Antiproliferative Activity of Chloroform and Methanol Extracts of *Piper attenuatum* (Buch-Ham)

A Thesis submitted to the Central University of Punjab

For the award of

Master of Pharmacy

In

Pharmacognosy and Phytochemistry

By

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Central University of Punjab, Bathinda

June, 2018

DECLARATION

I declare that the thesis entitled **"Antiproliferative Activity of Chloroform and Methanol Extracts of *Piper attenuatum* (Buch-Ham)"** has been prepared by me under the guidance of Dr. Raj Kumar, Associate Professor, Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this thesis has formed the basis for the award of any degree or fellowship previously**.**

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CERTIFICATE

I certify that Ms. Neha Pathak has prepared her thesis entitled **"Antiproliferative Activity of Chloroform and Methanol Extracts of *Piper attenuatum* (Buch-Ham)"** for the award of M. Pharmacy (Pharmacognosy and Phytochemistry) degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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ABSTRACT

"Antiproliferative Activity of Chloroform and Methanol Extracts of *Piper attenuatum* (Buch-Ham)"

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Degree for which submitted: Master of Pharmacy (Pharmacognosy and Phytochemistry)

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Department : Pharmaceutical Sciences and Natural Products

School of Studies : Basic and Applied Sciences

Key words: Piper attenuatum, Piper species, Cancer, Antiproliferative,

Indian traditional medicinal plant Piper attenuatum (Buch-Ham) has been investigated for its antiproliferative activity. Dried powder of fruits of Piper attenuatum (Buch-Ham) was subjected to maceration to prepare various extracts using different solvents in the order of increasing polarity. In vitro antiproliferative activity of all the extract was carried out using MTT assay against MDA-MB-231(Breast cancer) cell line. The Chloroform and Methanol extracts were found to be the most active fractions. The results from MTT assay of isolated compounds from Chloroform extract, NP7C was found to be the most potent antiproliferative agent with IC_{50} value of 3.83 μM which is comparable to etoposide 2.37 μM. Compound NP7L also exhibit significant antiproliferative activity (IC_{50} of 6.44 μM) which was comparable to colchicine (IC_{50} = 6.3 μM). Thus, the present study indicated that isolated compounds of Piper attenuatum (Buch-Ham) possess great potential to be developed as anticancer agent in future.

(Neha Pathak) **(Dr. Raj Kumar)**

Dedicated

To

My Beloved Family

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(Neha Pathak)

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List of Abbreviation

Sr. no	Full form	Abbreviation
1.	Traditional Chinese medicine	TCM
2.	Traditional Korean medicine	TKM
3.	Piper attenuatum	P. attenuatum
4.	Piper nigrum	P. nigrum
5.	Piper auritum	P. auritum
6.	Piper sarmentosum	P. sarmentosum
7.	Staphylococcus aureus	S. aureus
8.	Escherichia coli	E. coli
9.	Pseudomonas aeruginosa	P. aeruginosa
10.	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid	ABTS
11.	Nitric oxide	NO
12.	P. attenuatum methanol extract	Pa-ME
13.	Prostaglandin E_2	PGE_2
14.	Lipopolysaccharide	LPS
15.	Inducible NO synthase	iNOS
16.	Cyclooxygenase 2	COX-2

CHAPTER-1

INTRODUCTION

1.1 INTRODUCTION

Natural products have been practiced since prehistoric times for the treatment of many human infectious diseases and illnesses. Ayurveda, traditional Chinese medicine (TCM), Kampo, traditional Korean medicine (TKM), and Unani systems are utilizing the natural products all over the world for hundreds or even thousands of years. Most of the currently available drugs for the treatment of various human and animal diseases are obtained of developed (through getting lead from natural sources) from natural products especially medicinal plants (Mishra and Tiwari, 2011). Such medications have been discovered after detecting the medicinal utilization of a specific plant or its parts (leaves, roots, barks, fruits or seed or whole plant) by botanists, and subsequent isolation of bioactive compounds from the plant or part of the plant that was utilized generally for treatment of various human sicknesses. Natural products still continue to provide exceptional structural diversity in comparison to standard combinatorial chemistry, which presents opportunities for discovering novel lead compounds (Dias et al., 2012).

Cancer is a vital universal health problem generally due to the lack of extensive early detection methods (Divisi et al., 2006). Thus, the struggle to combat cancer is one of the greatest challenges of mankind. In spite of modern techniques and therapeutics, we are unable to combat cancer and alternatives therapies are needed (Ohlyan et al., 2013). From last 40 years, small organic molecules derived naturally from microbes and plants have provided a number of useful cancer chemotherapeutic drugs. The search of lead compounds from natural sources has continued in recent years, with the constituents from plants and microorganisms being investigated for their anti-cancer activities. Some antitumor agents widely used throughout the world are plant-derived compounds, including, the camptothecins, Vinca alkaloids, the epipodophyllotoxins, and taxol derivatives (Kinghorn et al., 2009). Piper plants (Piperaceae) constitute one major class of medicinal plants commonly used as a food in almost all Indian and African regions. These are also a potential source of some Piper based drugs used in traditional medicines.

Piper attenuatum (Buch-Ham) (fruit) is another species of Piper which has a significant pharmacological profile, though, much data is not reported on the anti-cancer properties of P. attenuatum (Buch-Ham). We herein report the antiproliferative activity of chloroform and methanol extracts P. attenuatum (Buch-Ham) against MDA-MB-231 (breast cancer) cell lines. Further, the cytotoxic potential of extracts and their isolated major constituents were compared.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Natural Products as source of anticancer medicines

Practices of natural product chemistry empowered the enormous bioactive secondary metabolites from terrestrial sources to be discovered (Cragg and Newman, 2013; Dias et al., 2012). Many of these natural products have gone on to become current drug candidates. Some antitumor agents widely used throughout the world are plant-derived compounds, including, the camptothecins, Vinca alkaloids, the epipodophyllotoxins, and taxol derivatives (Kinghorn et al., 2009). Among the anticancer drugs approved in 19^{th} - 20^{th} century approximately 54% were isolated from natural products or drugs inspired from knowledge related to natural products (Newman and Cragg, 2007). For instance, the Vinca alkaloids from Catharanthus roseus, and paclitaxel from Taxus baccata, are among successful anticancer drugs originally derived from plants (Yuan et al., 2016).

Introduction and advancement of a few new and highly specific in vitro bioassay procedures, chromatographic strategies, spectroscopic systems and other institutionalized pharmacological techniques have additionally made it significantly less demanding to screen, isolate and identify potential medication compounds rapidly and accurately from natural sources to ease human ailments (Keifer, 2000). In spite of the fact that characteristic items (e.g., medicinal plants) have numerous therapeutic uses, there are several reasons that require isolation and characterization of bioactive compounds from them. Some of the reasons are (i) distribution of medicinal plants is not uniform throughout the world to be utilized by individual everywhere; (ii) the greater part of the therapeutic plants are under risk of extinction because of atmospheric changes and population pressure (Brower, 2008): (iii) Separation and purification of compounds from natural sources is tedious, costly and time consuming process (Bhandari et al., 2011); and (iv) the need to identify the chemical compounds that are responsible for the observed medicinal value of the plant (Altemimi et al., 2017). In addition, the amount of bioactive compounds isolated from medicinal plants are very small (Li and Vederas, 2009). All these facts require isolation and characterization of bioactive compounds from therapeutic plants.

2.2 Piper genus

2.2.1 History of Piper

Piper species had been used in medicinal systems from ancient time, including Indian as well as Chinese systems, and in medicines of of Latin America and West Indies described in folk tales (Kirtikar and Basu, 1993). Cookery use of pepper plants is proven as early as 9,000 years ago. The remains of peppercorn were found among the food refuse left by Hoabinhian artisans at Spirit Cave, Thailand. Still, there are not sufficient proof that these plants of piper were purposely grown in spite of collection from forest (Gorman, 1971; Gorman, 1969). Peppercorns are used as pungent spice significantly on an international scale. In ancient times, the vital trade of spices including black pepper (P. nigrum) from South Asia to Europe was done. The Apicius (a recipe collection book), mentions "pepper" as a spice for most main dishes. Other than the use of the seeds of Piper in cooking as spices, West African Pepper leaves, known locally as uziza, are used as flavouring vegetable in Nigerian stews. Mexican pepper leaf (P. auritum) are used to give flavour too. In Southeast Asia, leaves of two species of Piper: lolot (P. lolot), used to wrap meat for grilling and wild betel (P. sarmentosum), used raw or cooked as a vegetable have major importance in cooking (Solomon and Solomon, 2010). A few Piper animal varieties from India, Southeast Asia and Africa are of high business, restorative and monetary significance since they are utilized as flavors.

2.2.2 Distribution

The Piper genus is pantropical and has about 1000-2000 species in the World and 93 of them exist in Costa Rica Only (Fleming, 1983). Piper plants are generally found in humid and forested areas within the underwood of rainforests. The greatest diversity of Piper species is found in the Neotropics, where about two-thirds of the mentioned species are found. Around 300 species are endemic to Southeast Asia, including the East Indian islands and northern Australia. Only two species are native to Africa (Marquis, 2004). In India, Kerala State produces the 97% of India's total output in black pepper and is known as the land of Black pepper. There are about 16 species of pepper exist only in Kerala (Parthasarathy et al., 2006). Other states include Tamilnadu, Karnataka, Andhra Pradesh and South region of India.

Piper is found from sea level to at least 2,000 meters in elevation. Most pipers are terrestrial, existing as small herbs, small trees, shrubs of about 2-3 meters high, and some as lianas (Fleming, 1983).

The most generally perceived types of the class Piper will be Piper nigrum followed by Piper longum, Piper caninum, Piper mullesua, Piper lolot, Piper argyrophyllum, Piper attenuatum, Piper umbellatum, Piper colubrinum and Piper chaba. Some images of few Piper species are showed in Fig 2.1.

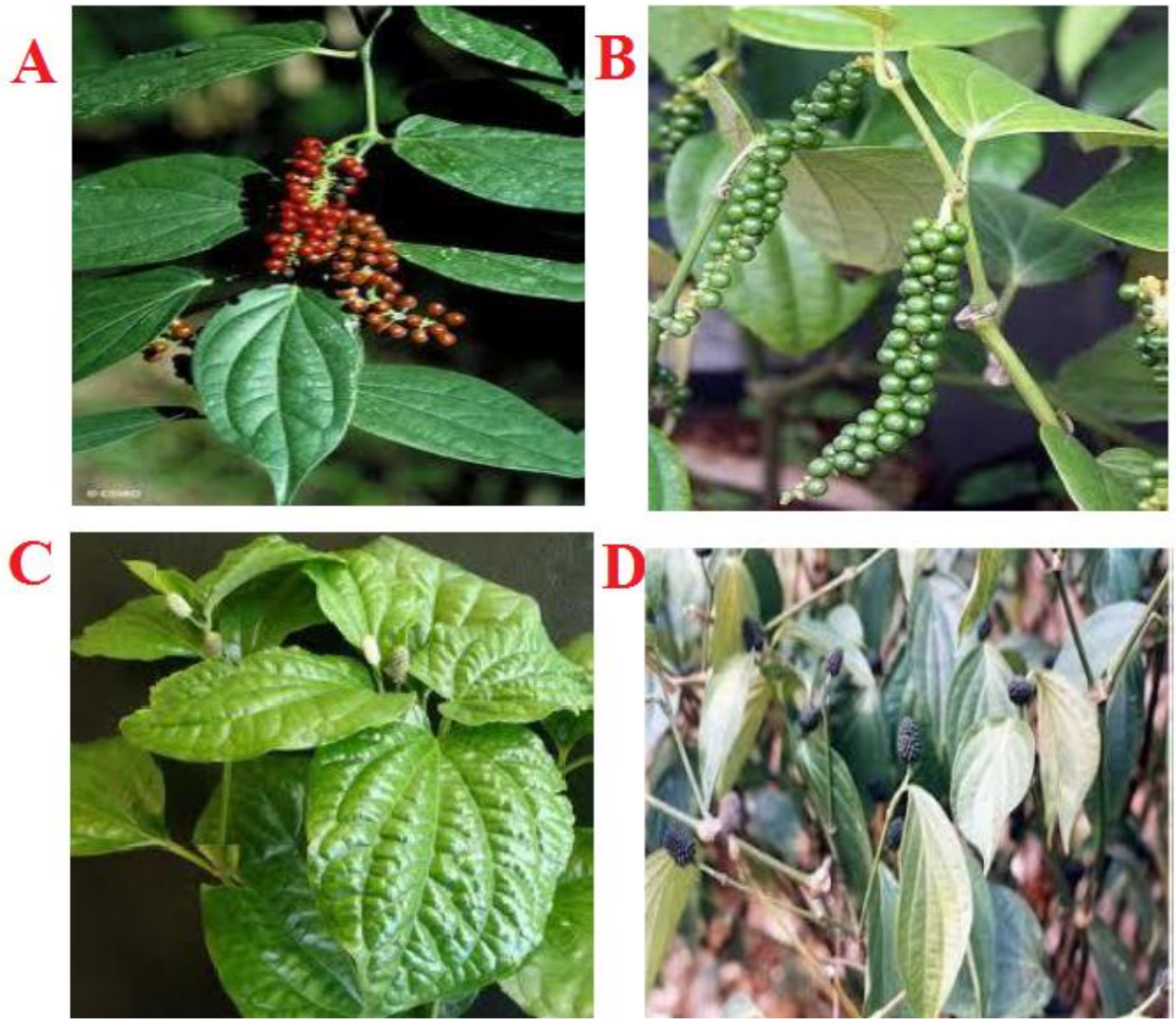


Fig 2.1: Medicinal plant of A) Piper caninum, B) Piper nigrum, C) Piper lolot and D) Piper mullesua (Ghosh et al., 2014)

They have been known as oriental therapeutic plants and reported to possess different pharmacological activities like, anti-inflammatory, anti-bacterial, anti-hypertensive,

antiplatelet, hepatoprotective, anti-thyroid, insecticidal, and anti-tumor (Dung et al., 2014; Gupta et al., 2010; Khushbu et al., 2011; Núñez et al., 2005).

The phytoconstituents acquired from different Piper species were described by classifying them into typical classes of compounds such as, chromenes, amides, phenylpropanoids, terpenes, benzoic acids, lignans, flavonoids, phenolic and a series of alkaloids. The chemistry of Piper species has been studied by chemists and botanists. Piperamides are secondary pungent metabolites present in the external part of the fruits, seeds of black pepper (P. nigrum) and other developed assortments and species (Govindarajan and Stahl, 1977). Their fruits, leaves and other plant parts are the ingredients of numerous formulations utilized in the Indian traditional system of medicines (Parmar et al., 1997). Piperine is the primary amide to be separated from the plants of Piper species followed by Piperlonguminine and Piperlongumine, as the significant alkaloids (Bezerra et al., 2013; Wu et al., 2004).

2.2.3 Various Piper species and isolated compounds

From last five decades, various reports on phytochemistry of Piper genus led to identification of various pharmacologically important scaffolds which have different structural skeleton (Reddy et al., 2015). It encouraged the discovery of various novel synthetic methodologies for synthesis of these novel scaffolds and further inspired many biochemists to determine reasonable biogenetic relationships among these diverse skeletal (Parmar et al., 1997; Parmar et al., 1998). Some of the piper species and isolated compounds from them are reported in Table 2.1

Table 2.1: Phytochemicals from Piper species

Species	**Plant part**	**Compound isolated**
P. acutisleginum	Stems + leaves	Beta-Sitosterol
	Petrol extract	
	Dichloromethane extract	Piperlonguminine

P. betle	Leaves Petrol extract Stems Petrol + Dichloromethane extract Roots Petrol + Dichloromethane Extract	Beta-Sitosterol Tritriacontane Stearic acid Piperine Piperlonguminine Beta-Sitosterol Beta-Sitosterol Beta-Sitosteryl palmitate
P. attenuatum	Stems + leaves Petrol extract	Beta- Sitosterol Kadsurin A Kadsurin B Crotepoxide 14-Benzo[1,3]dioxol-5-yl-tetradecan-2-ol
P. brachystachyum	Stems + leaves Petrol extract Dichloromethane-methanol Extract Fruits Petrol extract	Sesamine (+)- Asarinine Beta-Sitosterol Elemicin Beta-Sitosterol 3-(3,4-Dimethoxyphenyl)-propanoylpyrrole Beta-Sitosterol Parsley apiole
P. falconeri	Leaves Petrol extract	Nerolidol
P. longum	Stems + Leaves Petrol extract	Asarinin Guineensine

		Retrofractamide A
P. manii	Stems Petrol extract Fruits Petrol extract	Retrofractamide A Apigenin dimethyl ether Tetratriacontanol
P. thomsoni	Stems + leaves Petrol extract Dichloromethane- methanol extract	Dotriacontanol Dotriacontanoic acid (-)-Galbelgin Beta-Sitosterol Piperine Cepharadione A

2.3 Anticancer potential Piper species

Extracts from Piper species are also found to have potential cytotoxic activity. Wang et al. reported the cytotoxic potential of extracts of 24 species of Piper genus (Wang et al., 2014). In literature, number of piper species had been documented which have anticancer potential. Some of them are described in Table 2.2.

Table 2.2: Piper species having anticancer potential

Latin name	**Part used**	**Country**	**Use**	**Ref.**
P. aduncum L.	Unknown	Mexico	Skin tumors	(Alonso-Castro et al., 2011; Calderón et al., 2006)
P. boehmeriifolium Wall.	Root	India	Tumor	(Kuete et al., 2013; Mahanta et al., 1974; Tang et al., 2010)
P. capense L. f.	Unknown	Cameroon	Cancer	(Kuete et al., 2011; Kuete et al., 2013)
P. cubeba L.	Seeds	Morocco	Cancer	(Daoudi et al., 2013)
P. guineense Schum and Thonn	Seed Unknown	Nigeria Cameroon	Cancer Cancer	(Soladoye et al., 2010)

P. longum L.	Leaf Unknown	Cook Island India	Breast cancer Tumor	(Kim et al., 2011)
P. nigrum L.	Root Fruit	Thailand China	Abdominal tumors Respiratory or gastric cancers	(Xin et al., 2009)
P. sylvaticum Roxb.	Root	India	Tumor	(Kim et al., 2011)

Piper aduncum L. is conventionally used to cure dermatological conditions like rashes, skin allergies and skin tumors in Mexico (Alonso-Castro et al., 2011). Piperaduncin A **(1)** is isolated from this plant. It is a dihydrochalcone compound which exhibited growth inhibitory activity against human nasopharynx carcinoma (KB) cells with IC_{50} value of 2.3 µg/ml (Orjala et al., 1994). On other hand dichloromethane extracts of P. aduncum leaf were not found potential cytotoxic to various cell lines (Calderón et al., 2006). P. boehmeriifolium Wall and Piper sylvaticum Roxb. (roots) are reported to have laxative, and carminative properties. These are also reported in the Ayurvedic system of Indian medicine to have beneficial effects against diseases of the spleen, and tumors (Mahanta et al., 1974). 1-[(9E)-10-(3,4-methylenedioxyphenyl)- 9-decenoyl] pyrrolidine **(2)**, a cytotoxic amide alkaloid isolated from the plant of P. boehmeriifolium was reported to have an IC_{50} of 2.7 µg/ml against human cervix adenocarcinoma (HeLa) cells (Tang et al., 2010), while piplartine (an amide alkaloid) **(3)** (Fig. 2.2) is responsible for the anticancer potential of P. sylvaticum (Bezerra et al., 2013). Piper capense L.f. is also reported for the treatment of cancer in Cameroon (Kuete et al., 2013). The seeds of Piper capense were subjected to extraction and methanolic extracts was found to have cytotoxic potential against many tumor cell lines and Piperine **(4)** might be an active constituent responsible for its cytotoxic potential (Kuete et al., 2011; Umadevi et al., 2013).

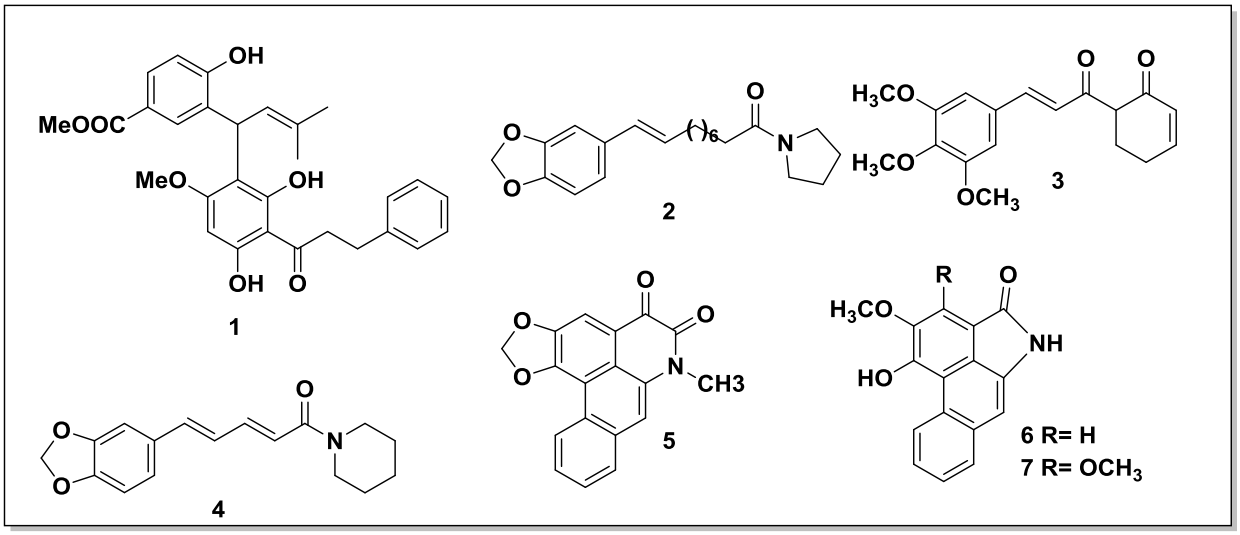


Fig 2.2 Various active constituents of Piper species reported for anticancer potential

Piper cubeba was found one of the most important plant against cancer in in vitro anticancer evaluation in Moroccan traditional medicine (Daoudi et al., 2013). (-)-Cubebin, a lignin is found major constituent of P. cubeba. Cubebin is have been reported to have significant therapeutic potential against halting the growth of prostate cancer by targeting number of features of the androgen-signaling pathway (Usia et al., 2005; Yam et al., 2008). Nigerian plant species of piper known as Piper guineense Schum and Thonn are reported to have anticancer properties (Soladoye et al., 2010). However, the active constituents of these species are still unknown but methanolic extract obtained from the seeds was found to have cytotoxic properties against leukemia CEM/ADR5000 cells with IC_{50} value of 8.20 µg/ml) in a study performed at Morocco (Kuete et al., 2011). Piper longum L. is the most well-known species of piper genus and medicinal plant. In ancient medical practice, it is reported that 12 leaves of Piper longum and 12 leaves of Thespesia populnea (L.) with little amount of water were crushed in a wooden bowl and the chest of a person with supposed breast cancer is washed with this solution (Holdsworth, 1991). P. longum is also reported in Indian Ayurveda to treat tumors. The major active principles reported of this plant are Piplartine, cepharadione A **(5),** and piperolactams A **(6)** and piperolactams B **(7)** (Kim et al., 2011). Piper nigrum L. (black pepper), is applied to abdominal tumors in various fomulations for releief. Black pepper is also used in number of formulations for treatment of various respiratory and gastric cancers in China (Xin et al., 2009).

2.4 *Piper attenuatum*

Indian medicinal plant Piper attenuatum (Buch-Ham) has a place with the family Piperaceae. It is found to exist in Vishakhapatnam of Andhra Pradesh, Madurai and Tirunnelveli of Tamil Nadu. It is a substitute for black pepper (P. nigrum). Distinctive parts of P. attenuatum have been utilized as herbal medicine for the treatment of muscular pain, headache and have been utilized as a rubefacient. The wood from plant has been used to treat throat pain (Ohlyan et al., 2014). The root part has diuretic activity. Leaves have been utilized for their wound healing property. The entire plant is reported to contain an uncommon phytoconstituent crotepoxide, which has been reported to exhibit significant antitumor activity against Lewis lung carcinoma cell line (Kupchan et al., 1969). Phytochemical studies have demonstrated that the plant contains pipoxide and chlorohydrins which are major chemical components. (−)-galbelgin and another aliphatic liquor; 8- hentriacontanol have additionally been segregated from the leaves of P. attenuatum. Roots of the plants have been reported to contain alkamides including guineensine, piperine and Piperlonguminine while areail parts are reported to have few aristolactams. The petroleum extract of stems and leaves of P. attenuatum have been reported to contain a novel long chain alcohol, 14-benzo [1, 3] dioxol-5-yl-tetradecan-2-ol (Parmar et al., 1998). Recently methanol extract of dry fruits has also been reported to contain antioxidant components with promising activity (Ohlyan et al., 2013).

Fig 2.3: Piper attenuatum Buch.-Ham. Ex Miq.
(https://flickr.com/photos/91314344@N00/16176193326)

2.4.1 Scientific name: Piper attenuatum Buch-Ham. Ex Miq. (Hassler, 2000; Sasikumar et al., 1999)

2.4.2 Synonyms: Piper malamiris Roxb. (Synonym)

Piper Karok Blume (synonym)

Chavica diffusa (Vahl) Miq. (Synonym)

Piper diffusum Vahl (synonym)

2.4.3 Common name: Flat-branched pepper, oval-leaved pepper plant **Malayalam:** Kattumulaka **Tamil:** arenukam, kattu-milaku (Piper attenuatum Buch.-Ham. ex Miq., Syst. Piperac. 306 1843.)

2.4.4Taxonomic classification

Table 2.3: Taxonomic classification of P. attenuatum

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Piperales
Family	Piperaceae

2.4.5 Distribution

Java, peninsular Malaysia, New Guinea (alpine), India, China (Yunnan), Bhutan, Sikkim and Myanmar [Burma] (Kachin, Mandalay, Sagaing, Yangon) (Sasikumar et al., 1999).

2.4.6 Macroscopy character (Sasikumar et al., 1999).

Colour : Grayish black

Odour : No characteristic odor

Taste : Pungent

Shape : Globular dry fruits with few striations

Size : The average diameter of dry fruits is 4-6 mm

Fig 2.4: External morphology of dry fruits of P. attenuatum

Adapted from (Ohlyan et al., 2014)

2.4.7 Microscopic characterization

Shrubby root-climber, up to 2.5 m long. Stem and branches hefty, however delicate, end up compacted notched when dry, greenish-yellow, glabrous, bring down internodes 8.0 – 8.5 cm, upper 2.0 – 3.5 cm long; stipules 0.5 – 0.65 cm, subulate. Lamina comprehensively cordate; bring down 8.0 – 9.5 x 7.0 – 10 cm, somewhat more extensive than length, orbicular-ovate; upper 4.5-7.0 x 2.5 – 5.0 cm, whole, pellucid, intense to taper with a mucro, base cordate-truncate, membranous, relatively glabrous above, hairy on veins, nerves for the most part 7, sometimes 7 – 9 from base, laterals disparate aside from center; petioles 2.5 – 6.5 cm long, angled, greenish-yellow, hairy upwards; plants dioecious; male spike c 0.03 cm long, clustered; Stamens 3, sessile, ;adnate to elongated basifixed bracts (1.0 – 0.2 cm), winged, decurrent (4.0 – 5.0 cm long) rachis; female spikes (up to 10 cm long) with adnate bracts bearing its end on ovoid ovary (0.05 cm long); stigma obscurely 4; drupes 0.2 x 0.15 cm, globose, loosely aggregated, glabrous, sessile, 0.35 – 0.4 cm across, black (Brach and Song, 2006).

2.4.8 Plant habitat

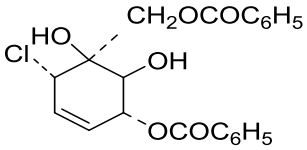
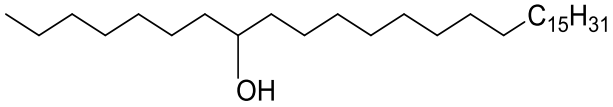
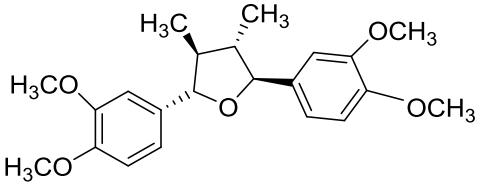
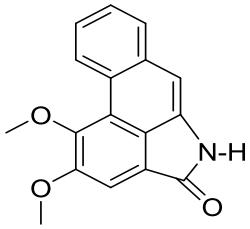
Flowering & Fruiting done in the month of August to March

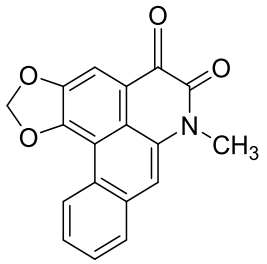
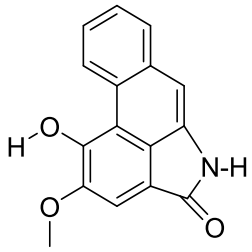
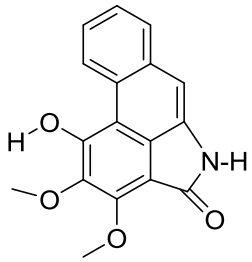
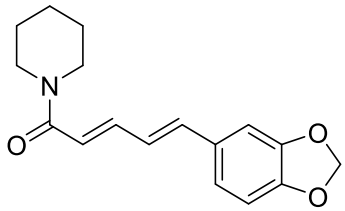
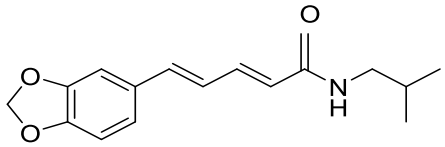
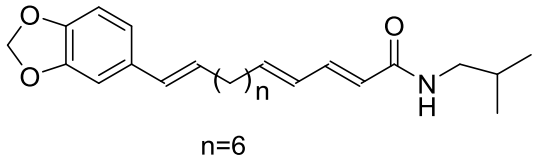
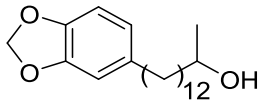
2.4.9 Phytochemical investigations

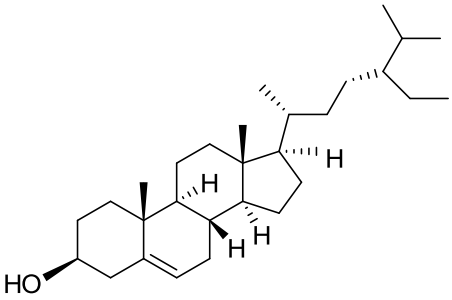
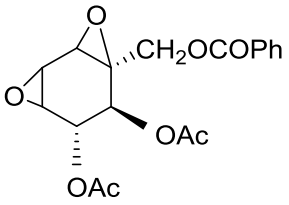
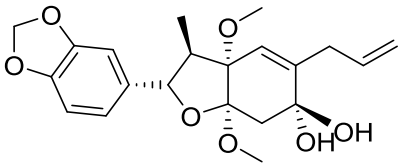
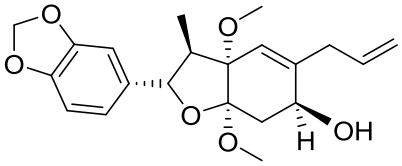
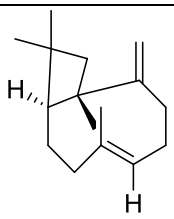
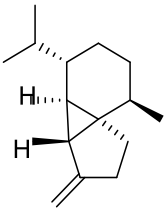
Phytochemical studies have demonstrated that the plant contains pipoxide chlorohydrins in a major amount (Joshi et al., 1979). (−)-galbelgin (Stevenson and Williams, 1977), aliphatic liquor and 8-hentriacontanol have also been isolated from the

leaves of P.attenuatum (Sumathykutty and Rao, 1991). There are number of aristolactams have been reported from the aerial parts of the plant which include cepharanone B, piperolactamA, piperolactam D, and cepharadione A (Desai et al., 1990), (Kumar et al., 2003). Roots have been testified to contain alkamides including piperine, Piperlonguminine and guineensine (Parmar et al., 1998). The petroleum extract of P.attenuatum have been reported to contain a novel chain alcohol, 14-Benzo [1, 3] dioxol-5-yl-tetradecan-2-ol, beta-Sitosterol, Kadsurin A, Kadsurin B and (+)-Crotepoxide (Parmar et al., 1998). Leaf oil of P.attenuatum contain beta-Caryophyllene and beta cubebene (Sumathykutty and Rao, 1990).

Table 2.4: Structure of chemical constituents of Piper attenuatum extract out from chloroform extract

Constituents	**Structure**	**References**
Pipoxide chlorohydrin		(Stevenson and Williams, 1977)
8-hentriacontanol		(Sumathykutty and Rao, 1991)
(−)-galbelgin		(Stevenson and Williams, 1977)
Cepharanone B		(Desai et al., 1990), (Kumar et al., 2003).

Cepharadione A		(Parmar et al., 1998)
PiperolactamA		(Desai et al., 1990), (Kumar et al., 2003).
piperolactam D		(Desai et al., 1990), (Kumar et al., 2003).
Piperine		(Parmar et al., 1998)
Piperlonguminine		(Parmar et al., 1998)
Guineensine	 n=6	(Parmar et al., 1998)
14-Benzo [1, 3] dioxol-5-yl-tetradecan-2-ol		(Parmar et al., 1998)

beta-Sitosterol,		(Parmar et al., 1998)
(+)-Crotepoxide		(Parmar et al., 1998)
Kadsurin A		(Parmar et al., 1998)
Kadsurin B		(Parmar et al., 1998)
Caryophyllene		(Sumathykutty and Rao, 1990).
beta cubebene		(Sumathykutty and Rao, 1990).

2.4.10 Traditional uses:

Distinctive parts of P. attenuatum have been utilized as herbal grown prescription for the treatment of migraine, muscular pain and have been used as a rubefacient. The wood of plant has been used to treat throat pain. The root part has diuretic activity

(Ohlyan et al., 2014). Leaves have been used for their wound healing property. The entire plant is accounted to contain an uncommon phytoconstituent crotepoxide, which has been reported to show significant antitumor activity against Lewis lung carcinoma cell lines (Kupchan et al., 1969).

2.5 Pharmacological activity of *Piper attenuatum*

2.5.1 Antibacterial activity

Samy et al. assessed antibacterial activity of dry fruits of Piper attenuatum (Buch-Ham) in which they prepared three distinct extracts by utilizing ethyl acetate, ethanol and Methanol. Each of the three extracts were screened for their antibacterial activity against S.aureus, E.coli and P. aeroginosa by agar diffusion method in which they found that Methanol extract showed comparatively good inhibition at higher doses at 200 and 500 µg against all organisms (Samy et al., 1998). Ethanol extract additionally demonstrated some restraint against E. coli at higher doses (200 and 500 µg).

2.5.2 Antioxidant activity

Auddy et al. explored antioxidant and anticancer potential of Piper attenuatum (Buch-Ham). Total three extracts of Piper attenuatum (Buch-Ham) were prepared by utilizing solvents ethyl acetate, ethanol and methanol. Further, in vitro antioxidant potential was evaluated using ABTS free radical scavenging method (Auddy et al., 2003), compared to the standard (Gallic acid) and they found that Methanol extract demonstrated greatest inhibitory impact with IC_{50} of 13.17µg which is around six-folds than that of Gallic acid (2.16µg). Both ethanol and ethyl acetate extract also showed inhibition of free radicals in concentration dependent manner with IC_{50} of 20.35µg and 68.06µg, respectively.

2.5.3 Antihyperglycemic activity

Reddy et al. examined the antihyperglycemic and free radicals scavenging constituents present in the fruit part of Piper attenuatum. Extracts having strong free radical scavenging activity were further selected for isolation of major constituent present. Chloroform extract of P. attenuatum was found to have strong radical scavenging

potential was further assessed for antihyperglycemic action. Nine neolignans specifically, denudatin B **(8)**, iso-4', 5'-dimethoxy-3, 4-methylenedioxy-2'-oxo-Δ'-8.1'-lignan **(9)**, lancifolin D **(10)**, denudatin A **(11)**, wallichinin **(12)**, piperenone **(13)**, lancifolin C **(14)**, 2-oxo-piperol B **(15)**, piperkadsin A **(16)** and a crotepoxide **(16)** (Fig 2.5) was recognized present in chloroform extract. From evaluation of free radical scavenging potential, it was found that all the neo-lignans present in chloroform extract have $ABTS^{+}$ radical scavenging activity, however just **16** showed the $DPPH^{\cdot}$ scavenging activity. The SAR studies revealed that the presence of methoxyl groups in the rings primarily influence $ABTS^{+}$ radical scavenging potential of the compounds as absence of methoxyl groups lead to the decreased DPPH activity (Reddy et al., 2015).

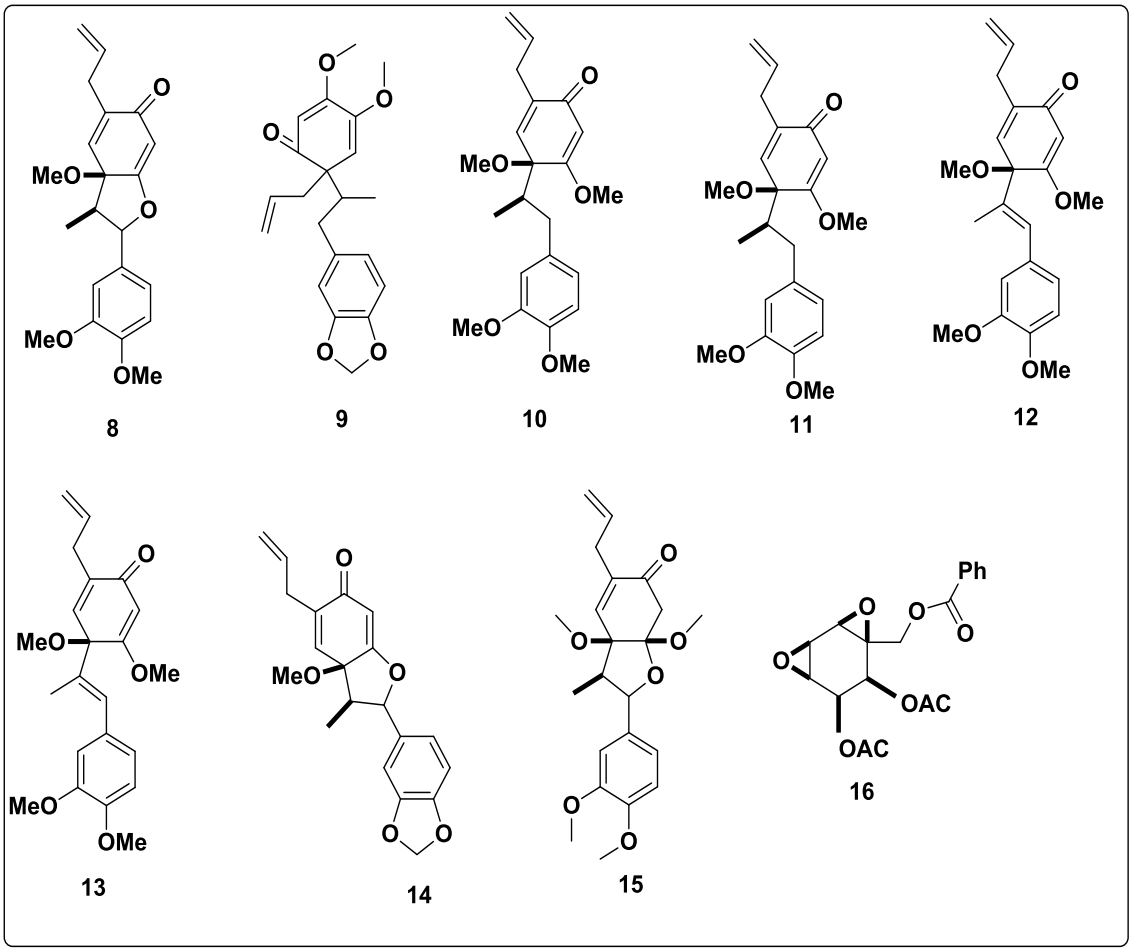


Fig 2.5: Reported compound isolated from fruits of P. attenuatum

2.5.4 Anti-Inflammatory effect of *Piper attenuatum*

Kim et al. evaluated the effect of methanol extract of P. attenuatum on the production of inflammatory mediator nitric oxide (NO) and prostaglandin E_2 (PGE_2), the expression of proinflammatory genes, the translocation level of transcription factors, and intracellular signaling activities were investigated using macrophages. Methanol extract of P. attenuatum was found to suppress the production of NO and PGE_2 in lipopolysaccharide- (LPS-), pam3CSK4-, and poly (I: C)-stimulated RAW264.7 cells without displaying cytotoxicity. The mRNA articulation levels of inducible NO synthase (iNOS) and cyclooxygenase 2 (COX-2) were diminished by Pa-ME. P-ME diminished the translocation of p50/NF-κB and AP-1 (c-Jun and c-Fos), and additionally the action of their upstream catalyst Src, Syk, and TAK1. Immunoprecipitation investigation demonstrated the failure of binding between their substrates, phospho- (p-) p85 and p-MKK3/6. P-p85 and p-MKK3/6, which were prompted by overexpression of Src, Syk, and TAK1, were likewise decreased by Pa-ME. In this manner, these outcomes recommend that Pa-ME applies its mitigating impacts by focusing on Src and Syk in the NF-κB signaling pathway and TAK1 in the AP-1 signaling pathway (Kim et al., 2017).

CHAPTER-3

RATIONALE

3.1 Rationale

From review of literature it has been confirmed that Piper plants are important sources for the development of new anticancer agents. Piper plants include about 2000 species, out of which 10 have been used as traditional medicines to treat cancer or cancer-like symptoms while, 35 extracts from 24 Piper species and number of compounds from Piper plants were found to have in vitro cytotoxic activity. Piper attenuatum (Buch-Ham) is one of piper species having significant pharmacological potential like antibacterial, anti-oxidant and anti-inflammatory effects but not much data is available on anti-cancer potential except few reports on crotepoxide (Kupchan et al., 1969) and extracts on MCF-7 cell lines (Ohlyan et al., 2013). Thus, further in vitro and in vivo anticancer research studies on Piper attenuatum (Buch-Ham) and its isolates are advisable for getting leads or development of potential ligands for treatment of cancer.

The novel molecules isolated from Piper attenuatum (Buch-Ham) may enhance the chances of getting potential leads for development of anticancer drug.

CHAPTER-4

AIM AND OBJECTIVES

4.1 Objectives

The major objectives of present study designed are:

- Preparations of chloroform and methanol extracts and their phytochemical investigations.
- Isolation and characterization of major chemical constituents of chloroform and methanol extracts of Piper attenuatum (Buch-Ham) (fruit).
- In-vitro antiproliferative activity of different extracts and isolated constituents of Piper attenuatum (Buch-Ham) against available cancel cell-lines.

CHAPTER-5

MATERIAL AND METHODS

5.1 Materials and methods

5.1.1 Plant material (procurement)

Dried fruits of Piper attenuatum nearly 1.1 kg were collected from Berikonda Chittoor district of Andhra Pradesh (12°37′ - 14°8′ north latitudes and 78°3′ - 79°55′ east longitudes). The authentication of sample was done by Dr. K. Madhava Chetty, Assistant Professor at Department of Botany, Sri Venkateswara University, Tirupati (Authentication certificate attached in Annexure-I).

Fig 5.1: Forest of Chittoor district, Andhra Pradesh (https://www.gettyimages.in/detail/photo/trail-passing-through-a-forest-tirupati-chittoor-royalty-free-image/140824080)

5.1.2 Chemicals

1. All the solvents and chemicals required for extraction and phytochemical investigation were purchased from S.D. Fine Chemicals, Avra synthesis and used without further purification.
2. Thin layer chromatography was done with silica gel G (Merck India) as the adsorbent. Ethyl acetate: Petroleum ether and Methanol: Chloroform mixtures at various concentrations were used as solvent system for the chromatographic

purification of compounds. Spots were visualized under UV light and iodine chamber.

3. Dulbecco's Modified Eagle's Medium (DMEM), antibiotic solutions, phosphate buffer saline (PBS) and fetal bovine serum (FBS), used in cell culturing were purchased from Gibco.
4. DMSO (Dimethyl sulfoxide) extrapure was purchased from HiMedia.

5.1.3 Instruments

1. Aspirator (2000 mL) of JSGW was used for extraction of plant material.
2. Rotavapour of IImvac purchased from Siskin Pvt. Ltd. was used for drying various fractions obtained from column chromatography.
3. Infrared spectra of compounds were recorded on Bruker IR spectrophotometer.
4. GC-MS spectra were recorded on GC-MS (ESI), at CIL, Central University of Punjab, Bathinda.
5. The ^{1}H and ^{13}C NMR of the compounds were recorded on Jeol and Bruker Advance II instruments at 400 MHz frequency, in $CDCl_3$ and TMS (δ=0) as internal standard at IIT Ropar, and Punjab University, Chandigarh respectively.
6. Other instruments used in biological evaluation are listed below in table:

Instruments Used	**Company**	**Purpose**
CO_2 Incubator	Galaxy, New Brunswick	Incubation
Centrifuge 5430 R	Eppendorf, Germany	Centrifugation
Laminar air flow	Klen Airflow	For aseptic condition
Micro plate Reader	Biotek	Fluorescence studies
Inverted microscope	Magnus, Olympus	Visualization of the cells

5.1.4 Cell lines

1**.** For anticancer evaluation of extracts and purified compounds MDA-MB-231 cell lines were used.

5.2 Extraction of plant material

1.1 kg of fruit parts of Piper attenuatum were collected, dried in shade and grounded to get fine powder for extraction. 400 gm of fine powder of fruit parts of Piper attenuatum was further used for extraction. Extraction was done using solvents petroleum ether, chloroform, ethyl acetate and methanol in increasing order of polarity. Plant material was macerated three times with 1500 mL each. Extracted fractions were dried and weighed. The detailed procedure is described in flow chart below (Fig 5.2):

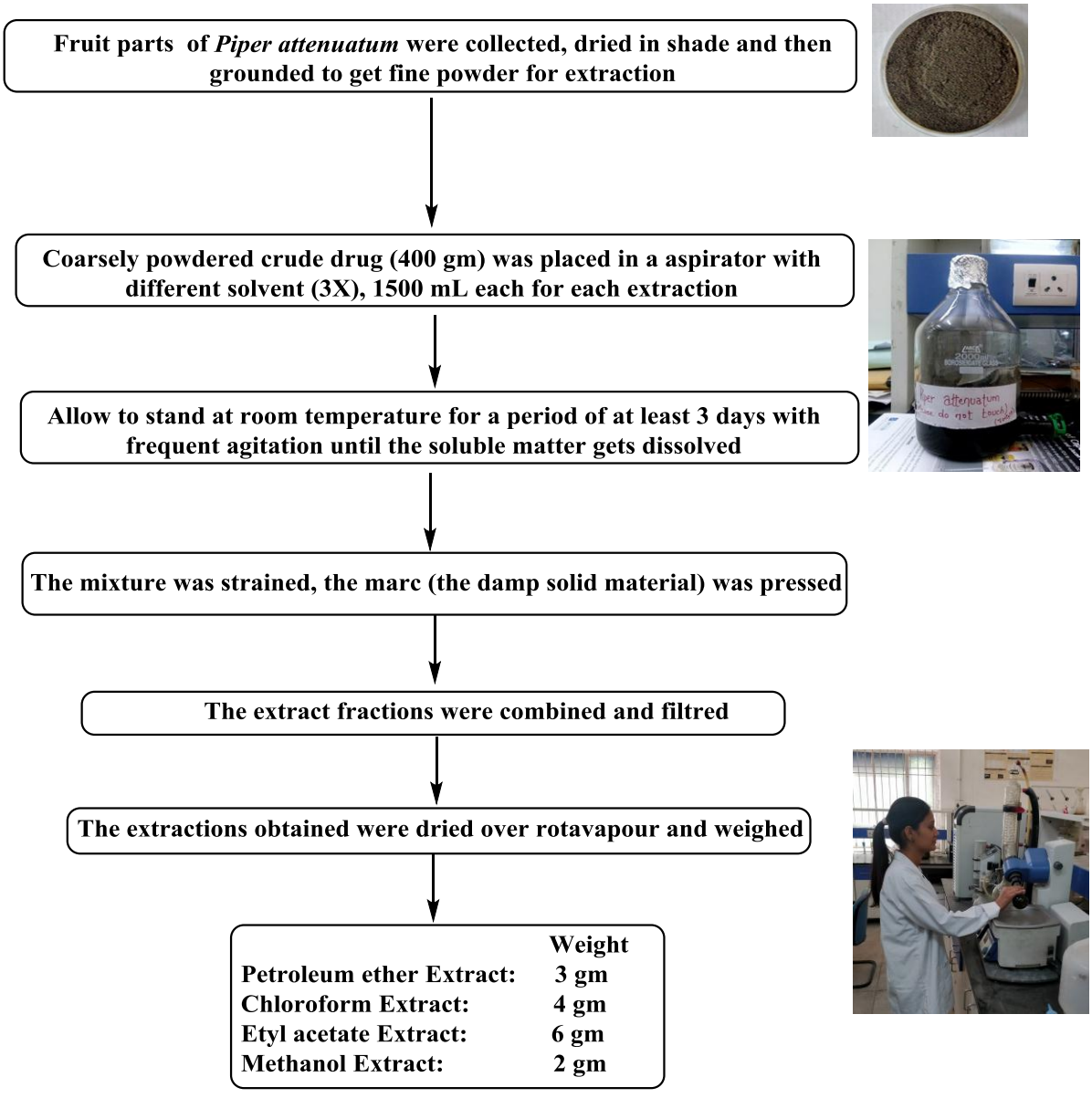


Fig 5.2: Schematic flow chart for extraction from Piper attenuatum

5.3 Phytochemical screening of extracts

Preliminary phytochemical analysis was carried out for the extract as per standard methods described by (Brain and Turner, 1975) and (Evans, 2002). Various testes performed for various phytoconstituent are listed below:

5.3.1 Alkaloids detection methods

5 mg of extracts were individually dissolved in 5 mL dilute hydrochloric acid and filtered. Then the filtrate was treated with following reagents to detect alkaloids.

Mayer's test: Filtrate was treated with potassium mercuric iodide solution (Mayer's reagent), Formation of a yellow or cream-colored precipitate indicated the presence of alkaloids.

Hager's test: Filtrate was treated with saturated aqueous solution of picric acid (Hager's reagent), Presence of alkaloids were confirmed by the formation of yellow colored precipitate (Fig 5.3).

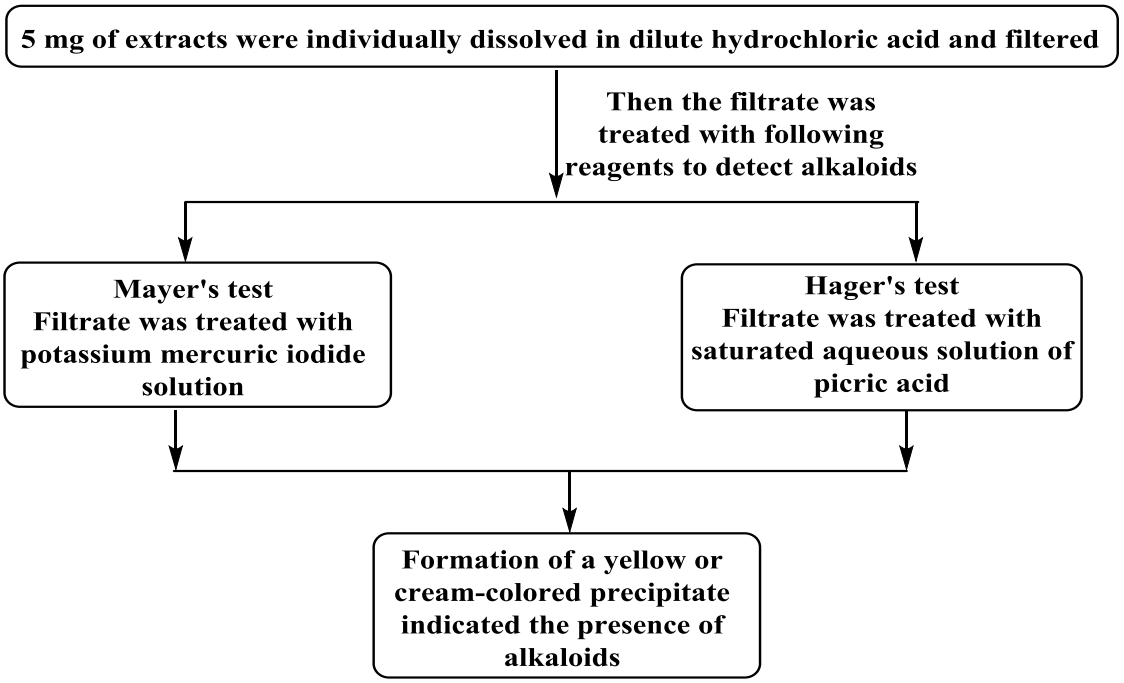


Fig 5.3: Schematic flow chart for alkaloids detection tests performed

5.3.2 Carbohydrate detection methods

5 mg of extract was dissolved in 5 mL distilled water and filtered. The filtrates were used to test the presence of carbohydrates.

Molisch's test: Filtrate was treated with 2 drops of alcoholic α-napthol solution in a test tube, shaken and add conc. sulphuric acid from the side of the test tube. Development of a violet ring at the junction of two liquid confirmed the presence of carbohydrates (Fig 5.4).

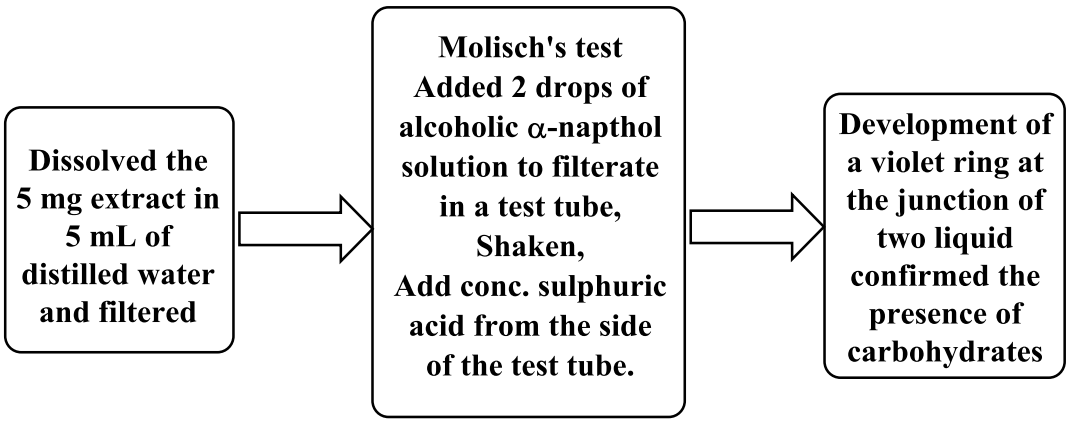


Fig 5.4: Schematic flow chart for Carbohydrates detection tests performed

5.3.3 Detection of reducing sugars

5 mg of extract was dissolved in 5 mL distilled water and filtered. The filtrates were used to test the presence of carbohydrates.

Benedict's test: Filtrate was treated with Benedict's reagent and boiled in a water-bath for 5 minutes. Formation of an orange red precipitate indicated the presence of reducing sugars.

Fehling's test: Filtrate was acidified with dil. HCl acid, neutralized with alkali and heated with Fehling's A and B solutions. Formation of red precipitate indicated the presence of reducing sugars (Fig 5.5).

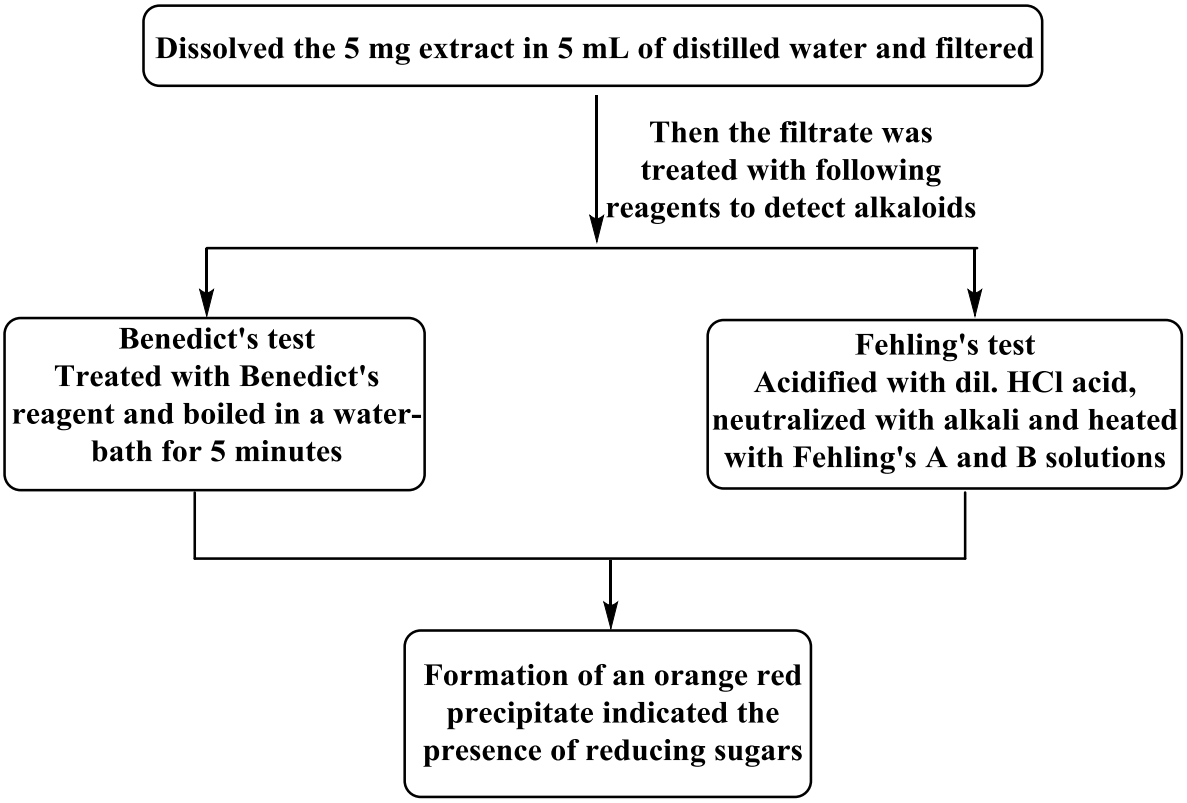


Fig 5.5: Schematic flow chart for reducing sugars detection tests performed

5.3.4 Detection of Saponins

Foam test: About 0.5 mg of the extract was shaken with 5 mL of distilled water. Formation of frothing (appearance of creamy miss of small bubbles) shows that the presence of Saponins (Fig 5.6).

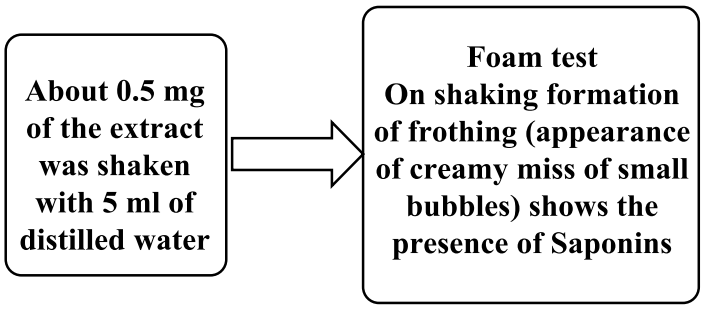


Fig 5.6: Schematic flow chart for saponins detection tests performed

5.3.5 Detection of Phytosterols

Liebermann Burchard Test: 2 mL of acetic anhydride was added to 5 mg of the extracts and added few drops of H_2SO_4. The colour was changed from violet to blue or green in some samples indicated that the presence of steroids.

Salkowski's Test: 5 mg of the extract was mixed with 2 mL of chloroform and concentrated H_2SO_4 (3 mL) was carefully added to form a layer. An appearance of reddish brown colour in the inner face indicated that the presence of terpenoids (Fig 5.7).

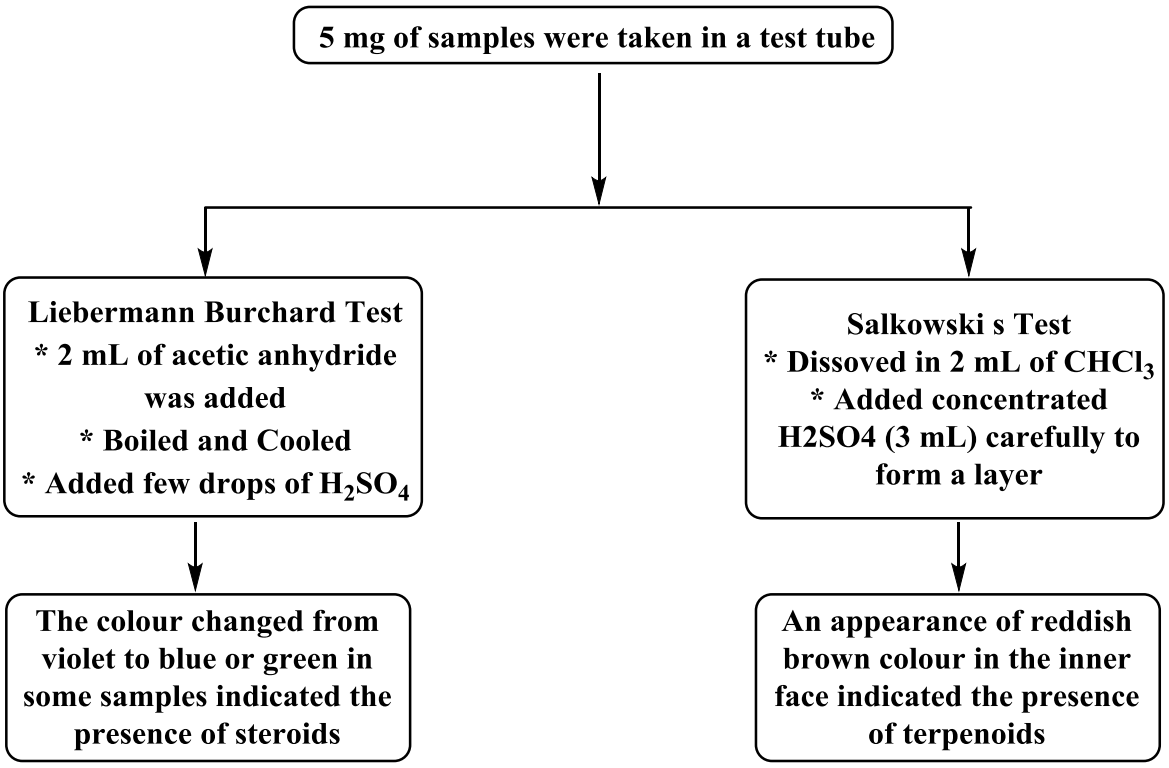


Fig 5.7: Schematic flow chart for Phytosterols detection tests performed

5.3.6 Detection of Oils and Resins

Spot test: Test solution was applied on filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and resins.

5.3.7 Detection of Tannins

0.5 mg extract was mixed with 5 mL of distilled water and heated on a water bath. The mixture was filtered and then ferric chloride was added to the filtrate. A dark green colour was formed which indicated the presence of tannins.

5.4 Isolation

Column chromatography technique was used for the isolation of compounds. TLC of all four extracts was checked using appropriate mobile phase and checked under UV chamber to visualize the presence of various compounds in each. Results from phytochemical tests and TLC indicated that chloroform extract (CEE) and methanol extracts (MCE) have large number of compounds. CEE and MCE extracts were chosen for further isolation and were subjected to column chromatography on a silica gel column (60-120 mesh, 60x3 cm) and eluted with a stepwise gradient of pet ether/ EtOAc, (99:1, 98:2, 97:3,96:4, 95:5, 93:7,90:10 by volume) to afford a total of 160 fractions of 20 mL each. Column fractions were analyzed by TLC (pet ether /EtOAc, 85:15), and fraction with similar TLC patterns were combined to give three major fractions (NP_1-NP_3). NP_1 fraction was further purified by column chromatography using solvent pet ether /EtOAc, 97:3 to give NPM-1 (10 mg). Fraction NP_2 was subjected to column chromatography by using solvent pet ether /EtOAc, 95:5 to give NP-7L (20 mg) and fraction NP_3 was purified using solvent pet ether /EtOAc, 90:10 to give NP-7C (25 mg).

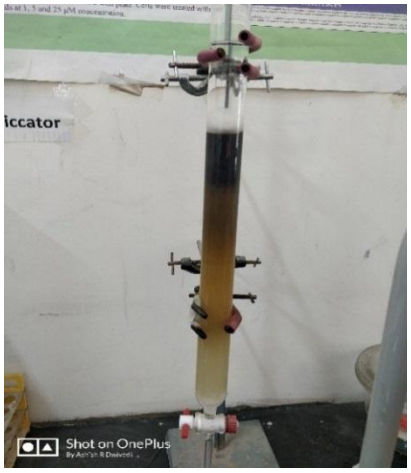


Fig 5.8: Image during column Chromatography of extracts

5.5 Characterization of isolated compounds

The characterizations of isolated compounds were done using NMR, and MASS spectroscopy.

5.6 *In-vitro* anticancer assay

5.6.1 Cell lines under study

For antiproliferative evaluation of extracts and isolated compounds MDA-MB-231 breast cancer cell line were used. **MDA-MB 231** is a human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma. MDA-MB-231 is a highly aggressive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification.

5.6.2 Culturing of cell lines

DMEM is used as a medium for culturing of the cancer cell lines as it is adherent cells, trypsin was added to remove them from the surface (trypsinization). Cells were harvested in 5 mL media containing serum which inactivates trypsin enzyme. Harvested cells were centrifuged at 1200 rpm at 4°C for 5 minutes and supernatant was removed and pellet was resuspended in media (2 mL). With automated cell counter, cell number was counted. The cells were moved to fresh media every two days.

5.6.3 Maintenance and sub-culturing of cell lines

The maintenance and culturing of cell lines was done in 25 cm^2 or 75 cm^2 flasks having DMEM medium supplemented with 10% fetal bovine serum (FBS), 1X antibiotic solution and incubated at 37° C in a humidified atmosphere containing 5% CO_2. Further sub-culturing of cells was done in 25 cm^2 flasks up to when the cancer cell lines have reached 70-80% of growth. The reagents vital for the procedure were placed in water bath maintained at 37° C for 10-15 minutes and trypsin was added for the detachment of adherent cells. The 1 mL of media containing serum was added after 5 minutes for

stopping the action of trypsin. Then, the cells were transferred to 15 ml centrifuged tubes and centrifuged for 5 min at 1200 rpm at 4° C. The supernatant was removed and the cell pellet was again re-suspended in complete media. The cell lines were transferred to fresh media every two days (cell passaging).

5.6.4 MTT assay

MTT (3-(4, 5-Dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was performed to evaluate the anti-proliferative activity of extracts and isolated compounds. MTT is a colorimetric assay used for the measurement of cell proliferation. The tetrazolium yellow compound MTT is reduced to an insoluble purple coloured formazan product by mitochondrial reductase or succinate dehydrogenase in metabolically active cells only. When formazan passes to the mitochondria it gets solubilized with DMSO and measured spectrophotometrically.

To carry out the MTT assay, cell lines MDA-MB-231 (8,000–10,000 cells) were seeded in each well of the 96 well plates. The plate was incubated at 37^0C with 5% CO_2 for 24 h followed by serum starvation. The treatment was given to the cells in triplicate concentrations of 1 μM, 5 μM and 25 μM and incubated for 48 h. MTT solution (5 mg/10 mL) was added after removing the media from each well and incubated in the dark for 4 h. At the end of 4 h, the MTT solution was removed from each well and the intracellular precipitate was dissolved in DMSO solution and the absorbance of the violet color formed as consequence of DMSO addition, is read spectrometrically at 570 nm and expressed as % inhibition (Mean ± S.D).

CHAPTER-6

RESULTS AND DISCUSSION

6.1 Results and discussion

Extensive phytochemical evaluation of dry fruits of P. attenuatum (Buch-Ham) was performed. We also performed the evaluation of antiproliferative potential of chloroform and methanol extracts along with major isolated compounds of dry fruit of P. attenuatum (Buch-Ham) using MTT assay.

6.1.1 Phytochemical screening

Preliminary phytochemical analysis was carried out for the extract as per standard method. The results of various testes performed for various phytoconstituent are listed below in Table 6.1:

Table 6.1: Results of phytochemical tests performed

Constituent	Test	Methanol	Chloroform	Ethyl acetate	Petroleum Ether
Alkaloids	Mayer's test	++	++	++	++
	Hager's test	++	++	++	++
Carbohydrate	Molisch test	++	++	++	--
Reducing sugars	Benedict test	--	--	++	--
	Fehling test	--	--	++	--
Saponins	Froth test	--	--	--	--
Phytosterols	Salkowski test	--	++	++	--
	Lieberman Burchard test	--	++	++	--
Oils and Resins	Spot test	++	++	++	++
Tannins	Ferric chloride test	--	--	++	--

++ Positive test, -- Negative test

6.1.2 Antiproliferative evaluation of extracts

All extracts obtained from extraction procedure on Piper attenuatum (Buch-Ham) were evaluated for their antiproliferative potential using MTT assay against MDA-MB-231 (breast cancer) cell lines. Methanol extract (MCE) and chloroform extract (CEE) were found to have potential antiproliferative properties. MCE and CEE extracts were found to reduce the cell viability to 50% at 10 μg/mL and 50 μg/mL concentrations respectively. It was found comparable to the results obtained from colchicine which reduced the cell viability to 50% at 50 μg/mL concentration (Fig 6.1). Thus, from these results it can be concluded that chloroform and methanol extracts of Piper attenuatum (Buch-Ham) contains the potential antiproliferative compounds.

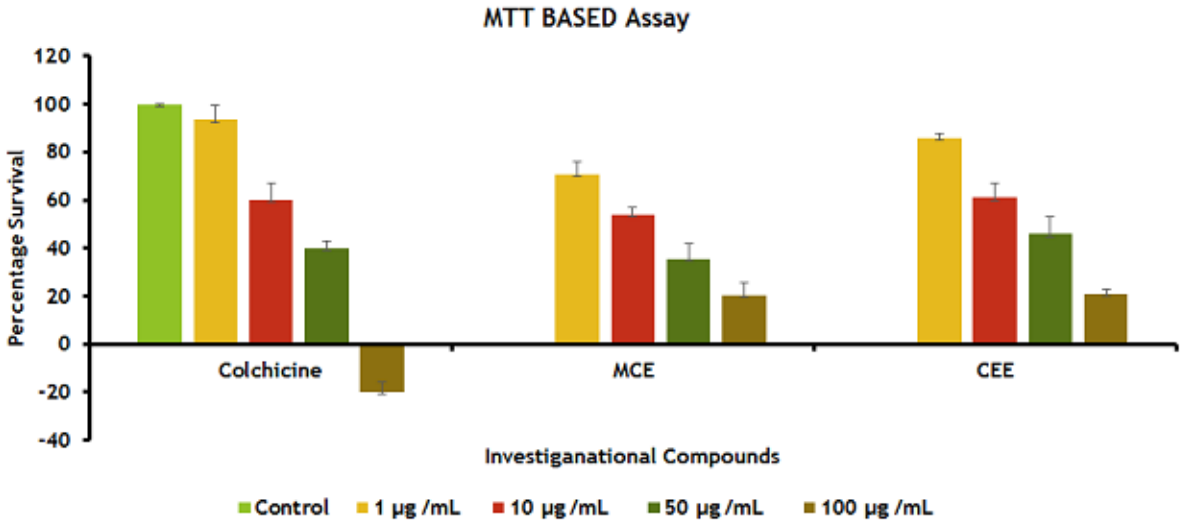


Fig 6.1 Antiproliferative evaluation of methanol and chloroform extracts of Piper attenuatum (Buch-Ham) against MDA-MB-231. Values are expressed as mean ± S.D (n=3)

6.1.3 Isolation of major constituents of chloroform and methanol extracts using column chromatography

Column chromatography technique was used for the isolation of major constituents of chloroform and methanol extracts. Results from antiproliferative evaluation, phytochemical tests and TLC indicated that chloroform extract (CCE) and methanol extract (MCE) have large number of compounds. CEE and MCE were chosen for further isolation and subjected to column chromatography on a silica gel column (60-120 mesh, 60x3 cm) and eluted with a stepwise gradient of pet ether/ EtOAc, (99:1,

98:2, 97:3,96:4, 95:5, 93:7,90:10 by volume) to afford a total of 160 fractions of 20 mL each. Column fractions were analyzed by TLC (pet ether /EtOAc, 85:15), and fraction with similar TLC patterns were combined to give three major fractions (NP_1-NP_3). NP_1 fraction was further purified by column chromatography using solvent pet ether /EtOAc, 97:3 to give NPM-1 (10 mg). Fraction NP_2 was subjected to column chromatography by using solvent pet ether /EtOAc, 95:5 to give NP-7L (20 mg) and fraction NP_3 was purified using solvent pet ether /EtOAc, 90:10 to give NP-7C (25 mg). Different compounds isolated from CEE and MCE extracts are listed below in Table 6.2.

Table 6.2: Isolated compounds from of chloroform and methanol extracts of Piper attenuatum (Buch-Ham)

Sr. no.	**Compounds**	**Amount**	**TLC solvent system**	**Extract (Isolated from)**
1.	NPM-1	10 mg	Pet ether: EtOAc (9.5: 0.5)	Methanol
2.	NP-7L	20 mg	Pet ether: EtOAc (9: 1)	Chloroform
3.	NP-7C	25 mg	Pet ether: EtOAc (8.5: 1.5)	Chloroform

6.1.4 Characterization of compounds

Three compounds isolated from CCE and MCE extracts were characterized using various spectral techniques like NMR, IR and Mass spectroscopy. The results of spectral analysis are listed below in Table 6.3

Table 6.3: Spectral analysis results of isolated compounds

Product code	**M. P. (°C)**	**IR (cm^{-1})**	**^{1}H NMR (δ)**	**^{13}C NMR (δ)**	**HRMS**
NPM-1	Semi-solid	2800-2900 (CH=O) 1000-1219 (C-O)	8.12 (1H, dd, J_1 = 8Hz, J_2 = 4Hz), 7.67 (2H, d, J = 8Hz), 7.52-7.42 (3H, m), 7.17 (2H, d, J = 8Hz), 6.93-6.90 (4H, m), 6.78 (1H, t, J = 8Hz), 5.81 (1H, q, J = 4Hz), 4.09 (1H, q, J =	134.82, 134.67, 133.38, 133.23, 133.01, 132.77, 129.96, 129.31, 129.19, 129.14, 128.61, 128.40, 128.26, 128.15, 127.71, 127.59,	374.1589

			4Hz), 3.83 (3H, s), 3.11 (1H, q, J = 4Hz)	127.42, 127.32, 125.56, 124.65, 124.59, 124.02, 119.71, 119.33, 114.27, 124.21, 124.13, 112.89, 112.76, 105.45, 82.64, 69.61, 60.76, 55.46, 55.41, 43.42, 29.79	
NP-7L	Liquid	2800-300 (CH)	7.31-7.25 (3H, m), 7.19 (2H, d, J = 8Hz), 7.14 (3H, d, J = 8Hz), 2.49-2.45 (1H, m), 1.66-1.49 (5H, m), 1.30-1.16 (30H, m), 0.91-0.82 (13H, m), 0.77 (1H, t, J = 8Hz)	146.54, 128.34, 128.32, 127.84, 127.76, 127.08, 125.80, 125.76, 47.99, 46.17, 45.96, 37.11, 37.07, 32.00, 29.89, 29.86, 29.44, 27.76, 27.72, 22.93, 22.78, 22.69, 22.44, 20.83, 14.28, 14.33, 12.32	567.2752, 263.4392, 241.0010, 137.0170
NP-7C	Liquid		7.31-7.25 (3H, m), 7.19 (2H, d, J = 8Hz), 7.14 (3H, d, J = 8Hz), 2.49-2.45 (1H, m), 1.66-1.49 (5H, m), 1.30-1.16 (30H, m), 0.91-0.82 (13H, m), 0.77 (1H, t, J = 8Hz)	146.54, 128.34, 128.32, 127.84, 127.76, 127.08, 125.80, 125.76, 47.99, 46.17, 45.96, 37.11, 37.07, 32.00, 29.89, 29.86, 29.44, 27.76, 27.72, 22.93, 22.78, 22.69,	557.2867, 489.2278, 415.1217, 353.1008

				22.44, 20.83, 14.28, 14.33, 12.32	

6.1.5 Antiproliferative activity of isolated compounds

Three isolated compounds were further subjected to MTT assay to evaluate their antiproliferative potential (Fig 6.2) against MDA-MB-231 (breast cancer) cell lines. Compound NP7C was found most potent antiproliferative agent with IC_{50} value of 3.83 μM which is comparable to etoposide 2.37 μM. Compound NP7L was also found potent antiproliferative agent with IC_{50} value of 6.44 μM which was comparable with colchicine (IC_{50} = 6.3 μM). So, from this study, it was found that isolated compounds of Piper attenuatum are potential antiproliferative agents.

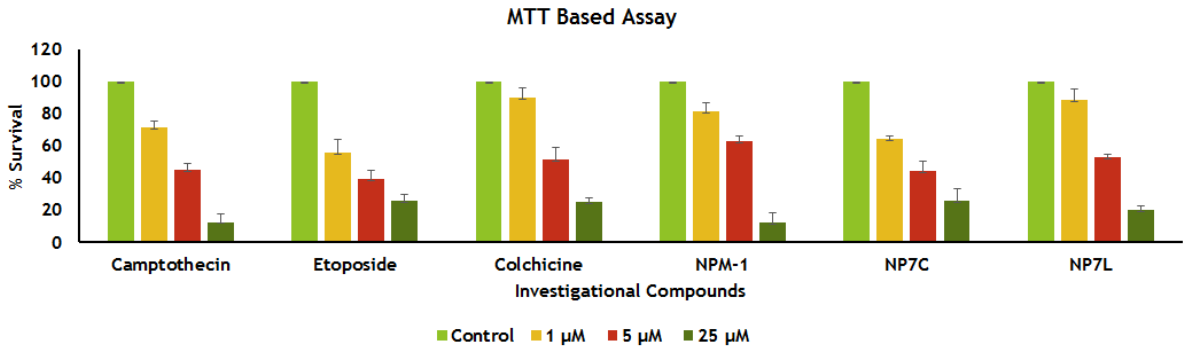


Fig 6.2 Antiproliferative evaluation of isolated compounds from Piper attenuatum (Buch-Ham) against MDA-MB-231. Values are expressed as mean ± S.D (n=3)

Table 6.4: IC_{50} values of synthesized compounds against MDA-MB 231 cell line

Compounds	**IC_{50} (μM)**
Camptothecin	4.16
Etoposide	2.37
Colchicine	6.3
NPM-1	10.21
NP7C	3.83
NP7L	6.44

CHAPTER-7

SUMMARY AND CONCLUSION

Ayurveda, traditional Chinese medicine (TCM), Kampo, traditional Korean medicine edicine (TKM), and Unani systems are utilizing the natural products from hundreds or even thousands of years. Currently most of the anticancer drugs used are obtained or derived from natural sources. Piper species also had been used in traditional medicinal systems from thousands of years, including Indian and Chinese systems, as well as in folklore medicines of Latin America and West Indies. Indian medicinal plant Piper attenuatum (Buch-Ham), a substitute for black pepper (P. nigrum), is one important species of Piper which has extensive pharmacological profile and had been utilized as herbal medicine for the treatment of muscular pain, headache and have been utilized as a rubefacient. The wood from plant has been used to treat throat pain.

We collected the dried fruits of Piper attenuatum (Buch-Ham) from Berikonda Chittoor district of Andhra Pradesh (12°37′ - 14°8′ north latitudes and 78°3′ - 79°55′ east longitudes) and authentication of sample was done by Dr. K. Madhava Chetty, Assistant Professor at Sri Venkateswara University, Tirupati. Extraction of the fine powder of dried fruits of Piper attenuatum (Buch-Ham) was done using solvents petroleum ether, chloroform, ethyl acetate and methanol in increasing order of polarity. Extracted fractions were dried and were evaluated for various phytochemicals present. Results from antiproliferative evaluation, phytochemical tests and TLC of extracts indicated that chloroform (CEE) and Methanol (MCE) extract have large number of compounds. Thus, further isolation of various compounds from CEE and MCE was done using column chromatography. From this extract three compounds (NP_{1-3}) were isolated. Spectral analysis of isolated compounds was performed using NMR and HRMS analysis. Structural characterization of isolated compounds from 2D-NMR will further help in determining the structure of compounds. The results from MTT assay of isolated compounds, NP7C was found to be the most potent antiproliferative agent with IC_{50} value of 3.83 μM which is comparable to etoposide 2.37 μM. Compound NP7L also exhibit significant antiproliferative activity (IC_{50} of 6.44 μM) which was comparable to colchicine (IC_{50} = 6.3 μM). Thus, the present study indicated that isolated compounds of Piper attenuatum (Buch-Ham) possess great potential to be developed as anticancer agent in future.

CHAPTER-8

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APPENDIX

ANNEXURE-I

SRI VENKATESWARA UNIVERSITY
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Date: 23-12-2016

AUTHENTICATION CERTIFICATE

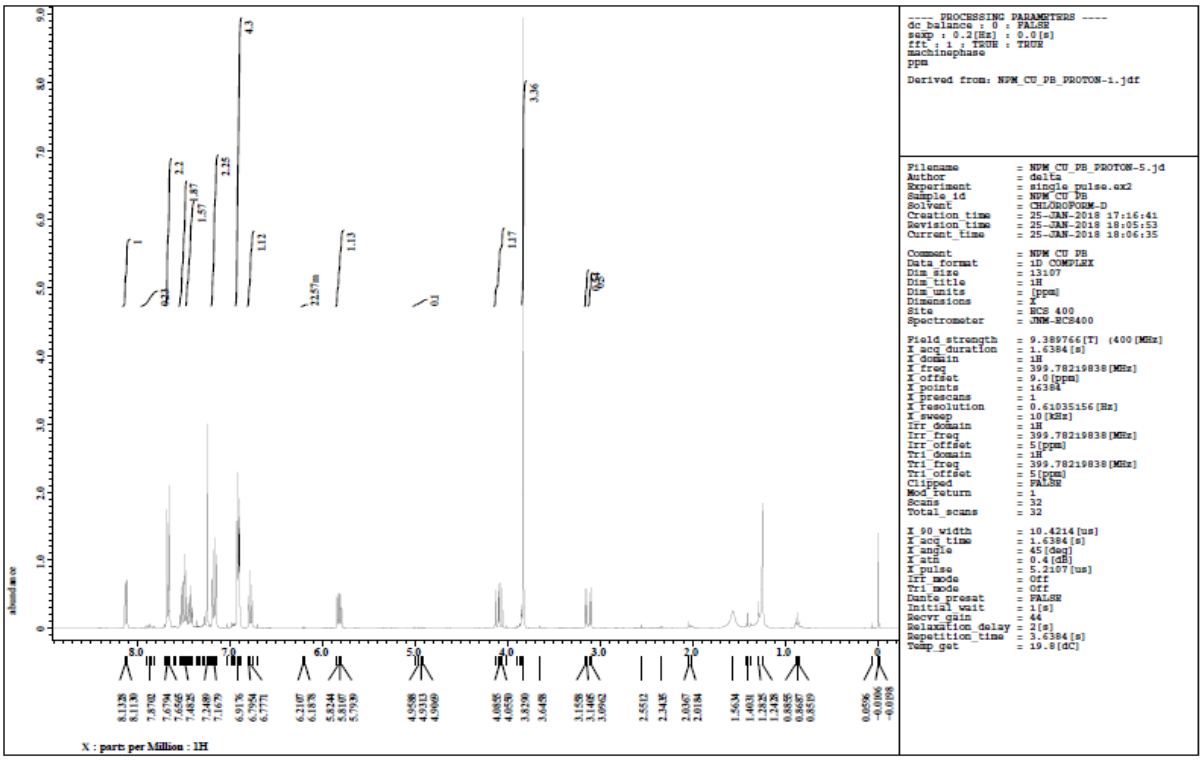
I hereby certify that the following plant species for pharmacognostical / pharmaceutical / pharmacological / phytochemical investigation research work is identified and their botanical name and family name is given.

Botanical Name	Voucher number	Family
Piper attenuatum Buch.-Ham. ex Miq.	3147	Piperaceae

Authenticated by
K. Madhava Chetty
(K. Madhava Chetty)
DR. K. MADHAVA CHETTY
M.Sc., M.Ed., M.Phil., Ph.D., PG DPD
ASSISTANT PROFESSOR
DEPARTMENT OF BOTANY
SRI VENKATESWARA UNIVERSITY
TIRUPATI-517 502, A.P. India

ANNEXURE-II

SPECTRAL ANALYSIS OF NPM-1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fft : 1 : TRUE : TRUE
machinephase
ppm
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Filename = NPM_CU_PB_PROTON-5.jd
Author = delta
Experiment = single_pulse.ex2
Sample_id = NPM_CU_PB
Solvent = CHLOROFORM-D
Creation_time = 25-JAN-2018 17:16:41
Revision_time = 25-JAN-2018 18:05:53
Current_time = 25-JAN-2018 18:06:35
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Data_format = 1D COMPLEX
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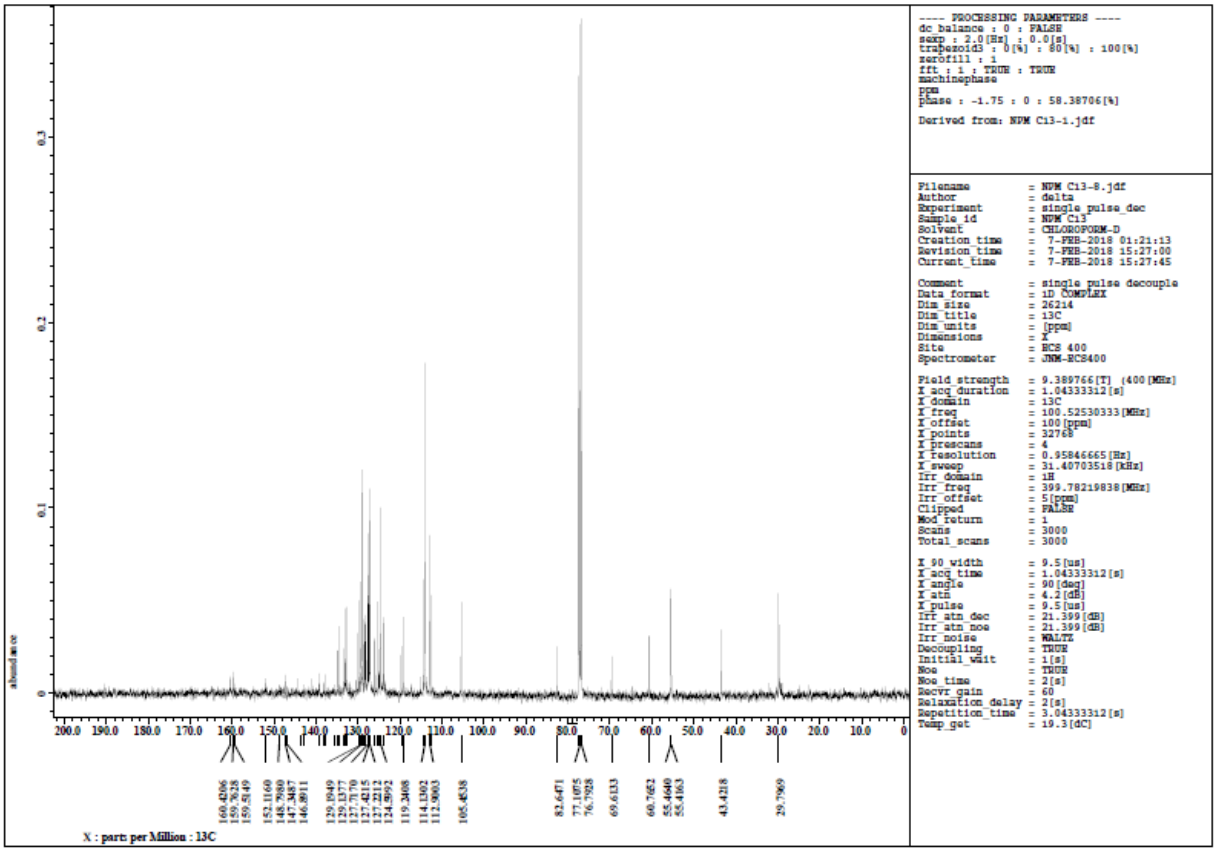
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = RCS 400
Spectrometer = JNM-RCS400
Field_strength = 9.389766[T] (400[MHz]
X_acq_duration = 1.6384[s]
X_domain = 1H
X_freq = 399.78219838[MHz]
X_offset = 9.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.61035156[Hz]
X_sweep = 10[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Tri_domain = 1H
Tri_freq = 399.78219838[MHz]
Tri_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 32
Total_scans = 32
X_90_width = 10.4214[us]
X_acq_time = 1.6384[s]
X_angle = 45[deg]
X_atn = 0.4[dB]
X_pulse = 5.2107[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
RecVr_gain = 44
Relaxation_delay = 2[s]
Repetition_time = 3.6384[s]
Temp_get = 19.8[dC]
X : parts per Million : 1H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phase : -1.75 : 0 : 58.38706[%]
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Filename = NPM C13-8.jdf
Author = delta
Experiment = single_pulse_dec
Sample_id = NPM C13
Solvent = CHLOROFORM-D
Creation_time = 7-FEB-2018 01:21:13
Revision_time = 7-FEB-2018 15:27:00
Current_time = 7-FEB-2018 15:27:45
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Dimensions = X
Site = RCS 400
Spectrometer = JNM-RCS400
Field_strength = 9.389766[T] (400[MHz]
X_acq_duration = 1.04333312[s]
X_domain = 13C
X_freq = 100.52530333[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.95846665[Hz]
X_sweep = 31.40703518[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 3000
Total_scans = 3000
X_90_width = 9.5[us]
X_acq_time = 1.04333312[s]
X_angle = 90[deg]
X_atn = 4.2[dB]
X_pulse = 9.5[us]
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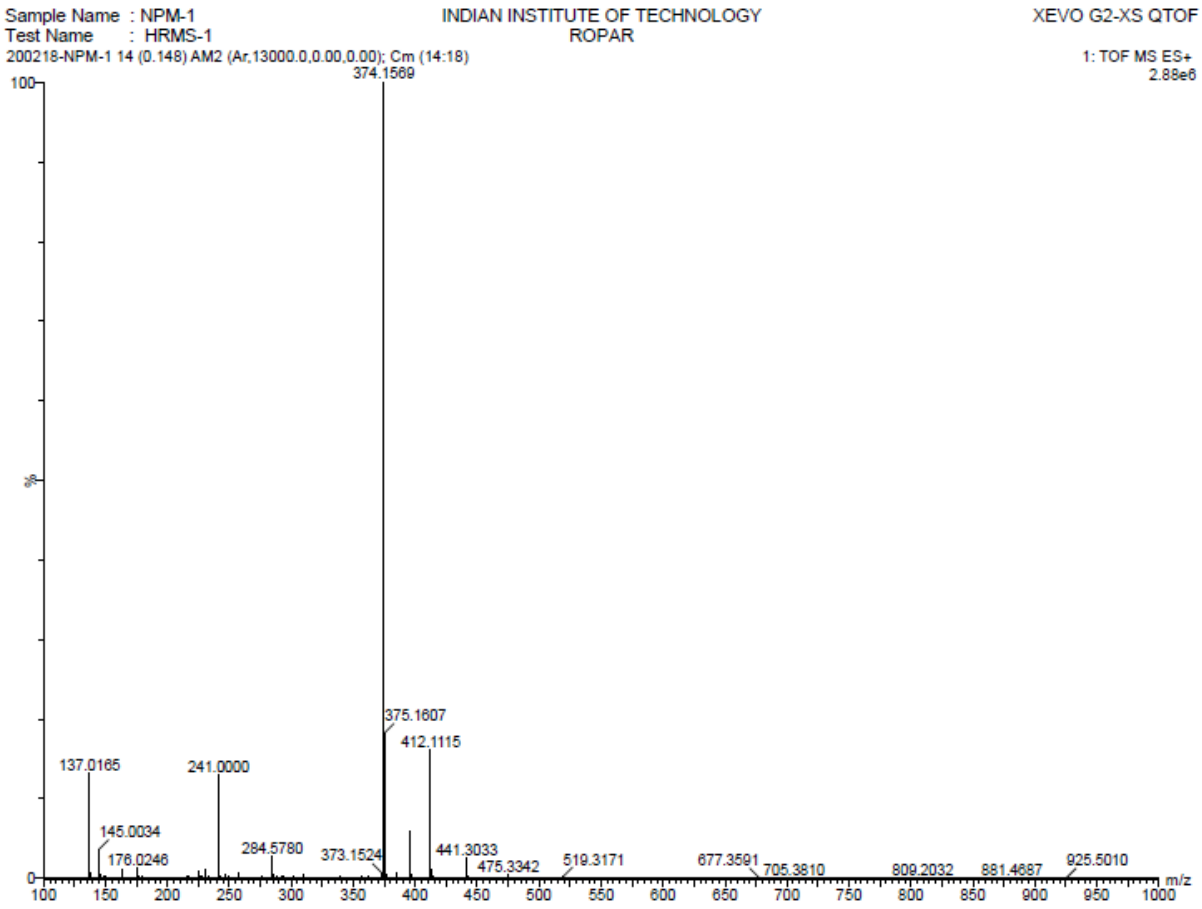
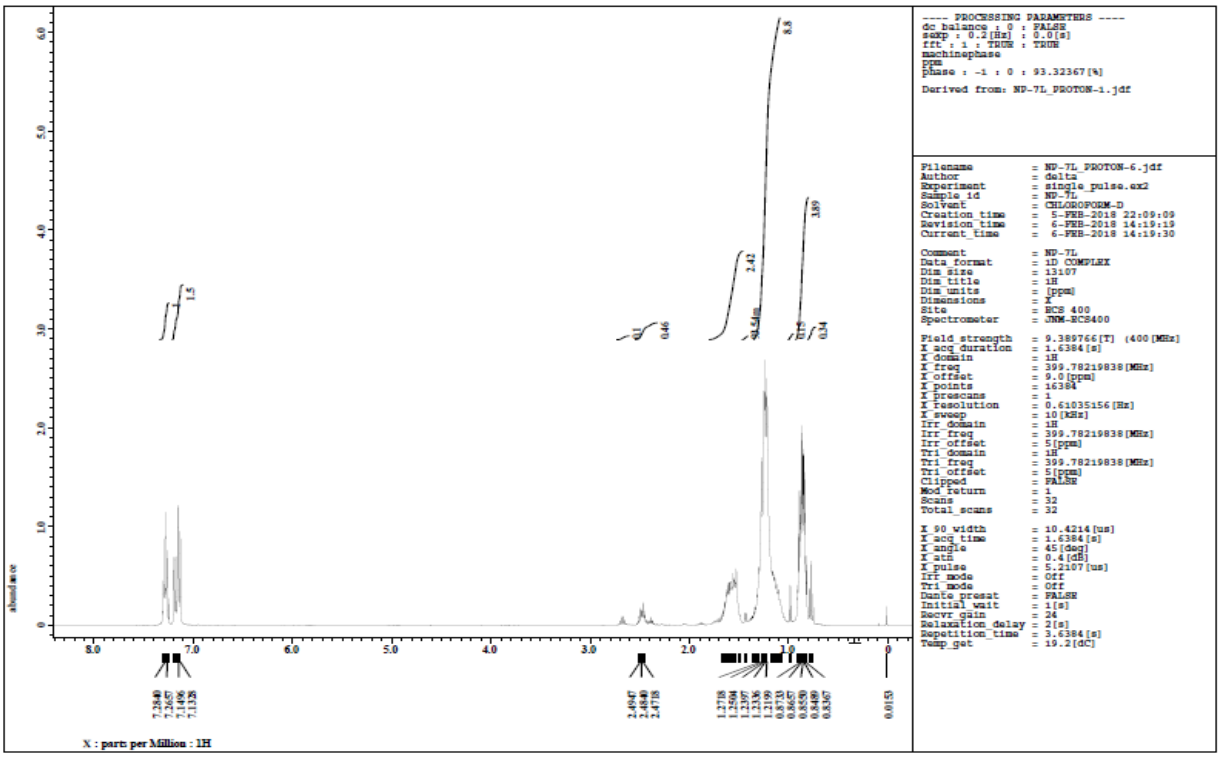
Irr_atn_noe = 21.399[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
RecVr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 3.04333312[s]
Temp_get = 19.3[dC]
X : parts per Million : 13C

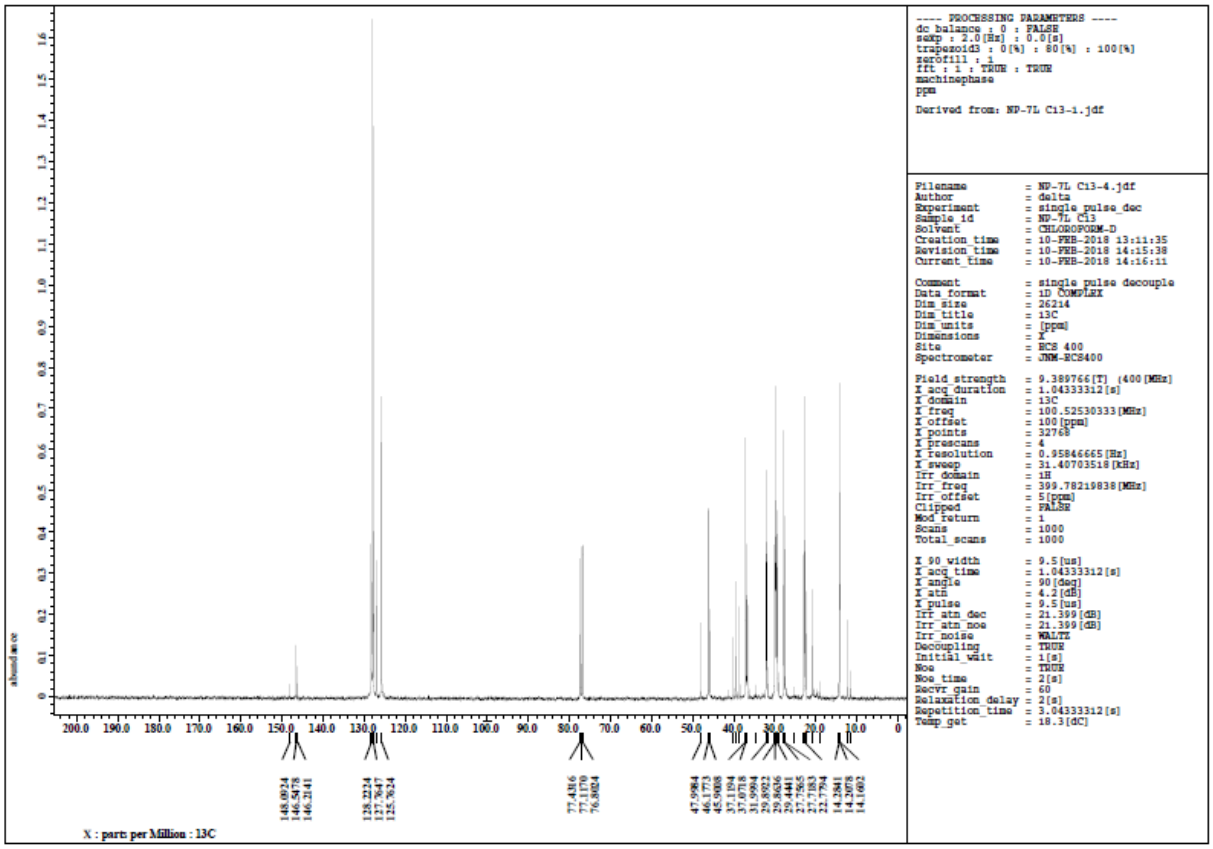

Sample Name : NPM-1
Test Name : HRMS-1
INDIAN INSTITUTE OF TECHNOLOGY
ROPAR
XEVO G2-XS QTOF
200218-NPM-1 14 (0.148) AM2 (Ar,13000.0,0.00,0.00); Cm (14:18)
1: TOF MS ES+
2.88e6
374.1569
375.1607
412.1115
137.0165
241.0000
145.0034
176.0246
284.5780
373.1524
441.3033
475.3342
519.3171
677.3591
705.3810
809.2032
881.4687
925.5010
%
m/z

SPECTRAL ANALYSIS OF NP-7L



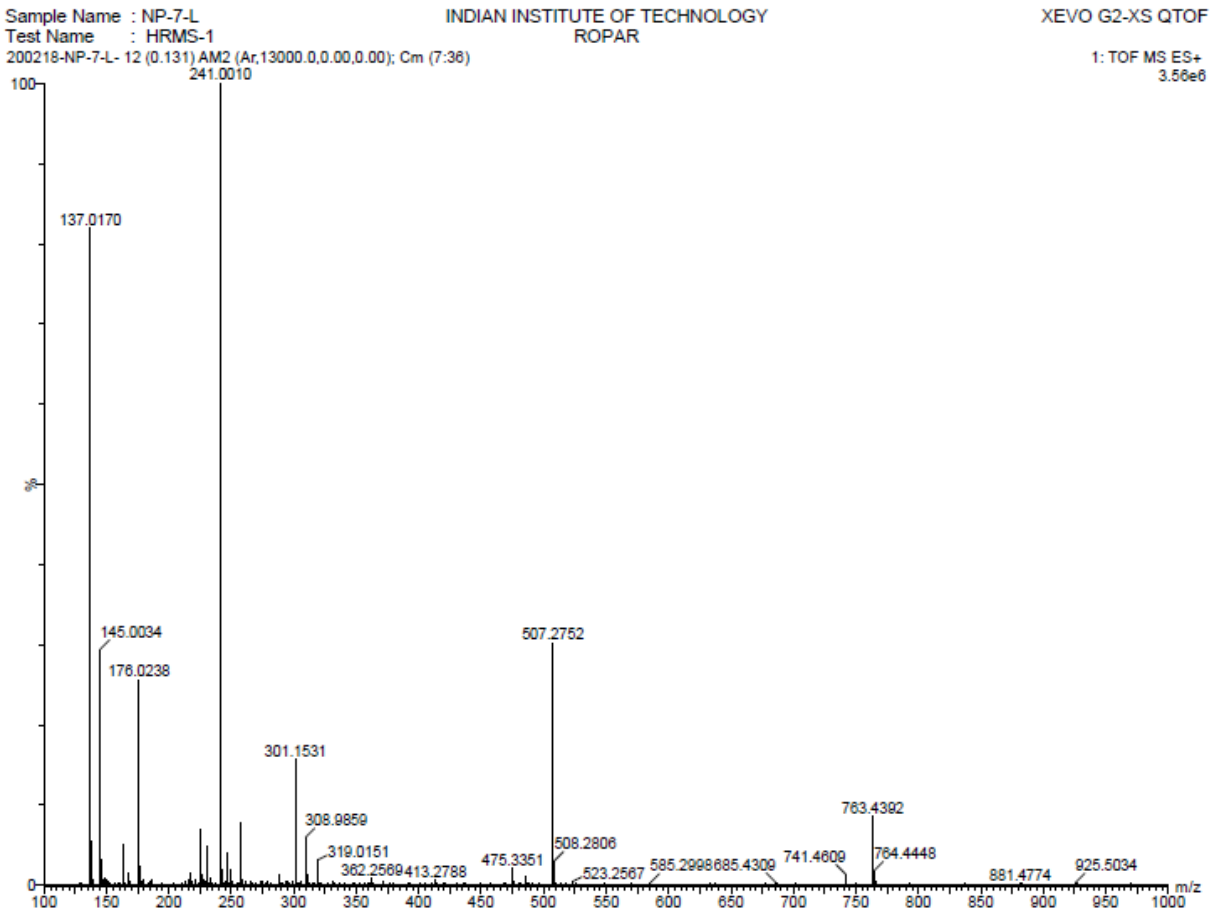

```
---- PROCESSING PARAMETERS ----
dc balance : 0 : FALSE
sexp : 0.2[Hz] : 0.0[s]
fft : 1 : TRUE : TRUE
machinephase
ppm
phase : -1 : 0 : 93.32367[%]

Derived from: NP-7L_PROTON-1.jdf
```

```
Filename            = NP-7L_PROTON-6.jdf
Author              = delta
Experiment          = single_pulse.ex2
Sample_id           = NP-7L
Solvent             = CHLOROFORM-D
Creation_time       =  5-FEB-2018 22:09:09
Revision_time       =  6-FEB-2018 14:19:19
Current_time        =  6-FEB-2018 14:19:30

Comment             = NP-7L
Data_format         = 1D COMPLEX
Dim_size            = 13107
Dim_title           = 1H
Dim_units           = [ppm]
Dimensions          = X
Site                = NCS 400
Spectrometer        = JNM-NCS400

Field_strength      = 9.389766[T] (400[MHz]
X_acq_duration      = 1.6384[s]
X_domain            = 1H
X_freq              = 399.78219838[MHz]
X_offset            = 9.0[ppm]
X_points            = 16384
X_prescans          = 1
X_resolution        = 0.61035156[Hz]
X_sweep             = 10[kHz]
Irr_domain          = 1H
Irr_freq            = 399.78219838[MHz]
Irr_offset          = 5[ppm]
Tri_domain          = 1H
Tri_freq            = 399.78219838[MHz]
Tri_offset          = 5[ppm]
Clipped             = FALSE
Mod_return          = 1
Scans               = 32
Total_scans         = 32

X_90_width          = 10.4214[us]
X_acq_time          = 1.6384[s]
X_angle             = 45[deg]
X_atn               = 0.4[dB]
X_pulse             = 5.2107[us]
Irr_mode            = Off
Tri_mode            = Off
Dante_presat        = FALSE
Initial_wait        = 1[s]
Recvr_gain          = 24
Relaxation_delay    = 2[s]
Repetition_time     = 3.6384[s]
Temp_get            = 19.2[dC]
```

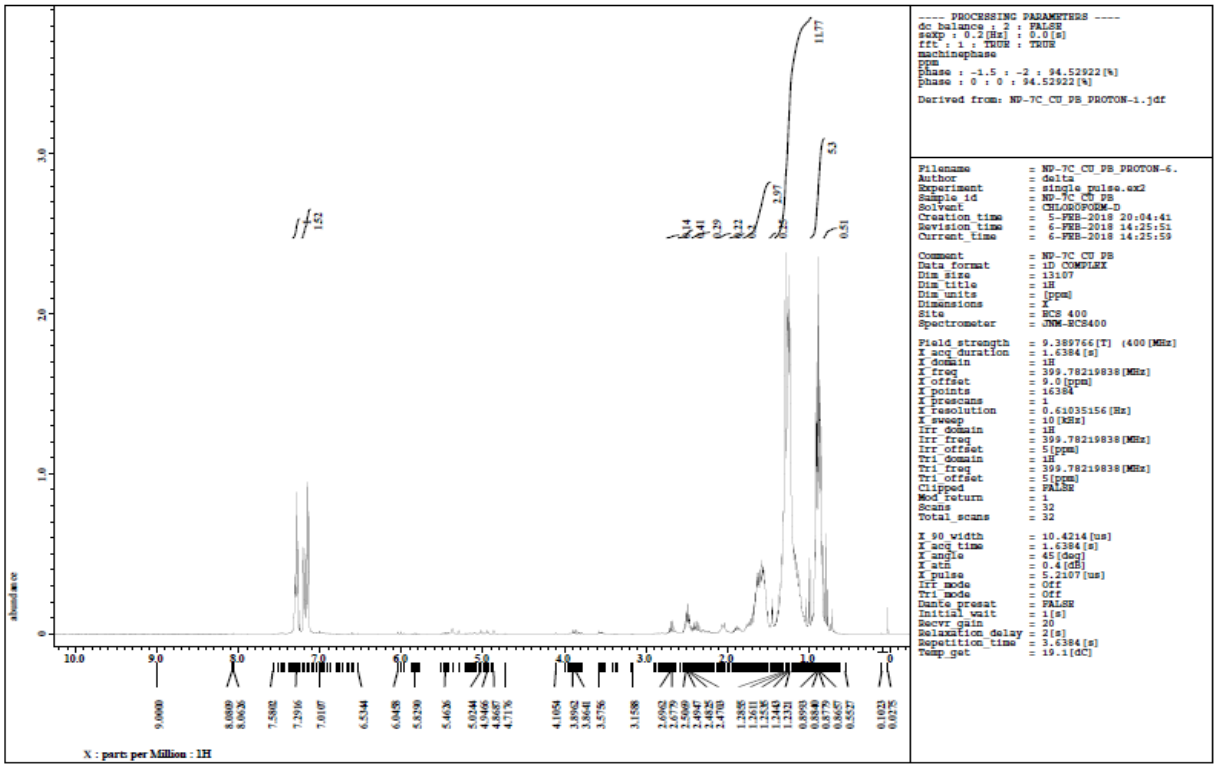


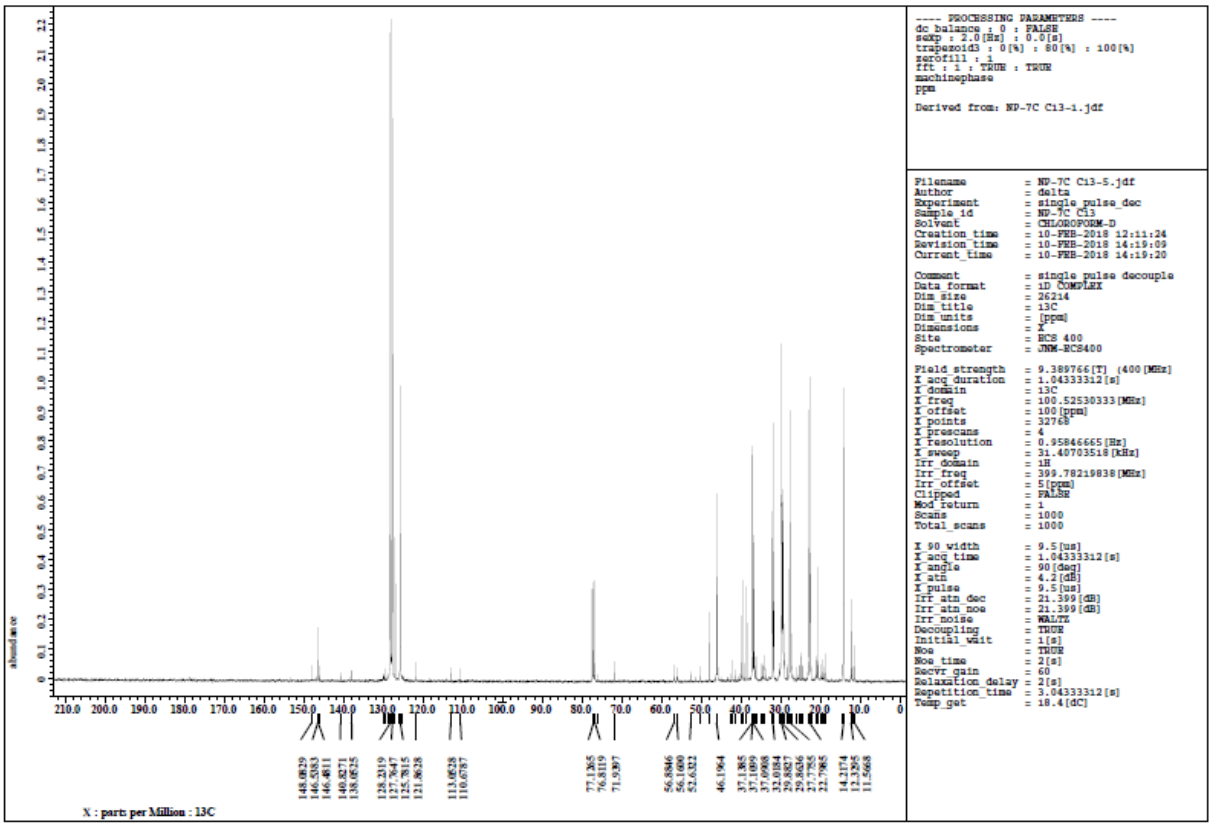

```
---- PROCESSING PARAMETERS ----
dc balance : 0 : FALSE
sexp : 2.0[Hz] : 0.0[s]
trapezoid3 : 0[%] : 80[%] : 100[%]
zerofill : 1
fft : 1 : TRUE : TRUE
machinephase
ppm

Derived from: NP-7L C13-1.jdf
```

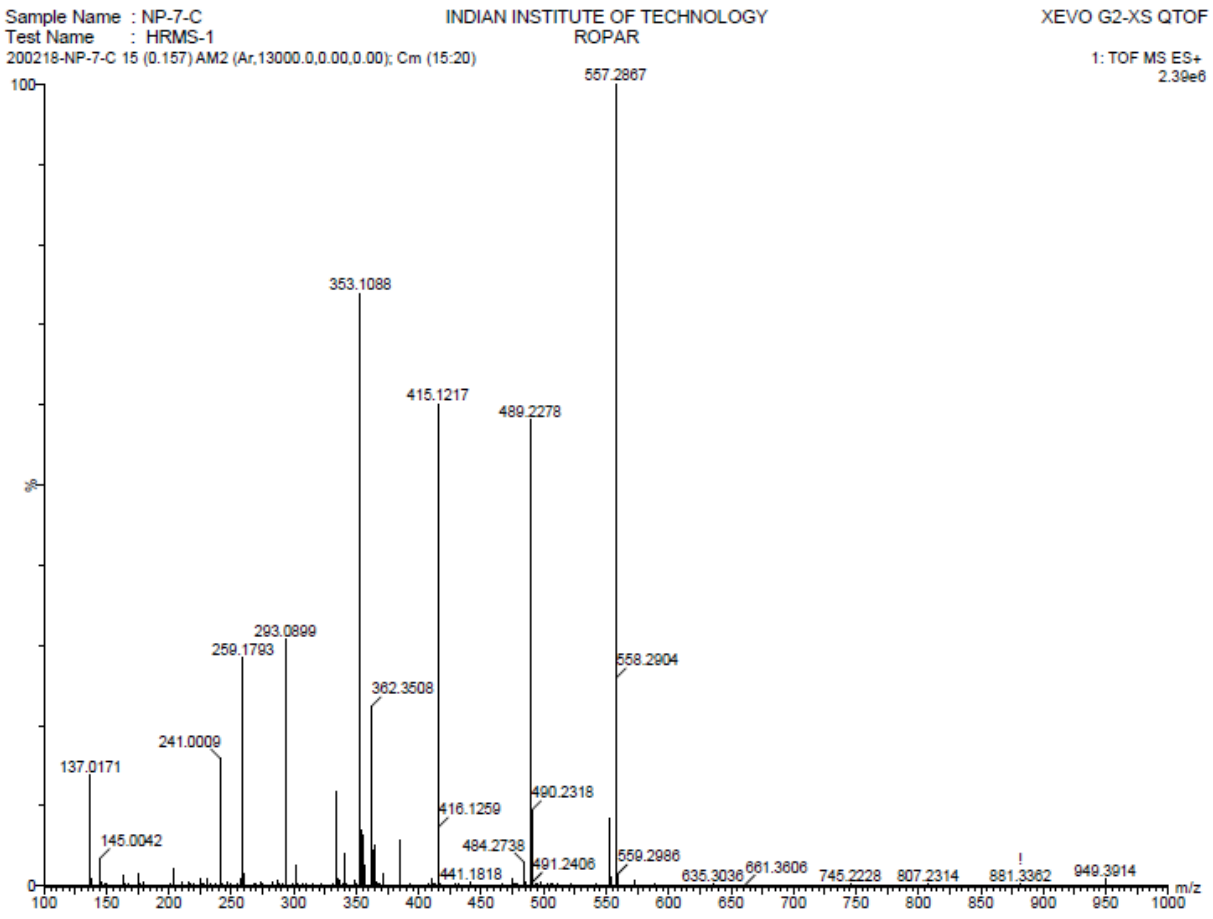
```
Filename            = NP-7L C13-4.jdf
Author              = delta
Experiment          = single_pulse_dec
Sample_id           = NP-7L C13
Solvent             = CHLOROFORM-D
Creation_time       = 10-FEB-2018 13:11:35
Revision_time       = 10-FEB-2018 14:15:38
Current_time        = 10-FEB-2018 14:16:11

Comment             = single pulse decouple
Data_format         = 1D COMPLEX
Dim_size            = 26214
Dim_title           = 13C
Dim_units           = [ppm]
Dimensions          = X
Site                = NCS 400
Spectrometer        = JNM-NCS400

Field_strength      = 9.389766[T] (400[MHz]
X_acq_duration      = 1.04333312[s]
X_domain            = 13C
X_freq              = 100.52530333[MHz]
X_offset            = 100[ppm]
X_points            = 32768
X_prescans          = 4
X_resolution        = 0.95846665[Hz]
X_sweep             = 31.40703518[kHz]
Irr_domain          = 1H
Irr_freq            = 399.78219838[MHz]
Irr_offset          = 5[ppm]
Clipped             = FALSE
Mod_return          = 1
Scans               = 1000
Total_scans         = 1000

X_90_width          = 9.5[us]
X_acq_time          = 1.04333312[s]
X_angle             = 90[deg]
X_atn               = 4.2[dB]
X_pulse             = 9.5[us]
Irr_atn_dec         = 21.399[dB]
Irr_atn_noe         = 21.399[dB]
Irr_noise           = WALTZ
Decoupling          = TRUE
Initial_wait        = 1[s]
Noe                 = TRUE
Noe_time            = 2[s]
Recvr_gain          = 60
Relaxation_delay    = 2[s]
Repetition_time     = 3.04333312[s]
Temp_get            = 18.3[dC]
```


Sample Name : NP-7-L
Test Name : HRMS-1
200218-NP-7-L- 12 (0.131) AM2 (Ar,13000.0,0.00,0.00); Cm (7:36)
INDIAN INSTITUTE OF TECHNOLOGY
ROPAR
XEVO G2-XS QTOF
1: TOF MS ES+
3.56e6
241.0010
137.0170
145.0034
176.0238
301.1531
308.9859
319.0151
362.2569
413.2788
475.3351
507.2752
508.2806
523.2567
585.2998
685.4309
741.4609
763.4392
764.4448
881.4774
925.5034
%
m/z

SPECTRAL ANALYSIS OF NP-7C


---- PROCESSING PARAMETERS ----
dc_balance : 2 : FALSE
sexp : 0.2[Hz] : 0.0[s]
fft : 1 : TRUE : TRUE
machinephase
ppm
phase : -1.5 : -2 : 94.52922[%]
phase : 0 : 0 : 94.52922[%]
Derived from: NP-7C_CU_PB_PROTON-1.jdf
Filename = NP-7C_CU_PB_PROTON-6.
Author = delta
Experiment = single_pulse.ex2
Sample_id = NP-7C_CU_PB
Solvent = CHLOROFORM-D
Creation_time = 5-FEB-2018 20:04:41
Revision_time = 6-FEB-2018 14:25:51
Current_time = 6-FEB-2018 14:25:59
Comment = NP-7C_CU_PB
Data_format = 1D COMPLEX
Dim_size = 13107
Dim_title = 1H
Dim_units = [ppm]
Dimensions = X
Site = NCS 400
Spectrometer = JNM-NCS400
Field_strength = 9.389766[T] (400[MHz])
X_acq_duration = 1.6384[s]
X_domain = 1H
X_freq = 399.78219838[MHz]
X_offset = 9.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.61035156[Hz]
X_sweep = 10[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Tri_domain = 1H
Tri_freq = 399.78219838[MHz]
Tri_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 32
Total_scans = 32
X_90_width = 10.4214[us]
X_acq_time = 1.6384[s]
X_angle = 45[deg]
X_atn = 0.4[dB]
X_pulse = 5.2107[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Recvr_gain = 20
Relaxation_delay = 2[s]
Repetition_time = 3.6384[s]
Temp_get = 19.1[dC]
X : parts per Million : 1H


---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp : 2.0[Hz] : 0.0[s]
trapezoid3 : 0[%] : 80[%] : 100[%]
zerofill : 1
fft : 1 : TRUE : TRUE
machinephase
ppm
Derived from: NP-7C C13-1.jdf
Filename = NP-7C C13-5.jdf
Author = delta
Experiment = single_pulse_dec
Sample_id = NP-7C C13
Solvent = CHLOROFORM-D
Creation_time = 10-FEB-2018 12:11:24
Revision_time = 10-FEB-2018 14:19:09
Current_time = 10-FEB-2018 14:19:20
Comment = single pulse decouple
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = NCS 400
Spectrometer = JNM-NCS400
Field_strength = 9.389766[T] (400[MHz])
X_acq_duration = 1.04333312[s]
X_domain = 13C
X_freq = 100.52530333[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.95846665[Hz]
X_sweep = 31.40703518[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 1000
Total_scans = 1000
X_90_width = 9.5[us]
X_acq_time = 1.04333312[s]
X_angle = 90[deg]
X_atn = 4.2[dB]
X_pulse = 9.5[us]
Irr_atn_dec = 21.399[dB]
Irr_atn_noe = 21.399[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recvr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 3.04333312[s]
Temp_get = 18.4[dC]
X : parts per Million : 13C


Sample Name : NP-7-C
Test Name : HRMS-1
INDIAN INSTITUTE OF TECHNOLOGY
ROPAR
XEVO G2-XS QTOF
200218-NP-7-C 15 (0.157) AM2 (Ar,13000.0,0.00,0.00); Cm (15:20)
1: TOF MS ES+
2.39e6
100
%
0
557.2867
353.1088
415.1217
489.2278
293.0899
259.1793
558.2904
362.3508
241.0009
137.0171
490.2318
416.1259
145.0042
484.2738
491.2406
559.2986
441.1818
635.3036
661.3606
745.2228
807.2314
881.3362
949.3914
100
150
200
250
300
350
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450
500
550
600
650
700
750
800
850
900
950
1000
m/z

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3

CHAPTER-1 Introduction

1.1 INTRODUCTION Natural products have been practiced since prehistoric times for the treatment of many human infectious diseases and illnesses. Ayurveda, traditional Chinese medicine (TCM), Kampo, traditional Korean medicine (TKM), and Unani systems are utilizing the natural products all over the world for hundreds or even thousands of years. Most of the currently available drugs for the treatment of various human and animal diseases are obtained of developed (through getting lead from natural sources) from natural products especially medicinal plants (Mishra and Tiwari, 2011). Such medications have been discovered after detecting the medicinal utilization of a specific plant or its parts (leaves, roots, barks, fruits or seed or whole plant) by botanists, and subsequent isolation of bioactive compounds from the plant or part of the plant that was utilized generally for treatment of various human sicknesses. Natural products still continue to provide exceptional structural diversity in comparison to standard combinatorial chemistry, which presents opportunities for discovering novel lead compounds (Dias et al., 2012).

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Cancer is a vital universal health problem generally due to the lack of extensive early detection methods (

Divisi et al., 2006). Thus,

0: Hemand SBS thesis 2016.docx 84%

the struggle to combat cancer is one of the greatest challenges of mankind.

In spite of modern techniques and therapeutics, we are unable to combat cancer and alternatives therapies are needed (Ohlyan et al., 2013). From last 40 years, small organic molecules derived naturally from microbes and plants have provided a number of useful cancer chemotherapeutic drugs. The search of lead compounds from natural sources has continued in recent years, with the constituents from plants and microorganisms being investigated for their anti-cancer activities. Some antitumor agents widely used throughout the world are plant-derived compounds, including, the camptothecins, Vinca alkaloids, the epipodophyllotoxins, and taxol derivatives (Kinghorn et al., 2009). Piper plants (Piperaceae) constitute one major class of medicinal plants commonly used as a food in almost all Indian and African regions. These are also a potential source of some Piper based drugs used in traditional medicines. Piper attenuatum (Buch-Ham) (fruit) is another species of Piper which has a significant pharmacological profile, though, much data is not reported on the anti-cancer properties of P. attenuatum (Buch-Ham). We herein report the antiproliferative activity of chloroform and methanol extracts P. attenuatum (Buch-Ham) against MDA-MB-231 (breast cancer) cell lines. Further, the cytotoxic potential of extracts and their isolated major constituents were compared.

CHAPTER-2 Review of literature

2.1 Natural Products as source of anticancer medicines Practices of natural product chemistry empowered the enormous bioactive secondary metabolites from terrestrial sources to be discovered (Cragg and Newman, 2013; Dias et al., 2012). Many of these natural products have gone on to become current drug candidates. Some antitumor agents widely used throughout the world are plant-derived compounds, including, the camptothecins, Vinca alkaloids, the epipodophyllotoxins, and taxol derivatives (Kinghorn et al., 2009). Among the anticancer drugs approved in 19th - 20th century approximately 54% were isolated from natural products or drugs inspired from knowledge related to natural products (Newman and Cragg, 2007). For instance, the Vinca alkaloids from Catharanthus roseus, and paclitaxel from Taxus baccata, are among successful anticancer drugs originally derived from plants (Yuan et al., 2016). Introduction and advancement of a few new and highly specific in vitro bioassay procedures, chromatographic strategies, spectroscopic systems and other institutionalized pharmacological techniques have additionally made it significantly less demanding to screen, isolate and identify potential medication compounds rapidly and accurately from natural sources to ease human ailments (Keifer, 2000). In spite of the fact that characteristic items (e.g., medicinal plants) have numerous therapeutic uses, there are several reasons that require isolation and characterization of bioactive compounds from them. Some of the reasons are (i) distribution of medicinal plants is not uniform throughout the world to be utilized by individual everywhere; (ii) the greater part of the therapeutic plants are under risk of extinction because of atmospheric changes and population pressure (Brower, 2008): (iii) Separation and purification of compounds from natural sources is tedious, costly and time consuming process (Bhandari et al., 2011); and (iv) the need to identify the chemical compounds that are responsible for the observed medicinal value of the plant (Altemimi et al., 2017). In addition, the amount of bioactive compounds isolated from medicinal plants are very small (Li and Vederas, 2009). All these facts require isolation and characterization of bioactive compounds from therapeutic plants.

2.2 Piper genus 2.2.1 History of Piper Piper species had been used in medicinal systems from ancient time, including Indian as well as Chinese systems, and in medicines of of Latin America and West Indies described in folk tales (Kirtikar and Basu, 1993). Cookery use of pepper plants is proven as early as 9,000 years ago. The remains of peppercorn were found among the food refuse left by Hoabinhian artisans at Spirit Cave, Thailand. Still, there are not sufficient proof that these plants of piper were purposely grown in spite of collection from forest (Gorman, 1971; Gorman, 1969). Peppercorns are used as pungent spice significantly on an international scale. In ancient times, the vital trade of spices including black pepper (P. nigrum) from South Asia to Europe was done. The Apicius (a recipe collection book), mentions "pepper" as a spice for most main dishes. Other than the use of the seeds of Piper in cooking as spices, West African Pepper leaves, known locally as uziza, are used as flavouring vegetable in Nigerian stews. Mexican pepper leaf (P. auritum) are used to give flavour too. In Southeast Asia, leaves of two species of Piper: lolot (P. lolot), used to wrap meat for grilling and wild betel (P. sarmentosum), used raw or cooked as a vegetable have major importance in cooking (Solomon and Solomon, 2010). A few Piper animal varieties from India, Southeast Asia and Africa are of high business, restorative and monetary significance since they are utilized as flavors. 2.2.2 Distribution The Piper genus is pantropical and has about 1000-2000 species in the World and 93 of them exist in Costa Rica Only (Fleming, 1983). Piper plants are generally

found in humid and forested areas within the underwood of rainforests. The greatest diversity of Piper species is found in the Neotropics, where about two-thirds of the mentioned species are found. Around 300 species are endemic to Southeast Asia, including the East Indian islands and northern Australia. Only two species are native to Africa (Marquis, 2004). In India, Kerala State produces the 97% of India's total output in black pepper and is known as the land of Black pepper. There are about 16 species of pepper exist only in Kerala (Parthasarathy et al., 2006). Other states include Tamilnadu, Karnataka, Andhra Pradesh and South region of India. Piper is found from sea level to at least 2,000 meters in elevation. Most pipers are terrestrial, existing as small herbs, small trees, shrubs of about 2-3 meters high, and some as lianas (Fleming, 1983). The most generally perceived types of the class Piper will be Piper nigrum followed by Piper longum, Piper caninum, Piper mullesua, Piper lolot, Piper argyrophyllum, Piper attenuatum, Piper umbellatum, Piper colubrinum and Piper chaba. Some images of few Piper species are showed in Fig 2.1.

(Ghosh et al., 2014) They have been known as oriental therapeutic plants and reported to possess different pharmacological activities like, anti-inflammatory, anti-bacterial, anti-hypertensive, antiplatelet, hepatoprotective, anti-thyroid, insecticidal, and anti-tumor (Govindarajan and Stahl, 1977). Their fruits, leaves and other plant parts are the ingredients of numerous formulations utilized in the Indian traditional system of medicines (Parmar et al., 1997). Piperine is the primary amide to be separated from the plants of Piper species followed by Piperlonguminine and Piperlongumine, as the significant alkaloids (Bezerra et al., 2013; Wu et al., 2004). 2.2.3 Various Piper species and isolated compounds From last five decades, various reports on phytochemistry of Piper genus led to identification of various pharmacologically important scaffolds which have different structural skeleton (Reddy et al., 2015). It encouraged the discovery of various novel synthetic methodologies for synthesis of these novel scaffolds and further inspired many biochemists to determine reasonable biogenetic relationships among these diverse skeletal (Parmar et al., 1997; Parmar et al., 1998). Some of the piper species and isolated compounds from them are reported in Table 2.1

2.3 Anticancer potential Piper species Extracts from Piper species are also found to have potential cytotoxic activity. Wang et al. reported the cytotoxic potential of extracts of 24 species of Piper genus (Wang et al., 2014). In literature, number of piper species had been documented which have anticancer potential. Some of them are described in Table 2.2.

Piper aduncum L. is conventionally used to cure dermatological conditions like rashes, skin allergies and skin tumors in Mexico (Alonso-Castro et al., 2011). Piperaduncin A (1) is isolated from this plant. It is a dihydrochalcone compound which exhibited growth inhibitory activity against human nasopharynx carcinoma (KB) cells with IC50 value of 2.3 µg/ml (Orjala et al., 1994). On other hand dichloromethane extracts of P. aduncum leaf were not found potential cytotoxic to various cell lines (Calderón et al., 2006). P. boehmeriifolium Wall and Piper sylvaticum Roxb. (roots) are reported to have laxative, and carminative properties. These are also reported in the Ayurvedic system of Indian medicine to have beneficial effects against diseases of the spleen, and tumors (Mahanta et al., 1974). 1-[(9E)-10-(3,4-methylenedioxyphenyl)- 9-decenoyl] pyrrolidine (2), a cytotoxic amide alkaloid isolated from the plant of P. boehmeriifolium was reported to have an IC50 of 2.7 µg/ml against human

cervix adenocarcinoma (HeLa) cells (Tang et al., 2010), while piplartine (an amide alkaloid) (3) (Fig. 2.2) is responsible for the anticancer potential of P. sylvaticum (Bezerra et al., 2013). Piper capense L.f. is also reported for the treatment of cancer in Cameroon (Kuete et al., 2013). The seeds of Piper capense were subjected to extraction and methanolic extracts was found to have cytotoxic potential against many tumor cell lines and Piperine (4) might be an active constituent responsible for its cytotoxic potential (Kuete et al., 2011; Umadevi et al., 2013). Piper cubeba was found one of the most important plant against cancer in in vitro anticancer evaluation in Moroccan traditional medicine (Daoudi et al., 2013). (-)-Cubebin, a lignin is found major constituent of P. cubeba. Cubebin is have been reported to have significant therapeutic potential against halting the growth of prostate cancer by targeting number of features of the androgen-signaling pathway (Usia et al., 2005; Yam et al., 2008). Nigerian plant species of piper known as Piper guineense Schum and Thonn are reported to have anticancer properties (Soladoye et al., 2010). However, the active constituents of these species are still unknown but methanolic extract obtained from the seeds was found to have cytotoxic properties against leukemia CEM/ADR5000 cells with IC50 value of 8.20 µg/ml) in a study performed at Morocco (Kuete et al., 2011). Piper longum L. is the most well-known species of piper genus and medicinal plant. In ancient medical practice, it is reported that 12 leaves of Piper longum and 12 leaves of Thespesia populnea (L.) with little amount of water were crushed in a wooden bowl and the chest of a person with supposed breast cancer is washed with this solution (Holdsworth, 1991). P. longum is also reported in Indian Ayurveda to treat tumors. The major active principles reported of this plant are Piplartine, cepharadione A (5), and piperolactams A (6) and piperolactams B (7) (Kim et al., 2011). Piper nigrum L. (black pepper), is applied to abdominal tumors in various fomulations for releief. Black pepper is also used in number of formulations for treatment of various respiratory and gastric cancers in China (Xin et al., 2009). 2.4 Piper attenuatum Indian medicinal plant Piper attenuatum (Buch-Ham) has a place with the family Piperaceae. It is found to exist in Vishakhapatnam of Andhra Pradesh, Madurai and Tirunnelveli of Tamil Nadu. It is a substitute for black pepper (P. nigrum). Distinctive parts of 2.4.1 Scientific name: Piper attenuatum Buch-Ham. Ex Miq. (Hassler, 2000; Sasikumar et al., 1999) 2.4.2 Synonyms: Piper malamiris Roxb. (Synonym) Piper Karok Blume (synonym) Chavica diffusa (Vahl) Miq. (Synonym) Piper diffusum Vahl (synonym) 2.4.3 Common name: Flat-branched pepper, oval-leaved pepper plant Malayalam: Kattumulaka Tamil: arenukam, kattu-milaku (Piper attenuatum Buch.-Ham. ex Miq., Syst. Piperac. 306 1843.) 2.4.4Taxonomic classification

2.4.5 Distribution Java, peninsular Malaysia, New Guinea (alpine), India, China (Yunnan), Bhutan, Sikkim and Myanmar [Burma] (Kachin, Mandalay, Sagaing, Yangon) (Sasikumar et al., 1999).

2.4.6 Macroscopy character (Sasikumar et al., 1999). Colour : Grayish black Odour : No characteristic odor Taste : Pungent Shape : Globular dry fruits with few striations Size : The average diameter of dry fruits is 4-6 mm

(Ohlyan et al., 2014)

2.4.7 Microscopic characterization Shrubby root-climber, up to 2.5 m long. Stem and branches hefty, however delicate, end up compacted notched when dry, greenish-yellow, glabrous, bring down internodes 8.0 – 8.5 cm, upper 2.0 – 3.5 cm long; stipules 0.5 – 0.65 cm, subulate. Lamina comprehensively cordate; bring down 8.0 – 9.5 x 7.0 – 10 cm, somewhat more extensive than length, orbicular-ovate; upper 4.5-7.0 x 2.5 – 5.0 cm, whole, pellucid, intense to taper with a mucro, base cordate-truncate, membranous, relatively glabrous above, hairy on veins, nerves for the most part 7, sometimes 7 – 9 from base, laterals disparate aside from center; petioles 2.5 – 6.5 cm long, angled, greenish-yellow, hairy upwards; plants dioecious; male spike c 0.03 cm long, clustered; Stamens 3, sessile, ;adnate to elongated basifixed bracts (1.0 – 0.2 cm), winged, decurrent (4.0 – 5.0 cm long) rachis; female spikes (up to 10 cm long) with adnate bracts bearing its end on ovoid ovary (0.05 cm long); stigma obscurely 4; drupes 0.2 x 0.15 cm, globose, loosely aggregated, glabrous, sessile, 0.35 – 0.4 cm across, black (Brach and Song, 2006). 2.4.8 Plant habitat Flowering & Fruiting done in the month of August to March 2.4.9 Phytochemical investigations Phytochemical studies have demonstrated that the plant contains pipoxide chlorohydrins in a major amount (Joshi et al., 1979). (−)-galbelgin (Stevenson and Williams, 1977), aliphatic liquor and 8-hentriacontanol have also been isolated from the leaves of P.attenuatum (Sumathykutty and Rao, 1991). There are number of aristolactams have been reported from the aerial parts of the plant which include cepharanone B, piperolactamA, piperolactam D, and cepharadione A (Desai et al., 1990), (Kumar et al., 2003). Roots have been testified to contain alkamides including piperine, Piperlonguminine and guineensine (Parmar et al., 1998). The petroleum extract of P.attenuatum have been reported to contain a novel chain alcohol, 14-Benzo [1, 3] dioxol-5-yl-tetradecan-2-ol, beta-Sitosterol, Kadsurin A, Kadsurin B and (+)-Crotepoxide (Parmar et al., 1998). Leaf oil of P.attenuatum contain beta-Caryophyllene and beta cubebene (Sumathykutty and Rao, 1990).

2.5 Pharmacological activity of Piper attenuatum 2.5.1 Antibacterial activity Samy et al. assessed antibacterial activity of dry fruits of Piper attenuatum (Buch-Ham) in which they prepared three distinct extracts by utilizing ethyl acetate, ethanol and Methanol. Each of the three extracts were screened for their antibacterial activity against S.aureus, E.coli and P. aeroginosa by agar diffusion method in which they found that Methanol extract showed comparatively good inhibition at higher doses at 200 and 500 µg against all organisms (Samy et al., 1998). Ethanol extract additionally demonstrated some restraint against E. coli at higher doses (200 and 500 µg).

2.5.2 Antioxidant activity Auddy et al. explored antioxidant and anticancer potential of Piper attenuatum (Buch-Ham). Total three extracts of Piper attenuatum (Buch-Ham) were prepared by utilizing solvents ethyl acetate, ethanol and methanol. Further, in vitro antioxidant potential was evaluated using ABTS free radical scavenging method (Auddy et al., 2003), compared to the standard (Gallic acid) and they found that Methanol extract demonstrated greatest inhibitory impact with IC50 of 13.17µg which is around six-folds than that of Gallic acid (2.16µg). Both ethanol and ethyl acetate extract also showed inhibition of free radicals in concentration dependent manner with IC50 of 20.35µg and 68.06µg, respectively.

2.5.3 Antihyperglycemic activity Reddy et al. examined the antihyperglycemic and free radicals scavenging constituents present in the fruit part of Piper attenuatum. Extracts having strong free radical scavenging activity were further selected for isolation of major constituent present. Chloroform extract of P. attenuatum was found to have strong radical scavenging potential was further assessed for antihyperglycemic action. Nine neolignans specifically, denudatin B (8), iso-4', 5'-dimethoxy-3, 4-methylenedioxy-2'-oxo-Δ'-8.1'-lignan (9), lancifolin D (10), denudatin A (11), wallichinin (12), piperenone (13), lancifolin C (14), 2-oxo-piperol B (15), piperkadsin A (16) and a crotepoxide (16) (Fig 2.5) was recognized present in chloroform extract. From evaluation of free radical scavenging potential, it was found that all the neo-lignans present in chloroform extract have ABTS+ radical scavenging activity, however just 16 showed the DPPH· scavenging activity. The SAR studies revealed that the presence of methoxyl groups in the rings primarily influence ABTS+ radical scavenging potential of the compounds as absence of methoxyl groups lead to the decreased DPPH activity (Reddy et al., 2015).

CHAPTER-3 Rationale

4.1 Rationale From review of literature it has been confirmed that Piper plants are important sources for the development of new anticancer agents. Piper plants include about 2000

species, out

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of which 10 have been used as

traditional medicines to treat cancer or cancer-like symptoms while, 35 extracts from 24 Piper species and

number of compounds from Piper plants

were found to have in vitro cytotoxic activity. Piper attenuatum (Buch-Ham) is one of piper species having significant pharmacological potential like antibacterial, anti-oxidant and anti-inflammatory effects but not much data is available on anti-cancer potential except few reports on crotepoxide (Kupchan et al., 1969) and extracts on MCF-7 cell lines (Ohlyan et al., 2013). Thus, further in vitro and in vivo anticancer research studies on Piper attenuatum (Buch-Ham) and its isolates are advisable for getting leads or development of potential ligands for treatment of cancer. The novel molecules isolated from Piper attenuatum (Buch-Ham) may enhance the chances of getting potential leads for development of anticancer drug.

CHAPTER-4 Aim and objectives

3.1 Objectives The major objectives of present study designed are: • Preparations of chloroform and methanol extracts and their phytochemical investigations. • Isolation and characterization of major chemical constituents of chloroform and methanol extracts of Piper

attenuatum (Buch-Ham) (fruit). • In-vitro antiproliferative activity of different extracts and isolated constituents of Piper attenuatum (Buch-Ham) against available cancel cell-lines.

CHAPTER-5 Material and METHODS

5.1 Materials and methods 5.1.1 Plant material (procurement) Dried fruits of Piper attenuatum nearly 1.1 kg were collected from Berikonda Chittoor district of Andhra Pradesh (12°37′ - 14°8′ north latitudes and 78°3′ - 79°55′ east longitudes). The authentication of sample was done by Dr. K. Madhava Chetty, Assistant Professor at Department of Botany, Sri Venkateswara University, Tirupati (Authentication certificate attached in Annexure-I).

5.1.4 Cell lines 1. For anticancer evaluation of extracts and purified compounds MDA-MB-231 cell lines were used.

5.2 Extraction of plant material 1.1 kg of fruit parts of Piper attenuatum were collected, dried in shade and grounded to get fine powder for extraction. 400 gm of fine powder of fruit parts of Piper attenuatum was further used for extraction. Extraction was done using solvents petroleum ether, chloroform, ethyl acetate and methanol in increasing order of polarity. Plant material was macerated three times with 1500 mL each. Extracted fractions were dried and weighed. The detailed procedure is described in flow chart below (Fig 5.2): 5.5 Characterization of isolated compounds The characterizations of isolated compounds were done using NMR, and MASS spectroscopy. 5.6 In-vitro anticancer assay 5.6.1 Cell lines under study For antiproliferative evaluation of extracts and isolated compounds MDA-MB-231 breast cancer cell line were used. MDA-MB 231 is a human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma. MDA-MB-231 is a highly aggressive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification. 5.6.2 Culturing of cell lines DMEM is used as a medium for culturing of the cancer cell lines as it is adherent cells, trypsin was added to remove them from the surface (trypsinization). Cells were harvested in 5 mL media containing serum which inactivates trypsin enzyme. Harvested cells were centrifuged at 1200 rpm at 4°C for 5 minutes and supernatant was removed and pellet was resuspended in media (2 mL). With automated cell counter, cell number was counted. The cells were moved to fresh media every two days. 5.6.3 Maintenance and sub-culturing of cell lines The maintenance and culturing of cell lines was done in 25 cm2 or 75 cm2 flasks having DMEM medium supplemented with 10% fetal bovine serum (FBS), 1X antibiotic solution and incubated at 37° C in a humidified atmosphere containing 5% CO2. Further sub-culturing of cells was done in 25 cm2 flasks up to when the cancer cell lines have reached 70-80% of growth. The reagents vital for the procedure were placed in water bath maintained at 37° C for 10-15 minutes and trypsin was added for the detachment of adherent cells. The 1 mL of media containing serum was added after 5 minutes for stopping the action of trypsin. Then, the cells were transferred to 15 ml centrifuged tubes and centrifuged for 5 min at 1200 rpm at 4° C. The supernatant was removed and the cell pellet was again re-suspended in complete media. The cell lines were transferred to fresh media every two days (cell passaging). 5.6.4 MTT assay MTT (3-(4, 5-Dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was

performed to evaluate the anti-proliferative activity of extracts and isolated compounds. MTT is a colorimetric assay used for the measurement of cell proliferation. The tetrazolium yellow compound MTT is reduced to an insoluble purple coloured formazan product by mitochondrial reductase or succinate dehydrogenase in metabolically active cells only. When formazan passes to the mitochondria it gets solubilized with DMSO and measured spectrophotometrically. To carry out the MTT assay, cell lines MDA-MB-231 (8,000–10,000 cells) were seeded in each well of the 96 well plates. The plate was incubated at 370C with 5% CO2 for 24 h followed by serum starvation. The treatment was given to the cells in triplicate concentrations of 1 µM, 5 µM and 25 µM and incubated for 48 h. MTT solution (5 mg/10 mL) was added after removing the media from each well and incubated in the dark for 4 h. At the end of 4 h, the MTT solution was removed from each well and the intracellular precipitate was dissolved in DMSO solution and the absorbance of the violet color formed as consequence of DMSO addition, is read spectrometrically at 570 nm and expressed as % inhibition (Mean ± S.D).

CHAPTER-6 Results and discussions

6.1 Result and discussion Extensive phytochemical evaluation of dry fruits of P. attenuatum (Buch-Ham) was performed. We also performed the evaluation of antiproliferative potential of chloroform and methanol extracts along with major isolated compounds of dry fruit of P. attenuatum (Buch-Ham) using MTT assay.

6.1.1 Phytochemical screening Preliminary phytochemical analysis was carried out for the extract as per standard method. The results of various testes performed for various phytoconstituent are listed below in Table 6.1: Table 6.1: Results of phytochemical tests performed Constituent Test Methanol Chloroform Ethyl acetate Petroleum Ether Alkaloids Mayer’s test

Hager’s test ++

++ ++

++ ++

++ ++

++ Carbohydrate Molisch test ++ ++ ++ -- reducing sugars

Benedict test

Fehling test --

-- --

-- ++

++ --

-- Saponins Froth test -- -- -- -- Phytosterols Salkowski test

Lieberman Burchard test --

-- ++

++ ++

++ --

-- Oils and Resins

Spot test ++ ++ ++ ++ Tannins Ferric chloride test -- -- ++ -- ++ Positive test, -- Negative test 6.1.2 Antiproliferative evaluation of extracts All extracts obtained from extraction procedure on Piper attenuatum (Buch-Ham) were evaluated for their antiproliferative potential using MTT assay gainst MDA-MB-231 (breast cancer) cell lines. Methanol extract (MCE) and chloroform extract (CEE) were found to have potential antiproliferative properties. MCE and CEE extracts were found to reduce the cell viability to 50% at 10 µg/mL and 50 µg/mL concentrations respectively. It was found comparable to the results obtained from colchicine which reduced the cell viability to 50% at 50 µg/mL concentration (Fig 6.1). Thus, from these results it can be concluded that chloroform and methanol extracts of Piper attenuatum (Buch-Ham) contains the potential antiproliferative compounds.

6.1.3 Isolation of major constituents of chloroform and methanol extracts using column chromatography Column chromatography technique was used for the isolation of major constituents of chloroform and methanol extracts. Results from antiproliferative evaluation, phytochemical tests and TLC indicated that chloroform extract (CCE) and methanol extract (MCE) have large number of compounds. CEE and MCE were chosen for further isolation and subjected to column chromatography on a silica gel column (60-120 mesh, 60x3 cm) and eluted with a stepwise gradient of pet ether/ EtOAc, (99:1, 98:2, 97:3,96:4, 95:5, 93:7,90:10 by volume) to afford a total of 160 fractions of 20 mL each. Column fractions were analyzed by TLC (pet ether /EtOAc, 85:15), and fraction with similar TLC patterns were combined to give three major fractions (NP1-NP3). NP1 fraction was further purified by column chromatography using solvent pet ether /EtOAc, 97:3 to give NPM-1 (10 mg). Fraction NP2 was subjected to column chromatography by using solvent pet ether /EtOAc, 95:5 to give NP-7L (20 mg) and fraction NP3 was purified using solvent pet ether /EtOAc, 90:10 to give NP-7C (25 mg). Different compounds isolated from CEE and MCE extracts are listed below in Table 6.2.

6.1.4 Characterization of compounds Three compounds isolated from CCE and MCE extracts were characterized using various spectral techniques like NMR, IR and Mass spectroscopy. The results of spectral analysis are listed below in Table 6.3

6.1.5 Antiproliferative activity of isolated compounds Three isolated compounds were further subjected to MTT assay to evaluate their antiproliferative potential (Fig 6.2) against MDA-MB-231 (breast cancer) cell lines. Compound NP7C was found most potent antiproliferative agent with IC50 value of 3.83 µM which is comparable to etoposide 2.37 µM. Compound NP7L was also found potent antiproliferative agent with IC50 value of 6.44 µM which was

comparable with colchicine (IC50 = 6.3 μM). So, from this study, it was found that isolated compounds of Piper attenuatum are potential antiproliferative agents.

CHAPTER-7 Summary and Conclusion

Ayurveda, traditional Chinese medicine (TCM), Kampo, traditional Korean medicine edicine (TKM), and Unani systems are utilizing the natural products from hundreds or even thousands of years. Currently most of the anticancer drugs used are obtained or derived from natural sources. Piper species also had been used in traditional medicinal systems from thousands of years, including Indian and Chinese systems, as well as in folklore medicines of Latin America and West Indies. Indian medicinal plant Piper attenuatum (Buch-Ham), a substitute for black pepper (P. nigrum), is one important species of Piper which has extensive pharmacological profile and had been utilized as herbal medicine for the treatment of muscular pain, headache and have been utilized as a rubefacient. The wood from plant has been used to treat throat pain. We collected the dried fruits of Piper attenuatum (Buch-Ham) from Berikonda Chittoor district of Andhra Pradesh (12°37′ - 14°8′ north latitudes and 78°3′ - 79°55′ east longitudes) and authentication of sample was done by Dr. K. Madhava Chetty, Assistant Professor at Sri Venkateswara University, Tirupati. Extraction of the fine powder of dried fruits of Piper attenuatum (Buch-Ham) was done using solvents petroleum ether, chloroform, ethyl acetate and methanol in increasing order of polarity. Extracted fractions were dried and were evaluated for various phytochemicals present. Results from antiproliferative evaluation, phytochemical tests and TLC of extracts indicated that chloroform (CEE) and Methanol (MCE) extract have large number of compounds. Thus, further isolation of various compounds from CEE and MCE was done using column chromatography. From this extract three compounds (NP1-3) were isolated. Spectral analysis of isolated compounds was performed using NMR and HRMS analysis. Structural characterization of isolated compounds from 2D-NMR will further help in determining the structure of compounds. The results from MTT assay of isolated compounds, NP7C was found to be the most potent antiproliferative agent with IC50 value of 3.83 μM which is comparable to etoposide 2.37 μM. Compound NP7L also exhibit significant antiproliferative activity (IC50 of 6.44 μM) which was comparable to colchicine (IC50 = 6.3 μM). Thus, the present study indicated that isolated compounds of Piper attenuatum (Buch-Ham) possess great potential to be developed as anticancer agent in future.

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of which 10 have been used as traditional medicines to treat cancer or cancer-like symptoms while, 35 extracts from 24 Piper species and number of compounds from Piper plants	of which 10 species have been used in traditional medicines to treat cancer or cancer-like symptoms. Studies have shown that 35 extracts from 24 Piper species and 32 compounds from Piper plants