12	indicates the presence of polysaccharides	Muruganantham, S., Anbalagan, G., & Ramamurthy, N. (2009).
	C-O stretching vibrations of polysaccharides	Mihoubi, Wafa, et al. 2017
719.318	methylene rocking vibration	Dubis, E. N., Dubis, A. T., & Morzycki, J. W. (1999).

Table 4.13. FTIR spectra analysis of EA Sox extract of *P. korintii*

Frequency (cm^{-1})	Component peaks	References
3420.14	OH stretching vibration	Lakshmanan, G et al., 2018
		Sharma, G et al, 2014
2919.7	C–H stretching vibrations of	Kumar, B et al, 2014
2850.27	methyl, methylene and methoxy groups	
1717.3	Stretching vibration of free keto group	Suresh, S., Karthikeyan, S., & Jayamoorthy, K. (2016)
1455.99	asymmetric bending vibration of methyl groups	Abraham, J. P et al., 2003
1382.71	stretching of O-H groups	Malaikozhundan, B et al ., 2016
1258.32	C-O group	Adib, A. M., & Abdullah, Z. (2018).
1166.72	C-C stretch	Sushma, N. J. (2013).
		Tarantilis, P. A et al., 1998
1045.23	presence of C-N functional group	Oktri Mulya Dewi, N., et al., 2019
607.467	C-H bend of alkynes	Vidhu, V. K., & Philip, D. (2014).

Table 4.14. FTIR spectra analysis of MET Sox extract of *P. korintii*

Frequency (cm^{-1})	Component peaks	References
3397.96	OH bond stretching	Chandra, H et al, 2019
2919.7	C–H stretching vibrations of	Kumar, B et al, 2014
2851.24	methyl, methylene and methoxy groups	
1620.88	C=C of aromatic ring	Asing, J., Wong, N. C., & Lau, S. (2009)
1456.96	bending vibration of C-H and the stretching vibration of aromatics	Oliveira, R. N et al, 2016
1384.64	C–H bending	Saravanakumar, A., et al., 2015
1284.36	vibration of C-O stretching	Megawati, W et al., 2020

Continued…

1168.65	C–N stretching vibration of the imidazolium ring	Ambika, S., & Sundrarajan, M. (2016).
1078.98	aliphatic amines	Sana, S. S., & Dogiparthi, L. K. (2018).
808.992	-	-
615.181	indicates aromatic compounds of alkanes.	Fazlzadeh, M. et al., 2017
470.646 451.261	corresponding to metal–oxygen (M–O) bond	Rajiv, P., Rajeshwari, S., & Venckatesh, R. (2013)

Table 4.15. FTIR spectra analysis of AQ Sox extract of *P. korintii*

Frequency (cm^{-1})	Component peaks	References
3854.04	O–H stretching vibration	Lingegowda, D. C et al., 2012
3385.42	asymmetric stretching H−O−H vibration (Hydroxy group, H-bonded OH stretch)	Sakib, A. A. M. et al., 2019 Khader, S. Z. A et al., 2018
2933.2	stretching vibrations of alkyl C–C	Ghaedi, M et al., 2015
1623.77	stretch vibration of –C=C-	Satyavani, K., Ramanathan, T., & Gurudeeban, S. (2011).
1457.92	C–H bend of alkanes	Janakiraman, N., & Johnson, M. (2015).
1278.57	Asymmetric stretching vibration in C-O-C	El Kaaby Ekhlas, A., Al Hattab Zahra, N., & AI-Anny Jenan, A. (2016).
1194.69	C–H bending vibrations in aromatic rings.	Singh, C., et al., 2019
1078.01	deviation of the nitrogen-carbon C-N bond stretching of aliphatic amine groups or to a combination of alcohols and phenols	Sadeghi, B., Mohammadzadeh, M., & Babakhani, B. (2015).
1048.12	C-N stretching vibration of aliphatic amines C–O bond	Wang, T. et al., 2014 Lingegowda, D. C et al., 2012
617.109	resemblance to alkenes	Jacob, S. J. P., Finub, J. S., & Narayanan, A. (2012).
456.082	corresponding to metal–oxygen (M–O) bond	Rajiv, P., Rajeshwari, S., & Venckatesh, R. (2013)

4.3.4.2 Gas chromatography-mass spectrometry (GC–MS) analysis

The GC–MS chromatogram of PE Sox extract recorded a total of 30 peaks (Table 4.16) and the compounds were identified from the National Institute of Standards and Technology (NIST) library. The major compound was Alpha-Curcumen (22.83 %). 34 compounds were identified in CHL Sox extract (Table 4.17). The major compounds were alpha-curcumen (12.03%) and 1-[2-Methyl-2-(4-methyl-3-pentenyl)cyclopropyl]ethanol (11.88%).

EA Sox extract contained 23 compounds (Table 4.18). Pentacosane (65.38 %) was the major metabolite. Among the 9 compounds identified from MET Sox extract, Tetratetracontane (50.19%) was the major compound (Table 4.19). The GC-MS chromatogram of extracts is presented in Appendix.

From the data it was evident that the extracts were rich in metabolites with antibacterial activity (Phytol, Pentacosane, Nonacosane, 9-Eicosene, (E)-) (Kalsum, N. et al., 2016; Konovalova, O.et al., 2013) , antifungal activity (Tau.-Cadinol) (Ho, C. L. et al., 2011), anticancer activity (β-elemene, Alpha-Curcumen, Spathulenol, Beta.-Caryophyllene Epoxide, Phytol, 9-Eicosene, (E)-) (Yang, H et al., 1997; Dahham, S. S. et al., 2015; Hadem, K. L., & Sen, A. 2017), anti-inflammatory activity (Phytol, vitamin E) (Karthikeyan, V.et al., 2016) and anti-oxidant activity (vitamin E, tetratetracontane) (Kalsum, N. et al., 2016).

Table 4.16 Compounds identified from GC-MS analysis of PE Sox extract

Sl. No.	Retention Time	Compound Name	Peak area (%)
1	14.336	Beta-Elemen-(2)	1.39
2	15.429	Alpha.-Bergamotene	1.76
3	16.618	Alpha-Curcumen	22.83
4	16.712	Cis-.Beta.-Farnesene	1.58
5	16.859	Eudesma-4(14),11-Dien	1.70
6	17.036	Alpha.-Selinene	0.97
7	17.264	Beta-Bisabolen	1.68
8	18.925	2,3-Dimethyl-2,3-Diphenylbutane	2.44
9	18.983	(-)-Spathulenol	6.16
10	19.113	(-)-.Beta.-Caryophyllene Epoxide	3.66
11	19.799	Beta.-Sesquiphellandrene	1.10
12	20.470	Tau.-Cadinol	1.68
13	20.786	Benzenebutanal, .Gamma.,4-Dimethyl-	2.64
14	20.857	Junipercamphor	3.58
15	21.425	Cis-Sesquisabinene Hydrate	0.94
16	21.640	6,10-Dodecadien-1-Yn-3-Ol, 3,7,11-Trimethyl-	3.82
17	21.967	(+)-Thunbergen	2.50
18	22.338	Aromadendrenoxid-(1)	2.34
19	22.784	Delta.-Cuparenol	2.68
20	23.475	7-Epi-Trans-Sesquisabinene Hydrate	1.35
21	24.102	1,6,10,14,18,22-Tetracosahexaen-3-Ol, 2,6,10,15,19,23-Hexamethyl-, (All-E)-	3.54
22	24.323	1-[2-Methyl-2-(4-Methyl-3-Pentenyl)Cyclopropyl]Ethanol	9.86
23	25.281	Tricyclo[20.8.0.0(7,16)]Triacontane, 1(22),7(16)-Diepoxy-	2.23
24	25.667	Beta-Cedren-9-Alpha-Ol	6.48
25	25.774	E-9-Hexadecenal	1.13

Continued…

26	25.872	2,2-Dimethyl-1-(3-Oxo-But-1-Enyl)-Cyclopentanecarboxaldehyde	2.43
27	23.327	Alpha.-Bisabolol	1.93
28	26.053	1S,3R,4S,5R,6S-1-Hydroxy-2,2,3,4,5,6-Hexamethyl-8-Oxo-7,9-Dioxatricyclo[4.2.1.0(3,5)]Nonane	2.82
29	27.125	2(1H)-Naphthalenone, Octahydro-4a,7,7-Trimethyl-, Trans-	1.39
30	31.486	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-, [R-[R*,R*-(E)]]- (T-Phytol)	1.38

Table 4.17 Compounds identified from GC-MS analysis of CHL Sox extract

Sl. No.	Retention Time	Compound Name	Peak area (%)
1	14.340	Beta-Elemen-(2)	2.7
2	15.138	(-)-Beta-Caryophyllen	1.51
3	15.428	.Alpha.-Bergamotene	1.77
4	16.605	Alpha-Curcumen	12.03
5	16.704	(E)-.Beta.-Famesene	1.34
6	16.858	(+)-.Beta.-Selinene	1.59
7	16.946	.Alpha.-Zingiberene	7.04
8	17.035	.Alpha.-Selinene	0.86
9	17.250	.Beta.-Methylionone	2.05
10	18.544	Germacrene B	1.13
11	18.978	(-)-Spathulenol	4.23
12	19.108	(-)-.Beta.-Caryophyllene Epoxide	1.83
13	19.264	9-Eicosene, (E)-	1.24
14	20.468	.Tau.-Cadinol	1.35
15	21.635	3,7,11-Trimethyldodeca-6,10-Dien-1-Yn-3-Ol	3.42
16	21.997	Dihydroionone	2.47
17	22.332	Aromadendrene Oxide-(1)	1.63
18	22.777	Alpha.-Chamigrene	2.16
19	23.326	Isoaromadendrene Epoxide	1.72
20	23.462	2,6,11-Tridecatrien-10-Ol, 2,6,10-Trimethyl-	2.65
21	23.728	9-Eicosene, (E)-	1.94
22	24.092	1-[2-Methyl-2-(4-Methyl-3-Pentenyl)Cyclopropyl]Ethanol	11.88
23	24.506	3,7,11,15-Tetramethyl-2,6,10,14-Hexadecatetraenyl Acetate	1.42
24	24.630	Neophytadiene	3.48
25	25.267	Beta-Cedren-9-Alpha-Ol	8.74
26	25.510	3,7,11,15-Tetramethyl-2-Hexadecen-1-Ol	1.54
27	25.771	2,2-Dimethyl-1-(3-Oxo-But-1-Enyl)-Cyclopentanecarboxaldehyde	2.93
28	25.863	Longipinocarveol, Trans-	2.22
29	26.045	Jatamansone, (+)-	1.96
30	28.047	1-Octadecene	1.52
31	31.486	2-Hexadecen-1-Ol, 3,7,11,15-Tetramethyl-, [R-[R*,R*-(E)]]- (T-Phytol)	1.92
32	34.402	1-Heneicosanol	1.05
33	39.477	Heptadecyl Trifluoroacetate	0.85
34	42.191	Pentacosane	3.83

Table 4.18 Compounds identified from GC-MS analysis of EA Sox extract

Sl. No.	Retention Time	Compound Name	Peak area (%)
1	16.607	Alpha-Curcumen	0.74
2	17.241	Phenol, 3,5-Bis(1,1-Dimethylethyl)-	0.35
3	19.263	1-Tetradecanol	0.69
4	20.490	1,4,2,5 Cyclohexanetetrol	3.76
5	20.787	Benzene, 1-(1,4-Dimethyl-4-Hexenyl)-2-Methyl-, (Z)-(.+-.)-	1.27
6	20.912	Cyclohexane, 2-(3-Iodopropyl)-1,1-Dimethyl-3-Methylene-	0.31
7	21.634	3,7,11-Trimethyldodeca-6,10-Dien-1-Yn-3-Ol	1.37
8	22.796	Cyclohexanol, 2-Methyl-3-(1-Methylethenyl)-, Acetate, (1.Alpha.,2.Alpha.,3.Alpha.)-	1.08
9	23.460	Nerolidol Z And E	0.97
10	23.728	9-Eicosene, (E)-	1.23
11	24.073	Alpha.-Bisabolol	1.06
12	24.633	Neophytadiene	9.22
13	24.717	2-Pentacosanone	1.56
14	25.252	Geranyl-.Alpha.-Terpinene	1.36
15	25.508	3,7,11,15-Tetramethyl-2-Hexadecen-1-Ol	2.43
16	25.768	2,2-Dimethyl-1-(3-Oxo-But-1-Enyl)-Cyclopentanecarboxaldehyde	0.61
17	26.041	1S,3R,4S,5R,6S-1-Hydroxy-2,2,3,4,5,6-Hexamethyl-8-Oxo-7,9-Dioxatricyclo[4.2.1.0(3,5)]Nonane	0.52
18	28.046	(Cis)-2-Nonadecene	1.24
19	31.491	Phytol, Acetate	3.10
20	34.400	1-Tetradecanol	0.42
21	42.188	Octadecanal	0.90
22	43.367	Pentacosane	65.38
23	45.808	Cyclopentane, (2-Hexyloctyl)-	0.41

Table 4.19 Compounds identified from GC-MS analysis of MET Sox extract

Sl. No.	Retention Time	Compound Name	Peak Area (%)
1	24.628	Neophytadiene	3.79
2	37.616	2-Methyloctacosane	9.38
3	38.652	3.Alpha.,5.Alpha.-Cyclo-Ergosta-7,9(11),22t-Triene-6.Beta.-Ol	9.37
4	41.477	Rhodopin	6.63
5	41.787	Tetratetracontane	50.19
6	42.575	Vitamin E	3.05
7	43.217	Stigmasterol	2.97
8	44.752	Gamma.-tocopherol	4.61
9	45.825	Dodecanoic acid, 1,2,3-propanetriyl ester	10.0

Thus, a good number of volatile compounds were identified from various extracts of *P. korintii* leaves by GC-MS. Most of the compounds have previously been reported from *Polyalthia* and other plant species.

The present study thus validates the pharmacognostic and physicochemical characteristics of *Polyalthia korintii* leaf samples to aid in identification, authentication, in establishing quality standards, and to rule out the presence of any adulterants or impurities. Among the three different methods employed to assess their effectiveness in extracting the *P. korintii* leaves to obtain the maximum yield, sequential soxhlation proved to be high yielding and less time consuming. The study thus helped to decide on the best extraction method and an ideal solvent to extract the phytoconstituents from *Polyalthia korintii* leaves and to employ them in bioprospecting.

Further the sequential soxhlated extracts subjected to qualitative phytochemical screening revealed the presence of plant metabolites like alkaloids, flavonoids, phenolics, tannins, carbohydrates, steroids and saponins and these were quantified to aid in extraction and purification of the bio actives. The extracts were standardised using HPTLC and spectroscopic studies. The spectroscopic analysis of *P. korintii* extracts revealed the presence of various classes of secondary metabolites and identified the presence of bioactive phytoconstituents. Thus, the identification of these biologically active metabolites in the extracts of the scientifically unexplored endemic species *Polyalthia korintii* urges for exploration of the pharmacological activities of these extracts.

5. *IN VITRO* PHARMACOLOGICAL ACTIVITY OF *Polyalthia korintii* LEAVES EXTRACTS

5.1 Assessment of *in vitro* antioxidant activities of the extracts

The imbalance in the oxidant: antioxidant levels, either due to increased free radical production or due to diminished inherent antioxidant amounts, results in oxidative stress. When the balance between the activity of these free radicals and the capacity of the body's antioxidant mechanism is disturbed, the biological functions like anti-carcinogenic, anti-aging, anti-mutagenic activities are also hampered. Using dietary antioxidants and providing medicinal supplements are safe ways to restore the lost balance.

So, recently more interest is drawn towards the naturally occurring antioxidants from plants and other living forms owing to their free radical scavenging abilities (Shukla, S., & Mehta, A. 2017). In this regard, to acquire comprehensive knowledge on the antioxidant potential of the *Polyalthia korintii* leaf extracts, different antioxidant testing methods were adopted including the cell-based antioxidants assays and the results are discussed below.

5.1.1 DPPH free radical scavenging assay

All the extracts from *P. korintii* exhibited DPPH free radical scavenging activity at the concentrations (50-400 µg/ml) tested (Fig. 5.1). It was observed that MET Sox and AQ Sox exhibited the highest scavenging activity, followed by PE Sox, CHL Sox and EA Sox. The DPPH radical scavenging abilities of the MET Sox and AQ Sox extracts were comparable to the activity shown by the reference compound, Ascorbic acid.

Table 5.1 summarises the comparison of the activity expressed in terms of IC_{50} values. The lower the IC_{50} value, the higher is its radical scavenging activity. Clearly the data shows that the extracts possessed proton donating ability and hence could be used as free radical scavengers. Since MET Sox and AQ Sox extracts had lowest IC_{50} values, they are the strongest DPPH free radical scavengers compared to other extracts.

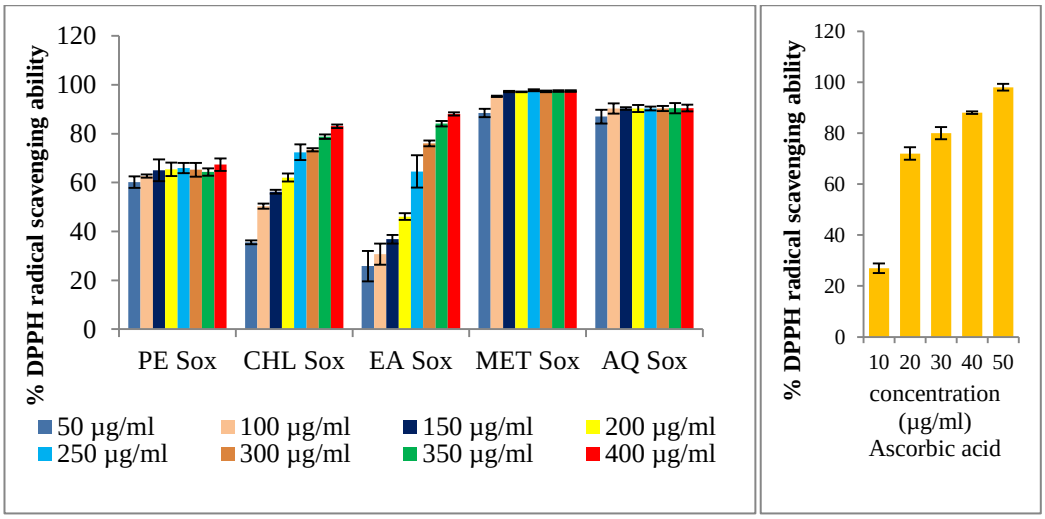


Fig. 5.1 Dose-response depiction of % DPPH radical scavenging activity of *P. korintii* leaf extracts compared with Ascorbic acid. All the extracts exhibited DPPH free radical scavenging activity. MET Sox and AQ Sox have the highest scavenging activity comparable to the activity shown by the reference compound, Ascorbic acid. All values are represented as mean ± SD.

Table 5.1 DPPH radical scavenging activity and IC_{50} values of extracts

Extracts	IC_{50} values Mean ± SD (µg/ml)
PE Sox	120.9 ± 8.5
CHL Sox	160.7 ± 1.4
EA Sox	198.8 ± 2.5
MET Sox	16.2 ± 1.8
AQ Sox	17.7 ± 2.4
Ascorbic acid	15.2 ± 1.7

5.1.2 ABTS radical scavenging assay

The *P. korintii* leaves extract were effective scavengers of the $ABTS^{\cdot+}$ radical at higher concentrations (Fig. 5.2). The ABTS cation scavenging activity of the extracts followed the order MET Sox > EA Sox > CHL Sox > AQ Sox > PE Sox (Table 5.2). The activity exhibited by the extracts was significantly lower than the reference compound as is evident from the large variations in IC_{50} values.

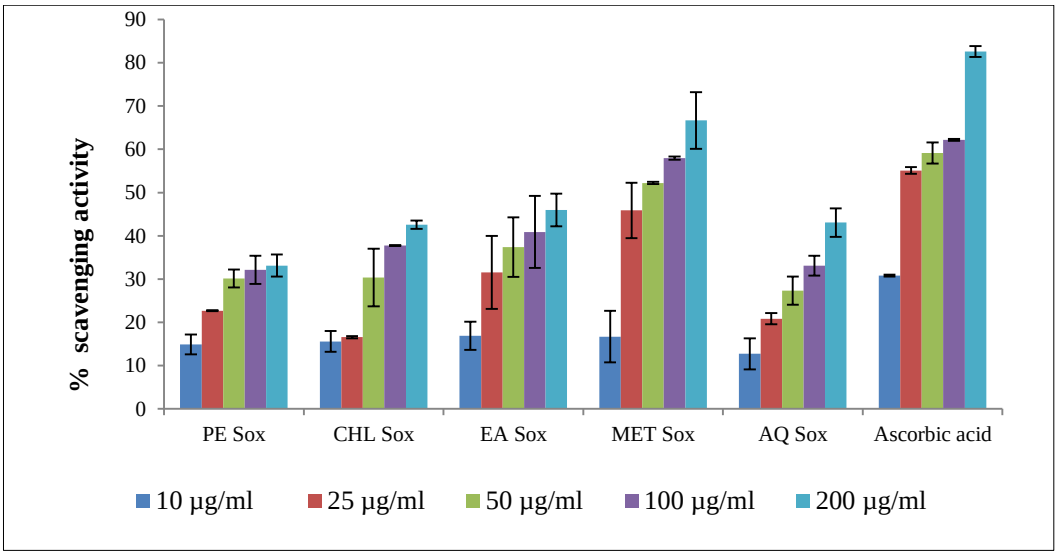


Fig. 5.2 Dose-response depiction of % ABTS radical scavenging activity of *P. korintii* leaves extract compared with Ascorbic acid. ABTS radical cation produced by reacting ABTS (7mM) with potassium persulfate (2.45 mM) was scavenged by the plant extracts. MET Sox exhibited higher scavenging potentials. Values are Mean ± SD of three independent experiments.

Table 5.2 ABTS radical scavenging activity and IC_{50} values of extracts

Extracts	IC_{50} values (µg/ml) Mean ± SD
PE Sox	303.5 ± 14.2
CHL Sox	207.5 ± 2.7
EA Sox	186.1 ± 16.3
MET Sox	101.9 ± 13.4
AQ Sox	215.5 ± 12
Ascorbic acid	18.3 ± 0.3

5.1.3 Fe (III) reducing power-based antioxidant capacity assays /ferric ion based antioxidant capacity

Single electron transfer based methods currently used for measuring reducing potential of antioxidants involves the use of ferric ions as such, as in potassium ferricyanide and those that are linked to ligands like 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ). The Fe (III) reducing ability of a sample is depended on the pH of the redox system. Ferric Reducing Antioxidant Power (FRAP) assay uses

TPTZ and is operational in acidic pH whereas Ferricyanide method of reducing power uses potassium ferricyanide and the reactions are carried out at the neutral pH of $[Fe(CN)_6]$(Berker, K. I et al., 2007). So both assays were employed to assess the reducing potential of antioxidants in the *P. korintii* leaf extracts.

5.1.3.1 Ferric Reducing Antioxidant Power (FRAP) assay

$FeSO_4.7H_2O$ at different concentrations (0.1-1 mM) was used to prepare an absorbance vs. concentration standard curve and the reducing ability of the extracts was calculated in terms of the reducing power of $FeSO_4.7H_2O$.

All the extracts showed considerable reducing power in FRAP assay (Fig. 5.3). Gallic acid, ascorbic acid and quercetin were also used to provide a comparison. Quercetin exhibited the highest reducing power with an activity equivalent to 1 mM $FeSO_4.7H_2O$ at a concentration of 0.36 ± 0.03 mg/ml followed by gallic acid and ascorbic acid at concentrations 0.43 ± 0.08 mg/ml and 0.69 ± 0.09 mg/ml respectively.

Among the *P. korintii* extracts, MET Sox had higher reducing power, even greater than the ascorbic acid. CHL Sox, EA Sox and AQ Sox extracts showed activity comparable to other extracts and the standard compounds used (Table 5.3). PE Sox had the least reducing ability.

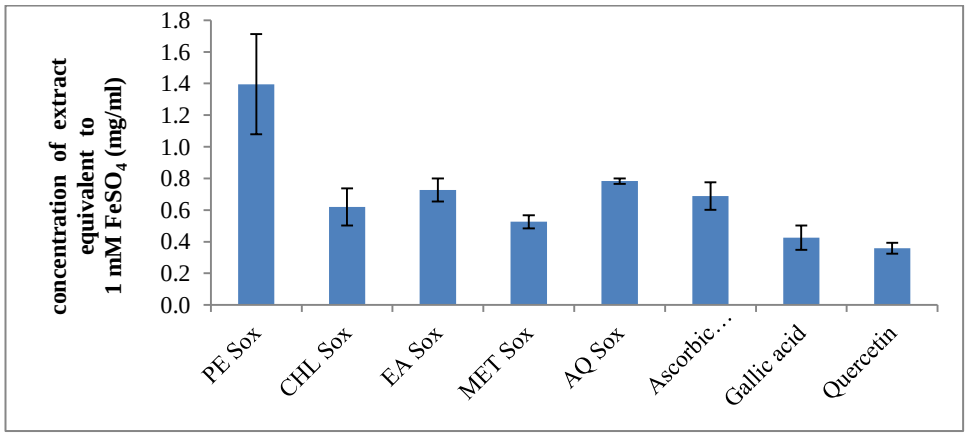


Fig. 5.3 Ferric Reducing Antioxidant Power (FRAP) of *P. korintii* leaf extracts. FRAP reagent containing TPTZ was treated with extracts; the increase in absorbance was measured and expressed as 1 mM $FeSO_4$ equivalents. MET Sox and PE Sox had the higher and the least reducing ability. Values are Mean ± SD of three independent experiments.

Table 5.3 Ferric Reducing Antioxidant Power (FRAP) of *P. korintii* leaf extracts

Extracts	1 mM $FeSO_4$ equivalents (mg/ml) Mean ± SD
PE Sox	1.40 ± 0.32
CHL Sox	0.62 ± 0.12
EA Sox	0.73 ± 0.07
MET Sox	0.53 ± 0.04
AQ Sox	0.78 ± 0.02
Ascorbic acid	0.69 ± 0.09
Gallic acid	0.43 ± 0.08
Quercetin	0.36 ± 0.03

5.1.3.2 Ferricyanide method of reducing power assay

As in Fig. 5.4, all the *P. korintii* leaf extracts showed reducing power in a dose dependent manner. AQ Sox extract gave the highest reducing power and is higher than that of all other extracts at corresponding concentrations studied. The order of reducing power of the extracts is as follows: AQ Sox > MET Sox > EA Sox> CHL Sox> PE Sox.

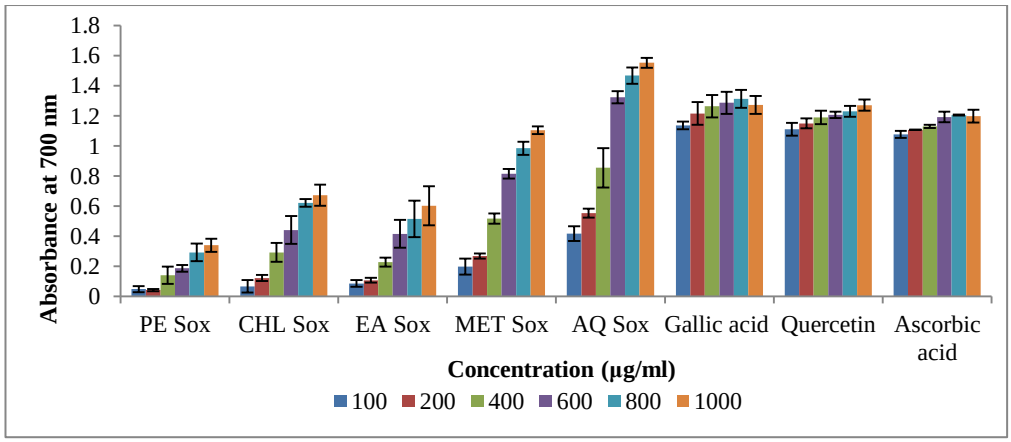


Fig. 5.4. Reducing power of *P. korintii* leaf extracts determined using ferricyanide. Higher absorbance value of the reaction mixture indicates greater reducing power. Values are Mean ± SD of three independent experiments.

The free radical scavenging activity and the reducing power property indicates that the compounds in *P. korintii* leaf extracts can act as electron donors and can reduce the oxidized intermediates. They are therefore useful as primary and secondary antioxidants.

5.1.4 Metal chelating activity: Chelation Power on Ferrous (Fe^{2+}) Ions

The chelating agents can be used to reduce the formation of reactive oxygen species and by virtue of this activity, they also possess antioxidant potential. As can be seen from Fig. 5.5, all five extracts demonstrated strong chelating activities in a concentration-dependent manner. EA Sox had the highest ferrous ion chelating activity (56.7 -79.2 %) followed by PE Sox (31.2- 75.9 %) and CHL Sox (26.5- 77.2 %) at 50-200 µg/ml. MET Sox and AQ Sox had 13.5- 93.7 % and 0.6- 84.8 %activity at concentration of 15.6-500 µg/ml. EDTA, the reference compound showed 13.6- 93.7 % activity in the tested range. IC_{50} values were calculated and used for comparison (Table 5.4). EA Sox had remarkable chelation power comparable to EDTA. In this assay, the extracts of *P. korintii* interfered actively with the formation of a ferrous and ferrozine complex, indicating its chelating potential by capturing the ferrous ions prior to ferrozine.

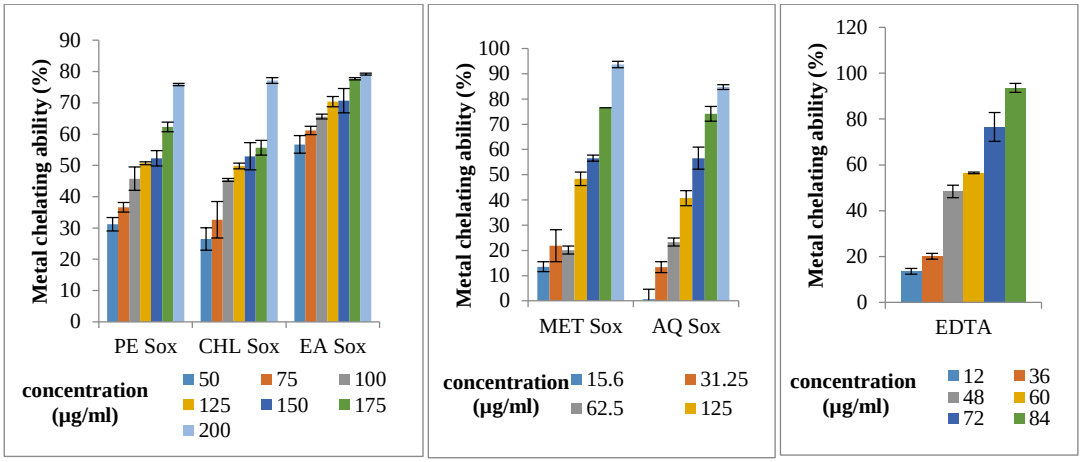


Fig. 5.5 Metal chelating activity of *P. korintii* leaf extracts determined as the chelation power on Ferrous (Fe^{2+}) Ions. Extracts demonstrates strong chelating activity in a concentration-dependent manner. EA Sox has the highest activity. EDTA was used as a reference standard. Values are Mean ± SD of three independent experiments.

Table 5.4 Comparison of metal chelating activity of *P. korintii* leaf extracts

Extracts	IC_{50} values (µg/ml) Mean ± SD
PE Sox	120 ± 1.4
CHL Sox	127.5 ± 0.8
EA Sox	47.5 ± 2.3
MET Sox	150 ± 1.2
AQ Sox	195 ± 0.6
EDTA	50.5 ± 3.4

5.1.5 Total antioxidant capacity assay - Phosphomolybdenum method

The method illustrates the antioxidant activity in terms of reduction of molybdate ions. Among the extracts, MET Sox showed higher antioxidant activity by reduction of molybdate ions followed by AQ Sox > EA Sox > PE Sox > CHL Sox (Fig 5.6 & Table 5.5).

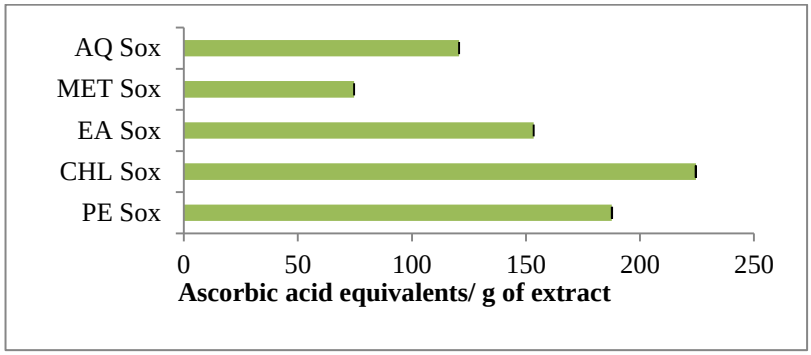


Fig.5.6. Total antioxidant capacity of *P. korintii* leaf extracts determined by Phosphomolybdenum method. The assay relies on the reduction of Mo (VI) to Mo (V) by the sample and concomitant formation of a green phosphate Mo (V) complex at acidic pH. The formation of coloured complex is measured at 695 nm. The reduction by ascorbic acid at different concentrations gave absorbance values which were plotted against concentration to obtain standard curve y=6.904x-0.0581, R^2=0.991. Using this equation, the total antioxidant capacity of extracts is expressed as ascorbic acid equivalents/ g of extract (AAE/g of extract). Values are Mean ± SD of three independent experiments.

Table 5.5. Comparison of Total antioxidant capacity of *P. korintii* leaf extracts

Extracts	AAE/g of extract Mean ± SD
PE Sox	187.68 ± 0.016
CHL Sox	224.47 ± 0.013
EA Sox	153.45 ± 0.002
MET Sox	74.61 ± 0.011
AQ Sox	120.72 ± 0.001

5.1.6 Assay of nitric oxide radical scavenging activity

In this study, ascorbic acid and quercetin were used as the reference NO radical scavenging compounds. CHL Sox , MET Sox and AQ Sox exhibited significant NO radical scavenging activity in a concentration dependant manner

(Fig. 5.7). Quercetin had an IC_{50} value of 1.825 ± 0.49 µg/ml whereas ascorbic acid appeared much less active than quercetin with an IC_{50} value of 3.1 ± 0.14 µg/ml. Of the *P. korintii* extracts AQ Sox (IC_{50} value of 1.235 ±0.26 µg/ml) showed highly appreciable activity than the reference compounds. MET Sox with IC_{50} value of 2.05 ± 0.07µg/ml had activity greater than ascorbic acid (Table 5.6).

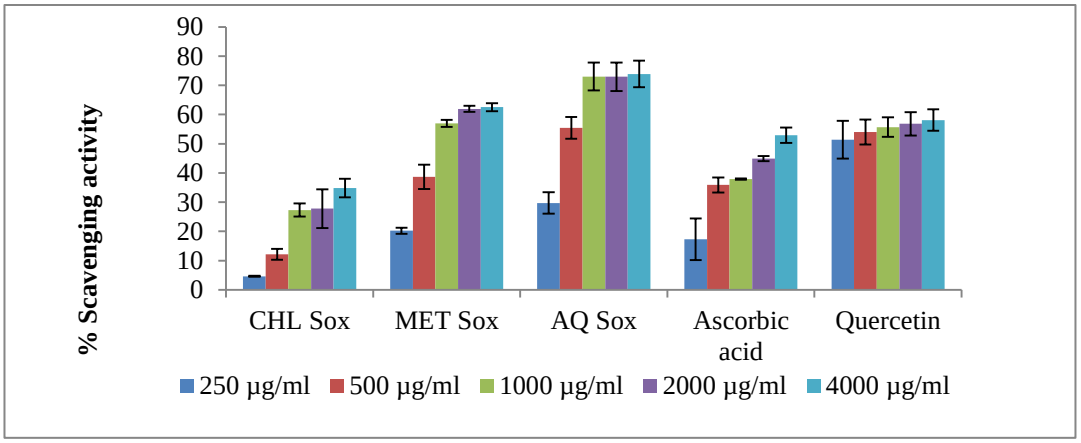


Fig. 5.7 Nitric oxide radical scavenging activity of *P. korintii* leaf extracts. The extracts exhibited significant NO radical scavenging activity in concentration dependant manner. Values are Mean ± SD of three independent experiments.

Table 5.6 Comparison of nitric oxide radical scavenging activity of *P. korintii* leaf extracts.

Extracts	IC_{50} values (µg/ml) Mean ± SD
CHL Sox	5.25 ± 0.07
MET Sox	2.05 ± 0.07
AQ Sox	1.235 ±0.26
Ascorbic acid	3.1 ± 0.14
Quercetin	1.825 ± 0.49

5.1.7 Assay of superoxide radical ($O_2 \cdot^-$) scavenging activity

Fig 5.8 shows the superoxide radical scavenging activity of the extracts in a dose dependant manner. All extracts exhibited moderate activity compared to the standard compound ascorbic acid. The PE Sox had higher scavenging ability with an

IC_{50} value of 48 ± 1.02 µg/ml followed by MET Sox >AQ Sox > CHL Sox > EA Sox (Table 5.7).

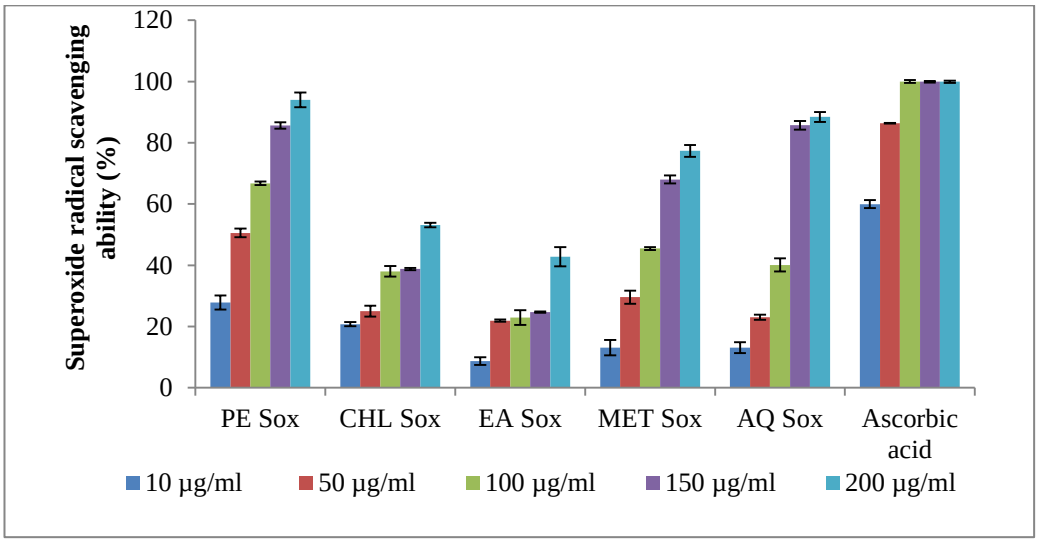


Fig. 5.8 Superoxide anion-scavenging activity of *P. korintii* leaf extracts observed with the riboflavin-light-NBT system. Photoreduction of flavins results in O^{2-} which can reduce the tetrazolium salt- Nitro Blue Tetrazolium (NBT) to form blue coloured formazan. The compounds capable of scavenging superoxide inhibit the formation of formazan and the decrease in colour intensity can be spectrophotometrically measured. The percentage scavenging was calculated and plotted against concentration. Values are Mean ± SD of three independent experiments.

Table 5.7 Comparison of Superoxide anion scavenging activity of *P. korintii* leaf extracts.

Extracts	IC_{50} values (µg/ml) Mean ± SD
PE Sox	48 ± 1.02
CHL Sox	190 ± 3.01
EA Sox	350 ± 1.3
MET Sox	110 ± 1.8
AQ Sox	114 ± 2.4
Ascorbic acid	4.5 ± 0.7

5.1.8 Assay of hydroxyl radical (˙OH) scavenging activity

The hydroxyl radical scavenging activity of the *P. korintii* extracts was dose dependent (Fig 5.9). Among the extracts, EA Sox had higher activity than other extracts. At the concentrations tested (5- 250 µg/ml), scavenging activity was in the

range 57.06 ± 1.25 % to 82.2 ± 0.49 % with an IC_{50} values of 4.3 ± 1.3 µg/ml which is significant compared to the activity of standard compound quercetin with an IC_{50} values of 3.6 ± 0.3 µg/ml. The activity of other extracts was in the order: PE Sox > MET Sox > CHL Sox > AQ Sox (Table 5.8).

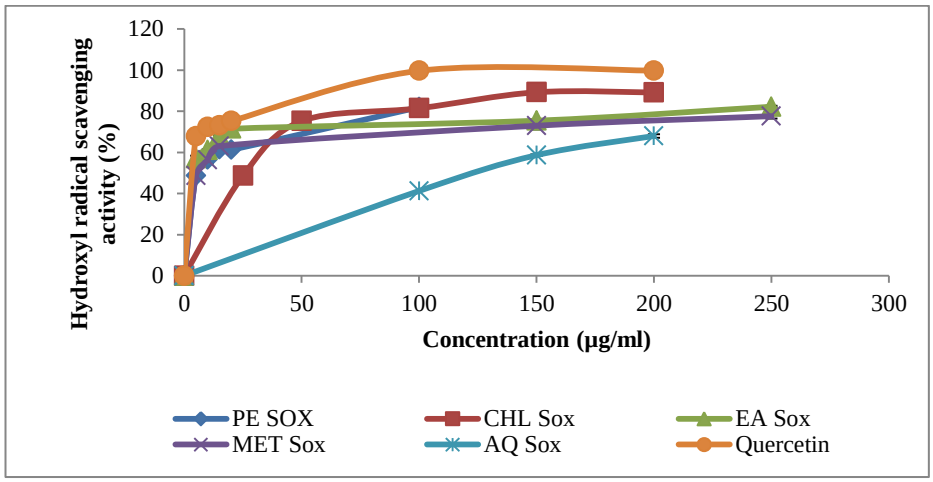


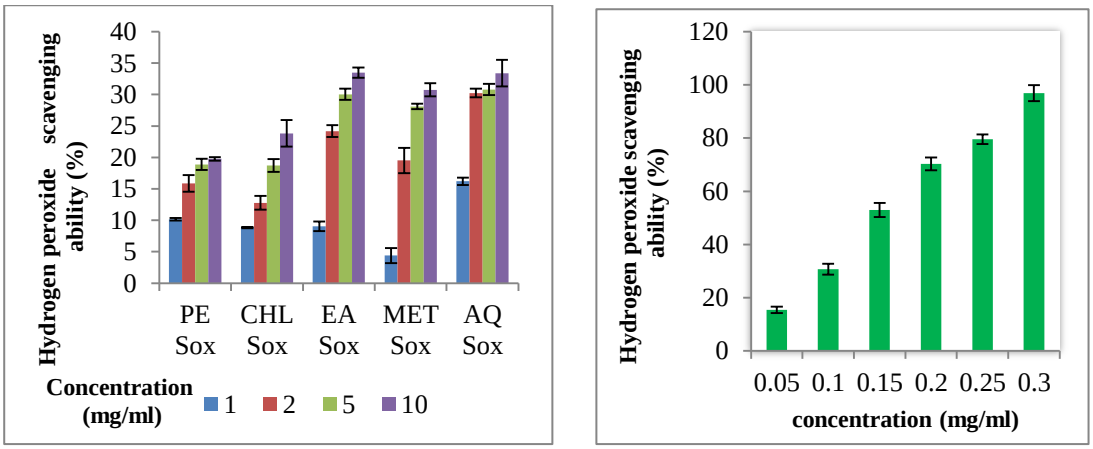
Fig. 5.9 Hydroxyl radical (˙OH) scavenging activity of *P. korintii* leaf extracts. The hydroxyl radicals generated by the Fenton reaction using Fe^{3+}-ascorbate-EDTA-H_2O_2 system reacts with 2-deoxy-2-ribose to generate a malondialdehyde-like product which upon heating with thiobarbituric acid, forms a pink chromogen at low pH. The extracts scavenged hydroxyl radicals thus preventing the reaction. Values are Mean ± SD of three independent experiments.

Table 5.8 Comparison of Hydroxyl radical scavenging activity of *P. korintii* extracts

Extracts	IC_{50} values (Mean ± SD) µg/ml
PE Sox	8.5 ± 1.03
CHL Sox	26 ± 0.78
EA Sox	4.3 ± 1.3
MET Sox	6 ± 0.4
AQ Sox	125 ± 1.9
Quercetin	3.6 ± 0.3

5.1.9 Determination of Hydrogen peroxide scavenging ability

The H_2O_2 scavenging ability of extracts is shown in Fig. 5.10 and compared with ascorbic acid. The extracts were capable of scavenging in a concentration dependant manner. None of the extracts showed good activity comparable to the standard used. Among the extracts, EA Sox and MET Sox had similar activity followed by AQ Sox, CHL Sox and PE Sox.



(a) **(b)**

Fig 5.10. H_2O_2 scavenging ability of (a) *P. korintii* leaf extracts (b) ascorbic acid. The assay relies on the reaction of H_2O_2 with dichromate in acetic acid to give a green colour with an absorbance maximum at 620 nm. The percentage scavenging was calculated and the values are Mean ± SD of three independent experiments.

Table 5.9. Comparison of hydrogen peroxide scavenging ability of *P. korintii* leaf extracts

Extracts	IC_{50} values (mg/ml) Mean ± SD
PE Sox	43.1 ± 1.7
CHL Sox	26 ± 2.3
EA Sox	16.3 ± 0.98
MET Sox	16.6 ± 1.4
AQ Sox	20.9 ± 1.06
Ascorbic acid	0.145 ± 0.32

5.1.10 Lipid peroxidation inhibition capacity assay

As shown in Fig. 5.11, the extracts demonstrated considerable amount of lipid peroxidation inhibition in a concentration dependant manner. The lipid peroxidation activity were in the range 17.08 ± 3.6 % to 82.9 ± 1.05 % for PE Sox, 19.76 ± 0.99 % to 86.13 % for CHL Sox, 23.54 ± 2.0 % to 76.47 ± 0.42 % for EA Sox, 42.99 ±1.76 % to 77.59 ± 0.87 % for MET Sox and 21.02 ± 2.13 % to 64.98 ± 2.03 % for AQ Sox. BHT was used as reference compound. IC_{50} values were determined (Table 5.10). Among the extracts, MET Sox (IC_{50} value: 14 ± 1.56 µg/ml) and AQ Sox (IC_{50} value: 140 ± 0.45 µg/ml) recorded the highest and lowest lipid peroxidation inhibition activity respectively.

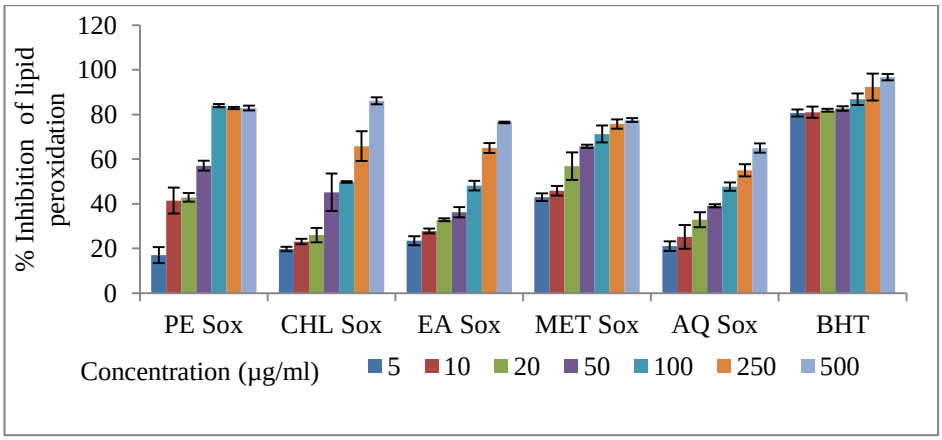


Fig. 5.11. Lipid peroxidation inhibition assay of *P. korintii* extracts by the thiobarbituric acid reactive substances (TBARS) method. The values are represented as Mean ± SD of three independent experiments.

Table 5.10. Comparison of lipid peroxidation inhibition by *P. korintii* extracts

Extracts	IC_{50} values (Mean ± SD) µg/ml
PE Sox	35 ± 1.34
CHL Sox	100 ± 0.98
EA Sox	120 ± 2.04
MET Sox	14 ± 1.56
AQ Sox	140 ± 0.45
BHT	6 ± 2.42

To sum up, *Polyalthia korintii* leaf extracts are effective in scavenging DPPH free radical, ABTS+ cation, nitric oxide radical, hydroxyl radical, superoxide radical and hydrogen peroxide. The extracts have considerable ferric reducing power, exhibits reduction of molybdate ions and possess ferrous chelating ability. They also showed significant inhibition of lipid peroxidation. All these ability dictates the antioxidant power of the phytoconstituents present in the extracts. Table 5.11 provides a comparative analysis of antioxidant activity of *P. korintii* extracts. MET Sox exhibited highest antioxidant potential for most of the assays. Hence, it was subjected to HR-LC-ESI-MS to profile its antioxidant phytochemicals.

Table 5.11. Antioxidant activity of *P. korintii* leaf extracts

Extracts	DPPH free radical scavenging activity (µg/ml)	Nitric oxide radical scavenging activity (µg/ml)	ABTS radical scavenging activity (µg/ml)	Ferric reducing antioxidant power (FRAP) assay 1 mM $FeSO_4$ equivalents (mg/ml)	Superoxide radical scavenging activity (µg/ml)	Metal (Ferrous) Chelating Ability (µg/ml)	Hydroxyl Radical Scavenging Activity (µg/ml)	Hydrogen peroxide scavenging activity (mg/ml)	Lipid peroxidation inhibition assay (µg/ml)	Total Antioxidant Capacity by Phosphomolybdenum Method *(AAE/g extract)
PE Sox	120.9 ± 8.5[a]	NA	303.5±14.2[a]	1.40 ± 0.32[a]	**48 ± 1.02[a]**	120 ± 1.4[a]	8.5 ± 1.03[a]	43.1 ± 1.7[a]	35 ± 1.34[a]	187.7± 0.02[a]
CHL Sox	160.7 ± 1.4[b]	5.25±0.07[a]	207.5 ± 2.7[b]	0.62 ± 0.12[b]	190 ± 3.01[b]	127.5± 0.8[b]	26 ± 0.78[b]	26 ± 2.3[b]	100 ± 0.98[b]	224.5 ± 0.01[b]
EA Sox	198.8± 2.5[c]	ND	186.1± 13.3[c]	0.73 ± 0.07[b]	350 ± 1.3[c]	**47.5 ± 2.3[c]**	**4.3 ± 1.3[ac]**	**16.3 ± 0.98[c]**	120 ± 2.04[c]	153.5 ±0.002[c]
MET Sox	**16.2 ±1.8[d]**	2.05±0.07[b]	**101.9 ± 13.4[d]**	**0.53 ± 0.04[b]**	110 ± 1.8[d]	150 ± 1.2[d]	6 ± 0.4[a]	16.6 ± 1.4[c]	**14 ± 1.56[d]**	**74.6 ± 0.011[d]**
AQ Sox	17.7 ± 2.4[d]	**1.235±0.3[b]**	215.5 ± 12[b]	0.78 ±0.02[b]	114 ± 2.4[d]	195 ± 0.6[e]	125 ± 1.9[d]	20.9 ± 1.06[d]	140 ± 0.45[e]	120.8 ± 0.001[e]
Standard compounds	Ascorbic acid 15.2 ±1.7[d]	Ascorbic acid 3.1± 0.14[b] Quercetin 1.825±0.5[b]	Ascorbic acid 18.3 ± 0.3[e]	Ascorbic acid 0.69 ± 0.09[b] Quercetin 0.36 ± 0.03[c] Gallic acid 0.43 ±0.08[bc]	Ascorbic acid 4.5 ± 0.7[e]	EDTA 50.5 ± 3.4[c]	Quercetin 3.6 ± 0.3[ac]	Ascorbic acid 0.145 ± 0.32[e]	BHT 6 ± 2.42[f]	-

* AAE/g extract: Ascorbic acid equivalent per gram of extract, NA- no activity, ND - not determined.
The values represent mean ± SD for three independent samples.
Means not sharing common letter in columns were significantly different at $P \leq 0.05$.
Superscript letters within the same column indicate significant ($P \leq 0.05$) differences in means within the extracting solvents.
Extracts with highest activity are in bold.

5.1.11 High-Resolution Liquid Chromatography Mass Spectrometry analysis of MET Sox extract

Twenty five major compounds were tentatively identified in the MET Sox extract (Table 5.12). Identification of compounds was established using on online mass libraries matching and comparison to published data. As shown in Table 5.12, 25 major compounds were identified from MET Sox extract. Most of them are already reported in different species and possess pharmacological activities. Glaucine was the abundant compound followed by caseadine and isoliensinine. The HR-LC-MS chromatogram data are presented in appendix.

Table 5.12 List of major chemical constituents identified from MET Sox extract of *Polyalthia korintii* using HR-LC-ESI-MS/MS.

Sl No.	Retention time	Compound Name	Biological activity reported in literature
1	1.167	(2R,3R,4R)-2-Amino-4-hydroxy-3-methylpentanoic acid	-
2	1.737	Unidentified	-
3	2.068	10-Isopropyl-2,7-dimethyl-1-oxaspiro[4.5]deca-3,6-diene	-
4	3.074	Ephedroxane	Anti-inflammatory (Konno C. et al., 1979)
5	3.443	5-Methoxytryptophol	Hormonal effects, immunomodulatory, anticancer, antigonadal, antioxidant (Ouzir, M et al., 2013)
6	4.906	Isonigerone	-
7	4.984	N-Feruloyltyramine	Antioxidant, anti-inflammatory (Aswad, M. et al., 2018)
8	5.151	Neoacrimarine G	-
9	5.234, 5.945	Caseadine	Antioxidant (Shrestha, R. L., & Adhikari, A., 2017)
10	5.479	Gossyrubilone	-
11	6.015,6.377, 6.674, 6.986	Glaucine	Antitussive, photoprotective and antioxidative activity (Spasova, M et al., 2008) anti-inflammatory , analgesic, antipyretic (PINTO, L et al., 1998)
12	6.015	Ethylmorphine	cough suppressant and an opioid analgesic (Dasgupta, A., 2020).

Continued….

13	6.096, 6.381	Isoliensinine	antitumor , cardiovascular protection, anti-HIV effects (Cheng, Y et al., 2021)
14	6.489	Unidentified	-
15	6.904	Fabianine	-
16	7.213	LysoPE(18:4(6Z,9Z,12Z,15Z)/ 0:0)	-
17	7.372	Papaveraldine	antimicrobial, antitussive, antispasmodic, and anticancer (Pyne, M. E. et al., 2016)
18	7.451	Thalicsessine	Anti-inflammatory (Perez, G. ,2001)
19	7.732	Deoxynupharidine	immunosuppressive (Zhang, L., et al.,1995)
20	9.041	Liriodenine	Antimicrobial and antifungal, Cardiovascular effects, Antitumor activity, Antiplatelet actions, sedative (Chen, C. Y. et al., 2013) DNA intercalation ability (Wiart, C., 2012) Antioxidant (Rajca-Ferreiraa, A. K. et al., 2017)
21	9.447	N-Methyl-14-Odemethylepiporphyroxine	-
22	10.53, 11.62	Unidentified	-
23	15.981	C16 Sphinganine	-
24	17.684, 19.293	Phenylethylamine	Antimicrobial (Irsfeld, M et al., 2013)
25	20.024	Oleamide	potent hypolipidemic agent; strong inhibitor of *Microcystis aeruginosa*, a cyanobacterium known for forming blooms resulting in lethal toxin production (Tanvir, R. et al., 2018)

The antioxidant compounds identified were 5-Methoxytryptophol, N-Feruloyltyramine, Caseadine, Liriodenine and Glaucine. The chemical structures of the compounds are depicted in Fig. 5.12.

5-Methoxytryptophol, is an indole which removes free radicals resembling melatonin in their antioxidant activity. They also prevent lipid and protein oxidations, increases superoxide dismutase and glutathione peroxidase levels (Şehirli AÖ and Savtekin G., 2018).

N-Feruloyltyramine, also known as Moupinamide is an alkaloid which has been previously isolated from the plants of the genus *Polyalthia*. The compound was

isolated from stem bark of *Polyalthia jucunda*. (Suedee, A. et al, 2007), stem of *Polyalthia suberosa* (Tuchinda, P et al., 2000), stem barks of *Polyalthia longifolia var. pendula* (Evidente, A., & Masi, M., 2021). The alkaloid is reported to work against the oxidative damage induced by H_2O_2 and maintains the integrity of mitochondria (Gao, X.et al., 2019).

Caseadine, is a protoberberine alkaloid earlier reported to have isolated from leaves and branches of *Guatteria elliptica* (*Annonaceae)* and *Dasymaschalon sootepense (Annonaceae)* (Rajca-Ferreiraa, A. K. et al., 2017). Caseadine at a concentration of 1.0 mM exhibited 23 % nitric oxide scavenging activity and at 0.5 mM, 27 % iron chelation activity (Shrestha, R. L., & Adhikari, A., 2017).

Liriodenine is an oxoaporphine alkaloid previously reported from *Polyalthia longifolia* (Wu, Y. C. et al., 1990), from stem bark of *Polyalthia cauliflora* var. *cauliflora (*Ghani, N. A et al., 2012*),* from aerial parts of *Pseudomalmea boyacana (J.F. Macbr.) Chatrou (Annonaceae)* and *Pseuduvaria setosa (King) J. Sinclair (Annonaceae)* (Zuhrotun, A., 2021), stem of *Polyalthia plagioneura Diels (Annonaceae)*(Nugraha, A. S. et al, 2019). At 100 μM concentration, the compound exhibited 12.9 % ABTS radical scavenging activity, 3.4 % metal chelating activity and reducing power (Liu, C. M et al., 2014).

Glaucine is an aporphine alkaloid reported to have isolated from leaves of *Annona reticulate (Annonaceae),* and leaves of *Annona squamosa (Annonaceae)* (Nugraha, A. S. et al, 2019). Glaucine, being a non-phenolic compound, their antioxidative property is due to the presence of the biphenyl system rather than the phenol group. They have highly significant hydroxyl radical scavenging activity which could make them potential leads for pharmacological purpose (Spasova, M et al., 2008).

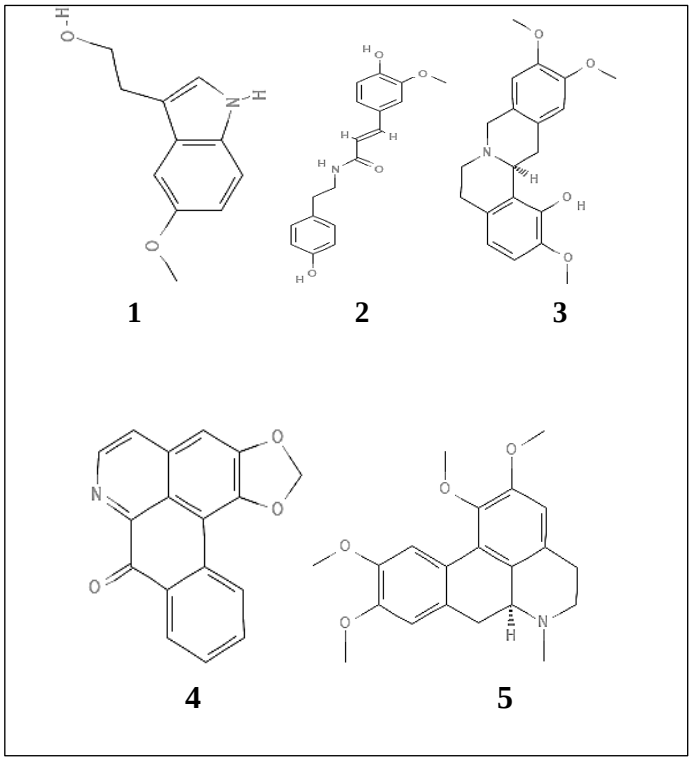


Fig. 5.12. Chemical structures of antioxidant compounds identified from MET Sox extract of *Polyalthia korintii*. The compounds are **1**- 5-Methoxytryptophol, **2**- N-Feruloyltyramine, **3-** Caseadine, **4-** Liriodenine, and **5-** Glaucine.

5.2. Protective effects of *P. korintii* extracts against H_2O_2-induced oxidative stress in Hep G2 cells - Cellular antioxidant assay

From the results (Fig. 5.13), it is evident that the extracts significantly decreased the cellular fluorescence when compared to the control indicating the presence of antioxidant compounds in the extracts to exhibit activity in cellular model. The dose–response curves for the cellular antioxidant activity (Fig. 5.14) and the calculated EC_{50} values (Table 5.13) show that EA Sox exhibits higher antioxidant potential against H_2O_2 induced oxidative stress in HepG2 cells followed by MET Sox, AQ Sox, CHL Sox and PE Sox. The observations are consistent with the results obtained for in vitro hydrogen peroxide scavenging ability shown by these extracts (Table 5.9).

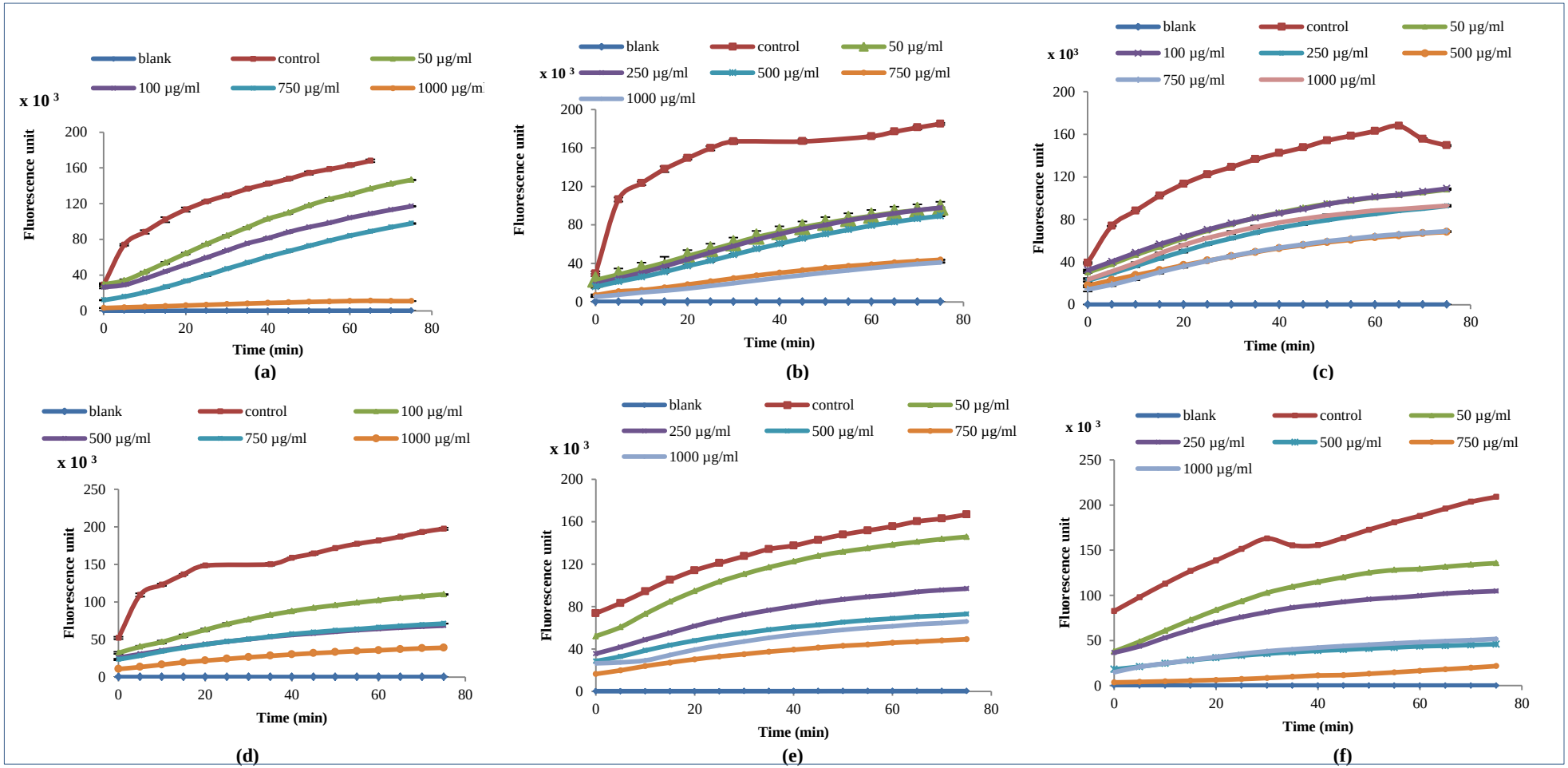


Fig. 5.13. Cellular antioxidant activity (CAA) assay in HepG2 cells. Kinetics of the conversion of DCFH to DCF and inhibition of oxidation in HepG2 cells in the presence of increasing concentrations of (a) PE Sox (b) CHL Sox (c) EA Sox (d) MET Sox (e) AQ Sox and (f) Quercetin (mean ± SD, n=3). Briefly, the cells were treated with different concentrations of extracts (0–1000 µg/ml) containing 25 µM DCFH-DA for 1 h. H_2O_2 (100 mol /l) was applied to the cells in Hank's Balanced Salt Solution (HBSS) and the emission at 538 nm was measured with excitation at 485 nm every 5 min for75mins.

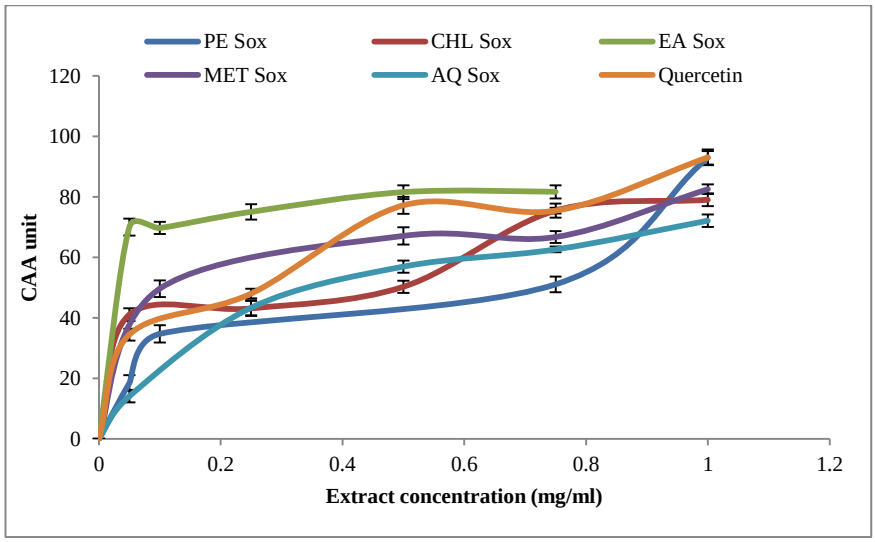


Fig. 5.14. CAA dose–response curves for *P. korintii* extracts and Quercetin. After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time for each extract and quercetin (Fig. 5.13) was integrated to calculate the CAA value using Microscoft excel. The median effective dose (EC50) was determined from the plot of CAA value versus dose.

Table 5.13. Cellular Antioxidant Activities of *P. korintii* extracts and Quercetin

Extracts	EC_{50} values Mean ± SD mg/ml
PE Sox	0.7 ± 2.87
CHL Sox	0.485 ± 2.1
EA Sox	**0.035 ± 2.6**
MET Sox	0.105 ± 2.85
AQ Sox	0.325 ± 2.21
Quercetin	0.270 ± 2.42

5.3 Antibacterial activity of the leaf extracts from *P. korintii*

Antibacterial activity of five different extracts from *P. korintii* were tested against four gram positive (*Streptococcus pyogenes* (ATCC 19615), *Enterococcus feacalis* (ATCC 29212), *Listeria monocytogenes* (clinical isolate), *Staphylococcus aureus* (MTCC 87)) and five gram negative (*Proteus mirabilis* (clinical isolate), *Chromobacterium violaceum* (ATCC 22019), *Pseudomonas aeruginosa* (clinical

isolate), *Klebsiella pneumoniae* (MTCC 3384), *Escherichia coli* (MTCC 41)) bacteria by broth microdilution method.

According to Bubonja-Šonje, Knežević S, & Abram M, 2020, broth microdilution methods have endpoints measured as turbidity which correlate with the concentration of viable microorganisms and can be used to determine not only MIC but also 50 % and 90 % inhibitory concentrations (IC50 and IC90, respectively). Fig. 5.15 (a-e) represents Inhibition of bacterial growth, as measured by the change in the OD600 following treatment with different extracts. The Inhibitory Concentration IC_{50} value is defined as the concentration of the extract at which 50% of the bacterial growth was inhibited, and is calculated from the curve.

Of the five extracts evaluated, PE Sox showed prominent antibacterial activity against all the organisms tested with an IC_{50} range of 4.24 ± 2.03 to 15.14 ± 3.4 mg/ml. The GC-MS analysis of this extract as already mentioned in Table 4.16 shows the presence of compounds with literature reported antibacterial activity. They are Alpha-curcumene (Silva, G. N. S. D et al., 2021), Cis-.beta.-farnesene (Turkez, H, et al., 2014), and Juniper camphor (Assaeed, A. etal., 2020). CHL Sox was active against *S. pyogenes* (IC_{50} 8.30 ± 2.3 mg/ml).

EA Sox inhibited *P. mirabilis, C. violaceum, S. pyogenes, and S. aureus.* MET Sox showed antibacterial activity against *P. mirabilis, C. violaceum* and *S. pyogenes*. *P. mirabilis* and *S. pyogenes* was susceptible to AQ Sox extract. Chloramphenicol was used as a positive control and exhibited an IC_{50} range of 0.0004 ± 1.06 to 0.0449 ± 2.1 mg/ml. *Streptococcus pyogenes* was susceptible to all the extracts. Among the extracts tested at the concentrations specified, only PE Sox effectively inhibited *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Enterococcus feacalis, and Listeria monocytogenes.*

Table 5.14 summarises the antibacterial activity demonstrated by the *P. korintii* extracts. Statistical analysis revealed that antibacterial activities of the extracts were significantly different in comparison with the control ($P< 0.001$). ANOVA showed significant differences and variations between the extracts against the microorganisms tested ($P<0.05$) (Table5.14).

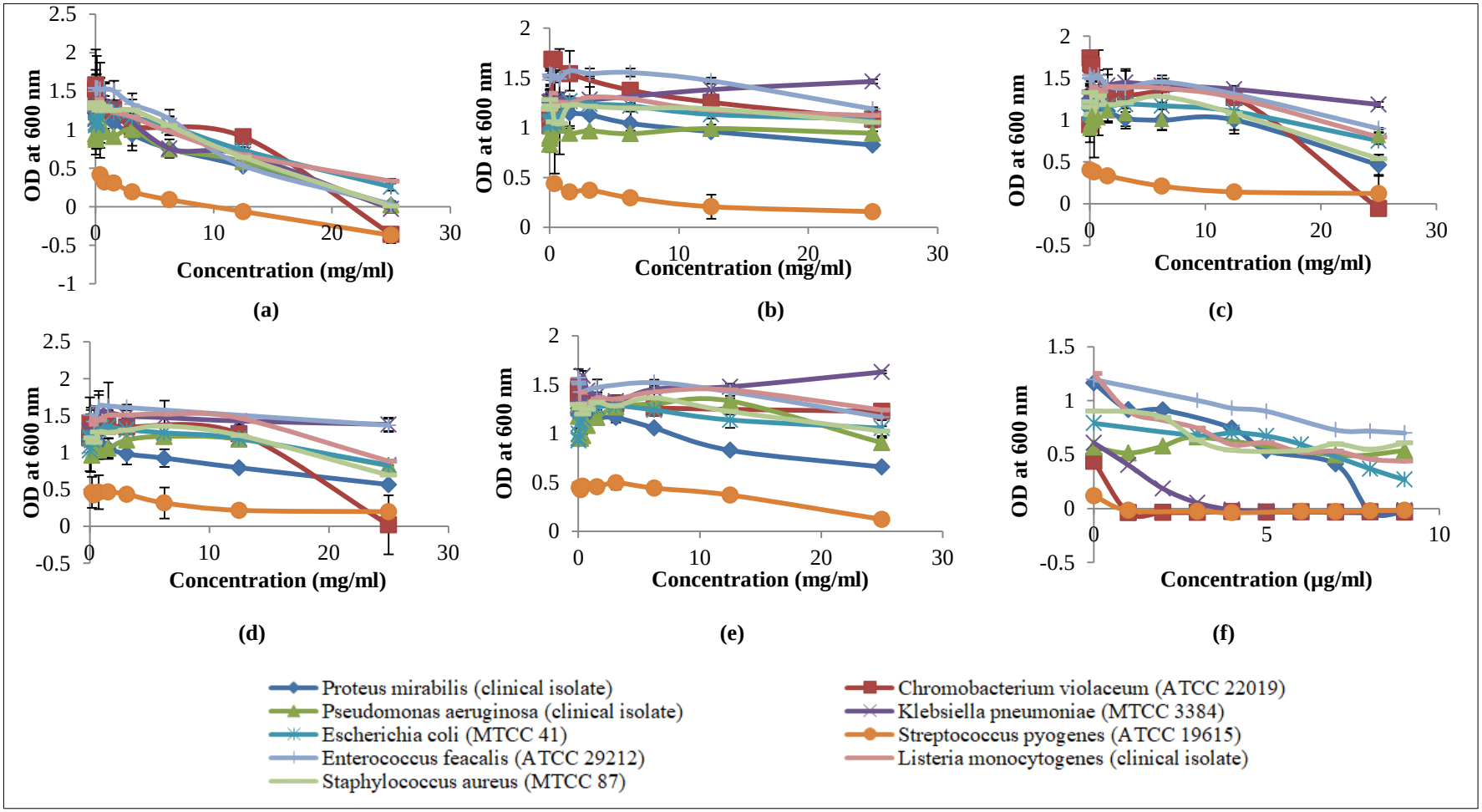


Fig. 5.15. Evaluation of antibacterial activity of *P. korintii* leaf extracts against human pathogenic bacteria. The assays were performed in sterile, 96-well microtitre plates after turbidity adjustments to 0.5 McFarland with sterile Mueller-Hinton broth. Sterility controls included in the assay contained media only and growth controls contained bacteria only. To control for the color of different extracts, absorbance at 600 nm (OD_{600}) measurements were taken prior to incubation at 37°C and after 12 hours. The figure depicts the growth, as measured by the change in the OD 600 following treatment with plant extracts (a) PE Sox (b) CHL Sox (c) EA Sox (d) MET Sox (e) AQ Sox and (f) chloramphenicol. The values represent mean± SD for three independent samples

Table 5.14. A**ntibacterial activity (IC_{50} values) of *P. korintii* leaf extracts against human pathogenic bacteria**

Organism	Extracts IC50 (mg/ml)					Chloramphenicol IC50 (mg/ml)
	PE Sox	CHL Sox	EA Sox	MET Sox	AQ Sox	
Proteus mirabilis (clinical isolate)	**11.25 ± 2.1** $_{\mathbf{b}}^{\mathbf{a}}$	> 25	20.96 ± 1.4 $_{c}^{a}$	20.70 ± 1.6 $_{c}^{a}$	24.70 ± 1.4 $_{d}^{a}$	0.0045 ± 2.4 $_{a}^{a}$
Chromobacterium violaceum (ATCC 22019)	**9.29 ± 3.2** $_{\mathbf{b}}^{\mathbf{b}}$	> 25	11.82 ± 1.06 $_{b}^{a}$	15.13 ± 2.4 $_{c}^{b}$	> 25	0.0005 ± 0.98 $_{a}^{b}$
Pseudomonas aeruginosa (clinical isolate)	**10.77 ± 2.6** $_{\mathbf{b}}^{\mathbf{ab}}$	> 25	> 25	> 25	> 25	0.0449 ± 2.1 $_{a}^{b}$
Klebsiella pneumoniae (MTCC 3384)	**11.47 ± 1.4** $_{\mathbf{b}}^{\mathbf{b}}$	> 25	> 25	> 25	> 25	0.0013 ± 1.6 $_{a}^{c}$
Escherichia coli (MTCC 41)	**15.14 ± 3.4** $_{\mathbf{b}}^{\mathbf{b}}$	> 25	> 25	> 25	> 25	0.0082 ± 1.84 $_{a}^{d}$
Streptococcus pyogenes (ATCC 19615)	4.24 ± 2.03 $_{b}^{b}$	8.30 ± 2.3 $_{c}$	**0.30 ± 2.04** $_{\mathbf{b}}^{\mathbf{b}}$	18.25 ± 0.9	16.06 ± 2.2 $_{c}^{a}$	0.0004 ± 1.06 $_{a}^{c}$
Enterococcus feacalis (ATCC 29212)	**11.96 ±1.34** $_{\mathbf{b}}^{\mathbf{b}}$	> 25	> 25	> 25	> 25	0.0101 ± 2.45 $_{a}^{ae}$
Listeria monocytogenes (clinical isolate)	**14.48 ± 2.13** $_{\mathbf{b}}^{\mathbf{b}}$	> 25	> 25	> 25	> 25	0.0053 ± 1.62 $_{a}^{a}$
Staphylococcus aureus (MTCC 87)	**12.46 ± 3.04** $_{\mathbf{b}}^{\mathbf{b}}$	> 25	24.72 ± 1.23 $_{c}^{c}$	> 25	> 25	0.0102 ± 1.94 $_{a}^{ce}$

The values represent mean± SD for three independent samples.

Superscript letters within the same column indicate significant ($P \leq 0.05$) differences in means between different organisms.

Subscript letters within the same row indicate significant ($P < 0.05$) differences in means among the extracts.

5.4 Heat induced protein denaturation inhibition potential of *P. korintii* extracts

The results of the heat-induced protein denaturation inhibition assay (Fig. 5.16) clearly showed that MET Sox and AQ Sox extracts were capable of inhibiting the denaturation in a concentration dependent manner and the potential is comparable to the standard compound diclofenac. At higher concentrations tested for PE Sox, CHL Sox and EA Sox, a decrease in denaturation inhibition potential was noticed.

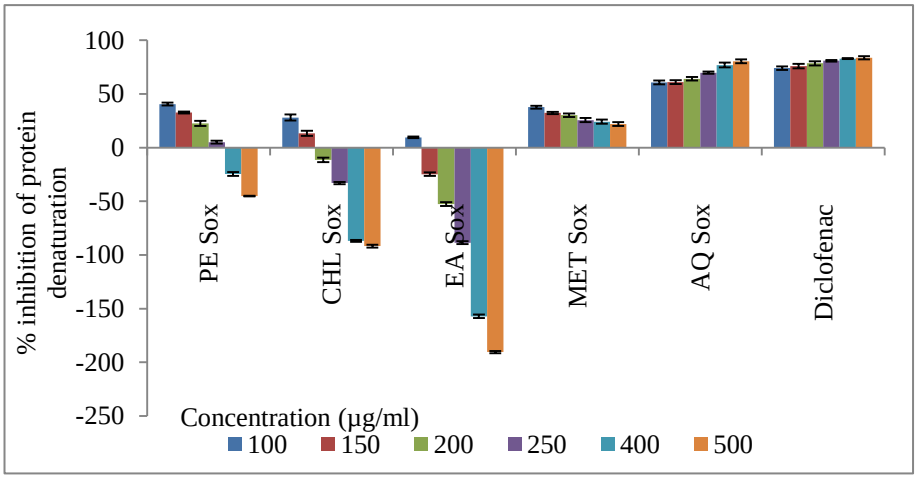


Fig. 5.16. Protien denaturation inhibition potential of *P. korintii* extracts. The reaction mixture consisted of bovine albumin and extracts at varying concentration in PBS, pH 6.4. Following an initial incubation at 37 ◦C for 15 min, heating was done at 70 ◦C for 5 min. On cooling, the turbidity was measured at 660 nm and the percentage inhibition of protein denaturation was calculated. The values represent mean± SD for three independent samples.

5.5 DNA interacting ability of *P. korintii* extracts

Since compounds that interact with nucleic acids are used as cancer chemotherapeutics and antimicrobials (Sinicropi, D. et al., 1994), there is great interest in detecting phytocompounds that can interact with nucleic acids exhibiting less toxicity than many of the currently used bioactive agents. The DNA-methyl green assay was thus employed to investigate the presence of *P. korintii* phytoconstituents interacting with DNA.

Absorbance decrease of 20% or more was considered a significant activity and therefore indicated positive reaction. The results obtained (Fig. 5.17 &Table

5.15) show that only PE Sox and CHL Sox extracts at higher concentration were active in displacing methyl green from the complex. None of the extracts at the tested concentrations showed 50% activity. Ethidium bromide at 50 µg/ml showed 93 ± 2.3 % activity.

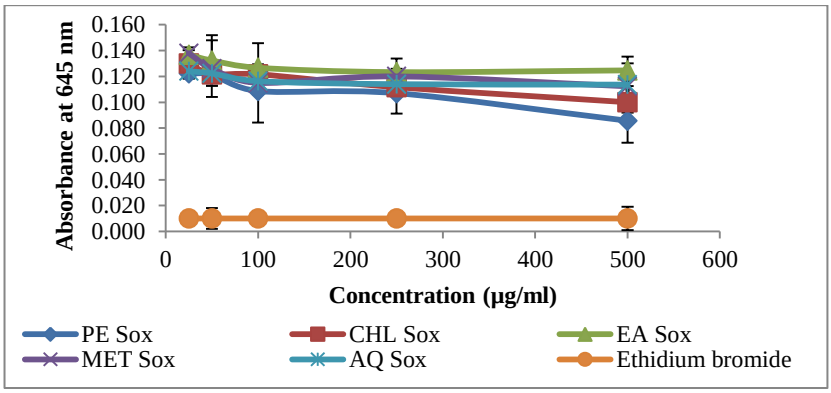


Fig. 5.17 DNA interacting activity of *P. korintii* extracts.

Table 5.15. DNA-MG displacement potential of *P. korintii* extracts

Extracts	concentration (µg/ml)	% of absorbance decrease (mean ± SD)	DNA interaction
PE Sox	25	12.6 ± 1.3	Negative
	50	13.3 ± 2.5	Negative
	100	22.4 ± 0.98	Positive
	250	23.6 ± 2.6	Positive
	500	38.8 ± 0.4	Positive
CHL Sox	25	7.1 ± 0.8	Negative
	50	13.1 ± 1.32	Negative
	100	13.1 ± 1.48	Negative
	250	20.2 ± 0.3	Positive
	500	28.6 ± 1.1	Positive
EA Sox	25	2.4 ± 0.42	Negative
	50	5.5 ± 1.2	Negative
	100	9.5 ± 0.8	Negative
	250	11.9 ± 4.3	Negative
	500	11 ± 3.8	Negative
MET Sox	25	1.4 ± 0.92	Negative
	50	10 ± 0.34	Negative
	100	17.9 ± 3.5	Negative
	250	14.3 ± 0.6	Negative
	500	19.8 ± 0.36	Negative
AQ Sox	25	11.4 ± 1.4	Negative
	50	12.6 ± 0.2	Negative
	100	16.9 ± 1.3	Negative
	250	18.6 ± 1.47	Negative
	500	18.8 ± 2.8	Negative
Ethidium bromide	50	93 ± 2.3	Positive

5.6 Cytotoxic potential of *P. korintii* extracts

The *P. korintii* leaf extracts viz. PE Sox, CHL Sox, EA Sox, MET Sox, and AQ Sox were tested against several human cancer cell lines as given in Table 5.16. Only PE Sox, CHL Sox, and EA Sox extracts exhibited cytotoxic activity against the tested cell lines whereas MET Sox and AQ Sox did not show decreased viability when tested up to a concentration of 250 µg/ml.

Apart from cytotoxic potential, an ideal chemotherapeutic agent should exhibit high specificity in discriminating the cancer cells and normal cells. It is mandatory to check for the potential toxicity of all formulations to assess their safety levels. To investigate the cytotoxic specificity of *P. korintii* extracts, assay on cells of normal origin - Vero, HEK-293, L929 and human peripheral blood lymphocytes (hPBL) were done. The results depicted in Fig. 5.17 exhibited negligible cytotoxic activity of PE Sox, CHL Sox and EA Sox extracts. MET Sox and AQ Sox extracts had no cytotoxic potential on these cells at the concentrations tested. All the extracts showed >90 % cell viability of all the tested normal cells at 50 µg/ml. The IC_{50} values (Table 5.16) were calculated using the ED50 plus v1.0 software.

Table 5.16 Cytotoxic activity of *P. korintii* extracts on cells.

Sl No.	Cell lines	Origin	IC_{50} values µg/ml (mean ± SD) of extracts		
			PE Sox	CHL Sox	EA Sox
1.	PC3	Grade IV Prostate cancer	61.4 ± 0.84	73.25 ±0.4	124.5 ± 3.2
2.	DU145	Prostate carcinoma	53.26 ±0.5	67.37 ±1.3	117.24 ±0.72
3.	LNCaP.FGC	Prostate carcinoma	85.8 ±1.51	98.03 ±3.5	124.6 ±2.4
4.	MCF-7	Breast adenocarcinoma	67.4 ±0.48	83.5 ±1.7	>150
5.	MDA-MB-231	Breast carcinoma	73.43 ±2.8	98.02 ±2.3	>150
6.	**K562**	**Chronic myelogenous leukemia**	**31.8 ± 3.1**	52.8 ± 4.3	118.9 ± 6.0
7.	**HL-60**	**Acute myeloid leukemia**	**35.32 ± 0.7**	55.5 ± 2.9	95.5 ± 0.56
8.	**THP1**	**Acute monocytic leukemia**	**28.04 ± 1.3**	48.6 ± 2.4	85.2 ± 2.6
9.	**Jurkat E6.1**	**Acute T cell leukemia**	**33.52 ±2.0**	57.5 ± 0.9	90.5 ± 2.1

Continued….

10.	Hep3B	Hepatocellular carcinoma	53.3 ± 2.76	68.7± 3.4	>120
11.	HepG2	Hepatocellular carcinoma	73.2 ± 3.01	66.5 ± 4.2	>120
12.	HeLa	Cervical carcinoma	84.5 ± 2.4	80.2 ± 3.0	>150
13.	SiHa	Cervical carcinoma	77.2 ± 3.6	74.5 ± 3.0	>150
14.	**HCT116**	**Colorectal carcinoma**	**40.72 ±1.1**	73.8 ± 5.3	>120
15.	**HT-29**	**Colorectal adenocarcinoma**	**43.8 ± 1.7**	77.8 ± 2.6	>120
16.	A549	Lung adenocarcinoma	58.3 ± 4.5	85.2 ± 3.2	>150
17.	HT-1080	Fibrosarcoma	67.4 ± 2.1	88.7 ± 2.4	>150
18.	PA-1	Ovarian teratocarcinoma	60.3 ± 3.2	93.5 ± 0.9	>150
19.	PANC-1	Pancreatic carcinoma	57.5 ± 4.3	89.4 ± 2.4	>150
20.	RAW 264.7	Monocyte/Macrophage cell line (BALB/c Mice)	55.3 ± 0.8	68.7 ± 1.6	>150
21.	HEK293	Human embryonic kidney cells	180.8 ± 3.2	365.1± 1.4	>2000
22.	L929	Murine fibroblast cell line	241.9 ± 4.4	387.8 ± 3.2	>2000
23.	VERO	Kidney epithelial cells from an African green monkey	349.7 ± 1.9	433.3 ± 3.9	>2000
24.	hPBLs	Human peripheral blood lymphocytes	464.05 ±3.7	454.81 ± 4.2	426.4 ±4.7

5.7. Haemolytic assay

Haemolysis is an important parameter in assessing the safety and utility of several formulations. Their abundance, availability and the ease of separation from plasma has made them good choice as normal human cells for several experimental studies. Phytocompounds like saponins acting on erythrocyte membrane can induce haemolysis (Karou, S. D.et al., 2011).

In the present study, 1 % Triton X-100 and PBS served as positive and negative controls respectively. By convention, any haemolysis value below 10% is considered to be non-haemolytic while values above 25% are considered to be haemolytic (Amin, K., & Dannenfelser, R. M., 2006). The extracts exhibited low

haemolytic effect (< 1 %) toward human erythrocytes in the tested concentration range. PE Sox extract exhibited the higher haemolytic potential and MET Sox had the least. Haemolytic percentage was found to be increasing with increase in concentration of the extracts.

Literature has documented several reports on the hemolytic activity caused by the plant extracts. Triterpene and steroid saponins present in the plants are the primary components possessing hemolytic properties (Mead, B. P. et al., 2016). In the present study, the qualitative phytochemical analysis have revealed the presence of saponins in all the extracts and the quantitative study had shown that the Diosgenin Equivalent saponin content in the extracts follows the given order PE Sox ≥ CHL Sox >AQ Sox >EA Sox > MET Sox. The haemolytic activity of the extracts also follow the same trend with PE Sox having comparatively higher potential followed by CHL Sox > AQ Sox > EA Sox > MET Sox. This preliminary investigation points to saponins in imparting the hemolytic activity to the *P. korintii* extracts.

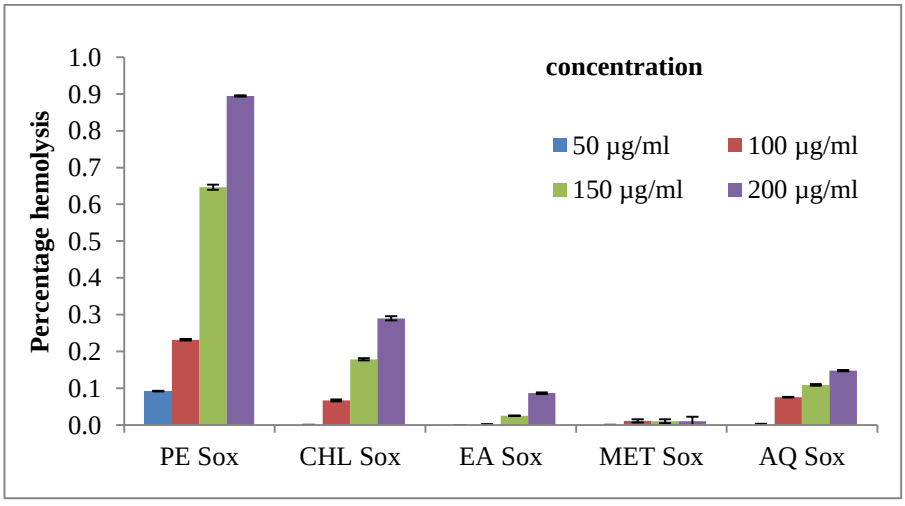


Fig. 5.18. Hemolytic assay on *P. korintii* extracts using human erythrocytes. Triton X-100 (1% v/v) and PBS served as positive and negative control respectively. Values represent Mean ± SD (n=3).

To sum up, the present investigation was effectual and worthwhile in revealing the unexplored potentials of *P. korintii* phytoconstituents. MET Sox and AQ Sox extracts had strong DPPH scavenging ability comparable to ascorbic acid (IC_{50} values of 16.2 ± 1.8, 17.7± 2.4 and 15.2 ± 1.7µg/ml respectively). The extracts showed lower ABTS radical scavenging activity compared to ascorbic acid. The extracts also exhibited strong reducing potential equivalent to the antioxidant compounds gallic acid, quercetin and ascorbic acid.

EA Sox extract was highly effective in chelating ferrous ions (IC_{50} value of 47.5 ± 2.3µg/ml) and possessed higher H_2O_2 scavenging potential (IC_{50} value of 16.3 ± 0.98 µg/ml) than other extracts. MET Sox extract exhibited relatively high molybdate ion reduction potential (IC_{50} value of 74.61 ± 0.011 AAE/g of extract). All the extracts were powerful in scavenging nitric oxide radical at lower concentrations.

PE Sox extract showed significant superoxide radical scavenging activity (IC_{50} value of 48 ± 1.02 µg/ml) compared to other extracts. The extracts exhibited dose dependant activity and EA Sox extract had higher potential (IC_{50} value of 4.3 ± 1.3 µg/ml). MET Sox extract effectively inhibited lipid peroxidation of egg yolk homogenate than other extracts. EA Sox extract exhibited significantly high protective effects against H_2O_2-induced oxidative stress in Hep G2 cells in cellular antioxidant assay. These results thus emphasises the potential of the *P. korintii* extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathologic damage. The study thus reports the significant antioxidant property of *P. korintii* extracts.

MET Sox extract which possessed higher antioxidative potential was subjected to HR LCMS analysis and identified the presence of antioxidant compounds like 5-Methoxytryptophol, N-Feruloyltyramine, Caseadine, Liriodenine and Glaucine.

PE Sox showed prominent antibacterial activity against *Streptococcus pyogenes* ATCC 19615 (IC_{50}: 4.24 ± 2.03 mg/ml), *Enterococcus feacalis* ATCC

29212 (IC_{50}: 11.96 ±1.34 mg/ml) , *Staphylococcus aureus* MTCC 87 (IC_{50}:12.46 ± 3.04mg/ml), *Chromobacterium violaceum* ATCC 22019 (IC_{50}:12.46 ± 3.04mg/ml), *Klebsiella pneumoniae* MTCC 3384 (IC_{50}: 10.77 ± 2.6mg/ml), *Escherichia coli* MTCC 41 (IC_{50}: 15.14 ± 3.4 mg/ml) and clinical isolates like *Listeria monocytogenes* (IC_{50}: 14.48 ± 2.13 mg/ml), *Proteus mirabilis* (IC_{50}: 11.25 ± 2.1 mg/ml), and *Pseudomonas aeruginosa* (IC_{50}: 10.77 ± 2.6 mg/ml).

Heat induced protein denaturation inhibition potential of the extracts were studied and compared to the drug diclofenac. AQ Sox and MET Sox extracts were active. DNA interacting ability was investigated using Methyl green displacement assay. Only PE Sox and CHL Sox extracts at concentrations higher than 100µg/ml and 250µg/ml respectively, were able to displace methyl green from the complex. This thus rules out the mutagenic potential of the extracts.

Cytotoxic/ anti-proliferative potential of extracts studied on an array of cell lines revealed that PE Sox, CHL Sox, and EA Sox extracts possessed the potential. PE Sox was the most effective against all the cell lines tested. But these were relatively ineffective on Vero, HEK293, L929 and lymphocytes. This observation substantiates the cytotoxic potential of extracts and further emphasises the cytotoxic specificity shown by them. Finally, the very weak haemolytic ability of extracts makes them potential prospect for further advanced studies.

Owing to the presence of highly potent anticancer agents from *Polyalthia* species, the unexplored *P. korintii* PE Sox extract which possess significant broad spectrum anticancer effect on a multitude of cancer cell lines with least toxicity towards normal human lymphocyte was selected for detailed investigation to clearly demonstrate its mode of action and to identify the anticancer agents.

6. ANTICANCER POTENTIALS OF *Polyalthia korintii* PE Sox EXTRACT

Preliminary screening of *P. korintii* extracts for antiproliferative activity revealed significant cytotoxic potential of PE Sox and CHL Sox extracts against all the cell lines tested. PE Sox had higher cytotoxic potential and it was selected for further studies. The activity was broad spectrum, Leukemia -K562, HL60, Jurkat E6.1, THP1 (suspension cells) and Colon cancer -HCT116, HT 29 (adherent cells) cells exhibited lower IC_{50} values compared to cancers of other origin.

6.1 Dose and time dependent cellular responses to PE Sox extract on leukemia and colon cancer cell lines.

In the present study, PE Sox extract at different concentrations (0- 100 µg/ml) were treated for 24, 48 and 72 hours duration on K562, HL-60, Jurkat E6.1, THP1, HCT-116 and HT-29 cancer cell lines for cytotoxicity evaluation. The cell viabilities were assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, which relies on the ability of the mitochondrial dehydrogenases in intact cells to reduce the dye to formazan which on solubilisation in DMSO gives purple coloured product quantified spectrophotometrically. The results are represented in terms of % cell viability (Fig.6.1) and the IC_{50} values (Table 6.1). Curcumin and cisplatin served as reference compounds. The extract showed dose and time dependant decrease in cell viability. The extract had higher cytotoxicity on the THP1 cells with IC_{50} values ranging from 45.52 ± 1.58µg/ml at 24 h treatment to 6.52 ± 0.72 µg/ml after 72 h treatment. Among the colon cancer cell lines, HCT116 showed lower IC_{50} values of 67.04 ±1.5, 40.72 ±1.06, and 24.63 ± 0.48 µg/ml at 24h, 48h and 72 h respectively. Since treatment of THP1 cells with the PE Sox extract resulted in lack of uniformity leading to slightly adherent morphology of cells resembling differentiation, this cell line was omitted and K562 was included for further investigations. Thus, one adherent- HCT116 and one suspension- K562 cell line showing increased sensitivity to PE Sox was selected to elucidate the cellular and molecular mechanisms.

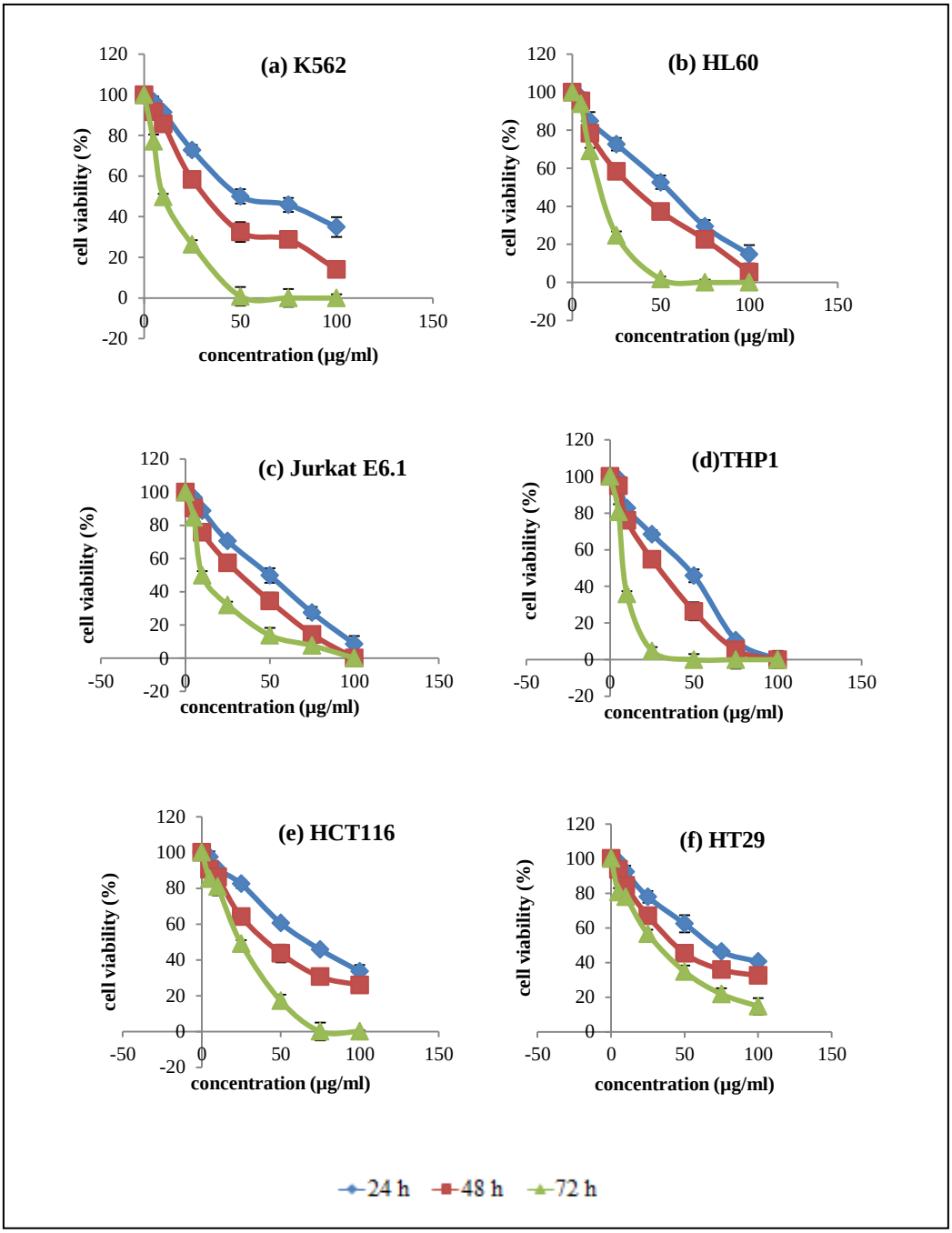


Fig. 6.1 (a) to (f) Dose- and time-dependent anti-proliferative activity of *P. korintii* PE Sox extract on leukemia and colon cancer cells (a) K562, (b) HL60, (c) Jurkat E6.1, (d) THP1, (e) HCT116 and (f) HT29. Cells were treated with extract at different concentrations (0 to 100 µg/ml) for 24, 48 and 72 h. The cell viability was determined by MTT assay. Values represent mean ± SD of three experiments.

Table 6.1 Cytotoxic activity (IC_{50} values) PE Sox on selected leukemia and colon cancer cell lines.

Cell lines used	IC_{50} values µg/ml (mean ± SD)				
	24 h	48 h	72 h	Curcumin	Cisplatin
Leukemia					
K562	50.4 ± 2.45	31.8 ± 3.1	10.4 ± 0.89	18.4 ±0.6	8.6 ± 2.6
HL60	52.05 ± 1.67	35.32 ± 0.7	11.48 ± 1.45	22.7±1.2	10.6 ± 3.7
Jurkat E6.1	50. 5 ±2.6	33.52 ±1.94	10.76 ± 2.3	26.3 ±3.1	13.4 ±0.8
THP1	45.52 ± 1.58	28.04 ± 1.3	6. 52 ± 0.72	6.4 ± 2.7	4.5 ± 1.7
Colon cancer					
HCT116	67.04 ±1.5	40.72 ±1.06	24.63 ± 0.48	25.8 ± 3.2	10.04 ± 2.6
HT29	70.5 ± 0.25	43.8 ± 1.7	30.58 ±0.23	21.56 ±4.1	14.5 ±0.89

IC_{50} values are mean of three independent experiments ± S.D.

6.2 Trypan blue dye exclusion assay

The cytotoxicity was also assessed simultaneously by Trypan blue dye exclusion assay. The live cells exclude the entry of acidic trypan blue dye in to the cells with intact membranes. The dye enters the non-viable cells through damaged membranes and stains the cytoplasm blue. K562 and HCT116 cells were treated with PE Sox extract for 24 h, 48 h, 72 h and stained with trypan blue dye (Fig 6.2). Results of trypan blue exclusion assay matched well with the observations of MTT assay for all the durations treated.

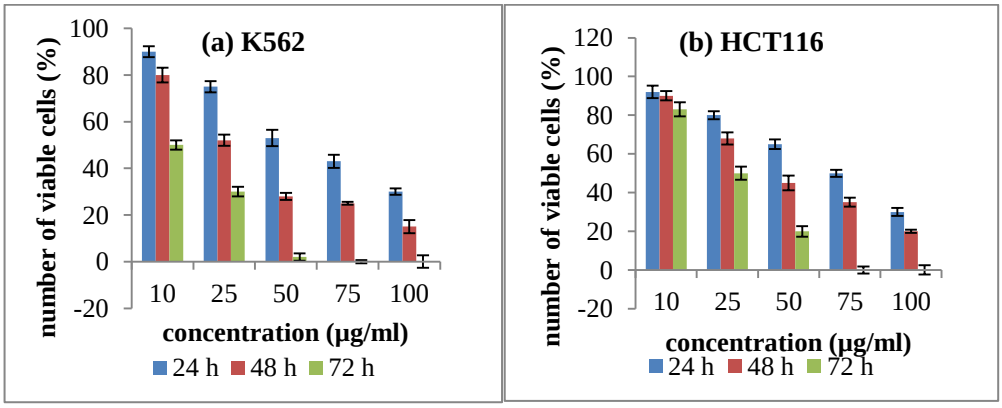


Fig 6.2. Trypan blue dye exclusion assay using *P. korintii* PE Sox extract on K562 and HCT116 cancer cell lines. Treated cells in suspension was mixed with dye, visually examined, nonviable cells with blue cytoplasm and viable cells without dye colour were counted. The results are expressed as percentage number of viable cells. Values represent mean ± SD of three experiments.

6.3 Clonogenic assay

Clonogenic assays are widely used in cancer research to evaluate cellular growth and the cytotoxic or genotoxic effects of various agents. Cancer cells which have tumor-initiating capabilities can form clones. Clonogenic assays thus determine the ability of cells to retain their reproductive integrity over a prolonged period of time revealing the phenotypic effects which may take time to develop. Since *in vitro* reproductive cell survival cannot be identified using short-term cytotoxicity assays, clonogenic assays represent the gold standard method (Franken, N. A. et al., 2006; Brix, N. et al., 2020). The cells were treated with PE Sox extract at its IC_{50} concentration, a lower and a higher IC_{50} concentration. The result is shown in Fig. 6.3 and Fig 6.4 depicts significant concentration dependent decrease in colony formation as compared to the untreated control and DMSO treated cells. An 80 % reduction in colony number was observed in K562 cells treated at IC_{50} concentration. HCT116 exhibited 50 % decrease in colony numbers at IC_{50} concentration compared to the controls. The extract significantly affected the clonogenic survival of both cell types. These results clearly demonstrated that the extract exhibited a cytostatic effect on cells thus interfering with the colony formation ability.

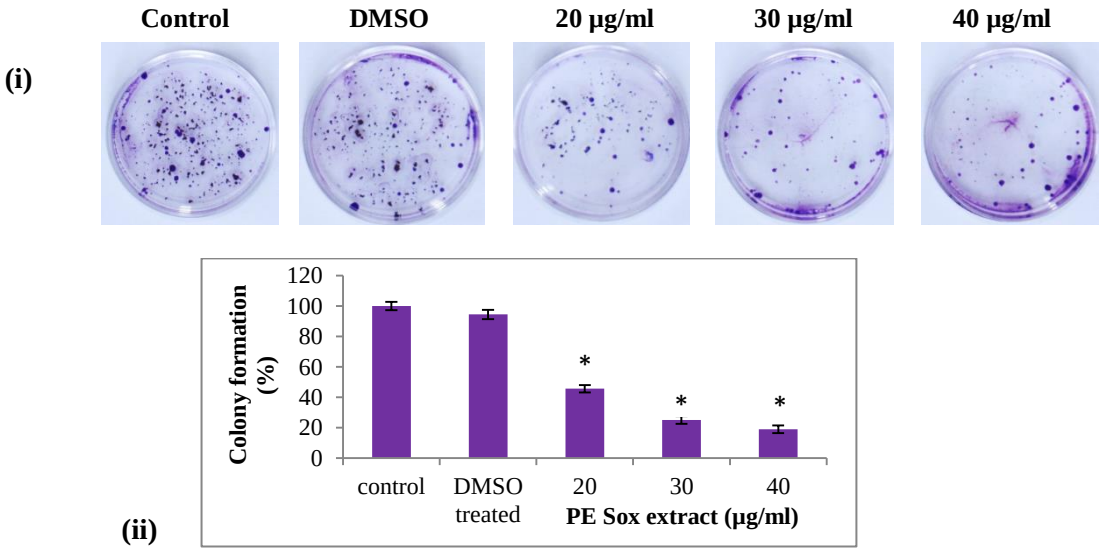


Fig 6.3 Clonogenic assay of *P. korintii* PE Sox treated K562 cells i) colonies stained with crystal violet (ii) histogram showing significant percentage reduction in number of colonies compared to untreated and vehicle (DMSO)-treated controls. The colonies were counted manually and expressed as percentage of treatment relative to the control. * $P < 0.05$

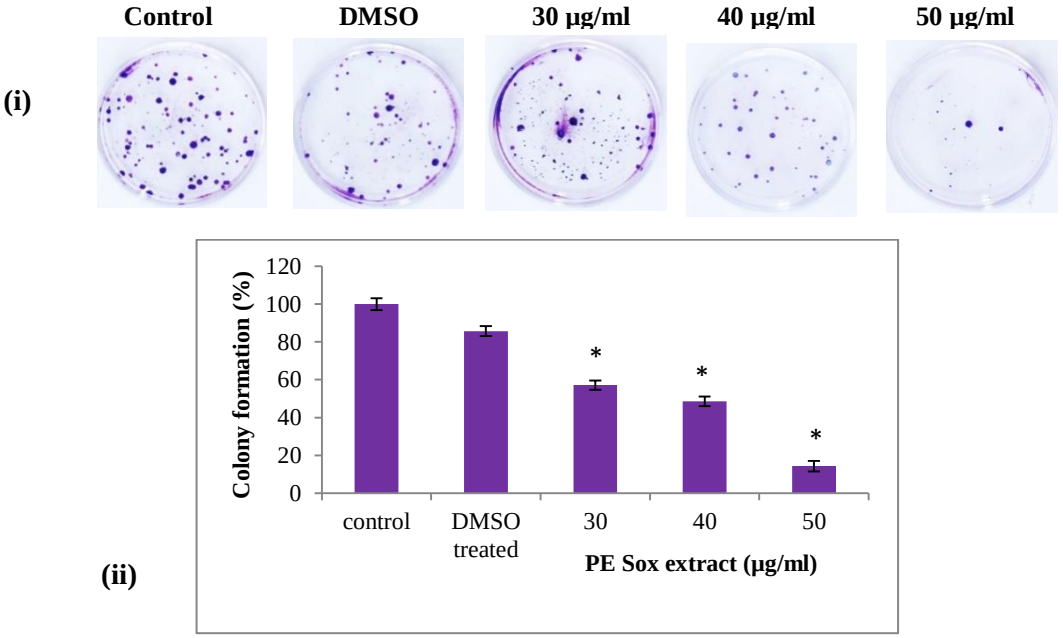


Fig 6.4 Clonogenic assay of *P. korintii* PE Sox treated HCT116 cells - (i) colonies stained with crystal violet (ii) histogram showing significant percentage reduction in number of colonies compared to untreated and vehicle (DMSO)-treated controls. The colonies were counted manually and expressed as percentage of treatment relative to the control. * $P < 0.05$

6.4 Assessment of cancer cell migration potential on treatment with PE Sox extract

The extract under study exerted significant anti-proliferative activity and was active in reducing the clonogenic potential. The next step in the investigation of cancer suppression activity of the extract was evaluation of the ability to affect stages of cancer progression namely migration and invasion. Metastasis empowers cancer cells to spread and is an important target for cancer chemotherapeutics. As migration is a primary step in cancer metastasis, the effect of PE Sox extract on migration of HCT116 cells were analysed using the *in vitro* scratch assay.

The extract treated cells showed decreased migratory potential even at low concentrations of the extract (Fig.6.5). The untreated and DMSO treated control showed 40% wound closure after 48 hours. No significant migration was observed in treated cells and as the time period increased, cell death was noted. The results clearly indicate inhibition of cancer cell migration by the extract.

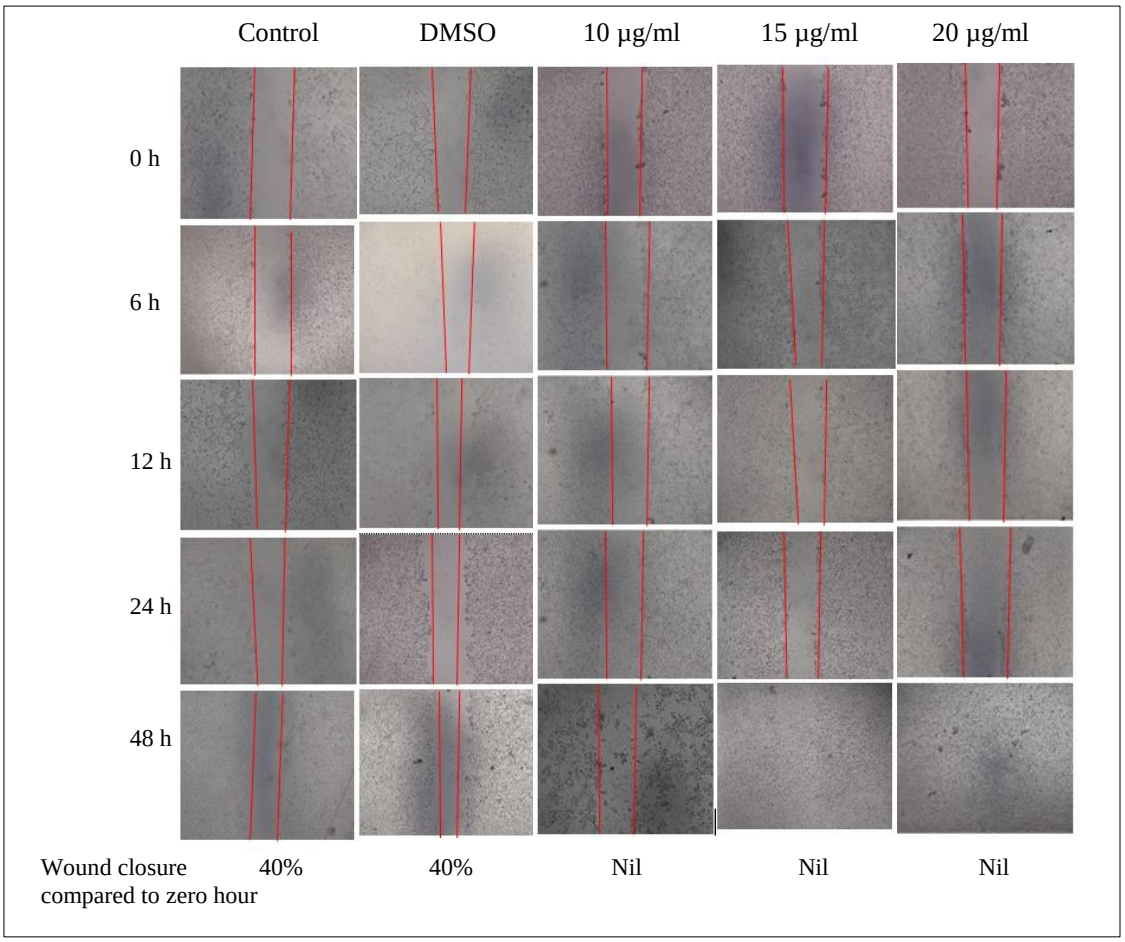


Fig. 6.5. Effect of *P. korintii* PE Sox extract on HCT116 cell migration by *in vitro* scratch assay. Scratch was made on near-confluent monolayer of cells grown in serum free media and cultured the cells in the absence and presence of various low toxic concentrations (10, 15 and 20 µg/ml) of *P. korintii* PE Sox extract and photographs were taken at different time points.

6.5 Evaluation of cancer cell invasion potential on treatment with PE Sox extract

Tumor invasion and metastasis represents a series of events that leads to proteolytic degradation of extracellular matrix components. The major components of the Basement Membrane Matrix include laminin, collagen IV, entactin, and heparin sulfate proteoglycan. An invasion assay was performed with PE Sox treated HCT116 cells on transmembranes coated with geltrex® matrix. The bottom layer of the transmembrane is fixed, stained with crystal violet, photographed and the number of cells was counted to determine their invasive potential.

The extract treated cells showed a dose dependant inhibition of cell invasion. Extract concentrations and cell number were optimised to decrease cytotoxic effects. Drastic decrease in cell invasion was observed compared to the control and DMSO treated cells (Fig. 6.6). At the highest concentration tested, 90 % inhibition in cell invasion was noted.

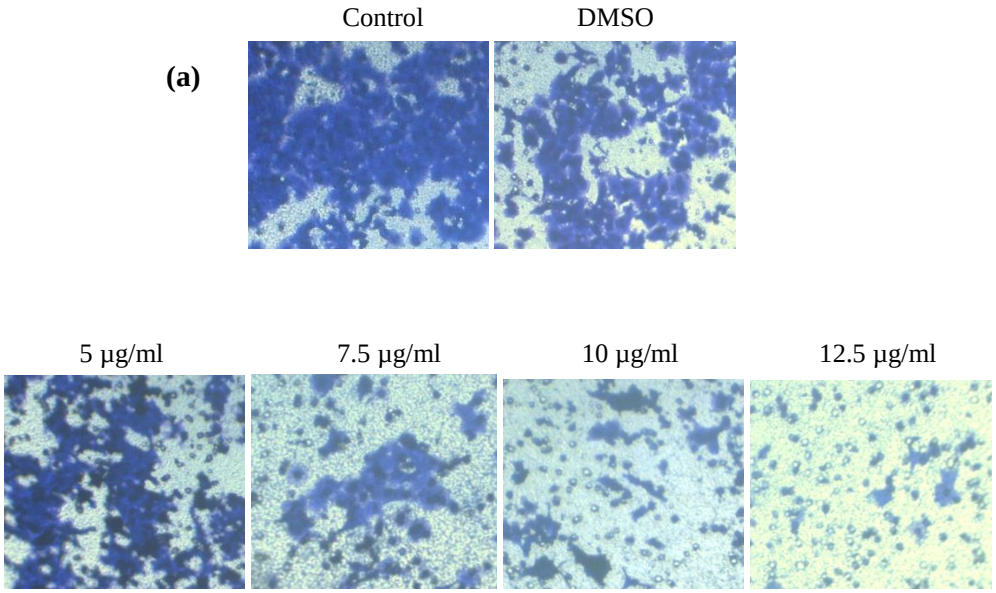


Fig. 6.6. Assessment of *in vitro* invasive potential of *P. korintii* PE Sox treated HCT116 cells using coated transwell membranes. The cells were stained with crystal violet and photographed under the microscope (a) shows cell invasion (b) the histogram. Data represented as mean ± SD of three experiments.

7. ELUCIDATION OF CYTOTOXICITY MECHANISM OF ACTION OF *P. korintii* PE SOX EXTRACT ON HCT116 AND K562 CELL LINES

There are a multitude of ways for cells to die and the Nomenclature Committee on Cell Death have listed several forms of cell death modalities identified and characterized by the differences in their corresponding stimuli, molecular mechanisms, morphologies etc. Despite these, the widely accepted and well characterized mechanisms based on the mode of execution and the end result are categorised as apoptotic cell death, necrotic cell death, autophagy and other forms of cell death. Understanding the mechanisms of cell death is of utmost importance as it aids in devising novel therapeutic strategies (Strasser, A., & Vaux, D. L., 2020; Yan, G., Elbadawi, M., & Efferth, T., 2020; Nirmala, J. G., & Lopus, M. 2020).

7.1 Assessment of cytomorphological alterations

Each type of cell death manifests with its morphological changes, for instance apoptotic cells show cytoplasmic shrinkage, pyknosis (chromatin condensation), karyorrhexis (nuclear fragmentation), plasma membrane blebbing, and generation of apoptotic bodies. Autophagy exhibits extensive cytoplasmic vacuolization and necrosis displays no distinctive features of others modes. Morphological analysis is still considered an essential step in studying the mechanism of action of drugs.

7.1.1 Phase contrast light microscopy for morphological analysis

Microscopy helped to detect the initial changes in the cells morphology on treatment with the extract. Microscopy images revealed obvious alterations in cell shape and morphology in both the tested cell lines in a concentration dependant manner compared to the control and DMSO treated cells (Fig. 7.1(a) & (b)). Considerable decrease in cell number indicating lower proliferation or cell death was observed. Morphological changes noticed includes reduction in the cell size (shrinkage), flattening of the cells, appearance of small vesicles inside cells, membrane blebbing, membrane disruption, and presence of ‘ghosts’ of cell with no

cellular contents present within cell. The control cells appeared normal as confluent monolayer. All these observations imply the induction of apoptosis in HCT116 and K562 cell lines by the PE Sox *P. korintii* extract. However, the first sign of apoptotic induction was observed much earlier at 12 hours of incubation.

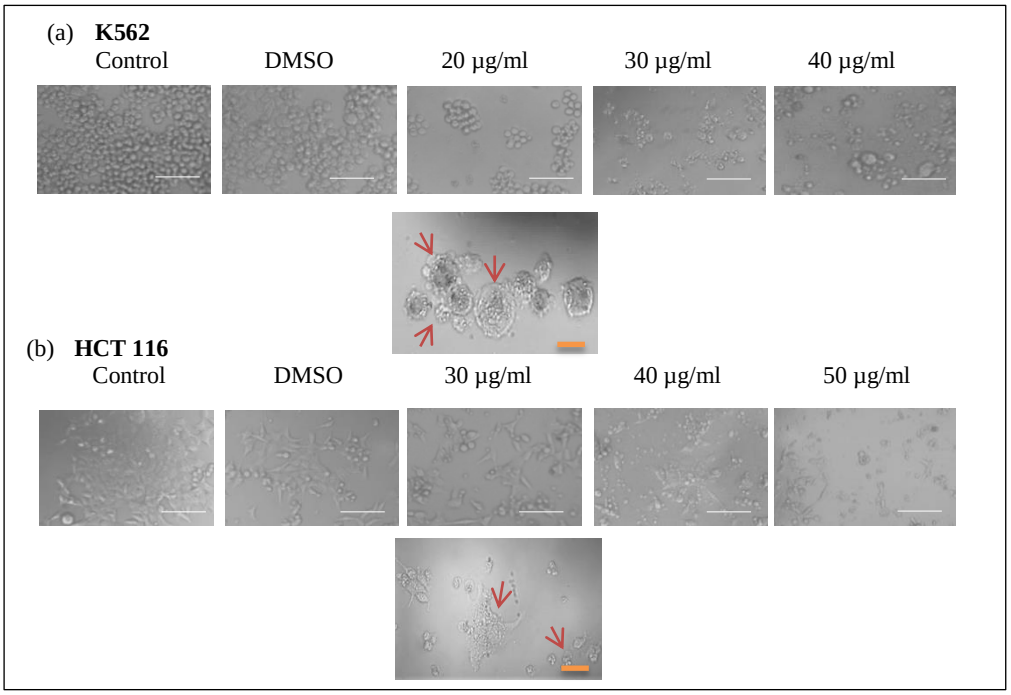


Fig. 7.1. Cytomorphological changes observed in (a) K562 and (b) HCT116 cancer cells on 48 hours treatment with *P. korintii* PE Sox extract. Magnified image of cells undergoing apoptotic induction are also shown. *White scale bars represent 50 µm and orange scale bars represent 15 µm in all images.*

7.1.2. Fluorescence microscopy for detection of apoptosis induction

a) Hoechst 33258 staining:

Apoptosis in mammalian cells are characterised by morphological changes like DNA condensation and nuclei fragmentation. These alterations are distinguished from the normal cells with the help of membrane permeable DNA binding fluorescent dyes like Hoechst 33258 which can bind to AT rich regions in the minor grooves of DNA. When excited at 350 nm, they exhibit enhanced fluorescence at 460 nm.

HCT116 and K562 cells treated with PE Sox extract at different concentrations and stained with Hoechst 33258 showed a concentration dependent increase in the number of cells with bright blue condensed nuclei when compared to the uniformly stained untreated and DMSO treated controls (Fig. 7.2.). The treated cells showed condensed and fragmented nuclei which indicate the induction of apoptosis and is marked with arrow heads in Fig.7.2.

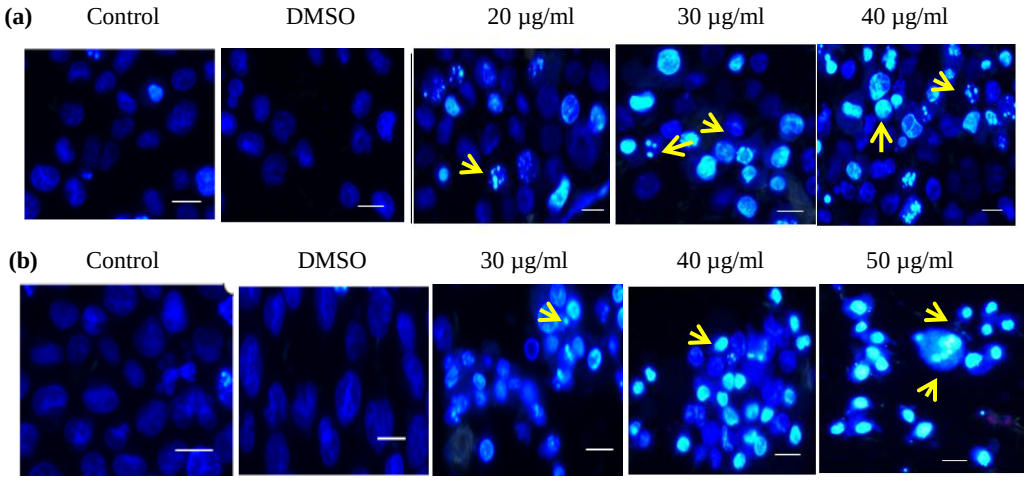


Fig. 7.2. Fluorescence staining with Hoechst 33258 (a) K562 and (b) HCT116 cells following 48 h treatment with *P. korintii* PE Sox extract *(arrow heads indicate apoptotic nuclear condensation and DNA fragmentation; scale bars represents 15 µm in all images)*.

b) Acridine orange - ethidium bromide dual staining:

The dual staining is a simple, reliable method to qualitatively and quantitatively measure apoptosis and to distinguish the early and late apoptotic stages from necrosis. Acridine orange being a permeable dye, crosses intact membranes of viable cells to stain its nuclei green and ethidium bromide being impermeable to intact membranes, enters disrupted cell membranes of apoptotic cells to bind DNA to emit yellow to orange fluorescence. Thus normal cells display uniform bright green fluorescent nuclei; early apoptotic cells show green nuclei with bright patches of condensed nuclei or fragmented DNA and late apoptotic cells exhibits highly fragmented and condensed orange to red nuclei. Necrotic cells have uniformly stained bright orange nuclei.

The staining of K562 and HCT116 cells showed a dose-dependent induction of apoptosis (Fig. 7.3). At the highest concentration of PE Sox extract tested, 60 % and 65 % of apoptotic cell population was observed for K562 and HCT116 cells respectively.

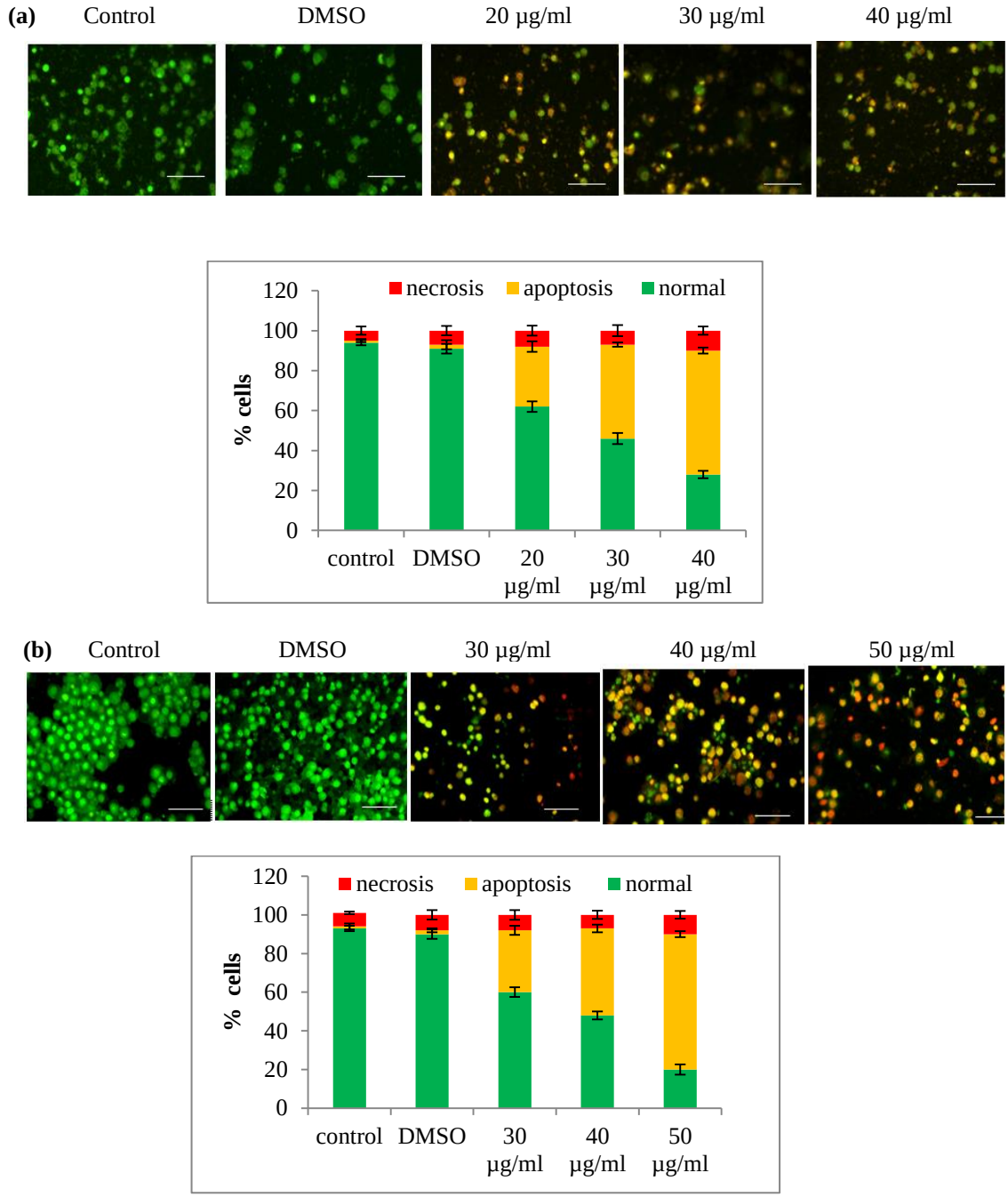


Fig.7.3. Fluorescence microscopy images of cells stained with acridine orange and ethidium bromide: Effect of PE Sox extract on (a) K562 and (b) HCT116 cells following 48h treatment and quantitative analysis of apoptotic cells. *Scale bars represent 90 µm in all images.*

c) Propidium Iodide staining:

The propidium iodide stain is impermeable to the live cells whereas the dead cells take up the dye and it binds to the nucleic acid to give red fluorescence. The nuclear morphology analysis of PE Sox extract treated cells clearly showed the presence of apoptotic cells with fragmented nuclei as shown in Fig.7.4 (a) & (b) and indicated by white arrows.

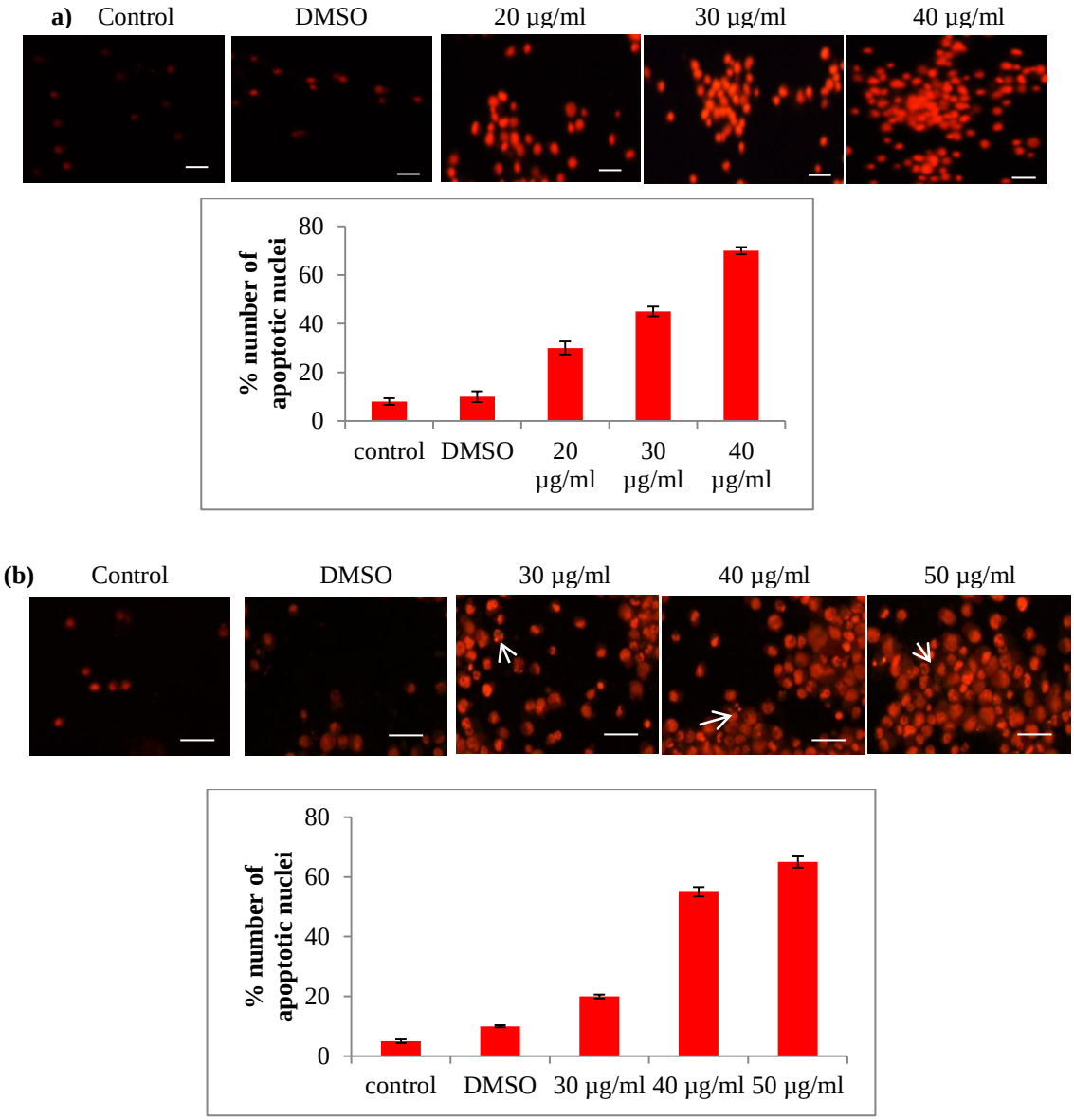


Fig.7.4. Fluorescence microscopy images of cells stained with propidium iodide: Effect of PE Sox extract on (a) K562 and (b) HCT116 cells following 48 h treatment and Quantitative analysis of apoptotic cells. *Scale bars in (a) represents 20 µm, in (b) represents 50 µm.*

7.2 Assessment of phosphatidylserine externalisation

Phosphatidylserine externalization is the biochemical hallmark of the early apoptosis. This can be detected by Annexin V / Propidium iodide staining and flow cytometric analysis to confirm the induction of apoptosis. . Annexin V/Propidium iodide co-staining distinguishes and detects late apoptotic / necrotic cells from early apoptotic cells due to Propidium iodide's ability to only enter into disrupted cells to bind with DNA and Annexin V to bind phosphatidylserine (Chan, C. K., et al., 2016).

P. korintii PE Sox extract treated K562 cells clearly showed dose dependent increases in apoptotic cells in fluorescence microscopic images (Fig. 7.5 (a)). As shown in Fig. 7.5 (b), (c), (d), the proportion of apoptotic cells (early and late apoptotic phases) significantly increased and reached at 54.7 %, corresponding to the highest concentration tested. Further, the proportion of viable cells also decreased with increasing concentration of extract.

Dose dependent increases in apoptotic cells were clearly visible in fluorescence microscopic images of PE Sox treated HCT116 cells (Fig. 7.6 (a)). The distribution of treated cells into different phases including viable ($annexin^-/PI^-$), early apoptosis ($annexin^+/PI^-$), late apoptosis ($annexin^+/PI^+$), and necrosis ($annexin^-/PI^+$) are depicted in Fig. 7.6 (b) & (d).

Concentration dependent increase of apoptotic cells were quantified (Fig. 7.6 (c)) and the highest concentration tested showed 38.3 % apoptotic cells. The results markedly indicate the externalisation of phosphatidylserine on treatment with *P. korintii* PE Sox extract on K562 and HCT116 cells, thus providing clear evidence for the induction of apoptosis.

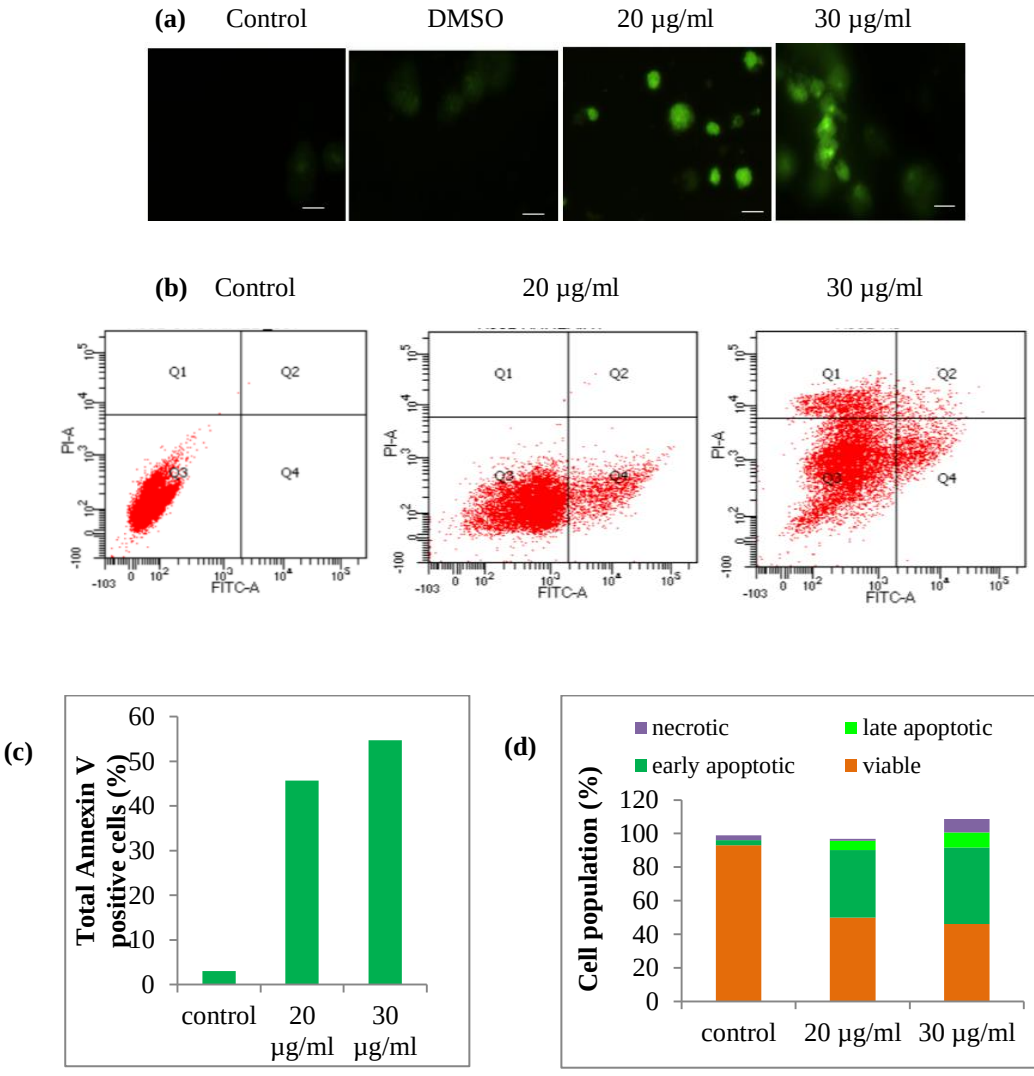


Fig. 7.5. Externalization of phosphatidylserine induced by different concentrations of *P. korintii* PE Sox extract on K562 cells detected using Annexin V-FITC/PI staining (**a**) displays the fluorescence microscopy images; (**b**) the flow cytometric fluorescence patterns; (c) The bar chart shows the percentage of Annexin V positive cells; (d) The bar chart summarizes the percentage of viable, early apoptotic, late apoptotic and necrotic cells. *Scale bars represent 15 µm in all images*

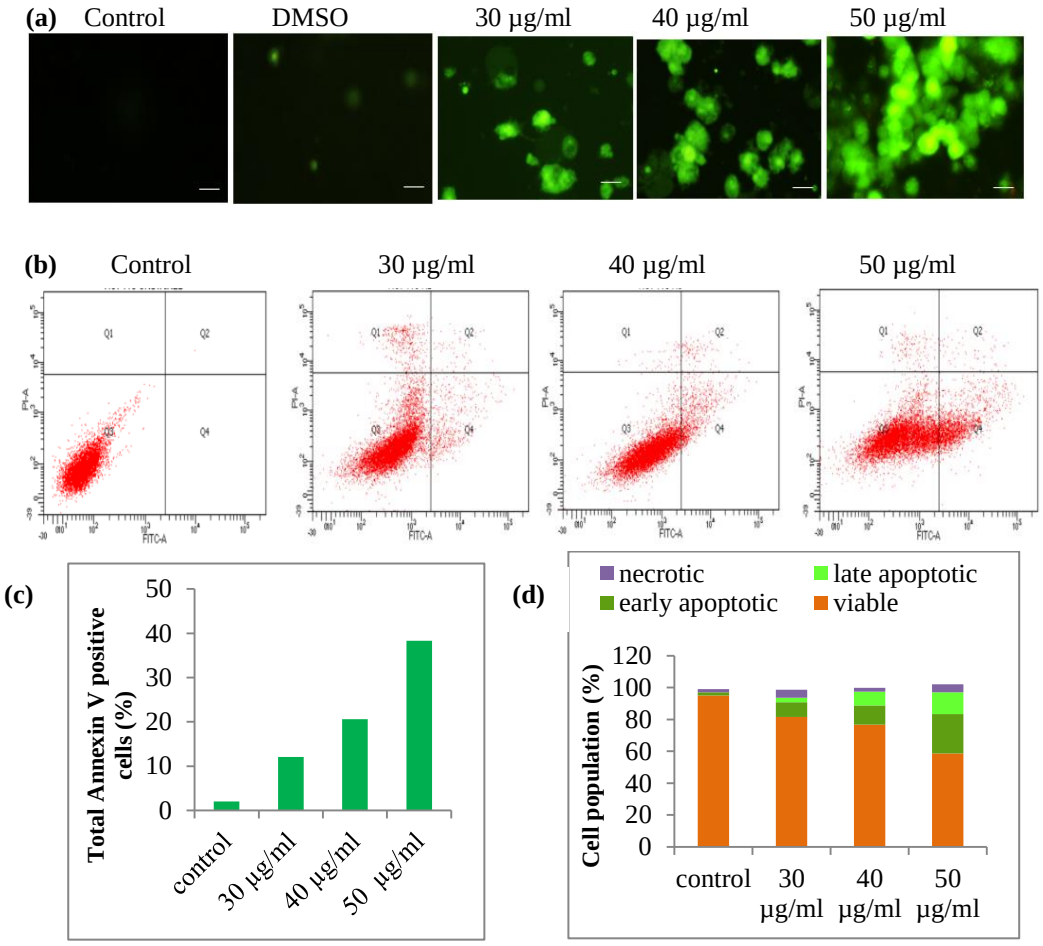


Fig. 7.6. Externalization of phosphatidylserine induced by different concentrations of *P. korintii* PE Sox extract on HCT116 cells detected using Annexin V-FITC/PI staining at 18 h. (**a**) displays the fluorescence microscopy images; (**b**) the flow cytometric fluorescence patterns; (c) The bar chart shows the percentage of Annexin V positive cells; (d) The bar chart summarizes the percentage of viable, early apoptotic, late apoptotic and necrotic cells. *Scale bars represent 15 µm in all images*

7.3 Evaluation of intracellular Reactive Oxygen Species (ROS) generation potential

Reactive oxygen species (ROS) are derived from oxygen that can oxidize other molecules. Commonly confronted and well-studied ROS in cancer research are superoxide, hydrogen peroxide, and hydroxyl radicals. Basal levels of ROS are essential for cell physiologic function whereas accumulation of ROS induces apoptosis and cell cycle arrest in cancer cells (Jamali, T. et al., 2018).

The results showed that the *P. korintii* PE Sox extract slightly elevates ROS levels about 0.3, 0.4, 0.7 fold in K562 cells treated with 10, 20 and 30 µg/ml of extract respectively. In HCT116 cells, 0.3, 0.5 and 1.7 fold increase of DCF fluorescence was noted on treatment with 30, 40 and 50 µg/ml of extract respectively.

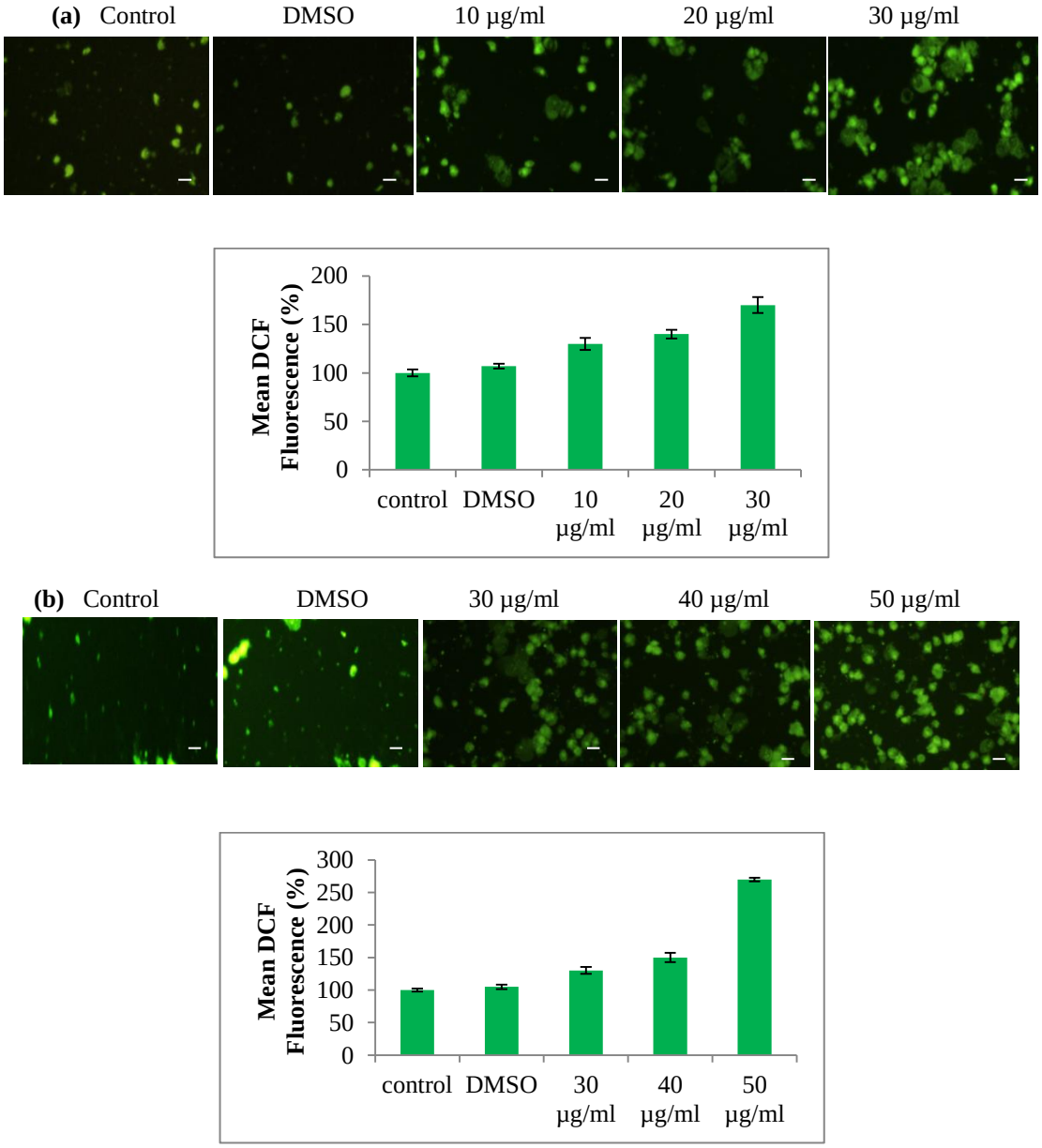


Fig. 7.7. Measurement of intracellular ROS in (a) K562 cells and (b) HCT116 cells using DCFH-DA following 48 h of treatment with PE Sox extract: fluorescence microscopy images and mean fluorescence intensity comparisons. S*cale bars represent 20 µm in all images.*

7.4 Evaluation of intracellular Ca^{2+} release

The intracellular Ca^{2+} level is highly regulated and it act as second messenger to control many cellular processes including transcription, apoptosis, proliferation etc. Several stimuli lead to release of Ca^{2+} from the ER thus overloading cytosolic Ca^{2+}. It has been reported that the disrupted Ca^{2+} levels could induce tumor cell apoptosis by activiation of caspase enzymes (Li, X. et al., 2020; Shin, D. H. et al., 2018). Intracellular Ca^{2+} levels in K562 and HCT116 cells after treatment with *P. korintii* PE Sox extract were determined by using cell permeant fluorescent dye based Ca^{2+} indicator, Fluo-3AM. The study showed that the treatment resulted in an increase in intracellular Ca^{2+} levels (Fig.7.8). At a concentration of 30 µg/ml, 2.3 fold increase of intracellular Ca^{2+} was observed in K562 cells and 5.3 fold increases in HCT116 cells at a concentration of 50 µg/ml compared to the control.

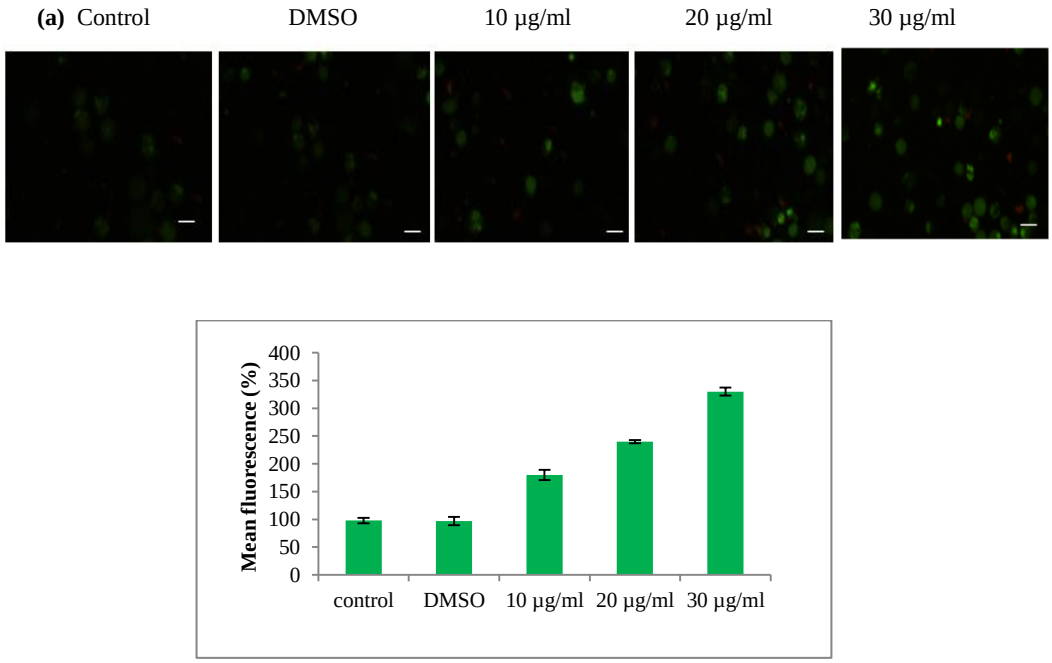


Fig.7.8. Determination of the intracellular Ca^{2+} levels in K562 cells on treatment with *P. korintii* PE Sox extract using Fluo-3AM fluorescent probe. Quantitative results of the fluorescent intensity of Fluo-3 are represented in bar diagram. Results are displayed as mean ± SD (n=3).

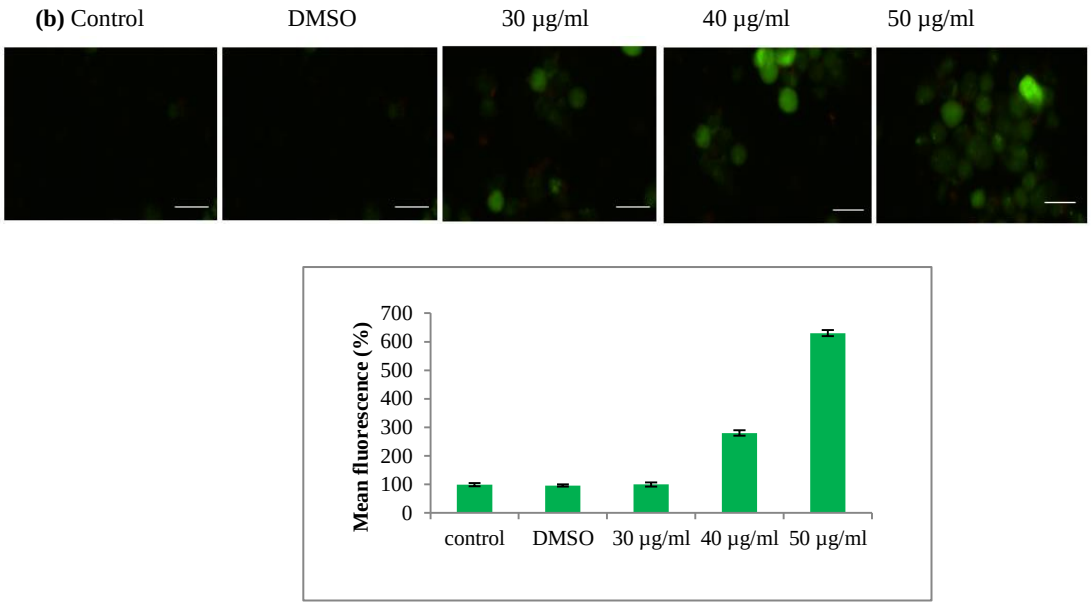


Fig.7.9. Determination of the intracellular Ca^{2+} levels in HCT116 cells on treatment with *P. korintii* PE Sox extract using Fluo-3AM fluorescent probe. Quantitative results of the fluorescent intensity of Fluo-3 are represented in bar diagram. Results are displayed as mean ± SD (n=3).

7.5 Evaluation of mitochondrial membrane potential changes

Depletion of the mitochondrial membrane potential (MMP, ΔΨm) is an event during the induction of apoptosis. The collapse in MMP will initiate cell apoptosis irreversibly. So, evaluation of MMP change is an essential move towards studying the induction of apoptosis. MMP changes can be detected using lipophilic cationic dyes capable of passing through the cell membrane to accumulate inside the mitochondrial matrix. Rhodamine123 is such a cationic fluorescence dye used as an indicator for transmembrane potential. Rhodamine123 can penetrate the cell membrane and enters the matrix of mitochondrion depending on the potential difference of the membrane.

In the present study, Rhodamine123 is applied to detect the MMP change in connection to proving the induction of cell apoptosis. The *P. korintii* PE Sox treated K562 showed an increase in fluorescence (Fig 7.10 (a)) and the fluorescence intensities were measured and expressed as % mean fluorescence compared to control. Treatment on HCT116 cells also exhibited dose dependant increase in

fluorescence which dictates the changes in mitochondrial membrane potentials (Fig 7.10 (b)).

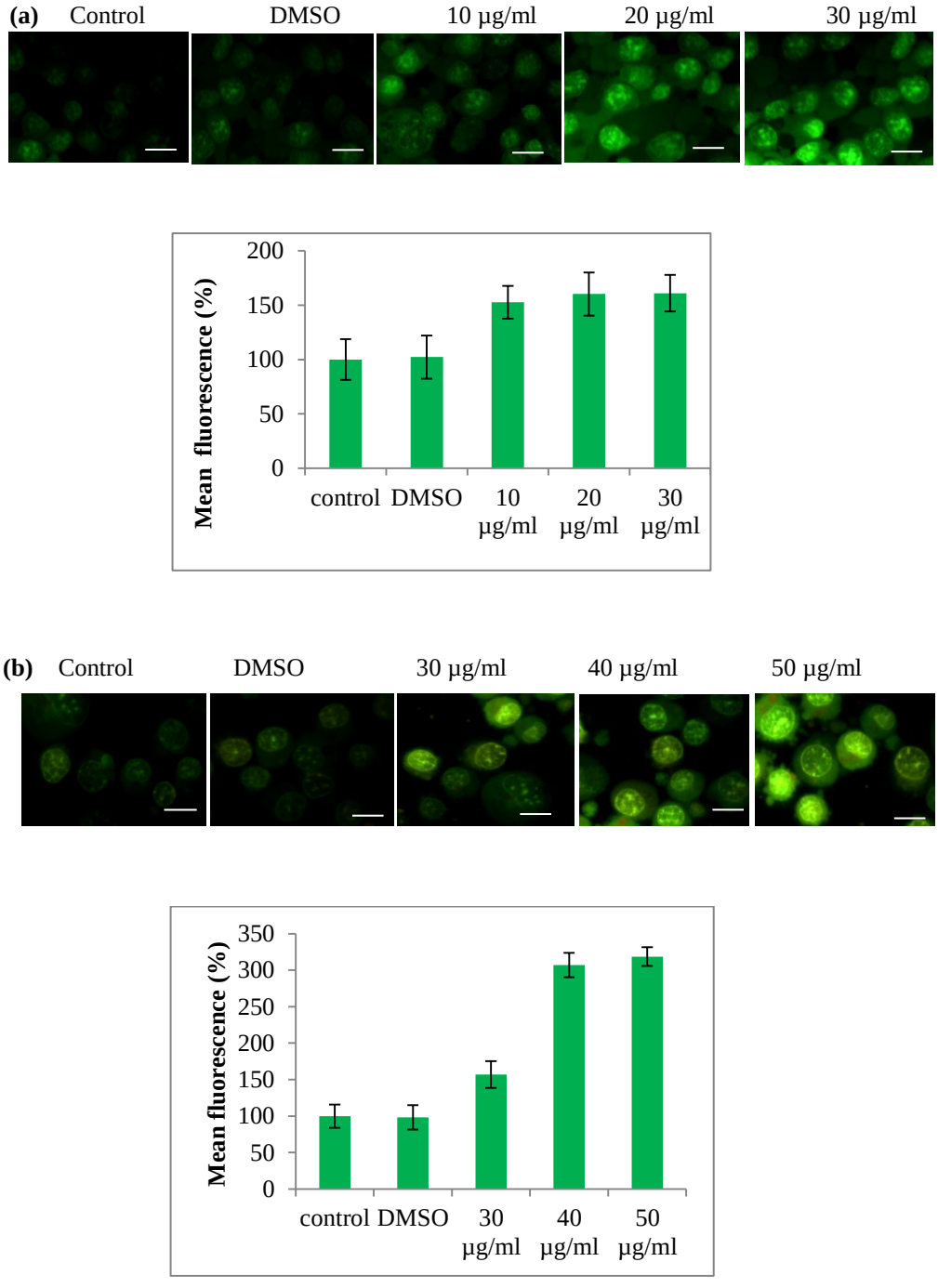


Fig.7.10. Measurement of mitochondrial membrane potential in (a) K562 and (b) HCT116 cells using rhodamine 123, following 48 h treatment with PE Sox extract: fluorescence microscopy images and mean fluorescence data comparison. *Scale bars represent 20 µm.*

7.6 Cell cycle analysis

Cell cycle distribution is studied to determine the state of DNA in response to treatment with specific drugs. As some cells die through apoptosis and others due to obstructions from cell-cycle mechanisms, cell cycle analysis is important to understand the targets and pathways involved (Mfengwana, P. H. et al., 2019).

The results discussed so far clearly indicate the induction of apoptosis by *P.korintii* PE Sox extract in K562 and HCT116 cells. In this regard, the cell cycle phase-specific distribution of PE Sox extract treated K562 and HCT116 cells population was studied by flow cytometric analysis on staining with DNA binding fluorescent dye, propidium iodide (PI). The analysis revealed the distribution of cells in three major phases of the cycle- G1, S and G2/M, and a sub-population of cells- sub-G_0 phase representing cells with fractional DNA content.

K562 cells treated with different concentrations of PE Sox extract for 12 hours showed a dose dependent increase in the sub G0 peak indicating induction of apoptosis (Fig. 7.11 (a)). The DNA content histogram (Fig. 7.11 (b)) showed an increase of sub G0 from 3.2 % of control to 10.3% at 30 μg/ml. The G1, S and G2/M phases exhibited concentration dependant decrease in cell population compared to the untreated control and DMSO treated cells.

HCT116 cells treated with PE Sox extract for 24 hours and analysed for cell cycle phase-specific distribution showed a prominent dose dependant increase in sub Go peak and a moderate increase in G2 peak (Fig.7.12 (a)). The DNA content histogram (Fig. 7.12 (b)) showed an increase of sub G0 from 6.7 % of control to 22.3% at 40 μg/ml.

To sum up, *P. korintii* PE Sox extract treatment increased the sub-G0 cell population, indicating the apoptosis induction in both the cell lines used. In HCT116 a marginal cell cycle arrest in G2 at higher concentrations was also observed.

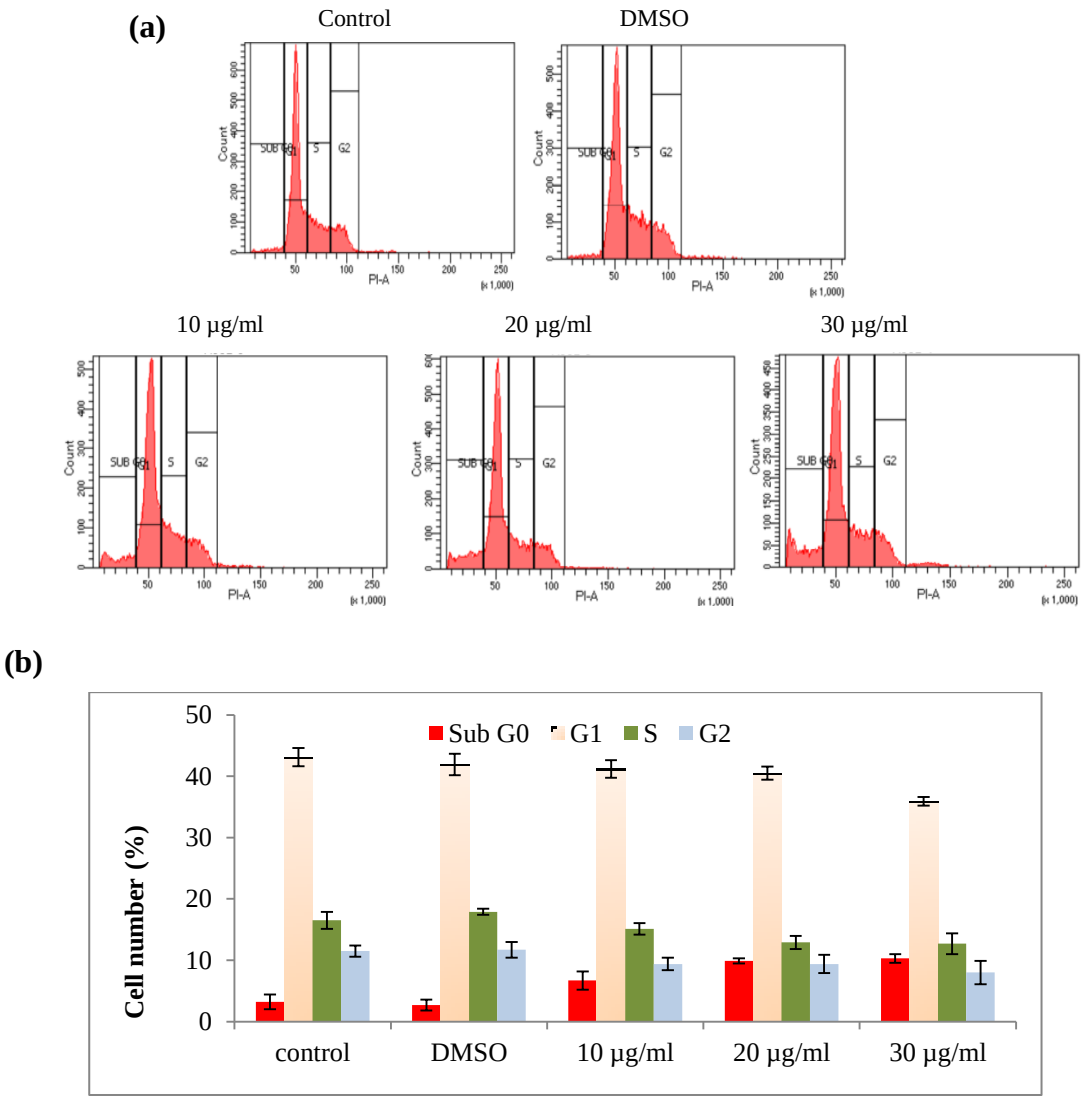


Fig.7.11. Effect of *P. korintii* PE Sox extract on cell cycle distribution of K562 cells after 12 hours treatment (a) Flow cytometric analysis following PI staining (b) quantification of cells in different cell cycle phases. Data represents mean ± SD of three independent experiments.

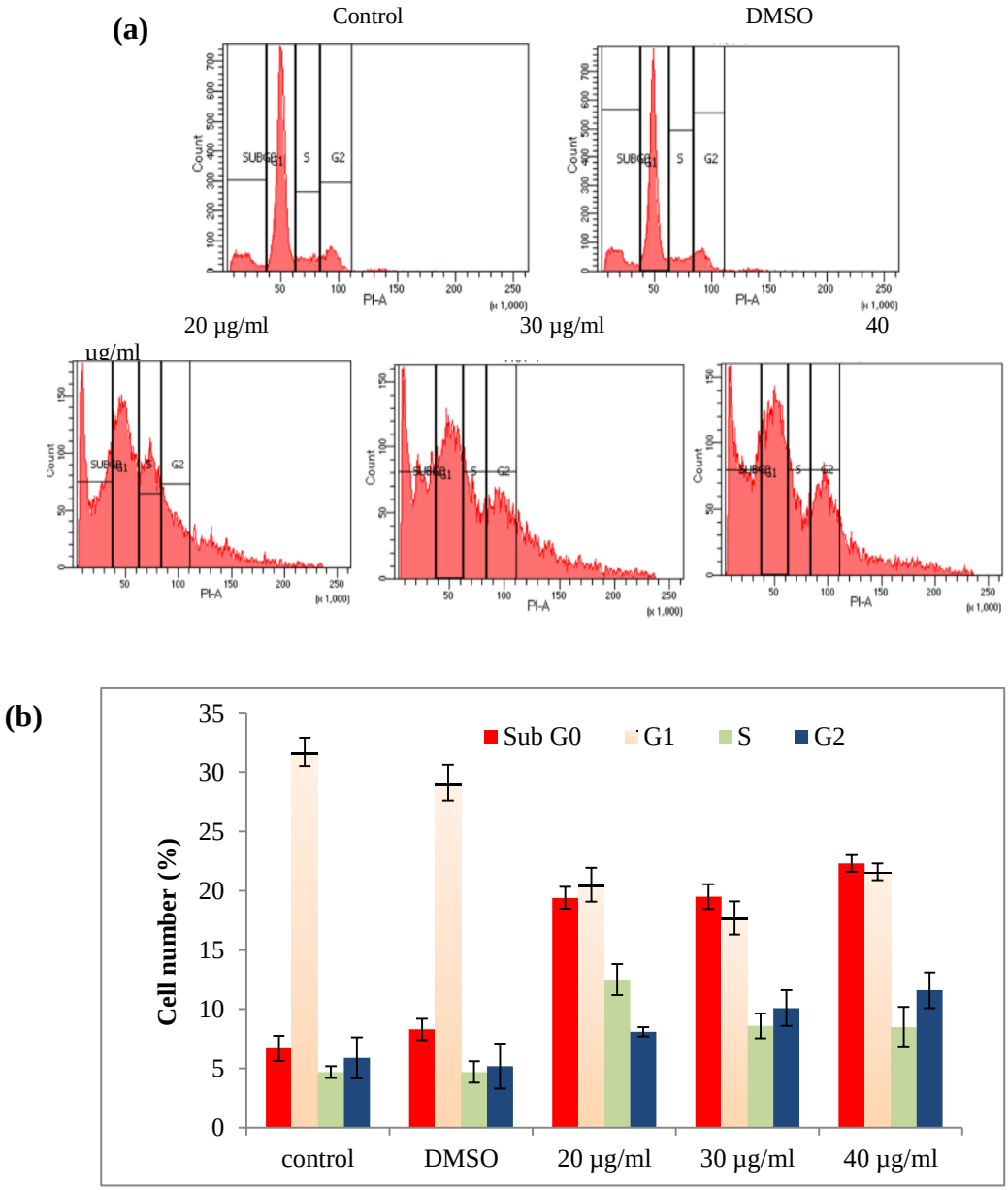


Fig.7.12. Effect of *P. korintii* PE Sox extract on cell cycle distribution of HCT116 cells after 24 hours treatment (a) Flow cytometric analysis following PI staining (b) quantification of cells in different cell cycle phases. Data represents mean ± SD of three independent experiments.

7.7 DNA Fragmentation/ laddering assay

Cleavage of chromosomal DNA into oligonucleosomal size fragments commonly known as DNA fragmentation is one among the many markers and biochemical hallmarks of apoptosis. These large fragments are then cleaved in to oligomers that are multiples of about 180 basepairs, which when run on agarose gels, appear as characteristic "DNA ladders" (Matassov, D.et al., 2004). In the present study, the DNA extracted from the control cells and the PE Sox extract treated cells, were electrophoresed on 1.8% agarose gel. A typical DNA laddering pattern was observed in treated samples compared to controls (Fig. 7.13). These observations confirm the generation of inter nucleosomal DNA fragmentation in course of apoptotic induction by *P. korintii* PE Sox in K562 and HCT116 cells.

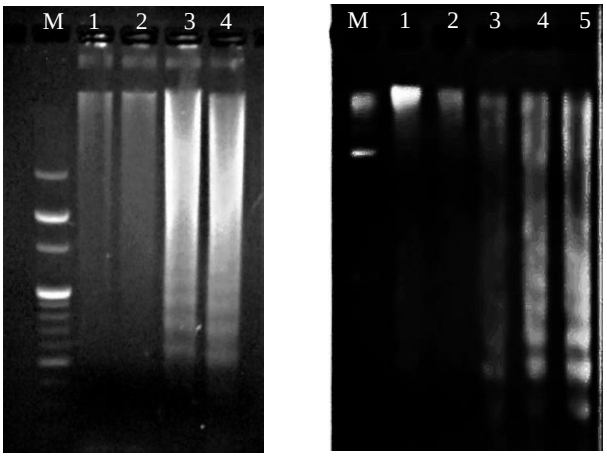


Fig.7.13 DNA fragmentation induced by *P. korintii* PE Sox extract in (a) K562 and (b) HCT116 cells. Lane M: 100bp DNA ladder, Lane 1: DNA from Control cells, Lane 2: DNA from DMSO-treated cells. Lane 3: DNA from PE Sox treated cells (a) 20 µg/ml (b) 30 µg/ml, Lane 4: DNA from PE Sox treated cells (a) 30 µg/mL (b) 40 µg/ml, Lane 5: 50 µg/ml.

7.8 Western blot analysis

PARP-1 is a nuclear enzyme which is specifically proteolysed by caspases to a 24 kDa (DNA-binding domain) and to 89 kDa (catalytic fragment) during the execution of the apoptotic program. PARP-1 cleavage promotes apoptosis by preventing both necrosis and cell survival (D'Amours, D et al., 2001). Cleavage of

PARP-1 by caspases is thus considered to be a hallmark of apoptosis (Chaitanya, G. V et al., 2010). To confirm the induction of apoptosis, PARP (Poly (ADP-ribose) Polymerase-1) cleavage was detected by western blot analysis in PE Sox treated K562 and HCT116 cells. The results showed that the treatment with PE Sox extract cleaved PARP and the 89 kDa fragment along with the intact protein was detected in the immunoblot. β- Actin was used as the loading control (Fig. 7.14).

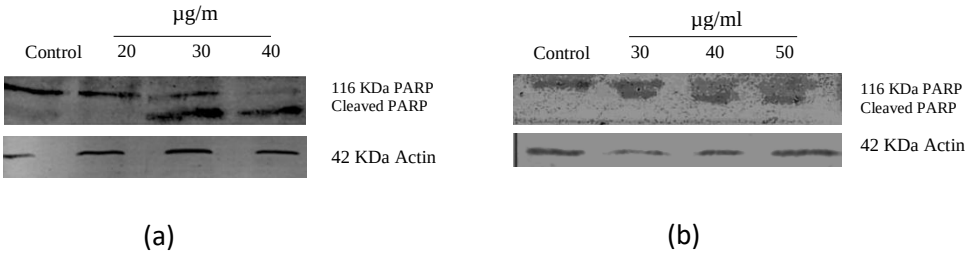


Fig. 7.14 Western blot analysis of PARP in (a) K562 and (b) HCT116 cells treated with PE Sox extract for 24 h. Immunostaining to monitor expression of cleaved PARP (visualized using TMB-HRP-H_2O_2 reaction). The protein was normalized against β-actin taken as the loading control.

To sum up, detailed investigations on leukemia and colon cancer cells showed a dose and time dependent cellular responses to PE Sox extract. PE Sox extract inhibited colony formation ability of both the cancer cells, and decreased migration and invasion ability of HCT116 cells. Microscopic investigations displayed cytomorphological changes in treated cells which involved membrane blebbing, cell shrinkage, condensation and fragmentation of nuclei, and phosphatidylserine externalization. The extract lead to slight elevation in ROS levels in both the cell lines and an increased level of intracellular Ca^{2+} was observed. Dose dependant loss of mitochondrial membrane potential was exhibited in both cell types, indicating mitochondrial apoptosis. An increase in the sub G0 peak indicating fractional DNA content, genotoxic damage as evidenced by DNA fragmentation assay and cleavage of PARP finally confirmed induction of apoptosis in PE Sox treated K562 and HCT116 cells. The present investigations have proven valuable in revealing the promising anticancer potential of this species for therapeutic use.

8. BIOACTIVITY GUIDED ISOLATION AND CHARACTERISATION OF ANTI-CANCER COMPOUNDS IN *Polyalthia korintii* PE SOX EXTRACT

As a part of the present study, a preliminary screening of the leaf extracts from *Polyalthia korintii*- an endangered endemic species found in Western Ghats was carried out to ascertain the *in vitro* pharmacological potentials. The PE Sox extract exhibited potent cytotoxic and anti-cancer potential by the induction of apoptosis. Subsequently, to isolate and characterise the anti-cancer phytochemical(s) present in the extract, bioactivity guided fractionation was carried out using chromatographic methods and characterisation was accomplished using spectroscopic techniques.

8.1 Extraction of PE Sox crude extract with different solvents for anti-cancer activity.

The PE Sox extract was extracted separately with each of the solvents viz. petroleum ether, toluene, acetonitrile, acetone, methanol and distilled water. The solvent soluble fraction (filtrate) and the insoluble portion (residue) were collected. Each of the fractions and residues were subjected to anticancer activity evaluation by MTT assay against K562 and HCT116 cell lines.

Of the various PE Sox extract fractions tested on K562 cells, most of the solvent soluble fractions exhibited anticancer activity (Fig. 8.1). Methanol soluble and acetone soluble fractions showed significant potential with comparable IC_{50} values of 29.32 ± 1.76 µg/ml and 24.2 ± 2.5 µg/ml (Table 8.1) after 48 hour treatment which is 2.48 µg/ml and 7.6 µg/ml less than the value obtained with PE Sox crude extract respectively. On HCT116 cells, the petroleum ether soluble fraction and acetone soluble fraction showed considerable activity (Fig. 8.2) with IC_{50} values of 66.18 ± 3.6µg/ml and 36 ± 2.85µg/ml respectively (Table 8.1). But only acetone soluble fraction exhibited a decrease in IC_{50} value. Considering these

results, acetone soluble portion of PE Sox extract, hereafter referred to as PE Sox ACTN fraction was used for subsequent studies and acetone insoluble fraction is referred as PE Sox ACTN insoluble.

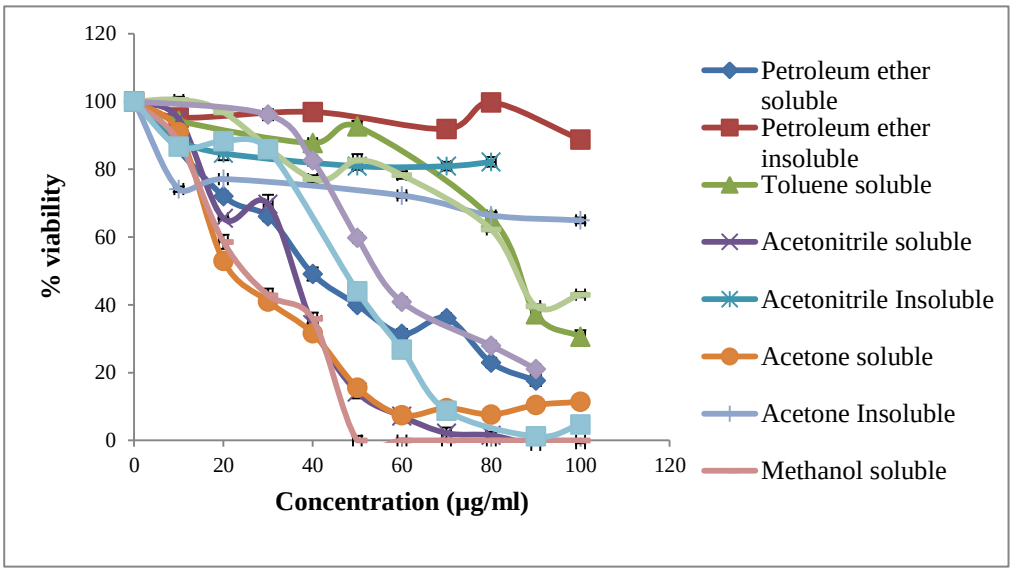


Fig. 8.1. Cytotoxicity of PE Sox extract fractions against K562 cells. Values represent mean± SD of three independent experiments.

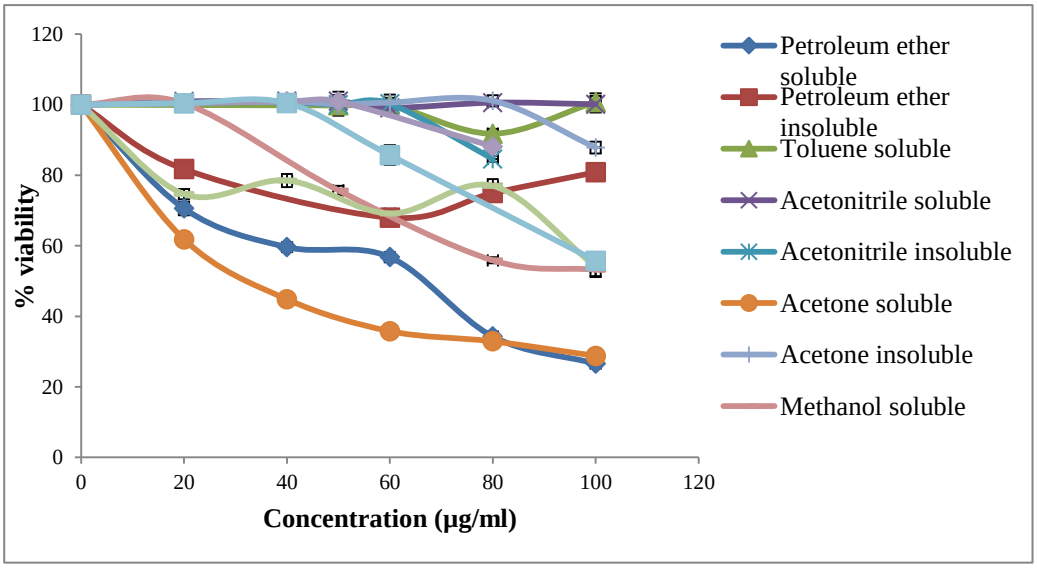


Fig. 8.2. Cytotoxicity of PE Sox extract fractions against HCT116 cells. Values represent mean± SD of three independent experiments

Table 8.1 Comparison of IC_{50} values of PE Sox extract fractions against K562 and HCT116 cells.

Solvents used	On K562 cells IC_{50}values µg/ml (Mean ± SD)		On HCT116 cells IC_{50}values µg/ml (Mean ± SD)	
	Soluble fraction	Residue	Soluble fraction	Residue
Petroleum ether	44.03 ± 1.54	>100	66.18 ± 3.6	>100
Toluene	86.16 ± 2.7	No activity	>100	No activity
Acetonitrile	44.2 ± 1.8	>100	>100	>80
Acetone	**24.2 ± 2.5**	>100	**36 ± 2.85**	>100
Methanol	29.32 ± 1.76	86.14 ± 2.82	>100	>100
Aqueous	55.42 ± 0.87	47.31 ± 0.84	>80	>100

PE Sox acetone insoluble fraction (residue) was obtained as cream coloured amorphous powder which was completely soluble in chloroform. It was then characterised by GC-MS analysis. The major compounds are Eicosane, Pentacosane, Hexacosane, and n-Nonacosane which are 20 Carbon(C), 25 C, 26 C and 29 C straight chain alkanes and accounts for 86.4% of the PE Sox ACTN insoluble fraction. Details are presented in appendix.

8.2 Evaluation of cytotoxic and apoptogenic potential of PE Sox ACTN Fraction

Fig. 8.3 depicts the cytomorphological changes associated with PE Sox ACTN treatment on K562 and HCT116 cells. The K562 cells showed decrease in cell number, cell shrinkage, membrane blebbing, cellular fragmentation, and spilling of cell contents. In HCT116 cells, the treatment resulted in loss of cell morphology, rounding of cells, cell detachment and floating, shrinking of cells, and cellular fragmentation. These characteristics are typical of apoptotic cells. Further to confirm

the apoptosis induction, acridine orange ethidium bromide dual staining was performed. As evident in Fig. 8.4, the apoptotic morphology was detected in both the cell lines on treatment with PE Sox ACTN. The untreated cells appeared with bright green normal nuclei. The arrow heads marks the late apoptotic cells showing chromatin condensation and fragmented nuclei. These results clearly indicate the induction of apoptosis in the treated cells.

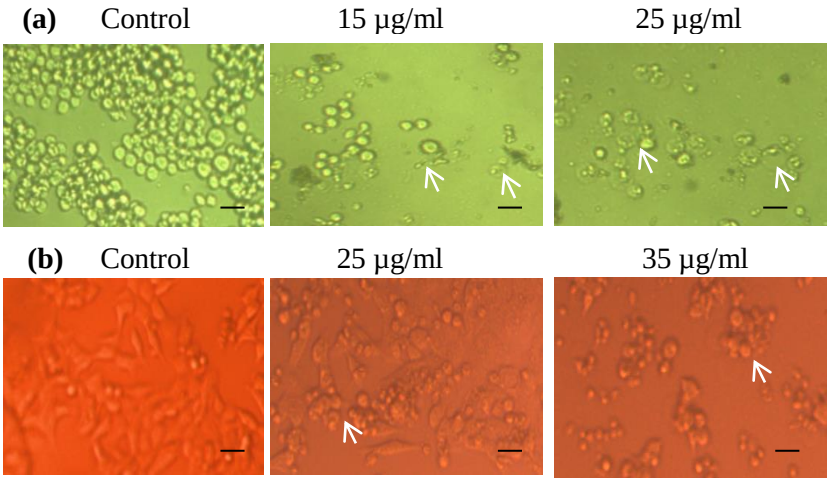


Fig. 8.3 Cytomorphological changes observed in (a) K562 and (b) HCT116 cancer cells on 48 hours treatment with *P. korintii* PE Sox ACTN. *Scale bars represent 20 µm.*

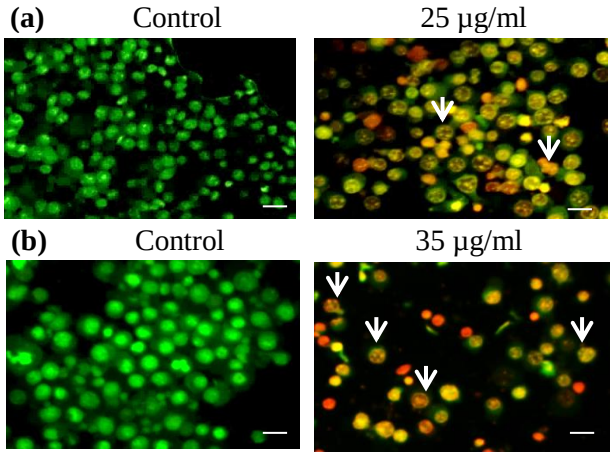


Fig. 8.4 Fluorescence images of (a) K562 and (b) HCT116 cells stained with acridine orange and ethidium bromide after treatment with PE Sox ACTN for 48 hours. *Scale bars represent 20 µm.*

Subsequently, the effect on the cell cycle phase-specific distribution of PE Sox ACTN extract treated K562 and HCT116 cells population was studied by flow cytometric analysis on staining with DNA binding fluorescent dye, propidium iodide (PI). The results revealed the distribution of cells in three major phases of the cycle- G1, S and G2/M, and the cells with fractional DNA content representing the sub-G_0 phase. K562 cells treated with specified concentrations of PE Sox ACTN for 12 hours showed a dose dependent increase in the sub G0 peak indicating induction of apoptosis (Fig. 8.5). The DNA content histogram (Fig. 8.6) showed an increase of sub G0 from 3.7 % of control to 12.4 % at 35µg/ml. The G1, S and G2/M phases exhibited concentration dependant decrease in cell population compared to the untreated control and DMSO treated cells. HCT116 cells treated with PE Sox ACTN for 24 hours and analysed for cell cycle phase-specific distribution showed a prominent dose dependant increase in sub G0 peak (Fig. 8.7). The DNA content histogram (Fig. 8.8) showed an increase of sub G0 from 16 % of control to 43.4 % at 45µg/ml. To sum up, *P. korintii* PE Sox ACTN treatment increased the sub-G0 cell population, indicating the apoptosis induction in both the cell lines used.

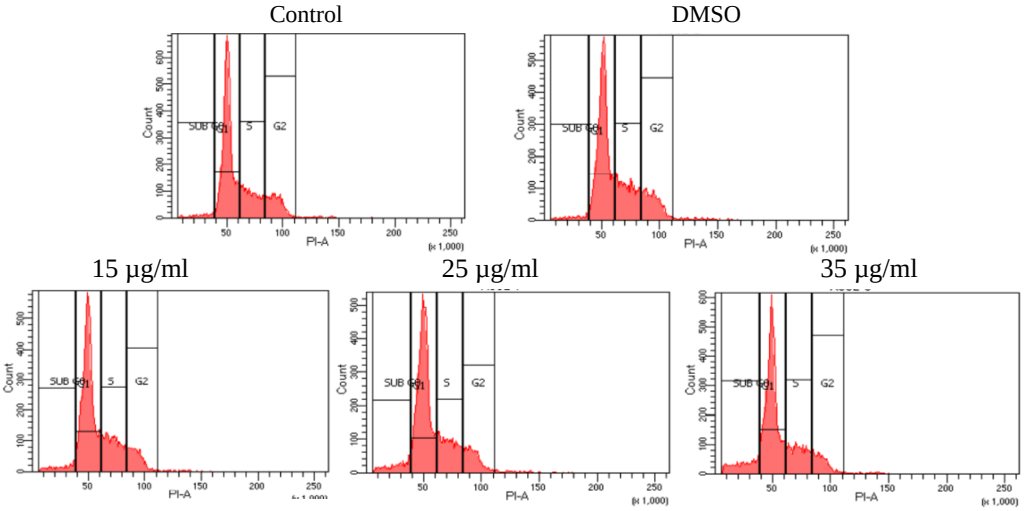


Fig. 8.5 Effect of *P. korintii* PE Sox ACTN fraction on cell cycle distribution of K562 cells after 12 hours treatment - Flow cytometric analysis following PI staining.

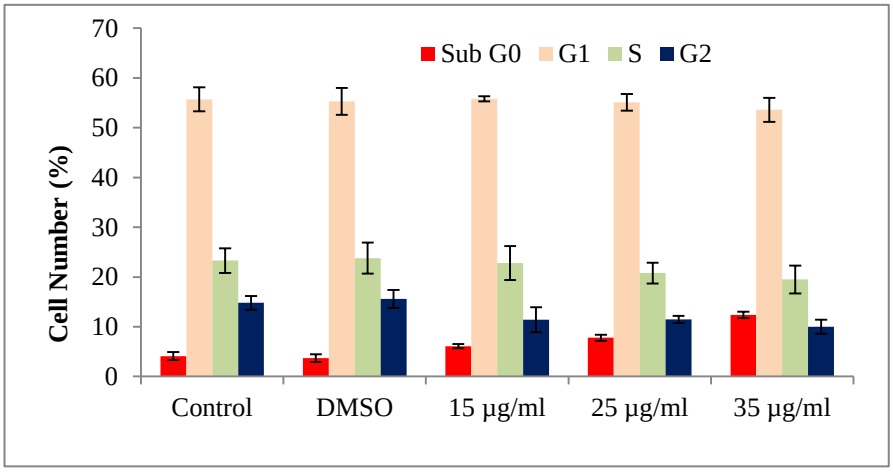


Fig. 8.6 Effect of *P. korintii* PE Sox ACTN fraction on cell cycle distribution of K562 cells after 12 hours treatment – DNA content histogram represents the quantification of cells in different cell cycle phases. Data represents mean ± SD of three independent experiments.

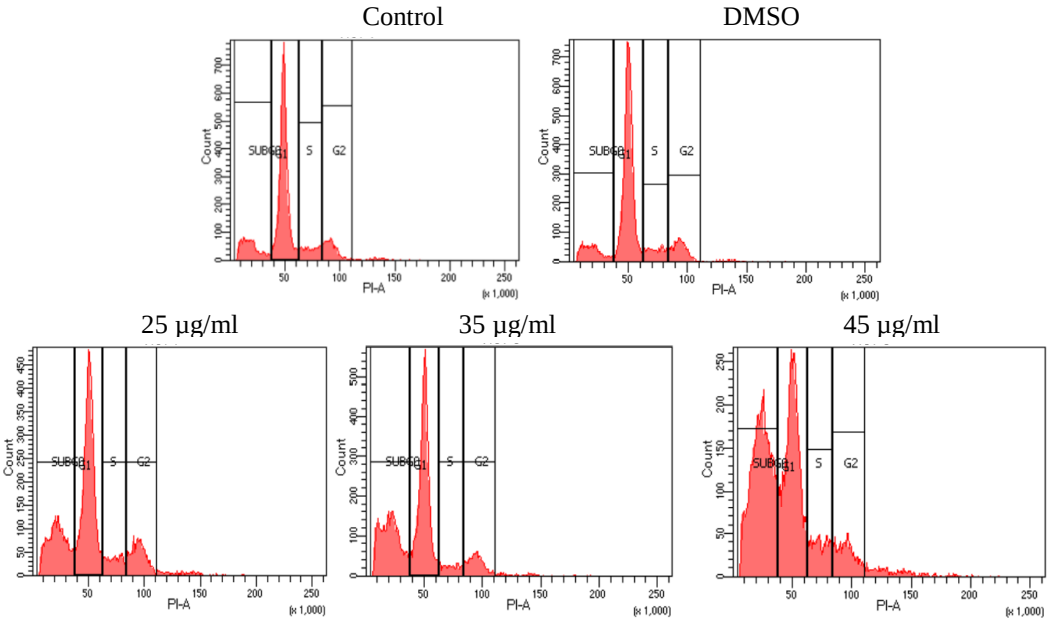


Fig. 8.7 Effect of *P. korintii* PE Sox ACTN fraction on cell cycle distribution of HCT116 cells after 24 hours treatment - Flow cytometric analysis following PI staining.

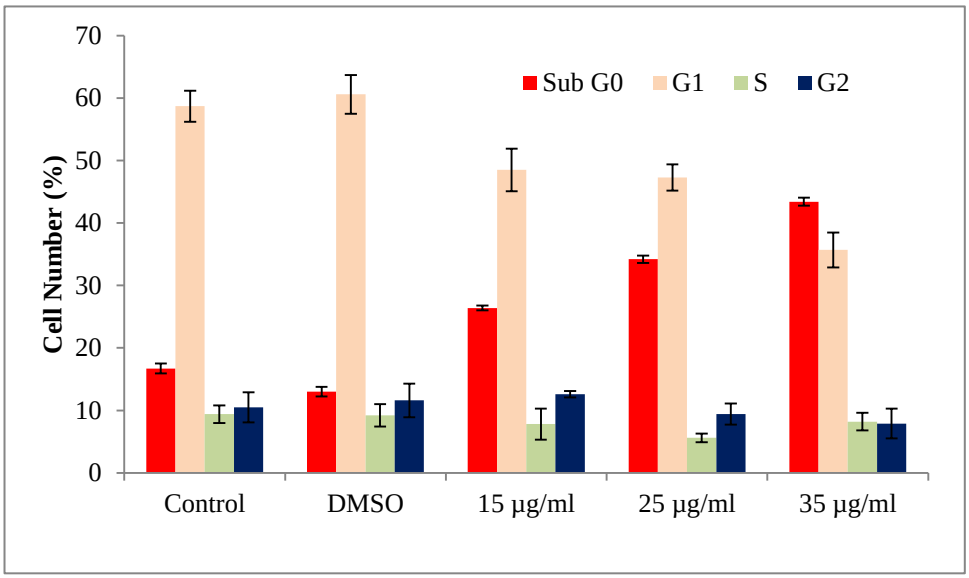


Fig.8.8 Effect of *P. korintii* PE Sox ACTN fraction on cell cycle distribution of HCT116 cells after 24 hours treatment. Histogram shows quantification of cells in different cell cycle phases. Data represents mean ± SD of three independent experiments.

8.3 Sub-fractionation of PE Sox ACTN by silver ion silica gel column chromatography

PE Sox ACTN was subjected to silver ion column chromatography as described in the Materials and Methods section. The sub-fractions were collected following elution with petroleum ether, various combinations of petroleum ether and ethyl acetate, ethyl acetate and methanol. A total of 50 fractions were obtained and the details of elution system profiles are noted in Table 8.2. MTT assay was performed to determine their cytotoxic activities against K562 and HCT116 cell lines after treatment for 48 hours. A comparison of cytotoxicity of sub-fractions in terms of IC_{50} values is depicted in Table 8.3. The sub-fractions SF25, SF27, SF31, SF35, SF36, SF37, SF38, SF40, SF42, SF43, SF47, SF49, and SF50 showed significant cytotoxic activity with prominent decrease in IC_{50} values on both the cell lines. SF40 exhibited highest anticancer activity and the lowest IC_{50} value of 12.5 ±

0.87µg/ml on K562 cells and 18.6 ± 1.2µg/ml on HCT116 cells. Very similar IC_{50} value was obtained for SF43 on K562, 13.5 ± 0.28µg/ml and 20.6 ± 1.4 µg/ml on HCT116 cells.

Table 8.2. Column chromatography elution profile of PE Sox ACTN

Elution solvents and combinations	Sub-fractions collected	
Petroleum ether	SF1	
Petroleum ether: Ethyl acetate (90:10)	SF2-SF5	
Petroleum ether: Ethyl acetate (80:20)	SF6-SF9	
Petroleum ether: Ethyl acetate (70:30)	SF10-SF13	
Petroleum ether: Ethyl acetate (60:40)	SF14-SF17	
Petroleum ether: Ethyl acetate (50:50)	SF18-SF21	
Petroleum ether: Ethyl acetate (40:60)	SF22-SF25	
Petroleum ether: Ethyl acetate (30:70)	SF16-SF29	
Petroleum ether: Ethyl acetate (20:80)	SF30-SF33	
Petroleum ether: Ethyl acetate (10:90)	SF34-SF37	
Ethyl acetate	SF38, SF39	
Methanol	SF40 On concentration to 1/4th volume, these sub-fractions showed phase separation.	
	Upper phase	Lower Phase
	SF41U	SF41L (brown)
	SF42 U	SF42L (oily)
	SF43 U	SF43L (oily)
	SF44 U	SF44L (oily)
	SF45 U	SF45L (oily)

U-upper phase & L- Lower phase

Table 8.3 Cytotoxicity of PE Sox ACTN sub-fractions

Fractions	IC_{50} values (Mean ± SD) µg/ml	
	K562 cells	HCT116 cells
SF25	57 ± 1.42	65 ± 0.67
SF27	44 ± 2.34	52.6 ± 1.49
SF31	56 ± 1.89	62.5 ± 1.3
SF35	**12 ± 2.08**	**20.2 ± 2.4**
SF36	56 ± 1.4	68.4 ± 1.4
SF37	32 ± 0.2	43.7 ± 2.8
SF38	50.5 ± 1.09	62.1 ± 0.6
SF40	**12.5 ± 0.87**	**18.6 ± 1.2**
SF42	18.5 ± 1.92	22.5 ± 0.8
SF43U	**13.5 ± 0.28**	**20.6 ± 1.4**
SF42L	23.5 ± 1.56	29.5 ± 1.5
SF44L	16.5 ± 1.08	23.5 ± 1.4
SF45L	19.5 ± 0.56	25.8 ± 0.9

8.4 Characterisation of sub-fraction SF35 of PE Sox ACTN by GC-MS analysis

Fig. 8.9 shows the GC-MS chromatogram of SF35 and Table 8.4 depicts the compounds identified from this fraction along with their retention time and abundance (Peak area %). The results presented an array of chemical compounds with potential biological activities. 1-octadecanol (48.61 % peak area) was the major compound. Also known as stearyl alcohol, it is a saturated fatty alcohol. Other major compounds were 7, 9-di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione (a lactone) (13.47 % peak area) and Dibutyl phthalate (13.87 % peak area).

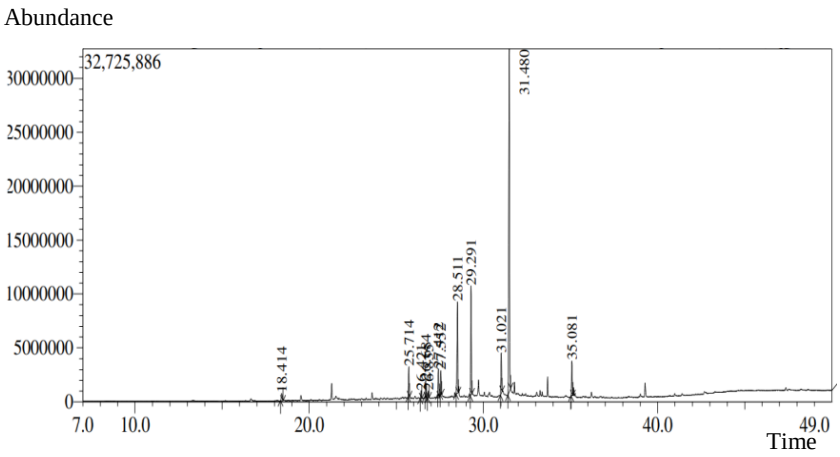


Fig. 8.9. GC-MS chromatogram of SF35.

Table 8.4 Chemical composition of SF35 by GC-MS analysis

No	Retention Time (Min)	Hit Name	Compound Formula	MW (Amu)	Peak Area (%)
1	18.414	2,6-di-tert-butyl-p-benzoquinone	$C_{14}H_{20}O_2$	220.31	1.54
2	25.714	1-octadecene	$C_{18}H_{36}$	252.5	3.40
3	26.421	Isopropyl tetradecanoate	$C_{17}H_{34}O_2$	270.5	0.98
4	26.684	3-methylene-7,11,15-trimethylhexadec-1-ene (Neophytadiene)	$C_{20}H_{38}$	278.5	1.71
5	26.835	6, 10, 14-trimethyl pentadecan-2-one (Phytone) (Hexahydrofarnesyl Acetone)	$C_{18}H_{36}O$	268.5	0.81
6	27.412	Di-iso-butyl phthalate	$C_{16}H_{22}O_4$	278.34	3.18
7	27.552	N-heptadecanol-1	$C_{17}H_{36}O$	256.5	2.96
8	28.511	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276.4	13.47
9	29.291	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	13.87
10	31.021	9-octadecen-1-ol, (z)-	$C_{18}H_{36}O$	268.5	5.13
11	31.480	1-octadecanol	$C_{18}H_{38}O$	270.5	48.61
12	35.081	1-iododododecane	$C_{12}H_{25}I$	296.23	4.35

The PASS prediction results provide Pa (Probable activity) and Pi (Probable inactivity) values to interpret the potential of a compound. Compounds with Pa > Pi is considered active. Pa values higher than 0.7 indicates the probability to obtain high activity experimentally and also there is a chance that the compound is an analogue of an existing active agent. If 0.5 < Pa < 0.7, usually less probable to obtain an activity experimentally and the substance is likely to be different from the existing active agents. Finally Pa values less than 0.5 implies least probability to find the activity experimentally but the probability of finding a novel chemical entity is high (Basanagouda, M. et al., 2011).

PASS predicted anticancer and related activities of the identified compounds are presented in Fig. 8.10. 1-octadecene (Pa: 0.649, Pi: 0.021) and 7, 9-di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione (Pa: 0.649, Pi: 0.021) exhibited activity as apoptosis agonist. N-heptadecanol-1 (Pa: 0.521, Pi: 0.030), (z) - 9-octadecen-1-ol (Pa: 0.512, Pi: 0.032), and 1-octadecanol (Pa: 0.521, Pi: 0.030) showed activity against Non-Hodgkin's Lymphoma.

6, 10, 14-trimethyl pentadecan-2-one (Pa: 0.509, Pi: 0.018) and Di-iso-butyl phthalate (Pa: 0.546 Pi: 0.011) was predicted to be antimetastatic. PASS predictions showed that 3-methylene-7, 11, 15-trimethylhexadec-1-ene and 6, 10, 14-trimethyl pentadecan-2-one were active against breast cancer (Pa: 0.646, Pi: 0.008) and cervical cancer (Pa: 0.552, Pi: 0.005) respectively. All other compounds exhibited lower Pa values (Pa < 0.5) for anti-cancer related activities which suggest a least probability to demonstrate the activity experimentally and they are not similar to the known active agents, may be new chemical entities.

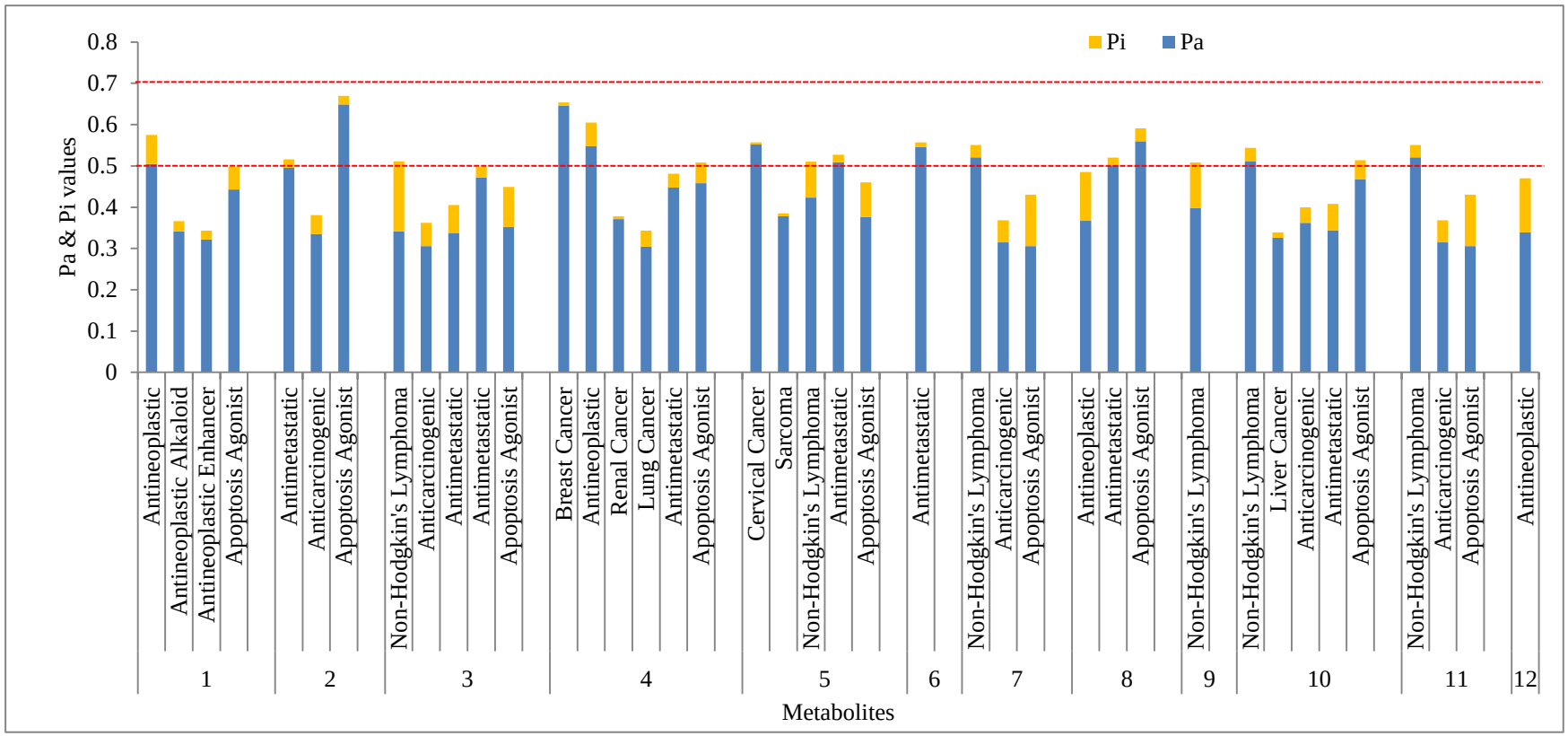


Fig. 8.10 PASS predicted anticancer and related activities of compounds identified in SF 35 sub fraction of *P. korintii* PE Sox ACTN extract. The compounds are **(1)** 2,6-di-tert-butyl-p-benzoquinone **(2)** 1-octadecene **(3)** Isopropyl tetradecanoate **(4)** 3-methylene-7,11,15-trimethylhexadec-1-ene (Neophytadiene) **(5)** 6,10,14-trimethyl pentadecan-2-one (Phytone) (Hexahydrofarnesyl Acetone) **(6)** Di-iso-butyl phthalate **(7)** N-heptadecanol-1 **(8)** 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione **(9)** Dibutyl phthalate **(10)** 9-octadecen-1-ol, (z)- **(11)** 1-octadecanol **(12)** 1-iodododecane

The literature reported biological activities of the identified compounds are summarised in Table 8.5. According to the literature, 1-octadecene, Di-iso-butyl phthalate, and Dibutyl phthalate are anti-cancer agents. A fraction of *Amomum xanthioides* containing 17.4% peak area of 1-octadecene reduced cell viability of human gastric cancer cells (AGS, KATOIII and SNU638) in a dose-dependent manner and increased intracellular Ca^{2+} concentration in SNU638 cells. So its cytotoxicity may be at least in part due to imbalance in intracellular Ca^{2+} homeostasis (Lee, Y. S. et al., 2007).

Wang, T. et al. (2012) reported the isolation of Di-iso-butyl phthalate and Dibutyl phthalate from stem of *Polyalthia rumphii.* They were cytotoxic on SPC-A-1 (IC_{50}:122 µg/ml), BEL-7402 (IC_{50}:122 µg/ml) and K562 (IC_{50}:55 µg/ml). Dibutyl phthalate effectively inhibited proliferation of DU145 (IC_{50}:27.32 µg/ml) and PC3 (IC_{50}:77.21 µg/ml) cells. Di-iso-butyl phthalate was moderately active against DU145 (IC_{50}: 449.74 µg/ml) and PC3 (IC_{50}: 785.80 µg/ml) cells (Kismali, G.et al., 2017). Dibutyl phthalate exhibited highly significant and potent antitumor activity (>100 µM) against HepG2 cell lines, cerebral glioma cells (U251), and ovarian cancer SK-OV-3/DDP cell line and the activity is ascribed to their ability to disrupt mitochondrial transmembrane potential and probable inhibition or activation of anti- or proapoptotic proteins (Wu, Z.et al., 2021).

PASS prediction has shown considerable Pa values for 3-methylene-7, 11, 15-trimethylhexadec-1-ene against breast cancer; for 6, 10, 14-trimethyl pentadecan-2-one against cervical cancer; and as apoptosis agonist for 7, 9-di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2,8-dione. But so far, these activities are not reported in literature.

Table 8.5 Biological activity of compounds identified from SF 35 sub fraction of *P. korintii* PE Sox ACTN extract.

No	Hit Name	Reported activity / uses	Reference	Type of compound
1	2,6-di-tert-butyl-p-benzoquinone	Di-*tert*-butyl benzoquinone derivatives are antiproliferative.	Misra, S. et al., 2013	Monocyclic monoterpenoid
2	1-octadecene	Used in the synthesis of colloidal quantum dots, Antibacterial, antioxidant, anticancer	Asokan, S et al., 2005; Mishra, P. M., & Sree, A. (2007); Lee, Y. S. eta l, 2007	A long-chain hydrocarbon and an alkene
3	Isopropyl tetradecanoate	Skin penetration enhancer, Used in cosmetics and topical medical preparations	Eichner, A.et al., 2017	Fatty acid ester
4	3-methylene-7,11,15-trimethylhexadec-1-ene (Neophytadiene)	An essential oil component, antibacterial activity, in treatment of headache, rheumatism and skin disease	Roy, S. et al., 2010; Lalitharani, S et al., 2010	Diterpene.
5	6,10,14-trimethyl pentadecan-2-one (Phytone) (Hexahydrofarnesyl Acetone)	An essential oil component. Volatiles rich in this compound exhibited antimicrobial activity, allopathic and pest control activities	Balogun, O. S. et al., 2017	Isoprenoid ketone
6	Di-iso-butyl phthalate	Anticancer activity against SPC-A-1, BEL-7402 and K562 cell lines.	Wang, T. et al., 2012	Oil
7	N-heptadecanol-1	Antifungal agent	Pradhan, S., & Dubey, R. C. 2021	Long-chain primary fatty alcohol
8	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	Antioxidant Found in essential oil	Merlin, N. J et al., 2009; Adeosun, C. B et al., 2013	Oxaspiro compound, lactone, an enone a cyclic ketone.
9	Dibutyl phthalate	Antibacterial, antifungal, anticancer activity against SPC-A-1, BEL-7402 and K562 cell lines, Anticancer activity on hepg2, U251 and SKOV-3 cell line	Roy, R. N. et al., 2006 ; Shobi, T. M., & Viswanathan, M. G., 2018 ; Wang, T. et al., 2012; WU, Z.et al., 2021	Oil
10	9-octadecen-1-ol, (z)-	Used in cosmetics, investigated as a carrier for delivering medications through the skin or mucus membranes	Karthikeyan, V. et al., 2016	Unsaturated fatty alcohol
11	1-octadecanol	Used as solidifying agent, in cosmetics,Antibacterial, antifungal, anti-larva	Arn, H., & Acree, T. E. 1998 ; El-Hawary, S. S. et al., 2013	Saturated fatty alcohol
12	1-iodododecane	No activity reported		Iodoalkane

Thus, the known anticancer agents identified from SF 35 are 1-octadecene, Di-iso-butyl phthalate, and Dibutyl phthalate; the other predicted compounds (Pa >0.5) with anticancer activity are 2, 6-di-tert-butyl-p-benzoquinone, 3-methylene-7,11,15-trimethylhexadec-1-ene; 6, 10, 14-trimethyl pentadecan-2-one; N-heptadecanol-1; 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione; 9-octadecen-1-ol, (z)- ; 1-octadecanol and that with Pa < 0.5 is Isopropyl tetradecanoate. The chemical structures of these compounds are shown in Fig 8.11.

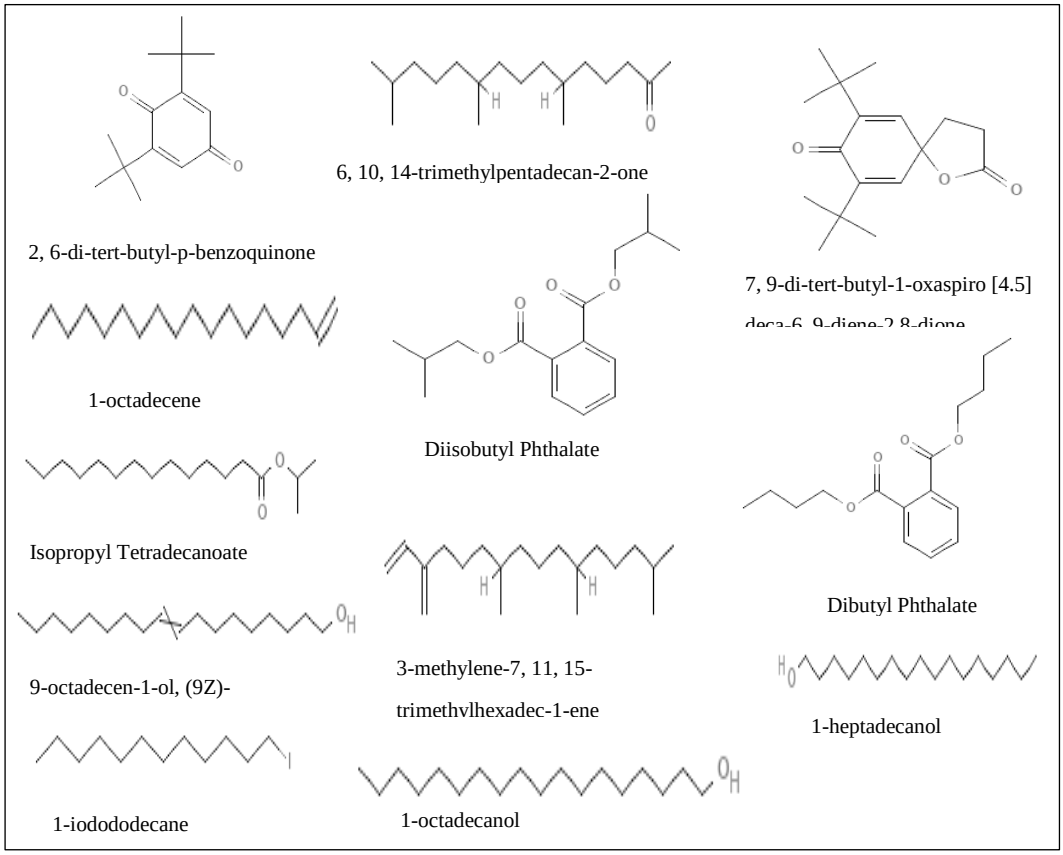


Fig 8.11. Chemical structures of anticancer compounds identified in SF35

8.5 Characterisation of sub-fraction SF40 of PE Sox ACTN by GC-MS analysis

The GC-MS analysis of sub-fraction SF40 (Fig 8.12) revealed it to be terpene and fatty acid rich fraction (Table 8.6). The terpenes 3-Methylene-7,11,15-trimethyl-1-hexadecene and 3,7,11,15- Tetramethyl-2-hexadecen-1-ol accounted to

48.21 % peak area and the long chain fatty acids and its esters were 40.15 % peak area comprising Methyl hexadecanoate and 15-methylhexadecanoate. The branched alkanes 3, 7- Dimethyldecane and 3, 3-Dimethylhexane accounted to 7.51 % peak area.

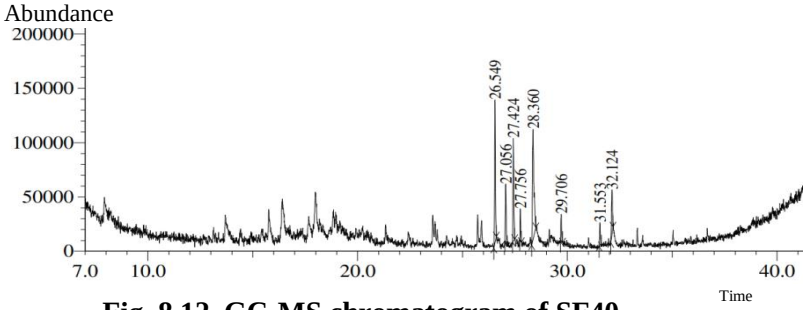


Fig. 8.12. GC-MS chromatogram of SF40.

Table 8.6 Chemical composition of SF40 by GC-MS analysis

No.	Retention Time (min)	Hit Name	Compound formula	MW (amu)	Peak area (%)
1	26.549	3-Methylene-7,11,15-trimethyl-1-hexadecene	$C_{20}H_{38}$	278.5	22.22
2	27.056 27.424	3,7,11,15- Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296.5	10.03 15.96
3	27.756	3,7- Dimethyldecane	$C_{12}H_{26}$	170.33	3.92
4	28.360	Methyl hexadecanoate	$C_{17}H_{34}O_2$	270.5	31.03
5	29.706	1-Iodo-2-methylnonane	$C_{10}H_{21}I$	268.18	4.12
6	31.553	3,3-Dimethylhexane	C_8H_{18}	114.23	3.59
7	32.124	Methyl 15-methylhexadecanoate	$C_{18}H_{36}O_2$	269.4	9.12

Fig. 8.13 represents the PASS predicted anticancer and related biological activities of metabolites identified from SF40. Among the metabolites, 3-Methylene-7, 11, 15-trimethyl-1-hexadecene; 3, 7, 11, 15- Tetramethyl-2-hexadecen-1-ol; 3, 7-Dimethyldecane; Methyl hexadecanoate and 1-Iodo-2-methylnonane had Pa values > 0.3.

3-Methylene-7, 11, 15-trimethyl-1-hexadecene; 3, 7, 11, 15- Tetramethyl-2-hexadecen-1-ol; and 3, 7- Dimethyldecane were predicted to exhibit potentials as anticancer agent (Pa: 0.646, Pi: 0.008), apoptosis agonist (Pa: 0.531, Pi: 0.035) and against Non-Hodgkin's lymphoma (Pa: 0.518, Pi: 0.030) respectively. The Table 8.7 summarises the reported biological activity of SF40 metabolites.

Literature reports the anticancer activity of 3, 7, 11, 15- Tetramethyl-2-hexadecen-1-ol on Hela cell line with IC_{50} value of 4.38 µg/ml (El-Fayoumy, E. A. et al., 2021). Also, Yu, F. R. et al. (2005) reported the effective inhibition of the proliferation of the human gastric cancer cell line (SGC-7901) by Methyl hexadecanoate (11.5%) containing methyl ester rich extract of *Euphorbia kansui* by induction of apoptosis and interfering with the progression of cells through the G1 phase of the cell cycle. So far no reports on anticancer potentials of 3-Methylene-7, 11, 15-trimethyl-1-hexadecene and 3, 7- Dimethyldecane is found.

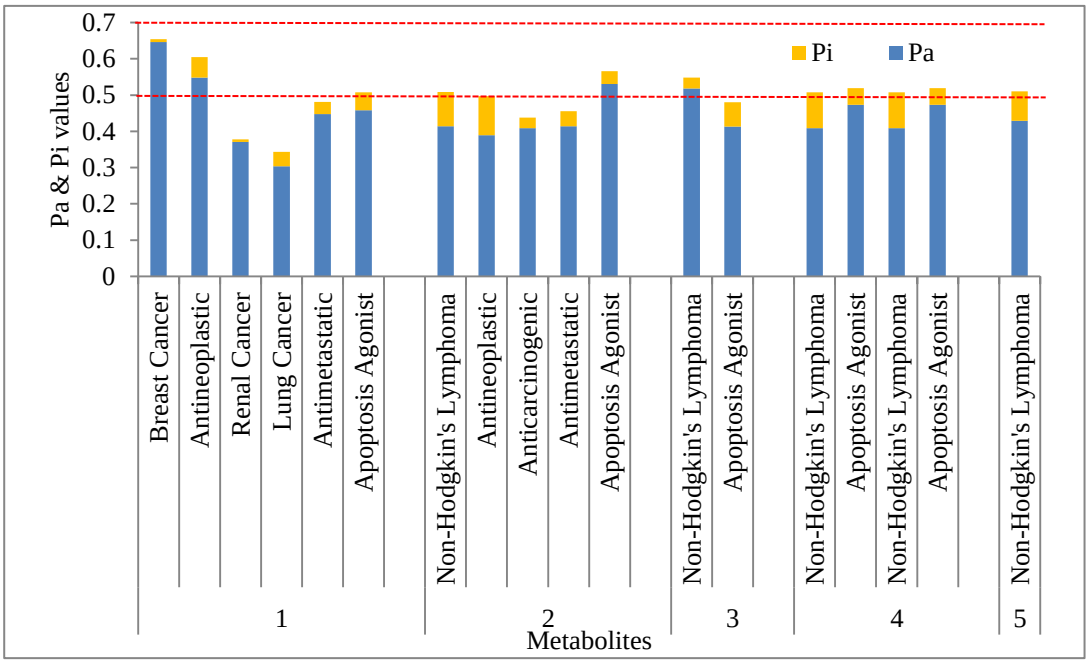


Fig. 8.13 PASS predicted anticancer and related activities of compounds identified in SF40 sub fraction of *P. korintii* PE Sox ACTN extract. The compounds are **(1)** 3-Methylene-7,11,15 trimethyl -1-hexadecene **(2)** 3,7,11,15- Tetramethyl-2-hexadecen-1-ol **(3)** 3,7- Dimethyldecane **(4)** Methyl hexadecanoate **(5)**1-Iodo-2-methylnonane

Table 8.7 Biological activity of compounds identified from SF40 sub fraction of *P. korintii* PE Sox ACTN extract.

No.	Hit Name	Reported activity / uses	Reference	Type of compound
1	3-Methylene-7,11,15-trimethyl-1-hexadecene	Present in essential oils exhibit antibacterial activity, in headache treatment, rheumatism and skin disease	Roy, S. et al., 2010; Lalitharani, S et al., 2010	diterpene.
2	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Antimicrobial, Anti-inflammatory, Anticancer, Diuretic	Karthikeyan, V.et al., 2016	Acyclic diterpene
3	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Antimicrobial, Anti-inflammatory, Anticancer, Diuretic	Karthikeyan, V.et al., 2016	Acyclic diterpene
4	3,7-Dimethyldecane	No activity reported		
5	Methyl hexadecanoate	Anti-oxidant, decrease blood cholesterol, anti-inflammatory Antibacterial and antifungal, antitumor, immunostimulant, chemopreventive and lipoxygenase inhibitor	Belakhdar, G et al., 2015; Chandrasekaran, M.et al., 2011; Rahbar,N et al., 2012	Fatty acid methyl ester
6	1-Iodo-2-methylnonane	No activity reported		
7	3,3-Dimethylhexane	No activity reported		hydrocarbon
8	Methyl 15-methylhexadecanoate	No activity reported		methylated fatty acid methyl ester

SF40 thus furnished two known anticancer agents (3, 7, 11, 15- Tetramethyl-2-hexadecen-1-ol and Methyl hexadecanoate) and three probable anticancer compounds (3-Methylene-7, 11, 15 trimethyl -1-hexadecene; 3, 7- Dimethyldecane and 1-Iodo-2-methylnonane). The chemical structures of these anticancer agents are presented in Fig. 8. 14.

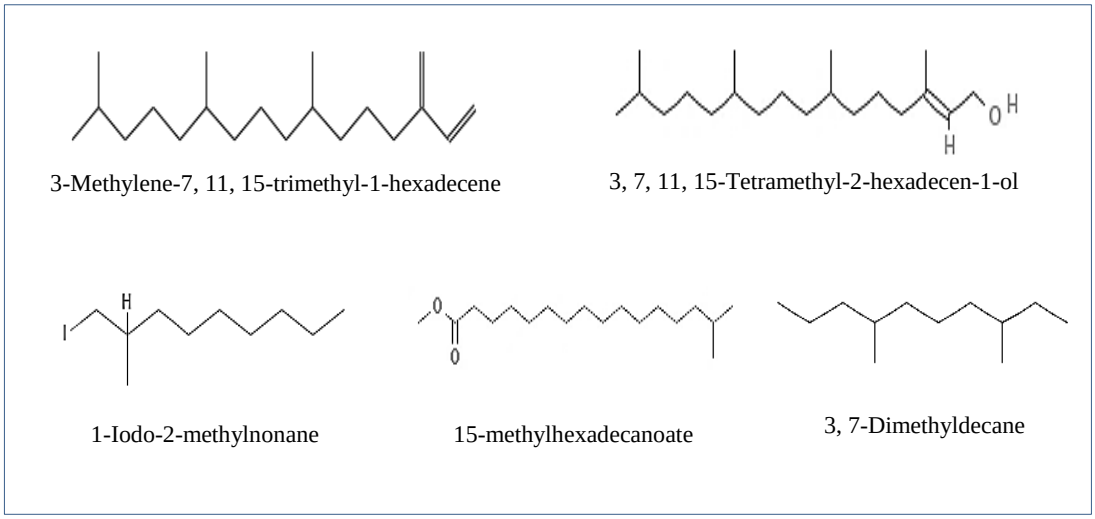


Fig 8.14. Chemical structures of anticancer compounds present in SF40

8.6 Characterisation of sub-fraction SF43U of PE Sox ACTN by GC-MS analysis

The GC-MS analysis of sub-fraction SF43U was done and it was found to be an alkane rich fraction (Fig. 8.15 & Table 8.8). Other constituents were fatty acid methyl esters (34.64 % peak area), an alcohol (6.39 % peak area) and a triterpene (7.11 % peak area).

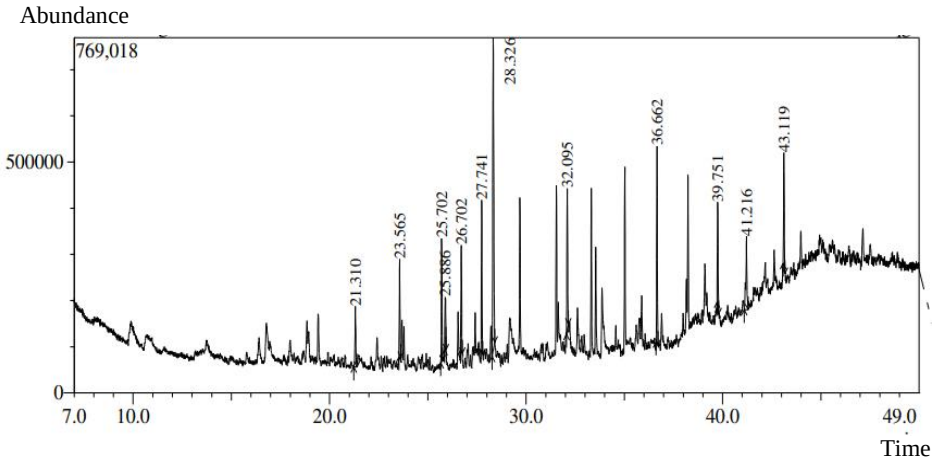


Fig. 8.15. GC-MS chromatogram of SF43U

Table 8.8 Chemical composition of SF43U by GC-MS analysis

No.	Retention Time (min)	Hit Name	Compound formula	MW (amu)	Peak area (%)
1	21.310	Hexadecane	$C_{16}H_{34}$	226.44	4.34
2	23.565	Heptadecane	$C_{17}H_{36}$	240.5	5.49
3	25.702	Octadecane	$C_{18}H_{38}$	254.5	6.93
4	25.886	2,6,10,14-Tetramethyl Hexadecane	$C_{20}H_{42}$	282.5	3.65
5	26.702	Kauran-13-ol	$C_{20}H_{34}O$	290.5	6.39
6	27.741	Nonadecane	$C_{19}H_{40}$	268.5	7.92
7	28.326	Methyl Hexadecanoate	$C_{17}H_{34}O_2$	270.5	26.22
8	32.095	Methyl Octadecanoate	$C_{19}H_{38}O_2$	298.5	8.42
9	36.662	Nonacosane	C29H60	408.8	11.15
10	39.751	Pentacosane	$C_{25}H_{52}$	352.7	5.67
11	41.216	Tetratetracontane	$C_{44}H_{90}$	619.2	6.7
12	43.119	Squalene	$C_{30}H_{50}$	410.7	7.11

Anti-cancer potential predicted with PASS software revealed considerable anticancer activity for all the constituents identified from SF43U (Fig.8.16). All the metabolites exhibited anticancer related potentials (Pa > Pi & Pa > 0.3). Kauran-13-ol exhibited antineoplastic potential (Pa: 0.709, Pi: 0.024), activity against lung cancer (Pa: 0.643, Pi: 0.008) and apoptosis agonist activity (Pa: 0.647 Pi: 0.021). Squalene showed antineoplastic activity (Pa: 0.803, Pi: 0.011), activity against lung cancer (Pa: 0.705, Pi: 0.006) and breast cancer (Pa: 0.738, Pi: 0.005); antimetastatic ability (Pa: 0.560, Pi: 0.009), and apoptosis agonist potential (Pa: 0.854, Pi: 0.005). Literature search for the biological activities of the metabolites (Table 8.9) showed that Hexadecane, Nonadecane, Methyl Hexadecanoate and squalene are known anticancer agents. In epidemiological animal models, oral consumption of squalene has shown to decrease the incidence of cancers in mammary cells and colon (Lozano-Grande, M. A.et al., 2018). Squalene is currently used as an adjunctive therapy for cancer treatment (Kelly, G. S., 1999). The chemical structures of the anticancer compounds identified from SF43U are shown in Fig. 8.17.

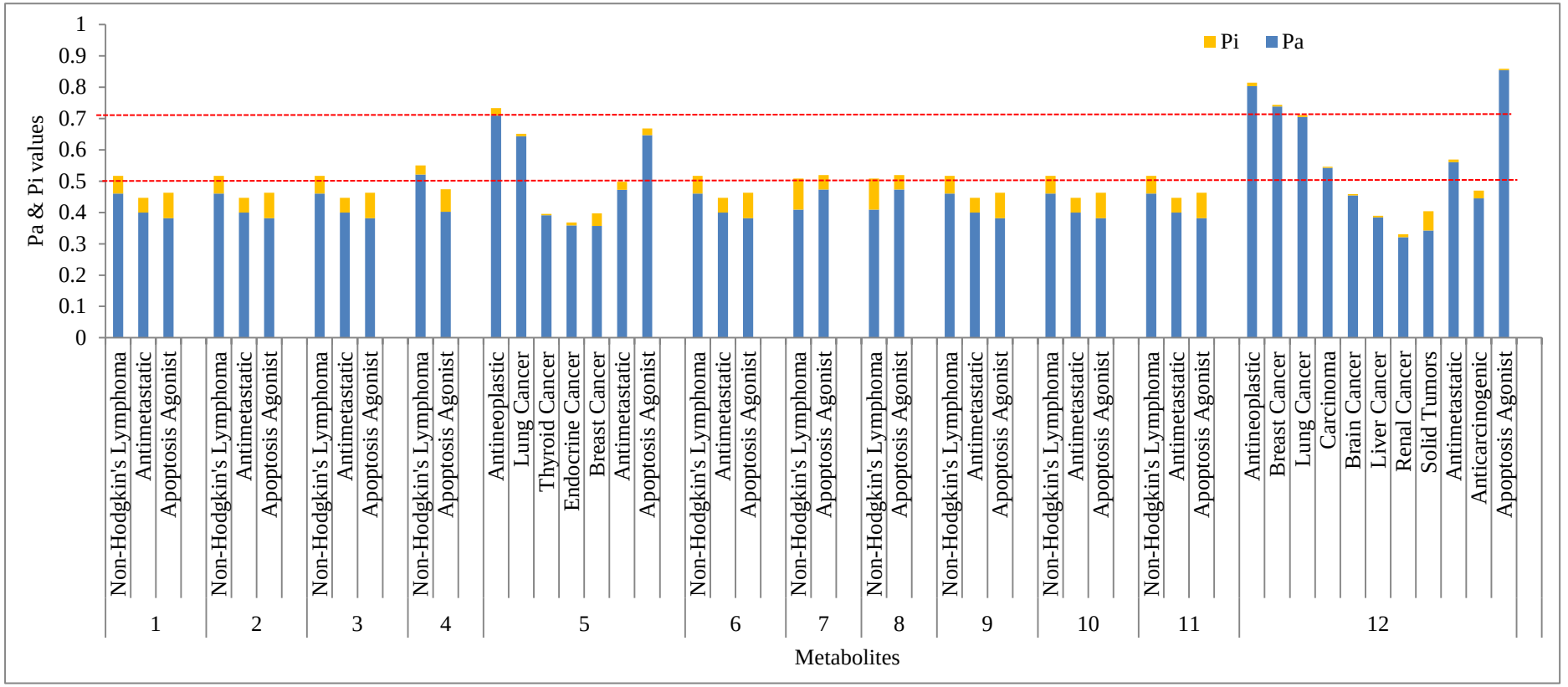


Fig. 8.16 PASS predicted anticancer and related activities of compounds identified in SF43U sub fraction of *P. korintii* PE Sox ACTN extract. The compounds are **(1)** Hexadecane **(2)** Heptadecane **(3)** Octadecane **(4)** 2,6,10,14-Tetramethyl Hexadecane **(5)** Kauran-13-ol **(6)** Nonadecane **(7)** Methyl Hexadecanoate **(8)** Methyl Octadecanoate **(9)** Nonacosane **(10)** Pentacosane **(11)** Tetratetracontane **(12)** Squalene

Table 8.9 Biological activity of compounds identified from SF43U sub fraction of *P. korintii* PE Sox ACTN extract

No	Hit Name	Reported activity / uses	Reference	Type of compound
1	Hexadecane	essential oil component, antimicrobial, antioxidant, cytotoxicity, Antipyretic, Antihelmentic, anti-tumour, anti-tuberculosis, Antidiabetic, Anti-inflammatory, Antidiarrhoeal	Yogeswari, S. et al., 2012; Banakar, P., & Jayaraj, M. (2018).	straight-chain alkane (hydrocarbon)
2	Heptadecane	Antimicrobial activity	Rahbar, N. et al., 2012	alkane
3	Octadecane	Antioxidant, Anti-inflammatory Anticorrosion agent , Antisepsis	Banakar P., & Jayaraj, M. (2018).	alkane
4	2,6,10,14-Tetramethyl Hexadecane	No activity reported		alkane
5	Kauran-13-ol	No activity reported		alcohol
6	Nonadecane	Essential oil component, Antimicrobial, Antioxidant, Anticancer, Anti HIV, Cytotoxic, Antimalarial	Banakar, P., & Jayaraj, M. (2018).	alkane
7	Methyl Hexadecanoate	Anti-oxidant, decrease blood cholesterol, anti-inflammatory Antibacterial, antifungal, antitumor, immunostimulant, chemopreventive and lipoxygenase inhibitor	Belakhdar, G et al., 2015; Chandrasekaran, M.et al., 2011; Rahbar, N. et al., 2012	Fatty acid methyl ester
8	Methyl Octadecanoate	Antioxidant activity	Abdel-Hady, H. et al., 2018	Fatty acid methyl ester
9	Nonacosane	Oil component ,anti-mutagenic, antibacterial	Kalsum, N. et al., 2016; Konovalova, O.et al., 2013	Alkane

Continued….

10	Pentacosane	Constituent of waxes, antibacterial	Konovalova, O.et al., 2013	alkane
11	Tetratetracontane	hypoglycaemic, anti-oxidant	Kalsum, N. et al., 2016	alkane
12	Squalene	Antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant	Amarowicz, R. 2009; Konovalova, O.et al., 2013	Triterpene

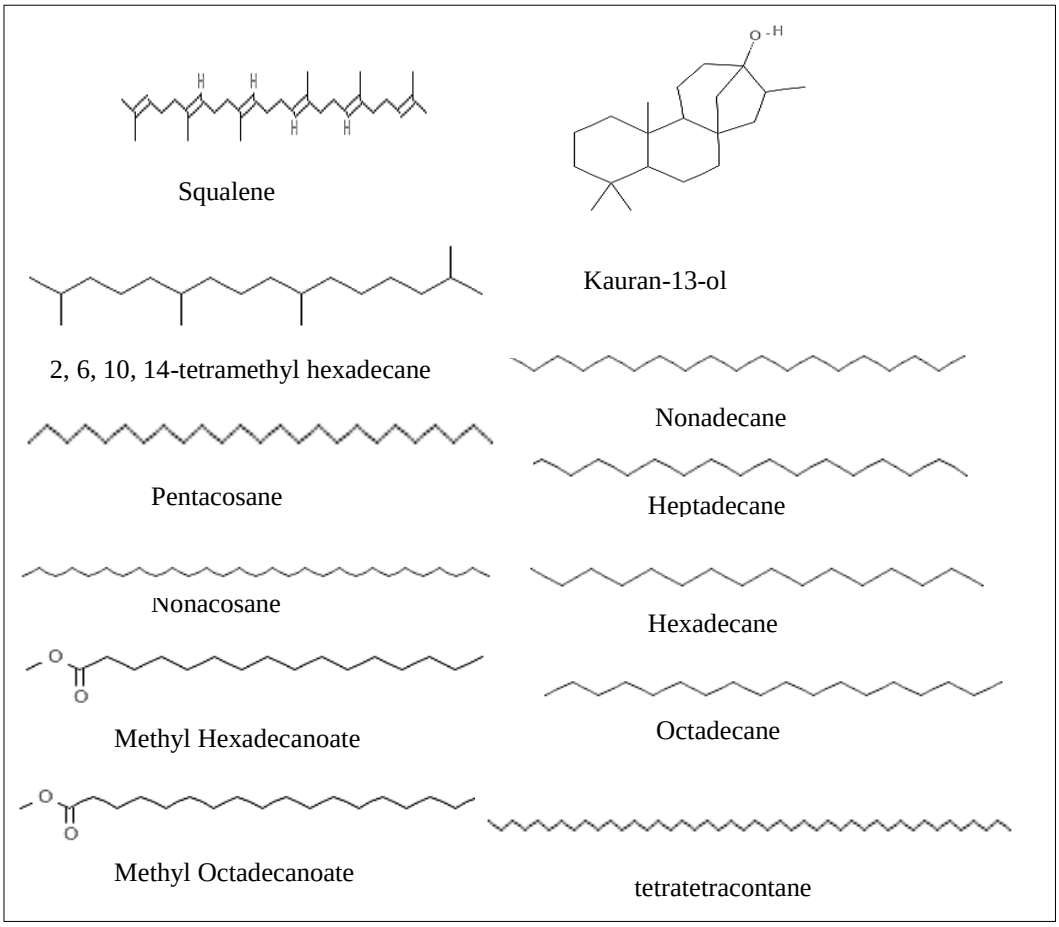


Fig 8. 17. Chemical structures of bioactive compounds present in SF43U

8.7 Molecular docking of anticancer compounds to various cancer related proteins employing Schrodinger Glide software

Molecular docking predicts the binding orientation of ligands to their protein targets in order to assess the affinity and activity of small molecules. In the present study, molecular docking interactions between selected cancer related protein targets involved in cell survival and death pathways and already known anticancer

compounds identified from the GC-MS analysis of fractions SF35, SF40 and SF43U - Squalene; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; Methyl hexadecanoate; Di-iso-butyl phthalate; Dibutyl phthalate; 2,6-Di-tert-butyl-p-benzoquinone; Nonadecane; 1-Octadecene and Hexadecane were studied using Schrödinger Glide software. The same procedure applied on known inhibitors of each protein enabled a comparison of docking efficiency. All the compounds tested showed interaction with the protein targets and the scores are shown in Table 8.10. Among these compounds, only three, namely, Di-iso-butyl phthalate, Dibutyl phthalate and 2,6-Di-tert-butyl-p-benzoquinone were found to display good docking on PI3K than the chosen known inhibitor.

Table 8.10. Molecular docking scores of anticancer compounds identified from sub fractions on protein targets

Ligands	Protein targets (Docking score in kcal/mol)						
	ABL Kinase	AKT	B-RAF	VEGFR2	EGFR	CDK2	PI3K
Squalene	-7.96	-3.847	-3.765	-7.447	-2.467	-5.698	-4.859
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	-7.804	-3.696	-4.626	-5.69	-4.832	-4.661	-4.288
Methyl hexadecanoate	-6.613	-4.424	-4.417	-5.851	-4.319	-2.877	-3.065
Di-iso-butyl phthalate	-6.392	-3.105	-6.958	-6.404	-5.194	-5.745	**-6.531**
Dibutyl phthalate	-6.352	-2.424	-6.14	-4.828	-6.136	-5.154	**-6.68**
2,6-Di-Tert-butyl-p-benzoquinone	-6.329	-3.299	-6.432	-4.653	-6.285	-5.687	**-5.311**
Nonadecane	-4.648	-0.944	No interaction	-3.916	No interaction	-3.396	-2.073
1-Octadecene	-3.833	-1.164	-1.402	-2.401	-0.179	-2.712	-1.631
Hexadecane	-3.236	-1.3	No interaction	-2.821	No interaction	-2.54	-0.936
Known Inhibitors	-16.362 (Nilotinib)	-7.856 (Ipatasertib)	-14.874 (Vemurafenib)	-9.309 (Axitinib)	-10.492 (Gefitinib)	-10.403 (Flavopiridol)	-5.308 (LY294002)

Molecular docking interactions between selected protein targets involved in cell survival and death pathways and already known anticancer compounds identified from the GC-MS analysis of fractions SF35, SF40 and SF43U. Ligands showing higher binding potential than the corresponding known inhibitor is marked in bold.

The ligand docking results of Di-iso-butyl phthalate -PI3K showed that the ligand displayed a greater docking score than the inhibitor of PI3K. The results also showed that the hydrophobic interaction of Di-iso-butyl phthalate- PI3K was also high (10 hydrophobic amino acids within 4Å) as depicted in the Fig. 8.18. It was observed that the ligand formed a pi-pi stacking with TYR836 besides the H-bond with VAL851. Similarly Dibutyl phthalate also formed an H-bond with VAL851 as shown in Fig. 8.19 and Table 8.11. Interaction of 2, 6-Di-tert-butyl-p-benzoquinone with PI3K is shown in Fig. 8.20.

Table 8.11. The binding site amino acid residues of PI3K (4JPS) interacting with the ligand compounds

Ligand	Bonds formed and amino acid residues involved	Binding site amino acids
Di-iso-butyl phthalate	1H-bond with VAL851 PI-PI stacking - TYR836	THR856, SER854**, VAL851**, VAL850, GLU849, ILE848, MET772, TRP780, **TYR836**, PHE930, ILE932, ASP933, MET922, ILE800, LYS802
Dibutyl phthalate	1 H-bond with VAL 851	HID855, SER854, ASN853, ARG852, **VAL851**, VAL850, GLU849, ILE 848, MET922, TRP780, MET772, TYR836, PHE930, ILE932, ASP933, ASP810, LYS802, ILE800
2,6-Di-tert-butyl-p-benzoquinone		ILE800 ,TRP780, MET772, MET922, SER919, GLN859, THR856, HID855, SER854, ASN853, ARG852, VAL851, VAL850, GLU849, ILE848, TYR836, PHE930, ILE932, ASP933

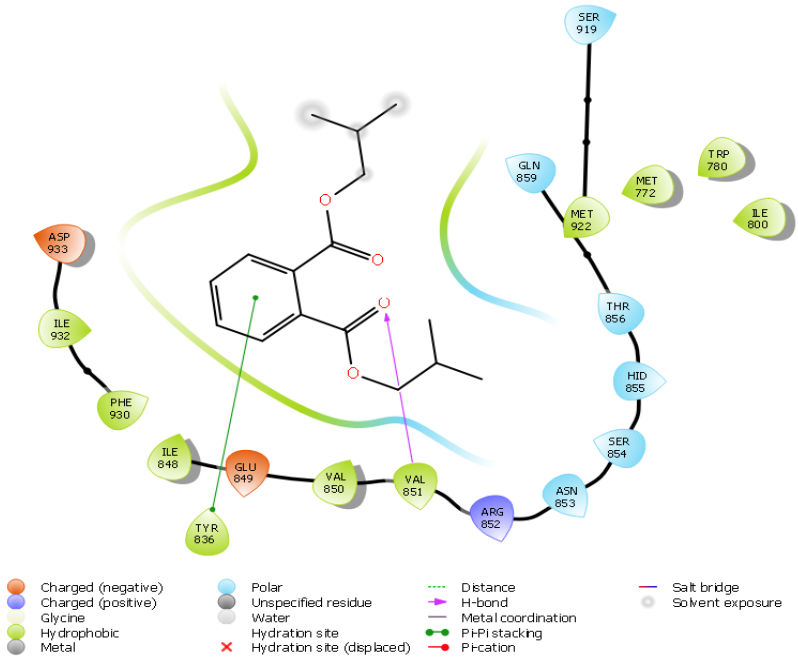


Fig. 8.18. 2D representation of the interaction of Di-iso-butyl phthalate with PI3K.
The properties of ligand interaction with amino acid are represented in different colors.

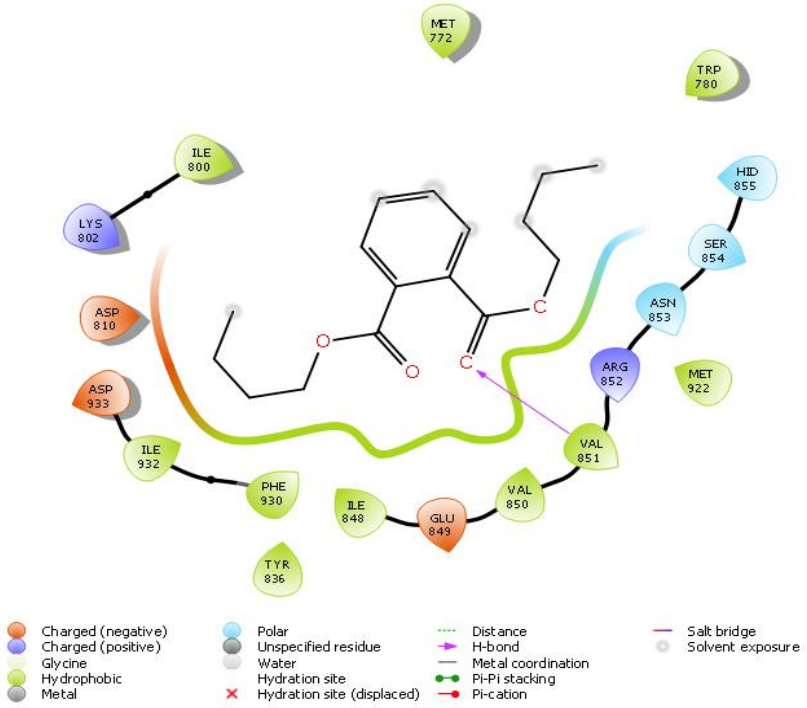


Fig. 8.19. 2D representation of the interaction of Dibutyl phthalate with PI3K.
The properties of ligand interaction with amino acid are represented in different colors.

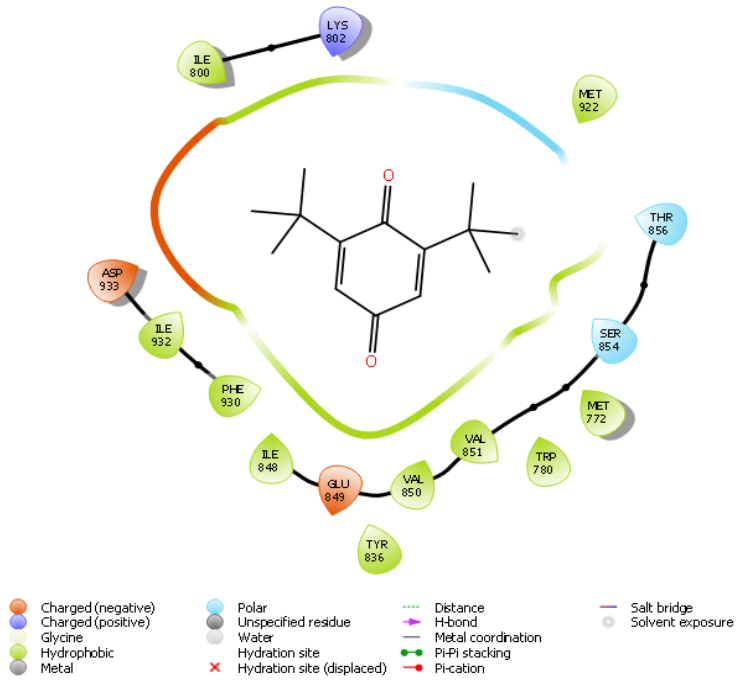


Fig. 8.20. 2D representation of the interaction of 2, 6-Di-tert-butyl-p-benzoquinone with PI3K. The properties of ligand interaction with amino acid are represented in different colours.

It was also noted that these three compounds- Di-iso-butyl phthalate, Dibutyl phthalate and 2,6-Di-tert-butyl-p-benzoquinone, does not violate any of the physicochemical parameters of Lipinski‘s ‘rule of five‘ which states that drug-like compounds should have molecular masses less than 500 daltons, logarithm of the octanol-water partition coefficient (log $P_{o/w}$) of less than 5, hydrogen bond donors should be 5 or fewer and hydrogen bond acceptors should be 10 or fewer. The details are presented in appendix. In other words, they possess drug-like properties. Therefore, the study unveils the potential of Di-iso-butyl phthalate, Dibutyl phthalate and 2, 6-Di-tert-butyl-p-benzoquinone as novel PI3-K inhibitors.

The present study thus emphasise the fact that similar to many other *Polyalthia* species, the scientifically unexplored *Polyalthia korintii* also possess anticancer phytoconstituents. This is the first time report of the presence of the mentioned compounds in this species. Most of the anti-cancer compounds identified from the sub fractions are already known active agents, which further limits the need of purification.

To sum up, from three sub fractions of PE Sox ACTN extract, nine known anticancer compounds were identified and with the use of PASS software, 18 other phytoconstituents were predicted to exhibit anticancer activity. Thus, the anticancer activity exhibited by the PE Sox extract is due to the synergistic action of the above mentioned compounds.

However, it was noted that SF35, SF40 and SF43U contained approximately 35%, 48% and 31% of essential oil components in their respective chromatograms. So in the quest for anticancer compounds from *P. korintii*, essential oil was also foreseen to be a prospect. Thus, essential oil was isolated from fresh leaves of *P. korintii* and evaluated for their anticancer potential on K562 and HCT116 cells.

9. **BIOPROSPECTING OF ESSENTIAL OIL FROM *Polyalthia korintii* LEAVES FOR ANTICANCER POTENTIAL**

Essential oils are natural metabolic secretions of plants rich in complex hydrocarbons mixtures which confer them the characteristic fragrance and flavor. Almost every part of the plant secretes essential oils which are extractable and extensively used owing to their biological properties (Sharifi-Rad, J. et. al., 2017).

9.1 Chemical composition of essential oil isolated from *Polyalthia korintii* leaves

Hydrodistillation of fresh leaves of *Polyalthia korintii* yielded 0.7 % v/w of aromatic volatile oil. The GC-MS chromatogram of the essential oil is shown in Fig. 9.1. The analysis of oil revealed the presence of a total of 16 phytoconstituents, all of which were identified and the details therein are presented in Table 9.1. The chemical structures of the identified compounds are shown in Fig. 9.2. Being the first report of the phytoconstituent analysis of essential oil from *P. korintii*, presence of these metabolites in other *Polyalthia* sp. was also reviewed. Most of the constituents like β -Elemene, β Caryophyllene, α-Bergamotene, Humulene, Curcumene, beta.-Famesene, Isocaryophyllene, (-)-Spathulenol, Caryophyllene oxide, Humulene epoxide II, tau.-Cadinol, tau.-Muurolol, Globulol, and alpha.-Bisabolol have been reported from several *Polyalthia* species (Table 9.2). The presence of trans-calamenene and 7-epi-cis-sesquisabinene hydrate were not reported from any *Polyalthia* sp. However, other Annonaceae species like *Artabotrys jollyanus* leaves essential oil was dominated by *trans*-calamenene (15.7%) (Gooré, S. G.et. al., 2017) and trace amounts were reported in *Annona squamosa* L. essential oils (Verma, R. S. et.al, 2016). Similarly, cis-sesquisabinene hydrate was reported in essential oil of *Bocageopsis multiflora (Annonaceae*) (Alcântara, J. M.et al., 2017).

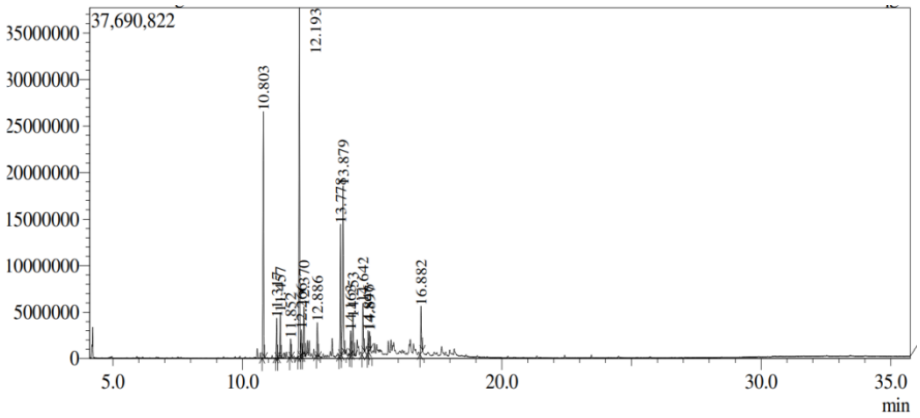


Fig. 9.1 GC-MS Chromatogram of *P. korintii* leaves essential oil

Table 9.1 *P. korintii* leaves essential oil composition

S.No.	Retention time (min)	Hit Name	MW (amu)	Peak area %
1	10.803	(-)-.beta.-Elemene	204.35	16.90
2	11.317	Caryophyllene	204.35	2.72
3	11.457	alpha.-Bergamotene	204.35	3.21
4	11.852	Humulene	204.35	1.35
5	12.193	Curcumene	202.33	25.81
6	12.266	(E)-.beta.-Famesene	204.35	2.00
7	12.370	Isocaryophillene	204.35	4.29
8	12.886	trans-calamenene	202.33	2.52
9	13.778	(-)-Spathulenol	220.35	10.27
10	12.879	Caryophyllene oxide	220.35	14.23
11	14.163	7-epi-cis-sesquisabinene hydrate	222.37	1.66
12	14.253	Humulene epoxide II	220.35	3.55
13	14.642	tau.-Cadinol	222.37	4.12
14	14.846	tau.-Muurolol	222.37	1.98
15	14.897	Globulol	222.37	1.64
16	16.882	alpha.-Bisabolol	222.37	3.75

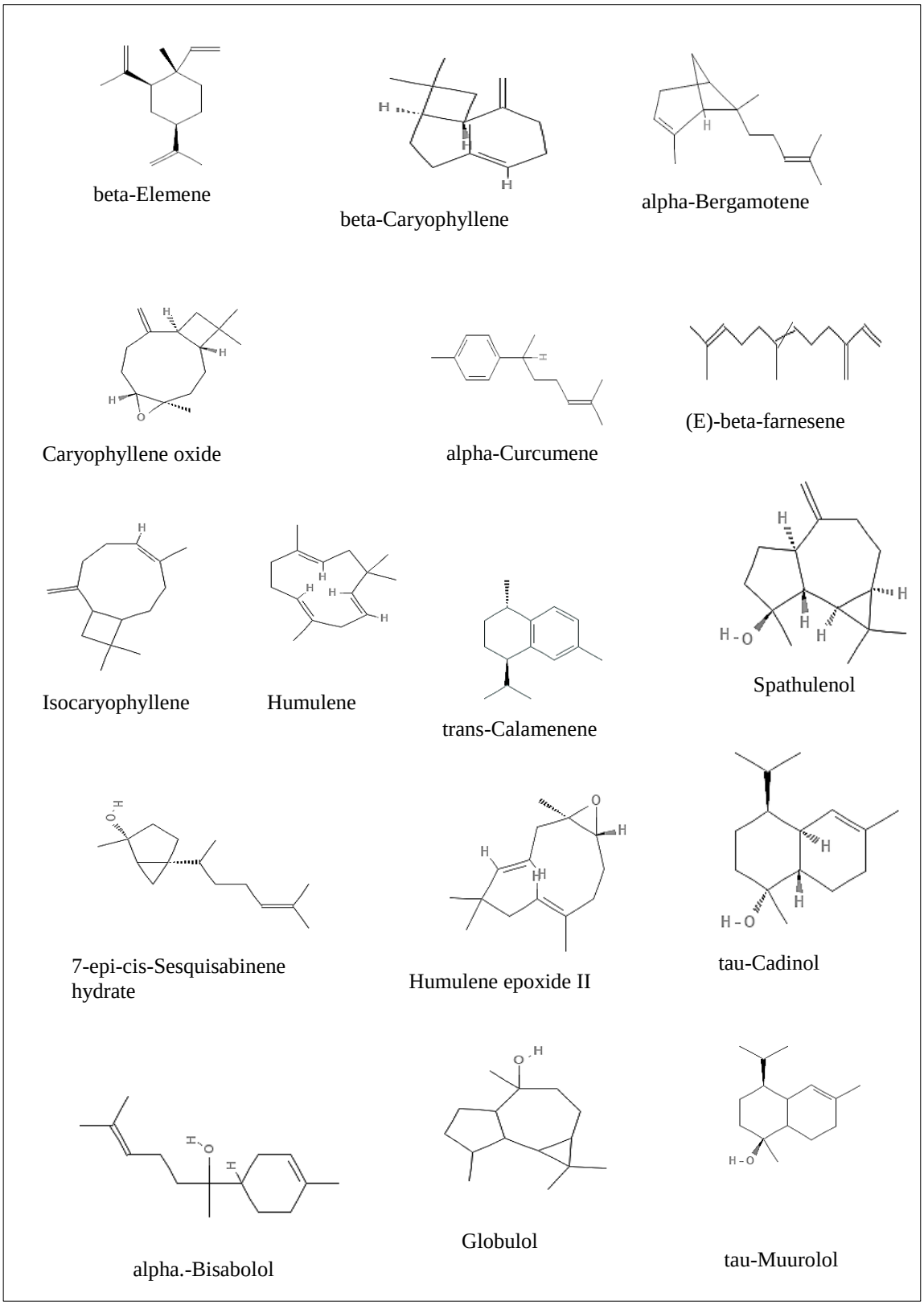


Fig. 9.2 Chemical structures of *P. korintii* essential oil components

Table 9.2 Biological activity and distribution of the *P. korintii* essential oil components in Polyalthia species

S.No.	Hit Name	Reported Biological Activity	Presence in other Polyalthia species
1	(-)-.beta.-Elemene	Anticancer , antitumor (Yang, H et al., 1997) antimicrobial (Sieniawska, E. et al., 2018)	*Polyalthia viridis* Craib (Son, N. T et al., 2021) , *P. harmandii* ,*P. jucunda P. thorelii* (Dai, D. N. et al.,2014) *P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020*) P. harmandii P. jucunda, P. thorelii* (Dai, D. N.,et al., 2014) *P. longifolia* (Thang, T. D et al.,2013)
2	β Caryophyllene	Anticancer, antimicrobial, antioxidant (Dahham, S. S. et al., 2015)	*Polyalthia viridis* Craib (Son, N. T et al., 2021*) P. harmandii* ,*P. jucunda,P. thorelii* (Dai, D. N. et al.,2014) *P. harmandii, P. jucunda, P. thorelii* (Dai, D. N.,et al., 2014) *P. longifolia* (Katkar, K. V. et al., 2010) *P. longifolia* (Thang, T. D et al.,2013) *P. oliveri* (Ouattara, Z. A et al.,2016)
3	alpha.-Bergamotene		*P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020)
4	Humulene (α-caryophyllene)	Antibacterial, antifungal, anticancer(Ali, N. A. A. et al., 2017)	*Polyalthia viridis* Craib (Son, N. T et al., 2021) *P. harmandii* ,*P. jucunda P. thorelii* (Dai, D. N. et al.,2014) *P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020) *P. harmandii, P. jucunda and P. thorelii* (Dai, D. N.,et al., 2014) *P. longifolia* (Katkar, K. V. et al., 2010) *P. longifolia* (Thang, T. D et al.,2013) *P. oliveri* (Ouattara, Z. A et al.,2016)
5	Curcumene	Antibacterial,(Silva, G. N. S. D et al.,2021) Antioxidant, larvicidal, pheromone (Afzal, A et al., 2013) cytotoxic, anti-tumor (Hadem, K. L., & Sen, A. 2017)	*P. longifolia* (Katkar, K. V. et al., 2010) (Thang, T. D et al.,2013)
6	(E)-.beta.-Famesene	DPPH free radical scavenging, anticarcinogenic, antibacterial and antifungal activity (Kendall, D., & Alexander, S. 2017).	*P. longifolia* (Thang, T. D et al.,2013)

Continued……

7	Isocaryophyllene (β-caryophyllene)	Anticancer (Legault, J., & Pichette, A. 2007).	*P. oliveri* (Ouattara, Z. A et al.,2016)
8	trans-calamenene	Anticancer, cytotoxic activities (Fajriah, S. et al.,2017)	
9	(-)-Spathulenol	antioxidant, anti-inflammatory, antiproliferative, antimycobacterial (Do Nascimento, K. F. et al., 2018)	*P. viridis* Craib (Son, N. T et al., 2021) *P. harmandii ,P. jucunda P. thorelii* (Dai, D. N. et al.,2014) *P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020) *P. harmandii, P. jucunda, P. thorelii* (Dai, D. N.,et al., 2014)
10	Caryophyllene oxide	Antifungal, insecticidal (Kendall, D., & Alexander, S. (2017) anticancer (Pan, Z. et al., 2016)	*P. harmandii ,P. jucunda P. thorelii* (Dai, D. N. et al.,2014) *P. harmandii, P. thorelii* (Dai, D. N.,et al., 2014) *P. longifolia* (Katkar, K. V. et al., 2010) (Thang, T. D et al.,2013) *P. oliveri* (Ouattara, Z. A et al.,2016)
11	7-epi-cis-sesquisabinene hydrate	Anti-cancer (Azhagu Madhavan S et al.,2021) Anti-inflammatory and anti-diarrheal (Hameed, I. H., Altameme, H. J., & Idan, S. A. ,2016))	--
12	Humulene epoxide II		*P. oliveri* (Ouattara, Z. A et al.,2016)
13	tau.-Cadinol	Antimicrobial, antifungal (Ho, C. L. et al., 2011) anticancer (Su, Y. C. et al., 2013)	*P. viridis* Craib (Son, N. T et al., 2021)
14	tau.-Muurolol	Antimicrobial, Antiinflammatory (Kala, S. M. J et al.,2012)	*P. viridis* Craib (Son, N. T et al., 2021) *P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020)
15	Globulol	Antimicrobial (Tan, M. et al., 2008)	*P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020)
16	alpha.-Bisabolol	Antibacterial, antifungal (De Lucca et al., 2011)	*P. sumatrana , P. stenopetalla, P. rumphii, P. cauliflora* (Shakri, N. M. et al.,2020)

It was also noted that beta-elemene was present in PE Sox (1.39 % peak area) and CHL Sox (2.7 % peak area) extracts and essential oil contained 16.9 % (peak area). Similarly, 3.21 % (peak area) of alpha.-Bergamotene was present in essential oil compared to 1.76 % (peak area) in PE Sox and 1.77 % (peak area) in CHL Sox.

The major component of essential oil was curcumene (25.81 % peak area). Likewise, curcumene was the abundant compound in both PE Sox (22.83 % peak area) and CHL Sox (12.03 % peak area). EA Sox also contained traces of curcumene (0.74 % peak area). Spathulenol was present in essential oil (10.27% peak area), PE Sox (6.16 % peak area) and CHL Sox (4.23 % peak area). Comparably, tau- cadinol was detected in essential oil (4.12 % peak area), PE Sox (1.68 % peak area) and CHL Sox (1.35 % peak area). Essential oil, PE Sox and EA Sox also contained alpha-bisabolol – 3.75 %, 1.93% and 1.06 % (peak area) respectively. Cis-sesquisabinene hydrate was present in essential oil (1.66 % peak area) and PE Sox (0.94 % peak area). Similar quantity of Beta-famesene was found in essential oil (2 % peak area) and CHL Sox (1.34 % peak area).

9.2 Evaluation of cytotoxic and apoptogenic potential of *P. korintii* essential oil

Antiproliferative activity of essential oil was evaluated on K562 and HCT116 cancer cell lines for 48 h. As expected, the essential oil exhibited potent cytotoxic activity with IC_{50} values of 28µg/ml and 30µg/ml respectively for K562 and HCT116 cells (Fig. 9.3). The light microscopic cytomorphological changes on treatment with IC_{50} concentration of the essential oil clearly exhibited surface features of apoptosis. Further fluorescence images of Hoechst 33258-stained cells confirmed the induction of apoptosis in the treated cells (Fig. 9.4).

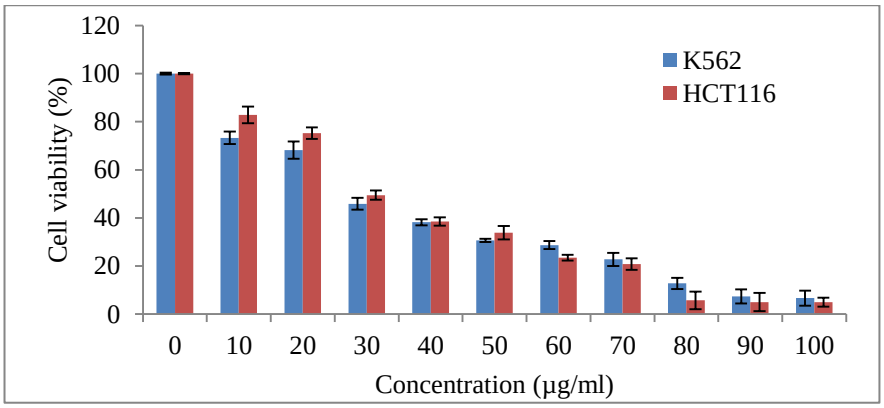


Fig. 9.3 Cell viability of K562 and HCT116 cells following 48 h treatment with *P. korintii* essential oil. Values represent mean ± SD (n=3)

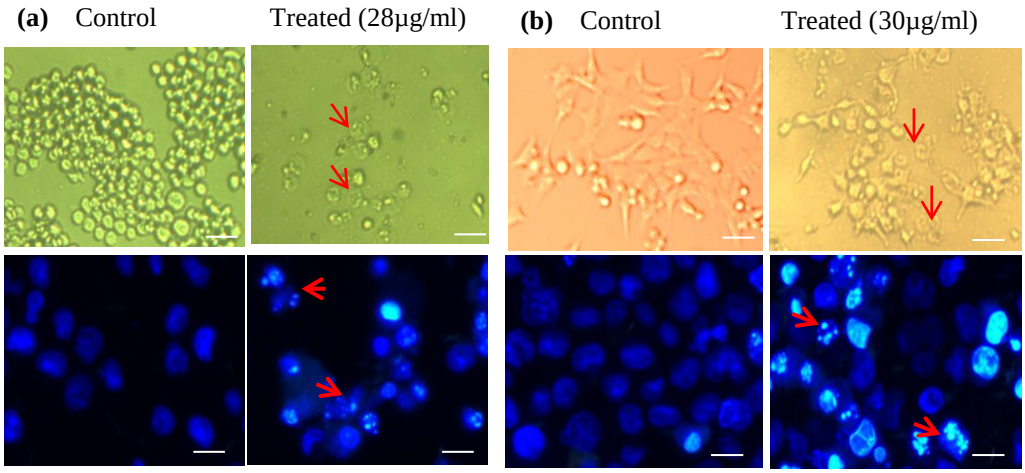


Fig. 9.4 *P. korintii* essential oil induced cytomorphological changes in (a) K562 and (b) HCT116 cells following 48 h treatment. In light microscopic images, arrow heads indicate cell shrinkage and in fluorescence images of Hoechst 33258-stained cells, arrow heads indicate apoptotic nuclear condensation and DNA fragmentation. *Scale bars represent 20µm.*

Cell cycle analysis - The results of flow cytometric analysis of cell cycle phase-specific distribution are shown in Fig. 9.5 & Fig. 9.6. Essential oil-treated K562 and HCT116 cells showed a 7% and 8.6 % increase in the sub-population of sub-G0 phase cells which represents the apoptotic cells with the fractional DNA content.

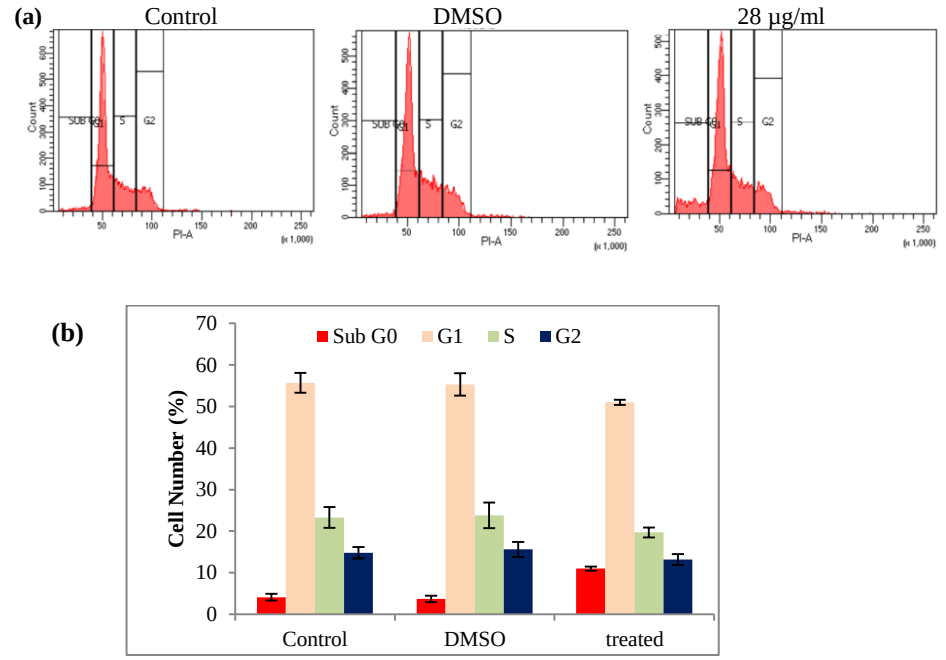


Fig 9.5. Cell cycle distribution analysis following treatment with IC_{50} concentration of *P. korintii* essential oil on K562 cells. (a) Flow cytometric data representing PI stained DNA content K562, treated for 12 hours. (b) Histogram showing percentage distribution of phase-specific cells K562 cells. Data represents mean ± SD of three independent experiments.

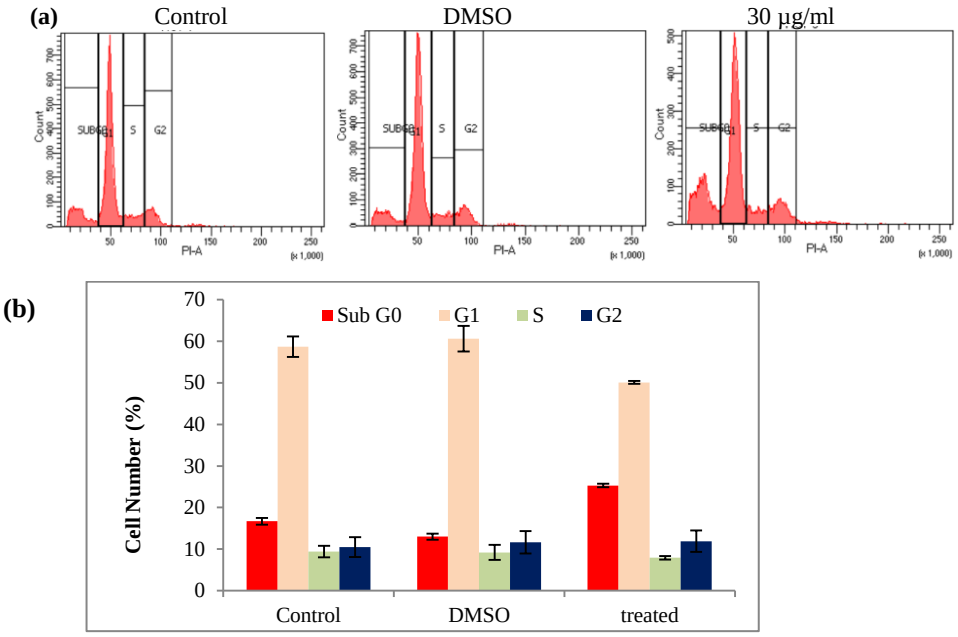


Fig 9.6. Cell cycle distribution analysis following treatment with IC_{50} concentration of *P. korintii* essential oil on HCT116 cells. (a) Flow cytometric data representing PI stained DNA content HCT116 cells, treated for 24 hours. (b) Histogram showing percentage distribution of phase-specific cells HCT116 cells. Data represents mean ± SD of three independent experiments.

Polyalthia korintii leaves essential oil contains sesquiterpenes and most of them are reported to have biological activity (Table 9.2). The essential oil contained 16.9 % β-elemene, an anticancer agent used clinically to treat cancers of lungs, liver, brain, breast, stomach, prostate, and other tissues (Xie, Q. et al., 2020). Literature reports that Humulene (1.35% in essential oil) and isocaryophyllene (4.29% in essential oil) has anticancer activity against several cancers and their activity is heightened by beta-caryophyllene, also present in the essential oil (2.72%) (Legault, J., & Pichette, A. 2007). Beta-caryophyllene, an anticancer and analgesic agent and its oxide affects the growth and proliferation of various cancer cells (Fidyt, K.et al., 2016). Similarly, other metabolites of the essential oil- Curcumene, (E)-. beta.-Famesene, trans-calamenene, (-)-Spathulenol, Caryophyllene oxide, 7-epi-cis-sesquisabinene hydrate and tau-cadinol are also highly researched anticancer agents. Thus the anticancer potential of the *P. korintii* essential oil can be attributed to the presence of the above mentioned metabolites.

SUMMARY AND CONCLUSIONS

Plants being a source of assorted and active phytochemicals, it is vital to explore their extensive biodiversity. The *Polyalthia* species (*Annonaceae*) consists of indigenous medicinal plants. Their crude extracts and isolated pure compounds possess various pharmacological activities like anti-oxidant, anti-microbial, anti-tumor, and anti-cancer, etc. The reported study thus aimed to screen leaves of *Polyalthia korintii*, an Indo-Sri Lankan endemic endangered and scientifically unexplored species from the Western Ghats (Kerala) of India for its antioxidant, antimicrobial, antiproliferative, and apoptogenic potentials.

Owing to the scarcity of scientific data on the aforementioned plant and due to limited availability of the plant specimen, pharmacognostic and physicochemical validation of *Polyalthia korintii* leaf samples were done to aid in identification, authentication, in establishing quality standards, and to rule out the presence of any adulterants or impurities. Macroscopic characters showed that the leaf had oval shape, entire margin, apiculate apex, rounded leaf base and short petiole. The adaxial surface was green, smooth and shiny, and abaxial was dull green. Matured leaf measured to about 7.5 - 9 cm long. No characteristic odour and taste was noted. Microscopic examination of the leaves transverse sectioned through the midrib showed cuticle covered epidermis with trichomes, collenchyma, stele and bundle sheath. Leaf lamina was narrow, dorsiventral with upper and lower epidermis covered with cuticle and hairs. Stomata were seen and mesophyll cells with palisade and spongy parenchyma were observed.

The leaf powder microscopy revealed the presence of fragments of trichomes, spiral vessels, lignified fibers, collenchyma cells etc. It also showed fragments of mesophyll in both surface and transverse view. The physicochemical parameters of *P. korintii* leaves determined were moisture content (11.16 %), Total ash (4.34 %), Acid-insoluble ash (0.008 %), Water-soluble ash (2.67 %), Sulphated ash (6.22 %), and crude fiber (19.63 %).

Different extraction methods viz. sequential maceration, sequential soxhlation, and acid-base extraction were employed to extract *P. korintii* leaves using solvents of varying polarity. The sequential maceration yielded Petroleum ether extract (4.0 ± 1.8 % w/w), chloroform extract (4.06 ± 2.3 % w/w), Ethyl acetate extract (2.0 ± 1.5 % w/w), methanol extract (14.0 ± 1.3 % w/w) and aqueous extract (3.9 ± 2.1 % w/w). Sequential soxhlation furnished Petroleum ether extract (6.3 ± 1.4 % w/w), chloroform extract (6.94 ± 2.0 % w/w), Ethyl acetate extract (3.2 ± 0.5 % w/w), Methanol extract (10.7 ± 2.2 % w/w) and Aqueous extract (19.0 ± 1.7 % w/w). Acid-base extraction yielded ethyl acetate extract (3.1 ± 0.8 % w/w), chloroform extract (2.5 ± 2.2 % w/w), chloroform: methanol extract (5.0 ± 1.5 % w/w) and methanol extract (8.0 ± 1.2 % w/w).

On assessing the effectiveness of different methods in terms of maximising yield and duration spent for extraction, sequential soxhlation was found to be the best method. The extracts thus obtained were further chosen for studying the biological properties and were designated PE Sox (Petroleum ether extract), CHL Sox (chloroform extract), EA Sox (Ethyl acetate extract), MET Sox (Methanol extract) and AQ Sox (aqueous extract). This study thus helped to decide on the best extraction method and on selection of ideal solvents to extract the phytoconstituents to employ them in bioprospecting.

Preliminary qualitative phytochemical screening revealed the presence of plant metabolites like alkaloids, flavonoids, phenolics, tannins, carbohydrates, steroids, and saponins in the extracts. AQ Sox extract contained higher amount of phenolic and flavonoid contents (11.072 ± 0.01 mg GAE/ g dry weight of extract and 139.291 ± 11.403 mg QE / g dry weight of extract respectively). Higher saponin contents were observed in PE Sox and CHL Sox extracts (309.7 ± 3.7 and 309.7 ± 1.2 mg DE / g dry weight of extract respectively). MET Sox extract had higher tannin content accounting to 48.233 ± 0.002 mg TAE/g dry weight of extract. The extracts were then standardised using HPTLC and spectroscopic studies (FTIR and GC-MS). The analysis revealed the presence of various classes of secondary metabolites and identified bioactive phytoconstituents from the extracts.

All the extracts exhibited DPPH scavenging ability. MET Sox (IC_{50} value of 16.2 ± 1.8 µg/ml) and AQ Sox (IC_{50} value of 17.7 ± 2.4 µg/ml) had the highest scavenging activity comparable to the activity shown by Ascorbic acid (IC_{50} value of 15.2 ± 1.7 µg/ml). The extracts possessed proton donating ability and hence could be used as free radical scavengers. The *P. korintii* leaves extract are effective scavengers of the $ABTS^{\cdot+}$ radical at higher concentrations. The dose-dependent ABTS radical scavenging activity of *P. korintii* leaf extracts were compared with ascorbic acid (IC_{50} value of 18.3 ± 0.3 µg/ml) and MET Sox (IC_{50} value of 101.9 ± 13.4 µg/ml) had higher scavenging potential. In an electron transfer based assay (FRAP assay), MET Sox again exhibited higher reducing power, even greater than the ascorbic acid. Whereas in an assay involving ferricyanide greater reducing power was shown by AQ Sox. These observations indicate that the compounds in *P. korintii* leaf extracts act as electron donors and can reduce the oxidized intermediates. They are therefore useful as primary and secondary antioxidants.

Chelating agents can be used to reduce the formation of reactive oxygen species and by virtue of this activity, they also possess antioxidant potential. Chelation power assay on *P. korintii* leaf extracts revealed their ability to bind the ferrous ions. All five extracts demonstrated strong chelating activities in a concentration-dependent manner and EA Sox (IC_{50} value of 47.5 ± 2.3 µg/ml) had the highest ferrous ion chelating activity. The Total antioxidant capacity assay (Phosphomolybdenum method) indicated high molybdate ion reducing potential of MET Sox.

The extracts also exhibited significant NO radical scavenging activity in a concentration dependant manner. AQ Sox (IC_{50} value of 1.235 ±0.26 µg/ml) showed highly appreciable activity than quercetin (IC_{50} value of 1.825 ± 0.49 µg/ml) and ascorbic acid (IC_{50} value of 3.1 ± 0.14 µg/ml). Superoxide is another harmful radical quite common in aerobic cells. All extracts exhibited moderate superoxide radical scavenging activity compared to ascorbic acid. The PE Sox had higher potential (IC_{50} value of 48 ± 1.02 µg/ml) followed by MET Sox >AQ Sox > CHL Sox > EA Sox.

The hydroxyl radical scavenging activity of the *P. korintii* extracts was dose dependent and EA Sox had higher activity (IC_{50} values of 4.3 ± 1.3 µg/ml) which is significant compared to quercetin (IC_{50} value of 3.6 ± 0.3 µg/ml). Penetration of biological membranes by H_2O_2 leads to oxidation of biomolecules. In vitro studies showed that EA Sox and MET Sox had considerable H_2O_2 scavenging ability than other extracts. The extracts demonstrated considerable amount of lipid peroxidation inhibition in a concentration dependant manner. Among the extracts, MET Sox (IC_{50} value: 14 ± 1.56 µg/ml) and AQ Sox (IC_{50} value: 140 ± 0.45 µg/ml) recorded the highest and lowest lipid peroxidation inhibition activity respectively.

Protective effects of *P. korintii* extracts against H_2O_2-induced oxidative stress in Hep G2 cells were determined from the dose–response curves for the cellular antioxidant activity. EA Sox (IC_{50} value 0.035 ± 2.6 mg/ml) exhibited highest protection against H_2O_2 in HepG2 cells followed by MET Sox, AQ Sox, CHL Sox and PE Sox. These observations are consistent with the results obtained for in vitro hydrogen peroxide scavenging ability shown by these extracts.

Since MET Sox exhibited highest antioxidant potential for most of the assays, it was subjected to HR-LC-ESI-MS/MS to profile its antioxidant phytochemicals. 25 major compounds were identified from MET Sox extract. Glaucine, caseadine and isoliensinine were the abundant compounds. The literature reported antioxidant compounds identified in the extract were 5-Methoxytryptophol, N-Feruloyltyramine, Caseadine, Liriodenine and Glaucine. Of these, N-Feruloyltyramine, Caseadine, Liriodenine and Glaucine are already reported from *Polyalthia* and other *Annonaceae* species.

Of the five extracts evaluated for antibacterial potential, PE Sox showed prominent activity against all the organisms tested :*Streptococcus pyogenes* ATCC 19615 (IC_{50}: 4.24 ± 2.03 mg/ml), *Enterococcus feacalis* ATCC 29212 (IC_{50}: 11.96 ±1.34 mg/ml) , *Staphylococcus aureus* MTCC 87 (IC_{50}:12.46 ± 3.04mg/ml), *Chromobacterium violaceum* ATCC 22019 (IC_{50}:12.46 ± 3.04mg/ml), *Klebsiella pneumoniae* MTCC 3384 (IC_{50}: 10.77 ± 2.6mg/ml), *Escherichia coli* MTCC 41 (IC_{50}: 15.14 ± 3.4 mg/ml) and clinical isolates like *Listeria monocytogenes* (IC_{50}:

14.48 ± 2.13 mg/ml), *Proteus mirabilis* (IC_{50}: 11.25 ± 2.1 mg/ml), and *Pseudomonas aeruginosa* (IC_{50}: 10.77 ± 2.6 mg/ml). The GC-MS analysis of this extract shows the presence of compounds with literature reported antibacterial activity. They are Alpha-curcumene, Cis-.beta.-farnesene, and Juniper camphor. CHL Sox was active against *S. pyogenes* (IC_{50}: 8.30 ± 2.3 mg/ml). EA Sox inhibited *P. mirabilis (*IC_{50}: 20.96 ± 1.4 mg/ml), *C. violaceum (*IC_{50}: 11.82 ± 1.06 mg/ml), *S. pyogenes (*IC_{50}: 0.30 ± 2.04 mg/ml), *and S. aureus (*IC_{50}: 24.72 ± 1.23 mg/ml). MET Sox showed antibacterial activity against *P. mirabilis (*IC_{50}: 20.70 ± 1.6 mg/ml), *C. violaceum (*IC_{50}: 15.13 ± 2.4 mg/ml) and *S. pyogenes (*IC_{50}: 18.25 ± 0.9 mg/ml). *P. mirabilis (*IC_{50}: 24.70 ± 1.4 mg/ml) and *S. pyogenes (*IC_{50}: 16.06 ± 2.2 mg/ml) was susceptible to AQ Sox extract. Chloramphenicol was used as a positive control. *Streptococcus pyogenes* was susceptible to all the extracts. Among the extracts tested at the concentrations specified, only PE Sox effectively inhibited *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Enterococcus feacalis, and Listeria monocytogenes.*

The MET Sox and AQ Sox extracts were capable of inhibiting the heat induced protein denaturation in a concentration dependent manner and the potential was comparable to the standard compound diclofenac. The DNA-methyl green assay employed to investigate the presence of *P. korintii* phytoconstituents interacting with DNA revealed that only PE Sox and CHL Sox extracts at higher concentration (>100 µg/ml) were active in displacing methyl green from the complex.

The cytotoxicity/antiproliferative activity was shown by PE Sox, CHL Sox and EA Sox extracts on all the cell lines tested. Among these PE Sox was the most potent and exhibited higher activity on leukemia (THP1, K562, HL60, Jurkat) and colorectal carcinoma cells (HCT116, HT29). The extracts were further tested for the cytotoxic specificity which included assay on Vero, HEK-293, L929 and human peripheral blood lymphocytes (hPBL). Negligible cytotoxicity was shown by PE Sox, CHL Sox and EA Sox extracts which emphasises the possession of selective toxicity for *P. korintii* extracts. Evidently, all the extracts also demonstrated negligible hemolysis (< 1%), providing additional evidence for the safety. Thus the PE Sox extract which possess significant broad spectrum anticancer effect on a

multitude of cancer cell lines exhibiting least toxicity towards normal cells was selected for investigations on its mode of action and identification of the anticancer agents and the studies were focussed on K562 (suspension cell line) and HCT116 (adherent cell line).

PE Sox extract was found to inhibit colony formation ability of both the cancer cells in a dose-dependent manner. The migration and invasion ability of HCT116 cells were hampered by treatment with PE Sox extract as evidenced by the scratch and transwell invasion assays. The extract thus inhibits colony formation ability, cell migration and invasion of the cancer cells.

Light and fluorescence microscopic investigations revealed that the extract treated cells displayed cytomorphological changes involved in apoptosis induction like membrane blebbing, cell shrinkage, condensation and fragmentation of nuclei. The extract treatment also led to phosphatidylserine externalization. The extract could only lead to slight elevation in ROS levels in both the cell lines. An increased level of intracellular Ca^{2+} was observed. At a concentration of 30 µg/ml, 2.3 fold increase of intracellular Ca^{2+} was noted in K562 cells and 5.3 fold increases in HCT116 cells at 50 µg/ml compared to the control. Dose dependant loss of mitochondrial membrane potential was exhibited in both cell types, indicating mitochondrial apoptosis. The extract treatment showed a dose dependent increase in the sub G0 peak indicating induction of apoptosis in both the cell lines used. The G1, S and G2/M phases exhibited concentration dependant decrease in cell population compared to the untreated control and DMSO treated cells. In HCT116, a marginal cell cycle arrest in G2 at higher concentrations was also observed. The extracts also induced genotoxic damage as evidenced by DNA fragmentation assay. Further, western blot analysis confirmed the apoptosis induction as shown by cleavage of PARP.

Bioactivity-guided fractionation of PE Sox extract was preceded by an enrichment step where the extract was re-extracted by acetone. The acetone soluble fraction of PE Sox (PE Sox ACTN Fraction) exhibited lower IC50 value and thus was more effective than PE Sox. The PE Sox ACTN insoluble Fraction was

subjected to GC-MS and was found to contain straight chain alkanes accounting for 86.4% peak area. The major compounds present were Eicosane, Pentacosane, Hexacosane, and n-Nonacosane.

The PE Sox ACTN Fraction treated cells were observed for cytomorphological changes by light and fluorescence microscopy and effect on cell cycle distribution was studied. The results also revealed that the PE Sox ACTN Fraction induced apoptosis in the cells studied. Bioactivity-guided sub-fractionation of PE Sox ACTN by silver ion silica gel column chromatography furnished three sub fractions with higher cytotoxic potential-SF35, SF40, and SF43U.

Characterisation of sub fractions by GC-MS analysis identified compounds with literature reported anticancer activity in all the fractions. Additional evidence to the anticancer properties of the identified compounds was obtained from predictions by PASS online software.

1-octadecene, Di-iso-butyl phthalate, and Dibutyl phthalate are known anticancer agents found in SF35. Di-iso-butyl phthalate and Dibutyl phthalate are compounds previously isolated from *Polyalthia rumphii.* The present study is thus the first time report on the presence of anticancer agents Di-iso-butyl phthalate and Dibutyl phthalate from leaf extract of *Polyalthia korintii*. PASS predicted anticancer activities for 3-methylene-7, 11, 15-trimethylhexadec-1-ene (Neophytadiene) on breast cancer; 6, 10, 14-trimethyl pentadecan-2-one (Phytone) on cervical cancer; and apoptosis agonist activity for 7, 9-di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8- dione.

SF40 was a terpene and fatty acid rich sub fraction. The terpenes accounted to 48.21% peak area and the long chain fatty acids and its esters were 40.15 % peak area, the branched alkanes accounted to 7.51 % peak area. The presence of already known anticancer agents 3, 7, 11, 15- Tetramethyl-2-hexadecen-1-ol, and Methyl hexadecanoate was found. Only 3-Methylene-7, 11, 15-trimethyl-1-hexadecene; 3, 7, 11, 15- Tetramethyl-2-hexadecen-1-ol; and 3, 7- Dimethyldecane were predicted to exhibit potentials as anticancer agent, apoptosis agonist and anti-Non-Hodgkin's

agent respectively. The results indicate that a new potential was predicted by PASS software for 3, 7- Dimethyldecane which needs to be validated.

The GC-MS analysis of sub-fraction SF43U revealed it to be an alkane rich fraction (50 % peak area). Other constituents were fatty acid methyl esters (34.64 % peak area), an alcohol (6.39 % peak area) and a triterpene (7.11% peak area). Considerable anticancer potentials were predicted for all the constituents identified from this subfraction. Among these, Kauran-13-ol and Squalene exhibited higher potential as anticancer agent and apoptosis agonist. Literature search for biological activities of the metabolites showed that Hexadecane, Nonadecane, Methyl Hexadecanoate and squalene are previously studied anticancer agents, of which squalene is a well-researched anti-cancer agent. PASS predictions demonstrated high anticancer potential for kauran-13-ol. To date no activity is ascribed for this compound.

Further *in silico* ligand-protein interaction (docking) study employing Schrodinger Glide software showed that Di-iso-butyl phthalate, Dibutyl phthalate and 2, 6-Di-tert-butyl-p-benzoquinone effectively binds PI3-K and the docking score was higher than the chosen inhibitor. Thus the study unveils the potential of Di-iso-butyl phthalate, Dibutyl phthalate and 2, 6-Di-tert-butyl-p-benzoquinone as novel PI3-K inhibitors. The *in silico* results need to be validated by *in vitro* or *in vivo* analysis.

The present study thus emphasise the fact that similar to many other *Polyalthia* species, *P. korintii* also possess biologically active phytoconstituents. This is the first time report on the presence of all the above mentioned compounds in this species. Most of the anti-cancer compounds identified from the sub fractions are already known active agents, which further limits the need of elucidation of the mode of action for these metabolites. Since the sub fractions contained higher amount of essential oil components, *P. korintii* leaf essential oil was isolated by hydro distillation yielding 0.7 % v/w of aromatic volatile oil and was investigated for their anticancer potential on K562 and HCT116 cells.

The chemical characterisation of *P. korintii* leaf essential oil identified 16 components and most of the constituents are already reported from several *Polyalthia* species and other *Annonaceae* species like *Artabotrys jollyanus*, *Annona squamosa* L, and *Bocageopsis multiflora*. The presence of these essential oil components in *P. korintii* extracts was also noted. As expected, the essential oil exhibited potent cytotoxic activity with IC_{50} values of 28µg/ml and 30µg/ml respectively for K562 and HCT116 cells. The light microscopic cytomorphological changes clearly exhibited surface features of apoptosis. Further fluorescence images of Hoechst 33258-stained cells confirmed the induction of apoptosis in the treated cells. The cell cycle phase-specific distribution study showed increase in the sub-population of sub-G0 phase in both cell lines representing the apoptotic cells with the fractional DNA content. The anticancer potential of the essential oil is due to β-elemene (16.9 %), a clinically used anticancer agent; Humulene (1.35%), isocaryophyllene (4.29%), beta-caryophyllene (2.72%), Curcumene, (E)-.beta.-Famesene, trans-calamenene, (-)-Spathulenol, Caryophyllene oxide, 7-epi-cis-sesquisabinene hydrate and tau-Cadinol.

To conclude, the present study accomplished the pharmacognostic and physicochemical validation of *Polyalthia korintii* leaf samples, identified a better extraction method and an ideal solvent for extracting of phytochemicals, standardised the extracts using phytochemical screening and estimations; HPTLC and spectroscopic studies (FTIR and GC-MS). The research determined a variety of important biological potentials like antioxidant, antibacterial, and anti-cancer ability. Five antioxidant compounds were identified from MET Sox extract. The ability of the PE Sox extract to induce apoptosis by mitochondria mediated pathway was confirmed. Nine known and 18 other putative anticancer compounds were identified from PE Sox extract. Thus the ethnomedicinal plant *P. korintii* harness a plethora of promising antioxidant and anticancer phytochemicals. This indeed is the first report on the evidence of these biological activities and the presence of the reported phytochemicals from *Polyalthia korintii*.

RECOMMENDATIONS

The present study on the plant *Polyalthia korintii* revealed the anti-bacterial, anti-oxidant, anti- cancer and various other biological potentials of its extracts. The pharmacognostic identity reported on this plant will serve in identification and standardisation of the plant. The pharmacognostical and physicochemical parameters reported may serve as valuable tool in identification of the plant in its powder form. Nine known anticancer compounds effective against leukemia and colon cancer cell lines were identified from the leaves extract of *P. korintii*. Of these, three compounds were identified as novel PI3-K inhibitors. Other phytoconstituents present in the extract were also predicted to exhibit anticancer activity. Thus, anticancer activity exhibited by the studied *P. korintii* extract is due to the synergistic action of these compounds. Further studies in detail may prove them to be ideal leads for consideration in cancer therapy.

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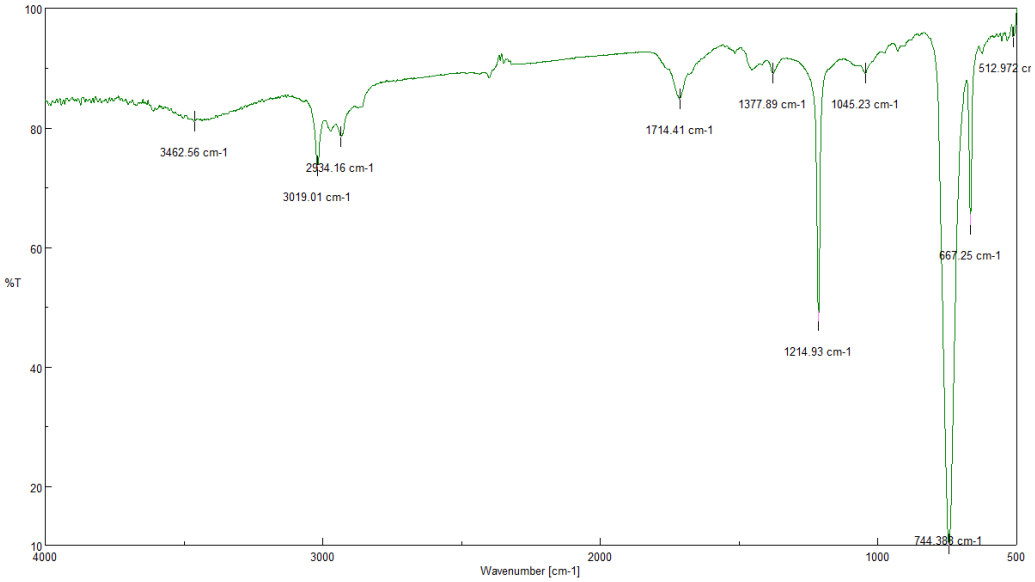
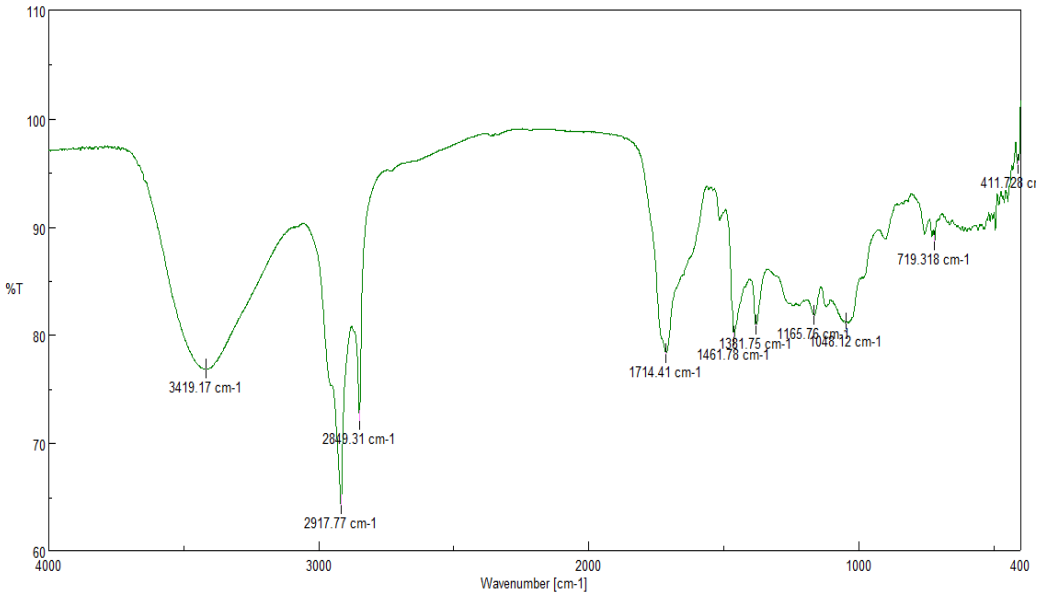
APPENDIX

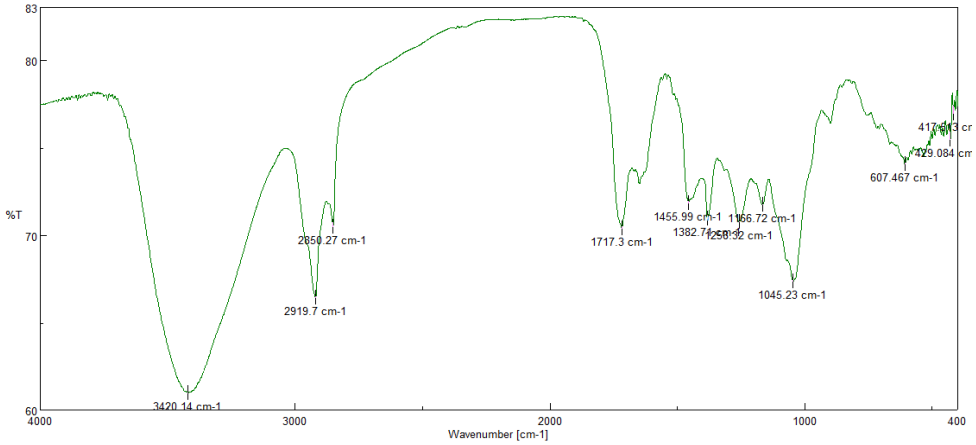
1. FTIR spectrum of extracts



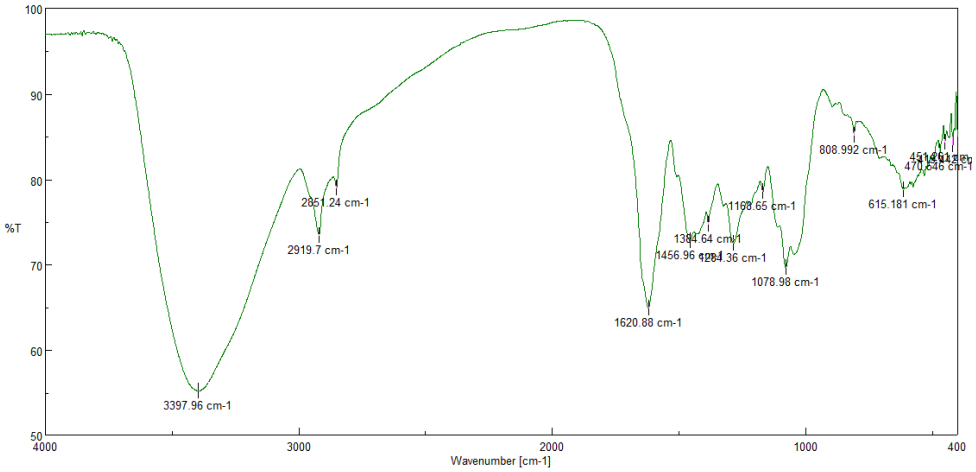
(a) PE Sox extract



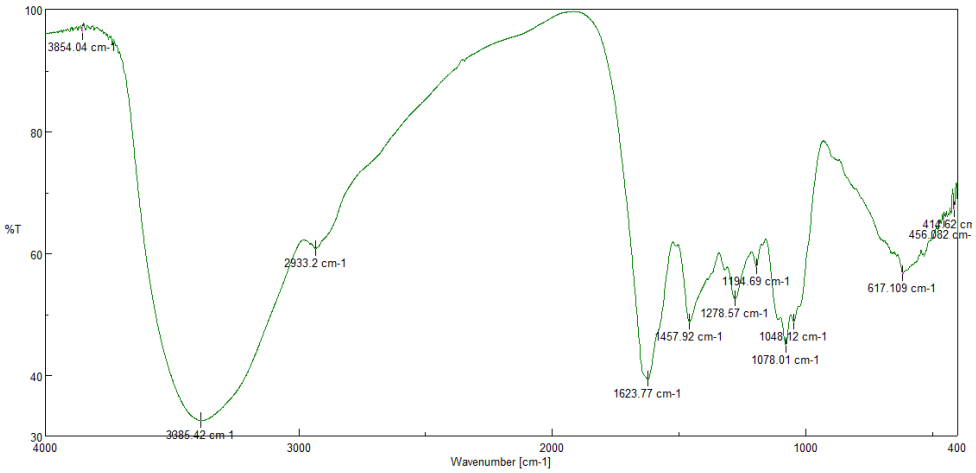
(b) CHL Sox extract



(c) EA Sox extract

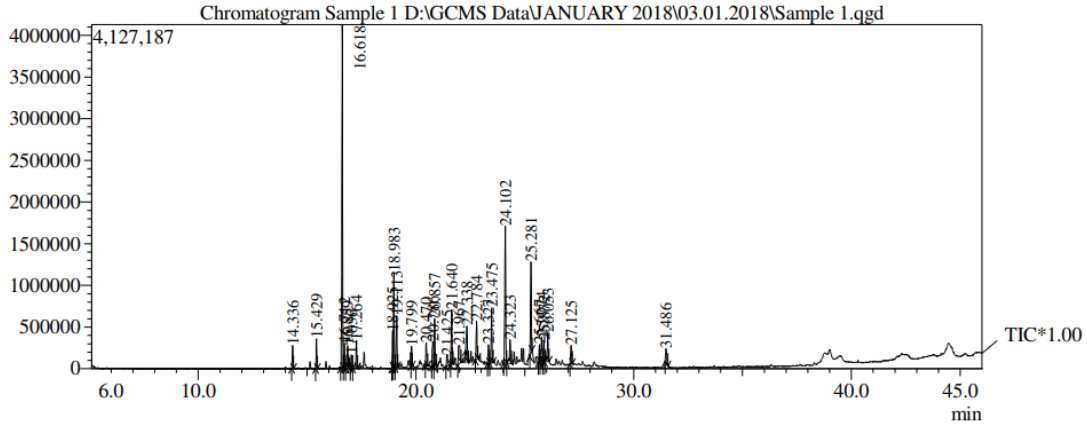


(d) MET Sox extract

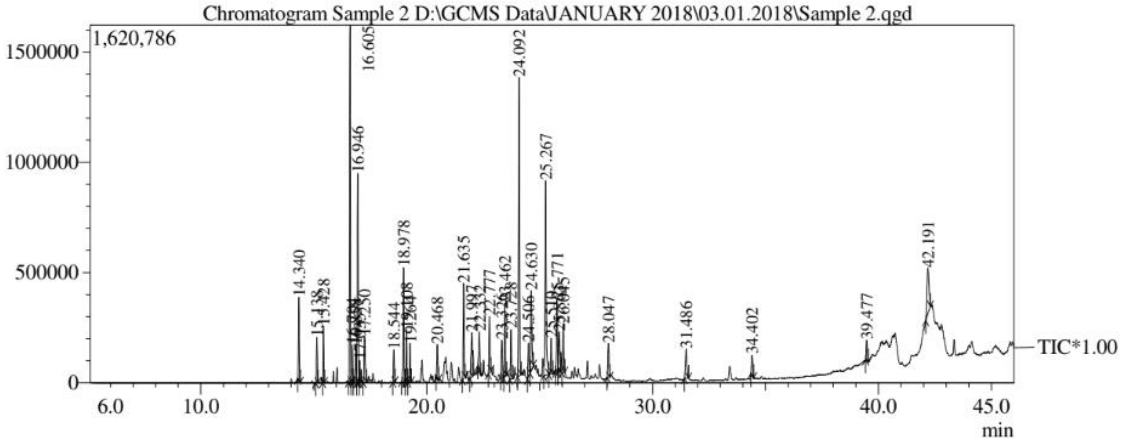


(e) AQ Sox extract

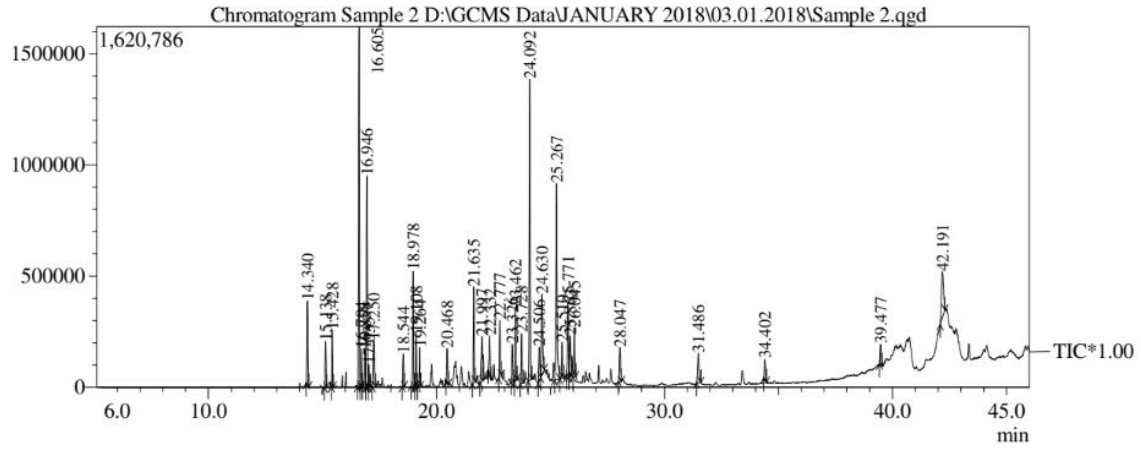
2. GC-MS Chromatogram of extracts



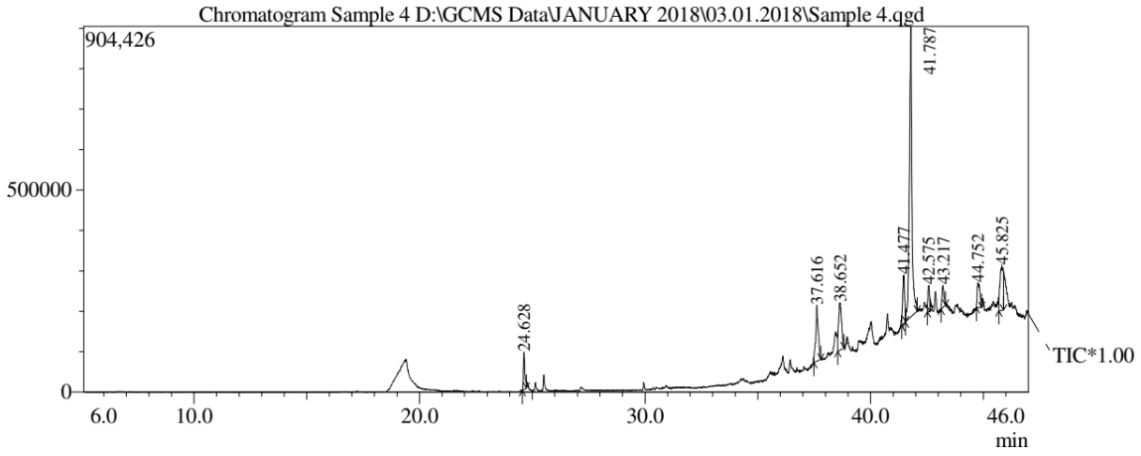
(a) PE Sox



(b)) CHL Sox



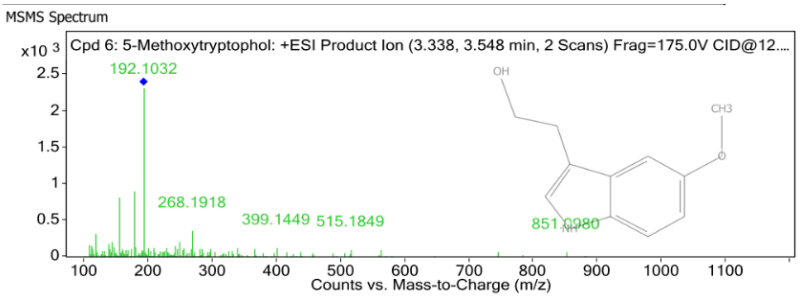
(c) EA Sox



(d) MET Sox

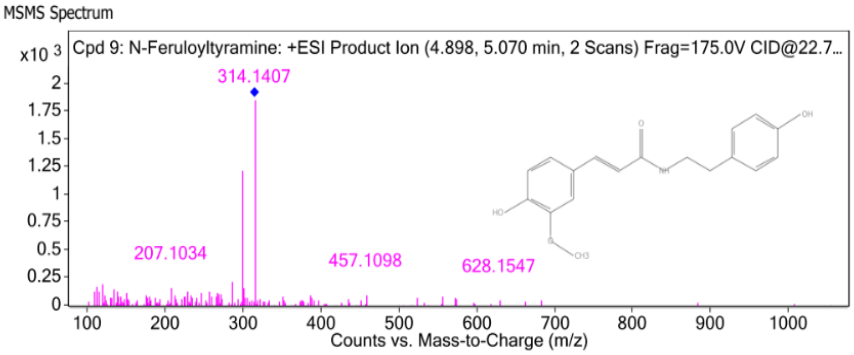
3. HR LC MS/MS Spectrum and MS/MS Spectrum Peak List of antioxidant compounds identified in MET Sox extract

MSMS Spectrum and MS/MS Spectrum Peak List of 5-Methoxytryptophol



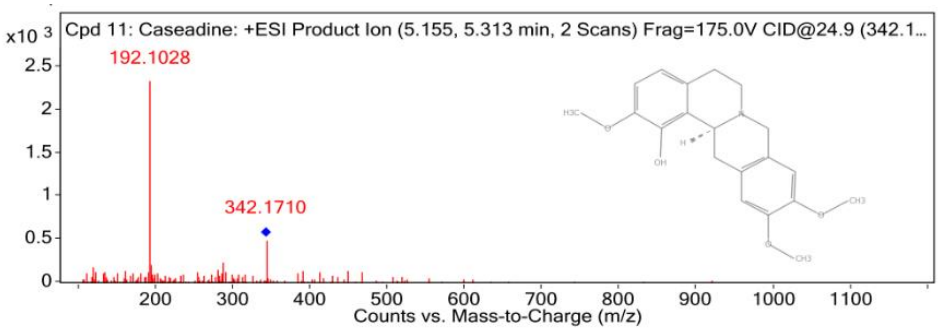
m/z	z	Abund
108.0818		171.18
118.0865		324.81
138.0542		177.89
143.0015		206.04
154.0508		821
177.0792		899.73
192.1032	2	2315.42
193.1038	2	214.07
247.1232		210.05
268.1918		363.93

MSMS Spectrum and MS/MS Spectrum Peak List of N-Feruloyltyramine



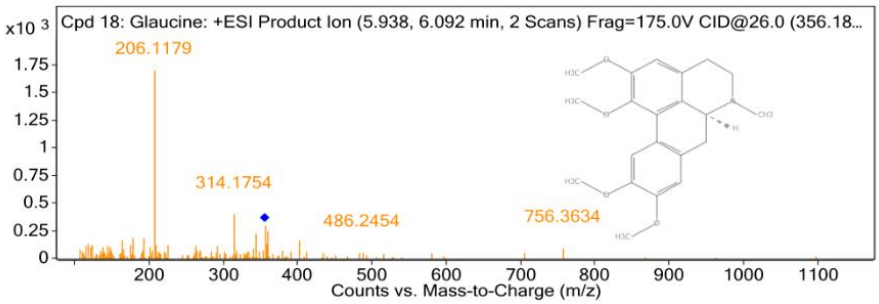
m/z	z	Abund
111.0447		173.81
118.0856		198.26
207.1034		170.35
285.1006		220.06
298.1076		1220.51
299.1163	1	1090.07
300.1185	1	171.68
314.1407	1	1856.88
314.175		227.61
315.0497		241.47

MSMS Spectrum and MS/MS Spectrum Peak List of Caseadine



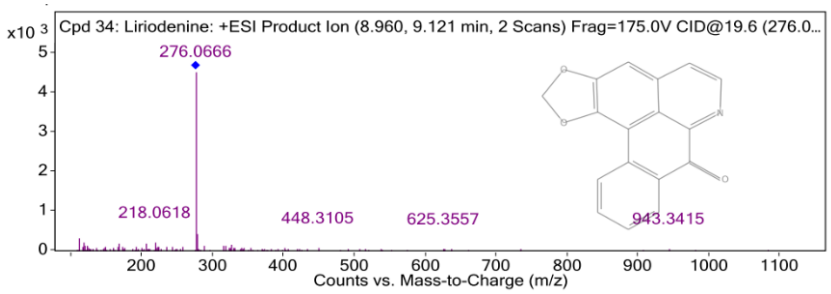
m/z	z	Abund
118.0867		174.82
160.0856		136.19
192.1028	1	2334.34
193.1073	1	207.87
280.1663		159.43
286.1448		241.83
342.171		485.17
343.1733	1	296.11
389.1774		144.11
448.3816		133.53

MSMS Spectrum and MS/MS Spectrum Peak List of Glaucine



m/z	z	Abund
163.1108		172.06
178.1676		193.34
192.1044		197.93
206.0899		169.21
206.1179	1	1709.37
314.1754	1	410.79
342.1688		233.63
356.1861	1	309.37
358.2023		259.08
401.1663		176.8

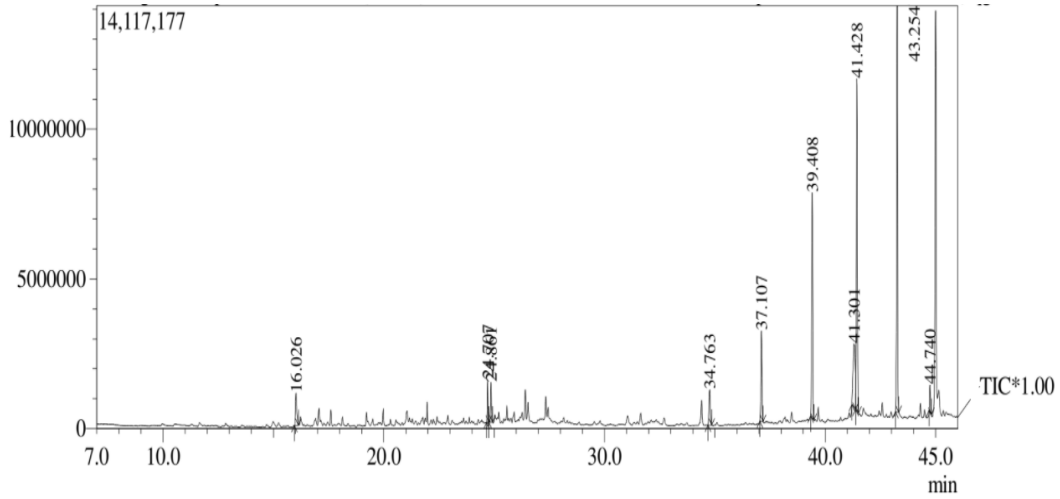
MSMS Spectrum and MS/MS Spectrum Peak List of Liriodenine



m/z	z	Abund
111.0454		324.82
118.0871		229.21
119.0856		130.03
167.0717		195.53
205.1232		178.32
218.0618		219.72
276.0666	1	4528.41
277.0696	1	422.45
317.1602		128.23
325.0952		173.04

4. GC MS Characterisation of PE Sox ACTN insoluble fraction of PE Sox extract

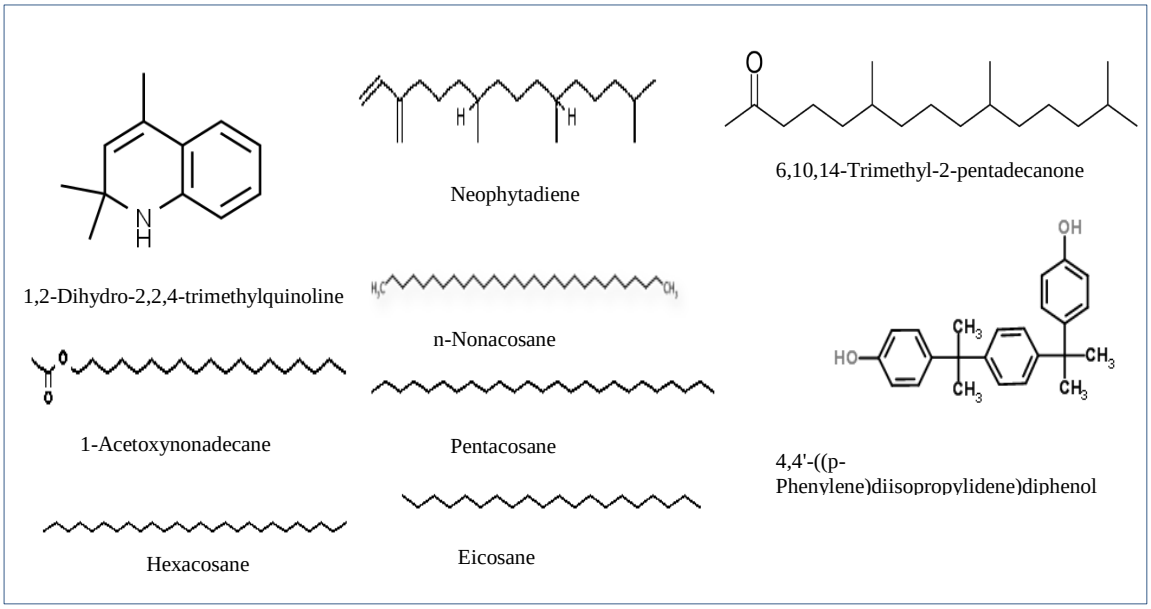
(a) GC-MS chromatogram of PE Sox ACTN insoluble fraction



(b) Chemical composition of PE Sox Acetone Insoluble fraction

Peak	R.Time	Area%	Name	Base m/z
1	16.026	3.29	1,2-dihydro-2,2,4-trimethyl-Quinoline	158.10
2	24.707	2.38	Neophytadiene	68.05
3	24.861	2.66	6,10,14-Trimethyl-2-pentadecanone	58.05
4	34.763	3.44	1-Acetoxynonadecane	83.05
5	37.107	7.04	n-Nonacosane	57.10
6	39.408	16.75	Eicosane	57.10
7	41.301	10.14	Hexacosane	57.05
8	41.428	24.90	n-Nonacosane	57.10
9	43.254	27.57	Pentacosane	57.10
10	44.740	1.83	4,4'-((p-Phenylene)diisopropylidene)diphenol	331.20

(c) Chemical structures of compounds present in PE Sox ACTN insoluble fraction



5. SWISS ADME Predictions of Lipinski's rule of five parameters

Compounds	molecular mass	Log P $_{o/w}$	H bond donors	H bond acceptors	Lipinski's rule violation
Squalene	410.72	9.72	0	0	1
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296.53	6.21	1	1	1
Methyl Hexadecanoate	270.45	5.54	0	2	1
Di-iso-butyl phthalate	278.35	3.56	0	4	0
Dibutyl phthalate	278.34	3.69	0	4	0
2,6-Di-Tert-butyl-p-benzoquinone	220.31	3.16	0	2	0
Nonadecane	254.49	7.18	0	0	1
1-Octadecene	252.48	7.2	0	0	1
Hexadecane	226.44	6.42	0	0	1
Lipinski's rule of five	less than 500 daltons	less than 5	should be 5 or fewer	Should be 10 or fewer.	

Log P $_{o/w}$: logarithm of the octanol-water partition coefficient

LIST OF PUBLICATIONS AND ABSTRACTS PRESENTED FROM THESIS

- **Rosalin, T.,** & Elyas, K. K. (2018). Pharmacognostical and physico-chemical evaluation of an indo-sri lankan ethnomedicinal plant species Polyalthia korintii (Dunal) Benth. & Hook F. *International Journal Of Pharmaceutical Sciences And Research*, *9*(10), 4355-4360.

- Solvent and temperature effect on the extraction of phenolics from *Polyalthia korintii* for its free radical scavenging ability. **Tancia Rosalin** & K.K.Elyas. Communicated.

- Non Polar Extract from *Polyalthia korintii* Leaves Inhibits Proliferation, Migration, Invasion and Induces Apoptosis in Human Colorectal Carcinoma Cells. **Tancia Rosalin** and K.K.Elyas 34th Kerala Science Congress - Abstracts, *10- 12 February 2022*, ISBN: 978-81-953863-1-4.

- Antioxidant and anti-proliferative potential of an unexplored endemic species – *Polyalthia Korinti* (Dunal) Benth. & Hook.F. **Tancia Rosalin** and K.K. Elyas, UGC Sponsored Two day International Seminar on Molecular Biology- An Underpinning to the Life Sciences organized by the Post Graduate & Research Department of Zoology, Farook College, Kozhikode, 8-9 January 2018.

- Antioxidant activity, total phenolic and flavonoid content evaluation of an endemic species - *Polyalthia korintii* (Dunal) Benth.& Hook. F. **Tancia Rosalin** and K.K. Elyas. Proceedings of Extended Abstracts, 29th KERALA SCIENCE CONGRESS, 28-30 January 2017.

- Antioxidant And Antiproliferative Potential Of An Unexplored Endemic Species – *Polyalthia Korintii* (Dunal) Benth. & Hook.F. **Tancia Rosalin** and K.K. Elyas Second International Conference on "Nutraceuticals and Chronic Diseases "(INCD- 2017) organized by Society for Nutraceuticals and Chronic Diseases and IIT Guwahati in Goa, 1-3 September 2017.

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- Thasneema, K. K., Thayyil, M. S., **Rosalin, T**., Elyas, K. K., Dipin, T., Sahu, P. K., Hadda, T. B. (2020). Thermal and spectroscopic investigations on three phosphonium based ionic liquids for industrial and biological applications. *Journal of Molecular Liquids, 307*, 112960.

- Thasneema, K. K., Dipin, T., Thayyil, M. S., Sahu, P. K., Messali, M., **Rosalin, T**., & Hadda, T. B. (2021). Removal of toxic heavy metals, phenolic compounds and textile dyes from industrial waste water using phosphonium based ionic liquids. *Journal of Molecular Liquids, 323*, 114645.

- Thomas, R., Megha, K., Surya, P., **Rosalin, T.**, Varghese, S., & Elyas, K. (2021). Investigation on the biological attributes of *Tragia involucrata* Linn. using in vitro methods. *J. Pharmacogn. Phytochem*, *10*, 398-404.

- Secondary metabolite profiling, antioxidant activity and anti-leukemic potential of the perennial peanut - *Arachis glabrata* Benth. **Tancia Rosalin**, Galiya , K.K. Elyas Communicated.

- Efficient removal of methyl orange and methylene blue using iron oxide nanoparticles synthesized from aqueous leaf extract of *Clerodendrum paniculatum L.* and *Polyalthia korintii*. Hana Mol K E, **Tancia Rosalin**, K.K. Elyas. International conference on advances and Innovations in Recycling Engineering (AIR-2021), October 21-22, 2021.

- Comparative study between catalytic and larvicidal activity of aqueous leaf extract of *Clerodendrum paniculatum L.* and its biosynthesised iron nanoparticles. Hana Mol K E, **Tancia Rosalin**, K.K. Elyas. Proceedings Of The International Conference On Advances in Biochemistry, Agriculture and Biological Sciences- 2021, September 24-25, 2021, ISBN: 978-93-90146-29-1.

- Synthesis, Characterization and Bioactivity Studies of *Clerodendrum paniculatum L*. Leaf Extract Derived Iron Oxide Nanoparticles. Hana Mol K E, **Tancia Rosalin**, K.K. Elyas. Proceedings Of The International Conference On Agriculture, Climate Change & Life Sciences 2021 Mar 05-06, 2021, ISBN 978-93-90146-15-4