“Phytochemical and biological evaluation of *Heracleum afghanicum* Kitamura.”

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By

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DECLARATION BY THE CANDIDATE

I hereby declare that, this thesis entitled "**Phytochemical and biological evaluation of *Heracleum afghanicum* Kitamura**" represents my own work and my ideas in my own words. This thesis is result of my own research work carried out by me under the guidance of Mr. Ashish Suttee, Associate Professor of Department of Pharmacognosy & Phytochemistry, School of pharmaceutical sciences, Lovely Professional University, Punjab, India. It is to mention that if other's notions, phrases or words were required to be included in this thesis, in that manner, I have surely cited them in term of their originality. I would like to make sure that I have obeyed all principles of academic honesty and integrity. I declare that no misrepresentation or fabrication or falsification of any idea/data or source have been done by me in my thesis. This submitted thesis encompasses all my own work and relevant data generated by me in laboratories of Lovely Professional University.

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Abstract

Human being has been dealing with and relied on plants from the starting time of their life on Earth. The ancient human used plants and plants derived substances/products as food, shelter, medicine, as fuel for baking food and also for hunting purpose. Certainly, plants are providing all life necessities which are required for maintenance of all kinds of life on Earth. Medicinal plants have been the primary source of diseases curing agents and still comprise promising sources of lead compounds for pharmaceutical companies.

This study was aimed to figure out the preliminary phytochemical profile and to evaluate basic biological activities (in vitro) of *Heracleum afghanicum* Kitamura indigenous to Afghanistan. *H. afghanicum* Kitamura is a robust plant in family Umbelliferae. The plant grows abundantly in several provinces and regions of Afghanistan. It has been used by rural people for several purposes. Younger stems and young leaves of the plant are used as beverage and salad since it bears a special aroma. Seeds of the plant are used as stomachic, appetizer, carminative and as condiment. Fruits of the plant are used as lactating agent by nursing mothers. Mature leaves of the plant are used as animal feed by local farmers claiming that the plant leaves enhance milk production in cows, goats and sheep. No scientific literature or publication is yet available regarding phytochemistry and biological activity of the plant, except the work of Karimi and Ito (2012). The authors have analyzed *Heracleum afghanicum* Kitamura seeds volatile oil and they reported presences of 33 compounds in the oil. They also reported significant sedative effects of the essential oil on mice when the essential oil vapors were inhaled by the mice.

Taking into account both the importance of work on, and the wildly abundance of *H. afghanicum* Kitamura which encourage selection of the plant for research work, decision was made to study the plant both chemically as well as biologically. Hence, this work entitled "**Phytochemical and biological evaluation of *Heracleum afghanicum* Kitamura**" comprises two main parts, while the first general part is dedicated to phytochemical screening of *H. afghanicum* leaves and the second one covers the biological evaluation of the said crud drug. The entire theoretical and practical aspects of this research work have been planned in different chapters as discussed below:

Chapter I contains introductory part where it includes general introduction, research on plants, some medicinal plants of Afghanistan and introduction of *Heracleum afghanicum* Kitamura.

Chapter II includes review of literature and prior arts related to general aspect of herbal drugs. Moreover, it embraces small topics on free radicals, oxidative stress, antioxidants, microbial infections and helminthiasis. In this part, different *Heracleum* species are introduced and reviewed.

Chapter III incudes the aim and objective of the research work. Simultaneously, the overall plans for conducting envisaged experiments are stepwise described and long term objectives are also discussed under this Chapter.

Chapter IV as the main chapter embraces materials and methodology of this research project. Main topics covered in sub- sections of this Chapter are; successive extraction of *Heracleum afghanicum* leaves, hydrodistillation of volatile oil, phytochemical screening of various extracts, GC-MS analysis of the volatile oil, in vitro antioxidant activity evaluation, evaluation, evaluation of in vitro antibacterial activity, evaluation of antifungal activity and evaluation of anthelmintic activity of various solvent extracts and volatile oil of *Heracleum afghanicum* leaves.

Chapter V includes results and discussions relevant to performed experiment during the project. Apart from textual data, tabulated, graphical and figured data are appropriately included under this Chapter. Analysis of the result and comparison of obtained data with previous work are included in this Chapter.

Chapter VI contains summary and conclusion, where the summarized summation of the entire project outcomes are presented thereunder. Proper justifications regarding the obtained data are provided and suggestions for the future work are presented in Chapter VI.

Chapter VII is dedicated to bibliography. All the original references from which ideas, methods or words were utilized in order to carry out this research project, are adequately cited and referenced under this Chapter. Appendixes at the end of this Chapter are included embracing GC-MS report of *H. afghanicum* leaves volatile oil, and other required certificates.

Preliminary phytochemical screening, in vitro antioxidant activity, in vitro antimicrobial and anthelmintic activities of *Heracleum afghanicum* leaves are reported for the first time in this study. However, it merits further research as well, to identify bioactive chemical ingredients of the crude drugs and to explore other biological effects of the same both in vitro and in vivo.

Key words: *Heracleum afghanicum* Kitamura, successive extractive, antioxidant activity, antimicrobial activity, anthelmintic activity. DPPH assay, FRAP assay, Total phenolic content, Total flavonoid content, disc diffusion method, agar well diffusion method.

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List of abbreviations

ACT	-	Artemisinin-based combined therapy
ANOVA	-	Analysis of Variance
AQ	-	Distilled water (Aqua distillate)
AQE	-	Aqueous extract
BOD	-	Bacteriological Oxygen Demand
^{o}C	-	degree Celsius
CFU	-	colony forming unit
DCM	-	Dichloromethane
DDM	-	Disc diffusion method
DMSO	-	Dimethyl sulfoxide
DPPH	-	2,2-dipheny-1-picrylhydrazil
EA	-	Ethyl acetate
EAE	-	Ethyl acetate extract
EHA	-	Egg hatch inhibition activity
EO	-	Essential Oil
EtOH	-	Ethanol
Ext.	-	Extract
FCR	-	Folin-Ciocalteu Reagent
FRAP	-	Ferric reducing activity power
g	-	gram
GAE	-	Gallic acid equivalent
GC-MS	-	gas chromatography-mass spectroscopy
GIN	-	Gastrointestinal nematode

Gram +ve	-	gram positive
Gram –ve	-	gram negative
HAK	-	*Heracleum afghanicum* Kitamura
HAKL	-	*Heracleum afghanicum* Kitamura leaf
hrs.	-	Hours
IC_{50}	-	inhibition concentration at 50%
Kg	-	kilogram
KUFS	-	Kabul University Faculty of Science
L	-	Liter
LDA	-	Larval development inhibition activity
LPU	-	Lovely Professional University
µ	-	micron
MDA	-	malondialdehyde
MeOH	-	Methanol
mg	-	milligram
MIC	-	Minimum inhibitory concentration
min	-	minute
ml	-	Milliliter
N	-	Normal
PA	-	Peak Area
Pet	-	Petroleum ether
PetE	-	Petroleum ether extract
PPS	-	Preliminary Phytochemical Screening
PS	-	Phytochemical Screening
RBF	-	Round Bottom Flask

RT	-	Retention time
SAR	-	Structure-activity relationship
SD	-	Standard deviation
SDA	-	Sabouraud Dextrose Agar
SOD	-	Superoxide dismutase
TLC	-	Thin layer chromatography
TPC	-	Total phenolic content
TPTZ	-	2,4,6-Tripyridyl-s-triazine
UV	-	Ultra violet
VO	-	Volatile oil
v/w	-	Volume per weight
WDM	-	Wells diffusion method
w/w	-	Weight per weight
WHO	-	World Health Organization
Wt.	-	Weight
ZOI	-	Zone of inhibition

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Chapter I

Introduction

1. General Introduction to medicinal plants

Life on earth depends on plants, and the first interaction the ancient human had have was with plant and plant products, since they relied completely on plant for maintaining their life on earth. Hence, our ancestors as the ancient humans at the beginning of their life adopted a plant-based (e.g. herbivorous) diet (Ansari, 2008). Plants sources are used since immemorial time by mankind to alleviate or cure illnesses (Amiri and Joharchi, 2013; Dolatkhahi et al., 2014; Gupta et al, 2012), and hence, for thousands of years plants have been the unique variable source of drugs (Aarti, 2014). Plants are really the most kindhearted creatures of the almighty Allah's. Because plants have provided life facilities for our ancestors, serve for us and definitely will do the same for all next generation, and hence they really worth to be called "*the main life-saving organisms*".

According to WHO estimation more than 80% of world populations still in case of their primary health care problems refer to only traditional medicines including medicinal plants, animals and mineral-based medicines, spiritual therapies and manual techniques or exercises (Bimlesh Kumar et al., 2011; Packer et al., 2004; Raju et al., 2013). Herbal drugs still retain their dignity even in this modern era. Plant-derived medicines comprise about 25% of drugs mentioned in modern pharmacopoeia (Kori, 2000). In many countries, special attention is paid to the values of ancient therapy systems based natural drugs.

China has written in its Constitution, that both Chinese medicine and modern medicine should be developed in china. Hence, in China, hospitals throughout the country should supply both services. Likewise, in India, the government has added 10 medicines from both Ayurvedic and Unani systems into their national family welfare program. However, India own a good reputation of its ancient traditional therapy system, the Ayurveda. Presently, several number of traditional herbal formulations and massage oils are abundantly used in India (Packer et al., 2004).

That is to say, a renaissance of herbal medicine is observed even in developed countries of the world and the acceptance of herbal medicine is augmenting again. In England, medicinal herbalism is practiced as one of the most popular forms of all complementary medicine and in Germany about 65% of the community people use herbal medicinal products each year (Packer et al., 2004).

1.1. Research on plants

Almighty Allah has descended the ***Holy Quraan*** as a complete and multidisciplinary "Guide book" for us. It is mentioned in verse No. 191 of *Sura AL-I Imran*, chapter 3 of this holy book "*rabbana ma khalaqhta haza batela*" means "Oh our Lord, you have not created (all) this in vain",. So, as per said verse, all of the creatures in our surrounding including plants contain at least some advantages, but exploitation of those advantages are the main responsibility of mankind. Without doubt, the ***Holy Quraan*** is clearly encouraging us toward carrying on continuous processes of multidisciplinary researches and investigations including researches on plants.

Plant kingdom represents an enormous reservoir of biologically active compounds called phytochemicals (Harborne, 1998). Phytochemicals with variable structural complexity and biological activity support their source plants to retain their historical dignity (Tripathee et al, 2011). So many plant-based chemical substances are reported for several biological activities such as antioxidant activity, antimicrobial activity, anti-inflammatory activity, anti-arthritic, radio-protective activity, cytotoxic activity, analgesic activity, hypoglycemic, hepatoprotective, anti-ulcerogenic, antipyretic, anti-leishmaniasis, anthelmintic, general tonic, and so forth (Gupta et al, 2012).

Taking into account that medicinal plants provide an enormous and cheaper source of new lead structures, current researches are very much devoted to the phytochemical investigation of higher plants (Harborne, 1988; Tripathee et al., 2011). Despite fast going researches in field of Phytochemistry, yet only a small portion of the total number of plants species (estimated to be around 200,000 to 250000 species of flowering plants) has been investigated (Trease and Evans, 2009). Luckily, the field of medicinal plants research and investigations in order to explore natural lead compounds is really very wide and is considered to be the most promising approach (Harborne, 1988; Gupta et al, 2012).

1.2. Afghanistan's medicinal plants

Afghanistan as a mountainous country in Asia is unique in term of its geo-climatic condition and owing to this specificity has a rich flora and fauna as parts of its natural resources. Diverse geological condition and climatic variation of this country has bestowed it a unique plant biodiversity. Although, Afghanistan's flora includes totally around 5000 plant

species (Sufiq Yunos, 1982), there is no enough publications available regarding pharmacognostic investigation of medicinal plants grow in Afghanistan.

In order to introduce only some important medicinal plant families of Afghanistan flora, few families along with popular members are enumerated in the following:

1. Compositae (Asteraceae): includes almost over 400 member species out of which 100 species pertain to *Artemisia* spp. *Taraxacum spp.*, *Silybium spp*., Chamomile, *Calendula spp.*, *Cichorium intybus*, etc.

2. Leguminosae (Fabaceae): comprises around 350 species such as; *Alhagi spp*., *Astragalus spp., Glycyrrhiza spp*., *Trigonella foenum-graecium*, *Medicago spp.*, etc.

3. Cruciferae (Brassicaceae): comprises around 200 species such as *Brassica spp*., *Raphanus sp*. etc.

4. Graminae (Poaceae): comprises more than 150 species including crop plants (wheat, corn, rice, sugarcane, etc.) as well as medicinal plants (e.g. *Cymbopogon spp*.)

5. Lamiaceae: comprises more than 160 species such as; *Mentha spp*., *Nepeta spp.*, *Thymus spp*., *Salvia spp*., *Origanum spp*., etc.

6. Umbelliferae (Apiaceae): comprises about 100 species such as; *Ferula spp.*, *Carum curvi L.*, *Cuminum cyminum, Anisum sativum*, *Foeniculum vulgare*, *Coriandrum sativum*, *Heracleum spp., Prangos pabularia* Lindl. etc. (Pimenov et al., 2014).

7. Liliaceae: comprises *Allium spp.*, *Eremurus spp*., *Iris spp*., etc.

8. Solanaceae: such as *Hyoscyamus spp., Datura spp*., *Solanum spp.*, *Withania spp.*, etc.

9. Polygonaceae: such as *Polygonum spp.*, *Rheum spp.*, *Rumex spp.*, etc.

10. Rosaceae: such as *Amygdalus spp.*

11. Iridaceae: such as *Crocus sativus.*

12. Ephedraceae: like *Ephedra spp.*

13. Plantaginaceae: such as *Plantago spp.*, and so forth.

However, because of the continuous anarchies and some critical political situation in Afghanistan since last few decades, floristic and pharmacognostic studies of its flora and medicinal herbs were almost ignored (Pimenov et al., 2014).

Assuredly, both the botanical diversity and geo-climatic variations of Afghanistan provide us with the wide opportunities of pharmacognostic researches on medicinal plants grow in this country. It is very much expected that Afghanistan's flora and especially its indigenous plants will award us potent and biologically active compounds which will serve as novel remedies.

1.3. *Heracleum afghanicum* Kitamura

Heracleum afghanicum Kitamura belongs to the genus *Heracleum*, family Umbelliferae, is an endemic plant of Afghanistan and grows wildly in different cold and mountainous regions of the country (Karimi and Ito, 2012; Pimenov et al, 2014).

1.3.1. Botanical description

Heracleum afghanicum is a fast growing perennial robust herbaceous plant, with an erect hollow stem up to 5 cm in diameter at the bottom and up to 1.5 m tall (Figure 1.). Its leaves are alternate, petiolate, large, broad and having 5 to 7 lobes. Its flowers are white in colour and aggregated in large flat and compound umbels up to 30 cm in diameter. The marginal flowers of the umbel are larger than those located in the center (Figure 2.). The plant starts blooming from April to August according to climatic condition of its natural habitat. As in cooler regions the plant blooms latter. The fruits are obovate achenes up to 8 mm long, and often mature in July and August. Fruit ripening also depend upon climatic condition (see Figure 3.).

1.3.2. Common local names

Local rural people call *H. afghanicum* by several vernacular names such as; Balderghan (Karimi and Ito, 2012), Balderghoo, Sofi and Safid Sarak (personnel communication with common villagers).

1.3.3. Geographical distribution

H. afghanicum prefers wet and cool mountainous regions rich in water and also it prefers sea sides and rivers sides (Figure 4.). The plant grows abundantly in some regions of different provinces of Afghanistan, which have aforesaid criteria or geo-climatic specification

such as; Schibar Pass of Badakhshan, north slope of Hindu Kush (Pimenov et al., 2014), Bamyan, Holang of Parwan, Darrah-i-Fringel of Parwan, Paghman of Kabul, Sang-lakh of Maidan Wardak, Khoshi of Logar, etc. (Karimi and Ito, 2012; Logacheva, 2007). However, data about geographical distribution of the plant in Afghanistan is still incomplete.

Figure 1. *Heracleum afghanicum* Kitamura with entire stem
(Photo was taken by Author in)

Figure 2. *Heracleum afghanicum* Kitam., inflorescence (A) and Leaf (B)

(Photo was taken by author)

Figure 3. Premature fruits of *Heracleum afghanicum* (Photo was taken by Author).

Figure 4. *Heracleum afghanicum* Kitamura in local habitat Holang, Afghanistan. (Photo of the author at the field was taken by his friend M. Saber)

1.3.4. Traditional usages

Heracleum afghanicum Kitamura has been traditionally used in rural regions of Afghanistan, by local people for several medicinal and non-medicinal purposes. However, sufficient published data regarding traditional usage of this plant is not available till now. *Heracleum afghanicum* recently has been reported for its fruits' volatile oil sedative effect on mice (Karimi and Ito, 2012) (see chapter II).

1.3.4.1. Medicinal usage of *H. afghanicum*

Young stem (before blooming of the plant) of *H. afghanicum* is however eaten as refreshing agent, but it is also claimed that it acts as controlling agent for high blood pressure (personnel communication). The young stems are also used as a digestive aid. *H. afghanicum* leaves are used as pain killer and antipyretic (Karimi and Ito, 2012). Fruits of the plant are collected in August and during early autumn. The aromatic fruits are used in culinary, and as carminative and appetizer. Rural people are using the fruits for soothing common cold, asthma and respiratory disorders. Lactating mothers use *H. afghanicum* seeds for increasing their lactation, thus fruits are claimed to have strong lactogenic activity (personnel communication).

1.3.4.2. Non-medicinal usages

H. afghanicum mature fruits are used as spices. Young stems (before blooming) of the plant are edible (Karimi and Ito, 2012). Younger leaves of the plant are used in preparation of spices. Moreover, *H. afghanicum* serve as a forage plant, and rural farmers collect mature leaves of the plant as animal feeds for feeding cattle, cow, horses, sheep and goats. Local farmers claim that *Heracleum afghanicum* leaves increase the milk productivity of cows and other animals fed by the leaves. *Heracleum* seeds are also used as spice by local people.

Heracleum afghanicum Kitamura like many other medicinal plants grow abundantly in some regions of Afghanistan. Despite chromatographic analysis and sedative activity evaluation of *H. afghanicum* fruits volatile oil on mice spontaneous locomotor activity (Karimi and Ito, 2012), published data regarding this plant is still very scarce. In the other hand, unfortunately, since almost three decades of war and political sever situation, Afghanistan's flora were almost ignored to be investigated (Pimenov et al. 2014). Thus, based on two important reasons; first – the importance of pharmacognostic investigation of a medicinal plant from ignored Afghanistan and, second – novelty of the work, *H. afghanicum* was selected for the title of present work. As far as ascertained, phytochemical screening and biological acidity evaluation of *H. afghanicum* leaves different solvent extracts and volatile oil is reported for the first time in present study.

Chapter II

Review of literature

2. Review of literature

Mankind possess unique intellectual, cultural, and communicative abilities. Humans are able to think, abstract reasons, and to apply the foundational logical principle for improvement their surrounding and all life necessities. Human mind alone develop propositions, formulate arguments, figure out inferences, identify universal principles, and value logical and rational subjects, coherence and truth. Human being by virtue of their unique aptitude not only are responsible to think for the current life improvement, but also have the responsibility to understand how life on earth have evolved and how to pave the ways for further improved, safe, and healthy life of next-coming generation, on Earth.

Fortunately, ample of munificent multidisciplinary resources are available which bestow us invaluable data to enhance our knowledge and understanding. Moreover, based on available knowledge one can easily grasp solutions for ever current as well as future problems. Taking into account the scope of this research project, not the complete but a little portion of related prior knowledge was reviewed as discussed later in this Chapter.

2.1. Plants as natural sources of drugs

Human life on Earth, started millions of years back, and without doubt, plants were the only supplier for maintenance of life on earth. Plants not only provide foods and shelters, but they also produce drugs in term of different phytopharmaceuticals and metabolites. However, the way how we use plants and how our ancient ancestors were using them, have got so many changes. Currently, still majority of people worldwide are using plants and plant product for their primary health problems.

Phytochemicals are chemical constituents synthesized by plants and deposited in their different cells and tissues. These constituents exert different pharmacological activities and hence play important role as curing agents. Phytochemicals are either used directly as drugs or they are used indirectly as lead compounds in drug development (Tripathee et al., 2011; Harborne, 1988).

Plant kingdom comprise around 200,000 to 250000 flowering plants species out of which just a small number are studied so far. Thus, a huge number of plants are still waiting for being analyzed and explored (Trease and Evans, 2009). Particularly, medicinal plants

growing in developing countries are still intact and are not investigated for their chemical and pharmacological profile (Amiri and Joharchi, 2013).

The continuous increase in world population in one hand, and the concomitant spread of severe diseases such as viral and infectious diseases, cancers, coronary and hearth diseases, etc. in other hand both oblige the medical scientists to strive in exploring new natural sources in order to overcome the aforesaid challenges. Luckily, lots of works have been done and ample of researches are going on biological evaluation of phytochemicals in multiple institutions and pharma industries (Packer et al., 2004).

Fortunately, the recent modern and sophisticated technological tools and qualified analytical approaches make more facile the investigation process of natural resources including plants. However, plants provide a wide area of researches with fruitful results and this field is really a promising approach (Gupta et al, 2012).

2.1.1. Some phytochemicals as potent drugs

Medicinal plants have shown to be the cheapest sources of new chemical entities (NCEs), which have been used in development of potent drugs. For instance, vincristine and vinblastine (from *Vinca roseus*), artemisen (from *Artemisia annua* L.) taxol (from *Taxus brevifolia*) (Harborne, 1988), and podophyllotoxin (from *Potophylum peltatum*), etc are phytochemicals reputed for being potent drugs for treatment of some critical diseases. Vincristine, vinblastine and taxol are potent anticancer drugs. Artemisinin as a reputed plant-based antimalarial drug, plays an invaluable role in the treatment of malaria (Kori, 2000; Packer et al., 2004). As per WHO, after the year 2001, it was strongly suggested that artemisinin-based combination therapy (ACT) shall be used in treatment of uncomplicated malaria produced by *Plasmodium falciparum*. Malaria produced by *P. falciparum* was hard to be eradicated by the monotherapic approach, but combination of both synthetic drug and artemisinin effectively treat malaria.

Nowadays, several ACTs produced by pharmaceutical industries are available in the market. Artesunate-mefloquine, artesunate-amodiaquine and artesunate-pyronaridine are examples of ACTs developed in fixed-dose combinations (FDCs), and recently produced and marketed (WHO, 2006).

Similarly, numerous modern medicines and pharmaceutical formulations originated from plants are available in today's market (Moazzem Hossen et al, 2014). For instance, an

extract of the ornamental tree *Ginkgo biloba*, which is rich in flavonoids (e.g. rutin, kaempferol, quercitin and myricetin), has been used since many years. The extract showed potent antioxidant activity in vitro (Cadenas et al., 2002). A standardized extract of Ginkgo by the name of EGB 761 (Extrait de *Ginkgo biloba* No. 761) is used in France, Germany and the United States, since more than 30 years. The standardized extract contains 24 flavonoids and 6 terpene lactones (Packer et al., 2004).

2.1.2. Market value of herbal/natural medicine

Medicines from natural sources have their own market values in different parts of the world. More than 30% of worldwide drug sale is reported to be accounted for drugs derived from plants and microbes (Goel et al. 2010). Annual sales of herbal medicines in the United States exceeds US$350 million. The global market for all herbal and homeopathic medicines has been accounted to be over US$4 billion in the United States, over US$6 billion in Europe and over US$2 billion in Asia. However, the World Conservation Union estimated that the annual global market for herbal products was about US$60 billion in 1997. In 1999, the global market for herbal medicinal products was around $19 billion. World Bank predicted that the market value of herbal drugs will expand up to US$5 trillion, by 2050 (Packer et al., 2004). Hence, it seems that even the modern world population are referring once again to medicinal plant-based therapy systems (Dolatkhahi et al., 2014).

2.2. Free radicals, oxidative stress and antioxidants

2.2.1. Free radicals

Any atom or chemical species capable of independent existence and having one or more unpaired electrons is defined a free radical (Cadenas et al., 2002; Kalita et al., 2013; Rehman et al., 2013). Free radical can be formed when a non-radical either gains or losses a single electron and continue to exist with that odd or unpaired electron. The odd or unpaired electron of free radicals is highly reactive as it seeks to pair with another free electron (Acworth et al., 2003; Cadenas et al., 2002).

Free radicals based on whether ($O^{\cdot}$) or nitrogen ($N^{\cdot}$) acts as their active centers, fall into two categories namely; reactive oxygen species (ROS) and reactive nitrogen species (RNS). In many books and literatures, both ROS and RNS are given a shared name “pro-oxidants” (Acworth et al., 2003).

2.2.1.1. Reactive oxygen species (ROS)

Oxygen centered free radicals are known as reactive oxygen species (ROS) (Packer et al., 2004; Rehman et al., 2013). ROS also include some non-radical oxygen containing reactive molecules. Examples of oxygen centered free radicals (ROS) are: superoxide anion ($O^{\cdot}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($OOH^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$), and so on. Non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3) singlet oxygen (Acworth et al., 2003; Amiri, 2012).

2.2.1.2. Reactive nitrogen species (RNS)

Free radicals having nitrogen atom (along with or without oxygen atom), are known as reactive nitrogen species (RNS) (Amiri, 2012). RNS includes nitric oxide (NO), peroxynitrite ($ONOO^{\cdot}$), nitrogen dioxide (NO_2), etc. (Acworth et al., 2003; Kalita et al., 2013; Rehman et al., 2013).

2.2.1.3. Production of free radicals in the body and their biological significance

In human body, free radicals are normally produced during normal metabolism and energy production or cellular respiration. The process of energy production in living cells/organisms occurred via a variety of oxido-reduction reactions also called *redox*-reactions. These oxido-reduction reactions occur in every living cell, and hence, are inevitably coupled with generation of certain pro-oxidants (ROS and RNS) in the cells. Moreover, other known factors stimulating free radicals production in body cells are; activated phagocytes, cigarette smoke, ionizing and ultraviolet radiation, drugs, pollutants, pesticides and toxins (Amiri, 2012; Borkataky et al., 2013; Cadenas et al., 2002; Packer et al., 2004; Rehman et al., 2013).

Free radicals are involved in many physiological and pathological processes (Borkataky et al., 2013). Appropriate levels of most pro-oxidants are beneficial to the body but their excess amount causes health problems. For example superoxide in suitable amount, helps the body in its defense against invading pathogens, but its excess amount causes health problem and oxidative stress (Acworth et al., 2003). For maintenance of normal health, a well-balanced level between pro-oxidants and antioxidants in the body is necessary (see Figure 5). A normal and healthy body will always owe some endogenous antioxidant defense systems (e.g. such as superoxide dismutase, the glutathione peroxidase/glutathione system, catalase, and peroxidase) which inhibit excess production of pro-oxidants and their damaging effects.

For example; the enzyme superoxide dismutase (SOD) is responsible for maintaining the cellular level of superoxide in body cells (Acworth et al., 2003; Cadenas et al., 2002; Packer et al., 2004).

Excess amounts of pro-oxidants (ROS and RNS) cause oxidative damage of body biomolecules (e.g. proteins, lipids, enzymes and DNA molecules), and ultimately alter the normal body functions. Pro-oxidants are proved to contribute in the etiology of various ailments such as cancer, diabetes, hearth related diseases, autoimmune disorders, mutagenesis, carcinogenesis, neurodegenerative diseases, premature aging, etc. (Amiri, 2012; Borkataky et al., 2013; Cadenas et al., 2002; Kalita et al., 2013; Packer et al., 2004 ; Rehman et al., 2013).

2.2.2. Oxidative stress

As discussed earlier, a normal and healthy body bears suitable antioxidant defense systems which control overproduction of pro-oxidants and maintain physiologically balanced level of pro-oxidants vs. antioxidants in body cells (Figure 5.). Overproduction of pro-oxidants if not controlled, results to a decreased level of endogenous antioxidants and a failure of antioxidant defenses in the body. Failure of body antioxidant defense systems, may further lead to tissue damage and oxidative pathologic condition known as oxidative stress (Acworth et al., 2003; Amiri, 2012; Borkataky et al., 2013; Packer et al., 2004).

Briefly; the term oxidative stress refers to the altered status of cell and tissue antioxidants by exposure to oxidants (Cadenas et al., 2002). Oxidative stress subsequently leads to other diseases such as neurodegenerative diseases, liver diseases, Alzheimer's disease, Parkinson's diseases, apoptosis (a regulated cell death), arthritis, atherosclerosis, cardiovascular diseases, AIDS/HIV, rheumatic diseases, cancer, diabetes, inflammation, viral infection, etc. (Acworth et al., 2003; Alok et al., 2014; Gerber, 2000; Packer et al., 2004).

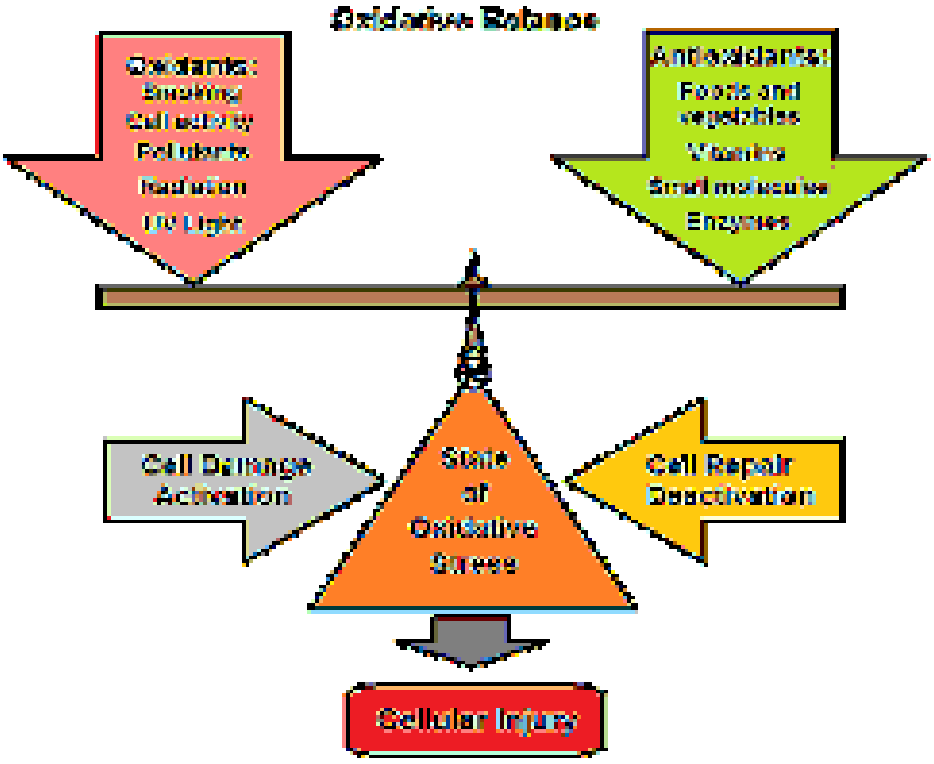

Figure 5. ***Oxidative balance between pro-oxidants and antioxidant species.*** *Under normal condition the level of pro-oxidants produced in the body is balanced by antioxidants. In case of over production of pro-oxidants, body antioxidants will not suffice to defense against damaging effects of pro-oxidants and hence, oxidative stress and cellular injury are coming off.*

2.2.3. Antioxidants

Antioxidants are compounds capable of scavenging free radicals and preventing or reducing their damaging effects on body biomolecules (e.g. lipids, proteins, enzymes, carbohydrates and DNA) (Kalita et al., 2013). Chemically, antioxidants are a diverse group of both natural as well as synthetic compounds. Based on their solubility they can be classified into water soluble antioxidants (e.g. albumin, ascorbic acid, GSH, plant phenolics, flavonoids, etc.) and lipid soluble antioxidants (e.g. carotenoids, vitamin K., tocopherols, bilirubin, etc.) (Acworth et al., 2003).

Based on origin, antioxidants can be endogenous and exogenous antioxidants. Endogenous antioxidants are present in the body (e.g. albumin, SOD, GSH, bilirubin, etc.), while exogenous antioxidants are of either synthetic or natural origins, particularly plants. Some phytochemicals such as tocopherols, flavonoids, coumarins and phenolic compounds

are natural exogenous antioxidative substances, found abundantly in plant kingdom (Acworth et al, 2003; Cetkovic et al., 2003).

Plant phenolics and polyphenols (e.g. ellagic acid) and flavonoids as natural antioxidants have several beneficial activities such as; metal chelating, reducing capability, hydrogen donating and radical scavenging and inhibiting chain reactions (Acworth et al., 2003; Akter et al., 2014; Amiri, 2012; Nayeem and Karvekar, 2010).

The antioxidative and free radical scavenging effects of flavonoids are attributed to their unique molecular structure importantly the position of phenolic hydroxyl (-OH) groups and other features in their molecule (Borkataky et al., 2013; Kalita et al., 2013; Packer et al., 2004; Rehman et al., 2013).

2.2.3.1. Role of antioxidants in prevention of diseases

Epidemiological studies show that a high dietary intake of antioxidants (e.g. dietary flavonoids in fruits and vegetables) and an augmented antioxidant status of body cells are contributing to reduce the risk of several diseases such as; cardiovascular diseases, carcinogenic diseases, rheumatoid arthritis, diabetes, etc. (Borkataky et al., 2013; Cadenas et al., 2002; Gerber, 2000; Packer et al., 2004; Thaipong et al., 2006). So, dietary supplementation of antioxidants is required in order to maintain body health (Amiri, 2012).

Although, the body owing to their endogenous antioxidant defense systems resist against hazardous effects of free radicals, but it will not suffice to diminish the damage entirely. Therefore, taking exogenous antioxidants of both natural (medicinal herbs and diet) and synthetic origins are imperative for protection of the body from hazards of oxidants (Packer et al., 2004; Rehman et al., 2013). Natural antioxidants of plants origin, based on their importance in maintaining normal health, are attracting attention of today's researchers worldwide (Amiri, 2012; Borkataky et al., 2013; Rusaczonek et al., 2007).

2.3. Medicinal plants as a source of antimicrobials

Microbial infections are the main cause of mortality and morbidity of human as well as animals worldwide. A large number of pathogenic bacteria and fungi are responsible for microbial infections and create a health burden worldwide. Medicals researchers are continuously carrying investigation for exploring and developing high quality medicines with

high efficacy and lowest or no toxicity. Many of the drugs currently used in treatment of bacterial and other infections were first obtained from natural sources including medicinal plants (Rebecca, 2007).

It is reported that many of todays' available synthetic antibiotics used in control and management of microbial infections are either very expensive (McGaw et al., 2000) or become indolent against resistant microbial pathogens (Block et al., 2000). It is well known that, plant have been used in treatment of diseases and infections since long back and still are used in many parts of the world. This reveals that, phytochemicals such as phenols, flavonoids, tannins, alkaloids, glycosides, terpenes, saponins and phytosterols exert potent biological actions and contribute in many of pharmacological activities including antimicrobial action (Nayeem and Karvekar, 2010; Rebecca, 2007).

Antibacterial activity of medicinal plants extracts and their essential oils are well known since long back. During recent decades, many works have been done regarding antibacterial activity evaluation of medicinal plants. Fortunately, in many of recent investigations, resistant bacterial strains have shown susceptibility to tested plant-originated antibacterial compounds (Flamini et al., 1999; Saleem et al., 2015). The antimicrobial potential of plant extracts and oils are counted for their multidisciplinary and wide applications including food preservation, pharmaceuticals, alternative medicines and natural therapies (Hammer et al., 1999).

It worth mentioning that, fungal resistance against some available antimicrobial drugs such as Fluconazole is reported worldwide, and this is an issue of concern for both patients as well as physicians. In the other hand, the number of antifungal drugs are very less than antibacterial drugs, and hence, curing of fungal diseases is still considered as a big concern. However, in recent years more attention have been paid toward exploring antifungal impacts of medicinal plants. Recently, number of plants are reported for their antifungal activity in vitro (Nayeem and Karvekar, 2010).

2.4. Medicinal plants as a sources of anthelmintic drugs

Helminths or worms being multicellular intestinal parasites, are accounted as the common cause of human parasitic infection called helminthiasis (Mukherjee and Ghosh, 2010). Helminths are divided into two phyla – Nematohelminths or Nematodes (roundworms) and platyhelminths or trematodes (flatworms). Gastrointestinal nematodes (GINs) such as *Ascaris lumbricoides*, *Trichuris trichiura*, *Nectator americanus* and

Ancylostoma duodenale are considered as the main cause of helminthiasis in human, worldwide (Bimlesh Kumar et al, 2011; Mukherjee and Ghosh, 2010).

Worm infection or helminthiasis as big health challenge, is very common in many developing and under-developed countries. As per WHO over two billion people worldwide are suffering from helminthiasis. Moreover, it is predicted that by the year 2025 over 57% of human population in developing countries will be infected by helminthiasis (A. Kumar et al., 2010; Bachaya et al., 2009; Bimlesh Kumar et al, 2011; Zhu et al., 2013; http://www.who.int/topics/helminthiasis/en/ cited on 29th April, 2015).

Intestinal worms mainly cause intestinal disorders, abdominal discomforts and general weakness in infected hosts. Helminthiasis can cause morbidity and sometimes death, and contribute in several other health problems such as parasitic and microbial infections, impairment of cognitive process, provoking intestinal obstruction, rectal prolapse, and so on (Bimlesh Kumar et al, 2011; Iqbal et al., 2004, 2005; Zhu et al., 2013).

Although, GIN infection is conventionally treated with synthetic anthelmintics (Zhu et al., 2013), according to WHO only few medicines are available for treatment of helminthiasis (A. Kumar et al., 2010). Moreover, in many parts of developing countries the modern anthelmintics are not available for all people and they are costly too. By the way, most of the presently available anthelmintics have shown to produce some unwanted effects such as nausea, loss of appetite, vomiting, headache, abdominal pain and diarrhea (Eguale et al., 2007; Satrija et al., 1995). Furthermore, GINs of both humans and live-stocks are reported to gain resistance against most of presently available synthetic anthelminthic drugs (Coles et al., 2006; Jabbar et al., 2007; Bachaya et al., 2009).

Luckily, some medicinal plants having anthelmintic activity, are known to the people and they are used as natural anthelmintic remedies since long back. These plant-based natural remedies are thought to play prominent role in control as well as treatment of worm infections of both humans and live-stocks (Bachaya et al., 2009; Jabbar et al., 2007; Satrija et al., 1995). In addition, medicinal plants have proved to serve as best natural sources of cheap and affordable, potent and safe, easily available, biodegradable and environmentally friendly alternative drugs. Therefore, researchers worldwide are motivated to investigate medicinal plants for their anthelmintic activity (Bimlesh Kumar et al, 2011; Eguale et al., 2011; Iqbal et al., 2004, 2005; Zhu et al., 2013).

2.5. *Heracleum* species at a glance

The genus *Heracleum* belongs to family Umbelliferae (Apiaceae) and consists of around 120 – 125 species worldwide out of which 70 species reported from tropical mountains and north temperate regions (Dincel et.al., 2013; Kala, 2010). *Heracleum spp*. are morphologically very similar to each other (Jakubska-Busse et al., 2013) and most of them are aromatic biennial and perennial herbs (Trease and Evans, 2009).

2.5.1. General botanical description

Heracleum spp. have robust stem more than 1.5 m tall, and the most stout one is *Heracleum mantegazzianum* which grows usually from 2 – 3 m but in certain cases reach up to 4 – 5 m tall (Klingenstein, F., 2007). *Heracleum sosnowskoyi* grows from 1 – 3 m tall (Kabuce and Priede, 2010). *Heracleum* species have large dissected leaves, large umbel-shaped inflorescence (flower head) with diameter up to 40 cm in diameter (up to 1 m in case of *H. mantegazzianum*) and dry fruits called achene. The ripe fruits have a strong aroma.

Common English names for the genus or its species include *hogweed* and *cow parsnip*. *Heracleum mantegazzianum* is called Giant hogweed or Giant cow parsnip (Klingenstein, F., 2007), (http://www.answers.com/topic/hogweed#ixzz2znb7kfWI , cited on 24 April 2014).

2.5.2. Distribution of *Heracleum spp.*

Heracleum spp. are thought to have their origin in Middle East, and south of Caucasian, but as ornamental plant they are diffused even to Northern Europe (Asgarpanah et al., 2012) and now with around 125 species they are found all around the world (Dincel et al., 2013). Some members of the genus grow abundantly in different countries. For instance, 23 species are found in India (Divya Nambath et al., 2014), 10 species grow in Iran (Asgarpanah et al, 2012), 23 species grow in Turkey (Dincel et al., 2013). Similarly *Heracleum* species are reported in Afghanistan (Karimi ant Ito, 2012; Pimenov et al., 2014), Poland, Iraq, Nepal, Bhutan, Sri-Lanka, Burma, Pakistan, Bangladesh, etc. (Kala, 2010), (http://www.answers.com/topic/hogweed#ixzz2znb7kfWI , cited on 24 April 2014).

2.5.3. Traditional use of *Heracleum* species

The genus *Heracleum* is reputed all over the world for its traditional and modern uses. The word "*Heracleum*" either is derived from a Latin word "*Hêraclêus*" or it came from a Greek word "*Hercules*" which means "glory" or "Hera" (Kala, 2010). Based on our literature survey, it was confirmed that, *Heracleum* spp. encompasses around 120 to 125 species found

worldwide (Dincel et al., 2013), and some of them are traditionally used for different purposes in different regions of the world.

***H. persicum* Deaf. ex Fischer** known as Persian Hogweed is reported for its use in folkloric and traditional medicine. The plant has been used for several purposes in Asian countries and also in Middle East. In Persian folkloric medicine, the fruits are used as analgesic (for chest pain), digestive aid and carminative, while leaves are used as lactogen by nursing mothers. Other usage of the plant in Persian folk medicines include anti-anorexia, anti-dropsy, anthelmintic, diuretic, remedy for tonsillitis and carminative. The seeds and also the young stems of *H. persicum* are used in preparation of pickles (Asgarpanah et al., 2012; Kousha and Bayat, 2012).

2.5.4. Phytochemistry and biological activities of *Heracleum spp.*

Heracleum spp. are aromatic plants and are excellent source of essential oils. Number of *Heracleum* species have been evaluated for their phytochemical profile biological activities. However, *Heracleum* species are distributed worldwide, their phytochemical nature extensively vary because of variations in their geographical origins and environmental or climatic conditions (Karimi ant Ito, 2012; Karuppusamy and Muthuraja, 2011). Based on literature survey, number of publications are now available reporting chemical composition and biological activities of different extracts and volatile oils obtained from different parts of *Heracleum* species.

***Heracleum afghanicum* Kitamura** fruits essential oil has been reported for its phytochemical profile and sedative activity on mice (Karimi and Ito, 2012). The authors reported 1.5% (v/w) volatile oil content in *H. afghanicum* fruits and presence of about 33 compounds in the oil, based on GC-MS analysis. Aliphatic esters composed most of the oil components, hexyl butyrate (34.3%) and octyl acetate (21.1%) were the major components. Sedative effect of vapor inhalation of the oil on mice spontaneous locomotor activity is also reported by the authors (Karimi and Ito, 2012).

Heracleum persicum found abundantly in Iran, have been reported for presence of various phytochemicals in different extracts obtained from different parts of the plant. Presence of terpenoids, triterpenes, alkaloids and quercitin is reported in seeds of the plants while presence of different furanocoumarins (e.g. pimpinellin, isopimpinellin, bergapten,

isobergapten and sphondin) is reported in different parts of the plant including roots (Asgarpanah et al., 2012; Hajhashemi et al., 2014). Kousha and Bayat, (2012) have reported presence of hexyl butyrate (56.5%), octyl acetate (16.5%), hexyl-2-methyl butyrate (5.2%) and hexyl isobutyrate (3.4%) in *H. persicum* essential oil. *H. persicum* mixed leaves and flowers methanolic extract has shown potent antimicrobial activity in vitro (Kousha and Bayat, 2012).

Similarly, Mojab and Nickavar (2003) have analyzed *H. persicum* volatile oils using capillary GC and the GC-MS. The authors found that, viridiflorol (23.05%), elemol (3.63%), B-maliene (3.07%), spathulenol (3.34%) and 2-tetradecanol (3.38%) comprised the major constituents of *H. persicum* roots volatile oil, while *trans*-anethol was found to be the major component of *H. persicum* leaves essential oil (Mojab and Nickavar, 2003).

Heracleum siamicum is reported to yeild 1.25% (v/w) in its fruits. The oil was analyzed using GC-MS, around 25 compounds were identified in the oil. The main components of the oil were identified to be *n*-octyl acetate (65.30%), *o*-cymene (10.35%), limonene (7.52%), δ-2-carene (6.87%), *cis*-thujone (1.92%), isobornyl acetate (0.94%), *n*-octanol (0.73%), 1,8-cineol (0.62%), *n*-tridecanol (0.44%), and safrole (0.37%). Antimicrobial activity of the oil is also reported (Nijsiri et al., 2010).

Heracleum sprengelianum seeds, leaves and roots volatile oils are reported to be composed of about 44 compounds. The oils showed potent antioxidant activity (Karuppusamy and Muthuraja, 2011).

Heralceum lasiopetalum Boiss a plant indigenous to Iran, is reported for its fruits essential oil chemical composition and antioxidant activity. The oil reported to contain around 39 compounds, and the major constituents were found to be α-pinene (4.82%) and n-Octanol (6.5%) (Ghasemi et al, 2013a, 2013b). The essential oil was for its antioxidant potential in vitro, using DPPH assay. The assay revealed an IC_{50} of 0.027 for the tested oil (Ghasemi et *al.*, 2013a).

Heracleum platytaenium, a Turkish endemic plant is reported for presence of about eight furocoumarins (e.g. psoralen, xanthotoxine, bergapten, pimpinellin, isopimpinellin, sphondin, byakangelicin and heraclenol) in petroleum ether extract of the plant (Dincel et al., 2013).

Heracleum lasiopetalum a plant reported to be endemic to Iran, yields 0.35% volatile oil in its fruits. Ghasemi et al., (2013b) have analyzed the oil by gas chromatography and GC-MS and they reported presence of 39 different compounds in the oil. The main compounds of the oil were found to be Octanol acetate (34.48%), n-Octanol (6.5%), Hexanol (5.12%), alpha-Pinene (4.82%), 1,8-Cineole (2.18%), Z-β-Ocimene (2.10%), and some other trace (Ghasemi et al, 2013b).

Heracleum sprengelianum collected from Western Ghats of India, has been reported for its volatile oil's constituents and antioxidant potential of the oil. Based on GC-MS analysis, β-Pinene, beta-Phellandrene, 1,8- Cineole and ρ-Cymen-8-ol were reported as the main components of *H. sprengelianum* essential oils (Karuppusamy and Muthuraja, 2011).

Briefly; based on literatue survey, during recent years numerous *Heracleum* species have been investigated and reported for their diverse active substances and different biological activities such as sedative (Karimi and Ito, 2012), antioxidant (Asgarpanah et al., 2012; Ghasemi et *al.*, 2013a; 2013b; Karuppusamy and Muthuraja, 2011), antimicrobial activity (Kousha and Bayat, 2012; Nijsiri et al., 2010; Sadeghi et al, 2014), appetizer, carminative, anthelmintic (Amiri and Joharchi, 2013; Hajhashemi et al., 2014), analgesic (Hajhashemi et al., 2009), anticonvulsant, anti-inflammatory activities, antihyperlipidemic activity (Hajhashemi et al., 2014; Karuppusamy and Muthuraja, 2011) and so forth.

2.5.5. Phototoxicity of *Heracleum spp*.

Some *Heracleum* species are reported for their photo-phytotoxic compounds which produce phototoxicity and dermatitis in allergic people. Furanocoumarins present in these species are known to be the main photo-allergen compounds which cause phototoxicity.

Furanocoumarins present in sap of *Heracleum sosnowskyi* and *Heracleum mantegazzianum* are reported to be the main causes of phototoxicity in sensitive people, if their naked skin is contacted with the plant sap. Moreover, some other toxic compounds such as isobutyl isobutyrate, isoamyl butyrate, hexyl hexanoate, 1-hexadecanol etc. are also reported to be present in volatile oil of both *Heracleum sosnowskyi* and *Heracleum mantegazzianum* (Jakubska-Busse et al. 2013). However, production of furanocoumarins differ from species to species and in some plants may be induced by exogenous factors. For instance; *Heracleum mantegazzianum* naturally contains high amounts of psoralen, bergapten and xanthotoxin which causes phototoxicity. While *Apium graveolens* is normally free from

furanocoumarins and only produces these compounds when is infected by fungal parasite *Sclerotinia sclerotiorum* (Katarzyna and Anna, 2012).

It should be noted that furanocoumarins despite causing phototoxicity, are also reported for their several beneficial biological activities such as anthelmintic effect (Jabbar et al., 2007), antimicrobial effects, antiproliferative effects and anti-psoriasis effect. Linear furanocoumarin (psoralen) is medicinally used in treatment of psoriasis and vitiligo, because psoralen stimulate skin pigmentation and promote synthesis of melanin, the skin pigment (Katarzyna and Anna, 2012).

As far as ascertained, no scientific publication except the work of Karimi and Ito, (2012), is available regarding *Heracleum afghanicum* Kitamura. However, the plant is used for several medicinal and non-medicinal purposes by local people (as discussed earlier under subsection 1.3.4.1 and 1.3.4.2. of Chapter I). As per our extent of knowledge, work on *H. afghanicum* Kitamura is considered to be a novel research and hence, it was selected for this project. It is very much hoped that this project will definitely open several other doors for further researches on the same plant.

Chapter III

Aim and Objective of the study

3. Aim and objective of the study

Present work entitled "**Phytochemical and biological evaluation of *H. afghanicum* Kitamura**" was planned to study phytochemistry and in vitro biological activities of the plant endemic to Afghanistan. However, as the title implies this project encompasses two goals namely; phytochemical screening of *H. afghanicum* leaves and its biological activity evaluation (in vitro). The main objectives of this project can be divided into two main sets of objectives; 1). Current objectives, and 2) Long-term objectives.

3.1. Current objectives

Taking into account the plan set for this project, two main goals are covered under the title. First, to figure out the phytochemical profile of *H. afghanicum* leaves by performing qualitative phytochemical tests, and second, to evaluate the crude drug for some of its biological activities using in vitro methods. In order to achieve these goals the following objectives were set as current objectives of present study:

1. Extraction of H. *afghanicum* leaves volatile oil

    1.1. GC-MS analysis of *H. afghanicum* leaves volatile oil

    1.2. Biological activity evaluation of *H. afghanicum* leaves volatile oil

        1.2.1. Evaluation of antioxidant activity of *H. afghanicum* leaves volatile oil.

        1.2.2. Evaluation of antimicrobial activity of *H. afghanicum* leaves volatile oil.

        1.2.3. Evaluation of anthelmintic activity of *H. afghanicum* leaves volatile oil.

2. Preparation of *H. afghanicum* leaves different solvent extracts

    2.1. Phytochemical screening of the extracts

    2.2. Biological activity evaluation of the extracts

        2.2.1. Evaluation of antioxidant activity of *H. afghanicum* extracts

        2.2.2. Evaluation of antimicrobial activity of *H. afghanicum* extracts

        2.2.3. Evaluation of anthelmintic activity of *H. afghanicum* extracts.

The overall plan and objective of the study is depicted in Figure 6.

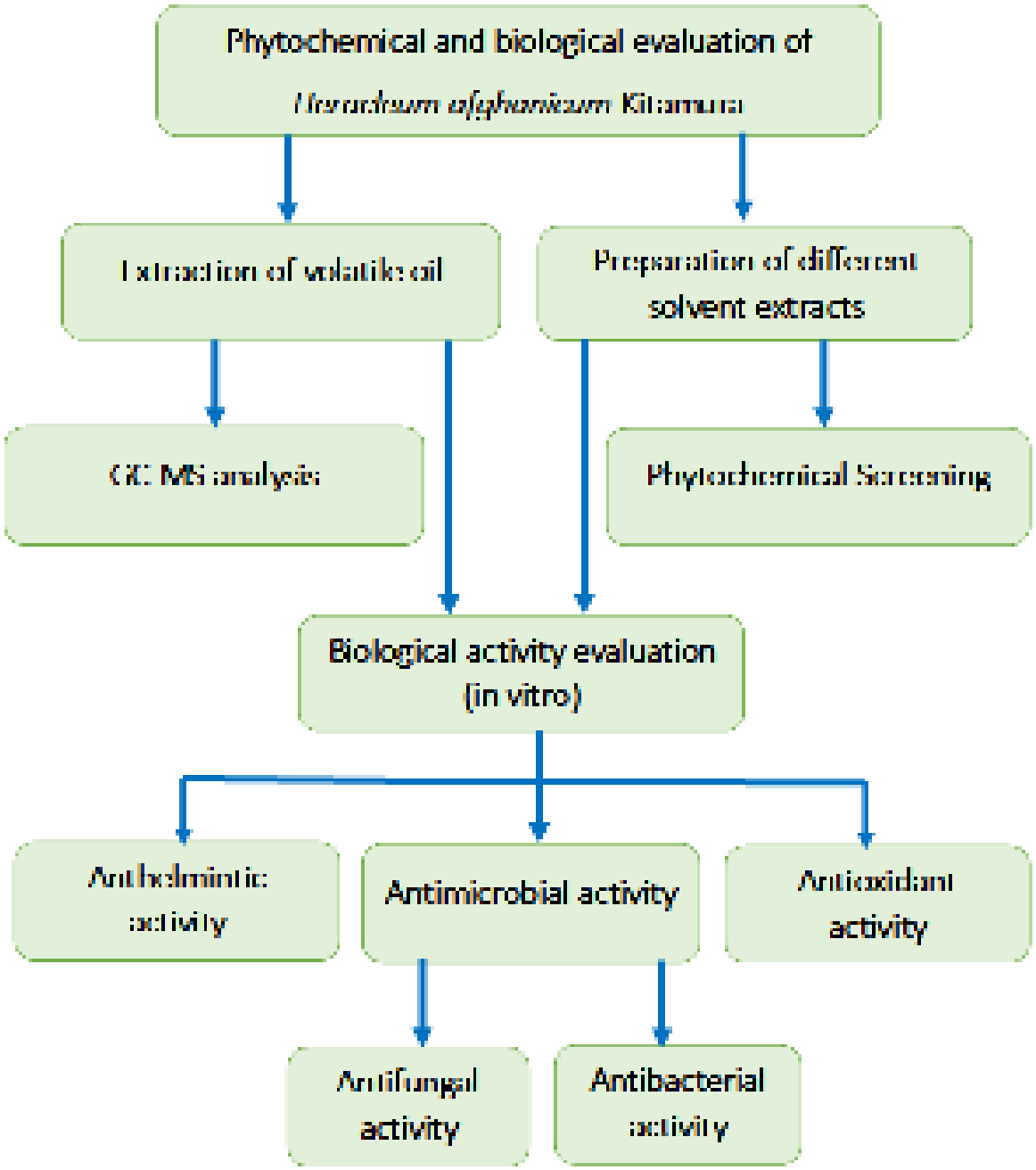

Figure 6. Flowchart of phytochemical and biological activity evaluation of *H. afghanicum* leaves.

3.2. Long-term objectives

As per our extent of knowledge, this study entitled "Phytochemical and biological activity evaluation of *H. afghanicum*" represents a novel work, and it is highly anticipated that new windows for subsequent researches will be opened. By compiling overall results and

outcomes of the study, the following items could be enumerate as long-term objectives of our research project:

1. Evaluation of further in vitro and in vivo biological activities of *H. afghanicum* Kitamura.

It is well known that medicinal plants contains a diverse array of bioactive phytochemicals based on which they exert different biological impacts. Since, present study on *H. afghanicum* Kitamura may only report few envisaged biological activities (namely antioxidant activity, antibacterial activity, antifungal activity and anthelmintic activity), exploring other pharmacological activities of this plant will be considered as future researches. Taking into account the traditional uses and phytochemical profile of HAKL, further works are suggested below:

a. Evaluation of *H. afghanicum* anti-inflammatory activity

b. Evaluation of *H. afghanicum* anti-proliferative activity

c. Evaluation of *H. afghanicum* anti-neurodegenerative activity

d. Evaluation of *H. afghanicum* memory enhancer activity

e. Evaluation of *H. afghanicum* lactogenic activity

2. Determination and identification of *H. afghanicum* bioactive phytopharmaceuticals

Composition of medicinal plants can considerably vary with changes in geographical and environmental conditions of their habitat, their stage of growth, time of collection and their different organs (Sadeghi et al., 2014; Saleem et al., 2015). In present study, based on preliminary phytochemical screening, different classes of phytochemicals are qualitatively reported to be present in HAKL. Meanwhile, potent antioxidant effects, antimicrobial activity in vitro and anthelmintic activity in vitro of HAKL are reported in present work. Therefore, determination, identification and structural elucidation of those potent unknown phytochemicals are emphasized.

Briefly; present study has opened several windows regarding further work on *H. afghanicum* Kitamura.

Chapter IV

Methodology

(Materials and methods)

4. Material and methods

To begin with a research project and successfully completion of the same, following a set of systematic and step-wise procedures, with appropriate materials and equipments are required. Moreover, as the key criteria for achieving the goals of a research project, it is more important to conduct the series of envisaged experiments from very simple phase toward more complicated step in a systematic manner and under prescribed guidelines and recommendations.

Steps involved in practical aspects of this research project were started from the field trips in Afghanistan and continued through collection of plant material, authentication of collected material, preparing herbarium sheets, drying the collected materials, grinding and packing of the dried materials, transfer of dried packed material to India, preparation of extracts, extraction of volatile oil, performing preliminary phytochemical screening, GC-MS analysis of the volatile oil, biological activity evaluation, analysis of obtained results and compilation of the entire work along obtained data in form of a compiled document termed "Thesis or Dissertation". Obviously, handling of all aforesaid materials and procedures and compilation of relevant results demand a talented mind and sound technical hands to successfully handle them in an accurate scientific manner.

4.1. Chemicals

Chemicals used in this projects were of analytical and Lab. grade and of high purity procured from reputed standard commercial suppliers in India (Table1).

Table 1. Chemicals used during research work

S. No.	**Name of chemicals**	**Manufacturer**
01	Acetic acid	Qualikems, India
02	Acetic anhydride	Qualigens, India
03	Acetone	Loba Chemie, India
04	Albendazole Oral Suspension 200 mg/5ml	Ranbaxy, India
05	Alph-naphthol	SD fine Chem., India
06	Aluminium chloride anhydrous	Loba Chemie, India

07	Ammonia solution	Loba Chemie, India
08	Amphoteracin-B 30µg/disc	Himedia, India
09	Ascorbic acid	Loba Chemie, India
10	Benzene	Loba Chemie, India
11	Chloroform	Loba Chemie, India
12	Dichloromethane	Loba Chemie, India
13	DPPH (2,2-dichlorophenyl-1-picrylhydrazyl)	SIGMA-ALDRICH, Germany
14	Dragendroff's reagent	Molychem, India
15	Ethanol	Changshu Yangyuan, China.
16	Ethylacetate	Molychem, India
17	Ferrous sulphate. $7H_2O$	Loba Chemie, India
18	Ferric chloride	CDH, India
19	Fluconazole 10 mcg/disc	Himedia, India
20	Fluconazole IP (Capsule 150 mg)	Alembic Pharma., India
21	Formic acid	Loba Chemie, India
22	Folin-Cioclateu Reagent	Loba Chemie, India
23	Gallic acid	Loba Chemie, India
24	Gelatin	Loba Chemie, India
25	Gentamicin 10 µg/disc	Himedia, India
26	Gentamicin Sulfate Inj. IP (80 mg/2ml)	INTAS Pharma, India.
27	Glycine	Loba Chemie, India
28	Hexane	CDH, India
29	Hydrochloric acid	Molychem
30	Iodine	CDH, India
31	KI	Loba Chemie, India
32	Mayer’s reagent	Loba Chemie, India
33	Methanol	Qualikems, India
34	Ninhydrin	SD-fine, India

35	Nutrient agar	CDH, India
36	Nutrient broth	CDH, India
37	Petroleum ether 60-80	Qualikems, India
38	Picric acid	Loba Chemie, India
39	Potassium hydroxide	CDH, India
40	Potato dextrose broth	HIMEDIA, India
41	Pyridine	Loba Chemie, India
42	Resorcinol	CDH, India
43	Rutin	Loba Chemie, India.
44	Sabouraud Dextrose Agar	CDH, India
45	Silica gel G (for TLC)	Loba Chemie, India
46	Sodium carbonate (anhydrous)	Loba Chemie, India
47	Sodium chloride crys.	Loba Chemie, India
48	Sodium hydroxide (pellets)	CDH, India
49	Sodium nitrite	RANKEM, India
50	Sodium nitroprusside	CDH, India
51	Sterile disc	HIMEDIA, India
52	Sucrose	Loba Chemie, India
53	Sulphuric acid	Loba Chemie, India
54	TPTZ (2,4,6-triazino-s-triazine)	Loba Chemie, India

4.2. Instruments

Instruments used in this project are listed in Table 2.

Table 2. Laboratory instruments used during research work.

S. No.	**Name of instruments**	**Manufacturer**
1	Aspirator	MEREX, India
2	Clevenger apparatus	PERFIT, India
3	Desiccator	PolyLab, India

4	Digital weighing balance (0.1 mg sensitivity)	Shimadzu, Japan
5	Drying oven	NAVYUG, India
6	Electronic weighing balance	CONTECH, India
7	Heating mantle (Size 1000 ml)	LABFIT, India
8	Heating mantle (Size 3000 ml)	NAVYUG, India
9	Hot Air oven	NAVYUG, India
10	DOB incubator	NAVYUG, India
11	Incubator for Fungi	NAVYUG, India
12	Refrigerator	Kelvinator, India
13	Freezer	Blue Star, india
14	Rotatory vacuum evaporator	POPULAR, India
15	Soxhlet extractor (500 ml)	PERFIT, India
16	Soxhlet extractor (3000 ml)	PERFIT, India
17	Triple distillation unit	PERFIT, India
18	TLC jars	PERFIT, India
19	UV chamber	POPULAR, India
20	UV spectrophotometer (UV-1800)	Shimadzu, Japan
21	Vacuum oven	NAVYUG, India
22	Water bath	NAVYUG, India

4.3. Plant material

4.3.1. Collection of plant material

Heracleum afghanicum Kitamura leaves was collected during flowering stage of the plant in July 2014 from Holang village, Salang district, Parwan province, Afghanistan.

4.3.2. Authentication of plant material

The collected plant material was authenticated and identified as *Heracleum afghanicum* Kitamura by Prof. Dr. Noor Ahamad Mirazai Head of biology Dept., Faculty of Science, Kabul University and Head of Kabul University Faculty of Science (KUFS) herbarium.

4.3.3. Herbarium preparation

Since *H. afghanicum* is a robust plant, its herbarium sheets were prepared on three sheets serially numbered as 1H-I (bearing inflorescence or flower umbel), 1H-II (bearing leaf) and 1H-III (bearing fruits with a stem part) (Figures 7., 8, and 9.). Each of the sheets sheets were prepared in triplicates (total 9 sheets), one copy (3 sheets) was deposited in KUFS herbarium as voucher specimens and two other copies (6 sheets) were transferred to India along with their authentication certificate (certificates are attached at the end of thesis). Transferred herbarium sheets were submitted to Dept. of Pharmacognosy and Phytochemistry, School of Applied Medical Sciences, Lovely Professional University to be used as references in the future.

4.3.4. Drying of plant material

Heracleum afghanicum leaves collected from the field were shade dried in open air at room temperature for several days. The dried material was then grinded manually into a coarse powder, and passed through a sieve to prepare a uniform coarsely powdered material. The dried powdered material was then packed appropriately in black-colored polyethylene bags and was stored at room temperature in shade until being transferred to India. The packed materials (7 kg), were then transferred to Lovely Professional University, Punjab, India, on 9th august 2014.

Figure 7. Herbarium sheet No. 1H-I, bearing flower head of *H. afghanicum* Kitamura.

Figure 8. Herbarium sheet No. 1H-II, bearing leaf of *H. afghanicum* Kitamura

Figure 9. Herbarium sheet No. 1H-III, bearing fruits and a part of stem of *H. afghanicum*.

4.4. Extraction of *H. afghanicum* leaves

H. afghanicum coarsely powdered lives was subjected to successive extraction process using different solvents with different polarity. As we know, fat soluble phytochemicals and non-polar compounds are easily extracted with non-polar solvents, some phytochemicals may show good affinity with moderately polar solvents while polar phytochemicals will dissolve well in polar solvents. Thus, by successive extraction method using several solvents of different polarity, in one hand, all of the phytochemicals present in the raw material will be extracted and on the other hand, these phytochemicals will be somehow divided into several groups based on their affinity with different solvents used in extraction process. For a researcher it is more interesting to first get information about different solvent extractives of the same crude material and then to choose and/or evaluate comparatively biological activity of each individual extractive. If any extractive found with the highest potency, than that solvent can be used as solvent of choice for extraction of potent phytochemical(s) found to be the most active.

4.4.1. Preparation of *H. afghanicum* different solvents extracts

Five different solvents (including water) with different polarity strength, as shown below in order of increasing polarity, were used in successive extraction of HAKL using hot maceration method or Soxhlet extraction (Figure 10.).

Petroleum ether < Dichloromethane < Ethyl acetate < methanol < Distilled water

Where, polarity of the solvents increases from left to right, meaning petroleum ether as a non-polar solvent was used first in extraction, followed by dichloromethane, ethylacetate, methanol and distilled water.

4.4.1.1. Petroleum ether extract

100 gram shade dried coarsely powdered HAKL was placed in the extractor of Soxhlet assembly and was extracted with 2.5 liter petroleum ether (60 – 80^{o} C) at 70^{o} C temperature using Soxhlet apparatus (Figure 10.). Extraction was continued for 5 days (every day from 9 : 00 AM to 5 : 00 PM) until it was complete and the colour of solvent circulated via syphon tube of the apparatus, was completely faded. Then, the marc was dried in open air before being subjected to extraction with the next solvent. Petroleum ether extract having dark green colour was filtered through whatmann No.1 filter paper using suction filtration. The filtrate

was concentrated and the solvent was recovered using rotatory evaporator until a dark green concentrated extract was obtained. The concentrated extract was transferred into a clean, dried and tarred china dish (weight of the empty china dish was recorded) and was further evaporated at about 50^{o} C on water-bath. Finally, after evaporation of the solvent a semi-solid residue having dark-greenish colour was obtained. The semi-solid residue was further dried in hot air oven at 40^{o} C until a constant weight was obtained. The dried extract was kept in refrigerator at 4^{o} C until being used in further experiments.

Figure 10. Soxhlet extractor and hot maceration method of extraction.

4.4.1.2. Dichloromethane extract

The dried marc of previous step was extracted with 2.5 liter dichloromethane (DCM), at 45 – 50^{o} C temperature using Soxhlet apparatus. Extraction was continued for 5 days until was completed. Then, the marc was dried again at room temperature before being extracted with the next solvent. The dark green extract was filtered and concentrated by simple distillation of solvent on water bath until a blackish-green concentrated extract was obtained. The concentrated extract was transferred into a dried tarred porcelain dish and was evaporated on water-bath until a semi-solid residue was obtained. The semi-solid residue was

further dried in hot air oven at 40° C until constant weight. The weight of dried extracts was noted down and then, it was stored at 4o C in refrigerator until further use.

4.4.1.3. Ethylacetate extract

The dried marc of previous step was extracted with 2.5 liter ethylacetate (EA) at 70° C using Soxhlet apparatus, for 3 days, until was completed. The marc was again dried at room temperature and the dark green extract was filtered, then was concentrated at 50° C, using rotatory evaporator, until a blackish-green concentrated extract was obtained. The obtained extract was then transferred into a tarred china dish and was evaporated on water-bath until a blackish-green residue was obtained. Similar to previous extracts, the residue was further dried in hot air oven at 40° C until getting a constant weight for the semisolid extract. Then the weight of the dried EA extract was noted, and was kept in refrigerator at 4° C for further use.

4.4.1.4. Methanol extract

The dried marc of previous extractions was extracted with 2.5 liter methanol (MeOH) for 2 days using Soxhlet apparatus. Then, the marc was dried at room temperature to be finally extracted with distilled water. Methanol extract having a greenish-brown colour, was concentrated by rotatory evaporator at 50° C, until a dark brownish-green concentrated extract was obtained. This extract was transferred into a tarred china dish and was further evaporated on water-bath followed by drying in hot air oven at 40° C temperature until getting a blackish-green semisolid residue with a constant weight. The weight of the dried MeOH extract was noted and then, it was stored in refrigerator until further use.

4.4.1.5. Aqueous extracts

This time, the dried marc being extracted with four precedent solvents, was extracted with 2.5 liter distilled for 2 days using Soxhlet apparatus. Then, the marc was discarded and the brown colored aqueous extract, was concentrated by rotatory evaporator at 5° C, until a dark brown concentrated extract was obtained. This extract was transferred into a tarred china dish and was further evaporated on water-bath followed by drying in hot air oven at 40° C temperature until getting a blackish-brown residue with a constant weight. The weight of the dried aqueous extract was noted and then, it was stored in refrigerator until further use.

The experiments were repeated three times, every time colour and the constant weight of the dried extracts were noted down. The percentage yield (% w/w) of each solvent

extractives was simply calculated by subtracting the weight of tarred china dish from that of total weight of china dish plus dried extract (because 100 g crude drug was used in the extraction process and the yield directly represents the percentage value). The percentage yield (w/w) for each solvent extractive was recorded as mean±SD of the three % yields obtained during the three experiments. The results are tabulated in Table 4.

4.5. Hydrodistillation of *H. afghanicum* leaves volatile oil

H. afghanicum leaves volatile oil was extracted by hydro-distillation method using Clevenger-type apparatus (Figure11).

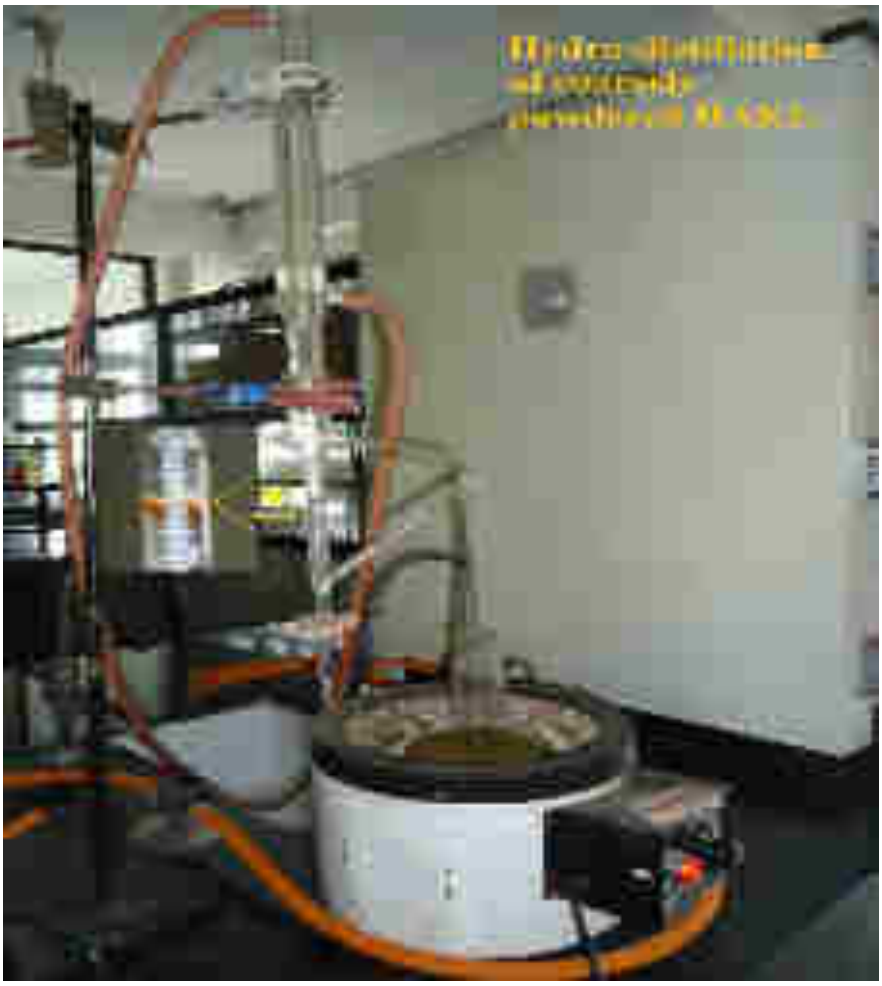

Figure 11. Hydrodistillation of essential oil using Clevenger-type apparatus.

Amount of 50 g coarsely powdered leaves was placed in a 1 liter round bottom flask (RBF), followed by addition of 600 ml distilled water. Then hydrodistillation was carried out and continued for about 4 hours until the volume of the collected volatile oil in the fleurentin tube (the graded tube) of the apparatus remain constant. Since the volatile oil was lighter than

water, it was collected as a yellow ring just above the collected water in the graded tube of the apparatus, while the excess of collected water was automatically directed back to the RBF via the designed connector tube of the apparatus.

Generally, after four hours of hydrodistillation process, the volume of collected oil in the graded tube was not increasing anymore, and hence, the plant material in RBF was considered to become completely free from volatile oil content. So, the heating was stopped and while the apparatus became cool, the content of RBF was substituted with another batch of 50 g leaves powder along with addition of 600 ml water. Then, the same process of hydrodistillation was stated again for another four hours as discussed above. By this way, volatile oil of 3 to 4 subsequent batches (50 g) of the powdered leaves were collected together in the graded tube and the amount (ml) of collected oil was noted from the readings of its volume in the graded tube.

The percentage yield (v/w) of *H. afghanicum* leaves volatile oil was calculated based on the volume of oil (in ml) collected in the graded collection tube of Clevenger apparatus and the amount (in gram) of the plant material used, applying the following formula:

$$\% \text{ VO} = \text{volume of VO (in ml)} / \text{weight of plant material (in g)}$$

Where, VO refers to volatile oil.

Thus, the volatile oil content of *H. afghanicum* leaves were found to be in range of 0.35 – 0.40% (v/w).

The collected volatile oil in graded tube of Clevenger apparatus, after every 3 to four days (4 to 5 run of hydrodistillation of different batches of plant material) was taken in a clean small Eppendorf tube and after being dried by few crystals of sodium sulphate, was transferred into brown-colored glass vials and stored at 4^{o} C in refrigerator until further use.

4.5.1. Physical characteristic of *H. afghanicum* volatile oil

The oil has transparent yellow colour and a sharp aromatic odour. The oil has lower density than water, and is easily soluble in methanol, ethanol, dimethyl sulfoxide (DMSO), petroleum ether, dichloromethane and ethyl acetate. However, exact density and solubility ratio of the oil was not determined.

4.5.2. GC-MS analysis of *H. afghanicum* leaves volatile oil

Amount of 1 ml *H. afghanicum* leaves volatile oil obtained by hydrodistillation were placed in brown colour vial, packed well and was sent for GC-MS analysis to IHBT research Lab., Palampur, Hemachal Pradesh, India, through courier. The sample was sent on 24th March, 2015 while it was reached there on 4th March, 2015. The volatile oil was analyzed at IHBT research Lab. using GC-MS and total 25 compounds were identified in the oil. The chemical constituents of *H. afghanicum* leaves volatile oil along with the percentage (peak area %) of the compound, adopted from the test report sent by IHBT, are presented in Table6. The test report in term of GC-MS chromatogram of *H. afghanicum* volatile oil was sent us by IHBT on 23rd April, 2015, and are attached as Appendix I at the end of this thesis.

4.6. Phytochemical screening of *H. afghanicum* leaves

4.6.1. Introduction

Medicinal value of a plant depends upon its bioactive phytoconstituents which are responsible for exerting pharmacological activity of source plant. Plants generally synthesize a diverse array of phytochemicals which may pertain to several chemical categories. Number of phytochemical qualitative tests (e.g. colour and precipitative tests) are adopted for qualitative detection or presence of various classes of phytochemicals in plant materials (Kokate, 2010; Houghton and Raman, 1998).

Preliminary qualitative determination of phytochemicals in crude samples, is considered as an indispensable step prior to isolation or quantitative identification phytochemicals (Harborne, 1988).

In present study, *H. afghanicum* leaves was subjected to preliminary phytochemical screening as discussed in the following:

4.6.2. Methodology

Prior to carry out phytochemical screening, small amount (10 mg) of *H. afghanicum* different solvent dried extracts (methanol and aqueous extract were dissolved in their own solvents) were separately dissolved in 10 ml DMSO to prepare a stock solution of 1 mg/ml. The prepared stock solution were then appropriately diluted with distilled water and were subjected to preliminary phytochemical screening. Different standard phytochemical

qualitative tests were performed using different colour and precipitative reagents as per described procedures (Ansari, 2009; Harborne, 1998; Houghton and Raman, 1998; Kokate, 2010; Trease and Evans, 2009).

4.6.2.1. Detection of alkaloids

Alkaloids give definite colour or specific precipitates with particular reagents. To detect alkaloids, small amount of test extracts were hydrolyzed by boiling for a while with few ml of dilute HCl. It was then, filtered through Whatmann No. 1 filter paper and the filtrate was subjected to the following tests (Ansari, 2009; Kokate, 2010; Trease and Evans, 2009).

a. **Mayer's test:** About 3 the filtrate was placed in a test tube followed by addition of 3 – 5 drop Mayer's reagent (solution of Potassium mercuric Iodide). It was observed for formation of a white, pale yellow or cream colored precipitate.

b. **Dragendorff's test:** About 3 the filtrate was placed in a test tube followed by addition of 3 drops of Dragendorff's test reagent (potassium Iodide + bismuth nitrate). Then, it was observed for formation of orange red/brown colour precipitate.

c. **Wagner's test:** About 3 the filtrate was placed in a test tube followed by addition of 3 to 4 drops of Wagner's test reagent (Iodine solution), and was observed for formation of a brown or reddish-brown precipitate.

d. **Hager's test:** A few drops of Hager's test reagent (Picric acid saturated solution) were dropped on to 3 ml of test filtrate in a test-tube, and observed for formation of characteristic crystalline yellow precipitate.

4.6.2.2. Detection of carbohydrates

For detection of carbohydrates the following qualitative tests were performed:

a. **Molisch's test:** About 3 ml of HAKL extract was placed in a clean test-tube followed by addition of 3 drops of Molisch's reagent (alpha-naphthol 20% in ethyl alcohol). Then about 2 ml of concentrated H_2SO_4 was carefully added into the test-tubes, along the side of test-tube. Formation of a reddish violet or dark purple colour ring at the junction of the two layers indicates presence of carbohydrates. Molisch's test gives positive result with both soluble as well as insoluble carbohydrates (Kokate, 2010).

b. Fehling's solution test: About 3 ml of test extract was placed in a clean test tube and to this an equal volume (3 ml) of a freshly prepared mixture (1:1) of Fehling A and Fehling B solutions was added. The mixture was shacked well and heated in water bath for a few minutes and was observed for formation of a brick red precipitate. Fehling's test is performed for detection of reducing sugars in the extracts (Kokate, 2010). Formation of the brick-red colored precipitate is due to formation of cuprous oxide which takes place in presence of reducing sugars (including all monosaccharides and also many disaccharides e.g. lactose, maltose, cellobiose and gentiobios) in the sample (Trease and Evans, 2009).

It should be noted that, in case of acidic extracts, prior to performing the test, neutralization of the extract by NaOH solution is required. Because in acidic environment Fehling's test will not give the result. Similarly, Fehling A and Fehling B solution should be mixed at equal proportions just at the time of performing the test.

c. Benedict's test: Two ml of HAKL extract was mixed with 2 ml of Benedict's reagent in a clean test tube. The mixture was heated on water-bath for few minutes and observed for formation of a yellow colored precipitate. Acidic extract needs to be neutralized before being tested.

d. Resorcinol test: A few small crystals of resorcinol was dissolved in about 2 ml of HAKL extract in a test-tube followed by addition of an equal volume concentrated HCl. The mixture was warmed on water-bath for a few minutes and was observed for formation of a rose colour. Resorcinol test is also known as Selivanoff's test and is performed for detection of ketoses (Trease and Evans, 2009).

4.6.2.3. Detection of glycosides

a. Legal's test: About 2 ml HAKL extract was treated with 2 ml of 2% solution of sodium nitroprusside in pyridine and few drops of 20% sodium hydroxide in methanol. The mixture was then observed for formation of pink to deep blood red colour. Legal's test is performed for detection of cardiac glycosides in plant extracts (Kokate, 2010).

b. Keller-Kiliani test: About 3 ml of test extract was mixed with 2 ml glacial acetic acid and 1 ml ferric chloride (5%) solution. The mixture was heated on water-bath, then cooled and was transferred to a test-tube containing 2 ml of concentrated H_2SO_4, and observed for formation of blue colour in acetic acid layer (Nair et al, 2013).

4.6.2.4. Detection of anthraquinone glycosides

a. **Borntrager test:** About 5 ml of HAKL acidic extract was extracted with 5 ml chloroform by mixing them in a separatory funnel and shaking for 15 minutes. Then, the mixture in the funnel was placed aside for being separated into two phases. The chloroform phase was taken in a clean test-tube and 2 ml of 10% ammonia solution was added to that. The mixture was shaken well, then it was stand till the two phases were separated. Appearance of a pink or reddish colour in aqueous phase indicates the presence of anthraquinones.

b. **Modified Borntrager's test:** About 3 ml of extract solution was mixed in a test tube with 1ml $FeCl_3$ 10% solution and 1 ml of concentrated HCl. The mixture was heated for a while, then cooled and filtered. The filtrate was shaken with chloroform in a separatory funnel and was left for a while for phase separation. Then, the chloroform phase was collected in a clean test tube and treated with 2 ml of 10 ammonia and was observed for appearance of a pink or deep red colour in the aqueous phase.

4.6.2.5. Detection of phenols

Phenols also called phenolic compounds embrace a wide range of plants substances which generally possess an aromatic ring bearing one or more hydroxyl substituents (Harborne, 1988).

a. **Ferric chloride test:** About 3 ml of HAKL extract was placed in a clean test tube followed by addition of 2 – 3 drops of 1% ferric chloride solution. It was observed for formation of an intense greenish-black colour.

4.6.2.6. Detection of flavonoids

Flavonoids change their colour when they are treated with alkali solutions (e.g. ammonia and/or NaOH solution). Thus, they are easily detected in plant extracts (Harborne, 1988).

a. **Alkali reagent test:** About 3 ml of HAKL diluted extract was taken in a test-tube and an alkali solution (e.g. solutions of NaOH, KOH ot ammonia) was added drop-wise on the extract. Along with addition of alkali, it was observed for increasing intensity of the yellow colour appeared by addition of alkali in the extract. The intensity of the formed yellow or orange colour will decrease back by addition of few drops of dilute HCl (Satheesh et al., 2012).

b. **Ammonia test:** A strip of filter paper was dipped in HAKL diluted extract and after being dried it was imposed to ammonia vapors. Appearance of an orange-red or yellow colour indicates the presence of flavonoids.

c. **Shinoda/Pew test:** About 4 ml of HAKL extract was placed in a clean test-tube and a small amount of magnesium turnings was added into that, followed by addition of 3 drops concentrated HCl. The test-tube was kept aside for completion of the reaction and after a while it was observed for formation of a reddish-pink or rose colour.

4.6.2.7. Detection of tannins

a. **Gelatin test:** About 2 ml of the HAKL extract was treated with 2 ml aqueous solution of 1% gelatin containing 10% sodium chloride and observed for formation of a white buff colour precipitate (a milky colour). However, suitable concentration of tannins (0.5 – 1%) in a test extract is required for revealing a positive result (Trease and Evans, 2009).

b. **Matchstick test for catechins:** A matchstick was dipped in HAKL extract and after being dried, was moisten with concentrated HCl. Then, it was warm near a flame of alcoholic lamp and was observed for appearance of a pink or red colour due to production of phloroglucinol.

   Catechins form phloroglucinol on heating with acids and are detected by performing a modified test for lignin (Trease and Evans, 2009).

4.6.2.8. Detection of saponins

a. **Foam test:** Small amount of HAKL extract was diluted with distilled water in a test-tube and vigorously shacked for a while. It was observed for formation of a persistent foam.

4.6.2.9. Detection of sterols/phytosterols

About 6 ml of HAKL extract was placed in a porcelain dish and evaporated on water-bath till dryness. The residue was first washed with petroleum ether and then with acetone and the remaining residue in porcelain was extracted with 10 ml chloroform. The chloroform extract was then subjected to the following tests for detection of sterols:

a. **Liebermann – Burchard's test:** About 5 ml of the chloroform extract was placed in a test-tube and few drops of acetic anhydride was added to that followed by carefully

addition of 2 ml concentrated Sulphuric acid from the side of test tube. It was observed for formation of brown to blood red colour at the junction of two phases.

a. **Salkowski Reaction:** About 5 ml of the chloroform extract was taken in a test-tube and about 2 ml of concentrated sulfuric acid was carefully added to that from the side of test-tube. It was observed for formation of yellow-red/brown colour ring at the junction, which turns red after a short time.

4.6.2.10. Detection of resins

Resins are insoluble in water and are rarely soluble in light petroleum (except colophony and dammar). They are generally soluble in alcohol, ether, acetone, chloroform, fixed oils and volatile oils, etc. (Ansari, 2009).

a. **Alcohol turbidity test:** About 3 ml of HAKL methanol extract was mixed with 3 ml distilled water in a test tube. As a control test, 3 ml of the same extract was mixed with 3 ml of methanol. Both test-tubes were comparatively observed for appearance of turbidity. Formation of turbidity in the test-tube containing mixture of extract and water, indicates the presence of resins in the extract (Ansari, 2009).

4.6.2.11. Detection of proteins and free amino acids

a. **Biuret test:** 5 ml of the extract was made alkaline by adding ammonia and heated for a while. Then 2 – 3 drops of 0.2% copper sulphate solution was added to that and was observed for appearance of red or purplish violet colour which indicates the presence of proteins and free amino acids (Ansari, 2009).

b. **Ninhydrin test**: To about 3 ml of the sample extract 3 or 4 drops of 0.25% Ninhydrin reagent was added and the mixture was heated in boiling water-bath for a few minutes. Then, it was observed for formation of blue or violet colour which indicates the presence of proteins and free amino acids in the sample (Ansari, 2009; Lalitha et al, 2012; Palanisamy et al, 2012)

4.6.2.12. Detection of gums and mucilage

a. About 25 ml of HAKL aqueous extract was stirred with 25 ml absolute alcohol, after constant stirring it was kept in refrigerator for a while to precipitate. The precipitate was collected and dried in open air. The dried precipitate was then examined for it swelling property and for presence of carbohydrates (Kokate, 2010).

4.6.2.13. Detection of Furanocoumarins

Furanocoumarins are detected in their solution under UV light by showing their blue, violet, brown, green or yellow colours which will intensify by adding drops of 10% KOH in methanol or antimony chloride in chloroform (Harborne, 1988).

a. **Fluorescence test:** Few ml of HAKL extract were taken in two clean test-tubes and observed in UV chamber for their fluorescence. Then, 2 – 3 drops of 10% KOH in methanol were added into one of the test tube and observed comparatively under UV light to observe if blue, violet, green or yellow fluorescence is appeared.

Results of phytochemical screening of *H. afghanicum* leaves different solvent extractives are presented in Table 5.

4.7. Evaluation of biological activity

The plant kingdom represents an enormous reservoir of natural pharmacologically active compounds. Plants are proved to be the major source of biologically potent substances such as flavonoids, alkaloids, phenolics, steroids, glycosides, essential oils and so on in their different organs and parts such as barks, leaves, seeds, roots, etc. (Kalita et al., 2013). Biological potential of crude drugs totally depends on presence of their active components. Presence and nature of active constituents in crude materials can be evaluated by performing biological screening of their extractives (Ansari, 2009).

Taking into account the proposal of present work, *Heracleum afghanicum* Kitamura leaves different solvent extracts and volatile oil were subjected to evaluation of antioxidant activity in vitro, antimicrobial activity in vitro and anthelmintic activity in vitro, as discussed later in this part of the Chapter.

4.7.1. Antioxidant activity evaluation of *H. afghanicum* leaves

4.7.1.1. Introduction

Any atom or chemical species capable of independent existence and having one or more unpaired electrons is defined a free radical (Kalita et al., 2013). Generally two types of free radicals are common; oxygen centered free radicals or reactive oxygen species (ROS), and those having nitrogen atom called reactive nitrogen species (RNS). A common name for both ROS and RNS is given as “pro-oxidants” (Acworth et al., 2003). Pro-oxidants cause

oxidative damage of body biomolecules and oxidative stress (Amiri, 2012), and hence, contribute in the etiology of critical health problems and different diseases including cancers (Borkataky et al., 2013; Kalita et al., 2013; Packer et al., 2004; Rehman et al., 2013). The only agents act against pro-oxidants are the antioxidants.

Antioxidants are compounds capable of scavenging free radicals and preventing or reducing their damaging effects on body (Kalita et al., 2013). Though, human body has some endogenous (internal) antioxidants (e.g. albumin, SOD, GSH, etc.) and an antioxidative defense systems inhibiting pro-oxidants and their damaging effects, but this is not sufficient to diminish the damage entirely (Acworth et al., 2003; Borkataky et al., 2013; Packer et al., 2004). Hence, for maintenance of normal health taking exogenous antioxidants is very necessary (Amiri, 2012). Medicinal plants and diets with ample amount of salads and vegetables are rich source of natural exogenous antioxidants.

Based on epidemiological surveys it was found that sufficient intake of external antioxidants and a high level of body cells antioxidant status, result in reducing the risk of several diseases such as cardiovascular diseases, carcinogenic diseases, etc. (Borkataky et al., 2013; Cadenas et al., 2002 ; Gerber, 2000; Packer et al., 2004; Thaipong et al., 2006). Therefore, antioxidants are proposed in prevention and treatment of various human disease caused by pro-oxidants including ROS and RNS (Gupta, 2013).

Fortunately, plants as natural reservoirs contain a diverse array of phytochemicals showing different biological activities including antioxidant potential (Rusaczonek et al., 2007). In this regard, plant phenolics and flavonoids due to their –OH groups or single electron donating properties, act as potent radical scavengers and antioxidants (Amiri, 2012; Borkataky et al., 2013; Kalita et al., 2013; Nayeem and Karvedar, 2010; Packer et al., 2004; Rehman et al., 2013).

Essential oils as a mixture of several simple and complex volatile compounds including aromatic phenolic compounds exert a diverse array of biological activities including antioxidant potentials (Amiri, 2012).

Heracleum spp. are commonly aromatic plants belongs to family Umbelliferae (Trease and Evans, 2009). Several *Heracleum species* have been reported for their different biological activities including antioxidant activity (as discussed in sub-section 2.6.4. of Chapter II).

In present study, *H. afghanicum* Kitamura leaves different solvent extracts and volatile oil were evaluated for their total antioxadative compounds and total antioxidant capacity. The extracts were subjected to determination of total phenolic content (TPC) and total flavonoids content (TFC) while the volatile oil was only tested for its TPC value. In addition, all of the extracts and volatile oil were evaluated for their total antioxidant activities using two in vitro methods namely; DPPH radical scavenging assay and FRAP assay. All of these evaluations are discussed later in detail.

4.7.1.2. Determination of total phenolic content

Total phenolic content (TPC) of *H. afghanicum* leaves different extracts and volatile oil was determined as per Folin-Ciocalteu spectroscopic method described in literature (Blois, 1958; Singleton et al., 1999). Gallic acid was used as standard phenolic compound for establishing a standard curve (Akter et al., 2014; Muniruzzaman et al., 2012; Namjooyan F et al, 2010; Valyova et al., 2012).

Main chemicals or reagents required for performing Folin-Ciocalteu method are as following:

a. Folin-Ciocalteu Reagent (FCR): Already made FCR is available in the market. The reagent can be diluted prior to performing the test as per desired modifications in the assay.

b. Sodium carbonate: Aqueous solution of Na_2CO_3 with known concentration (e.g. 7.5% or 15% in distilled water) according to modification of the method is used (Borkataky et al., 2013). Solutions of sodium bicarbonate ($NaHCO_3$) and sodium hydroxide (NaOH) also can be used (Singleton et al., 1999).

c. Gallic acid: a stock solution of gallic acid with known concentration is to be prepared by dissolving an accurately weighed amount of gallic acid in distilled water or methanol. Then, from the stock solution, different aliquots or serial dilutions in a known concentration range are to be prepared for making a standard curve (Akter et al., 2014).

Alcoholic or aqueous (if soluble in distilled water) solution of the test sample with a known concentration (e.g. 100 µg/ml, 200 µg/ml, 300 µg/ml, etc.) is used in this assay.

Principle of Folin-Ciocalteu method

Folin-Ciocalteu method is an electron transfer-based method (Apak et al., 2007). In this method, Folin-Ciocalteu reagent form a blue colour with phenolics or polyphenols present in test sample solution. The formed blue colour can be measured spectrophotometrically at 760 or 765 nm using a UV spectrophotometer.

The reaction equation of Folin-Ciocalteu assay can be summarized as bellow:

$$\text{Folin: Mo (VI) (yellow)} + e^- \text{ (from AH)} \rightarrow \text{Mo (V) (blue)}$$

Where, AH stands for antioxidants.

In this assay, the oxidizing agent is a molybdophosphotungstic hetropolyacid ($3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$).

Procedure of determining TPC of *H. afghanicum* leaves

Total phenolic content of test sample were determined as per following steps adopted from Folin-Ciocalteu method.

Briefly:

1) Aliquots of 1 ml test sample (extracts and volatile oil) methanolic solution at concentration of 300 µg/ml, were placed in separate test-tubes.

2) One ml of Folin-Ciocalteu reagent diluted (1/10) with distilled water was added into each test-tube and the mixtures were shacked well and placed at room ambience for 6 minutes.

3) Then, 3 ml of Na_2CO_3 7.5% solution in distilled water was added into the test-tubes and shacked well. While milky turbidity was produced in the test-tubes, they were again incubated at room ambience for 60 minutes.

4) Blank was concurrently prepared as per the same procedure by mixing 1 ml distilled water (instead of sample solutions), 1 ml FCR (1/10) and 3 ml 7.5% Na_2CO_3 aqueous solution.

5) Aliquots of gallic acid (1 ml) at concentrations of 5, 10, 20, 30, 40, 50 and 60 µg/ml in methanol were used according to same procedure, to establish the standard calibration curve (Table 7. and Figure 14.).

6) After 90 minutes, the whitish precipitates were sediment and blue colored supernatant of each test-tube was carefully pipetted and transferred into separate clean test-tubes. If required, the mixture were centrifuged for removing the suspended particles which will interfere with the UV readings.

7) Then, absorbance of the blue colored mixtures was measured at 765 nm wavelength against the blank.

The experiments were performed in triplicates and the results were recorded as mean±SD of the triplicates (Table 8.).

TPC of tested samples (different extracts and volatile oil) were calculated from the regression equation ($y=0.0168x + 0.0675$, $R^2=0.994$) obtained from gallic acid standard curve. The TPC values were expressed in terms of gallic acid equivalent (mg of GAE/g of extract) (Table 8.).(Figure 15.).

4.7.1.3. Determination of total flavonoid content

Determination of medicinal plants total flavonoid content (TFC) is of significant importance in predicting and correlating their antioxidant potential. Colorimetric methods (e.g. aluminium trichloride colorimetric method) are commonly used to determine TFC of crude drugs (Borkataky et al., 2013). A standard flavonoid (e.g. rutin, quercetin, etc.) is used for making a standard calibration curve and TFC of test sample is expressed as equivalent of standard flavonoid used is the assay. For instance, in present study, rutin is used as standard flavonoid to establish a standard curve and hence TFC of test sample is expressed as mg of RUE/g of tested sample.

Principle of Aluminium Chloride colorimetric method

Aluminum trichloride forms acid stable complexes with the C4 keto group and either the C3 or C5 hydroxyl group of flavones and flavonols. $AlCl_3$ also forms acid labile complexes with the ortho-dihydroxyl groups of A- or B-ring of flavonoids (Kalita et al., 2013). The amount of formed complexes are spectrophotometrically measurable by taking their absorbance at 415 nm.

Before proceeding to perform the assay, preparation of test reagents, sample solution and standard flavonoid solution are necessary which are discussed below:

a. $AlCl_3$ solution 2% (w/v) in methanol: 2 g of aluminium trichloride is to be accurately weighed and dissolved in 100 ml of methanol.

b. Stock solution of a standard flavonoid: a methanolic solution of a standard flavonoid such as rutin, quercetin, hesperidin and so on (whichever is in access) with a known concentration (e.g. 200 µg /ml, 300 µg /ml, etc.) is to be prepared and labelled properly.

c. Test/sample solution: an accurately weighed amount of sample is to be dissolved in appropriate volume of methanol to prepare a sample stock solution. For instance, if 10 mg of HAKL extract is dissolved in 10 ml methanol, then a stock solution of 1000 µg/ ml is obtained.

Procedure of determining TFC of *H. afghanicum* leaves

Total flavonoid content (TFC) of *H. afghanicum* leaves extracts were determined as per aluminium chloride colorimetric method previously described, with slight modifications (Amiri, 2012; Borkataky et al., 2013; Kalita et al., 2013; Ramamoorthy and Bono, 2007; Rosalind et al., 2013; Roy, 2012; Valyova et al., 2012).

Briefly:

1. Two milliliter of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume (2 ml) of extract solution (300 µg/ml) in clean test-tubes.

2. The mixtures were shacked well and were kept in dark at room ambience for 30 min.

3. After the incubation period, absorption readings at 415 nm wavelength were taken against a blank sample consisting of the mixture of 2 ml of sample/standard solution with 2 ml methanol without aluminium trichloride.

4. Rutin serial dilutions at different concentrations (5, 10, 20, 30, 40 and 50 µg/ml) in methanol were used as per the same procedure to establish a standard curve (Table 9. and Figure 16.).

The experiment was carried out in triplicates and the results were recorded as mean±SD of the three readings. The total flavonoid content of tested extracts were determined according to the following regression formula obtained from rutin standard curve:

$$\text{Absorbance } (y) = 0.021x - 0.2033, \quad (R^2: 0.9973)$$

The amount of flavonoids in *H. afghanicum* leaves different solvent extracts were expressed as rutin equivalent (mg of RUE / g of extract) (Table 10. Figure 17.)

4.7.1.4. DPPH free radical scavenging activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity is a widely used, simple, fast and highly sensitive method for evaluation of plant extracts anti-oxidant activity *in vitro* (Amiri, 2012). The sole equipment necessary for the method is a UV-spectrophotometer which is easily available in most of laboratories (Thaipong et al., 2006).

To perform DPPH radical scavenging assay method, the following reagents and solutions are required:

a. DPPH solution: an accurately weighed amount of DPPH is dissolved in methanol or ethanol to prepare a solution having concentration in mM level (e.g. 0.5 mM). Since DPPH has a molecular weight of 394.32 g/mol, then, 19.7 mg DPPH is accurately weighed and dissolved in 100 ml methanol to give 0.5 mM solution having an intense purple colour.

b. Standard antioxidant stock solution: Compounds commonly used as standard antioxidant in DPPH assay method are ascorbic acid, α-Tocopherol, butylated hydroxyl toluene (BHT), etc. An accurately weighed amount of the standard antioxidant is dissolved in methanol to prepare a stock solution having a known concentration. For example; ascorbic acid crystals (4 mg) are accurately weighed and

dissolved in 10 ml methanol to give 400 µg/ml solution. The prepared solution is used to prepare different aliquots in order to establish a standard curve for DPPH assay. Standard solution shall be freshly prepared on the day of performing the experiment.

c. Sample stock solutions: an accurately weighed or measured amount of test sample (e.g. extracts or volatile oil) is dissolved in methanol (or ethanol) to prepare a stock solution with known concentration. For example; if the amount of 20 mg of a test sample is dissolved in 10 ml solvent (methanol), a stock sample solution at 2000 µg/ml concentration is obtained. Then, different aliquots or serial dilutions are to be prepared from the obtained sample stock solution. It is better to prepare the stock solution freshly on the day of performing the experiment, because antioxidants present in the solution, especially if present in less quantities, will be oxidized during storage of prepared solutions.

Principle of DPPH free radical scavenging assay

DPPH dissolved in methanol produce free radicals in the solution and gives and intense purple colour. DPPH free radicals absorb UV light at 517 to 520 nm wavelength. DPPH free radicals are stable but they are scavenged and reduced by hydrogen donating antioxidants. When solution of DPPH come into contact or mixed with samples containing antioxidant compound(s), they became reduced, their odd electron is pairing off (Figure 12.). As the consequence of DPPH radical reduction and pairing off its odd electron, the purple colour of solution changes to yellow (Amiri, 2012; Kalita et al., 2013).

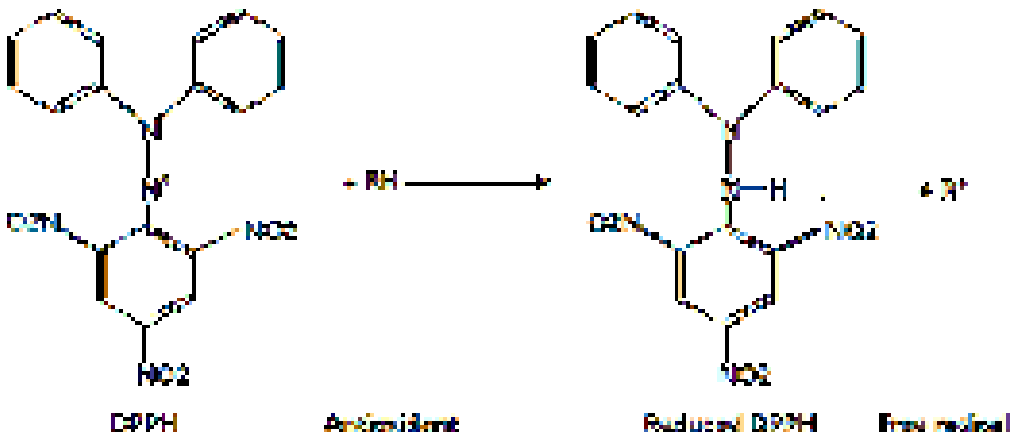

Figure 12: Reduction of DPPH by antioxidant.

The degree of discoloration and decrease in the solution absorbance at 517 nm indicate the degree of DPPH reduction by antioxidant molecules present in sample. Radical scavenging potential of a test sample (e.g. plant extract) is expressed as percentage inhibition of DPPH radicals. The higher is the % inhibition value of a test sample, the greater will be its radical scavenging potential.

Procedure of DPPH free radical scavenging assay

HAKL different solvent extracts and volatile oil were evaluated for their radical scavenging activity using DPPH radical scavenging assay method described previously, with slight modifications (Amiri, 2012; Muniruzzaman et al., 2012; Suttee and Rana, 2012; Valyova et al., 2012; Kalita et al., 2013).

Briefly;

1. Serial dilutions of ascorbic acid at different concentrations (0.25, 0.50, 0.75, 1.00, 1.25, 1.5, 2.00 and 2.50 µg/ml) were prepared and 4 ml of the dilutions were mixed with 0.5 ml DPPH solution to make a standard curve (Figure 18.).

2. Control was prepared by mixing 4 ml methanol (instead of extract/standard solution) with 0.5 ml DPPH (0.5 mM) solution.

3. Four ml of extracts serial dilutions (10, 25, 40, 55, 70, 85 and 100 µg/ml) and volatile oil serial dilutions (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µl/ml) in methanol were placed into separate clean test-tubes and mixed with 0.5 ml DPPH solution (0.5 mM) in methanol. In order to remove the interference of extract component with the test readings, blank dilutions of all extracts were concurrently prepared and their absorbencies were checked at 517 against methanol as blank, these values were subtracted from the same copy of these blank but treated with DPPH.

4. All of the resultant mixtures were shaken well and incubated at room temperature for 30 min in the dark for completion of the reaction process.

5. After incubation period, absorbance of the mixtures was measured at 517 nm wavelength against methanol as blank, using UV-spectrophotometer.

The experiments were performed in triplicates and the readings for standard (ascorbic acid) as well as test samples dilutions were recorded as mean±SD of the triplicates (Tables 11., 12., 13, 14.).

% Inhibition of DPPH free radical were calculated by using the following formula:

$$\% \text{ inhibition} = 100 \times [(A_c - A_s) / A_c]$$

Where, A*c* is absorbance of control

A*s* is absorbance of sample/standard

Antioxidative capacity of standard ascorbic acid and tested samples were calculated from their related graph of % inhibition, and expressed as inhibition concentration of 50% DPPH free radicals (IC_{50}) (Table 15.).

Note: it should be reminded that in case of measuring DPPH free radical scavenging activity of sample containing complex colored compounds (e.g. plant extracts, juices, etc.), the blank of sample dilutions should be checked for their absorbencies at 517 nm. These values shall be then subtracted from those of corresponding DPPH-treated dilutions. Otherwise because of interference of the sample absorbance no accurate or satisfactory result will be achieved in DPPH assay method.

4.7.1.5. Ferric reducing antioxidant power (FRAP) assay

FRAP assay is a simple, fast and inexpensive method, and gives reproducible results with minimum sample preparation (Benzie and Strain, 1996; Shetty et al., 2007; Thaipong et al., 2006).

To carry out FRAP assay, the following solutions and reagents are required to be prepared and mixed properly to make FRAP reagent:

a. Acetate buffer 300 mM at pH 3.6: accurately weighed 3.1 g sodium acetate trihydrate and 16 ml glacial acetic acid are dissolved in water to make 1 liter buffer. Prepared buffer solution having pH 3.6 need to be stored at 4^{o} C in refrigerator.

b. HCl diluted solution (40 mM): 0.9 ml of conc. HCl (11 M) is diluted with distilled water to prepare 250 ml diluted (40 mM) HCl solution. The diluted acid solution require to be stored at room temperature.

c. 2,4,6-Tripyridyl-s-triazine solution 10 mM in 40 mM HCl: 0.031 g TPTZ after being accurately weighed is dissolved in 10 ml of 40 mM HCl. (MW. of TPTZ is 312.33 g/mol). This solution needs to be freshly prepared before performing the experiments.

d. $FeCl_3$ 20 mM solution: 0.0324 g ferric chloride anhydrous after being accurately weighed are dissolved in 10 ml distilled water (MW of $FeCl_3$ = 162.2 g/mol). This solution also needs to be freshly prepared before performing the experiment.

FRAP reagent is prepared from above solutions as per the following formula:

- TPTZ 10 mM in 40 mM HCl …….…… 2.5 ml
- Acetate buffer (300 mM) ……. 25 ml
- $FeCl_3$ (20 mM) solution …….……. 2.5 ml

For FRAP assay, acidic condition (pH 3.6) and presence of 2,4,6-tripyridyl-s-triazine (TPTZ) are the main requirements.

In FRAP assay method FRAP reagent is necessary to be freshly prepared and incubated at 37^o C for 15 min each time before use.

Principle of FRAP assay method

FRAP assay is an indirect method based on the reduction of Fe^{3+} to Fe^{2+} and measures the ferric (Fe^{3+}) to ferrous (Fe^{2+}) reduction in the presence of antioxidants (Benzie and Szeto, 1999).

TPTZ (2,4,6-Tripyridyl-*S*-triazine) is an analytical reagent widely used for spectrophotometric determination of phenols, phenolic drugs and Iron (Sudha, 2013). In FRAP assay, extracts or samples having antioxidant compounds are allowed to react with ferric tripyridyltriazine (Fe-III-TPTZ) complex at low pH. Reduction of Fe^{3+} complex of tripyridyltriazine ($Fe(TPTZ)^{3+}$) to Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants (standard/sample) produces an intensely blue colour in the solution. This blue colored Fe-$(TPTZ)^{2+}$ complex has absorbance maximum at 593 nm (Sudha, 2013; Shetty et al., 2007; Gupta, 2013). Actually, in FRAP assay, the antioxidant potential of a sample can be determined by measuring the absorbance of Fe^{2+}-TPTZ complex. Increase in the absorbance is directly related to the reducing potential of the electron donating antioxidants present in

test sample (Gupta, 2013), and indicates the reducing capacity of a tested sample/standard drug (Muniruzzaman et al., 2012).

The reaction equation of FRAP assay is as following:

$$Fe\ (TPTZ)_2^{3+} + ArOH \rightarrow Fe\ (TPTZ)_2^{2+}\ (\text{blue colour}) + ArO + H^+$$

In FRAP assay, $FeCl_3$ in FRAP reagent acts as a strong oxidizing agent, which under proposed condition (low pH) oxidizes the antioxidant(s) and itself will be reduced to ferrous [Fe^{+2}] having a blue colour.

Procedure of FRAP assay method

HAKL different solvent extracts and volatile were subjected to FRAP assay as per method developed by Benzie and Strain (1996) with slight modification (Anbarasu et al., 2014; Cristina M. Mihai et al. 2011; Hakiman and Maziah, 2009; Rehman et al., 2013).

Briefly:

a. The amount of 0.5 ml of different serial dilutions of the extracts at concentration of 20, 40, 60, 80 and 100 µg/ml were mixed with 4 ml of freshly prepared FRAP reagent and warmed at 37^o C.

b. In case of volatile oil, 0.5 ml of different dilutions (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µl/ml) in methanol were used.

c. For establishing a standard curve (Figure 21.), 0.5 ml of serial dilutions of ferrous sulphate ($FeSO_4.7H_2O$, MW=278 g/mol) aqueous solution at different concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM were mixed with 4 ml of FRAP reagent in separate test-tubes (Gupta, 2013).

d. The resultant mixtures were shacked well and incubated in hot air oven at 37^o C for 30 min for completion of the reaction.

e. After incubation period absorbance of the blue colored mixtures was measured at 593 nm wavelength using UV-spectrophotometer.

f. All of the experiments were performed in triplicates and the results were recorded as mean±SD of the triplicates (Table 16., Table 17).

FRAP values of tested samples were calculated by the regression formula obtained from $FeSO^4$ standard curve (y=1.7882x – 0.046, R^2 : 9907). FRAP values for HAKL extracts and volatile oil were expressed as both mM and mg equivalent of $FeSO_4$ as tabulated in Table 17. and presented in Figure 22.

Note: The main drawback of FRAP method is that it is more suitable for water soluble antioxidants and on the other hand, it cannot measure those antioxidants which have –SH groups (e.g. gluthathion) (Apak et al., 2007).

4.7.2. Antibacterial activity evaluation of *H. afghanicum* leaves

4.7.2.1. Introduction

Microbial infections are the main cause of mortality and morbidity of human as well as animals worldwide. Many of todays' available synthetic antibiotics used in control and management of microbial infections are either very expensive (McGaw et al., 2000) or become indolent against resistant microbial pathogens (Block et al., 2000). Furthermore, all of the people do not have equal and easily access to the modern synthetic antibiotics since a large portion of population worldwide still rely on medicinal plants for their primary health problems. However, production of medicines and curing pathogenic conditions began with the use of herbs. Plant metabolites have shown to contribute in many of pharmacological activities (Nayeem and Karvekar, 2010). Many of the currently used drugs in treatment of bacterial and other infections were first obtained from natural sources including medicinal plants. Antimicrobial activity of medicinal plants extracts and their essential oils are well known since long back (Flamini et al., 1999). Recently, much more attention has been attracted to scientifically prove the efficacy of traditional medicinal herbs and to explore phytochemicals of potent bioactivity from plant source.

Evaluation of antibacterial activity is one of the important approach by which one can estimate the ability of a sample to inhibit different bacterial pathogens (Tripathee et al., 2011). Antimicrobial activity of plant extracts and volatile oils is commonly evaluated by using disc diffusion method and/or well diffusion method (Flamini et al., 1999; Hammer et al., 1999; McGaw et al., 2000).

4.7.2.2. Methodology

H. afghanicum different solvent extracts and volatile oil were subjected to in vitro antibacterial activity assay using both disc diffusion method (DDM) and agar wells diffusion method (WDM) adopted from previous literatures (Hammer et al., 1999; Flamini et al., 1999; McGaw et al., 2000; Mukherjee and Ghosh, 2010).

4.7.2.3. Microbial strains

Microbial strains were already procured from MTCC center, Chandigarh, Punjab, India, by microbiology department of Lovely Professional University. Bacterial strains were maintained in nutrient broth while fungal strains were maintained in potato dextrose broth media. Bacteria used in the assay comprised two Gram-negative bacteria (*Proteus vulgaris* MTCC 744, *Escherichia coli* MTCC 1551) and two Gram-positive bacteria (*Staphylococcus aureus* MTCC 6908 and *Bacillus subtilis* MTCC 8141). Fungi used in antifungal assay were namely; *Aspergillus fumigatus* MTCC 780, *Candida albicans* MTCC 227 and *Malassezia furfur* MTCC 1765.

4.7.2.4. Preparation of sample stock solutions

H. afghanicum different extracts (2 g of each extract) were accurately weighed and dissolved in small amount of dimethyl sulfoxide (DMSO) and transferred into 10 ml volumetric flasks separately. Sufficient amount of DMSO was carefully added until it was matched with the mark of 10 ml and the stock solutions of 200 mg/ml were prepared for all of test extracts. Aqueous extract stock solution (200 mg/ml) was prepared in sterile distilled water. In case of volatile oil, 2 ml of the oil was placed in 10 ml volumetric flask and was diluted with DMSO to get a stock solution of 200 µl/ml. All of the stock solutions prepared so, were stored at 4^{o} C in refrigerator, until being used in antimicrobial activity assays.

4.7.2.5. Preparation of standard antibiotics stock solutions

Amount of 0.5 ml gentamicin sulfate was taken from gentamicin vial (80 mg/2 ml) using a sterile 1 ml syringe and was transferred into 100 ml volumetric flask. It was then diluted to 100 ml by adding distilled water to prepare a stock solution of gentamicin 400 µg/ml. Itraconazole (400 µg/ml) fluconazole (400 µg/ml) as standard antifungal stock solutions, were prepared in DMSO. All of the prepared standard stock solutions were stored at 4^{o} C in refrigerator, until being used in respective antimicrobial assays.

4.7.2.6. Preparation of culture media

Nutrient broth media and potato dextrose broth media were used for sub culturing of bacterial strains and fungal strains, respectively. Nutrient agar and Sabouraud dextrose agar (SDA) was used in antibacterial and antifungal tests, respectively. Sabouraud dextrose agar is the medium of choice for most fungi. Preparation and sterilization of the media were carried out as per described procedures (Mukherjee and Ghosh, 2010).

Briefly;

Required amount of the dehydrated media was accurately weighed, placed in a suitable conical flask and dissolved in appropriate amount of distilled water (as per instructions provided on the bottle by manufacturer). Broth media (nutrient broth and potato dextrose broth) were easily solubilized in distilled water, while in case of nutrient agar and SDA, the media were heated till boiling on a heater mantle and were shacked periodically to facilitate proper dissolution of the agar present in the medium. Then, the prepared media were sterilized in autoclave at 121^{o} C and 15 lbs. pressure for 15 minutes. Sterilized broth media is used in sub-culturing of microbial strains while sterilized nutrient agar and SDA are used for preparation of agar plates to perform antimicrobial activity assays.

4.7.2.7. Preparation of microbial subculture and inoculum

Bacterial subcultures were prepared by inoculating a loopful of corresponding bacterial stock suspension (maintained in microbiology laboratory of Lovely Professional University) in about 7 ml of sterilized nutrient broth liquid medium in separate test tubes. The fungal strains were sub-cultured by inoculating a small portion of fungal colony into 7 ml sterilized potato dextrose broth in separate test tubes. Sub-culturing was carried out as per "*technique of aseptic transfer*" described by Mukherjee and Ghosh (2010), under sterile aseptic condition of laminar chamber, and at a distance of 10 cm from the flame. Inoculated test tubes were tightly caped with sterile cotton and covered further with aluminium foil. Then, the test tubes containing bacterial inoculated broth were incubated in BOD incubator at 37^{o} C for overnight (24 hours), while those containing fungal inoculated broth were incubated separately in Fungi incubator at 25^{o} C for 48 hours. These subcultures were then used for preparation of microbial inoculum.

4.7.2.8. Preparation of McFarland standards

The McFarland equivalence standards are used for adjusting densities of bacterial suspension used in inoculation during microbiological experiments (e.g. identification of microbes and susceptibility testing). The standard is used to visually approximate the concentration of cells in a suspension in term of colony forming unit or CFU/ml. In antibacterial susceptibility tests, both the size of the inoculum and the concentration of microbial suspension matter with the reliability of antimicrobial test result. Higher amount and higher density of microbial suspension result in less efficacy of the antimicrobial substance while less amount of inoculum or lower density of microbes revealed stronger efficacy of the tested sample. Hence, it is very important to adjust the population of microorganism in the microbial suspension aimed to be used in inoculation for antimicrobial test. A standardized level of bacterial population in a bacterial suspension before being used in inoculation is required to be equivalent with 0.5 unit of McFarland standard (Mukherjee and Ghosh, 2010). The unit 0.5 may not work properly with fungi (e.g. yeast and molds) since their mass differs from that of bacteria.

McFarland standards are generally labeled 0.5 through 10 and filled with suspension of barium sulphate.

McFarland standards of turbidity were prepared in laboratory as per following:

1. Solution of 1% anhydrous $BaCl_2$ in distilled water was prepared.
2. Solution of 1% H_2SO_4 was also prepared.
3. Both of the solutions were mixed as per designed proportion presented in the following Table 3., in a series of 11 clean and equal sized test-tubes.

Table 3. McFarland standards of turbidity

Solution	**Tube No.**										
	0.5	1	2	3	4	5	6	7	8	9	10
$BaCl_2$	0.05 ml	0.1 ml	0.2 ml	0.3 ml	0.4 ml	0.5 ml	0.6 ml	0.7 ml	0.8 ml	0.9 ml	1.0 ml
H_2SO_4	9.95 ml	9.9 ml	9.8 ml	9.7 ml	9.6 ml	9.5 ml	9.4 ml	9.3 ml	9.2 ml	9.1 ml	9.0 ml

All of the mixtures prepared as per Table 3 give precipitates of barium sulphate as suspension of different turbidity. The turbidity of the mixture increase with the increase in

Tube No. So, the Tube No. 0.5 acts as a standard for 1 X 10^8 cells/ml (interpreted also as 10^8 CFU/ml) (Mukherjee and Ghosh, 2010).

The turbidity of microbial suspension before being inoculated on agar plates is compared with that of tube 0.5 of McFarland standard.

How to compare turbidity of bacterial suspension with that of McFarland Tube No. 0.5?

Microbial suspension can be prepared either in broth medium or in normal saline. To compare the turbidity, both the standard and microbial suspension placed in separate test tubes of same diameter are held beside each other, and are observed against a white background with a contrasting black line (Mukherjee and Ghosh, 2010). Shacking of both the tubes in order to disperse the $BaSO_4$ precipitate as well as the bacterial colonies in relative tubes is required before comparison.

How to adjust turbidity of microbial suspension with that of McFarland Tube No. 0.5?

If the turbidity of microbial suspension is less than that of McFarland Tube No. 0.5, then incubation of the microbial suspension for a longer period is required to facilitate the growth of microbes and increase the microbial population or microbial density in the tube. If microbial suspension is more turbid than, then, it should be diluted by either normal saline or by broth medium until it became equivalent to the McFarland 0.5 unit. McFarland standards in test tube, sediment by time, so, every time it should be shaken well to make sure of equally suspending the precipitated $BaSO_4$ before being used as standard of turbidity.

McFarland standards once prepared, will work for 6 months, and after this duration they will expire.

4.7.2.9. Antibacterial activity evaluation using disc diffusion method

Disc diffusion method (DDM) is a simple and commonly used method of antimicrobial susceptibility testing. To evaluate the antibacterial potential of *H. afghanicum* different solvent extracts and volatile oil, disc diffusion method was carried out as per standard microbiological procedure adopted from previous literature (Hammer et al., 1999; McGaw et al., 2000; Mukherjee and Ghosh, 2010; Kousha and Bayat, 2012; Raju et al., 2013; Saleem et al., 2015).

Briefly;

1. About 25 ml of sterilized nutrient agar media was poured in sterile petri plates near (at about 10 cm) the flame and under sterile condition of laminar chamber (sterilized by UV light for 15 minutes before starting the work).
2. Then, plates were allowed for a while, to solidify at the same aseptic area of laminar chamber.
3. Amount of 100 µl bacterial inoculum (equivalent to tube No. 0.5 of McFarland standard) was add on the surface of solidified agar plates and was uniformly streaked on agar surface, using a sterile glass-rod spreader. Inoculated petri plates were allowed for a while for surface drying.
4. Sterile discs (6 mm diameter) impregnated with sample dilutions of three different concentration (100 mg/ml, 50 mg/ml and 25 mg/ml) were placed on the surface of seeded agar plates, at suitable distances from each other. Flam-sterilized forceps was used for dipping the discs in sample solution and placing them on the surface of agar plates (Mukherjee and Ghosh, 2010). Standard antibiotic disc gentamicin (10µg/disc), was used as positive control and a sterile blank disc impregnated in pure solvent (DMSO) was used as negative control (Kousha and Bayat, 2012).
5. Each plate on its backside was named by the name of its inoculated microbial strain (e.g. *Bacillus subtillis, Staph. aureus,* etc.), and numbered/marked properly.
6. Then, the plates were kept at room temperature (under laminar chamber) for one hour to make sure the diffusion of sample/standard discs loaded compounds into the agar media (Saleem et al., 2015).
7. After one hour, agar plates were inversed and incubated at 37^{o} C for 24 hours in incubator.
8. After incubation period (the next day) the plates were observed for bacterial growth inhibition by the samples as well as standard discs. The diameter of the clear circular area (the area without bacterial growth) around each disc, was measured in mm using a ruler and recorded as zone of inhibition* (ZOI) (Figure 23.).

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* : Zone of inhibition (ZOI) is defined as the area around the discs where the sample or standard drug has inhibited growth of the inoculated bacteria on the surface of agar plate. The antibacterial potential of test samples are judged by comparing the diameter of test sample ZOI with that of standard antibiotic used in the assay. The larger ZOI indicates stronger antibacterial activity.

The experiments were performed in triplicates and the results (ZOIs) were recorded as mean±SD of the three experiments (Table 19.).

Activity index (AI) for each sample disc were calculated by the following formula:

AI = ZOI of sample / ZOI standard

AI values for the tested samples are tabulated in Table 21.

4.7.2.10. Antibacterial activity evaluation using Well diffusion method

Well diffusion method (WDM) same like disc diffusion method is commonly used by many researchers in testing antimicrobial activity of plant extracts, volatile oils and other synthetic compounds (Saleem et al., 2015). In present study, *H. afghanicum* leaves different solvent extracts and volatile oil were evaluated for their antibacterial potential using Agar well diffusion method as per described procedure (Saleem et al., 2015):

1. Sterilized (autoclaved) nutrient agar media (25 ml) was poured in sterile petri plates near the flame (at about 10 cm distance) and under sterile ambience of laminar chamber. The plates were allowed for solidification of the media at room ambience.
2. Fixed amount (100 µl) of bacterial inoculum (equivalent to 0.5 McFarland standard) was pipetted on the surface of solidified agar plates and was spread uniformly using a sterile glass spreader. The spreader was sterilized using 70% alcohol before spreading next (different) microbial inoculum on another plate.
3. The inoculated plates were allowed for a while for surface drying.
4. Then, wells (5 wells per plate) of 5 mm diameter were made in all seeded agar plates using a sterile cork borer. The borer was flamed and cooled back each time before being used in making wells in the next agar plate inoculated with different microbial strain.
5. All the seeded plates were named by the name of seeded bacteria and the wells were marked/numbered properly on the backside of agar plates.
6. Amount of 50 µl of test sample solutions of three different concentrations (100, 50 and 25 mg/ml) were poured into corresponding numbered wells (1, 2 and 3, respectively), using a micropipette. As a result, the three wells numbered as 1, 2 and 3 contain doses of 5, 2.5 and 1.25 mg/well of the test samples, respectively. In

another well marked as *G,* 5 µg/well gentamicin (50 µl of 100 µg/ml gentamicin in distilled water) was added as standard antibiotic or positive control. Amount of 50 µl DMSO (the solvent of test samples solution) as negative control was placed in a separate well marked as *C*. (Kousha and Bayat, 2012).

7. To ensure sufficient diffusion of the poured samples/standard into the media, all plates were allowed at room temperature for about 1 hour under the same sterile condition of laminar chamber (Saleem et al., 2015).
8. The petri plates were then inversely incubated at 37^{o} C for 24 hrs.
9. After incubation period, the plates were observed for the zone of inhibition (ZOI) produced around the wells. The diameter of ZOI around all the wells, if produced, were measured in mm using a ruler. The diameter of a well (5 mm) was subtracted from the value of total diameter of each ZOI.

The experiments were performed in triplicates and the results were recorded as mean±SD of the three experiments (n=3), as presented in Table 20.

4.7.3. Antifungal activity evaluation of *H. afghanicum* leaves

4.7.3.1. Introduction

Fungi are organisms survive abundantly almost everywhere (Hawksworth, 2001), and they are generally different from bacteria, in their morphology. Fungi unlike bacteria are eukaryotic organisms (Mukherjee and Ghosh, 2010). Fungi despite being accounted as low virulent organisms as compared with bacteria, around 300 fungal species are deemed to contribute in fungal infections in humans particularly in elderly persons and as well as in immunocompromised patients (Hawksworth, 2001; Mukherjee and Ghosh, 2010). Fungal infections are claimed to be the cause of 1.35 million deaths per year.

Both the limited classes of available antifungal agents compared to antibacterial medicines, and their higher toxicity to human cells are considered to be the big challenges in control and treatment of fungal diseases. In addition, treatment of fungal diseases with available medicines require longer course, and hence the patient will be imposed to the toxic drugs for longer time. Hence, searching for new antimicrobial agents with broad spectrum of activity particularly against fungal pathogens are from the urgent need to treat fungal infections (Mukherjee ant Ghosh, 2010).

In present study, *H. afghanicum* different solvent extracts and volatile oil were screened for their antifungal activity. As far as ascertained, no scientific report is available till now about antifungal potential of this medicinal plant.

4.7.3.2. Methodology

The antifungal assay of HAKL different solvent extracts and volatile oil was carried out using both disc diffusion method (DDM) and agar wells diffusion method (WDM), as per the same procedure used for antibacterial tests and discussed earlier (in sub-section 4.7.2.9. and 4.7.2.10., Chapter IV).

4.7.3.3. Fungal strains

Fungal strains were obtained from the stock cultures of microbiology department of LPU, previously procured from Chandigarh MTCC center. The fungal strains were sub-cultured and maintained in potato dextrose broth media. Fungal strains used in the assay were as below:

1. *Aspergillus fumigatus* MTCC 870
2. *Candida albicans* MTCC 227
3. *Malassezia furfur* MTCC 1765

4.7.3.4. Preparation of fungal inoculum

To prepare inoculum, a small portion of fungal colony was sub-cultured in about 7 ml of sterilized potato dextrose broth medium and the tubes were incubated at 25^{o} C for 48 hours. Then, sufficient portion of the colony were suspended in liquid potato dextrose broth to be used in inoculation of agar plates.

4.7.3.5. Preparation of standard antifungal stock solutions

Stock solution of Itraconazole (antifungal) at concentration of 400 µg/ml were prepared in DMSO and was stored at 4^{o} C till further use.

4.7.3.6. Preparation of culture media

Potato dextrose broth media was used for sub-culturing and preparing inoculation of fungal strains while Sabouraud dextrose agar (SDA) was used as culture medium in antifungal activity evaluation. The media were prepared as per directions provided by their

supplier (written on the bottle label), and were sterilized by autoclaving at 121° C and 15 lbs. pressure for 15 minutes (Mukherjee and Ghosh, 2010).

4.7.3.7. Antifungal activity evaluation using disc diffusions assay

Antifungal activity of *H. afghanicum* leaves different solvent extracts and volatile oil were evaluated using disc diffusion method as per procedure discussed earlier (see sub-section 4.7.2.9. of Chapter IV), and adopted from previous literatures (Mukherjee and Ghosh, 2010; Saleem et al., 2015; Hammer et al., 1999; Nijsiri et al., 2010; Oluduro, 2012).

Briefly;

a. Sabouraud dextrose agar was used as culture medium.
b. Amphotericin B 20 µg/disc and fluconazole 10 µg/disc were used as standard antifungal drugs.
c. The plates were incubated in Fungi incubator for 48 hours at 25° C temperature.

The experiments were carried out three time and the results in term of ZOI and activity index (AI), were recorded as mean±SD of the three experiments (n=3) (Table22, Table 24) (Figure 25.)

4.7.3.8. Antifungal activity evaluation using Well diffusion method

H. afghanicum leaves different extracts and volatile oil were evaluated for their antifungal activity, using wells diffusion method as per procedure described earlier (see sub-section 4.7.2.10. of Chapter IV) and adopted from literatures (Saleem et al., 2015). All steps performed in this assay were identic with those carried out for disc diffusion assay performed in antifungal activity evaluation of the same samples (discussed earlier). Sabouraud dextrose agar was used as the culture medium and wells of 0.5 mm diameters were made by a sterile cork borer. Amount of 50 µl of the extracts dilutions at different concentrations of 25 mg/ml, 50 mg/ml and 100 mg/ml in DMSO, were pipetted in separate wells, meaning the extracts at actual dose of 1.25, 2.5 and 5 mg/well were use in the assay. In case of *H. afghanicum* volatile oil, 50 µl of three different dilutions (25, 50 and 100 µl/ml in DMSO), meaning doses of 1.25, 2.5 and 5 µl/well were used.

Itraconazole at dose 20µg/well (50 µl of Itraconazole 400 µg/ml DMSO) was used as standard antifungal drug (positive control), while pure DMSO (50µl/well) was used as negative control. All of the plates were incubated at 25 ° C for 48 hours. After incubation

period, the plates were observed for ZOI produced around the wells. The zones of inhibition were recorded in mm while the well diameter (5 mm) were subtracted from each value. The experiments were performed three times and the results (ZOI and AI) were recorded as mean±SD of the three experiments (Table 23, Table 24), (Figure 26.).

4.7.4. Anthelmintic activity evaluation of *H. afghanicum* leaves

4.7.4.1. Introduction

Helminths or worms are multicellular intestinal parasites (Mukherjee and Ghosh, 2010) and a common cause of human parasitic infection called helminthiasis, particularly in developing countries (Iqbal et al., 2004, 2005). Helminths are divided into two phyla – Nematohelminths or Nematodes (roundworms) and platyhelminths or trematodes (flatworms) (Mukherjee and Ghosh, 2010; Bimlesh Kumar et al, 2011). Gastrointestinal nematodes (GINs) such as *Ascaris lumbricoides*, *Trichuris trichiura*, etc. are well understood to be the most important cause of the prevalent helminthiasis for human worldwide (Bachaya et al., 2009; http://www.who.int/topics/helminthiasis/en/ cited on 29th April, 2015). As per WHO over two billion people worldwide are suffering from helminthiasis (A. Kumar et al., 2010). Helminthiasis can cause morbidity and sometimes death (Zhu et al., 2013).

According to WHO only few medicines are available for treatment of helminthiasis in human being (Kumar et al., 2010). Moreover, most of the presently available anthelmintics have shown to produce some unwanted effects such as nausea, vomiting, headache, etc. and hence, searching for new anthelminthic drugs is very necessary (Eguale et al., 2007; Satrija et al., 1995;). Without doubt, some natural remedies of plant origin are thought to play prominent role in control as well as treatment of worm infections (Bachaya et al., 2009; Bimlesh Kumar et al, 2011; Jabbar et al., 2007; Satrija et al., 1995).

Taking into account the increasing demand for anthelmintic drugs worldwide, *Heracleum afghanicum* leaves was evaluated for its anthelminthic activity in vitro

4.7.4.2. Methodology

Anthelmintic activity of *H. afghanicum* leaves different solvent extracts and volatile oil was carried out as per prescribed method (Bhakta et al., 2013; Bimlesh Kumar et al., 2011; Iqbal et al., 2004; Kumar et al., 2010; Partap et al., 2012).

4.7.4.3. Earthworms

Adult earthworms (*Eisenia foetida* L.) was used as a model for anthelmintic studies (Bimlesh Kumar et al., 2011; Pawar et al., 2014; Shintal et al., 2014). *Eisenia foetida* L. resembles in anatomical and physiological aspects as that of human intestinal parasitic roundworms. The healthy adult *Eisenia foetida* L. were procured from MAHAVIR ORGANIC MANURE vermi-compost farm, Phillaur, Jalandhar, Punjab, India.

4.7.4.4. Preparation of samples stock solutions

Stock solution of the extracts were prepared at concentration of 200 mg/ml in DMSO while that of volatile oil was prepared at concentration of 200 µl/ml DMSO, in separate small (10 ml) volumetric flasks. Aqueous extract solution (200 mg/ml) was prepared in normal saline (0.9% NaCl solution). All of the prepared stock solutions were stored at 4° C in refrigerator, until being used in preparation of diluted working formulations used in anthelmintic activity assay.

4.7.4.5. Preparation of standard anthelmintic stock solution

Albendazole oral suspension IP 200 mg/5ml (Ranbaxy Lab. Ltd. New Delhi) was procured from local pharmacy and was diluted with normal saline to prepare a suspension of 20 mg/ml as stock suspension. The suspension was then used in preparation of working suspensions of different concentrations (2 – 10 mg/ml) as standards in anthelminthic assay.

4.7.4.6. Preparation of diluent (normal saline) for preparation of working formulations

Amount of 9 g NaCl was accurately weighed and dissolved in 1000 ml distilled water to prepare one liter of normal saline solution. The solution was then used in preparation of standard/sample different dilutions (in range of 2– 10 mg/ml) designed for anthelmintic activity assay.

4.7.4.7. Anthelminthic assay

In present study, anthelmintic activity assay of HAKL was carried out against adult earthworms *Eisenia foetida* as per method described in previous literatures (Bimlesh Kumar et al., 2011), with slight modifications. Albendazole was used as standard anthelminthic drug and the worms used in the assay were observed for their paralysis time and death or mortality time at 6h post exposure (Iqbal et al., 2004).

Briefly;

1. Amount of 15 ml of the test samples (extract/volatile oil) dilutions at 5 different concentrations (2, 4, 6, 8 and 10 mg/ml) in normal saline were prepared from related stock solution and placed into a series of 5 petri plates (8 cm diameter). The volatile oil dilutions were prepared in range of 2 – 10 µl/ml.
2. Standard Albendazole dilutions at the same concentration range (2, 4, 6, 8 and 10 mg/ml) in normal saline were prepared and poured into 5 petri plates (15 ml per plate), separately.
3. Normal saline (15 ml/plate) and mixture of DMSO (0.5 ml) with normal saline (14.5 ml) were placed into two separate petri plates as negative controls (higher doses of DMSO also exert anthelminthic effect on earth worms).
4. The adult earthworms (*Eisenia foetida* L.) having 6 – 10 cm length and 0.2 – 0.3 mm diameter, were first rinsed with tap water to remove the clay and then were sorted into different groups of 3 worms of almost equal size in each group.
5. Then, in each petri plate containing 15 ml of either standard/sample solutions or negative controls, one group of the worms (3 worms) were released (Figure 27.).
6. The earthworms were observed individually at room ambience for recording the time (in minute) taken for paralysis and the time taken for death of each individual worm. Paralysis of the worms was confirmed when they did not show sensitivity in terms of motion, contraction or bending, except when pressed vigorously by finger at one of their ends. Death of the worms was confirmed by complete loss of their motility even by keeping them in warm water at 50^{o} C and subsequently fading of their body colour (A. Kumar et al., 2010; Bimlesh Kumar et *al*., 2011).

The inhibition of motility (paralysis) and/or mortality of the earthworms subjected to the treatment were considered as the criteria for anthelmintic activity of tested samples. For all tested samples, the experiments were carried out in triplicates and the results (paralysis time and death time) were recorded as mean±SD of the three experiments (n=3), (Table 25., Figure 28., Figure 29.).

Chapter V

Results and discussions

5. Results and discussions

5.1. Phytochemical screening of *H. afghanicum* leaves different solvent extracts

Table 4., represents the % extractive yield of HAKL obtained by different solvents namely petroleum ether, dichloromethane, ethylacetate, methanol and water. As shown the highest extractive value (%w/w) was recorded for aqueous extract (12.2%), followed by methanol extract (11.66%), petroleum ether extract (5.9%), ethyl acetate extract (3.45%) and dichloromethane extract (1.3 %).

Table 5., represents the results preliminary phytochemical screening of *H. afghanicum* leaves different solvent extracts. Phytochemical screenings were performed as per standard described procedures (Ansari, 2009; Houghton and Raman, 1998; Kokate, 2010; Satheesh et al., 2012; Trease and Evans, 2009). The purpose of these qualitative phytochemical tests was to comparatively figure out the chemical profile of every individual extract (Ansari, 2009). As shown in Table 4. Different classes of phytochemical were qualitatively found to be present at different level in *H. afghanicum* different solvent extracts. Interestingly, Molisch's test was positive for all tested extracts but intensely positive for methanol extract which indicates that sugars are well dissolving in methanol rather than other non-polar solvent used in extraction. Test for cardiac glycosides were negative, but for flavonoid glycosides were positive for all extracts, moderately positive for ethylacetate and methanol extract.

Phenolics were found to be in more amount in methanol extract, moderately present in ethylacetate a rare in DCM and aqueous extract but absent in phenolic. Since the phenolic extract was itself intensely green colored the $FeCl_3$ test was not well confirmed. Petroleum ether extract was rich in phytosterols, and tests were positive for detection of resin and terpenoids and furanocoumarins in pet ether extract. Flavonoids were found to be present in all tested extracts but more in methanol and ethylacetate extracts. However, this data cannot be considered sufficient, more phytochemical tests specific for some potent phytochemicals will provide further understanding regarding chemical profile of the plant.

Conclusively; preliminary phytochemical tests on HAKL different extracts collectively revealed presence of carbohydrates, flavonoids, phytosterols, terpenoids, phenolic compounds and furanocoumarins in the crude materials. However, presence of furanocoumarins in the extracts has been judged based on fluorescence which was produced

by sample solution under UV-light. Blue, bluish-green or yellowish fluorescence was observe for diluted solutions of the extracts while it intensified by addition of alkali solution.

Presence of furanocoumarins in many other *Heracleum spp*. are reported in previous works (see sub-sections 2.6.4. and 2.6.5. of Chapter II) (Sadeghi Nejad et al., 2014; Karuppusamy and Muthuraja, 2011).

Table 4. Data showing the % yield of *Heracleum afghanicum* leaves successive extractives.

S. No.	Solvent used	Extractive % (w/w)	Extract colour
1	PET ether	5.9	Dark green
2	Dichloromethane	1.3	Dark green
3	Ethylacetate	3.45	Dark green
4	methanol	11.66	Blackish-green
5	Distilled water	12.2	Dark brown

Table 5. Results of phytochemical screening of *H. afghanicum* different solvents extracts.

S. No.	Phytochemicals	PET extract	DCM extract	EA extract	MeOH extract	Aqu extract
01	**Alkaloid**	**-**	**-**	**-**	**-**	**-**
02	**Carbohydrate**					
	Molisch's test	**+**	**+**	**+**	**+++**	**+**
	Fehlings test	**-**	**-**	**-**	**+**	**-**
	Benedict's test	**-**	**-**	**-**	**++**	**-**
03	**Glycosides**					
	Legal test	**-**	**-**	**-**	**-**	**-**
04	**Saponin**	**-**	**-**	**-**	**-**	**-**
05	**Flavonoids**					
	Alkali R. Test	**+**	**+**	**++**	**++**	**+**
	Shinoda Test	-	**-**	**++**	**+**	**-**
	Lead Acetate Test	**+**	**-**	**+**	**++**	**+**
06	**Anthraquinone**	**-**	**-**	**-**	**-**	**-**
07	**Phenols**					
	FeCl3 Test	-	**+**	**++**	**+++**	**+**
08	**Tannin**					
	Gelatin test	**-**	**-**	**-**	**-**	**-**
09	**Sterols**					
	Liebermann – B test	**++**	**-**	-	**-**	**-**
	Salkowski test	**++**	**-**	-	**-**	**-**
10	**Terpenoids**					
	Lieberman – B test	**+**	**-**	-	**-**	**-**
	Salkowski's test	**+**	**-**	-	**-**	**-**
11	**Resins**					
	Alcohol-turbidity T.	**++**	**+**	**+**	**-**	**-**
	Acetone-turbidity T.	**++**	**+**	**+**	**-**	**-**
	$FeCl_3$ test	**+**	**+**	**-**	**-**	**-**
12	**Protein & amino acids**					
	Ninhydrin test	**-**	**-**	**-**	**-**	**-**
13	**Gum and Mucilage**	**-**	**-**	**-**	**-**	**-**
14	**Furanocoumarins**					
	Fluorescence test	+*	+**	+***	**-**	**-**

Aqu; aqueous, DCM; dichloromethane, EA; ethylacetate, MeOH; methanol, Pet; petroleum ether,
'+' Present, '++'moderately present, '+++'highly present, '-' absent, MeOH; methanolic,
* showed bluish fluorescence, ** Bluish fluorescence, *** yellowish fluorescence.

5.2. GC-MS analysis of *Heracleum afghanicum* leaves volatile oil

Shade dried coarsely powdered HAKL yields 0.3 - 0.4 % (v/w) volatile oil having aromatic odour, yellow colour and sharp spicy taste. The oil was lighter density than water. However, Karimi and Ito (2012) have reported presence of 1.5% volatile oil in mature fruits of the plant .*Heracleum afghanicum* Kitamura and this kind of discrepancy between same plants of different geographical regions is very common.

In present study, HAKL volatile oil was sent for GC-MS analysis at Institute of Himalayan Bioresource Technology (IHBT) - Palampur, Himachal Pradesh, India, using gas chromatography coupled with mass-spectrometry (GC-MS), and 25 compounds were identified in the tested oil (Figure 13.). Table 6, represents these compounds listed in order of their elution time from the column during GC-MS analysis (copy of the test report along with GS-MS chromatogram are attached as Appendix at the end of this thesis.). As shown in Table6., among 25 components identified in the oil, anethole is reported as the main component comprising 66.12% of the oil.

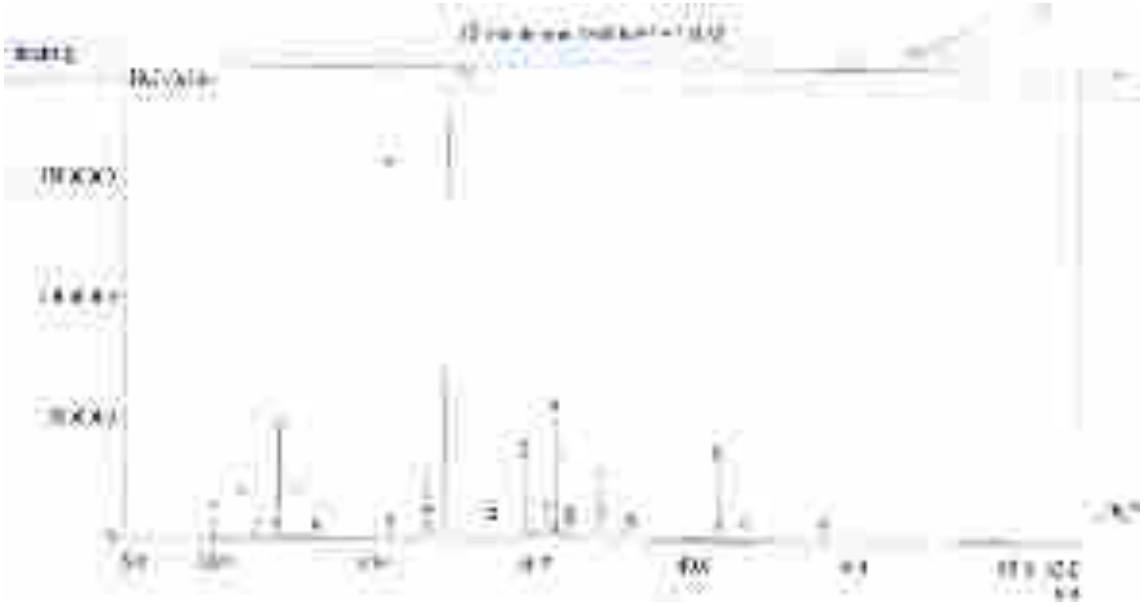

Figure 13. GC-MS chromatogram of *H. afghanicum* volatile oil.

Table 6. Data showing chemical composition of *H. afghanicum* leaves' volatile oil.

S. No.	Compounds	RT[a]	PA[b](%)	Chemical class
1	dl-Limonene	9.9	0.77	monoterpene
2	2-beta-Pinene	11.8	1.30	Monoterpene bicyclic
3	Beta-Myrcene	12.5	0.21	Monoterpene acyclic
4	p-Methylcumyl alcohol	13.9	0.31	Aromatic compound
5	dl-limonene	14.1	3.87	Monoterpene monocyclic
6	cis-Ocimene (3,7-dimethyl-Z-1,3,6-octatriene)	14.5	0.48	Monoterpene acyclic
7	beta-Ocimene (3,7-dimethyl-E-1,3,6-octatriene)	14.9	1.34	Monoterpene acyclic
8	Alpha-Terpinolene	16.4	0.26	Monoterpene monocyclic
9	p-Allylanisole (1-methoxy-4-(2-propenyl)-benzene)	21.0	0.46	Aromatic compound
10	p-Anethole (1-methoxy-4-(1-propenyl)-benzene), cis-Anethole	23.1	2.69	Aromatic compound
11	Anisaldehyde (4-methoxy-benzaldehyde)	23.4	0.71	Aromatic compound
12	Trans-Anethole 1-methoxy-4-(1-propenyl)-benzene.	24.6	66.12	Aromatic compound
13	Alpha-Copaene	27.4	0.51	Sesquiterpene tricyclic
14	Alpha-Bergamotene	29.4	2.79	Sesquiterpene bicyclic
15	Germacrene D	30.9	0.70	Sesquiterpene monocyclic
16	Trans-alpha-Bergamotene	31.3	4.30	Sesquiterpene bicyclic
17	Bicyclogermacrene	31.4	0.48	Sesquiterpene bicyclic
18	Farnesene	31.7	2.34	Sesquiterpenes acyclic
19	Cadinene	32.2	0.39	Sesquiterpene bicyclic
20	Spathulenol	34.1	6.40	Sesquiterpene triicyclic
21	Phenethyl isobutyrate, Isobutyric acid, Propionic acid, 2-methyl-2-phenylethyl ester.	36.0	0.22	Aromatic compound
22	Neophytadiene (2,6,10-trimethyl, 14-ethylene-14-pentadecne)	41.4	2.61	Aliphatic volatile hydrocarbon
23	6,10,14-trimethyl-2-Pentadecanone (Hexahydrofarnesyl acetone)	41.6	0.24	volatile hydrocarbon
24	9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)	43.1	0.23	Aliphatic ester (volatile hydrocarbon)
25	9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)	48.1	0.28	Aliphatic ester (volatile hydrocarbon)

The difference in composition of *H. afghanicum* leaves essential oil with that of its' fruits volatile oil reported by Karimi and Ito (2012) may be due to several factors such as geographical and geological variations, difference in climatic condition of plant local habitat, variations in season or time of collection, and chemotypic variation of the plant species. These factors are well understood to have considerable effects on both the amount as well as nature of plants secondary metabolites (Amiri, 2012).

It should be noted that Karimi and Ito (2012) have collected *H. afghanicum* fruits on July 2008, from Darrah-i-Fringel, Ghorband, Parwan, Afghanistan. While we have collected *H. afghanicum* mature leaves on 16th July, 2014 from Holang, Parwan, Afghanistan and the two regions are different in term of climate and geography.

5.3. Antioxidant activity evaluation of *H. afghanicum* leaves

Phytochemicals with antioxidant action are present in many plants including some fruits, vegetables, spices and herbs (Alok et al., 2014). Phenolics and flavonoids are reputed for being potent antioxidants of plant origin. Quality of botanicals totally depend upon amount of phytochemicals with potent biological activity. Determination of total phenolic content and total flavonoid content of some medicinal plant serve as quality test approach. Variety of in vitro antioxidant tests based on oxidation-reduction reactions are used to estimate the antioxidant potentials of different chemical and biological samples (Amiri, 2012).

In present work, *H. afghanicum* leaves was subjected to total phenolic content evaluation and total flavonoid content evaluation, in order to determine the two categories of phytochemicals in *H. afghanicum* leaves. Moreover, each individual extract and volatile oil of the crude material were subjected to two in vitro antioxidant assay methods namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and ferric ion reducing antioxidant power (FRAP) assay method.

5.3.1. Total phenolic content (TPC) of *H. afghanicum* leaves

TPC determination is used to evaluate existence of tannins, anthocyanin, simple phenolic compounds and polymeric pigments in tested sample (Gupta, 2013). In present study, TPC of HAKL different solvent extracts and volatile oil were evaluated for their phenolic content using Folin-Ciocalteu method (Gupta, 2013; Singleton et al., 1999).

Table 7., and Figure 14., represent UV-absorbance of gallic acid (765) which was used at concentrations range of 5 – 50 µg/ml for establishing a standard curve in Folin-Ciocalteu method. Aliquots of HAKL different solvent extracts (300 µg/ml) in methanol and aliquot of volatile oil (3000 µg/ml) in methanol, were use in TPC assay method. Table 8., represents the UV absorbance (mean±SD) of test samples at 765 nm wavelength and their relative TPC values (mg GAE/g sample). TPC values were calculated by reference to the regression formula (**y=0.0168x + 0.0675, R^2: 994**) obtained from gallic acid standard curve. Comparative TPC values for the tested samples are represented in Figure 15. As depicted in Figure 15, the order of TPC amount in HAKL extracts was highest for methanol extract >ethylacetate extract >dichloromethane extract >petroleum ether extract > aqueous extract >volatile oil.

As a result, among the extract methanolic and ethyl acetate extracts found to have the highest amount of 81.25±0.99 mg GAE/ g extract and 59.23±2.57 mg GAE/g dry extract respectively.

The results obtained regarding TPC value in this study, were found in compliance with many of previous works. Several authors reported the higher amount of TPC and significant antioxidant activity of methanolic and ethylacetate extracts of several plant leaves. Gawad et al, (2014) reported high phenolic contents and strong antioxidant properties of ethyl acetate fraction of defatted methanolic extract of *Allium* species. In another study by Gupta, (2013), the methanolic extracts of Cinnamon and Cloves exhibited strong antioxidant properties owing to their high phenolics and flavonoids content (Gupta, 2013). Rusaczonek et al. (2007) have evaluated total polyphenolic content and antioxidant activities of alcoholic extract and aqueous extract of five different culinary herbs (e.g. *Ocimum basilicum* L., *Melissa officinalis* L., *Origanum vulgare* L., *Rosmarinus officinalis* L. and *Thymus vulgaris* L.). The authors have reported higher TPC value and higher antioxidant activity for the alcoholic extract with positive correlation between TPC and antioxidant potential of tested samples (Rusaczonek et al., 2007).

In present study also a positive linear correlation was found between TPC and antioxidant activity of HAKL different solvent extracts (Table18.). Similarly, the TPC content of HAKL and the DPPH and FRAP assay results were very much correlated to each other.

The lower TPC value of aqueous extract (22.70±0.60) and petroleum ether extract (19.53±0.59) is also reasonable, because in present study, aqueous extract was obtained from the marc being already extracted by all other used solvents including methanol and hence it is poor in phytochemicals. In case of petroleum ether extract, the solvent being nonpolar is not able to dissolve adequately the polar phenolics and hence it is poor in phenolics. However the TPC value may be due to some phytochemicals present in pet extract and by having specific structure are reacting with FCR reagent in the assy. Volatile oil was found to very poor in phenolic compounds. However, based on GC-MS data the oil has 66.12% anethole and traces of other mono- and sesquiterpenes and aliphatic hydrocarbons. These compound are not phenolic and hence the TPC for volatile oil is the lowest one.

Briefly; methanol or ethylacetate could be the solvent of choice for extraction of phenolics from HAKL and both methanol extract and ethylacetate extract of HAKL could be further analyzed for identification of their chemical constituents and isolation of bioactive compound(s). Petroleum ether extract also showed moderate TPC value, but lower DPPH radical scavenging activity

Table 7: Data showing gallic acid absorbance in Folin-Ciocalteu method.

GA conc. (µg/ml)	Ab at 765 nm
5	0.127±0.003
10	0.245±0.006
20	0.391±0.003
30	0.624±0.007
40	0.745±0.005
50	0.883±0.008
60	1.073±007

GA = gallic acid, Ab = absorbance

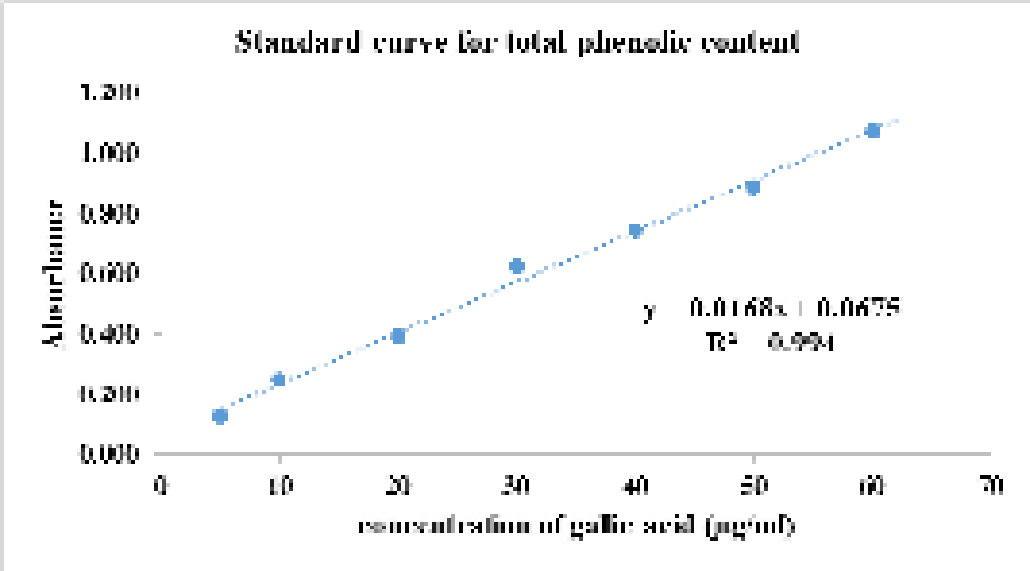

Figure 14: Graph showing standard curve of gallic acid in Folic-Ciocalteu method.

Table 8: Data showing the mean+SD absorbance and TPC values of tested samples in TPC assay.

Extract name	**Conc.(µg/ml)**	**Mean±SD at 765 nm**	TPC[a] (mg GAE/g extract)
Pet extract	300	0.166±0.003	19.54±0.59
DCM extract	300	0.343±0.008	54.67±1.58
EA extract	300	0.366±0.013	59.23±2.57
MeOH extract	300	0.477±0.005	81.25±0.99
Aqu. Extract	300	0.182±0.003	22.71±0.60
Volatile oil	3000	0.121±0.005	0.02±0.16*

Results are presented as mean±SD of triplicates.

Ab; absorbance, Aqu; aqueous, Conc.; concentration, DCM; dichloromethane, EA; ethylacetate, MeOH; ethanol, GAE; gallic acid equivalent, Pet; petroleum ether.

[a] TPC values are calculated as per gallic acid standard curve formula **y=**0.0168x + 0.0675; R^2=994).

*expressed as mg GAE/ml of volatile oil.

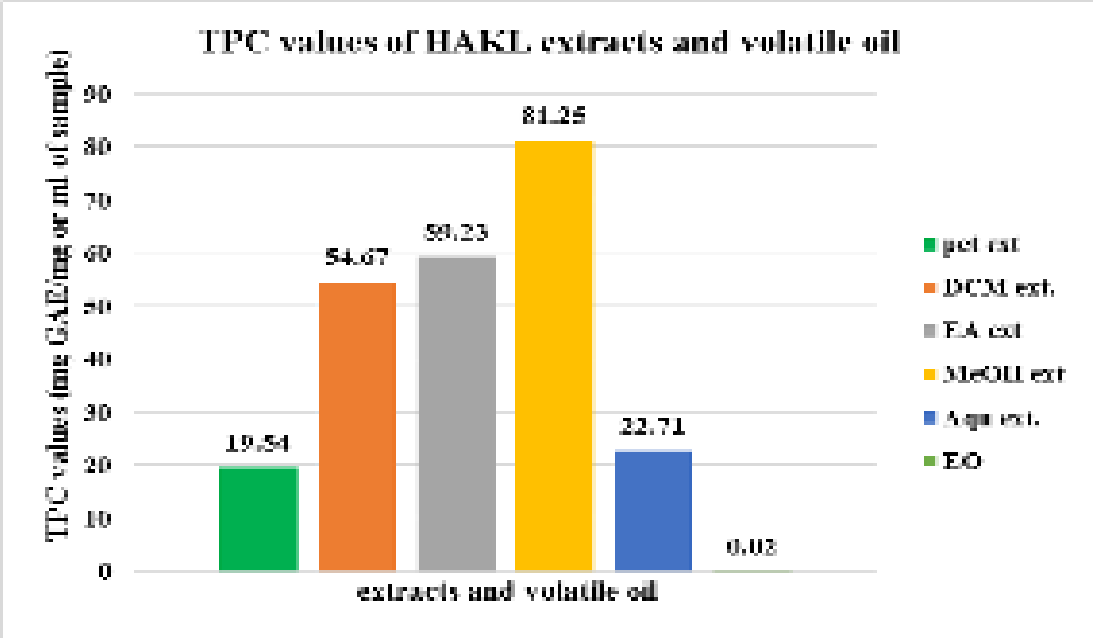

Figure 15. Chart showing comparative total phenolic contents of HAKL different extracts and volatile oil.

5.3.2. Total flavonoid content (TFC) of *H. afghanicum* leaves

H. afghanicum leaves extracts were evaluated for their total phenolic content using aluminium chloride method. Rutin at concentration range of 10 – 50 µg/ml was used to establish a standard curve. The mean±SD absorbance of rutin aliquots at 415 nm wavelength are presented in Table 9. HAKL extracts were used at concentration of 300 µg/ml in the assay. The TFC value for the tested extracts were determined based on regression formula ($y=0.021x - 0.2033$, R^2; 0.9973) obtained from rutin standard curve (Figure 16.). TFC values were therefore expressed as rutin equivalent (mg of RUE/ g of extract) as presented in Table10., and depicted in Figure 17.

As shown in both Table 10. and Figure 17., TFC of the extracts varied from 63.7±4.27 mg RUE/g extract to 123.07±0.80 mg RUE/g extract. The highest flavonoid content (123.07±0.80 mg RUE/g extract) was found for methanol extract followed by ethylacetate extract, petroleum ether extract, dichloromethane extract and aqueous extract with TFC values of 110.2±1.27, 97.5±1.27, 82.73±0.43 and 63.07±4.27 mg RUE/g extract, respectively.

Table 9: Data showing the mean±SD absorbencies of rutin serial dilutions at 415 nm in aluminium chloride assay method.

Rutin conc. (µg/ml)	Ab at 415 nm
10	0.025±0.003
20	0.207±0.004
30	0.411±0.002
40	0.626±0.004
50	0.867±0.005

Results are noted as mean±SD of three readings

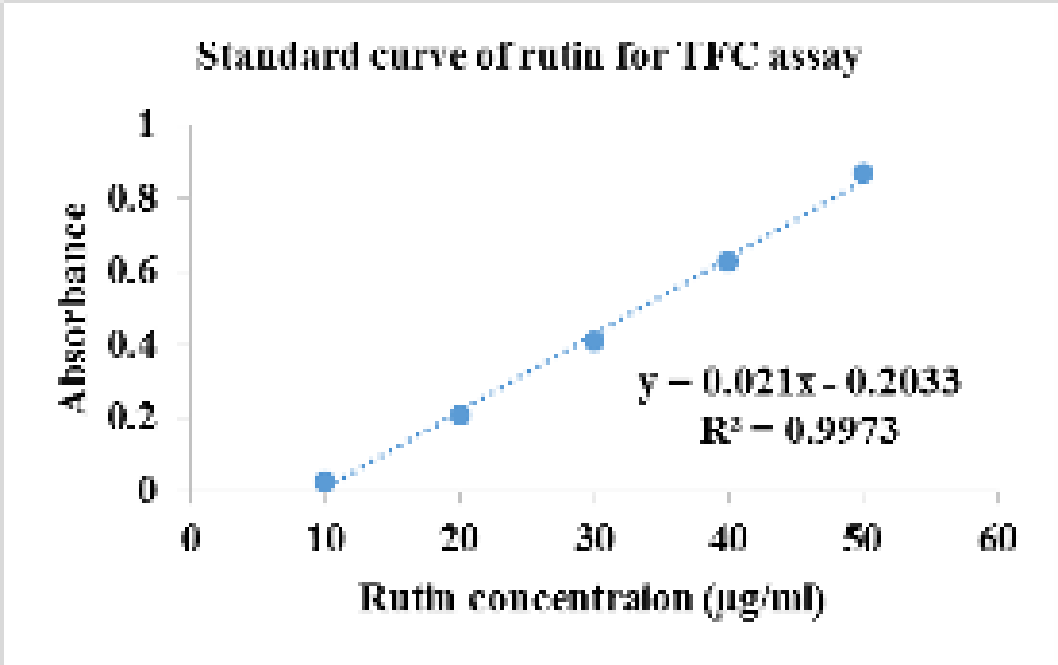

Figure 16: Graph showing the standard curve of rutin absrobance vs. concentration in aluminium chloride method.

Table 10: Data showing the total flavonoids content of HAKL extracts.

Extract type	Conc.(µg/ml)	Mean Abs. at 415 nm	TFC as mg RUE/mg dried extract
Pet extract	300	0.411±0.008	97.5±1.27
DCM extract	300	0.318±0.009	82.73±0.43
EA extract	300	0.491±0.008	110.2±1.27
MtOH extract	300	0.572±0.005	123.07±0.80
Aqu. Extract	300	0.198±0.027	63.7±4.27

Abs=absorbance, Aqu=aqueous, Conc.=concentration, DCM=dichloromethane, EA=ethylacetate, MtOH=Methanol, RUE=rutin equivalent, Pet=petroleum ether.

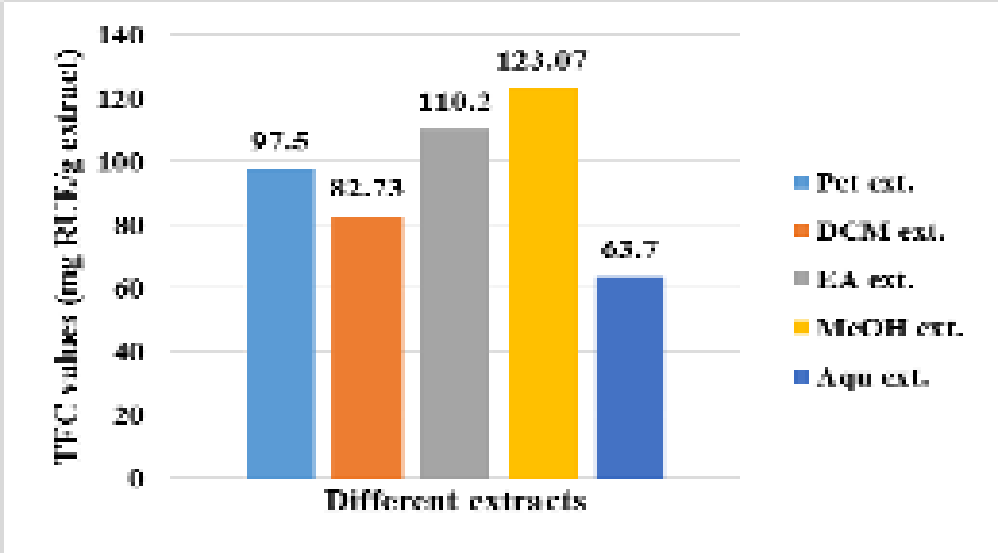

Figure 17: Chart showing the TFC values of *H. afghanicum* leaves extracts as mg RUE/g of extract

In present study, *H. afghanicum* methanol extract and ethylacetate extract exhibited higher amounts of flavonoid compounds and hence, are suggested for further analysis, purification and identification of their flavonoids.

Flavonoids are reputed for their broad spectrum biological activities including antioxidant and free radical scavenging potentials (Borkataky et al., 2013).

5.3.3. DPPH radical scavenging activity of *H. afghanicum* leaves

H. afghanicum different extracts (at concentration range of 10 – 100 µg/ml methanol) and volatile oil (0.5 – 3.0 µl/ml) were evaluated for their DPPH radical scavenging activity. Ascorbic acid dilutions (0.25 – 2.5 µg/ml), was used as standard antioxidant.

Table 11. represents the mean±SD absorbencies of ascorbic acid dilutions at 517 nm. While Figure 18 and Figure 19., depict ascorbic acid standard curve in DPPH assay and its %inhibition of DPPH free radical, respectively. The radical scavenging activity of the test extracts as well as standard ascorbic acid was calculated as percentage inhibition of DPPH free radical, applying the following formula:

$$\% \text{ inhibition} = [\ (Ac - As) / Ac\ ] \times 100$$

Where, Ac is the absorbance of control (containing all of the reagents except the sample) and As is the absorbance of sample solution.

Table 12 and Table 13., represent the absorbance of tested extracts dilutions and %inhibition free radical for the test extract, respectively. Figure 20., indicates %inhibition of DPPH free radical for the tested extracts. Percent inhibition of DPPH free radicals were increasing by increase in concentration of test samples. Hence, a dose dependent free radical scavenging activity was observed for all the extracts and volatile oil.

Table 14., shows the absorbance of tested volatile oil aliquots and %inhibition of free radical, for the oil. Table 15., represents IC_{50} values for all tested extracts, volatile oil and ascorbic acid.

IC_{50} stands for the concentration of sample/standard required for inhibition of 50% of the DPPH free radicals used in the assay (Muniruzzaman et al., 2012; Suttee and Rana, 2012; Valyova et al., 2012). Lower IC_{50} value of a sample indicates its higher radical scavenging activity (Borkataky et al., 2013).

IC_{50} values for both the samples and standard were calculated from their plotted graphs of % inhibition vs. concentrations. IC_{50} value for the extracts were in the range of 16.6 **µg/ml**

to 89.36 µg/ml. IC_{50} for ascorbic acid was 1.69 µg/ml. Lower IC_{50} indicates higher antioxidant activity (Ramamoorthy and Bono, 2007).

Among the tested extracts, methanolic extract exerts the highest antioxidant capacity (IC_{50} = 16.12). The remaining extracts DPPH radical scavenging capacity decreased in order of ethylacetate extract > dichloromethane extract > petroleum ether extract > aqueous extract with IC_{50} values of 28.34> 37.34 > 54.41> 89.36, orderly.

In DPPH assay, the stronger activity of methanolic extract may be due to a higher amount of phenolic and flavonoid compounds in this extract. Similarly, in many previous works also, methanolic fraction have shown highest values in DPPH radical scavenging activity assays.

Table 11: Data showing the mean±SD absorbance of ascorbic acid serial dilutions and its % inhibition of DPPH free radicals.

S. No.	Ascorbic acid (µg/ml)	Ab at 517 nm	% Inhibition
1	2.5	0.126±0.003	72.36±0.65
2	2	0.193±0.005	57.67±1.09
3	1.5	0.244±0.002	46.49±0.44
4	1.25	0.283±0.004	37.93±0.87
5	1	0.321±0.002	29.60±0.43
6	0.75	0.353±0.004	22.58±0.87
7	0.5	0.370±0.003	18.86±0.66
8	0.25	0.417±0.003	8.55±0.66
9	Control	0.456	-

Results are recorded as mean±SD of the three readings

Abs = absrobance, SD= standard deviation

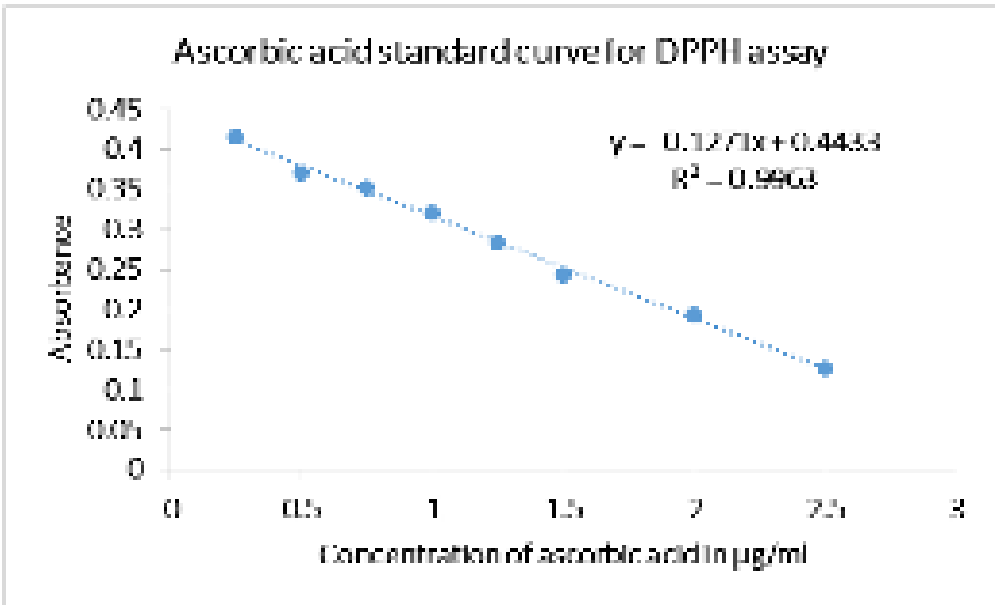

Figure 18: Graph showing ascorbic acid standard curve for DPPH assay method.

Table 12: . Data showing absorbance of *H. afghanicum* different extracts in DPPH assay.

S. No.	concentration (µg/ml)	Absorbance at 517 nm				
		Pet ext.	**DCM ext.**	**EA ext.**	**MeOH ext**	**Aqu. Ext.**
1	10	0.427±0.003	0.354±0.003	0.361±0.002	0.274±0.002	0.388±0.002
2	25	0.357±0.010	0.310±0.003	0.245±0.003	0.123±0.004	0.342±0.001
3	40	0.289±0.005	0.252±0.003	0.135±0.002	0.100±0.022	0.308±0.003
4	55	0.204±0.008	0.192±0.002	0.055±0.001	0.077±0.005	0.287±0.004
5	70	0.148±0.011	0.117±0.006	0.038±0.002	0.072±0.004	0.249±0.015
6	85	0.101±0.002	0.060±0.003	0.040±0.006	0.061±0.001	0.218±0.003
7	100	0.095±0.005	0.055±0.002	0.041±0.002	0.056±0.003	0.185±0.003
8	**control**	0.447	0.510	0.455	0.415	0.415

Aqu= aqueous, DCM=dichloromethane, EA=ethaylacetate, Ext.=extract, MeOH=methanol, Pet = petroleum ether.

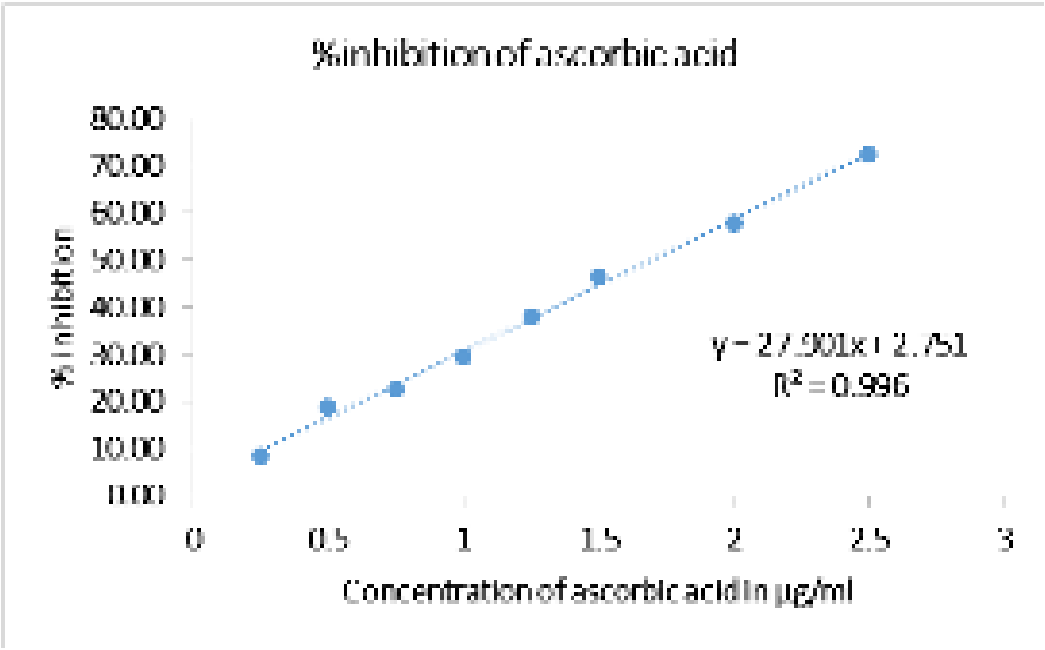

Figure 19: Graph showing percentage inhibition activity vs. concentration of ascorbic acid.

Table 13: Data showing the % inhibition of HAKL different solvent extracts in DPPH assay

S. No.	concentration (µg/ml)	% inhibition				
		Pet ext.	**DCM ext.**	**EA ext.**	**MeOH ext**	**Aqu. Ext.**
1	10	4.47±0.60	30.59±0.58	20.65±0.41	33.97±0.48	6.50±0.48
2	25	20.13±2.23	39.21±0.58	46.15±0.59	70.36±0.96	17.59±0.24
3	40	35.35±1.12	50.58±0.59	70.32± 0.41	75.90±5.30	25.78±0.72
4	55	54.36±1.79	62.35±0.39	87.91±0.22	81.44±1.20	30.84±0.94
5	70	66.897±2.46	77.06±1.18	91.64±0.41	82.65±0.96	40.00±3.62
6	85	77.40±0.44	88.23±0.58	91.21±1.32	85.30±0.24	47.46±0.72
7	100	78.75±1.12	89.21±0.39	90.98±0.41	86.50±0.72	55.42±0.72

Aqu= aqueous, DCM=dichloromethane, EA=ethaylacetate, Ext.=extract, MeOH=methanol, Pet = petroleum ether.

Table 14: . Data showing absorbance of *H. afghanicum* volatile oil aliquots and the % inhibition of DPPH radicals.

S. No.	concentration (µl/ml)	Mean±SD Absorbencies at 517 nm	%inhibition
1	0.5	0.448±0.002	13.01±0.48
2	1.0	0.401±0.005	22.13±0.97
3	1.5	0.364±0.001	29.32±0.19
4	2.0	0.320±0.001	37.86±0.19
5	2.5	0.291±0.008	43.50±1.56
6	3.0	0.225±0.003	56.31±0.58
7	control	0.515±0.005	-

Results are recorded as mean±SD of the triplicates

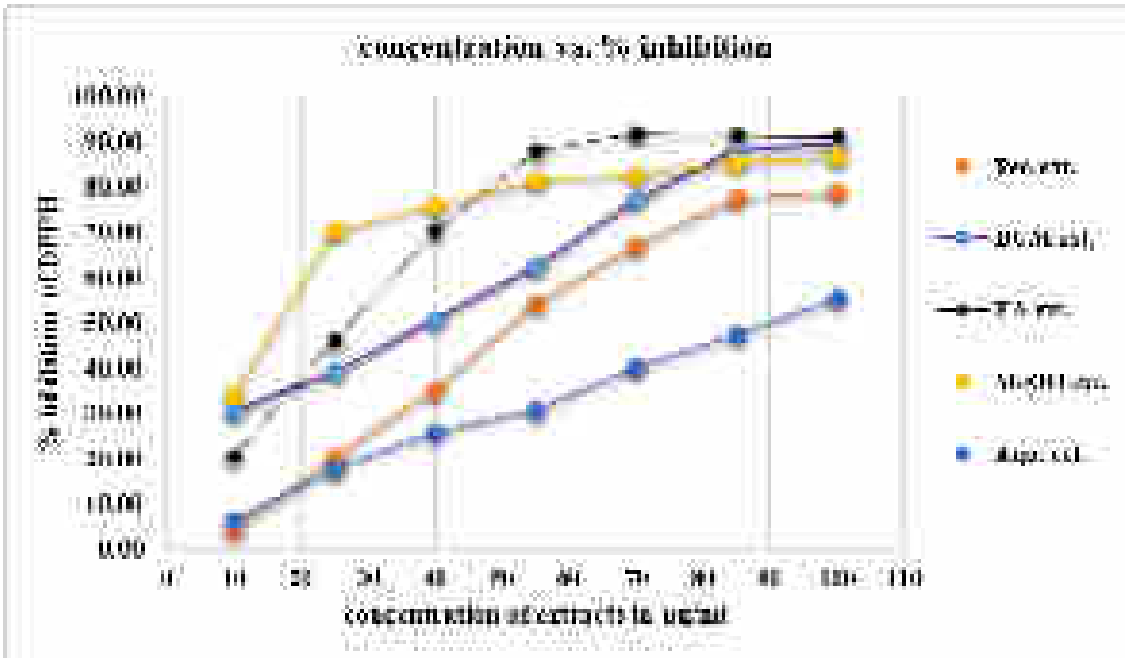

Figure 20: Graph showing % inhibition of DPPH radical scavenging, for HAKL different extracts.

Table 15: Data showing the IC_{50} of HAKL different solvent extracts, volatile oil and ascorbic acid.

IC_{50} value (µg/ml)						
Pet ext.	**DCM ext.**	**EA ext.**	**MeOH ext**	**Aqu. Ext.**	**Volatile oil**	**Ascorbic acid**
54.41	37.34	**28.34**	**16.60**	89.36	2.73 µl/ml	1.69

Aqu. = aqueous, DCM = dichloromethane, EA = ethylacetate, IC_{50}= inhibitory concentration of 50% radical, MeOH = methanol, Pet = Petroleum ether,

5.3.4. Ferric ion Reducing Antioxidant Power (FRAP) assay of *H. afghanicum*

H. afghanicum leaves different solvent extracts and volatile oil were subjected to FRAP assay method as per described method (Anbarasu et al., 2014). $FeSO_4$ solution at concentration range of 0.1 – 1.0 mM in water, was used to establish a standard curve (Table16. Figure 21.). HAKL extracts at concentration of 100 µg/ml in methanol and the volatile oil at concentration of 1µl/ml in methanol were used in FRAP assay method. Absorbencies of the sample mixtures with FRAP reagent were measured at 593 nm using UV spectrophotometer. All experiments were carried out in triplicate and results were recorded as mean±SD of the triplicates as presented in Table 17.

Table 17, represents FRAP values for the tested samples both in term or Mm FeSO4 eq/mg extract and mg FeSO4 eq/g extract.

As in FRAP assay, the antioxidant capacity of a test sample is measured based on Fe^{+3} reducing ability of the sample. *H. afghanicum* leaves different extracts and volatile oil being subjected to FRAP assay, showed different Fe^{+3} reducing potential. Since the samples were use in the assay at dose of 100 µg/ml concentration, then any extract exhibiting higher absorbance at 593 nm will be considered as the sample with strong reducing power and potent antioxidant activity.

FRAP values of HAKL different solvent extracts and volatile oil were calculated from the regression formula (Y=1.7882x – 0.046) obtained from $FeSO_4$ standard curve. The FRAP values for the tested extracts and volatile oil were expressed as mM equivalent of $FeSO_4$ (Cristina M. Mihai et al. 2011).

Taking into account the molecular mass of FeSO4 . 7H2O (278g/mol), FRAP values for the samples were calculated in mg too, as are tabulated in the same Table 17.

As shown in Table 17, and Figure 22., FRAP values for HAKL extracts varied from 1100 up to 3080 mM/g dried extract equivalent to 305.8 to 856.2 mg/g extract, expressed as equivalent of $FeSO_4$. The highest FRAP value of 3080 mM/g extract (or 856.2±5.56 mg/g extract), was found for methanol extract followed by ethylacetate extract, petroleum ether extract, dichloromethane extract and aqueous extract with FRAP values of 2330 mM (647.74 mg), 1430 mM (397.54 mg), 1260 mM (350.28 mg), and 1100 mM (305.8 mg) $FeSO_4$ Eq/g extract, respectively. The aqueous extract and the volatile oil exhibited the lower and lowest FRAP values of 1100 mM $FeSO_4$ Eq/g extract, and 417 mM (115.9 mg) $FeSO_4$ Eq/ml volatile oil), respectively.

However, the reason for lower FRAP value of the aqueous extract is well known in present work, because the marc was already washed out successively by four precedent used solvents including methanol. Methanol is a strong polar solvent and most of polar as well as non-polar compounds are soluble in this solvent. Low FRAP value for the volatile oil can be attributed to the terpenic nature (monoterpens and sesquiterpenes) and aliphatic compounds in the oil which may not react with the reagents used in FRAP assay (see GC-MS analysis report).

Table 16: Data showing Triplicate and mean**±SD** absorbencies of $FeSO_4$ dilutions at 593 nm wavelength.

S. No.	$FeS0_4$ conc. (mM)	Absorbance at 593 nm in FRAP assay			
		1st batch	2nd batch	3rd batch	mean±SD
1	0.1	0.059	0.058	0.055	0.057±0.002
2	0.2	0.347	0.344	0.328	0.340±0.010
3	0.4	0.699	0.687	0.676	0.687±0.012
4	0.6	1.119	1.094	1.080	1.098±0.020
5	0.8	1.412	1.426	1.413	1.417±0.008
6	1.0	1.676	1.669	1.661	1.669±0.008

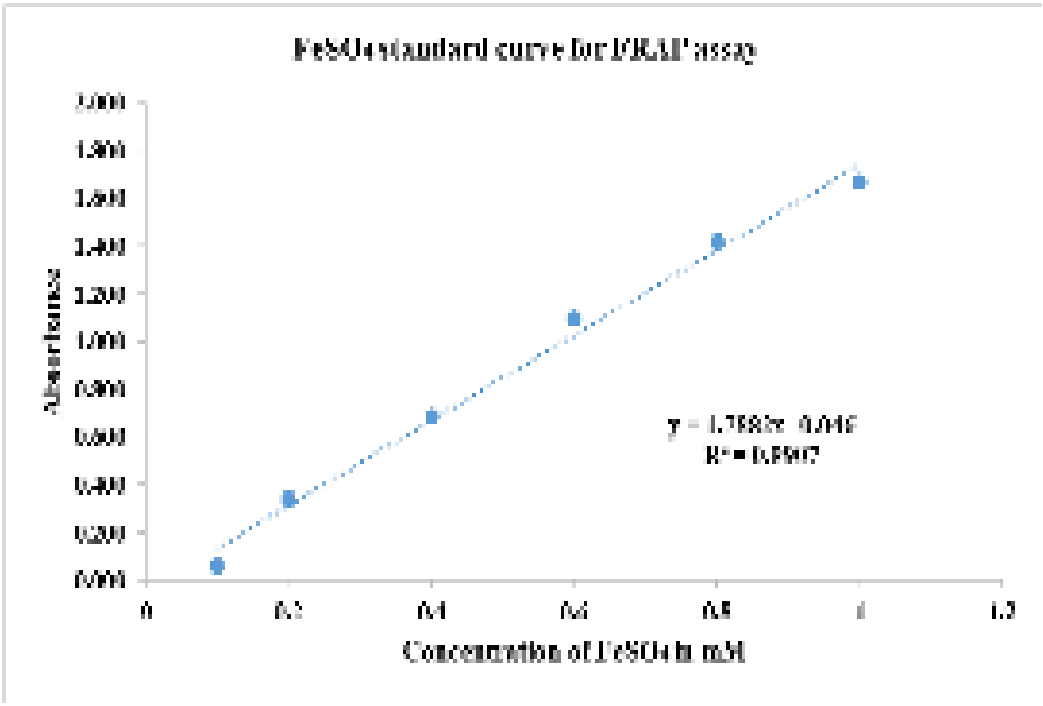

Figure 21: Graph showing standard curve of $FeSO_4$ for FRAP assay method.

Table 17: Data showing the FRAP values of HAKL different solvent extracts and volatile oil.

S. No.	Sample Code	Concentration (µg/ml)	Absorbance (593 nm)	FRAP for used dilutions	FRAP values (mM $FeSO_4$/ g of ext.)	FRAP values* (mg $FeSO_4$/g of ext.)
1	Methanol extract	100	0.504±0.005	0.308±0.002	3080 ±20	856.24±5.56
2	Ethylacetate extract	100	0.370±0.002	0.233±0.001	2330 ± 10	647.74±2.78
3	Petroleum ether extract	100	0.210±0.002	0.126±0.000	1430 ± 10	397.54±2.78
4	Dichloromethane extract	100	0.179±0.001	0.126±0.000	1260 ± 0.0	350.28±0.00
5	Aqueous extract	100	0.151±0.001	0.110±0.000	1100 ± 0.0	305.80±0.00
6	Volatile oil	1µl/ml	0.700**±0.018**	0.417**±0.010**	417.0 ± 10 **	115.93±2.78 ***

Results are calculated from the mean absorbencies of three different experiments.

* mg of $FeSO_4$ was calculated from its mM data, MW of FeSO4 . 7H2O is 278.01 g/mol.

** expressed as mM of $FeSO_4$/ml of Volatile oil

*** expressed as mg of $FeSO_4$/ml of volatile oil

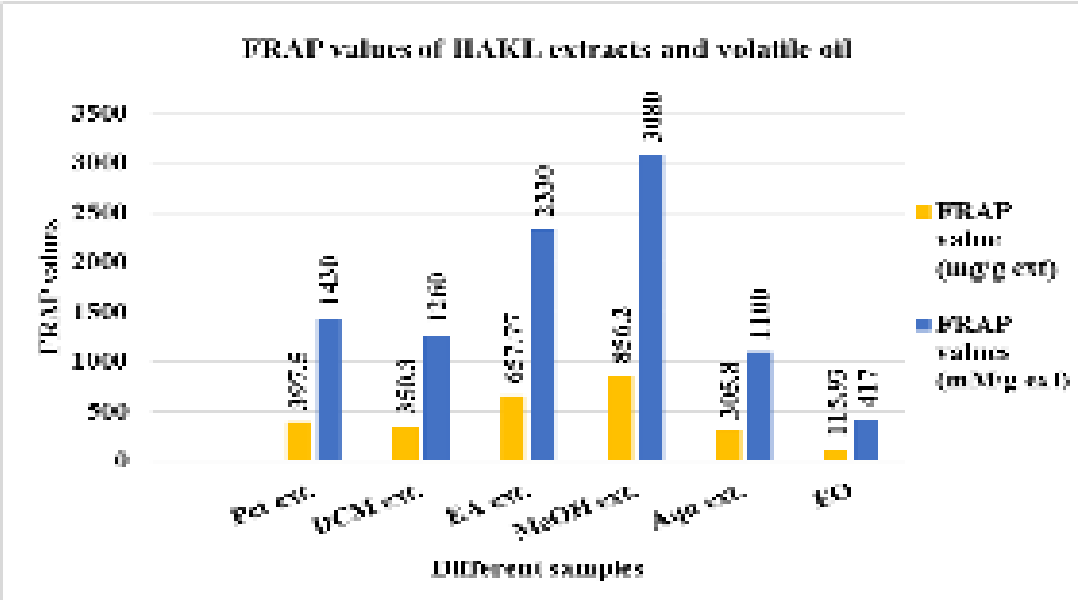

Figure 22: Chart showing FRAP values of *H. afghanicum* different extracts and volatile oil.

Based on obtained data, methanolic extract exhibited higher TPC and TFC values as well as stronger antioxidative capacity. Our results showed well compliance with those reported in literature. In a previous report, significant correlation has been reported between total phenolic contents and antioxidant activity of *Chrysanthemum morifolium* Ramat., extract (Packer et al., 2004). Rusaczonek et al (2007) have reported a comparative evaluation of TPC and antioxidant activity of leaves, stalk and stem of five different herbs. Alcoholic extracts of the tested plant leaves were reported to have highest TPC value and higher antioxidant capacity (Rusaczonek et al., 2007).

Statistical analysis

All the tests were carried out in triplicates and values were recorded as mean ± standard deviation (n=3). The data related to TPC, TFC, DPPH and FRAP assays of HAKL were statistically analyzed using Microsoft excel ANOVA function. Tukey's least significant difference (LSD) test was used to determine the significance of the results. $P<0.05$ was considered statistically significant (Table 18.)

Correlation

Correlation between all assays were calculated using Microsoft excel correlation function.

As per correlation studies, positive correlations were revealed between samples' % inhibition of DPPH radical and both their TPC and TFC values. As shown in Table 18, between all of the values obtained from performed antioxidant assays, highly positive correlations exist. While the strongest positive correlation (r = 9998) was recorded TPC value and FRAP value of ethylacetate extract and among the data, lower correlation (r = 0.7964) was recorded for TFC vs. DPPH of methanolic extract. All other correlations fall between these two values.

Table 18. Data showing correlations between samples DPPH % inhibition activity and FRAP values with both TPC and TFC values of HAKL extracts.

Sample code	Statistics	TFC* vs. DPPH %I	TFC vs. FRAP assay	TPC vs. DPPH %I	TPC vs. FRAP assay
Pet extract	r = R^2 = P value =	0.9772 0.955	0.9881 0.9763	0.995 0.9904	0.997 0.994
DCM extract	r = R^2 = P value	0.9941 9883 < 0.05	0.9717 0.9441	0.9923 0.9847 < 0.05	0.9815 0.9633 < 0.05
EA extract	r = R^2 = P value =	0.9526 0.9074 < 0.05	0.9988 0.9977 < 0.05	0.9613 0.924 < 0.05	0.9998 0.9997 < 0.05
MeOH extract	r = R^2 = P value =	0.7964 0.6342	0.9929 0.986 < 0.05	0.8175 0.6683	0.9913 0.9827 < 0.05
Aqu extract	r = R^2 = P value =	0.9748 0.9500 < 0.05	0.9836 0.9676 < 0.05	0.9791 0.9600	0.9825 0.9653 < 0.05
Volatile oil	r = R^2 = P value =	- - -	- - -	0.9884 0.9770 < 0.05	0.9884 0.9935 < 0.05

r: Pearson correlation coefficient, **R^2**:Rgression coefficient, **P**: Probability values, **TPC**: total phenolic content, **TFC:** total flavonoid content, **FRAP**: ferric reducing antioxidant power, **DPPH**: 1,1-diphenyl-2-picrylhydrazyl. P<0.05 was considered significant.

As shown in Table 18., correlation between samples TPC and DPPH assay; TPC and FRAP assay; TFC and DPPH assay and TFC and FRAP assay results were found to be positively high. Between TPC and FRAP assay and between TFC and FRAP assay of ethylacetate extract higher correlations (r=0.9998) and (r=0.9988) were found respectively. For methanolic extract, correlation between TFC and DPPH assay and TPC and DPPH assay were found (r=0.7964) and (r=0.8175), respectively. However, the correlations between both TFC and TPC values and FRAP assay for methanol extract were also high, r=0.9929 and r=0.9913 respectively. From the correlations values it is clear that antioxidant potential of the extracts pertain to their phenolics and flavonoid contents. Moreover, methanolic extract may contain other potent antioxidants also, since the higher values in both DPPH scavenging and FRAP assays were recorded for methanol extract.

Briefly; these positive correlations reveal that both phenolics and flavonoids are mainly involved in antioxidative properties of *H. afghanicum* leaves.

5.4. Antimicrobial activity evaluation of *H. afghanicum* leaves

Antimicrobial activity of *H. afghanicum* leaves' different solvent extracts and volatile oil were tested against two Gram+ve bacteria (*Bacillus subtillis* and *Staphylococcus aureus*), two Gram –ve bacteria (*E. coli* and *Proteus vulgaris*) and three fungal strains (*Aspergillus fumigatus*, *Candida albicans* and *Malassezia furfur*).

5.4.1. Antibacterial activity evaluation of *H. afghanicum* leaves

Table 19 and Table 20 represent the results (ZOIs) of *H. afghanicum* leaves antibacterial activity in disc diffusion method (DDM) and agar wells diffusion method (WDM), respectively. In both methods, *H. afghanicum* leaves different solvent extracts and volatile oil were screened against two Gram+ve bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), and two Gram –ve bacteria (*E. coli* and *Proteus vulgaris*). The antimicrobial strength of tested samples and standard Gentamicin were determined in term of their ZOIs measured in millimeter. Moreover, activity index (AI) for the every used concentrations of the tested samples were calculated as per the following formula:

AI = ZOI of sample / ZOI produced by standard

Where, AI stands for activity index of the tested sample at used concentration.

The higher the AI value for a sample the greater is its antimicrobial efficacy.

AI values of tested samples in antibacterial assay are represented in Table 21

As shown in Table 19, *H. afghanicum* tested samples discs impregnated in solutions of 25 – 100 mg/ml, produced ZOIs in range of 3.0 – 14.67 mm in disc diffusion assay while in well diffusion assay (Table 20), ZOIs produced by the test samples the test samples used at doses of 1.25 – 5 mg/well, were recorded to be in range of 3.0 – 27.83 mm. Gentamicin (10 µg/disc) in disc diffusion assay produced ZOI in range of 25.7 – 33.4 mm while in agar well diffusion assay at dose of 5 µg/well produced ZOI of 26.0 – 27.0 mm against tested bacteria. However gentamicin was found to exhibit almost same antimicrobial effect against all tested bacteria, and *Proteus vulgaris* with ZOI of 33.4±0.63 was more susceptible to gentamicin. Considering the doses of gentamicin 10 µg/disc used in DDM and 5 µg/well used in WDM, the drug showed slightly lower antibacterial action in WDM rather than in DDM, whilst half of the dose used in DDM is used in WDM (Figure 24.). This discrepancy clearly indicate that drug used in solution form in the wells diffuse more efficiently rather than drug used in solid form or incorporated in micro-discs used in DDM. Diffusion of drug through agar media in disc diffusion method can be considered as a main drawback of DDM. It is very much possible that the drug loaded on the discs may not be entirely released and completely diffuse through agar medium, and hence produce smaller ZOI in the assay.

Similarly, all of test samples at used doses were found to produce larger ZOIs in WDM (3.0 – 27.83 mm) rather than in DDM (3.0 – 14.67 mm) while were tested against the same bacteria on same nutrient medium (Figure 23). Activity index (AI) for the tested sample in DDM were recorded to be in range of 0.09 – 0.52 while in WDM this criteria for the tested samples were found to be in range of 0.10 – 0.94 (Table 22 and Table 23). In a general term, the samples showed almost double activity in WDM as compared with the results of DDM (Figure 23., Figure 24.). However, in case of the extracts and volatile oil this discrepancy in the results of DDM and WDM may also be in certain extent due to the dose variations used in the two

antimicrobial assays. In WDM, surely the samples at the exact doses of 1.25, 2,5 and 5 mg /well (1.25, 2.5 and 5 µl/well volatile oil) were placed in the wells but in DDM sterile discs were impregnated in sample solution of different strength (25 mg/ml, 50 mg/ml and 100 mg/ml) and then were placed on seeded agar plates. In this case, it can be claimed that the impregnated discs might not absorbed sufficient amount of the extract solution to be equivalent with the doses used in WDM (1.25, 2.5 and 5 mg/well). Hence, no one can guarantee the exact amount of extract loaded on the discs, and hence this lower amount of extracts and volatile oil loaded on sterile disc give lower ZOI in DDM as compared with ZOI produced in WDM. But still we will insist on efficacy of WDM in antimicrobial susceptibility assays based on the results of Gentamicin standard disc and its solution used in our work.

In present study, among all of the tested samples ethylacetate extract was found more potent against all tested bacteria, in both antibacterial assays (Table19 and Table20). The highest antibacterial activity of ethylacetate extract was observed in agar wells diffusion assay. Actually, ethylacetate extract exhibited the higher antibacterial effect against all tested bacteria in both antibacterial assay methods. The disc impregnated with the highest concentration of ethylacetate extract (100 mg/ml) produced ZOIs of 14.67±0.29, 14.05±0.5, 12.67±0.29 and 12.5±0.5 mm against *P. vulgaris*, *S. aureus*, *B. subtillis* and *E. coli,* respectively. Interestingly, ethylacetate extract at the highest dose of 5 mg/well in WDM produced ZOIs of 27.83±0.29, 27.33±1.15, 22.0±0.5 and 21.67±0.29 mm against tested bacteria namely *P. vulgaris, S. aureus, B. subtillis* and *E. coli,* respectively. The results shown by ethylacetate extract against the same tested bacteria in both the assays are very much similar and both the assays P. vulgaris found more susceptible against the extract followed by *S. aureus*, *B. subtillis* and *E. coli*. The antibacterial activity of ethylacetate extract was dose dependent since with decrease of the doses, exhibited lower ZOI and vice versa.

The highest activity of ethylacetate extract can be due to richness of the extract in various phytochemicals. Ethylacetate is said to be the best solvent for extraction of most phytochemicals since in easily penetrate into the cells and tissues of plants and dissolve the bioactive intracellular compounds of plants. However, further work in order to analyze the extract for its chemical ingredients is emphasized.

H. afghanicum leaves dichloromethane extract was the second potent sample revealed significant antibacterial action in both the assays. The extract in DDM at the higher used dose (disc dipped in 100 mg/ml solution) revealed ZOIs of 7.66±0.29, 6.75±0.35, 4.83±0.29 and 4.17±0.29 mm against *S. aureus*, *B. subtillis, E. coli* and *P. vulgaris*, respectively. The same extract at high dose (5 mg/well) used in WDM, produced ZOIs of 17.67±0.58, 11.0±0.87, 7.33±0.29 and 6.17±0.29 mm against *S. aureus*, *B. subtillis*, *E. coli* and *P. vulgaris*, respectively (Table 19 and Table 20).

The activity of DCM extract in both antibacterial assay was very much identic in term of susceptible bacteria, since in both assays *S. aureus* was found more susceptible. In present work, dichloromethane extract was found to show better antibacterial impact on Gram+ve bacteria (*S. aureus* and *B. subtillis*) rather than Gram – ve bacteria (*E. coli* and *P. vulgaris*). However, as shown in Tabel 21. Activity index (AI) for DCM extract at high treatment dose in disc diffusion assay was found to be in range of 0.12 – 0.27 while in well diffusion method ranged between 0.2 – 0.58. The highest index was found against *S. aureus* while the lowest was for *P. vulgaris*. Although, the antibacterial activity of DCM extracts was dose dependent, *P. vulgaris* was found less sensitive to the extract. At lower treatment doses in disc diffusion assay, the extract did not produce any ZOI against Gram – ve *P. vulgaris* (Figure 23.).

This higher activity of DCM extract against Gram-positive bacteria may be due to more affinity of the lipophilic phytochemicals present in the extract with the bacteria cell wall. Further study regarding chemical analysis of the extract is required to justify the specific action of the extract on Gram+ve bacteria.

Antibacterial impact of petroleum ether extract was negligible in DDM with AI value of 0.11 (ZOI of 3.67±0.58) and 0.15 (ZOI of 3.83±0.28) at highest treatment dose against *P. vulgaris* and *S. aureus*, respectively, and no activity against remaining bacteria. In WDM, petroleum ether at highest treatment dose (5 mg/well) revealed comparable antibacterial impact against *S. aureus*, *B. subtillis* and *E. coli* with respective ZOI of 15.83±0.29 (AI = 0.48), 12.00±0.5 (AI = 0.52) and 7.0±0.5 (AI = 0.30). *P. vulgaris* was found to be resistant against petroleum ether extract. The results in agar well method show that pet ether extract also indicate antibacterial action against Gram+ve tested bacteria rather than Gram-ve bacteria. This can be

attributed to the more lipophilic constituent of the extract. Based on phytochemical screening of the extract, presence of phytosterols, diterpenes and furanocoumarins were qualitatively confirmed in pet extract, but further quantitative analysis of the extract will adequately reveal its constituents.

Antibacterial effects of methanol extract was ignorable while aqueous extract did not produce any ZIO at all.

H. afghanicum leaves volatile oil same like other samples, produce weaker activity in DDM and moderate antimicrobial activity in WDM. The oil at highest treatment dose (5 mg/well) in agar well diffusion method reveal moderate antibacterial action against all tested bacteria. At 5 mg/well, *P. vulgaris* with ZOI of 13.66±0.76 (0.58) was found to be more susceptible, followed by *S. aureus*, *B. subtillis* and *E. coli* (Table 20.).

The activity of volatile oil can be attributed to its complex chemical nature which may exert a synergistic antimicrobial effect. GC-MS analysis of *H. afghanicum* leaves volatile oil confirmed presence of 25 different compounds as presented in Table 6. Anethole comprise 66.12% of HAKL volatile oil and based on GC-MS analyses. Anethole is reported for deferent biological activities including antimicrobial action. The compound has shown to enhance fungicidal effect of other antifungal drugs.

Biological activities of plant extracts depends upon the complex chemical nature of the tested extract. Higher antibacterial activity of HAKL reveals that the crude drug is rich in potent phytochemicals.

In present study, *H. afghanicum* ethylacetate extract was found more potent against the tested bacteria, followed by dichloromethane extract, petroleum ether extract and the volatile oil. Considering the excellent antimicrobial potential of *H. afghanicum* leaves ethylacetate extract, further study of the extract is suggested regarding isolation, identification, and biological activity evaluation of its isolated compounds. The potent phytochemicals present in *H. afghanicum* leave may serve as lead compounds to develop pharmaceutical formulations with strong antimicrobial action.

Some other *Heracleum* species are previously reported for their potent biological effects including antibacterial activity.

Table19: Data showing ZOIs produced by *H. afghanicum* leaves extracts and volatile oil in **disc diffusion method** of antibacterial assay.

Sample code	Concentration*	ZOI in mm			
		Gram + ve bacteria		Gram – ve bacteria	
		B. subtillis	***S. aureus***	***E. coli***	***P. vulgaris***
Pet extract	100mg/ml	3.83±0.28	0	0	3.67±0.58
=	50 mg/ml	0	0	0	0
=	25 mg/ml	0	0	0	0
DCM extract	100mg/ml	6.75±0.35	7.66±0.29	4.83±0.29	4.17±0.29
	50 mg/ml	4.83±0.29	6.0±0.50	3.0±0.0	0
	25 mg/ml	3.0±0.00	4.66±0.29	2.33±0.29	0
EA extract	100mg/ml	**12.67±0.29**	**14.05±0.5**	**12.5±0.5**	**14.67±0.29**
=	50 mg/ml	8.0±0.5	10.66±0.29	10.0±0.5	9.0±0.50
=	25 mg/ml	7.17±0.76	6.33±0.29	7.67±0.29	5.83±0.29
MeOH ext.	100 mg/ml	0	4.33±0.29	3.83±0.29	0
=	50 mg/ml	0	0	3.17±0.29	0
=	25 mg/ml	0	0	0	0
Aqu. extract	100 mg/ml	0	0	0	0
=	50 mg/ml	0	0	0	0
=	25 mg/ml	0	0	0	0
Volatile oil	100 mg/ml	4.67±0.28	4.0±0.5	3.5±0.5	9.0±0.5
=	50 mg/ml	4.17±0.57	3.6±0.76	0	5.8±0.76
=	25 mg/ml	4.17±0.57	0	0	3.0±0.0
Gentamicin	10µg/disc	25.7±0.49	28.14±0.6	25.8±0.57	33.4±0.63

Results are the mean±SD of the three experiments.
(disc diameter 5 mm is not subtracted)

Table20: Data showing ZOIs produced by *H. afghanicum* extracts and volatile oil in agar **wells diffusion method** of antibacterial activity.

Sample code	Concentration	ZOI (in mm)			
		Gram + ve bacteria		Gram – ve bacteria	
		B. subtillis	***S. aureus***	***E. coli***	***P. vulgaris***
Pet extract	5mg/well	12.00±0.5	15.83±0.29	7.0±0.5	0
	2.5 mg/well	9.17±0.29	9.67±0.29	6.17±0.29	0
	1.25 mg/well	7.17±0.29	8.17±0.29	4.67±0.29	0
DCM extract	5mg/well	11.0±0.87	**17.67±0.58**	7.33±0.29	6.17±0.29
	2.5 mg/well	10.17±0.29	11.33±0.58	7.0±0.5	4.33±0.29
	1.25 mg/well	8.17±0.29	10.0±0.50	6.33±0.29	3.00±0.0
EA extract	5mg/well	**22.0±0.5**	**27.33±1.15**	**21.67±0.29**	**27.83±0.29**
	2.5 mg/well	14.67±0.29	15.33±0.58	13.33±0.29	14.83±0.29
	1.25 mg/well	9.67±0.29	12.67±0.29	10.67±0.29	9.83±0.29
MeOH extract	5mg/well	4.83±0.28	0	0	0
	2.5 mg/well	0	0	0	0
	1.25 mg/well	0	0	0	0
Aqueous extract	5mg/well	0	0	0	0
	2.5 mg/well	0	0	0	0
	1.25 mg/well	0	0	0	0
Volatile oil	5mg/well	**10.16±0.28**	**11.0±0.5**	**8.0±0.5**	**13.66±0.76**
	2.5 mg/well	7.0±0.5	6.33±0.29	4.83±0.28	5.83±0.28
	1.25 mg/well	5.83±0.28	4.17±0.76	4.0±0.0	3.83±0.28
Gentamicin	5µg/well	26.0±0.6	27.0±0.58	26.5±0.9	26.5±0.53

Results are the mean±SD of the three experiments.

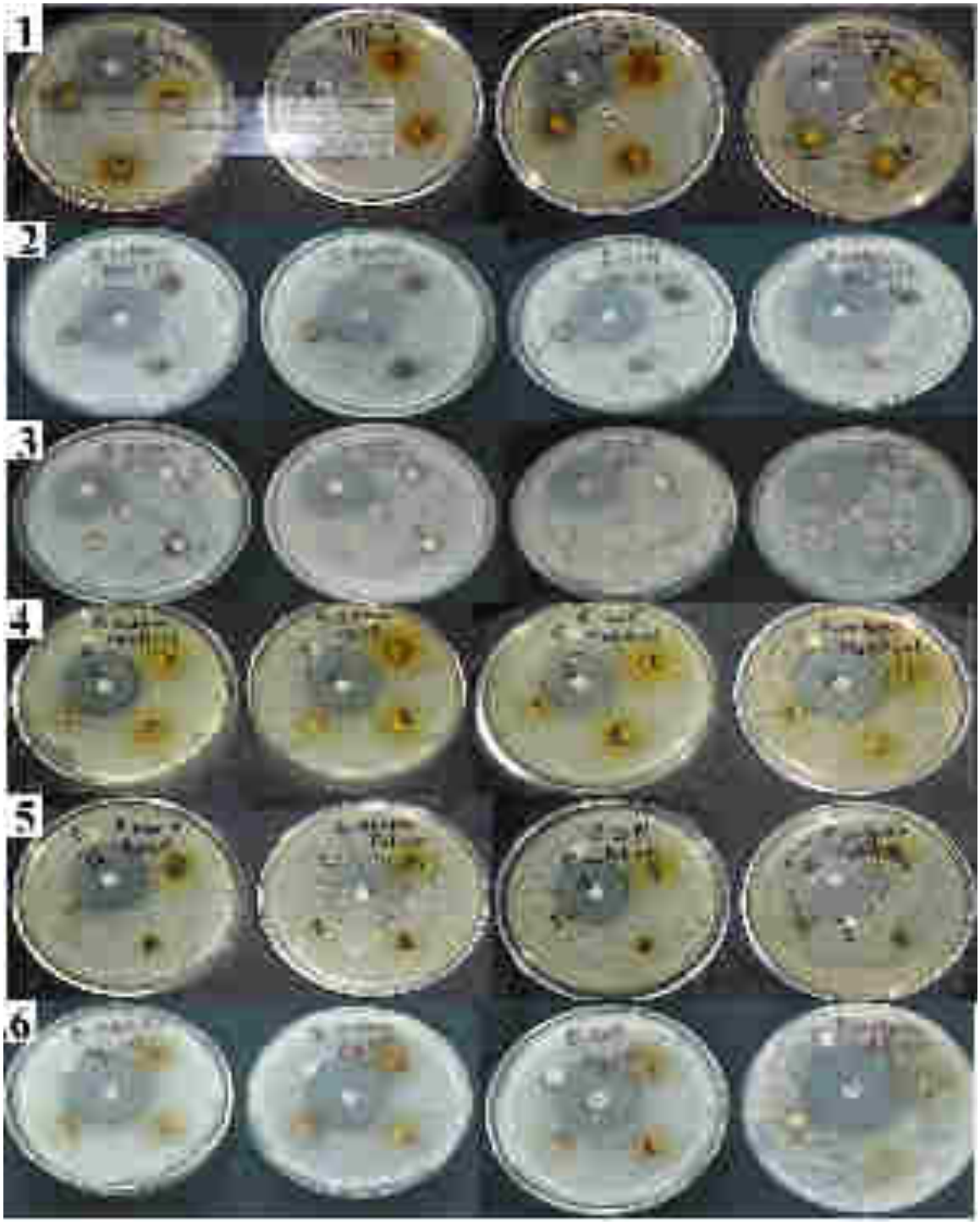

Figure 23. Antibacterial assay of *H. afghanicum* using disc diffusion method:
From left to right; *B. subtillis, S. aureus, E. coli* and *P. vulgaris.*
1, ethylacetate extract, 2. Dichloromethane extract, 3. Essential oil, 4. Methanol extract, 5. Pet ether extract, and 6. Aqueous extract.

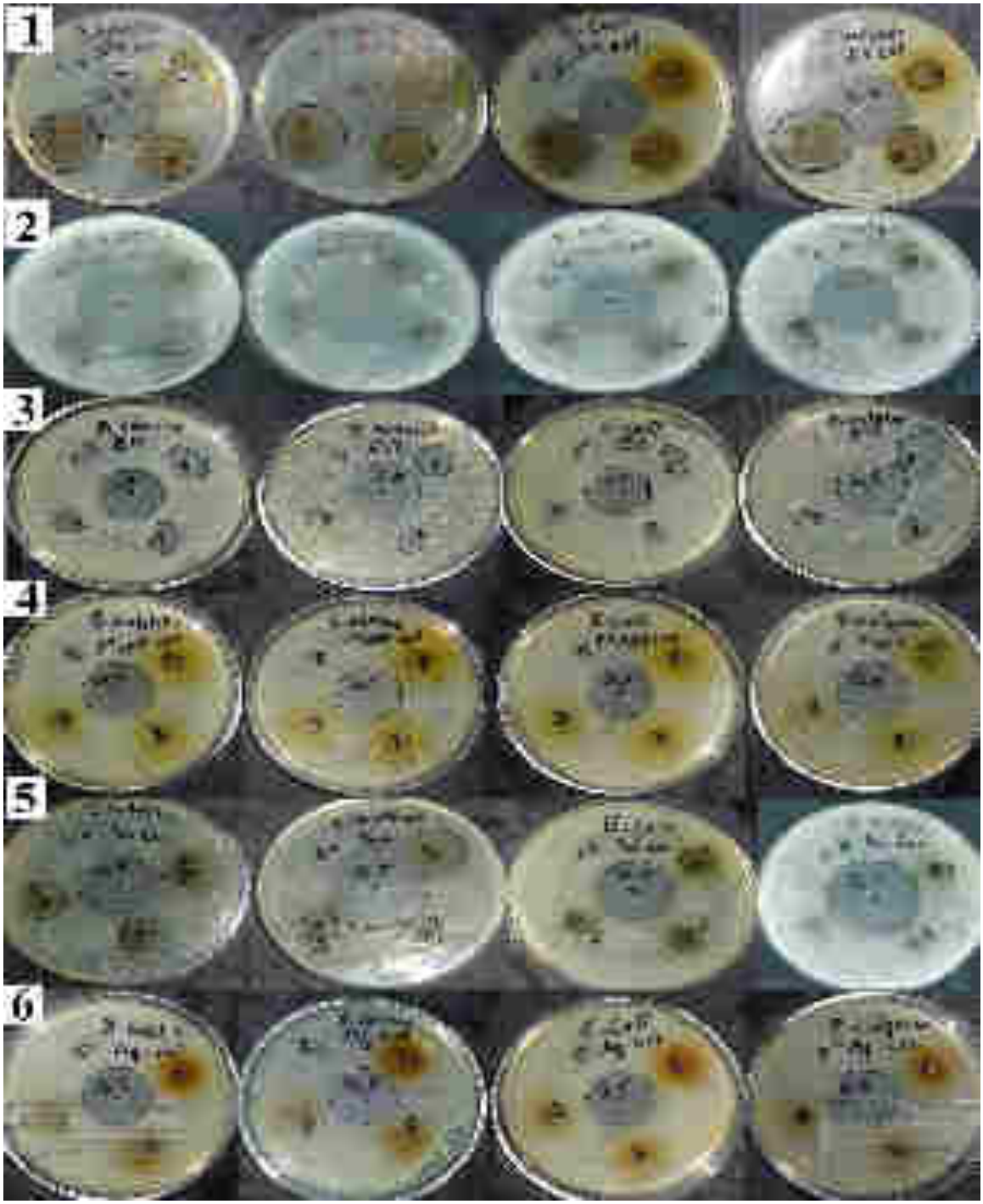

Figure 24. Antibacterial assay of *H. afghanicum* using agar wells diffusion method; from left to right; *B. subtillis, S. aureus, E. coli and P. vulgaris.*
1, ethylacetate extract, 2. Dichloromethane extract, 3. Essential oil, 4. Methanol extract, 5. Pet ether extract, and 6. Aqueous extract.

Table 21. Data showing activity index of *H. afghanicum* extracts and volatile oil in DDM and WDM of antibacterial assay

S. No.	microbe	Sample concentration		AI of tested samples										Mean ZOI of Gentamicin*	
		Disc impregnated	mg/well	Pet ext.		DCM ext.		EA ext.		MeOH ext.		VO			
				DDM	WDM	DDM	WDM	DDM	WDM	DDM	WDM	DDM	WDM	DDM	WDM
1	B. subtilis	100 mg/ml	5	0.15	0.52	0.26	0.35	0.49	0.94	0	0.21	0.18	0.45	25.7±0.49	24.3±0.52
		50 mg/ml	2.5	0	0.40	0.19	0.32	0.31	0.62	0	0	0.16	0.31		
		25 mg/ml	1.25	0	0.31	0.12	0.26	0.28	0.41	0	0	0.16	0.26		
2	S. aureus	100 mg/ml	5	0	0.48	0.27	0.58	0.52	0.96	0.15	0	0.14	0.43	28.14±0.6	27.5±0.7
		50 mg/ml	2.5	0	0.29	0.21	0.37	0.38	0.54	0	0	0.13	0.25		
		25 mg/ml	1.25	0	0.25	0.17	0.33	0.23	0.45	0	0	0	0.16		
3	E. coli	100 mg/ml	5	0	0.30	0.19	0.32	0.48	0.95	0.15	0	0.14	0.36	25.8±0.57	22.03±1.5
		50 mg/ml	2.5	0	0.26	0.12	0.30	0.39	0.58	0.12	0	0	0.22		
		25 mg/ml	1.25	0	0.20	0.09	0.28	0.30	0.47	0	0	0	0.18		
4	P. vulgaris	100 mg/ml	5	0.11	0	0.12	0.20	0.44	0.94	0	0	0.27	0.58	33.4±0.63	26.8±0.64
		50 mg/ml	2.5	0	0	0	0.14	0.27	0.50	0	0	0.17	0.25		
		25 mg/ml	1.25	0	0	0	0.10	0.17	0.33	0	0	0.09	0.16		

5.4.2. Antifungal activity evaluation of *H. afghanicum* leaves

Table 22, and Table 23, represent the data (ZOIs) pertaining to *H. afghanicum* leaves antifungal activity using disc diffusion method (DDM) and wells diffusion method (WDM), respectively. *H. afghanicum* different solvent extracts and volatile oil were screened against three fungal strains namely; *A. fumigatus*, *C. albicans* and *M. furfur*. Antifungal potential of the tested samples were determined in term of zone of inhibition (ZOI) and activity index (AI). ZOI as the diameter of the clear circular area where fungal growth was inhibited by the sample/standard, was measured in mm. AI value for each sample were calculated by dividing the ZOI produced by sample by the ZOI produced by standard antifungal drug, as per the following formula:

AI of sample = ZOI produced by standard drug / ZOI produced by test sample

Table 24 represent the AI of the tested sample against tested fungi. As shown in Table 24., the standard antifungal drugs amphotericin-B (20µg/disc) and fluconazole (10µg/disc) used as positive controls in disc diffusion method, did not produce measurable ZOI against *Malassezia furfur*, and hence, the AI for the samples showing activity against *M. furfur* were not determined and presented as ND (not determined). However, amphotericin-B produced ZOI of 11.0±0.5 and 13.6±1.45 respectively against *C. albicans* and *A. fumigatus* in disc diffusion method, whilst contrarily, the test samples except the volatile oil, did not produce ZOI against the two fungi (Table 24).

Two reasons can be given that why amphotericin –B (20 µg/disc) did not exert activity against *M. furfur* and fluconazole (10 µg/disc) did not show antifungal effect at all. First; these tested fungal strains may be resistant against the said drugs. Secondly; the used dose of the drugs my not exert observable antifungal impact.

In wells diffusion method, Itraconazole (20 µg/well) was used as positive control, and produced ZOIs of 15.53±0.45, 14.36±0.56 and 6.25±0.43 against *Aspergillus fumigatus*, *Candida albicans*, and *Malassezia furfur*, respectively. Thus, AI values for those samples producing ZOI were calculated based on ZOI produced by Itraconazole in WDM (Table23). Tested samples at used doses exhibited stronger activity rather than Itraconazole (20 µg/well) against *M. furfur* in wells diffusion assay.

In present work, the tested samples except the aqueous extract, showed different antifungal activities. *Malassezia furfur* was found to be the most susceptible fungi against all applied doses of all tested samples except the aqueous and methanolic extracts. Whilst the two other fungal strains (*Aspergillus fumigatus* and *Candida albicans*) were found sensitive in both assay methods against all applied doses of the volatile oil only. All tested extracts were found to be inactive against *A. fumigatus* and *C. albicans* in disc diffusion method. However, in wells diffusion method, ethylacetate extract at higher doses (2.5 and 5 mg/well) was found to be active against *A. fumigatus* by producing ZOI and AI of 7.17±0.29 (AI; 0.46) and 3.0±0.0 (AI; 0.19) mm, respectively. The effect of dichloromethane extract at the highest applied dose (5 mg/well) against *C. albicans* with ZOI of 3.83±0.29 mm (AI; 0.29) was ignorable.

As shown in both Table 22 and Table23, ZOIs for the susceptible fungi, *M. furfur* are recorded to be in range of 6.83±0.28 – 19.67±1.26 and 3.17±0.29 – 17.33±0.58 mm, in disc diffusion method and wells diffusion method, respectively. The highest ZOI in both the assays were recorded to be produced by ethylacetate extract.

In disc diffusion assay, ethylacetate extract at three increasing doses (in term of sterile discs impregnated in extract solutions of 25, 50 and 100 mg/ml concentration) produced ZOI of 14.67±0.76, 17.83±0.29 and 19.67±1.26 mm, respectively against *Malassezia furfur*. Since in disc diffusion method none of the used standard antifungal drugs produced measurable ZOI against *Malassezia furfur*, the activity index (AI values) for the test samples were not determined and hence shown as ND (not determined) in Table 24. Ethylacetate extract in agar wells diffusion assay, showed similar dose dependent activity against *M. furfur* too, and at doses of 1.25, 2.5 and 5 mg/well, it produced ZOIs and activity index (AI) of 11.00±0.50 (AI; 2.77) 13.33±0.29 (AI; 2.13) and 17.33±0.58 mm (AI; 1.76), respectively.

The excellent and dose dependent antifungal activity of ethylacetate extract against *Malassezia furfur* in both assays may be due to its richness in potent lipophilic phytochemicals with particular fungicidal property against *Malassezia furfur*. In wells diffusion assay, *Aspergillus fumigatus* was found resistant against all samples except ethylacetate extract and volatile oil. Volatile revealed ZOI of 11.5±0.29 (AI; 0.74), 8.5±0.5 (AI;0.54) and 5.5±0.5 (AI; 0.34) at dose of 5, 2.5 and 1.25 mg/well. Ethylacetate showed mild antifungal activity with ZOI

of 7.17±0.29 (AI; 0.46) and 3.00±0.00 (AI; 0.19) at used doses of 5 mg/well and 2.5 mg/well, respectively, against *A. fumigatus*.

Generally, the potent activity of the volatile oil may be due to its complex chemical nature. Since the GC-MS analysis of the oil revealed presence of 25 different compounds along with traces of other unknown compounds. *Trans*-anethole was found to be the major compound comprising 66.12% of the oil, then this compound along with other oil constituents exert the strong antifungal effects against tested fungi. Anethole is reported to be a broad antimicrobial agent and its activity differs against specific microorganisms (Fujita et al., 2006).

Our data match well with those of previous works which reported the synergistic effects of anethole. In a previous study, Fujita et al., (2006) have reported that anethole as a potent antimicrobial synergist, enhances antimicrobial activities of other antimicrobial agents against various microorganisms. For instance, it enhances fungicidal action of dodecanol against *Saccharomyces cerevisiae*. Similarly, anethole when was combined at a sub-lethal dose (equivalent to ½ MIC) with sorbic acid, was found to decrease the MIC of sorbic acid against *Saccharomyces cerevisiae* from 1600 to 200 µg/ml, and hence, 8-fold increased the antifungal effect (Fujita et al., 2006). In another study, anethole as a strong antifungal synergist when combined (at a sub-lethal dose) with polygodial, was found to enhance128-fold the fungicidal activity of the compound against *Candida albicans* and *Saccharomyces cerevisiae* (Kubo and Himejima, 1991).

The discrepancy seen in volatile oil activity against *A. fumigatus* in the two assays may be due to difference in the diffusion process of the oil volatile components through agar medium from both oil solution and disc impregnated in the oil. Since discs were directly placed on agar plate surface, the diffusion of the oil component in superficial areas of the seeded agar plate may be faster and easier rather than diffusion of the hydrophobic constituents of the oil solution placed in the wells through agar medium. In addition, diffusion of the oil component from the loaded disc through superficial area of agar medium may be more efficient than placing of oil solution in the wells. Because from the wells the oil component at higher extent my diffuse at the beneath or bottom of the agar media and hence my not reach properly to the fungi streaked on the surface of agar plate to efficiently inhibit fungal growth. It can also be estimated that the

volatile hydrophobic compound of the oil placed in wells may need more time to diffuse through the medium and maintain a concentration gradient in superficial areas around the wells. But from the impregnated discs the oil component may easily disperse into surrounding area of the disc and hence produce their antifungal activity against seeded fungal strain.

Briefly; the standard fluconazole (10 µg/disc) did not exhibit antifungal activity at all, against tested fungi. Amphotericin-B (20 µg/disc) was active against *A. fumigatus* and *C. albicans* but inactive against *M. furfur*. Among the tested fungal strains the most susceptible fungi was *M. furfur* inhibited by all tested samples except aqueous extract and doses < 2.5 mg/well methanol extract. Ethylacetate extract was found to be the most potent, followed by dichloromethane extract, petroleum ether extract and the volatile oil. However, *H. afghanicum* leaves' volatile oil exhibited a wider range of antifungal activity. *Aspergillus fumigatus* was inhibited only by the volatile oil at all used doses in disc diffusion assay, while it was also inhibited by ethylacetate extract at doses > 2.5 mg/wells in well diffusion assay. Similarly, *C. albicans* was inhibited only by the volatile oil in both assays (Figure25 and 26).

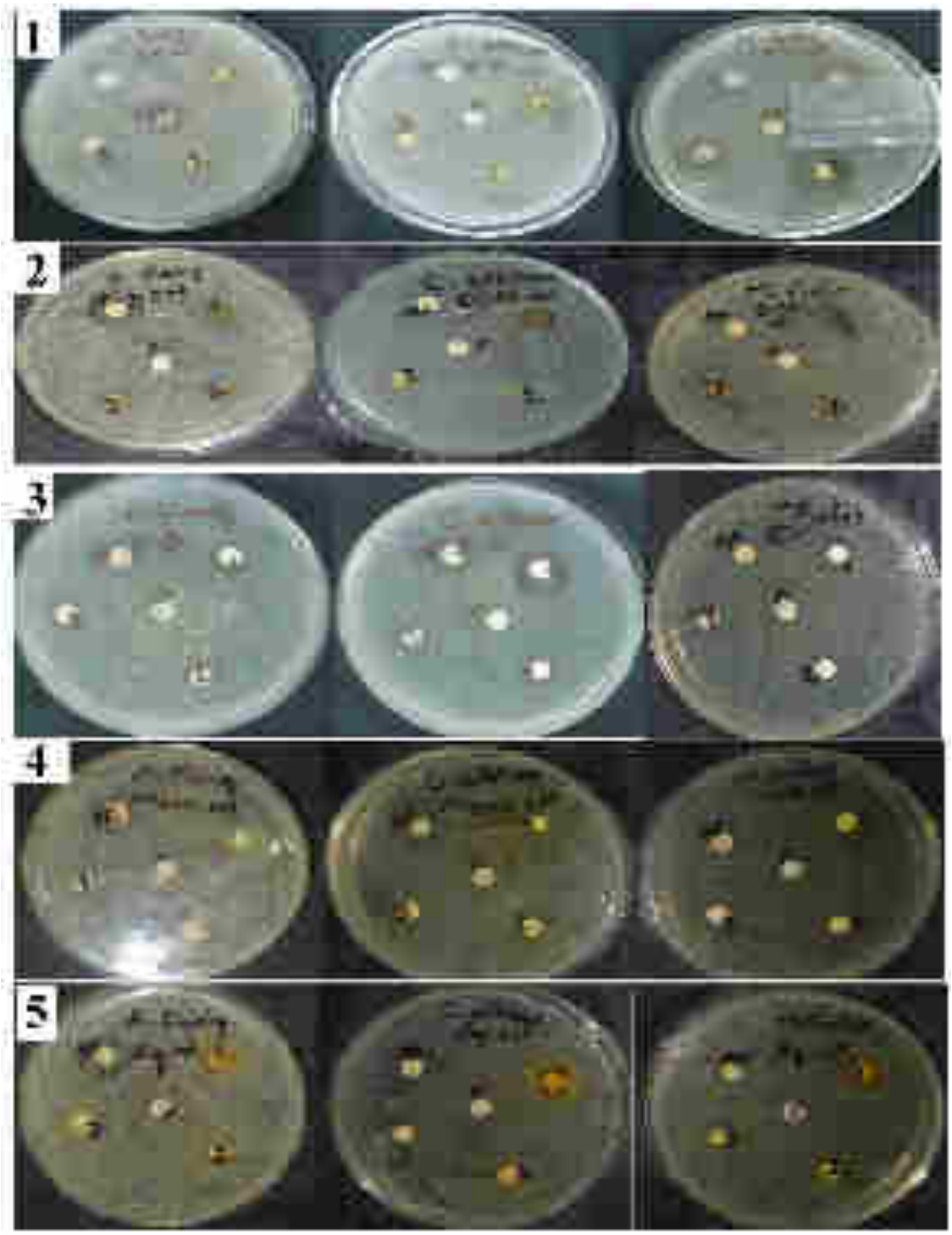

Figure 25. Antifungal assay using Disc diffusion method:
From left to right; *A. fumigatus, C. albicans,* and *M. furfur.*
1, Ethylacetate extract., 2. Dichloromethane extract, 3. Essential oil, 4. Methanol extract, 5. Aqueious extract.

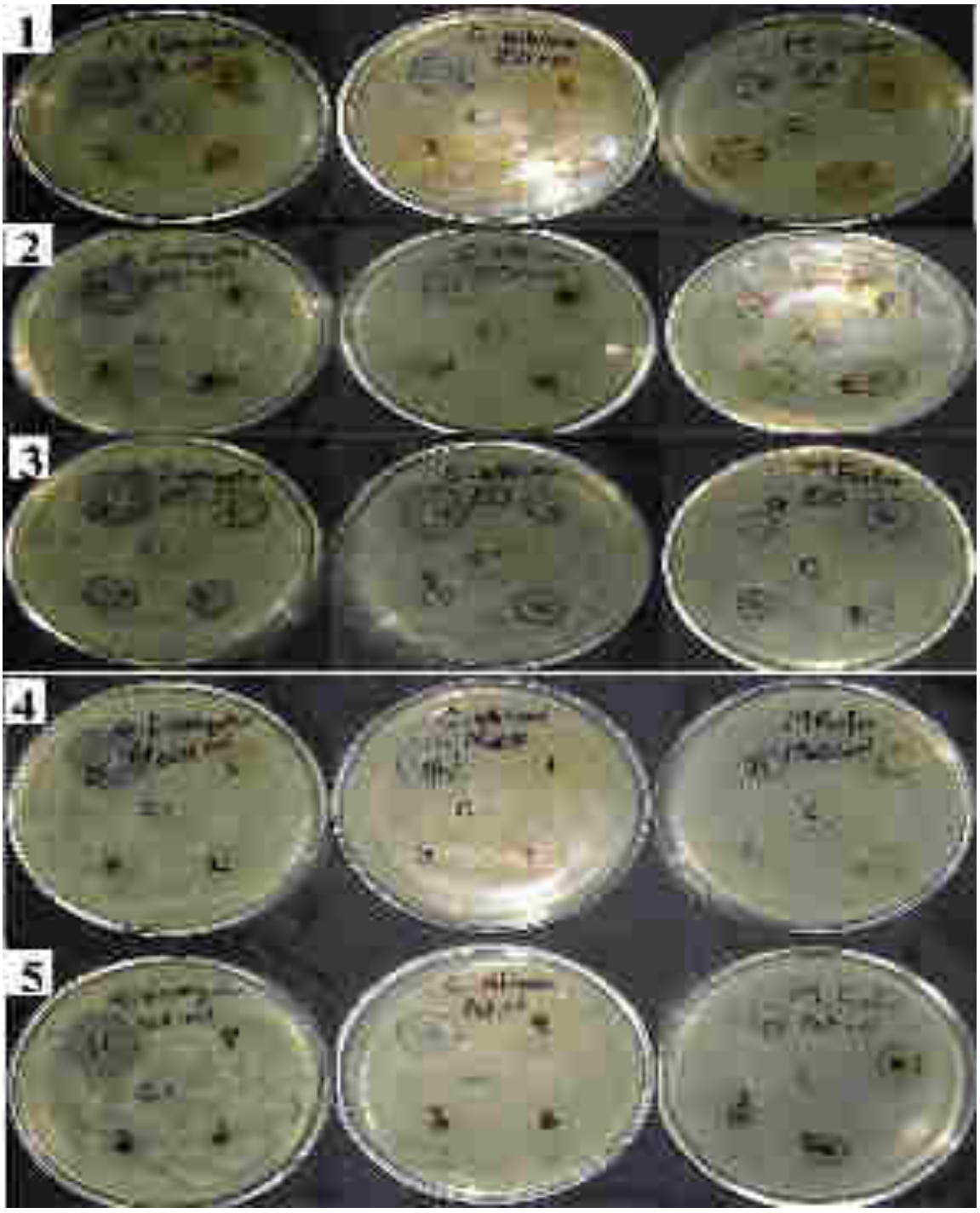

Figure 26. Antifungal assay using wells diffusion method.
From left to right; *A. fumigatus, C. albicans,* and *M. furfur.*
1, Ethylacetate extract., 2. Dichloromethane extract, 3. Essential oil, 4. Methanol extract, 5. Petroleum ether extract.

Table22: Data showing ZOIs of samples in disc diffusion method of antifungal activity evaluation.

Sample code	Concentration* (mg/ml)	ZOI (in mm)		
		Fungi		
		A. fumigatus	***C. albicans***	***M. furfur***
Pet extract	100	-	-	10.33±0.76
	50	-	-	9.17±0.76
	25	-	-	6.83±0.28
DCM extract	100	-	-	10.0±0.86
	50	-	-	9.5±0.50
	25	-	-	7.83±0.29
EA extract	100	-	-	**19.67±1.26**
	50	-	-	**17.83±0.29**
	25	-	-	**14.67±0.76**
MeOH extract	100	-	-	-
	50	-	-	-
	25	-	-	-
Aqueous extract	100	-	-	-
	50	-	-	-
	25	-	-	-
Volatile oil	100	10.33±0.29	13.0±0.5	9.67±0.29
	50	9.16±0.29	9.6±0.28	8.50±1.00
	25	7.83±0.57	7.5±0.5	7.50±0.50
Amphotericin-B	20µg/disc	13.6±1.45	11.0±0.5	0.33±0.58
Fluconazole	10µg/disc	-	-	-

Results are the mean±SD of the three experiments.

* : concentration of the extract solution in DMSO in which sterile discs were dipped.

Table 23. Data showing ZOIs of samples in wells diffusion method, antifungal activity evaluation.

Sample code	Concentration* (mg/well)	ZOI (in mm)		
		Fungi		
		A. fumigatus	***C. albicans***	***M. furfur***
Pet extract	5	0	0	8.83±0.29
	2.5	0	0	6.17±0.29
	1.25	0	0	3.17±0.29
DCM extract	5	-	3.83±0.29	12.33±0.29
	2.5	-	0	10.17±0.29
	1.25	-	0	8.17±0.29
EA extract	5	7.17**±0.29**	**0**	**17.33±0.58**
	2.5	3.00**±0.00**	**0**	**13.33±0.29**
	1.25	0	**0**	**11.00±0.50**
MeOH extract	5	0	0	4.83±0.29
	2.5	0	0	3.50±0.50
	1.25	0	0	0
Aqueous extract	5	0	0	0
	2.5	0	0	0
	1.25	0	0	0
Volatile oil	5	11.53±0.29	11.67±0.58	10.33±0.29
	2.5	8.5±0.50	10.50±0.50	7.5±0.50
	1.25	5.5±0.58	4.33±0.58	4.83±0.29
Itraconazole	20µg/well	15.53±0.45	14.36±0.56	6.25±0.43

Results are the mean±SD of the three experiments.

* : sample/standard solutions prepared in DMSO were placed in wells.

Table 24. Data showing activity index (AI) of *H. afghanicum* extracts and volatile oil in DDM and WDM of antifungal assay

S. No.	Microbial strains	Sample concentration		AI of tested samples										Mean ZOI of AP-B*	Mean ZOI of Itra*
		Disc impregnated	mg/well	Pet ext.		DCM ext.		EA ext.		MeOH ext.		VO			
				DDM	WDM	DDM	WDM	DDM	WDM	DDM	WDM	DDM	WDM	DDM	WDM
1	*A. fumigatus*	100 mg/ml	5	0	0	0	0	0	0.46	0	0	0.78	0.75	13.19±1.18	15.53±0.45
		50 mg/ml	2.5	0	0	0	0	0	0.19	0	0	0.69	0.54		
		25 mg/ml	1.25	0	0	0	0	0	0	0	0	0.59	0.35		
2	*C. albicans*	100 mg/ml	5	0	0	0	0.29	0	0	0	0	1.22	0.81	10.67±0.50	14.36±0.56
		50 mg/ml	2.5	0	0	0	0	0	0	0	0	0.91	0.73		
		25 mg/ml	1.25	0	0	0	0	0	0	0	0	0.70	0.30		
3	*M. furfur*	100 mg/ml	5	ND	1.41	ND	1.97	ND	2.77	0	0.77	ND	1.65	0	6.25±0.43
		50 mg/ml	2.5	ND	0.99	ND	1.63	ND	2.13	0	0.56	ND	1.20		
		25 mg/ml	1.25	ND	0.51	ND	1.31	ND	1.76	0	0	ND	0.77		

5.5. Results of anthelmintic activity evaluation of HAKL

Table 25. shows the data regarding in vitro anthelmintic activity of *H. afghanicum* leaves different solvent extracts (except aqueous extract which did not have anthelmintic activity) and volatile oil against adult earthworms, *Eisenia foetida* L. The tested extracts and standard albendazole were used at five different treatment doses of 2 – 10 mg/ml. The volatile oil was used at the same concentration range but in v/v or 2 – 10 µl/ml. By performing the assay, the order of vermicidal strength for tested samples was found as below:

Volatile oil > ethylacetate extract > DCM extract > pet ether extract > methanol extract

As shown in Table 25, and Figure 27 and 28, the volatile oil and ethylacetate extract, both exhibited stronger vermicide activity than standard albendazole at all treatment doses. At treatment doses 10 mg/ml (10 µg/ml of volatile oil), paralyses time and death time for albendazole, ethylacetate and volatile oil were recorded to be 14.97±1.06 and 82.11±8.23; 4.83±0.17 and 10.34±0.29; and 4.17 ± 0.29 and 10.0±0.58 minutes, respectively. With decrease of the treatment doses increases in both paralyses time and death time are observed. At the lowest dose of 2 mg/ml, paralyses time and death time decrease considerably for albendazole (153.67±1.86, 352.22±5.42), moderately for ethylacetate (71.11±9.67, 146.67±9.91) and slightly for volatile oil (13.89±0.38, 37.56±0.20). This revealed that albendazole at lower doses did not show potent activity against the earthworms, while teste samples produced shorter post-exposure paralysis time and death time as compared with the same dose (10 mg/ml) of standard albendazole suspension.

Methanol extract showed lowest activity while aqueous extract was inert at all against the earthworms.

Performing in vitro anthelmintic activity assay is really an economic approach for anthelminthic activity evaluation of natural as well as synthetic products. Moreover, the worms under treatment are directly under observation and the anthelmintic activity of test samples is easily measured by recording the time of worm paralysis and subsequently their death time. Here, interference of any internal physiological factors of the host is totally prohibited and potent products can be assuredly distinguished from those with lower or no anthelminthic activity.

Earthworms because of their resemblance with human intestinal roundworms, are commonly used in *in vitro* anthelminthic activity assays. Earthworms move by ciliary movement and their outer layer is composed of complex polysaccharides providing a mucilaginous nature for the layer. The mucilaginous outer body layer of the worms play prominent role in their freely movement. Any damage to this slimy mucopolysaccharide layer will impair the earthworm movement and may eventually result to their paralysis. However, further irritation of the outer layer results to death of the worms (Bimlesh Kumar et *al*., 2011).

During in vitro anthelminthic activity assay of *H. afghanicum* leaves against adult earthworms (*Eisenia foetida* L.) it was found that the crude drug is rich in phytochemicals exhibiting strong vermicidal activity. *H. afghanicum* leaves' volatile oil and extracts not only paralyze the earthworms (*Eisenia foetida* L.) but also kill them in shorter post-exposure time as compared to the standard albendazole suspension. The earthworms were found more sensitive against HAKL volatile oil as compared to standard albendazole (Table 25., Figure28.). Albendazole was used in suspension form, so, it may not easily penetrate through external layer of the earthworms, and hence, its activity is delayed as compared to sample volatile oil.

In general, at highest treatment dose (10 mg/ml) all of the test samples (except methanolic extract) revealed potent in vitro vermicide activity as compared with the standard albendazole. The volatile oil exhibited the highest activity, producing paralysis time of 4.17 ± 0.29 min and death time of 10.0±0.5 min. at treatment dose of 10 µl/ml. At treatment dose of 10 mg/ml the post-treatment paralysis time and death time for ethylacetate extract, dichloromethane extract, petroleum ether extract and methanol extract were found to be 4.83±0.17 and 10.33±0.2; 13.78±0.75 and 25.78±0.9; 33.89±1.8 and 53.11±1.3, and 206.67±8.35 and 348.11±7.07, respectively. Standard albendazole suspension at dose 10mg/ml exerts paralysis time and death time of 14.97±1.06 and 82.11±8.2 minutes, respectively (Table 25.), (Figure 29.). Among the tested samples the methanolic extract showed very weak anthelminthic activity. At doses of 8 mg/ml and 10 mg/ml paralyses time and death time for methanol extract were recorded to be 264.67±4.06, 441.89±5.42 and 202.67±8.35, 348.11±7.07, respectively. At lower doses the activity was ignorable for methanolic extract since the worms were actively moving in petri dishes even after 7 hours of

post-treatment time. Worms treated with aqueous extract, normal saline and mixture of 0.5 ml DMSO with 10 ml normal saline were alive and active till next day of the experiment.

In present study, the strongest activity of essential oil can be attributed to the complex phytochemistry of the oil and lipophilic nature of its constituents. Studies shown that lipophilic anthelminthic exhibit stronger activity as compared with hydrophilic anthelmintics. Therefore, anthelminthic activity of volatile oils generally could be attributed to the lipophilic nature of their ingredients which easily interfere with the glycolipids of the worms' body cells and by doing so, cause cell damage and death of the worms. Moreover, volatile oils are proved to be comprised of several (more than ten to hundreds compounds) compounds which may contribute in exerting biological activities of the oils.

H. afghanicum leaves volatile oil GC-MS analysis indicate presence of 25 compounds in the oil (Table6.), which as synergistic compounds, may contribute in anthelminthic activity against tested earthworms.

Volatile oil of various other plants are previously reported for their anthelmintic potential. Zhu et al. (2013), reported promising in vitro anthelmintic activity of essential oils obtained from two chines plants namely *Arisaema franchetianum* and *Arisaema lobatum* against *Haemonchus contortus* of sheep (Zhu et al., 2013). Volatile oil of *Chenopodium ambresioides* inhibited 100% of *Haemonchus contortus* egg hatching at 1.33 µl/ml concentration (Ketzis et al., 2002). *Eucalyptus globulus* essential oil while was tested in egg hatching inhibition assay and larval development assay, showed the maximum inhibition rates of 99.3% at 21.75 mg/ml and 98.7% at 43.5 mg/ml concentration, respectively (Zhu et al., 2013). The essential oils of *Croton zehntneri* and *Lippia sidoides* showed more than 98% egg hatching inhibition activity and 90% larval development inhibition activity against *Haemonchus contortus* at doses of 1.25 mg/ml and 10 mg/ml, respectively (Camurca-Vasconcelos et al., 2007).

The essential oil of *H. afghanicum* leaves tested in present study showed excellent vermicidal activity against adult earthworms. However, effect of the oil on egg hatching, larval development and adult worm mortality on other parasitic nematodes of human as well as livestock is suggested as further works. Since *H. afghanicum* leaves essential oil was found stronger than marketed albendazole suspension, it is very much emphasized to evaluate isolated oil constituents anthelmintic activity both in vitro and in vivo and to study toxicity of

the oil and its components in order to make use of this essential oil as possible anthelmintic drug.

Among the extracts, ethylacetate extract was found more potent against the earthworms as compared with standard albendazole. Ethylacetate is known to be a best organic solvent for many bioactive compounds, since it easily diffuses into the cells of plant crude drugs and dissolved their intracellular bioactive compounds. However, further work is suggested for identification of ethylacetate extract ingredients to find out the responsible component(s) exerting the vermicidal activity.

Anthelminthic activity of dichloromethane extract was also found potent than that of standard Albendazole at all treatment doses (Figure 27. And Figure 28.). Dichloromethane extract is also deemed to be rich in lipophilic phytochemicals showing efficient anthelmintic activity against the earthworms.

Anthelminthic action of petroleum ether extract against the earthworms, was different as compared with that of standard Albendazole. The extract at doses of 8 mg/ml and 10 mg/ml showed delayed paralytic time than albendazole. Paralyses time for the extract were recorded to be 42.67±0.88 and 33.89±1.87 min while for albendazole were 34.44±3.10 and 14.97±1.06 min at doses of 8 and 10 mg/ml, respectively. Interestingly, at doses of 2mg/ml, 4 mg/ml and 6 mg/ml, petroleum ether extract exhibited shorter post-exposure paralysis time as compared to albendazole. At all treatment doses, post-exposure death time produced by the extract were shorter than those produced by albendazole (Table 25, Figure 29). This activity again can be attributed to the easily penetration of lipophilic constituents (sterols, diterpenes, furanocoumarins, etc.) present in the extracts and their synergistic activity against the worms. Moreover, the first solvent used in successive extraction of *H. afghanicum* leaves was petroleum ether, and hence it is deemed to be rich in active lipophilic phytochemicals.

H. afghanicum methanol extract despite being rich in phenolics and flavonoids, exhibited feeble anthelminthic activity against the earthworms. This issue could be described that the lipophilic phytochemicals present in HAKL exhibit stronger vermicidal activity than the hydrophilic compounds (e.g. phenolics and flavonoids) which were found in higher amount in methanol extract.

H. afghanicum leaves' aqueous extract was tested against the earthworms but, even at higher treatment doses did not exhibit anthelmintic activity against the earthworms. In our

work, this was the last extract obtained from the same marc being already extracted by four precedent solvents including methanol. Though the aqueous extract gives the highest yield (12.20% w/w), it may contain some water soluble inert substances not showing potent biological action.

It can be claimed that *H. afghanicum* leaves exhibited considerably strong anthelmintic activity in vitro against the tested earthworms. The worms treated with all doses (2 – 8 mg/ml) of the test samples were found dead within 6 hours post-exposure time, except in case of methanol extract at doses ≤ 8mg/ml the worms were alive even after 6 h post-exposure time.

H. afghanicum leaves volatile oil and all extracts (except aqueous and methanolic extracts) were found to be significantly potent than marketed albendazole suspension (Albendazole oral suspensions IP, 200 mg/5 ml). All of the tested samples showed dose dependent vermicidal activity against earthworm (*Eisenia foetida* L.). It can be claimed that non-polar ingredients of *H. afghanicum* leaves exhibit stronger anthelmintic activity against earthworms.

Ethylacetate extract was found more potent as compared to rest of the tested samples. In present study, also the methanolic extract exhibited stronger antioxidant activity in both DPPH assay and FRAP assay, but lower anthelmintic activity and antimicrobial activity.

The discrepancy between anthelmintic potential of *H. afghanicum* leaves different extracts is said to be due to presence of different phytochemicals with different degree of bioactivity in each extracts. Since the crude drug was successively extracted by different solvents of increasing polarity, different categories of phytochemicals are extracted in each step based on their affinity with the used solvent.

In general, our results are very much in compliance with those reported by other researchers in previous works. Most of previous works reported that the organic solvent extracts show better anthelmintic potential than aqueous extracts. For instance, Egaule et al., (2007) evaluated in vitro anthelmintic activity of *Coriandrum sativum* seeds hydro-alcoholic and aqueous extracts against *Haemonchus contortus.* The authors found that the hydro-alcoholic extract due to its richness in lipophilic phytochemicals revealed higher anthelmintic potential than the aqueous extract (Eguale et al., 2007).

Jabbar et al., (2007) have studied both in vitro and in vivo anthelmintic activities of *Caesalpinia crista* (L.) seed kernel and *Chenopodium album* (L.) whole plant. The authors found higher activity for the alcoholic extract as compared to aqueous extract, and they justified that the alcoholic fraction due to its richness in lipophilic phytochemicals (e.g. ascaridole a component with potent anthelmintic activity in *Chenopodium ambrosioides* oil) exhibited stronger vermicidal activity (Jabbar et al., 2007).

It should be noted that, in case of our work, apart from the volatile oil, the three successive extracts precedent to methanolic extract are claimed to be richer in lipophilic compounds and hence revealed higher vermicidal activity than that both of methanolic and aqueous extracts. Since, methanol and aqueous extracts were obtained respectively at fourth and last step of successive extraction, they are poor in lipophilic phytochemicals.

Statistical analysis

All the experiments were performed in triplicates and the data are recorded as the mean value ± standard deviation (SD) of the triplicates. Statistical analysis were carried out by one way analysis of variance (ANOVA) using Microsoft excel (version, 2013), followed by Dunnet's *t*-test. A probability level of $p<0.05$ were considered to be statistically significant.

Conclusion

It can be concluded that *H. afghanicum* leaves volatile oil, ethylacetate extract, dichloromethane extract and petroleum ether extract revealed high vermicide activity against earthworms (*Eisenia foetida* L.). Lipophilic phytochemicals of *H. afghanicum* leaves may work as potent anthelmintics against other parasitic worms, and hence, more experiments (in vitro and in vivo) regarding anthelmintic activity evaluation of the crude drug against gastrointestinal nematodes and other worm species are suggested. Likewise, further studies regarding isolation and identification of the active principles involved in vermicidal activity and toxicity studies of *H. afghanicum* ethylacetate extract and volatile oil are also emphasized, since they were found more potent than standard albendazole. Isolated compounds of *H. afghanicum* are estimated to serve as potent leads for developing anthelmintic formulations.

Table 25 Data showing anthelmintic activity of *H. afghanicum* leaves different solvent extracts, volatile oil and Albendazole.

Conc.* mg/ml	Standard/test samples											
	Albendazole		Volatile oil		Ethylacetate ext.		Dichloromethane ext.		Pet. extract		Methanol ext.	
	PT	DT	PT	DT	PT	DT	PT	DT	PT	DT	PT	DT
2	153.6±1.86	352.2±5.42	13.8±0.38	37.5±0.19	71.1±9.67	146.6±9.9	72.5±4.37	207.3±4.4	79.2±9.8	233.3±15.1	-	-
4	100.7±3.8	194.2±1.26	12.4±0.21	35.3±0.58	34.7±2.62	82.1±2.34	38.8±2.59	89.5±4.67	64.7±1.8	162.7±5.05	-	-
6	68.6±5.29	152.8±3.4	10.7±0.84	31.4±0.59	12.6±0.60	21.6±0.67	27.2±1.26	83.2±2.22	54.6±2.6	153.2±5.06	368.5±2.22	-
8	34.4±3.01	91.1±2.87	7.4±0.69	13.4±0.35	7.89±0.25	13.2±0.75	23.1±2.50	78.4±4.35	42.6±0.8	80.6±1.33	264.6±4.06	441.8±5.42
10	14.9±1.06	82.1±8.23	4.17±0.29	10.0±0.58	4.83±0.17	10.3±0.29	13.7±0.75	25.7±0.96	33.8±1.8	53.1±1.35	206.6±8.35	348.1±7.07

Values are expressed as mean ± SD.
PT= paralysis time, DT = death time
* Volatile oil was used in μl/ml.
‘ – ’ = not determined.

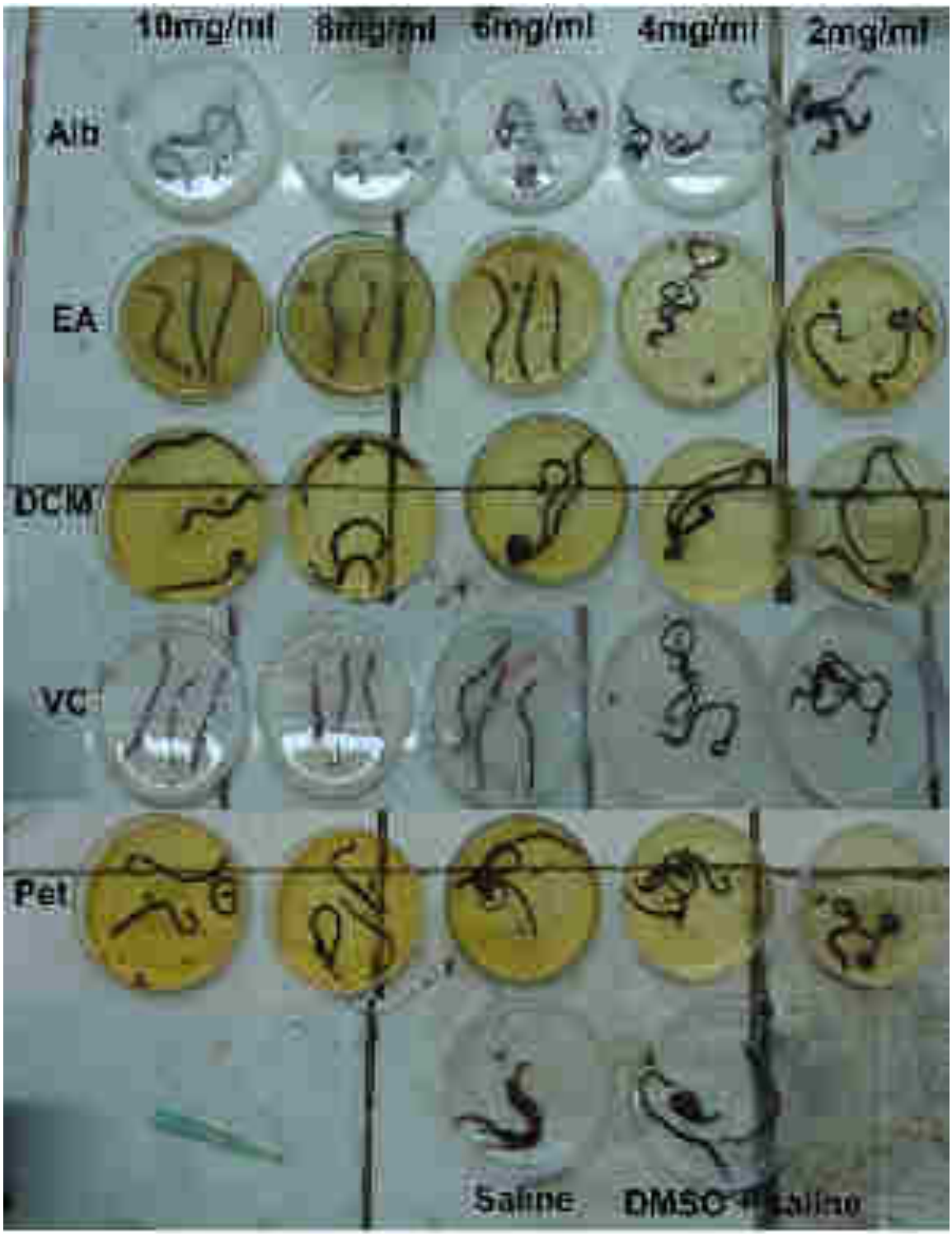

Figure 27: Petri plates of anthelmintic assay: from left to right; doses increase.
Alb; albendazole, EA, ethylacetate extract, DCM; dichloromethane extract, VO; volatile oil, Pet; petroleum ether extract. Saline; aqueous solution of 0.9%NaCl, DMSO; dimethyl sulfoxide.

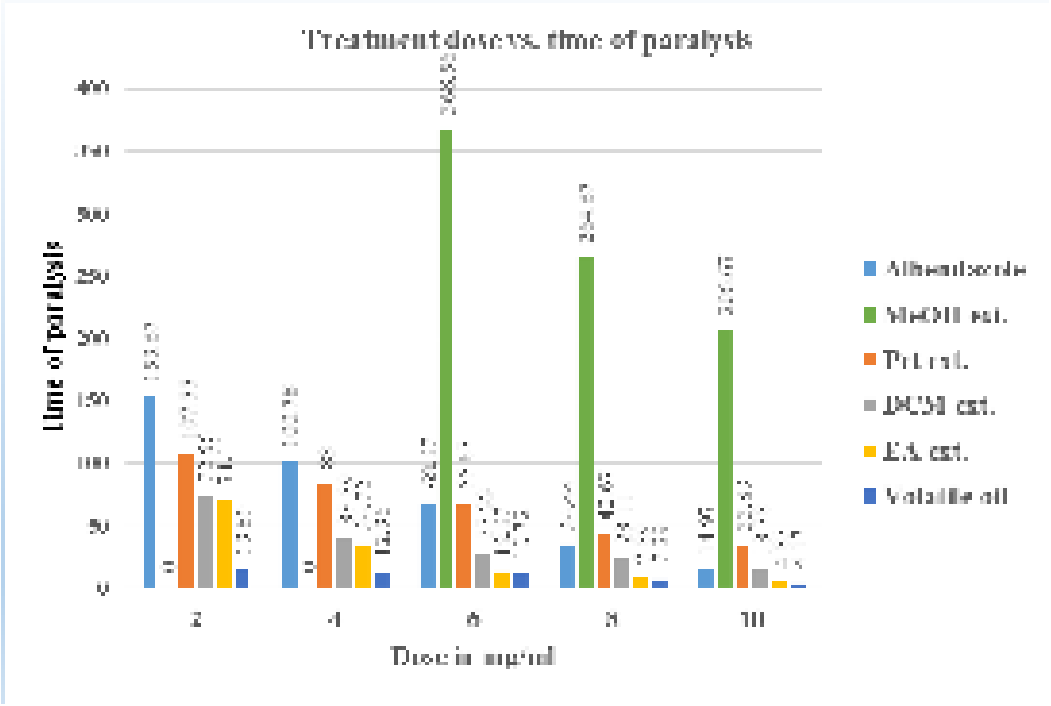

Figure 28: Bar diagram indicating the time taken for paralysis of *Eisenia foetida* during anthelmintic assay:

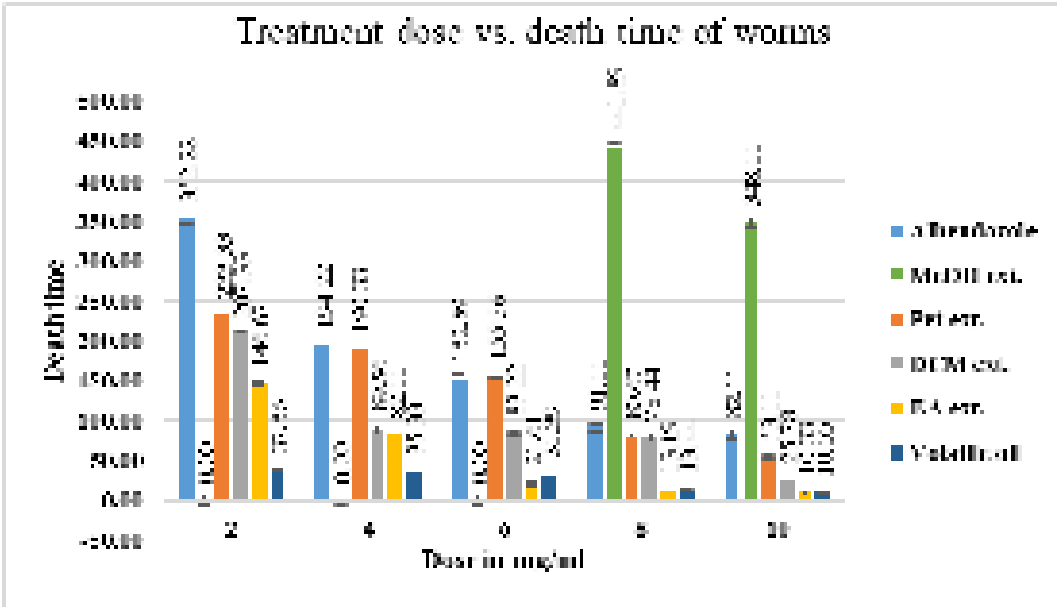

Figure 29: Bar diagram indicating the time taken for death of *Eisenia foetida* during anthelmintic assay.

Chapter VI

Summary and conclusions

6. Summary and conclusions

Present study was basically designed to evaluate *H. afghanicum* Kitamura leaves (HAKL) both chemically and biologically. The plant is quite robust and produces large leaves which are generally in some areas used as a best forage and animal feed. However, the plant is used in different rural areas of Afghanistan for different medicinal and non-medicinal purposes but no adequate scientific data regarding chemical composition of the plant were available.

In present study five different extracts were prepared using pet ether, DCM, ethylacetate, methanol and water as solvent in successive extraction by Soxhlet apparatus. Preliminary phytochemical screening of the plant extracts revealed that leaves of HAKL are rich in different categories of phytochemicals with potent biological activity. Presence of high amount of sterols, flavonoids, phenolics, diterpenes and furanocoumarins were confirmed based on qualitative tests. Pet ether extract was found to be richer in sterols, resins and diterpenes. Methanol extract and ethylacetate extracts were found rich in flavonoids and phenolics. DCM gives moderate results but aqueous extract despite giving higher extractive value was found poor in active phytochemicals such as phenolics, flavonoids, etc.

The results of TPC and TFC were different for each test sample. Methanol extract produced the highest values while volatile oil volatile oil and aqueous extract give the lowest values (Table 8.).

In in vitro antioxidant activity assay, methanol extract was found the most potent among other extracts in both DPPH assay and FRAP assay. Ethylacetate extract fall in second rank followed by DCM extract, and pet extracts. Aqueous extract and volatile oil were of low value samples. These data indicate that the polar compounds of *Heracleum afghanicum* leaves are mainly involved in antioxidant effect of the leaves.

In antimicrobial activity evaluation against the four bacterial strains (*B. subtillis, S. aureus, E. coli and Proteus vulgaris*) and three fungal strains (*A. fumigatus, C. albicans, and M. furfur*), ethylacetate extract was potent rather than other tested sample. Methanol extract as compared to its antioxidant action did not show satisfactory anti-microbial activity. Interestingly, HAKL volatile oil was found active against all tested fungi and at al use doses. Among tested bacteria, *Proteus vulgaris* and *Bacillus subtillis* were found susceptible against the oil, while only at higher dose produced a little bit ZOI against *S. aureus,* but *E. coli* was

resistant against used dosed (discs impregnated in sample solution of 25, 50 and 100 mg/ml concentration). *Malassezia furfur* was the most susceptible fungi to all tested samples (except methanolic and aqueous extract). M. furfur was resistant to both amphotericin-B (20 mcg/disc) and Fluconazole (10mcg/disc).

Ethylacetate extract among all other extract was found more potent than other extract, against all tested bacteria but only against *M. furfur* among the tested fungal strains.

These results indicated that, antimicrobial activity of *Heracleum afghanicum* is appreciable against some microorganisms. Moreover, antimicrobial activity of HAKL can be attributed largely to non-polar phytochemicals rather than to polar compounds such as phenolics and flavonoids. Because the methanolic extract rich in phenolics and flavonoids did not exert measurable antimicrobial action. Similarly, volatile oil with the lowest or ignorable TPC and TFC values and minor FRAP value, was found active against *M. furfur, P. vulgaris* and *Bacillus subtillis*.

While HAKL extracts and volatile were tested for their anthelmintic activity at dose range of 2 – 10 mg/ml on earthworms. All of the tested sample (except the aqueous extract) cause paralysis and subsequently death of the worms. In this case, also both the volatile oil and ethylacetate extract showed the strongest activity than standard albendazole at the same used doses. At dose 10 mg /ml both the volatile oil and EA extract was almost 3.5-fold potent than albendazole suspension used at the same dose against the earthworms. Paralyses time for the volatile oil and ethylacetate extract was recorded 4.17±0.29 and 4.83±0.17 min, respectively, at dose 10 mg/ml while for standard albendazole paralyses time was 14.9±1.0 min (Table 25.). DCM extract produces comparable (13.7±0.75 min) slightly stronger activity and pet ether was weaker but again methanol extract was found very weak against the tested worms. These data also indicate that anthelmintic action of HAKL is totally due to potent lipophilic components to the crude drug extracted by ethylacetate extract, dichloromethane extract and petroleum extract. Volatile oil constituent are all lipophilic compounds which may contribute in strong anthelmintic as well antifungal effects of the volatile oil

Briefly; it can be concluded that, *Heracleum afghanicum* leaves poses a diverse array of pharmacologically active constituents and 0.3 – 0.4 % (v/w) essential oil. Methanol as a solvent of choice is suggested for extraction of phenolics, flavonoids and other antioxidative compounds from HAKL. Ethylacetate is considered to be the most appropriate solvent for

extraction of pharmacologically potent lipophilic components of HAKL. Lipophilic phytoconstituents of the crude drug revealed significant antimicrobial and anthelmintic activities in vitro, as compared with the used standards. HAKL volatile oil can be developed into potent anthelmintic and antifungal formulations. Anethol forms 66.12 % of HAKL volatile oil. Anethole is reported to be a potent synergistic compound which significantly enhance antifungal activity of other molecules.

However, we suggest performing further research in order to isolate and identify HAKL bioactive compounds including phenolics and flavonoids and to evaluate independently their corresponding biological activities in vivo and vitro, to find out compounds with maximum activities. Similarly, further work is required to find out whether the biological activities of HAKL extracts are related to synergistic action of their compounds or to isolated compounds present in them.

Chapter VII

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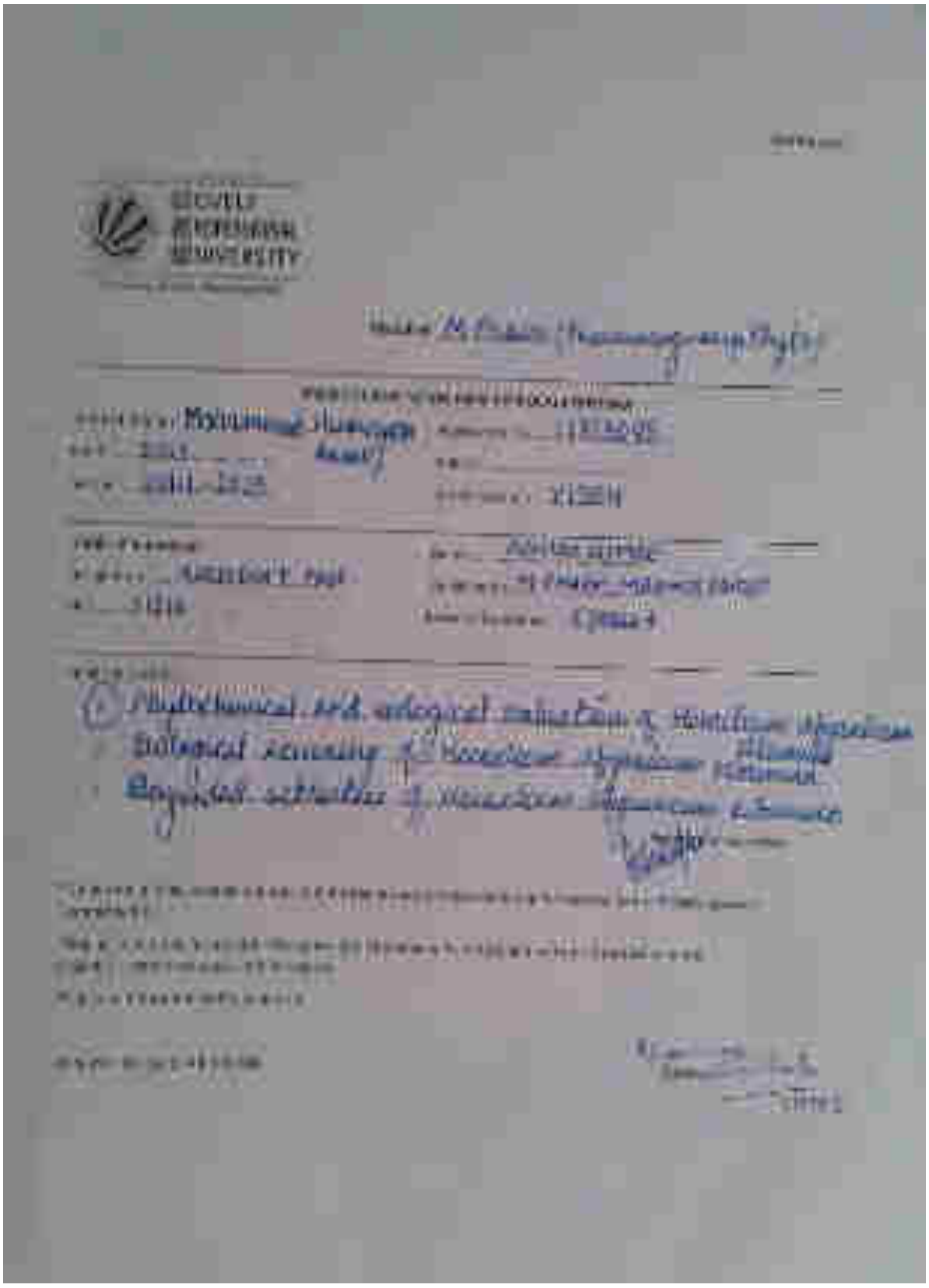
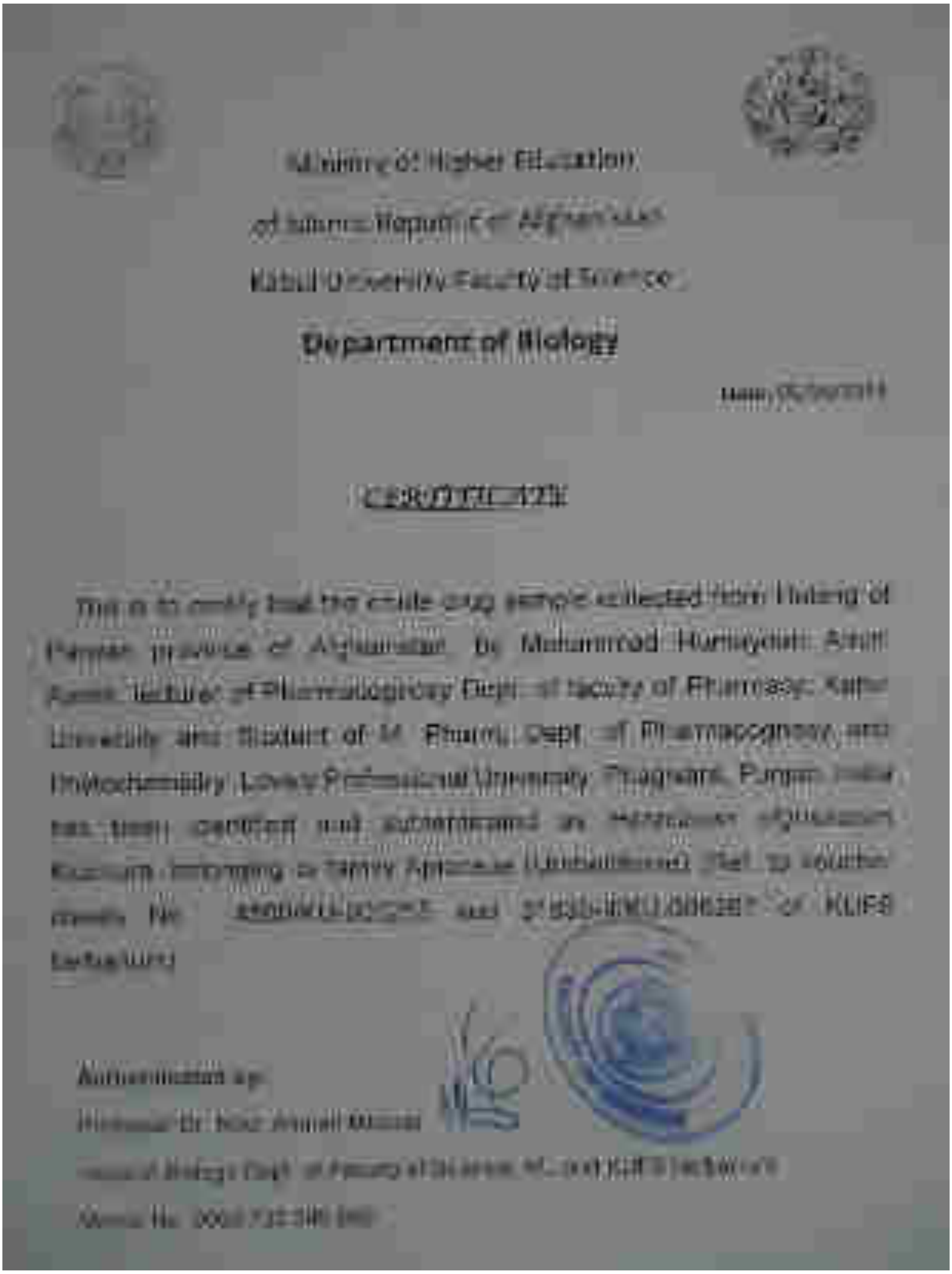
Appendixes

Approval sheet of the research project



Plant authentication certificate



Ministry of Higher Education

of Islamic Republic of Afghanistan

Kabul University Faculty of Science

Department of Biology

CERTIFICATE

Earthworm authentication certificate

MAHAVIR ORGANIC MANURE

CERTIFICATE

Report is not valid for legal purpose

GC-MS analysis report and chromatogram