Integrated *in-vitro* antioxidant and *in-silico* anti-apoptotic study of essential oil components of *Aconitum heterophyllum* Wall.

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BY

Yashika Bhalla

Administrative Guide: Prof. P. Ramarao

Dissertation Coordinator: Dr. Vikas Jaitak

Centre for Chemical and Pharmaceutical Sciences

School of Basic and Applied Sciences

Central University of Punjab, Bathinda

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CERTIFICATE

I declare that the dissertation entitled "**Integrated *in-vitro* antioxidant and *in-silico* anti-apoptotic study of essential oil components of *Aconitum heterophyllum* Wall.**" has been prepared by me under the guidance of Dr. Vikas Jaitak, Assistant Professor, Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

Yashika Bhalla

Centre for Chemical and Pharmaceutical Sciences

School of Basic and Applied Sciences,

Central University of Punjab,

Bathinda – 151001.

Date:

CERTIFICATE

I certify that Yashika Bhalla has prepared her dissertationentitled **"Integrated *in-vitro* antioxidant and *in-silico* anti-apoptotic study of essential oil components of *Aconitum heterophyllum* Wall. "**, for the award of M.Pharmacy degree of the Central University of Punjab, under our guidance. She has carried out this work at the Centre for Chemical and Pharmaceutical Sciences, School of Basic and Applied Sciences, Central University of Punjab.

(Dr. Vikas Jaitak)

DissertationCoordinator

Assistant Professor

Centre for Chemical and Pharmaceutical Sciences,

School of Basic and Applied Sciences,

Central University of Punjab,

Bathinda – 151001

Date:

(Prof. P. Ramarao)

Administrative Guide

Dean, COC,

Centre for Chemical and Pharmaceutical Sciences,

School of Basic and Applied Sciences,

Central University of Punjab,

Bathinda – 151001

Date:

ABSTRACT

"Integrated *in-vitro* antioxidant and *in-silico* anti-apoptotic study of essential oil components of *Aconitum heterophyllum* Wall."

Name of student	:	Yashika Bhalla
Registration Number	:	CUP/MPhil-PhD/SBAS/CPS/2011-2012/09
Degree for which submitted	:	Master of Pharmacy
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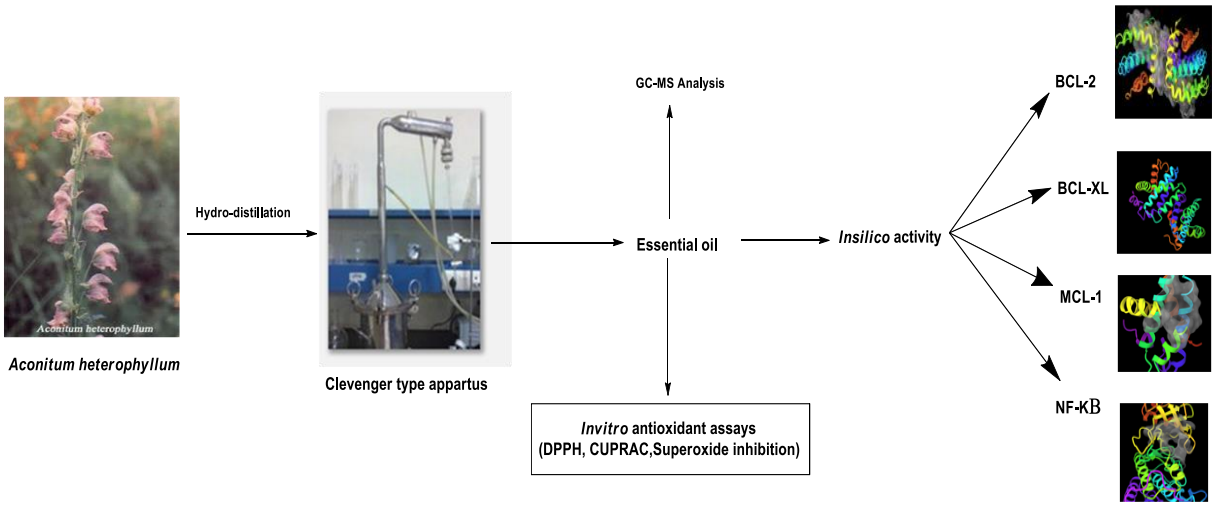
ABSTRACT



Aconitum heterophyllum Wall. is consumed for its promising medicinal properties in several parts of the world. Present study consists of hydrodistillation, antioxidant potential and *in-silico* antiapoptotic study of *A. heterophyllum* oil. Antioxidant activities were evaluated by *in-vitro* assays namely DPPH, Superoxide anion scavenging and CUPRAC. It was found that the anti-oxidative effect of *A. heterophyllum* oil was dose dependent up to 200 µg/ml. For studying the apoptotic nature of the volatile constituents, *in silico* studies were carried out using BCL-2 anti-apoptotic receptors (BCL-2, BCL-XL, MCL-1). To understand the cascade of mechanisms leading to apoptosis, NF-κB was also considered. From the comparative study of the constituents

with that of the standard inhibitor it has been observed that the constituents show favorable binding affinity for the receptors as in the case of BCL-2 receptor, α--longipinene has a dock score of -4.26 kcal/mol as comparable to that of standard inhibitor ABT 263 (-4.67 kcal/mol); BCL-XL receptor, neryl acetate has a dock score of -4.05 kcal/mol as compared to ABT 737 (standard inhibitor) which was -9.47 kcal/mol. Best results were observed in the case of NF-κB with β-fenchol, having the dock score of -4.36 kcal/ mol which shows higher binding affinity of the β-fenchol molecule for the receptor site as compared to the selective inhibitors parthenolide whose dock score was -3.04 kcal/ mol. In summary, based on our *in silico* and *in vitro* results, it can be postulated that essential oil of *A.heterophyllum* could be used as functional antiapoptotic inhibitor and as natural antioxidant.

(Yashika Bhalla) **(Dr. Vikas Jaitak)** **(Prof. P. Ramarao)**

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(Yashika Bhalla)

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LIST OF ABBREVIATIONS

S.No	Full form	Abbreviation
1.	Essential oil	EO
2.	Gas chromatography-Mass spectroscopy	GCMS
3.	Nuclear Magnetic Resonance	NMR
4.	B cell Lymphoma	BCL-2
5.	B cell Lymphoma extra large	BCL-XL
6.	Myeloid cell leukemia sequence 1	MCL-1
7.	Nuclear factor kappa beta	NF KAPPA B
8.	Reactive oxygen species	ROS
9.	IkappaB kinase beta	IKK BETA
10.	TNF receptor associated factor	TRAF 6
11.	Tumour necrotic factor	TNF
12.	Natriuretic polypeptide b	NPPB
13.	Action potential	AP
14.	Protein kinase C-alpha	PKCα
15.	High pressure liquid chromatography	HPLC
16.	Electron impact	EI
17.	Retention factor	Rf
18.	Thin layer chromatography	TLC
19.	Ultra violet visible	UV-Vis
20.	2, 2diphenyl-1- picryl hydrazyl	DPPH
21.	Cupric ion reducing antioxidant assay	CUPRAC
22.	Premenstrual syndrome	PMS
23.	Nicotinamide adenine dinucleotide	NADH
24.	Root-mean-square deviation	RMSD
25.	Leucine	LEU
26.	Valine	VAL
27.	Phenylalanine	PHE
28.	Methionine	MET
29.	Tyrosine	TYR
30.	Alanine	ALA
31.	Aspartine	ASP
32.	Glutamate	GLU
33.	Tryptophan	TRP
34.	Arginine	ARG
35.	Research Collaboratory for Structural Bioinformatics	RCSB
36.	Isopentenyl diphosphate	IPP
37.	Farnesyl diphosphate	FPP
38.	3-hydroxy-3-methylglutaryl coenzyme A	HMG-CoA
39.	Dimethylallyl diphosphate	DMAPP
40.	Geranylgeranyldiphosphate	GGPP
41.	Geranyl diphosphate	GPP
42.	3-hydroxy-3-methylglutaryl coenzyme A	HMGCoA

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CHAPTER ONE

1.0 INTRODUCTION

Nature has blessed the world with its endless treasures leading to basic comforts of human life. It has provided multiple parameters of beautification and attraction. The most common and popular is the nature's sense of smell which one realizes in many ways. The blooming gardens, growing crops and running creatures, even the mosquitoes and flies spread different odors in atmosphere. The odor which brings feelings of pleasure, environment of freshness and even imbibes the soul in the extreme of joy is probably perfume. A perfume is normally a complex mixture of natural or synthetically produced substances which are blended along with the odorless solvent such as ethanol to produce the typical odor. The core ingredients of these perfumes are Essential oil.

Essential oils (EOs) also called volatile or ethereal oils; are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). The term 'Essential oil' is thought to derive from the name coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim; he named the effective component of a drug Quinta essential. An estimated 3000 EOs are known, of which about 300 are commercially important destined chiefly for the flavors and fragrances market. EO belongs to the so called natural products of trade (Hegnauer, 1982), are a heterogeneous group of complex mixtures of organic substances. There is barely any group of naturally occurring substances where the number of possible components is as great as for the EO constituents.

The term 'Essential oil' is open to wide explanation and may include not only products obtained by traditional methods of distillation but also those obtained by very discriminating solvent extraction or mechanical expression. These include: concretes, absolutes, resinoids, and various extracts most of which are more applicable to the field of perfumery than to flavorings and seasonings (Meyer-Warnod, 1984). EOs are found in all distinctly aromatic plants and occur in about 60 botanical families, more significantly in *Asteraceae, Lamiaceae*, *Lauraceae, Myrtaceae, Rutaceae, Geraniaceae,*

Poaceae, Fabaceae, Caesalpiniaceae and Apiaceae (Samba Murthy and Subrahmanyam, 1989).

EO's which were once considered inactive waste products of plant metabolism and had no significant biological function, are now being realized for their importance as a means of chemical communication which the plants keep itself against competitors, predator and pathogens.

Nevertheless, there are an almost uncountable number of single substances and a remarkable variation in the composition of EOs. Many of these volatile substances have varied ecological functions. Among plant secondary metabolites terpenoids are structurally most assorted group; they function as as signals in indirect defense responses which involves herbivores and their natural enemies (Cheng *et al.,* 2007).

In recent years, attention has been paid towards investigation of ecological role of plant terpenoids (Cheng *et al.,* 2007). The compounds are mainly derived from three biosynthetic pathways, the mevalonate pathway leading to sesquiterpenes, the methyl-erithrytol-pathway leading to mono- and diterpenes, and the shikimic acid pathway *en route* to phenylpropenes. Terpenoids are synthesized from a common precursor in all organisms, i.e. the first isoprene isopentenyl diphosphate (IPP; C_5). IPP is formed generally from acetyl coenzyme A (acetyl-CoA) via 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and mevalonate, whereas an alternative pathway appears to exist in several bacteria or green algae (Pandian *et al.,* 1981). IPP is summarized to dimethylallyl diphosphate (DMAPP) by IPP isomerase (Anderson *et al.,* 1989). DMAPP is condensed with IPP to generate geranyl diphosphate (GPP; C_{10}), which undergoes further condensation with IPP to form farnesyl diphosphate (FPP; C_{15}). This sequential elongation reaction is catalyzed by FPP synthase (Anderson *et al.,* 1989) Geranylgeranyl diphosphate (GGPP; C_{20}) is formed from FPP by GGPP synthase, which enables further addition of IPP (Kuntz *et al.,* 1992). GGPP is the substrate for the biosynthesis of carotenoids, gibberellins and other various isoprenoid compounds.

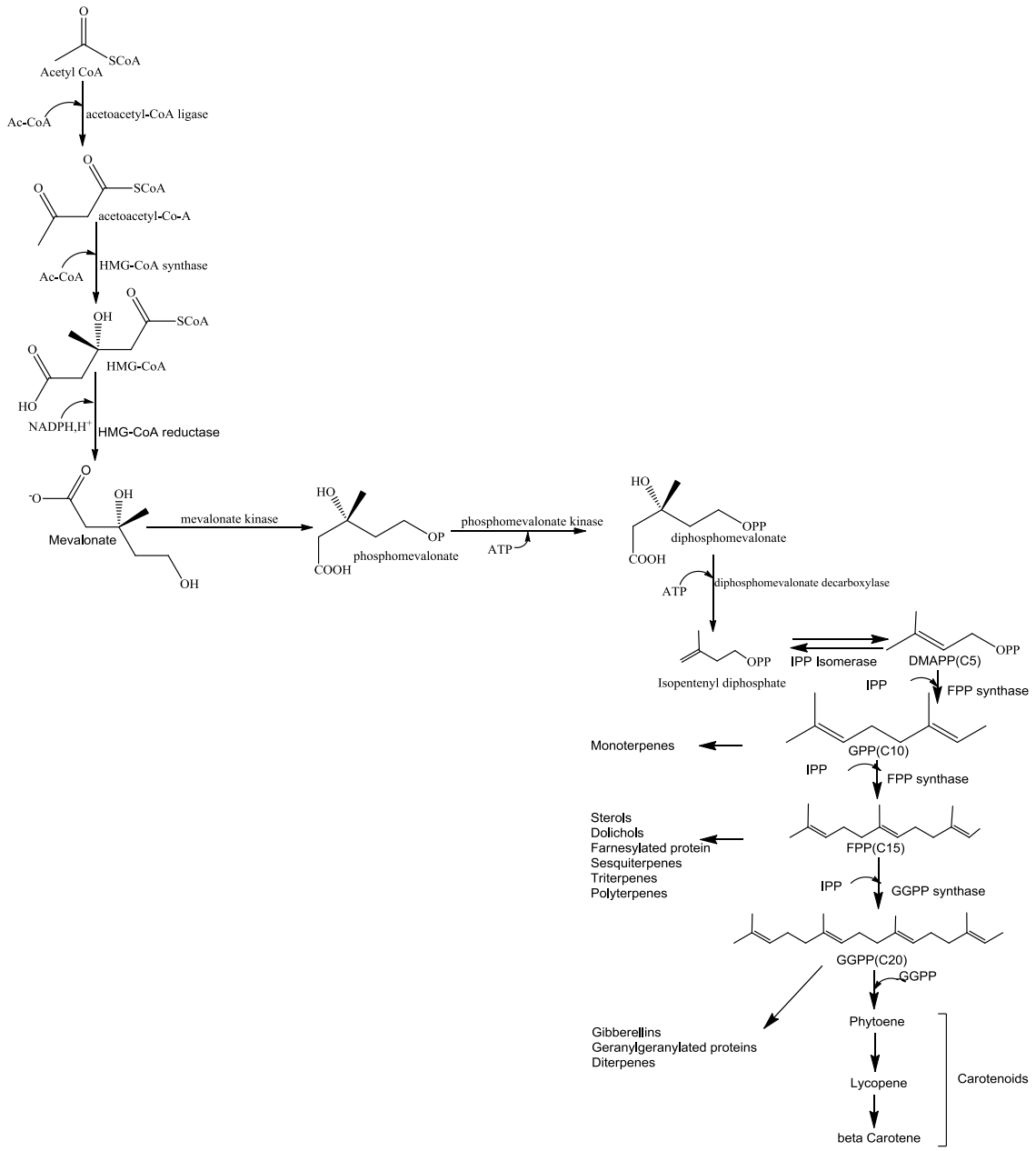

Figure 1: The Mevalonate biosynthesis pathway of isoprenoids in plant cell

IPP, isopentenyl diphosphate; acetyl-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate.

1.1 Sources of EOs

The differences in the complex composition of two EOs of one kind may sometimes be complicated to assign to precise chemo types or to differences arising in the

consequence of the reactions of the plants to specific environmental conditions, for example, to different growing locations(Chamorro *et al.,* 2012).

EOs protects plants from microbial attack and facilitates pollination by attracting insects. Thus the EO is implicated in fitness for life. In other words, secondary metabolites are more involved in the ecology than in the physiology of plants (Hegnauer, 1982).

1.2 Extraction of EOs

a. Steam distillation is the most commonly used method for producing EOs on a commercial basis.
b. Extraction by means of liquid carbon dioxide produces a more natural organoleptic profile but is much more expensive (Moyler, 1998).
c. Enfleurage method is used for the isolation of EO from rose petals.
d. The microwave irradiation or microwave assisted process (MAP) has also been developed and reported as a technique for extraction of EOs in order to obtain a good yield of the essence, and to reduce the time of extraction (Chiasson *et al.,* 2001).

1.3 Chemical analysis of EOs

Chemical analysis is generally performed using Gas Chromatography (GC) quantitative analysis) and Gas Chromatography/Mass Spectroscopy GC/MS (qualitative analysis). Identification of the main components is carried out by comparison of both the GC retention times and MS data against those of the reference standards (with known source) (Lahlou and Berrada, 2001).

In addition to mass spectra (electronic impact), sometimes identification by GC-MS must be confirmed by retention indices (Kovats indices) on two columns of different polarity, on the same column, but at different temperature and for the identification of new constituents by co-injection or spiking with authentic compounds. The compounds which are not easily separated by GC and similar molecular structures like stereo-isomeric compounds of EOs are analyzed by ^{13}C NMR. This technique is also applied to the study of the chemical intraspecific variation and could also be used in the quality control of volatile oils (Salgueiro *et al.,* 1997).

1.4 Medicinal attributes of EOs

Besides antibacterial properties (Mourey and Canillac, 2002), EOs or their components have been shown to exhibit antiviral (Bishop, 1995), antimycotic (Kivanc and Akgul, 1986), antitoxigenic (or non-toxigenic) (Ultee and Smid, 2001), antiparasitic (Pessoa *et al.,* 2002), and insecticidal (Karpouhtsis *et al.,* 1998) properties.

These characteristics are possibly related to the function of these compounds in plants (Mahmoud and Croteau, 2002). Phenolic components are chiefly responsible for the antibacterial properties of EOs (Cosentino *et al.,* 1999). EOs also have hepatoprotective activity for e.g. Nutmeg, (*Myristica fragrans*), showed a potent hepatoprotective activity against liver damage caused by certain chemicals (Morita *et al.,* 2003). There is an increasing worldwide interest in screening aromatic plants to study biological activities of their essential oils with particular focus on their chemical, pharmacological and therapeutic properties (De Martino *et al*., 2009). Many volatile constituents have been reported to possess potent antioxidant activity and to have anticancer or anticarcinogenic /antimutagenic /antiproliferation effects (Giaginis and Theocharis, 2011, Patil *et al.,* 2009, Abdolmohammadi *et al.,* 2009, Hardin *et al.,* 2010). Together these data strongly support the view that essential oils have potential therapeutic applications in the prevention of cancer. Various mechanisms like antioxidant, antimutagenic, anti-proliferative, enhancement of immune functions and surveillance, enzyme induction and enhancing detoxification, modulation of multi-drug resistance and synergistic mechanism of volatile constituents are responsible for their chemo preventive properties.

1.5 Mechanisms of action of essential oils as anticancer

1.5.1 Antimutagen:

Antimutagenic property of EOs is attributed to certain mechanisms including inhibition of penetration of the mutagens into the cells (Kada and Shimoi, 1987), inactivation of mutagens by direct scavenging, antioxidant capture of radicals produced by mutagens, activation of cell antioxidant enzymes (Sharma *et al.,* 2001), inhibition of metabolic conversion by P450 of promutagens into mutagens (Ramel, 1986, De Flora and Ramel,

1988) or activation of enzymatic detoxification of mutagens by EO constituents (Kada and Shimoi , 1987).

Antimutagenic compounds are effective by promoting error-free DNA repair or by inhibiting error-prone DNA repair (Kada and Shimoi, 1987). There has been no investigation on the type of antimutagenicity involving DNA repair by terpenic and phenolic compounds from EOs since the work by Kada and Shimoi on *E. coli* (Kada and Shimoi, 1987). Chemical compounds extracted from aromatic plants, such as α-terpinene, α-terpineol, 1,8-cineole, d-limonene, camphor, citronellal, and citral modulated hepatic mono-oxygenase activity by interacting with promutagen or procarcinogen xenobiotic biotransformation (De-Oliveira, 1999).

1.5.2 Enhancement of immune functions and surveillance:

Mechanisms that help improve immune system are effective when certain factors are taken into consideration such as stress reduction, supporting good intestinal bacteria, and promoting better blood and lymph quality. A predominantly successful strategy is the use of aromatherapy. Aromatherapy is the use of EOs through various mediums towards improving immune function. It has an important role to play in enhancement of immune function. It works in several ways such as by controlling the hormones secreted by the adrenal glands consequently resulting in alleviation of stress, stimulating the immune response by supporting the lymph to remove toxin and by stimulating the production of immune boosting cell, destruction of harmful micro-organisms. One study evaluated *Citrus limonum* and *Lavendula angustifolia* for the effects of aromatherapy on human immune function. It found that lemon EO inhalation enhanced positive mood and boosts norepinephrine release, but other immunological data gathered did not indicate the effectiveness of either EO (Seo, 2009).

1.5.3 Enzyme induction and enhancing detoxification:

The consumption of *Allium* species has been associated in epidemiologic studies with a reduced risk of cancer incidence (Buiatti *et al*., 1989, Dorant and Brandt, 1996, Steinmetz *et al.,* 1994, You *et al.,* 1989). The anticarcinogenic properties of Allium can be attributed to organosulfur compounds derived from these plants. Experimental

studies on animal models have shown an inhibition of chemically induced carcinogenesis in different organs by sulfur-containing compounds (Hayes *et al.,* 1987, Hong *et al.,* 1992, IP *et al.,* 1992). Sulfur containing agents inhibiting carcinogenesis and alter the metabolism of procarcinogen. These agents can increase detoxification by increasing the levels of phase II enzymes such as glutathione S-transferase (GST), UDP-glucuronyl transferase (UGT) or quinone reductase (QR) or by decreasing the levels of some phase I enzymes such as cytochromes P450 (Peter Guengerich ,1992, Sparnins *et al*., 1988). Allyl sulfides derived from garlic (*Allium sativum*) have been shown to increase phase II enzymes such as GST, UGT and epoxide hydrolase (EH) 80-83 and to inhibit cytochrome P450 2E1 (Kim *et al.,* 1994, Brady *et al.,* 1991, Kwak *et al.,* 1994, Reicks and Crankshaw,1996).

1.5.4 Modulation of multidrug resistance:

The success of chemotherapeutic agents is often hindered by the development of drug resistance, with multidrug resistant (MDR) phenotypes reported in a number of tumors, generally involving reduced intracellular drug accumulation and/or impairment of one or more steps of the apoptotic signaling cascades due to increased drug efflux by energy dependent drug transporters belonging to ABC (ATP-binding cassette) superfamily (Gottesman and Pastan, 1993).

Ravizza *et al.* (2008) reported that linalool which is an acyclic monoterpene alcohol found in the EOs from many aromatic plants, including lavender (*Lavandula officinalis*), coriander seeds (*Coriandrum sativum*), sweet basil (*Ocimum basilicum*) and *Mentha citrata* reverses doxorubicin resistance in MCF7 WT and MCF7 Adr(adriamicin-resistant) human breast adenocarcinoma cell lines (Todd and Murray, 1968).

In another study Neidnicht and Schobert, (2011) reported that the para-benzoquinone thymoquinone which is the active principle of thyme and black seed (*Nigella sativa*) EOs, responsible for the antioxidant, anti-neoplastic activity can modulate the multidrug resistance of doxorubicin. *In-vitro* anti-tumoral activity of Tea tree oil (*Melaleuca alternifolia*) was analyzed against human melanoma M14 wild type (WT) cells and their drug-resistant counterparts, M14 adriamicin-resistant (ADR) cells, selected by prolonged exposure to doxorubicin.

1.5.5 Antiproliferation:

Lampronti *et al* (2006) studied *in-vitro*and*in-vitro*anti-proliferative activities of *Satureja hortensis, Satureja montana, Salvia officinalis, Lavandula officinalis, Thymus vulgaris, Calamintha origanifolia, Foeniculum vulgare* and *Mentha arvensis*on on human erythroleukemic K562cells. It is reported that EOs derived from *S. Montana* (IC50 value 56 µM/ml) and *M. arvensis* (IC_{50} value 85 µM/ml) showed the most interesting biological activity in inhibiting the cell growth of K562 cell line. Common active principles of *Satureja hortensis* and *Satureja Montana* i.e α-pinene, γ-terpenene, 4-terpineol and caryophyllene showed antiproliferative activity between IC_{50} value 329-98 µM/ml (Lampronti *et al.,* 2006).

In other study, the active constituent eugenol from *Syzigium aromaticum* (cloves), nutmeg, basil, cinnamon and bay leaves showed antiproliferative activity against various cancer cell lines and animal models (Jaganathan and Supriyanto, 2012).Patil *et al.,* (2009) reported the apoptosis mediated proliferation inhibition of human colon cancer cells by volatile constituents of *Citrus aurantifolia.* This oil showed 78% inhibition of human colon cancer cells (SW-480) with 100 µg/ml concentration at 48 h. Lime volatile oil showed DNA fragmentation and induction of caspase-3 up to 1.8 and 2 folds after 24 h and 48 h, respectively, which may be due to the involvement of apoptosis. Analysis of apoptosis-related protein expression further confirmed apoptosis induction by lime volatile oil and suggested that lime volatile oil has potential benefits in colon cancer prevention (Patil *et al.,* 2009).

1.5.6 Antioxidant:

Oxygen free radicals induce damage as a result of peroxidation to bio membranes and also to DNA, which bring about tissue damage, thus cause occurrence of a number of diseases (Halliwell *et al.,* 1984). Antioxidants neutralize the effect of free radicals through different ways and may avert the body from various diseases. Antioxidants may be synthetic or natural. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have recently been reported to be dangerous for human health. Thus, the search for effective, non-toxic natural compounds with antioxidative activity has been intensified in recent years (Gupta *et al.,* 2006). Natural

antioxidants may function (a) as reducing agents, (b) as free radical scavengers, (c) as complexers of pro-oxidant metals, and (d) as quenchers of the formation of singlet oxygen (Pratt, 1992).

Recently, many researchers have been investigating the antioxidant activity of different EOs in order to search for safe natural antioxidants. Consequently, various studies have shown that EOs are ideal natural sources of antioxidants.

In eukaryotes, hydroxyl radicals which are highly damaging to mitochondrial DNA are produced by superoxide anions and hydrogen-peroxide (Figure 2). Damaged mitochondrial DNA inhibits the expression of electron transport proteins leading to the accumulation of ROS. EOs penetrate cell wall and cytoplasmic membrane and damage mitochondrial membranes. (Bhalla *et al.*, 2013).

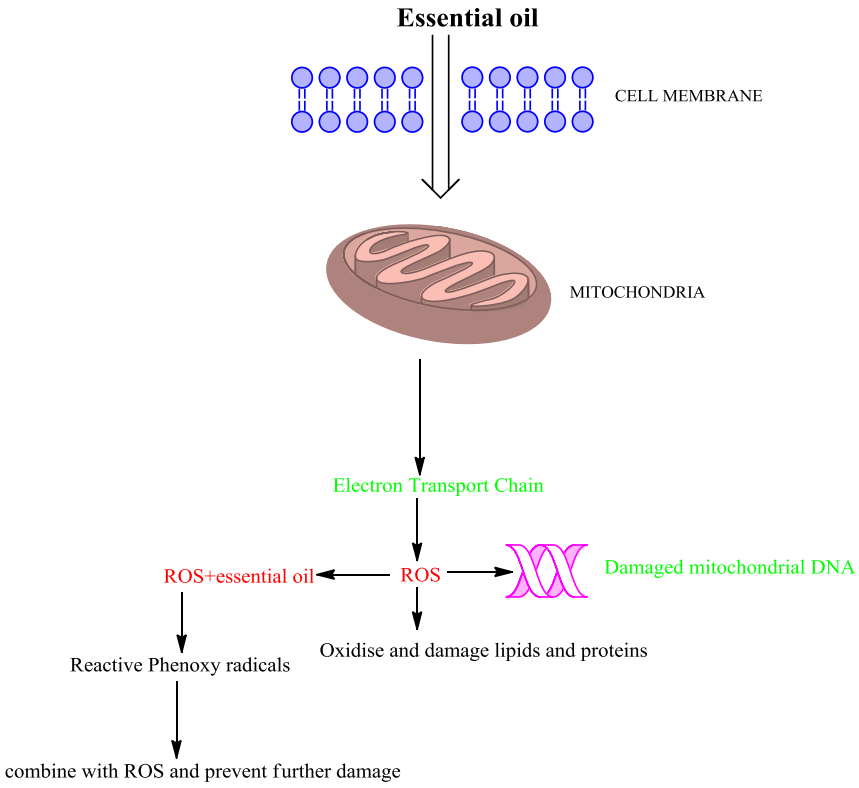

Figure 2: Antioxidant mechanism of Essential oil

Recent research by Misharina *et al.,* in 2009 and the literature data show that EOs are effective natural antioxidants, which are able to compete with synthetic ones (Misharina *et al.,* in 2009). The antioxidant properties of EOs are determined by their composition. Oils with high contents of substituted phenols are able to significantly hinder the oxidation processes of labile unsaturated aldehydes even in low concentrations. The antioxidant properties of EOs consisting of terpene hydrocarbons and alcohols are determined by α and γterpinenes and their sesquiterpene analogs (Misharina *et al.,* 2009).

1.6 Apoptosis and EOs:

Apoptosis represents a physiological process cell death involved in the regulation of tissue homeostasis (Rossi and Gaidano, 2003). During the progression of cancer, there is a malfunction of apoptosis as there is the upregulation of anti-apoptotic proteins and/or down regulation of proapoptotic signaling pathways.This eventually leads to poor response to conventional chemotherapy (Li *et al.,* 2011).The BCL-2 (B-cell lymphoma/leukemia-2) families including pro- and antiapoptotic proteins are key regulators of apoptosis. There are six antiapoptotic members of the BCL-2 family in humans (BCL-2, BCL-xl, BCL-B, BCL-W, Bfl-1, and MCL-1) and there is a hydrophobic cleft in these proteins that share homology in BH3-only proteins and to the pro-apoptotic BCL-2 family members Bad, Bak, and Bax to inhibit apoptosis.In order to suppress the expression of BCL-2 antiapoptotic proteins the inhibitors of the BCL-2 class proteins are designed.The NF-κB pathway is responsible for expression of genes required in control of immune and inflammatory responses(Barkett and Gilmore, 1999)apart from that NF-κB also is a key mediator of genes involved in the control of the cellular proliferation and apoptosis (Gloire *et al.,* 2006). By increasing the expression of antiapoptotic cellular proteins, NF-κB activation can thus reduce apoptosis in response to treatment with different chemotherapeutic agents. It has been reported that overexpression of NF-κB is due to ROS (Ukani *et al.,* 1996) and in order to suppress NF-κB, antioxidants should be administered. The cascade of the mechanism by which NF-κB and BCl-2 antiapoptotic protein family causes apoptosis is depicted in Figure3.The demand for medicinally

important plant species has been increased in the past few years globally due to a resurgence of interest in herbal medicine.

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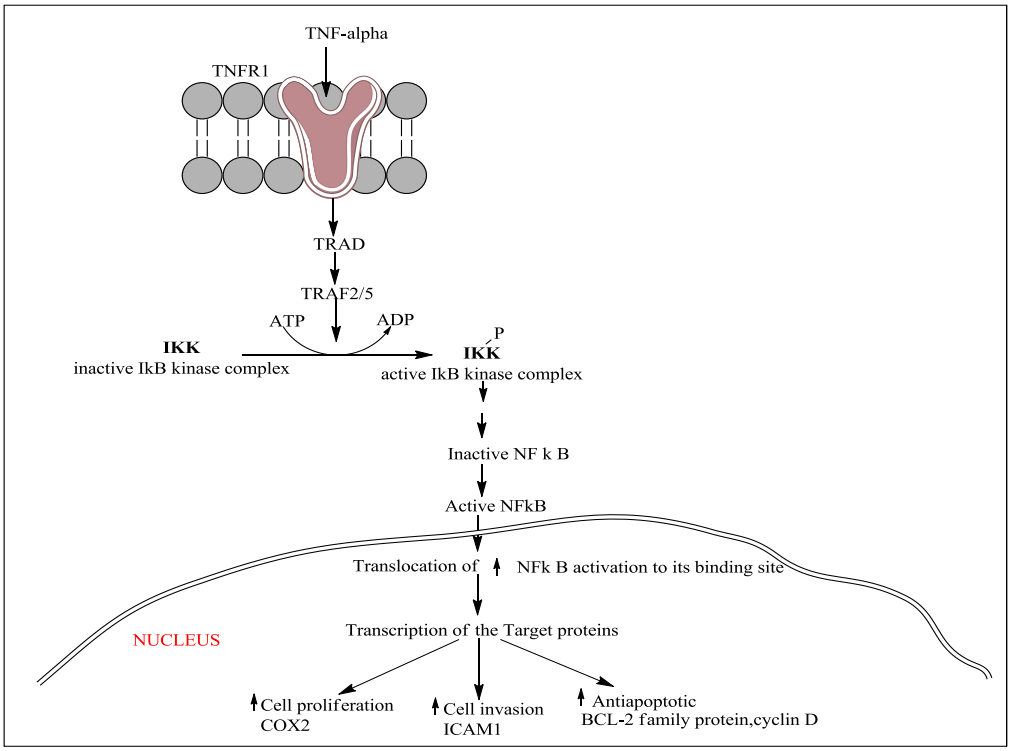

Figure 3: Mechanism leading to apoptosis

Aconitum heterophyllum (family: Ranunculaceae) commonly known as Atis, occurring in alpine and sub alpine regions of temperate Himalayas, is a reputed plant for its medicinal and pharmaceutical values. Ativisha (*A. Heterophyllum W*all) is an Ayurvedic herb known for significant medical properties. The roots of the plant have been used in various Ayurvedic preparations (Uniyal *et al.,* 2002). It is used for the treatment of diseases like the nervous system, digestive system, fever and rheumatism (Wang *et al.,* 2006). Being rich in substances having potential biological significance, such as benzoylmesaconine, mesaconitine, aconitine, hypaconitine, heteratisine, heterophyllisine, heterophylline, heterophyllidine, atidine, isotisine, hetidine, hetisinone, benzoylheteratisine and other nutrients (Anwar *et al.,* 2003), plant has been reported to

possess antifungal (Pandey *et al.,* 2004) , cytotoxic (Atal *et al.,*1986), antiviral (Pelletier *et al.,* 1968) and immune-stimulant properties.Considering the traditional uses and demonstrated medicinal properties of *A. heterophyllum*, the present study was undertaken to investigate the EOs apoptotic potential, which was hydrodistilled from *A. heterophyllum* by analyzing the *in-vitro* antioxidant and *in-silico* studies of anti-apoptotic BCL-2, BCL-XL, and MCL-1 proteins with the essential oil molecules of the plant. This study was conducted with the objective of exploring the anticancer activity of EO, with special reference to its potential to inhibit anti-apoptotic pathways.The objectives of the study were:

- Hydro-distillation and GC-MS analysis of essential oil from *Aconitum heterophyllum* Wall.
- *In-vitro* antioxidant activity of the essential oil.
- *In-silico* studies of anti-apoptotic BCL-2, BCL-XL, and MCL-1 proteins with the essential oil molecules of *Aconitum heterophyllum* Wall.

CHAPTER TWO

2.0 Review of Literature

2.1 *Aconitum heterophyllum*

Aconitum, known as aconite, monkshood, Wolfsbane, leopard‘s bane, women‘s bane, devil‘s helment or blue rocket, is a genus of over 250 species of flowering plant belonging to the buttercup family, Ranunculaceae. *Aconitum heterophyum* Wall.is a medicinally important endangered plant (Jain and Sastry, 1984). The roots and tubers of *A. heterophyllum* contain major aconite alkaloids that are used as a bitter tonic and febrifuge in the treatment of fevers, diarrhea, dyspepsia, and cough and also as astringent and antidiabetic. Aconite is an herbaceous perennial plant, chiefly native of the northern hemisphere, growing in moisture retentive but well-draining soils of the mountain meadows.

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2.2 Cultivation

Atees is an herbaceous, erect, biennial; leaves more or less hetronorphom; flower blue or violet, fruits follicles. Root tuberous in the pair’s whitish or grey breaks very easily and taste very bitter. The plant is found in sub-alpine and alpine zone of the Himalayas, between 2000-5000 m altitudes.

Common names: Aruna, Ativasa, Visa.

Location: Hills of Himachal Pradesh, Uttaranchal, Jammu & Kashmir, Arunachal Pradesh & Sikkim

Part used: Tuberous root

This plant thrives in most soils and in the shade of trees. This plant is so tough it even grows marvelously in heavy clay soils and well in the open woodlands. Most of all it prefers a moist soil which contains the characteristic of calcium carbonate, calcium, or limestone, also in the sun or semi shade.

2.3 *Aconitum heterophyllum (ATIVISHA)* IN AYURVEDA

It is tall herb and its roots are tuberous and paired. Based on morphology and anatomy, several forms of *A. heterophyllum* are recognized (White, Yellow, Black and Red) amongst a white variety which is commonly available is the best. The white tuberous roots are plump with a pale yellow color. *A. Heterophylum* grows in the Himalayas at an attitude of 2000 to 5000 meters. It is a characteristic species of Sikkim, Nepal and chamber area (Ukani *et al.*, 1996). *A.heterophyllum* is used both externally as well as internally for various ailments. The underground stem and root as such are used in traditional system of medicine. The root powder of Ativihsa with honey is prescribed for cough irritations and bronchitis. It is effective in blood-pressure. The aqueous pulp of *A. Heterophyllum* as well as its main constituent namely atisine produces a marked hypotensive effect. It is prescribed in malarial fevers but cannot replace quinine (Ukani *et al.*, 1996)

2.4 External uses

Antidote for poison:The dasanga agada formulated by KASYAPA cures the poison of all kinds of insects. Ghee prepared with Ativisa and cow's milk is orally administered or as a nasal drops in case of acute poisoning. Ativisa is made into a paste by grinding with honey and administered orally.

2.5 Internal uses

Digestive System: It has appetizing, digestive, astringent, antihaemorrhoidal, and antihelmentic due to its bitter, pungent and ushna properties. Useful in diarrhea and dysentery.

Circulatory System: It is a blood purifier, hemostatic and anti-inflammatory because it alleviates pita.

Respiratory System: It reduces phlegm and clears airways.

Temperature: Febrifuge, useful as a prophylactic in intermittent fever

Satmikarana: Reduces body weight due to ruksha guna, bitter tonic and antidote for poisons, useful in scorpion

2.6 Chemical constituents

The roots contain non-toxic amorphous alkaloids and it is one of the best bitter tonics for children. Alkaloid atisine, aconitic acid, tannic acid, palmitic acid, stearic acid Abundant starch, fat, vegetable mucilage, cane sugar, glycerides and Ash atisinol, heterophyllisine, entatisine dipterpenoid lactone, F-dishydroatisine, benzylleteratisine, hetisine, hetratisine, hetidine, atinide, hestinone, carotene, Diterpene alkaloid. Four Diterpenes viz., heterophyllisine, aetidine, atidine, hetisinone, atisine (Srivastava *et al.,* 2010).

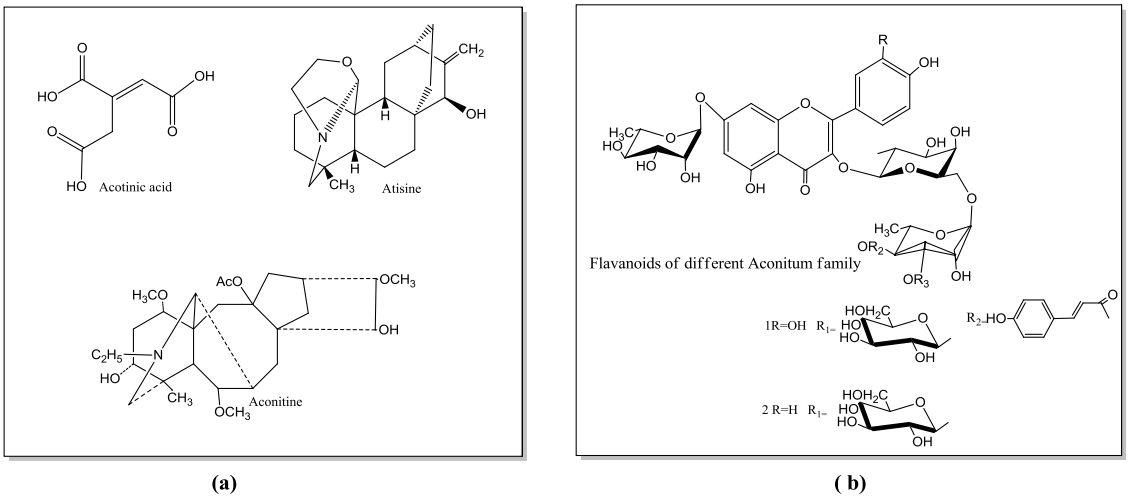


Figure 4 (a): Alkaloids (b) Flavanoids in *Aconitum heterophyllum*

2.7 PHARMACOLIGICAL ACTIVITIES

2.7.1 Aconitine-induced arrhythmias

The effect of Cl^- channel blocker 5-Nitro-2-(3 phenyl-propylamino) benzoic acid and niflumic acids were investigated on langendorff perfused rat hearts. An addition to the

Na^+ channel agonist accounting to the perfusion solution produced polymorphic ventricular arrhythmias with a latent period NPPB and NFA reversibly depressed the upstroke of the AP in a dose-dependent manner (Zhou *et al.,* 2005).

2.7.2 Aconitine-mediated phosphorylation

The band intensity of phosphorylated Cx43 & non-phosphorylated Cx43 in cultured and aconitine-treated cardiomyocytes of neonatal rats were determined by Western blot analysis. The changes in phosphorylation status occurring in PKCα in cultures were revealed by quantitative immunoflorescent microscopy (Liu *et al.,* 2011).

2.7.3 Anti-inflammatory activity

The anti-inflammated activity of *Aconitum heterophyllum* has been evaluated in cotton pellet-induced granuloma in rats. The extract has reduced inflammation as evidenced by decreased weight of cotton pellet in cotton pellet-induced granuloma in rats (Verma *et al.,* 2010).

2.7.4 Anti-diarrhoeal and anti-microbial activity

A.heterophyllum wall has previously been reported to anti-diarrheal activity along with astringent and tonic properties. It is now used as a key ingredient of anti-diarrheal medicine DIAREX (Mitra *et al.,* 2001).Antimicrobial activity of the crude extract *A.chasmanthum* along with brime shrimp lethality and insecticidal activity was evaluated. Antibacterial activity was tested against gram negative and gram positive bacteria (Zhang *et al.,* 2009).

2.7.5 Enzyme inhibition activity

Enzyme inhibitors are very useful. Tyrosinase inhibitors are involved in treating dermatological disorders associated with melanin hyperpigmentation and also important in cosmetics for whitening and de-pigmentation after sunburn (Shiino *et al.,* 2001). Tyrosinase inhibition studies on 5 alkaloids showed that only lappaconite and puberanine exhibited mild inhibition against the enzyme with IC_{50} of 93.33 and 205.21 µM, respectively (Shaheen *et al.,* 2005). Heterophylline A and heterophylline B alkaloids

from *Aconitum heterophyllum* Wall which inhibited muscle contracting enzyme acetylcholineatrase and butylcholinestrase responsible for Alzheimer disease (Nisar *et al.,* 2009). Both compounds were 13 times more specific to butylcholinestrase.

Aconitum genus is reputed for its medicinal and pharmaceutical value. The genus *Aconitum* comprises of 400 species, including some ornamental and medicinal plants (Utelli *et al.,* 2000).These medicinal plant species are a rich source of diterpene alkaloids and flavanoids, many of which exhibit broad spectrum activities. Various studies have been carried since last two decades on the isolation, identification, structural elucidation of active constituents of *Aconitum* species and their pharmacological and biological activity. *A.heterophyllum* Wall is one of the most important medicinal herb species of this genus. It is presently identified as critically endangered herb and calls for its sustainable utilization and conservation (Srivastava *et al.,* 2010).

CHAPTER THREE

3.0 MATERIAL AND METHOD

3.1 Plant material:

Aerial parts of *A. heterophyllum* were collected in August 2012 from the wild in Gattey village 4400–4500m above sea level N 32^{o} 18’ 14” and E 78^{o} 01’ 10” in the cold desert area of Spiti valley of Western Himalaya, India. Aerial parts of the plant were collected because with this we can utilize this golden plant wholly without any slaying. The plant materials collected were cleaned and shade-dried at room temperature as shade drying reduces the weight of the herb of the fresh weight and maximizes the oil yield without affecting the quality of the oil.

3.2 Reagents:

HPLC grade dichloromethane was purchased from M/S SD fine chemicals (Mumbai, India). Anhydrous Na_2SO_4 of analytical grade was procured from M/S Merck Chemicals (Mumbai, India). DPPH (2, 2’ Diphenyl-1-picrylhydrazyl), phenazine methosulphate, nitro blue tetrazolium, copper (II) chloride, neocuproine and ammonium acetate buffer were procured from Sigma Chemical Co. (St. Louis, MO, USA).

3.3 Hydrodistillation of EO:

Aerial parts of the plant were subjected to hydro-distillation in the Clevenger type apparatus. It is a method where the plant material is suspended in boiling water and steam is generated *in situ*, using a heat source from below the vessel. EO is carried away in the steam through tubes which was then cooled. EO is then removed from the top of the hydrosol. Hydrodistilled oil (0.5% v/w) was then dried over anhydrous Na_2SO_4 and stored at 4 ^{o}C for further analysis.

3.4 Isolation

The Hydrodistilled oil (0.5% v/w) was column chromatographed using petroleum ether and increasing amounts of ethyl acetate. Fractions of 100 ml were collected, and the fractions with similar TLC profiles were pooled. Each fraction was tested by TLC.

S.No.	Name of the compounds	Weight	Color	Odour
1.	VAM-4	226 mg	Light brown	Pleasant
2.	VAY-1	142 mg	Brownish yellow	Pleasant

3.5 GC-MS Analysis

EO from *A. heterophyllum* was analyzed by GC–MS. Analysis of the EO was performed using capillary column CP Si 8 CB (30 m X 0.32 mm X 0.25 um Thick film) and a 70 eV EI detector. For GC–MS detection, an electron ionization system was used with ionization energy of 70 EV. Helium was the carrier gas at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 280ºC. The spectrum was scanned with acquisition mass range 50 to 600 m/z, scan rate 1.6 scans s^{-1}. Column oven temperature was at 100ºC held for 5 min, and then finally increased to 250ºC at a 10 ºC /min rate, held for 20 min. 0.50 µL oil was injected.

3.6 Antioxidant activity

3.6.1 DPPH assay

The measurement of the hydrogen donating capability of oil was assessed using DPPH (2, 2' diphenyl-1-picrylhydrazyl) radical as substrate, following the method described by Bois (Blois, 1958). In this assay, 0.3ml of oil was added to 2.7 ml of 0.1mM methanolic DPPH solution in a cuvette and absorbance was read at 517nm.The decrease in absorbance at ambient temperature was correlated with the scavenging action of the test compound. The radical scavenging activity was calculated using the formula: $(1-A_S/A_C) \times 100$. Here,A_C=Absorbance of control, A_S=Absorbance of Sample solution.

3.6.2 Superoxide anion scavenging assay

For assessing the superoxide anion scavenging ability of EO, method described by Nishikimi *et al*. (1972) was followed with slight modifications. The superoxide anions were generated non-enzymatically in a PMS-NADH system comprising of phenazine methosulphate and reduced nicotinamide adenine dinucleotide, and assayed by development of blue coloured formazan dye upon reduction of nitro blue tetrazolium. Briefly, EO of different concentrations (200µg/ml) was mixed with 156µM NADH (1ml), 60µM NBT (1ml) and 468µM phenazine methosulphate (1ml) in phosphate buffer (pH = 8.3). The reaction was initiated by the addition of PMS. The reaction mixture was incubated at 25˚C for 10 minutes. The absorbance of colored complex was measured at 560 NM and inhibition percentage was calculated using the formula $(1- A_S/A_C)\times100$; A_C=Absorbance of control, A_S=Absorbance of sample solution.

3.6.3 Cupric ion reducing antioxidant assay - CUPRAC

The cupric ion reducing potential of different fractions was determined spectrophotometrically (Apak *et al.,* 2004). To the mixture of 1 ml (10 mM) copper (II) chloride, 1 ml (7.5 mM) neocuproine and 1ml (1.0 M, pH 7) ammonium acetate buffer solution, added 100 µl of extract solution with 1ml of distilled water as a dilution factor to different concentrations of extract/fractions. The reaction mixture was allowed to stand for 30 minutes at room temperature and absorbance was measured at 450 nm. An increase in absorbance indicates the increased reduction ability. The percentage reduction was calculated using the formula:

Percentage reduction: $[1 - (1 - A_S/A_C)] * 100$. Here, A_C = absorbance of standard (ascorbic acid) at maximum concentration tested i.e. 200µg/ml and A_S = absorbance of the sample.

3.7 In-silico studies

Molecular Docking is a computational method to determine probable binding modes of a ligand to the active site of a receptor. Docking studies were executed with a set of EO molecules using Maestro 9.3 on BCL-2, BCL-XL, MCL-1 complexes and NF-κB, IKK beta, TRAF 6. The X-ray structures were accessed from the protein data bank (PDB). The starting structures for virtual study experiments of BCL-2, BCL-XL, MCL-1

complexes and NF-κB, IKK beta, TRAF 6 were retrieved from the RCSB with the protein data bank (codes 4AQ3, 2YXJ, 4G35, ,4DN5, 3BRV,1LB5).

3.7.1 Ligand preparation

The ligand used were sketched by using ChemBioDraw ultra 12.0 and saved in SDF format. The molecules were converted to 3D structure from the 2D using Lig Prep version 2.5 (Maestro version 9.3., 2012). Lig Prep is a collection of programs designed for the preparation of high quality, all atom 3D structures for large numbers of drug-like molecules, starting with the 2D structures in SDF format. The Lig Prep produces a single, low energy, 3D structure with correct chiralities for each input structure. During the performance of this step, chiralities were determined from 3D structure and original states of ionization were retained. Ligprep application of the maestro 9.3 utilizes OPLS-2005 force field. The inhibitors of BCL-2, BCL-XL, MCL-1 and NF-κB were ABT 263, ABT 737, Obatoclax, Parthenolide were taken from PUBCHEM (codes CID 24978538, CID 11228183, CID 16681698, CID 5353864).

3.7.2 Protein preparation

The starting structures for virtual study experiments of BCL-2, BCL-XL, MCL-1 complexes and NF-κB , ikk beta, Traf 6 and were retrieved from the RCSB with the protein data bank (codes 4AQ3, 2YXJ, 4G35, ,4DN5, 3BRV,1LB5).The protein structure with polar hydrogen was prepared using the protein preparation wizard in Maestro 9.3(Maestro version.,2012).In this step, bond orders were assigned, all hydrogens were added, and bonds to metals were deleted and formal charges were adjusted on the metal and the neighboring atoms and waters molecules were deleted that were more than the 5 A ˚ specific distance. Any missing disulphide bonds were added. The H-bonds were optimized using protassingn at pH7.0. With generated Het states options, prediction of ionization, and tautomeric states of the het group at pH 7 was achieved. In protein preparation, reorienting hydroxyl group, water molecules, and amino acids lead to the optimization of hydrogen bond network. The refinement of the structure was the final step in the protein preparation, with the help of restrained minimization. It was initiated in the imperfect minimization with the 0.3 A˚ RMSD for the minimization

OPLS_2005 force field. All bound ligands (small molecules and BH3 peptides), waters beyond 5 angstrom and ions, molecules and heteroatoms were removed from the complexes.

3.7.3 Docking

SiteMap was used to determine the potential top ranked binding site of the protein MCL-1,NF-κB in which the ligand is not available in the co-crystal structure. In the parameters we went for the default parameters in the sitemap, which included the use of the more restrictive definition of hydrophobicity and the use of standard grid. OPLS-2005 force field was used. Five sites were generated the top ranked site was chosen on the basis of Sitescore, size, D Score and volume for grid generation (Maestro version 9.3.,2012). Glide examines favorable interactions between one or more ligand molecules and a receptor molecule, usually a protein. The shape and properties of the receptor were represented on a grid by several different sets of field that provide progressively more accurate scoring of the ligand poses.Ligand molecule was picked in case of so it could be excluded from the grid generation in which Van der Waals scalling was reduced to 1.0 to soften the potential for non-polar parts of the receptor with partial atomic charge cutoff of 0.25. It identifies and measures pockets and pocket mouth opening as well as cavities. The length of the ligands to be docked was increased to 36 Å.EO molecules and the inhibitors docking were done using XP (extra precision), XP descriptors were written. Ligand was taken as flexible. Sample nitrogen inversions ans sample ring conformation was taken into account. Bias sampling of torsions was one only for amides and non-polar conformations were penalized. Epikpenalties were added to the docking score. Vander waalsscalling was adjusted to 0.8 and partial charge cutoff to 0.15 to soften the non-polar parts of the ligand. 10000 poses per docking run were allowed to run and 1 pose per ligand was allowed to be written. In the post docking minimization number of poses per ligand to be included was taken to be 10. The threshold energy below which the pose to be rejected was 0.5 kcal/Mol. After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and most energetic favorable conformation of each ligand was selected.

CHAPTER FOUR

4.0 RESULT AND DISCUSSION

4.1 EO composition

Essential oil was obtained from aerial parts by hydrodistillation in 0.5% (v/w) on fresh weight basis. Oil was musky, woody and having yellow color. Chemical composition of the oil was studied by gas chromatography mass spectroscopy (GC-MS) using non-polar column.Nineteen constituents were identified representing 100.01% of the total oil identified of which linalyl acetate (48.276%), linalool (18.334%) were the major constituents followed by isoborneol (5.509%), cis-ocimene (5.298%), caryophyllene oxide (2.927%) and spathulenol (2.707%) (Table 1)(Spectra 1).

Clevenger apparatus assembly for hydrodistillation of Essential oil

Table1: Essential Oil Composition of *Aconitum heterophyllum*

S.No.	R.T*(min)	Compounds Identified	Percentage Composition	Ki value(Literature)
1.	5.925	Cis-Ocimene(beta)	5.298	1040
2.	6.001	Limonene	1.663	1031
3.	6.141	Eucalyptol	0.652	1024
4.	6.331	Dihydrotagetone	3.469	1050
5.	6.911	Linalool oxide-A	0.960	1076
6.	7.258	Linalool oxide-B	0.893	1088
7.	7.427	Linalool	18.334	1098
8.	9.176	Isoborneol	5.509	1158
9.	9.511	β Fenchyl alcohol	2.294	
10.	10.245	Linalyl acetate	48.276	1257
11.	10.782	Neryl acetate	2.238	
12.	11.587	β-Citral	0.804	1244
13.	11.823	β-Pinnoxide	1.858	
14.	13.097	Santalene	0.723	1446
15.	13.254	Caryophyllene	0.573	1418
16.	14.342	α Longipinene	0.444	1358
17.	14.459	Germacrene B	0.379	1543
18.	15.472	Spathulenol	2.707	1572
19.	15.473	Caryophyllene oxide	2.927	1572
Monoterpenes hydrocarbons:		**7.765**		
Oxygenated Monoterpenes:		**84.483**		
Sesquiterpene hydrocarbons:		**2.119**		
Oxygenated Sesquiterpenes:		**5.634**		
Total		**100**		

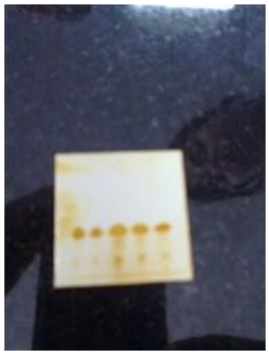
**R. T. = Retention time*

The brown colored oil (1.5 mL) was transferred to a column of silica gel (70g). The column was eluted successively with petroleum ether and ethyl acetate (increasing polarity). Volumes of 100 mL were collected. The collected volumes were monitored by TLC and divided into different fractions.

Fractions that showed single separate spots with the same Rf values on TLC were pooled. The active compounds were identified as, respectively, by comparison of GC-MS data with those reported in the GC-MS NIST (National Institute of Standard Technology) library.

VAM-4
Linalool(85%)
caryophyllene oxide(14%)

Spots showing two different
Rf signifying two different compounds



VAY-1
Mixture of components

The isolated components were analyzed by GC-MS.The compounds were identified as Linalool (85%),caryophyllene oxide (14%) in VAM-4 (GC-MS Spectra 2) and in VAY-1 mixture of components were obtained.

4.2 In-vitro antioxidant assay

4.2.1 DPPH Assay

The DPPH scavenging activities of different concentration of EO extracted from the *A. heterophyllum* plant are shown in Figure 5 (a). The concentration at 200µg/ml showed higher DPPH radical scavenging activity of 12.563% whereas at concentrations of 40, 80,120 and 160µg/ml showed DPPH radical scavenging activity of 3.565%, 4.036%, 10.797% and 12.396% respectively. Furthermore, the EO showed radical scavenging ability in a dose dependent manner.

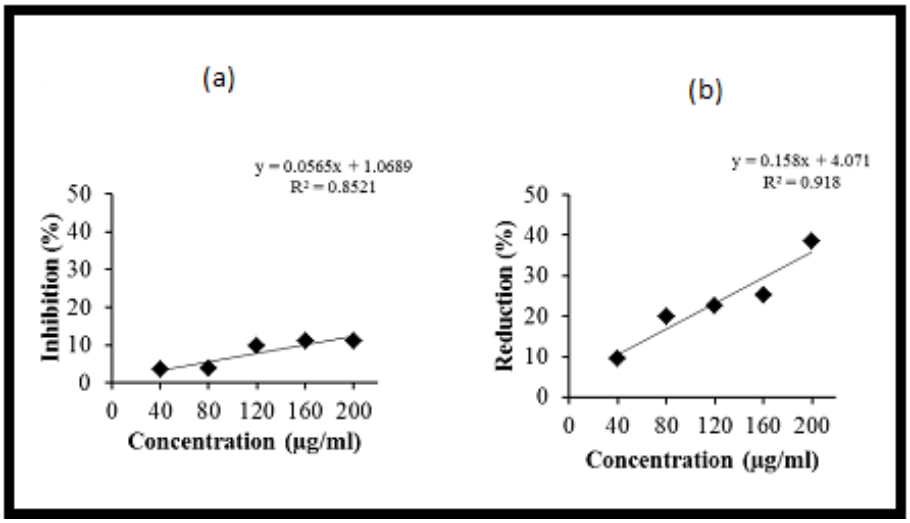


Figure 5: Shows DPPH radical scavenging (A) and cupric ion reducing potential (B) of EO isolated from *A. heterophyllum*

4.2.2 CUPRAC assay

The reducing ability of compounds generally depends on the presence of reductants, which have been reported to exhibit antioxidant potential by breaking the free radical chain. Values of different concentrations are as 9.42% at 40 µg/ml, 19.91% at 80µg/ml, 22.49% at 120 µg/ml, 25.29% at 160 µg/ml and 38.47% at 200 µg/ml, in comparison with standard ascorbic acid that is supposed to exhibit 100% reduction at 200 µg/ml. The result of *in-vitro* antioxidant activity of the oil has been depicted in Figure 5 (b).

4.2.3 Superoxide inhibition assay

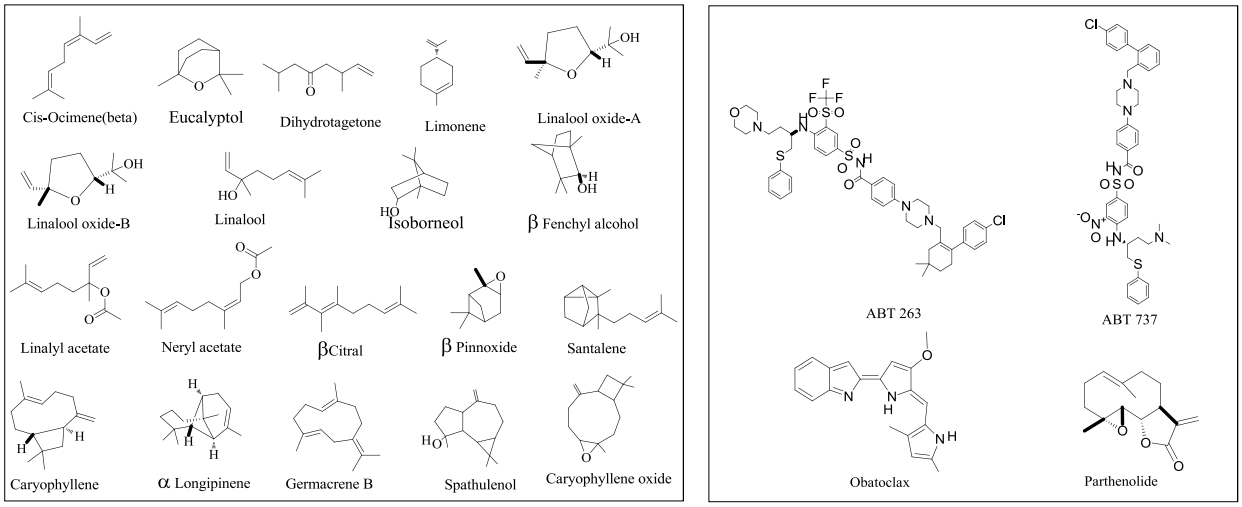
The superoxide radical is known to be produced *in vivo* and can result in the formation of H_2O_2 via dismutation reaction. Moreover, the conversion of superoxide and H_2O_2 into more reactive species, e.g., the hydroxyl radical, has been thought to be one of the unfavourable effects caused by superoxide radicals. From this experiment using different concentrations of the EO of *A. heterophyllum*, it was noted that the EO lacks superoxide anion radical scavenging ability. It was found that there was an increase in absorbance in a dose dependent manner that highlights its pro-oxidant nature (Table 2).

Table 2: Superoxide Anion Scavenging potential of Essential Oil extracted from *Aconitum heterophyllum*.

Concentration (µg/ml)	Mean±SE
Blank	0.403±0.028
40	0.413±0.019
80	0.424±0.012
120	0.426±0.010
160	0.428±0.017
200	0.440±0.014

4.3 *In-silico activity of EO constituents*

From the results of molecular modeling we could observe the type of interaction EO molecules makes with the receptors. It has been reported that there is an over-expression of NF-κB and BCL-2 anti-apoptotic proteins in cancer (Yip, 2008). Figure 6 depicts the structures of EO molecule and the standard inhibitors which are used for docking.



(a) **(b)**

Figure 6 (a) Structures of Essential oil molecules (b) Standard inhibitors used for docking

4.3.1 The Docking result of BCL-2 receptor with essential oil molecules

Phytochemicals like αlongipinene revealed strong binding affinity with BCL-2 anti-apoptotic protein but less as compared to the selective inhibitor of the BCL-2 protein i.e,

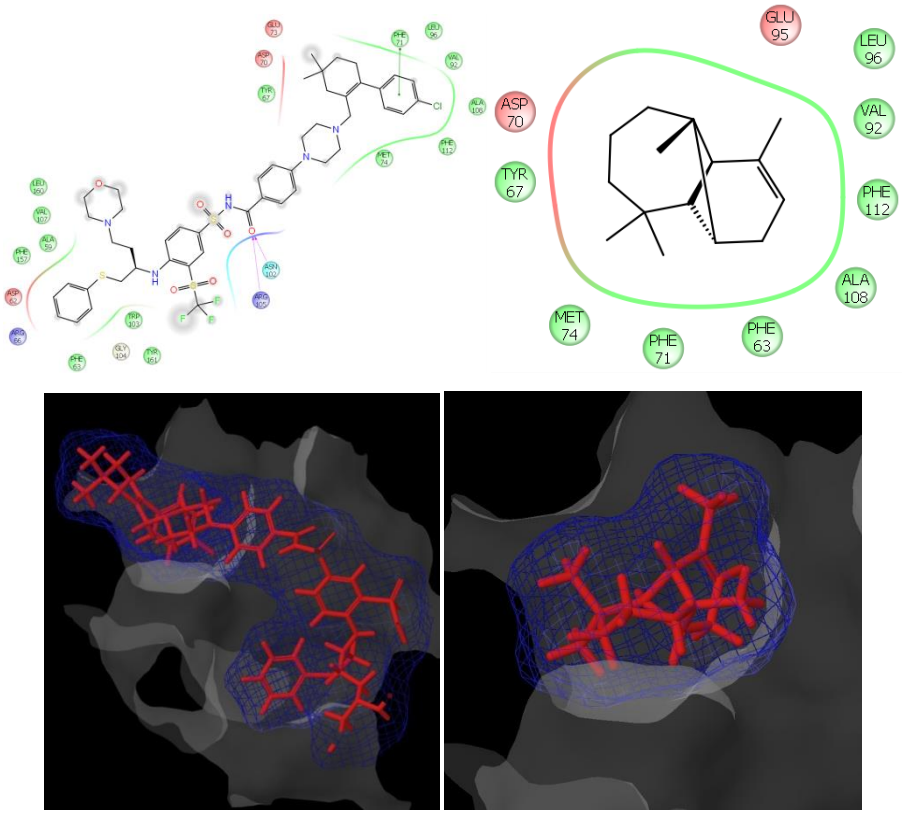
ABT 263. As α-Longipinene, eucalyptol have a glide dock score of -4.26 kcal/mol, -4.18 kcal/mol respectively and that of selective inhibitor ABT 263 is -4.67 kcal/mol, which depicts that α longipinene has strong binding affinity for the receptor site but less than the selective inhibitor ABT 263. α-Longipinene binds with the receptor site via hydrophobic interaction with the amino acid residue of LEU 96, VAL 92, PHE 71, PHE 63, PHE 112, MET 74, TYR 67 and ALA 108 and electrostatic interaction with ASP 70 and GLU 95 amino acid residue. The interaction reveals that the electrostatic forces and hydrophobic bonding are the forces which are responsible for the binding affinity of α Longipinene with the receptor site. The results of the docking of molecules with BCL-2 receptor are depicted in Table 3.

Table 3: Docking scores of EO with BCL-2 Receptor

S.No.	**Ligand**	**DockScore(**kcal/mol)	**LipophilicEvdW**	**HBond((**kcal/mol)	**Electro(**kcal/mol)
1.	**ABT263(inhibitor)**	-4.67	-5.76	-1.05	-0.57
2.	α-Longipinene	-4.26	-3.44	0	-0.01
3.	Eucalyptol	-4.18	-2.7	0	-0.02
4.	(-)-Spathulenol	-4.04	-3.35	0	-0.01
5.	Linalool oxide A	-4.03	-2.88	-0.48	-0.02
6.	β-Caryophyllene	-3.94	-3.49	0	-0.03
7.	Limonene	-3.69	-3.04	0	0
8.	(-)-Pulegone	-3.65	-2.38	0	0.02
9.	Germacrene B	-3.6	-2.75	0	0.02
10.	Linalool oxide B	-3.56	-2.85	0	-0.1
11.	Santalene	-3.55	-2.85	0	0
12.	(-)-β Fenchol	-3.49	-2.54	0	0.02
13.	α-Pinnenoxide	-3.43	-2.53	0	-0.02
14.	Dihydrotagetone	-3.43	-3.33	0	0.02
15.	β cis Ocimene	-3.34	-3.22	0	-0.05
16.	Isoborneol	-3.21	-2.16	-0.43	-0.1
17.	Linalool	-3.17	-2.62	-0.7	-0.09
18.	Caryophyllene oxide	-3.14	-2.66	0	0.04
19.	Linalyl acetate	-3.12	-2.55	0	-0.04
20.	Neryl acetate	-3.09	-2.41	-0.85	-0.23

Interaction profile of the ligands ABT 263 and α longipinene are depicted in Figure 7. It can therefore be concluded that the molecules having a higher dock score like alpha

longipinine can easily bind to the receptor site which blocks survival mechanisms and efficiently kill cancer by apoptosis.



(a) **(b)**

Figure 7: Interaction profile and docked poses of ABT-263 (a) and α-Longipinene (b) with BCL-2 receptor

4.3.2 The Docking result of BCL-XL receptor with the essential oil molecules

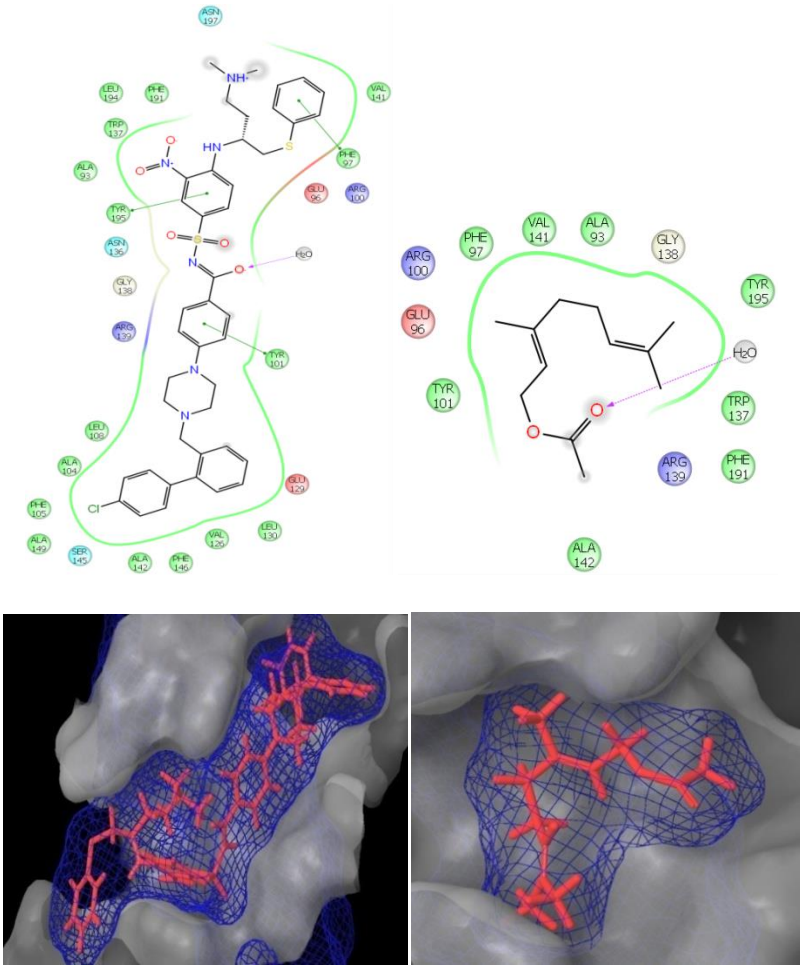
Neryl acetate, pulegone, caryophyllene have shown a comparable binding affinity for BCL-XL receptor but less than that of the selective inhibitor of BCL-XL i.e., ABT 737. The neryl acetate molecule binds with the BCL-XL receptor via hydrogen bonding with water molecule present in the receptor site while it shows hydrophobic interaction with ALA 142, VAL 141, TRP 137, TYR 195, PHE 191, ALA 93, PHE 97 and TYR 101 amino acid residue and it also shows electrostatic forces of interaction with GLU 96, ARG 139 and ARG 100 amino acid residue on the receptor site of BCL-XL**.** The interaction profile

reveals that due to large hydrophobic interactions with the receptor site the neryl acetate molecule shows more dock score and hence better interaction than other molecules.

Table 4: Docking scores of EO with BCL-XL Receptor

S.No.	Ligand	DockScore(kcal/mol)	LipophilicEvdW	HBond(kcal/mol)	Electro(kcal/mol)
1.	**ABT_737(Inhibitor)**	-9.47	-7.25	-0.7	-0.41
2.	Neryl acetate	-4.05	-3.22	-0.7	-0.18
3.	(-)-Pulegone	-3.95	-2.36	0	0.05
4.	β-Caryophyllene	-3.58	-2.82	0	-0.02
5.	Germacrene B	-3.46	-2.99	0	0.02
6.	Limonene	-3.44	-2.85	0	0.01
7.	Eucalyptol	-3.43	-2.73	0	-0.07
8.	Linalyl acetate	-3.37	-2.71	-0.7	-0.23
9.	(-)-β-Fenchol	-3.25	-2.15	-0.62	-0.08
10.	(-)-Spathulenol	-3.23	-2.7	0	0.01
11.	Caryophyllene oxide	-3.21	-2.75	0	0.01
12.	Linalool	-3.21	-2.64	-0.7	-0.07
13.	α-Longipinene	-3.21	-2.88	0	0.01
14.	Santalene	-3.18	-2.84	0	0.03
15.	Linalool oxide B	-3.17	-2.32	-0.61	-0.2
16.	Linalool oxide A	-3.13	-2.54	-0.48	-0.07
17.	Isoborneol	-2.8	-2.39	0	-0.01
18.	α Pinnenoxide	-2.74	-2	0	0.04
19.	β cis Ocimene	-2.71	-3.01	0	0.04
20.	Dihydrotagetone	-2.46	-1.98	-0.63	-0.2

Interaction profile of ABT 737 and neryl acetate with BCL-XL are depicted in Figure 8. The results of the docking of molecules with BCL-XL receptors are reported in Table 4. From the interaction profile it can be concluded that molecules such as neryl acetate and pulegone having higher dock scores can easily fit into the receptor site cavity inhibiting the cascade of pathways leading to apoptosis.



(a) **(b)**

Figure 8: Interaction profile and docked poses of (a) ABT-737 and (b) Neryl acetate with BCL-XL receptor.

4.3.3 The Docking result of MCL-1 receptor with the essential oil molecules

Whereas the phytochemical Linalool oxide B and α Longipinene had a low affinity for MCL-1 receptor as compared to the selective inhibitor Obatoclax as reflected by the little difference in the dock score. The results of the docking of molecules with the MCL-1 receptor are depicted in Table 5. Linalool oxide B molecule interacts with the receptor site hydrophobic interaction with the VAL 274, LEU 278, LEU 290, PRO 289 and PHE246 amino acid residues of the receptor site. Linalool oxide B shows electrostatic forces of attraction with the LYS 244, LYS 234 amino acid residues of the receptor site. Linalool oxide B shows polar interaction with SER 245 amino acid residue of the

receptor site. These interaction profiles are responsible for the binding affinity which this molecule shows with the receptor site. The interaction profile reveals that due to large hydrophobic interactions with the receptor site the Linalool oxide B molecule shows more dock score.

Table 5: Docking scores of EO with MCL-1 Receptor

S.No.	Ligand	DockScore(kcal/mol)	LipophilicEvdW	HBond(kcal/mol)	Electro(kcal/mol)
1.	**Obatoclax(inhibitor)**	-6.77	-3.11	-0.7	0.22
2.	Linalool oxide B	-3.73	-1.69	-1.54	0.35
3.	α-Longipinene	-3.53	-2.39	0	0.02
4.	Neryl acetate	-3.4	-2.11	-1.01	0.33
5.	Isoborneol	-3.38	-2.29	0	0.01
6.	(-)-Spathulenol	-3.36	-2	-0.42	0.16
7.	α-Pinnenoxide	-3.34	-2.26	0	0.02
8.	Limonene	-3.23	-2.45	0	0.01
9.	(-)-β-Fenchol	-3.14	-2.09	0	0.01
10.	(-)-Pulegone	-3.14	-2.49	0	0.11
11.	Linalool oxide A	-3.05	-1.95	-0.96	0.03
12.	Linalool	-3.02	-2.17	-0.7	0.37
13.	Germacrene B	-2.96	-2.41	0	0.02
14.	Santalene	-2.91	-2.57	0	0.02
15.	Linalyl acetate	-2.83	-2.05	-0.67	0.33
16.	Eucalyptol	-2.7	-1.69	-0.7	0.18
17.	β cis Ocimene	-2.47	-2.45	0	0.01
18.	Dihydrotagetone	-2.08	-2.27	0	0.02
19.	Caryophyllene-oxide	0.05	-1.19	0	0.01
20.	β-Caryophyllene	0.39	-1.19	0	0

Interaction profile of Obatoclax and Linalool oxide B with MCL-1 are depicted in Figure 9. It can be hereby be concluded that molecules such as linalool oxide b which are having a higher dock score can easily fit into the receptor cavity resulting in inhibition of the signaling pathway.

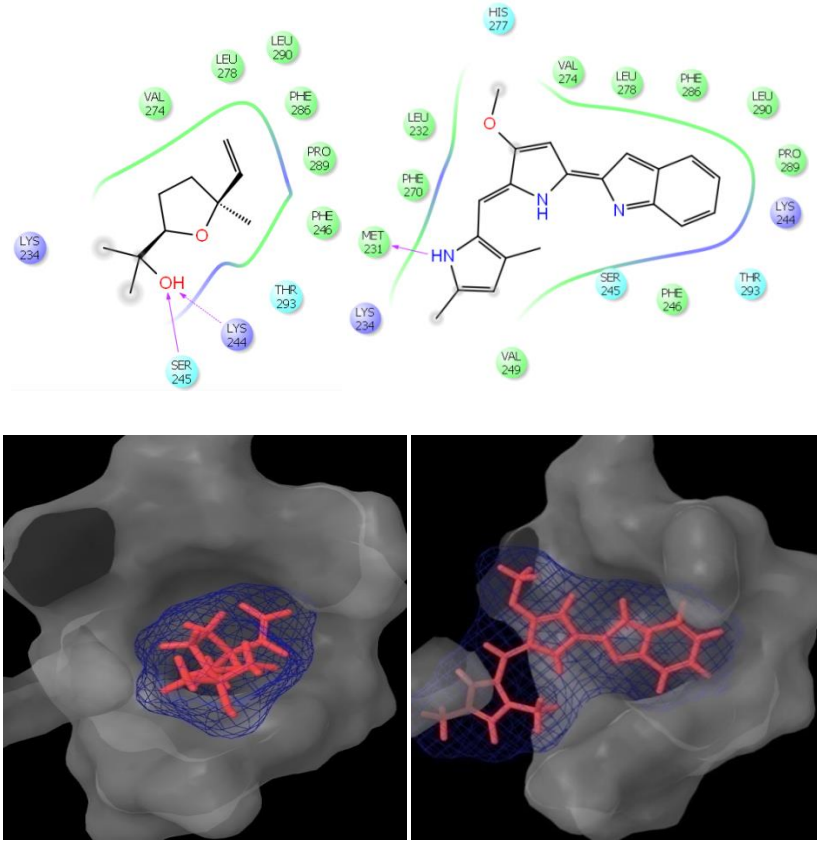


Figure 9: Interaction profile and docked poses of (a) Linalool oxide b and (b) Obatoclax with MCL-1 receptor.

4.3.4 Docking result of NF-κB receptor with the essential oil molecules

It has been observed that the β Fenchol, Linalool oxide A, Linalool oxide B, Neryl acetate, Linalyl acetate, Pulegone, Santalene, Eucalyptol, Isoborneol, Caryophyllene oxide, Limonene, Germacrene B, α Pinnenoxide had a high binding affinity for the NF-κB receptor than the selective inhibitor i.e., parthenolide. The dock score of the β Fenchol molecule with the receptor site of NF-κB was found to be -4.36 kcal/ mol which shows higher binding affinity of the β Fenchol molecule for the receptor site as compared to the selective inhibitors parthenolide whose dock score was -3.04 kcal/ mol. β Fenchol molecule interacts with the receptor site showing hydrogen bond interaction with the water molecule present on the receptor site and hydrophobic interaction with the VAL 414, ALA 427, LEU 471, LEU472, LEU 522 and MET 469 amino acid residues

of the receptor site. β Fenchol shows electrostatic forces of attraction with the GLU 430, ARG 416, ARG 408 amino acid residues of the receptor site. β Fenchol shows polar interaction with GLN 479, SER 475, HIS 415 amino acid residues of the receptor site. These interaction profiles are responsible for the highest binding affinity which this molecule shows with the receptor site. The interaction profile reveals that due to high hydrophobic, electrostatic, polar interaction and hydrogen bonding interactions there is a high affinity of the molecules with the receptor cavity. The results of the docking of the molecules with the NF-κB receptor are depicted in Table 6.

Table No.6 Docking score of EO molecules with NF Ƙappa B

S.No.	**Ligand**	**DockScore(**kcal/mol)	**LipophilicEvdW**	**HBond(**kcal/mol)	**Electro(**kcal/mol)
1.	(-)-β-Fenchol	-4.36	-2.88	-0.7	-0.29
2.	Linalool oxide A	-3.84	-2.6	-0.96	-0.24
3.	Linalool oxide B	-3.83	-2.66	-0.96	-0.17
4.	Neryl acetate	-3.72	-2.61	0	-0.61
5.	Linalyl acetate	-3.65	-3	-0.52	-0.08
6.	(-)-Pulegone	-3.57	-2.53	-0.35	-0.2
7.	Santalene	-3.51	-3.54	0	0.03
8.	Eucalyptol	-3.48	-2.38	-0.47	-0.12
9.	Isoborneol	-3.45	-1.96	-0.7	-0.29
10.	Caryophyllene oxide	-3.4	-2.47	-0.35	-0.08
11.	Limonene	-3.38	-3.19	0	-0.02
12.	Germacrene B	-3.26	-2.81	0	0.04
13.	α-Pinnenoxide	-3.18	-2.18	-0.35	-0.15
14.	**Parthenolide (Inhibitor)**	-3.04	-2.01	-0.43	-0.1
15.	Linalool	-2.84	-2.54	-0.7	-0.16
16.	Dihydrotagetone	-2.82	-2.85	-0.35	-0.19
17.	β-Caryophyllene	-2.65	-2.17	0	0.02
18.	α-Longipinene	-2.58	-2.11	0	0.03
19.	β cis Ocimene	-2.51	-3	0	0.01
20.	(-)-Spathulenol	-2.25	-1.83	0	0.08

Interaction profile of Parthenolide and β fenchol with NF-κB is depicted in Figure 10. Hence from the interaction profile it can be apparently observed that molecules like beta

fenchol and other molecules having a higher dock score can easily bind to the receptor site and hit the signalling pathways in the cascade resulting in apoptosis.

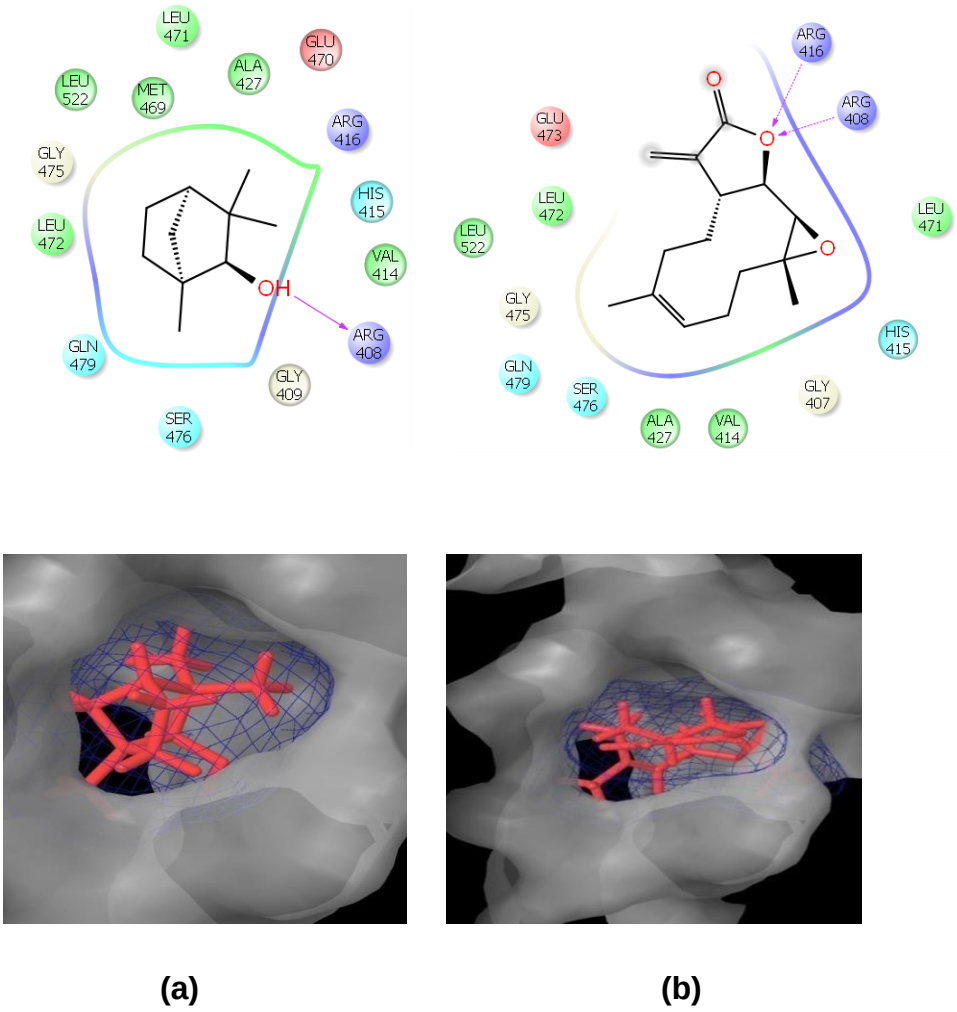


Figure 10: Interaction profile and docked poses of (a) β Fenchol and (b) with Parthenolide receptor.

CONCLUSION

EOs have shown to positively affect the immune system on a chemical level despite their direct effect on tumour cell. For removing foreign material and microbes from the body, EO enhances the activity of white blood cells making them more efficient. It is likely that the activity of the main components of the EO is modulated by other minor molecules. Moreover, it is probable that several components have a significant role in the fragrance, the density, the texture, the color and above all, cell penetration, lipophilic or hydrophilic attraction and fixation on cell walls and membranes, and cellular distribution. However, for biological purposes, it is more important to study an entire EO rather than some of its components because the theory of synergism appears to be more significant. Therefore, like many plant medicines, EOs work on a number of stages of many diseases, and cancer being one of them.

The EO extracted from *A. heterophyllum* contains linalool and linalyl acetate as the major components, which are also considered as the major components of fragrance and perfume industries so in future this plant can be utilized as a source for the production of linalool. The antioxidant activity of the essential oil reveals that the oil is antioxidant at dose dependant concentrations. From the outcome of this study the phytochemicals have shown a high affinity for the BCL-2 anti-apoptotic proteins including the BCL-2, BCL-XL and MCL-1. Due to small molecular size these molecules can easily fit into the receptor site cavity and elicit a desirable response. From the virtual screening it can be analyzed that the components of the oil show favorable binding for the apoptotic receptors and the *in-vitro* antioxidant activity of oil showed that the oil components suppress the NF-κB activity. In summary, based on our *in silico* and *in vitro* results, it can be postulated that essential oil of *A.heterophyllum* could be used as functional antiapoptotic inhibitor and as natural antioxidant.

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2. Gupta, V. K., Bhalla, Y., and Jaitak, V. (2013). Impact of ABC transporters, glutathione conjugates in MDR and their modulation by flavonoids: an overview. *Medicinal Chemistry Research*, 1-15. DOI 10.1007/s00044-013-0612-6.
3. Sapra, S., Bhalla, Y., Sharma, S., Singh, G., Nepali, K., Budhiraja, A., and Dhar, K.L. (2013).Colchicine and its various physicochemical and biological aspects.*Medicinal Chemistry Research, 22*(2), 531-547.
4. Bhalla, Y., Singla, R., Kaur, R., Arora ,S., and Jaitak, V.(2013). Integrated in-vitro antioxidant and *in-silico* antiapoptotic study of *Aconitum heterophyllum* Wall(Comminicated).
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GC-MS Spectra

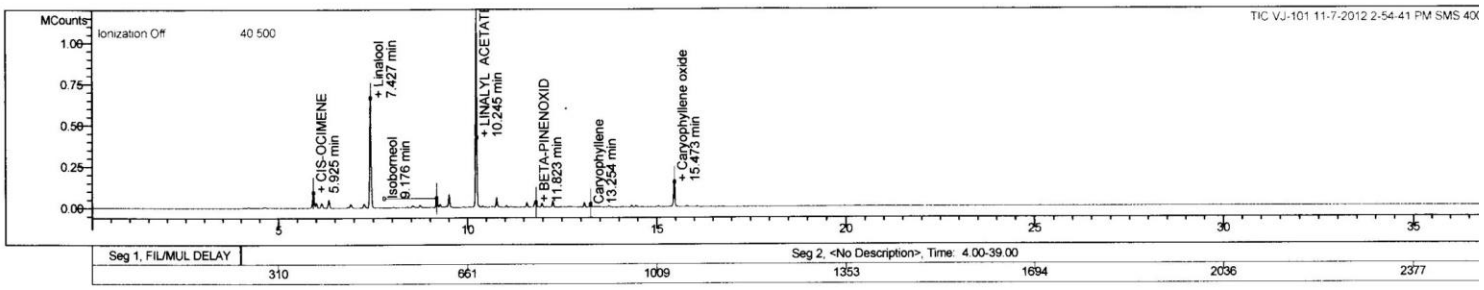
1. GC-MS Spectra of the EO

Print Date: 14 Dec 2012 10:11:02

Sample Report for ...01 11-7-2012 2-5

INSTRUMENTATION DIVISION, IIIM, JAMMU

Sample ID:	VJ-101	Operator:	Varian	Instrument ID:	Varian GC/MS #1
Vial:	TR: Tray1 VL: 2	Acquisition Date:	11/7/2012 2:54 PM	Data File:	... 11-7-2012 2-54-41 pm.sms
Calculation Date:	12/14/2012 10:10 AM	Method:	c:\varianws\fast method-vj-101.mth		
Inj. Sample Notes:	None			Injection:	1
Volume:	0.50 uL				



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Amount/RF	Area
1	5.925	CIS-OCIMENE	5.298	48103
2	6.001	Limonene	1.663	15098
3	6.141	Eucalyptol	0.652	5917
4	6.331	DIHYDROTAGETONE	3.469	31493
5	6.911	Linalool oxide-A	0.960	8716
6	7.258	Linalool oxide-B	0.893	8108
7	7.427	Linalool	18.334	166449
8	9.176	Isoborneol	5.509	50015
9	9.511	beta FENCHYL ALCOHOL	2.294	20823
10	10.245	LINALYL ACETATE	48.276	438276
11	10.782	Neryl acetate	2.238	20321
12	11.587	beta -Citral	0.804	7295
13	11.823	BETA-PINENOXID	1.858	16868
14	13.097	Santalene	0.723	6560
15	13.254	Caryophyllene	0.573	5198
16	14.342	alpha -Longipinene	0.444	4029
17	14.459	GERMACRENE B	0.379	3438
19	15.472	(-)-SPATHULENOL	2.707	24575
20	15.473	Caryophyllene oxide	2.927	26572

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Method Notes

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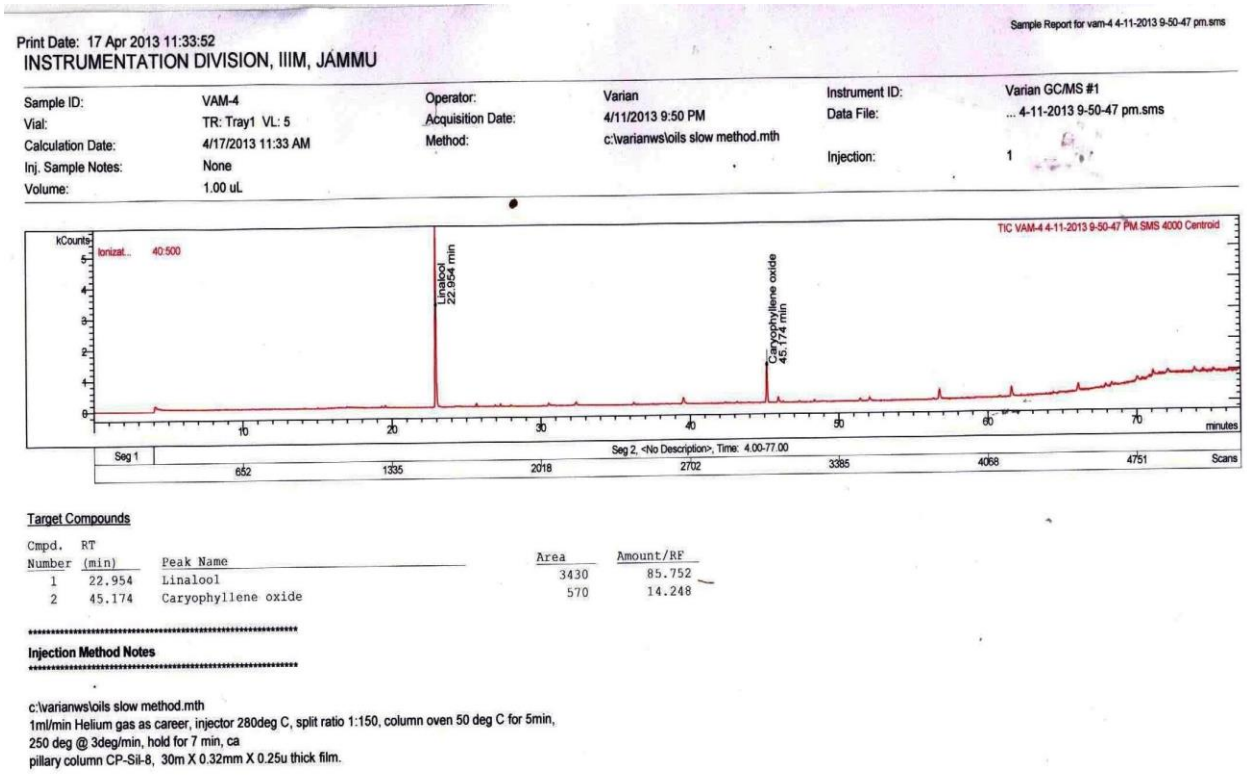
Injection Method: c:\varianws\fast method.mth

1ml/min Helium gas as career, injector 280deg C, splitl ratio1:50 , column oven 100 deg C for 5min,

2. GC-MS Spectra of the isolated molecules

VAM-4



Sample Report for vam-4 4-11-2013 9-50-47 pm.sms

Print Date: 17 Apr 2013 11:33:52
INSTRUMENTATION DIVISION, IIIM, JAMMU

Sample ID:	VAM-4	Operator:	Varian	Instrument ID:	Varian GC/MS #1
Vial:	TR: Tray1 VL: 5	Acquisition Date:	4/11/2013 9:50 PM	Data File:	... 4-11-2013 9-50-47 pm.sms
Calculation Date:	4/17/2013 11:33 AM	Method:	c:\varianws\oils slow method.mth		
Inj. Sample Notes:	None			Injection:	1
Volume:	1.00 uL				

Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	22.954	Linalool	3430	85.752
2	45.174	Caryophyllene oxide	570	14.248

Injection Method Notes

c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.32mm X 0.25u thick film.