Phytochemical Screening of Major Constituents of Various Folklore Medicinal Plants of Kashmir Valley

DISSERTATION

Submitted in partial fulfillment of the requirements provided for the award of the degree of

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In

Chemistry

By

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CERTIFICATE

This is to certify that **Mr. Sofi Mubashir** worked under our joint supervision for his M.Phil, studies "**Phytochemical Screening of Major Constituents of Various Folklore Medicinal Plants of Kashmir Valley".** His work embodied in this dissertation is original. Mr. Sofi Mubashir has fulfilled all the formalities prior to submission of this dissertation. His work and conduct has been satisfactory. The dissertation is recommended for the award of M.Phil degree.

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DEDICATION

This study is dedicated to my parents who have always been there for me.

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Sofi Mubashir

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CHAPTER 1

Natural Product Chemistry – An Introduction

1.1 GENERAL INTRODUCTION

Primitive men and women brewed strange positions from plants and small animals, because they thought that drinking these crude liquids would alleviate pain and cure disease. Many of the compounds they unwittingly employed, for example caffeine, cocaine and morphine, which are still in use today both in their native state and in the form of simple chemical derivatives. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs. Plants are the source of natural pesticides, cytotoxic agents that make excellent leads for new drug development [1]. According to World Health Organization (WHO), medicinal plants can be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy [2].

These plants play a vital role in maintaining human health and contribute towards improvement of human life. They act as important source of medicine, cosmetics, dyes, beverages etc. Plants have been used as source of medicine since the dawn of human civilization. In spite of tremendous development in the field of allopathy during the 20^{th} century, plants still remain one of the major source of drugs in the modern as well as in traditional system of medicine throughout the world. There is vast anecdotal information about the biological activity of plants which includes anti-carcinogen, anti-bacterial, anti-fungal and anti-oxidant type of activities [3]. Over 60% of pharmaceutical agents are plant based [4]. Plants are considered as state of art chemical laboratories capable of biosynthesizing number of biomolecules of different chemical classes. Many of these are proved to be precursors for the

development of other drugs [5].

India is one of the most medico-culturally diverse countries in the world where the medicinal sector is part of a time-honoured tradition which is respected even today. Ethno-botanical and ethno-pharmacological studies of medicinal plants continue to attract investigators through out the world. In current scenario focus on plant research has increased all over the world and a large body of evidence collected has shown immense potential of medicinal plants used in various traditional systems of medicine.

Natural product chemistry has advanced regularly on a broad front during this century. The main interest in natural product research is now gradually changing from problem of purely chemical character to those of biochemical and biological phenomena, thus changing the pattern of thinking of the biochemist, psychologist, botanist, zoologist, entomologist and microbiologist. The original impetus to natural product chemistry came from use of natural drugs which has been partly scientific and partly commercial in character. One of the world health organization (WHO) survey indicated that about 75-80% of the world's population rely on non conventional medicine, mainly of herbal source, in their primary health care. There has been an explosion of scientific information concerning plant crude extracts and various substances from plants as medicinal agents during last 30-40 years. Until the beginning of nineteenth century, man continued to use a very wide range of plant distillates as medicine and as mind-altering drugs without understanding their magic properties. As science advanced, however, it became possible to determine rigorously the active components of these extracts through painstaking and laborious chemical methods. This rational approach to the discovery of drugs inaugurated an era of bio-prospecting that is, raiding nature's storehouses of plant and microbiological life. Bio-

Prospecting literally involves exploring the forests, diving in the oceans and digging in the dirt to obtain environmental samples. The study of the compounds discovered by these methods has become a major area of research in organic chemistry, and has led to the isolation and identification of thousands of different structures, mostly extracted from plants and more recently from micro-organisms, with the animal kingdom contributing rather sparsely to the total.

Standard cancer chemotherapy is frequently compromised by the development of drug resistance and unwanted, partly life threatening side effects. There is therefore, an urgent need for novel treatment options with improved features. Interestingly many plant derived compounds like paciltaxel, vinblastine or vincristine and teniposide are used as anti-cancer drugs. As pointed out recently, natural products from medicinal plants represent a fertile ground for the development of novel anti-cancer agent [6]. Given the enormous medicinal and economic impact of natural-product pharmaceuticals, we have obvious incentives both to improve the activities of existing compounds and to discover new metabolites.

An important recent success from the plant kingdom is the discovery of Taxol, a constituent of the pacific yew tree, and its subsequent development as a treatment for cancers of the ovary, breast, head and neck. This wonder drug operates by inhibiting the uncontrolled growth of cancerous cells. Taxol halts mitotic division in a counter-intuitive manner: unlike many other anti-cancer drugs that act by inhibiting polymerization of microtubules, killing the cells instead by arresting the disassembly of cytoskeleton

Most of the progress in the 20th century modern medicine in surgery, cancer chemotherapy and organ transplantation is attributed to the use of antibiotics. The emergence and dissemination of resistant bacteria to antibiotics, however are major

health problems leading to drawbacks for a large number of drugs [7, 8]. Consequently there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy [9]. This approach suggests co-administration of anti-microbial agents and has an advantage of extending the usefulness of antibiotics with known pharmacological, toxicological and treatment properties [10, 11]. In this regard interest has increased in natural products to combat infectious diseases [12-14].

Keeping in view global and national scenario of medicinal plants, it is worthwhile to undertake the phytochemical investigations of rare and threatened medicinal plants of the Kashmir valley, especially existing at high altitudes with proven folklore medicinal claim.

This prompted us to undertake the phytochemical investigations of medicinal plants namely *Parthenium hysterophorus, Myricaria germanica* and *Caltha palustris.*

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2.1 INTRODUCTION

Parthenium hysterophorus L. is a species of flowering plant belonging to family *Asteraceae*. The plant is erect, branched, leafy, with a rigid herbaceous stem that persists usually for one growing season. The roots, however, can persist for at least three years and produce new shoots. Leaves are alternate, highly divided and covered on both sides with trichomes which are also present on the stems. The capitulum (Flowering head) is heterogamous and composed of five fertile pistillate ray florets, and about forty fertile staminate disk-florets. The mature fruits (achenes) with two disk-florets attached at the base and the subtending brack of female floret fall of together as a unit called an achene complex. The achenes alone are abovate, black, crowned by the persistent remnants of corolla, appendages and style [1]

Parthenium hysterophorus is widely distributed throughout the tropics, occurring primarly in areas disturbed by man [2]. According to Rollins (1950), the species is native to the region around the Gulf of Mexico, West Indies and Argentina. In Australia it was first recorded in Queens land in 1955, then it was eradicated but accidentally re-introduced again in 1958 [3]. In India *Parthenium hysterophorus* was first reported in 1956 from Pune.

The detrimental properties of *Parthenium hysterophorus* have been attributed mainly to the presence of a particular sesquiterpene lactone, Parthenin. Parthenin was found to be the major constituent of *Parthenium hysterophorus* from U.S.A, Mexico, India and West Indies.

In this study parthenin has been isolated from *Parthenium hysterophorus* using various chromatographic techniques. The structural elucidation has been done using spectral techniques such as ^{1}HNMR, ^{13}CNMR and Mass spectrometry. The isolated sesquiterpene lactone (Parthenin) was evaluated for its in-vitro and in-vivo

anthelmintic activities. However, the antioxidant activity of the parthenin has also been performed under in-vitro conditions. Further, the structure activity relationship (SAR) of the compound has also been carried out in the present studies.

Nematode infections of gastrointestinal tract adversely affect productivity of small ruminants all over the world especially in tropical and sub-tropical countries. Options of using synthetic anthelmintics are decreasing due to development of resistance in gastrointestinal nematodes of small ruminants against several families of drenches [4, 5]. This global problem has created interest in researches on alternates to the use of synthetic chemicals for the control of nematodes [6]. In this regard, traditionally used ethno-botanicals with anthelmintic properties are considered among the novel approaches particularly in temperate and tropical countries [7, 8]. Majority of the ethno-veterinary medicine surveys and validation studies indicate much wider and effective use of plants as anthelmintics compared with other diseases/conditions [9]. Considerable research has shown that some plants not only affect the nutrition of animals, but also have antiparasitic effects. The vast majority of studies investigating the effects of plant extract on helminth parasites, either in experimental or in grazing conditions, have been conducted using sheep. Various plant species have been screened for anthelmintic efficacy against different helminth parasites of ruminants. Keeping this in view, the present study was carried out with the objective of evaluating the anthelmintic efficacy of parthenin against gastrointestinal nematodes of sheep under in-vitro and in-vivo conditions [10-13].

2.2 REVIEW OF LITERATURE

Parthenium hysterophorus linn (*Compositae*) an obnoxious weed grows wild in different regions of India including Jammu & Kashmir. It is an aggressive weed, endemic to American content and has found its way to Australia, Africa & Asia. In

India it was first reported in 1956 from Pune [14]. The plant is commonly known as "Congress Grass", "Gajjar Grass" or "White Top". This obnoxious weed is responsible for contact dermatitis [15], hey fever [16] allergic rhinitis [17] erythematous papulovesicular eruptions on face and lesions [18]. Parthenin being hazardous to animals causes ulceration of the muzzle dental pads, dorsum of the tongue, and the upper palate. This has been observed in all the *Parthenium hysterophorus* fed animals [19]. Its allelopathic [20] effects on plants have also been observed. On the other hand plant extract is used as folk remedy against skin diseases, ulcerated sores, facial neuralgia fever & anemia [21]. The aerial portion of Indian variety of *Parthenium hysterophorus* contains Parthenin as the major component along with several sesquiterpene lactones. These sesquiterpene lactones contain an alpha-methylene-gamma lactone moiety which plays a vital role for bioactivity of the compounds especially for cytotoxicity [22]. In literature following chemical constituents have been reported from *Parthenium hysterophorus* (table 2.1).

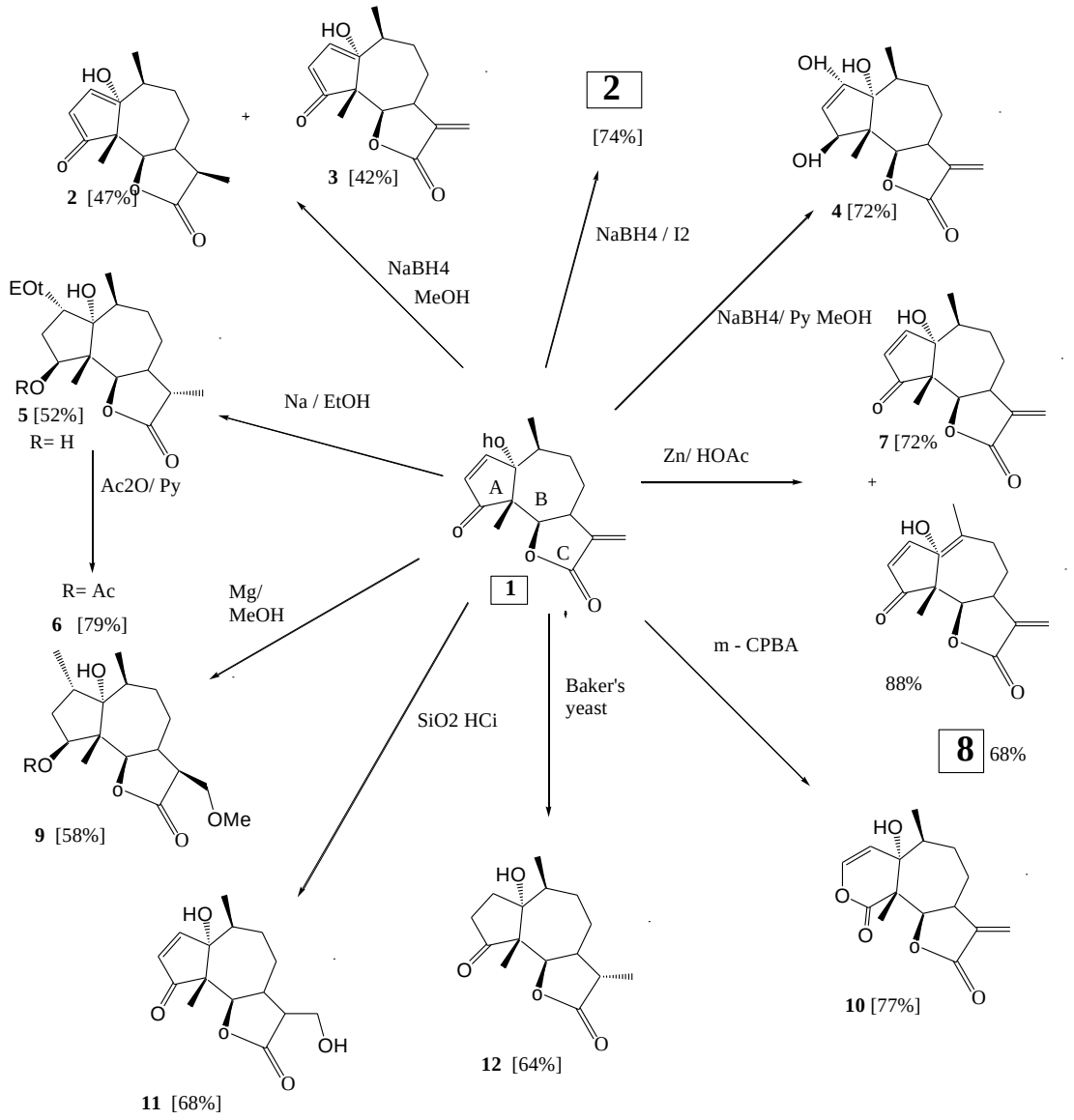
Table 2.1- Compounds reported from Parthenium hysterophorus.

S.No.	***Name***	***Reference***
1.	Parthenin	23
2.	Hymenin	24
3.	Ambrosin	25
4.	Coronopilin	26
5.	Damsin	27
6.	Tetraneurin-A	26
7.	Chiapin-B	24
8.	8β - Hydroxyparthenin	27
9.	8β Acetoxy parthenin	28
10.	Isoguaiene	29
11.	11-Hydraxy Iso quaiene	29
12	2β - Hydroxy coronopilin	30
13.	8β - Hydroxy coronopilin	30
14.	Dihydroparthenin	31
15.	Anhydro Parthenin	27
16.	Dihydro isoparthenin	32
17.	11H, 13-Hydroxy parthenin	30
18.	13-Methoxydihyro colonopilin	33
19.	Hysterin	25,32
20.	Confertdiolids	24
21.	Betulin	34
22.	Quercetagetin3-7,dimethyl ether	35
23.	6-Hyderoxy Kaempferol-3-7-dimethyl ether	35
24.	Quercitin-3-0-glucoside	35
25.	Kaempferol 3-o-glucoside	35
26.	Kaempferol-3-0-ara-bine glucoside	35

Besides the above mentioned compounds hexacosanol myracylalchol, β-sitosterol, β-o-glucoside of sitosterol, stigmasterol, compesterol, ursolic acid & saponin have been reported [36] from the leaves of *Parthenium hysterophorus*. Five free amino acids (Arginine, Proline, methionine, amino caprilic acid & hystidene) have also been reported from the pollen of the plant [37].

Parthenin has been subjected to various modifications and converted to different analogues by different workers. These various modifications have been summarized in the form of scheme 1 (Figure 2.1). The compound was treated with various common reducing agents including $NaBH_4$, $NaBH_4/I_2$, Na/EtOH, Mg/MeOH and Zn/AcOH as well as with different oxidizing agents including m-CPBA and dilute HCI under different reaction conditions. The retention of the α-methylene and γ-lactone moiety which plays a vital role for bioactivity of the compound was observed in some of the reaction products. The reduction of parthenin-1 with $NaBH4/I_2$ in THF is known to form diene-2. The mixture of $NaBH_4$ and I_2 produces borane which transfers hydrogen from parthenin-1 to saturate its exocyclic bond forming the β-methyl at C-11.Treatment of parthenin with sodium in ethanol produces compound-5 with saturation of both A and C rings. Acetylation of compound-5 with acetic anhydride and pyridine affords monoacetate-6. (Figure 2.1).

Therefore, the important bioactive natural sesquiterpenoid, parthenin undergoes several regioselective and steroselective modifications to different and interesting analogues by various readily available and inexpensive reducing and oxidising agents.



Scheme 1 : Reactions of Parthenin

Figure 2.1: Reactions of parthenin with different chemicals.

2.2.1 Biological activities of sesquiterpene lactones

Sesquiterpene lactones exhibit a variety of activities against different types of organisms. The major findings of individual types of biological activities of these compounds especially Parthenin are summarized below.

2.2.1.1 Antimicrobial activity

Most of the sesquiterpene lactones including parthenin have been reported to inhibit growth of bacteria and/or fungi [38-44]. It has been suggested that the unsubstituted cyclopentenone ring is a prerequisite for antimicrobial activity of sesquiterpene lactones and is independent of the presence or absence of α-methylene-gamma- lactone moiety [45, 46].

2.2.1.2 Allelopathic activity

Parthenium hysterophorous, a hazardous widespread weed that has infested agricultural lands in many parts of India and Australia, causes a serious reduction of many economically important crop species. It has been found by various researchers that the growth and yield of several crop species were considerably affected when they were grown in soil containing dried root and leaf materials of this weed [47]. They also observed that dried plant material or aqueous extracts from roots of *P. hysterophorous* caused suppression in the growth and colonization of Rhizobia in leguminous plants. Parthenin and water extracts of *P. hysterophorous* inhibited seed germination and growth of seedlings of *phaseolus vulgaris*, wheat, and ragi. Later, it has been proved that extracts of this plant showed inhibitory activity. On the basis of this and other supporting data it has been suggested that *P.hysterophorous* contains a complex mixture of inhibitors with parthenin and some phenolic acids as the prominent constituents [48].

2.2.1.3 Insecticidal effects

Parthenin from *P. hysterophorous* inhibits heart beat of grasshopper most likely by blocking thiol containing compounds important for normal heart activity. This is indicated by the fact that the activity of parthenin - arrested hearts can be restored by thiol addition [49].

2.2.1.4 Effect on mammals

Parthenium hysterophorous, when fed to cattle and buffaloes in excess quantity, has been found to cause illness or death of the animals, most likely because of parthenin present in this species [50]. The extract of *P.hysterophorous* from which parthenin and other sesquiterpene lactones has been removed could be used as a protein rich fodder [51], clearly showing that sesquiterpene lactones are responsible for the poisoning action of this plant.

2.2.1.5 Allergic contact dermatitis

Allergic contact dermatitis due to *parthenium hysterophorus* develops from repeated contacts with this plant or possibly with its disseminated trichomes and dried plant parts [52]. It has been established that the exomethylene on the lactone ring of sesquiterpene lactone is responsible for allergenicity, however this group alone is not always immunologically sufficient [53-56]. Since parthenin undergoes a reaction with cysteine via the exomethylene on the lactone ring as well as via the C_2-C_3 double bond [57]. The presence of these two active sites in a molecule of parthenin could be responsible for its strong allerginicity

2.2.1.6 Cytotoxic activities

Sesquiterpene lactones are of great interest in cancer research because many of these compounds have been shown to exihibit antileukemic, cytotoxic and/or tumor inhibitory activity. The relationship between chemical structure of sesquiterpene

lactones and their cytotoxic activity was investigated by many researchers. In their review of antineoplastic agents of plant origin, Hartwell and Abbott (1969) concluded that all known active sesquiterpene lactones possess an α, β -unsatured lactone ring. Later it was established that the conjugated exomethylene group on the lactone is an essential requisite for cytotoxicity [58, 59].

2.2.1.7 Mutagenic activities

Parthenin, a sesquiterpene lactone from *P.hysterophorous* has been reported to have the ability to break human leucocyte chromosomes in-vitro and to induce micronuclei formation in the polychromic erythrocytes of Mice in vivo [60]. The exact mechanism underlying the observed cytogenetic damage caused by parthenin is not known.

2.2.1.8 Anti-inflammatory activities

Hall et al. in 1979 tested some sesquiterpene lactones for anti-inflammatory activities in the rodents. In the carageenan inflammation screening tests and in the tests for the inhibition of the writhing reflex, the exomethylene on the lactone ring of the sesquiterpene lactones was found to be required for potency.

2.2.1.9 Anti-malarial activity

Parthenin and some of its derivatives have been shown to possess significant anti-malarial activity against a multi-drug resistant strain of *Plasmodium falciparum.* The activity of parthenin and its derivatives against malarial parasite may be due to their differential effects on host and Plasmodium membranes [61].

2.3 RESULTS AND DISCUSSIONS

2.3.1 *Observations based on literature studies / present studies.*

Parthenium hysterophorus L. is a rich source of sesquiterpenoids. Several pseudo guainaolide sesquiterpene lactones were reported by previous workers (table-

2.1). However the present investigation afforded the isolation of Parthenin from *Parthenium hysterophorus* using column chromatography, TLC and Co-TLC techniques. The structures were elucidated using various spectral techniques such as ^{1}HNMR, ^{13}CNMR and Mass spectrometry. The isolated sesquiterpene lactone (Parthenin) was evaluated for its antioxidant activity along with in-vitro and in-vivo anthelmintic activities. The structure activity relationship (SAR) of the compound was also carried out. Further, the theoretical studies of the molecule were also carried out and different parameters like ionization energy, electron affinity, dipole moment, polarizibility and total energy of the molecule was studied.

Parthenin (1) is the major sesquiterpenoid constituent of the obnoxious weed *Parthenium hysterophorus*. It was isolated as crystalline white solid, from the Hexane:Ethyl acetate (1:1) extraction of the flower portion of the plant. Melting point of the compound was recorded on Kofler block and was found to be 164-165^{0}C (MeOH). Molecular formula was found to be $C_{15}H_{18}O_4$ using mass spectrum. The structure of the compound was identified as the sesquiterpene lactone parthenin (1) through spectroscopic analysis and by comparing it directly with that of an authentic sample. The ^{1}HNMR spectrum of the compound was recorded on Varian Gemini 200 MHz in $CDCl_3$ is shown in Figure 2.2. ^{1}HNMR (200MHz, $CDCl_3$ with TMS): 7.50(d, H-2), 6.25(d, H-3), 6.24 (d, H-13b), 5.56(d, H-13a), 5.02 (d, H-6), 1.35(d, C-10 Me). These values correspond to those reported for parthenin by Herz et al. (1962) [62]. The mass spectrum of the compound showed molecular ion peak at m/z 285 $[M+Na]^{+}$ (Figure 2.3).

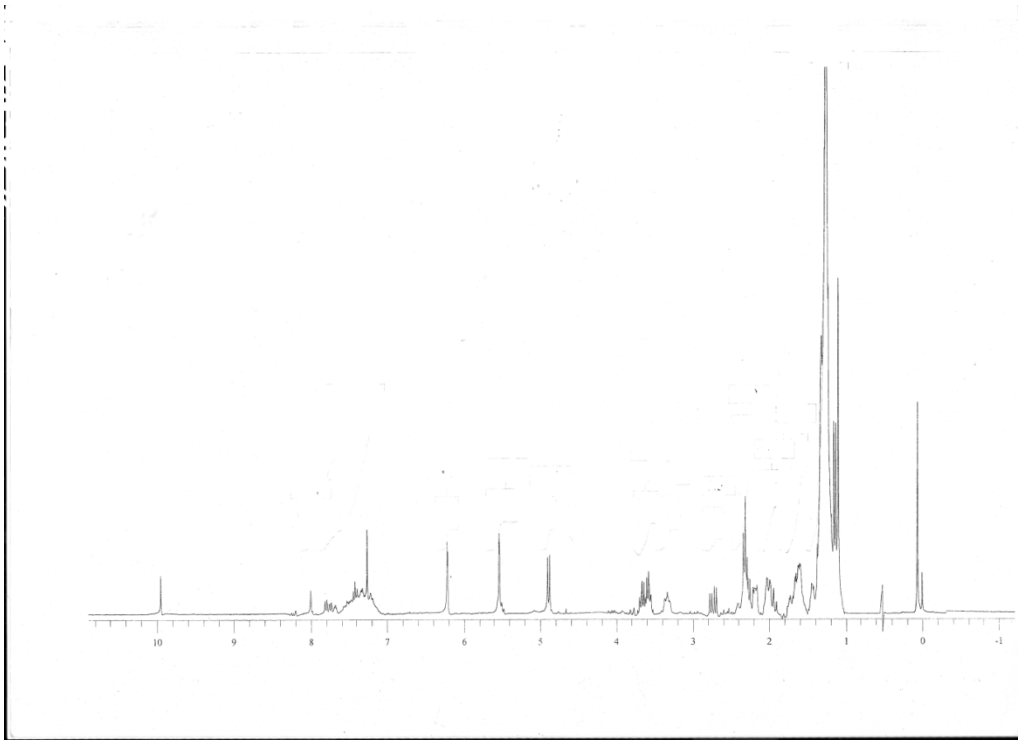


Figure 2.2 : ^{1}HNMR spectrum of Parthenin in $CDCl_3$

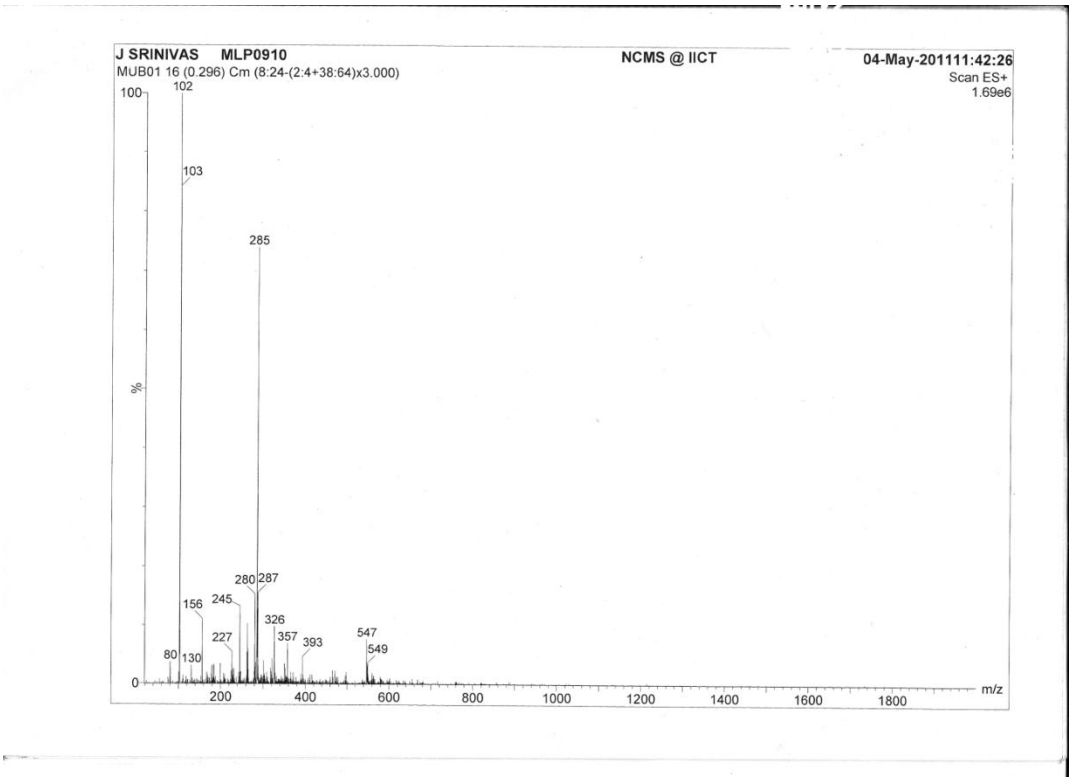
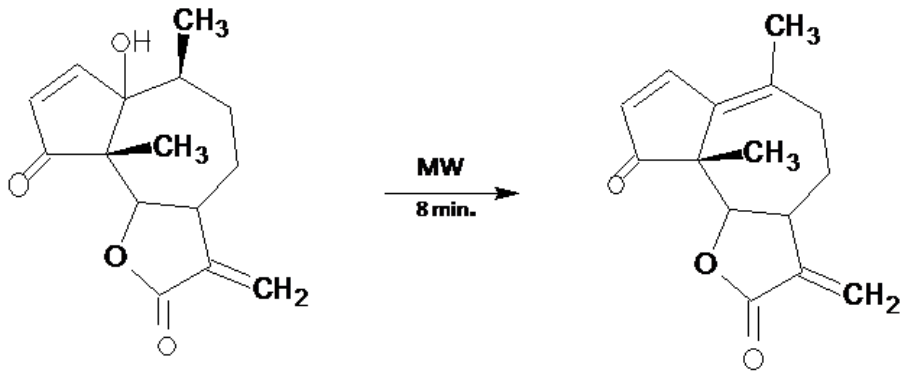


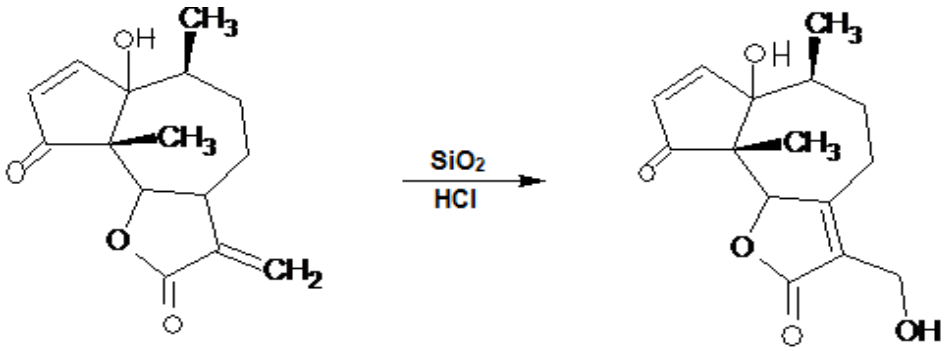
Figure 2.3: Mass spectrum of sesquiterpene lactone parthenin.

2.3.2 Chemical and Biochemical Transformations of Parthenin

Chemical or biochemical modification of an active molecule is a tool to obtain more active molecule form its natural counterpart [63]. Several natural bioactive molecules have been converted to their analogues in recent years by chemical or biochemical means. Medicinally the Parthenin has been found to be of interest for its anticancer [64, 65] anti bacterial [66], antiamoebic [67] and antimalarial properties. As an allelochemical the compound acts as an inhibitor of seed germination [68, 69] and possess antifungal activity [70]. However the compound is toxic and known to create allergic contact dermatitis in human and animals. In the present studies various modifications of parthenin has been carried out in order to ascertain SAR studies with respect to anthelmintic and antioxidant activities. Firstly, Parthenin (1) was converted into anhydroparthenin (2) under microwave conditions following the procedure of Biswanath et al, 1999. Secondly, parthenin adsorbed on silica gel was treated with dilute HCl in order to generate analogue (3) (Figure 2.4).



(1) (2)



(1) (3)

Figure 2.4 Conversion of parthenin (1) into its derivatives (2) and (3)

2.3.3 ANTHELMINTIC ACTIVITY

2.3.3.1 Faecal egg count reduction test

The in-vivo anthelmintic activity in terms of egg count percent reduction of the Parthenin (dissolved in methanol) in sheep naturally infected with mixed species of GI nematodes demonstrated significant anthelmintic activity. The compound exhibited a dose dependent anthelmintic activity (Table 2.2). The maximum reduction of 85.07% in faecal egg counts was recorded for compound @ 1mg/kg body weight at day 18 post-treatment.

Table 2.2. Mean faecal egg counts and percentage reduction in egg counts for Parthenin-treated sheep compared with untreated controls

Treatment	Solvent	Mean ± SEM of eggs per gram of faeces pre- and post-treatment	
		Pre-	Post-treatment

		treatment Day 0	Day 6	Day 12	Day 18
GroupI. Parthenin	Methanol	912.0±6.04	768.0±12.17 (15.78)	378.0±14.7 (58.55)	127.0±1.94 (85.07)
GroupII. Levamisole (Positive control)	-	880.4±10.03	60.8±12.37 (93.09)	31.6±20.46 (96.41)	12.6± .25 (98.56)
GroupIII. Untreated (Negative control)	-	742.6 ±3.93	705.0±2.79 (5.06)	697.6±2.92 (6.05)	682.6±4.95 (8.07)

Figures in parenthesis indicate mean egg count percent reduction (FECR %); SEM, standard error of mean; bw, body weight

2.3.3.2 Adult motility assay

Effect of Parthenin dissolved in methanol was dose-dependent. Highest mortality (98.00%) of worms was observed 8 hours post-exposure @ 50 mg/ml (Table 2.3). The compound Parthenin resulted in mean percentage worm motility inhibition (%WMI) of 94.44%, as observed after the worms were put in lukewarm PBS for 30 min after exposure to different treatments. There was 100% mortality of worms in Levamisole (used as a reference drug) within 4 hours post-exposure. There was no complete mortality of worms kept till 8 hours post-in PBS experiment.

Table 2.3. In-vitro anthelmintic efficacy of methanol dissolved Parthenin on *Haemonchus contortus* of sheep

Treatment	Conc. mg/ml	Mean ± SEM of number of Haemonchus contortus worms showing motility				
		0 hours	2 hours	4 hours	6 hours	8 hours

Parthenin	**50.00**	50 ± 0.00	20±0.10 (60.00)	13±0.20 (74.00)	8±0.20 (84.00)	1±0.40 (98.00)
	25.00	50 ± 0.00	25±0.10 (50.00)	22±0.04 (56.00)	12±0.05 (76.00)	4±0.33 (92.00)
	12.50	50 ± 0.00	32±0.570 (36.00)	28±0.04 (44.00)	13±0.10 (74.00)	6±0.40 (88.00)
Levamisole	**0.55**	50 ± 0.00	18 ± 0.57 (64.00)	0 ± 0.0 (00.00)	0 ±0.00	0 ±0.00
PBS	**0.9%**	50 ± 0.00	50 ± 0.00	50 ± 0.00	46 ± 0.00	42 ± 0.00

2.3.3.3 Egg hatch test

Inhibitory effect of the compound on percent egg hatching was very low as compared to Levamisole. The compound dissolved in methanol exhibited lethal concentration LC_{50}= 2.13mg/ml. The data of correlation of regression revealed a dose dependent response of the compound tested. Lethal concentration 50 (LC_{50}) analysis for the inhibition of egg hatching are shown in Table 2.4.

Table 2.4. Regression values and correlation of regression of the effect of the compound on egg hatching

Treatment	**LC_{50}**	**Regression values and correlation of regression**
Parthenin	2.13	$y = -0.0002x + 4.6324$, $r^2 = 0.9689$
Levamisole	1.88	$y = -0.2159x + 6.2447$, $r^2 = 0.775$

Further, when compound (2) and (3) (Figure 2.4) were subjected to similar anthelmintic type of activity procedures, it was shown that no significant change was observed in case of compound (2) as compared to parthenin, when treated against GI nematodes of sheep. However when compound (3) was tested for anthelmintic activity, a reduction in anthelmintic activity was observed as compared to parthenin. This is most probably because the former contains same exocylic methylene group like that of parthenin while latter does not contain this exocyclic methylene group, thereby showing reduction in anthelmintic activity. From this scenario, it can be concluded that the anthelmintic activity of the compound is either exclusively due to exocylic methylene group or due to synergistic effect between exocyclic methylene group with ester linkage.

2.3.4 Antioxidant activity.

DPPH free radical scavenging capacity of the compound parthenin and its derivatives were measured by DPPH assay under in-vitro conditions. The ability of the examined compound and its derivatives to act as donor for hydrogen atoms in the transformation of DPPH radical into its reduced form $DPPH_2$ was investigated. The examined samples were able to reduce the stable purple coloured DPPH radical into yellow coloured $DPPH_2$. Parthenin (1) and its analogue (3) showed most promising radical scavenging activity at concentration of 100μg/ml. It is suggested that the compound (3) contains two hydroxyl groups and probably that is why it showed high degree of scavenging activity. However in case of anhydroparthenin (2) negligible amount of antioxidant activity was observed, the most probable reason behind this could be removal/absence of hydroxyl group. These results are shown in table 2.5 and plotted in the form of graph (fig 2.5).

Table 2.5: Antioxidant activity of parthenin and its derivatives.

S.NO	Samples	Concentration(µg/ml)	Activity (% inhibition)
1	Parthenin	100	47.23
2	Standard	100	67.42
3	Analogue (2)	100	8.09
4	Analogue (3)	100	54.42

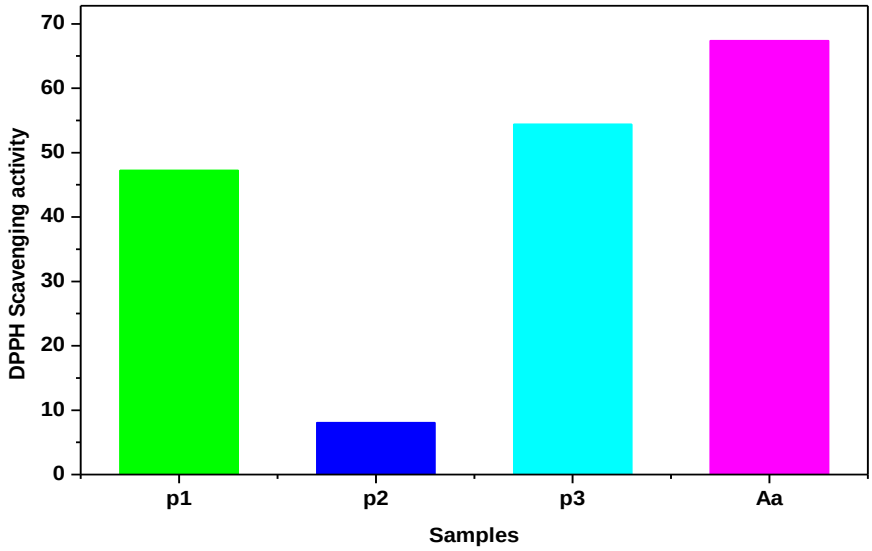


Fig 2.5: Antioxidant activity of parthenin and its derivatives

Where, P1=parthenin; p2= Parthenin analogue (2); p3=Parthenin analogue (3); Aa=Ascorbic acid

2.3.5 Computational studies

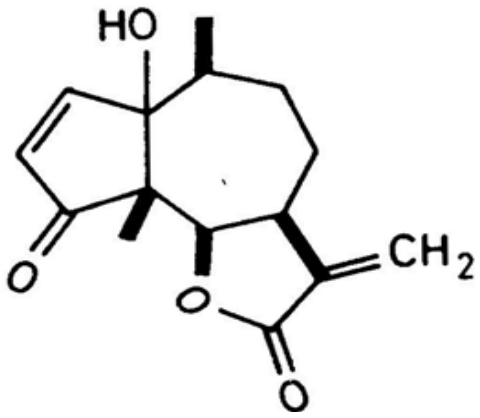
The computational studies of the sesquiterpene lactone parthenin involving different calculations and 3D modeling, were performed using Gaussian 03 chemistry package. The intial geometry was optimized by DFT method by employing B3LYP(24-25) and the 631G(d,p) basis set. Frequency analysis was performed on the

optimized structure at the same level of theory and no imaginary frequency was found. The main parameters obtained are as follows:

1	Ionisation energy	-6.67ev
2	Electron affinity	-1.59ev
3	Dipole moment	7.5D
4	Energy	-804.659028hatree
5	Polarizibility	58.81

2.4 EXPERIMENTAL

PARTHENIN (1)



(1)

Figure 2.6: Structure of Parthenin

- Melting point 164-165^{0}C (MeOH)
- Molecular formula $C_{15}H_{18}0_4$
- R_f 0.44 (1:1 Hexane: Ethyl acetate)

- ^{1}HNMR — 7.50 (d, H-2), 6.25 (d,H-3), 6.24 (d, H-13b), 5.56 (d, H-13a), 5.02 (d, H-6), 1.35 (d, C-10 Me).
- Mass — Molecular ion peak at m/z 285 $[M+Na]^{+}$

2.4.1 Plant Material

The *Parthenium hysterophorus* plant material was collected from the district Anantnag, J & K India. Voucher specimen of *Parthenium hysterophorus* bearing specimen no. 810 was deposited at KASH herbarium in centre of plant taxonomy, Kashmir University, Srinagar, J&K India.

2.4.2 Extraction and Isolation

The *Parthenium hysterophorus* plant material was ground to a fine powder and was later extracted via Soxhlet extractor directly with Ethyl acetate: Methanol mixture (1:1), followed by pure methanol. These combined extracts were concentrated on rota-vapor. The concentrated extract was further extracted with hot water. Water soluble fraction was collected and insoluble one was discarded. Ethyl acetate was added to water fraction in excess and vigorously shaken at 0^{0}C. Ethyl acetate fraction was collected and concentrated on rota-vapor. This extract was subjected to column chromatography over silica gel with increasing order of solvent polarity. The compound got eluted in Hexane: Ethyl acetate (1:1) combination and the fraction was collected and concentrated from which needle like crystals were obtained.

2.4.3 Identification of Compound

The isolated compound was identified as the sesquiterpene lactone parthenin (1) through spectroscopic analysis and by comparing it directly with that of an authentic sample. The ^{1}HNMR spectrum of the isolated compound in $CDCl_3$ with tetramethyl silane (TMS) as internal standard is shown in table 2.6. Further, ^{13}CNMR data of this compound is shown in table 2.7. These values correspond to those reported for parthenin by Herz et al. (1962) [62]. The mass spectrum of the compound showed molecular ion peak at m/z 285 $[M+Na]^+$.

Table 2.6: ^{1}HNMR data of Parthenin ($CDCl_3$) 200 MHz.

Proton	Chemical shift (δ)	Multiplicity	J (Hz)
H-2	7·50	d	6.2
H-3	6·25	d	6.2
H-4	-	-	-
H-6	5.02	d	8.2
H-7	3.46	m	-
H-8	2.37-2.18	m	-
H-9	1.84, 1.63	m,m	-
H-10	2.10	m	-
H-11	-	-	-

H-13	6.24, 5.56	d, d	2, 2
H-14	1.24	s	
H-15	1.12	d	7

Table 2.7 ^{13}CNMR data of Parthenin ($CDCl_3$) 200 MHz

Carbon	Chemical shift (δ)	Multiplicity
C-1	84.8	s
C-2	163.4	d
C-3	131.5	d
C-4	211.2	s
C-5	59.2	s
C-6	78.8	d
C-7	44.7	d
C-8	28.4	t
C-9	30.2	t
C-10	40.0	d
C-11	140.5	s
C-12	170.8	s
C-13	121.6	t

C-14	17.7	q
C-15	18.2	q

2.4.4.1 Microwave irradiation of parthenin (1)

Parthenin was converted into anhydroparthenin under microwave conditions following the procedure of Biswanath et al, 1999 [71]. 100mg of parthenin (1) was taken in an Erlenmeyer flask and placed in an alumina bath inside a commercial microwave oven (BPL BMO 700T). The compound was irradiated at 233 Watt for 8min. The reaction mixture was taken from oven and was cooled to room temperature. The mixture was shaken with CH_2Cl_2 (10ml) and filtered. The filtrate was concentrated to a gummy mass. This was purified by column chromatography over silica gel, the column being eluted with solvents of increasing polarity using hexane and EtOAc. The fraction eluted with hexane–EtOAc (3:2) afforded a solid which was crystallized from C_6H_6 to yield anhydroparthenin (2) (60mg), mp 124-125^{0}C, ^{1}HNMR($CDCl_3$,200MHz); δ 8.01 (d, j=6.0Hz, H-2), 6.30 (d, j=3.0Hz, H-13), 6.09 (d=6.0Hz, H-3), 5.58 (d, j=3.0Hz, H-13), 4.45 (d, j=7.0Hz, H-6), 3.19 (m, H-7), 2.72 and 2.45 (m, H-9), 2.38 and 1.80 (m, H-8), 2.03 (s, Me-10) and 1.36 (s, Me-5); Mass (70eV):m/z 244(M^+), 149, 145, 122, 105. Therefore molecular formula corresponds to $C_{15}H_{16}O_3$. The formation of product (2) was further confirmed by thin layer chromatography (TLC)

2.4.4.2 Treatment of parthenin adsorbed on silica gel with dilute HCl.

This reaction was carried out as per the procedure of Biswanath et al, 1999[72]. To a solution of parthenin (100mg) in CH_2Cl_2 (10 ml) silica gel (100-200 mesh, 3g) was added and CH_2Cl_2 removed under reduced pressure to adsorb the compound on silica gel. Dilute HCl (5%,0.1ml) was added and was thoroughly mixed to make a

homogeneous mass. The mixture was kept overnight at room temperature. This was shaken with CH_2Cl_2 (20ml) and filtered. The residue was again shaken with CH_2Cl_2 – MeOH (1:1, 20 ml). The filtrate after filtration was concentrated and dissolved in water (40ml), concentrated and purified by column chromatography to produce a new compound in the form of viscous oil (60mg); ^{1}HNMR ($CDCl_3$): δ 7.53 (d, j=6.0Hz, H-2) , 6.10 (d, j=6.0Hz, H-3), 5.44 (s, H-6), 4.30 (s, H-13), 2.98 (t, j=6Hz, H-8), 2.35-2.10 (m, H-9, H-10), 1.07 (d, j=7.0Hz, Me-10), 0.91 (s, Me-5); MS m/z: 260 (M^+), 245, 161, 145, 113, 117. Molecular formula corresponds to $C_{15}H_{18}O_5$. The formation of product (3) was further confirmed by thin layer chromatography (TLC).

2.4.5 In-vivo experiment

2.4.5.1 Animals and experimental design

The in-vivo studies were performed using the faecal egg count reduction (ECR) assay in sheep harbouring a naturally acquired GI nematode infection. The animals were pre-adapted to the pen conditions for 18 days prior to the start of the study. Water, hay and feed were provided regularly to the study animals. The study continued for a period of 15 days post-treatments. Before the start of the study, the animals were confirmed positive with an infection of mixed GI nematodes by faecal examination using the standard parasitological procedures applicable to detection of nematode eggs in sheep faeces [73]. Faecal samples were cultured to cultivate the L3 larvae and identified for dependable diagnosis of mixed GI nematode infection in sheep as per the methods of Coles *et al*. (2006) [74]. The animals used for the study were randomly divided into three treatment groups identified as group-1, group-2 and group-3, of two animals each and assigned to different treatments which were administered orally using a syringe. Group 1 received a single dose of compound at 1.0 $mgkg^{-1}$ body weight (bw). Group 2 received Levamisole at 0.5 $mgkg^{-1}$ body

weight (bw) as positive control. Group 3 received no treatment and served as negative control. Each group was isolated from other groups and no physical contact was possible between animals from different treatment groups.

2.4.5.2 Laboratory procedure:

To determine the faecal egg count reductions of GI nematodes in sheep, faecal samples of each animal in the respective treatment groups were collected in the morning, starting from day 0 and at days 5, 10 and 15 post-treatment (PT). The faecal samples were homogenized so that the eggs were uniformly distributed throughout the faeces prior to counting. The total numbers of nematode eggs (faecal egg counts) were determined using Mac Master Egg counting technique; with each egg count representing 50 eggs per gram of faeces. Faecal egg count percent reduction (FECR%) was calculated using the following formula:

FECR % = 198[(pretreatment egg count per gram - Post treatment egg count per gram) / (pretreatment egg count per gram)]100

2.4.5.3 Recovery and preparation of eggs

The recovery and preparation of eggs, egg hatch assay and larval development assay were done following the method by Bizimenyera et al. (2006) [75]. Faecal pellets were collected from the lambs using sterilized harnesses and collection bags. Water was slowly added to the faeces and pellets mashed in a blender until a relatively liquid suspension (slurry) was obtained. The slurry was then filtered through sieves of 150, 90, 63 and 38 μm. The eggs on the 38 μm were then backwashed and transferred into 50 ml centrifuge tubes using distilled water. The suspension was centrifuged for 5 min at the rate of 1200rpm, supernatant was decanted, and the sediments were suspended in saturated salt solution. The suspension was transferred into another set of tubes, centrifuged similarly again for 5 min and the supernatant was washed through a 38 μm pore mesh sieve using distilled water. The eggs were then washed off from the 38

μm sieve with distilled water into a 1 litre conical flask where they were allowed to sediment for 2 h. The eggs were then siphoned from the bottom of the conical flask into a beaker that was then subjected to electromagnetic stirring for egg suspension. The concentration of eggs was estimated by counting the number of eggs in 3 aliquots of 50 μl of the suspension on a microscope slide, replicated three times and the average number of eggs per 200 μl determined.

2.4.5.4 Egg hatch assay (EHA)

Egg hatch assay was conducted according to procedure described by Coles et al. (2006). Approximately, 50 eggs were collected per tube; each tube contained 1 ml of PBS and 1 ml of increasing concentrations of plant compound (75, 150, 300, 600, 1200 and 2400 μg/ml) prepared with PBS. In addition, positive (levamisole at 0.125 mg/ml) and negative (PBS) controls were considered. The tubes were covered, and the eggs were incubated for 48 hours at temperature of 27°C. Thereafter, the number of the first stage larvae (L1) present per tube was counted using a dissecting microscope. Each concentration was tested on five replicates. An inhibition percent (%) of egg hatching was calculated for each extract concentration using the following modified formula [76]

Inhibition (%) = 100 (1 - X1/X2)

Where X1=number of eggs hatched in test sample, and X2= number in PBS control.

2.4.6 Evaluation of *in-vitro* anthelmintic activity.

In-vitro anthelmintic activity of the plant compound was evaluated by exposing the adult *Haemonchus contortus* to compound isolated from plant. Five petridishes were used i.e. four for compound to be tested and one for 0.95% of PBS as control. Adult motile *H. contortus* were collected from the gastrointestinal tract of sheep slaughtered at Srinagar slaughterhouse and immediately transferred to the petridishes containing plant compound and PBS. Observations were made on the motility/survival of worm at 0, 2, 4, 6 and 8 h post-exposure (PE). The number of

dead worms after 8th hour of post exposure was compared statistically with those exposed to control group.

2.4.6.1 Determination of 50% lethal concentration (LC_{50})

LC_{50} values of the individual plant compound tested for anthelmintic activity was calculated by the following formula, as given by Assis et al. (2003): [77]

LC_{50} = No. of living (mobile) worms/Total no. of worms in PBS

2.4.7 Determination of antioxidant activity

The free radical scavenging activity of the compound parthenin (1) along with its derivatives (2) and (3) were measured in terms of hydrogen donating or radical-scavenging abilityusing the stable radical DPPH. 0.1 mM solution of DPPH in Methanol was prepared and 1.0 ml of this solution was added to 1.0 ml of the test solution in methanol at concentrations of 100 μg/ml. Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was used as the reference compound. Lower absorbance of activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ scavenging activity} = ((A_0\text{-}A) / A_0 \times 100).$$

Where A_0 was the absorbance of the control (blank, without compound) and A was the absorbance of the reaction mixture. All the tests were performed in triplicate and the graph was plotted with the mean values.

2.4.8 Computational Analysis

The molecule was studied with 3-D modeling and calculations were performed using the Gaussian 03 quantum chemistry package. The initial geometry were optimized by the DFT method by employing Becke's three-parameter hybrid functional (B3LYP) and the 6-31G(d,p) basis set; Frequency analysis was performed on the optimized structures at the same level of theory and no imaginary frequencies were found [78-80] .

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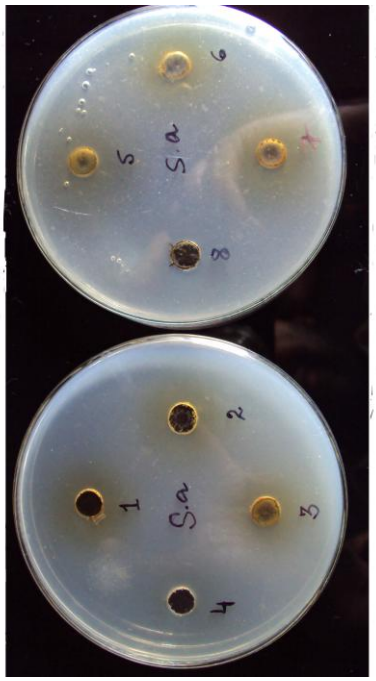
CHAPTER 3

Phytochemical Investigation of *Myricaria germanica*

(a)



(b)

THE FIGURE SHOWS:

a) ***Myricaria germanica*** **(plant)**

b) **Culture plates showing zones of inhibition for *S.aureus* (bacterial strain)**

3.1 INTRODUCTION

The genus Myricaria comprises of 13 species distributed in Europe, Central Asia, China, Sikkim, India, Russia, Afghanistan, Iran & Pakistan. *Myricaria germanica* L. is a pioneer shrub on the open spaces and usually found along the river sides. Other representatives of the same family, e.g. *Tamarix gallica* and *T. pentandra*, are frequently cultivated as ornamentals or planted along roadsides and dunes as protection against wind erosion. The scale-like leaves of *M. germanica* are covered by an array of tubular wax crystals on the cuticle surface, which scatter visible light and give the plant a bluish-green appearance [1]. On various plant species, epicuticular wax crystals have been described [2]. Their presence enlarges the exposed hydrophobic surface, thereby rendering the leaf highly unwettable [3]. This has two important ecological functions: firstly, forcing water droplets to go down and wash away dirt particles [4] and secondly it prevents the formation of macroscopic water drops and inhibiting germination of pathogenic micro-organisms [5].

In literature the following13 species are reported from different parts of the world.

- *Myricaria albiflora*
- *Myricaria bracteata*
- *Myricaria elegans*
- *Myricaria germanica*
- *Myricaria laxa*
- *Myricaria laxiflora*
- *Myricaria paniculata*
- *Myricaria platyphylla*
- *Myricaria prostrata*
- *Myricaria pulcherrime*

- *Myricaria eosea*
- *Myricaria squamosa*
- *Myricaria wardii*

Most of the species are threatened by increasing habitat fragmentation and anthropogenic disturbances like dam and high way construction & over grazing.

Myricaria germanica L is an erect shrub, stem yellowish grey to pinkish, glabrous with inconspicuous ridges and furrows. Leaves simple, sessile, larger on the main branches than on lateral, linear lanceolate to narrowly ovate, to subacute, more or less rounded at the base. Racemes mostly terminal rarely lateral. Flowers pink or pinkish white, pedicel 2-4mm long, each flower subtended by a bract, bracts broadly ovate, trapezoid, long acuminate, irregularly dentate recurved at the opex; sepals 5, united at the base, lanceolate to lancolate-ovate 4-4.5 mm long 1mm broad,obtuse-subacute, scarious at the margin petals oblong, 5-7 long, slightly matched on one side stamens united, free portion dilated at base. Long stamens' 4-5mm long, shorter 3-4 mm anthers dorsifixex, 0.5mm long obtuse carpel 5-8 mm long, ovary pyramidal with sessile capitates, stigma, capsule elongated pyramidal 8-10mm long, 3 mm broad dehiscing by 5 longitudinal halves.

3.2 REVIEW OF LITERATURE

The genus Myricaria (*Tamaricaceae*) is comprised of small deciduous shrubs that occur along river sides in mountains usually 2400-4600m above sea level. The branches and leaves of these plants are used in the folk medicine for treatment of cold, asthema, measles, scorpion poison, and for limiting the effects of poison [6]. Most of the plants from the genus *Myricaria* were found to contain phenolic compounds and flavonoids. It is known that polyphenol natural products are a major group of compounds with widespread distribution and a broad pharmacological profile,

including anti-inflammation, anti-oxidant, anti-bacterial, anti-allergic, anti-histamine and anti-asthma activity [7, 8]. Flavonoids are the major and active constituents of the genus. Their effects include dilation of the coronary artery, reducing blood pressure and blood fat, inhibition of platelet aggregation, delaying the formation of thrombi, improving humoral and cellular immunity, increasing synthesis of DNA, and anti-aging [9]. They are also used to treat rheumatism, chronic tracheitis, infantile paralysis, and neurasthenia in some clinical practices [10]. Several references have been published on the studies of chemical constituents of genus Myricaria. Capillary electrophoresis (CE) has been efficiently used for separation of active compounds from few Myricaria species and has been increasingly accepted as an attractive method for separation and identification of the components of traditional medicine [11-15]. *Myricaraia germanica* has been reported to contain long chain alkanediols in its leaf cuticular waxes [16]. Antimicrobial activities have been evaluated in few species of Myricaria [17]. Reviews of literature of some of the species of Myricaria have been briefly reproduced in the tabular form (Table 3.1).

Table 3.1: Reported Chemical Constituents of *Myricaria Species.*

Plant species	**Compounds isolated**	**Reference**
Myricaria-elegans	Corsolic acid, Betulin, Ursolic acid, Erythrodiol, Eleganene –A, Eleganene-B	18
Myricaria-paniculate	Myriconal, 28-hydroxy-14-tara-xeren-3-one, Epifriedelanol, Betasitosterol, 4-methyl stigmast-7-en-3-ol, 12-hentriacontanol, 1-	19

	triacontanol, Myricarin-A, Myricarin-B	
Myricalia - bracteata	3,4-Dimethoxgygallic acid, Gallic acetate, Gallic acid, Kaemferol-3-o- rhamnoside,	20
Myricaria wardii	3,4-dimethoxy gallic acid, 7- methoxy quercetin, kaemferol-3-o- rhamnoside, gallic acid	20
Myricalia alopecuroides	Ellagic acid 3,3^{1},4- Trimethylether, Ellagic acid 3,3^{1}-Dimethylether, Isorhamnetin, Kaemferol,3,5-Dihydroxy-4methoxybenzoic acid, Daucosterol, 6,7,10-Trihydroxy-8-octadecenoic and, Quercetin, Gallic acid, Palmitic acid, Hexadecanoic acid, 2,3-Dihydroxypropylester.	21

3.3 RESULTS AND DISCUSSIONS

3.3.1 Phytochemical screening

The phytochemical screening is an important step in the chemical and pharmacological study of a medicinal plant. It may suggest possible pharmacological effects of the extracts or fractions in comparison to identified phytochemical groups, highlighting a close relationship with its main therapeutic uses. Keeping this in view, the extracts of *Myricaria germanica* were phytochemically screened for the presence of Alkaloids, Flavonoids, Tannins and Saponins. However it was observed that *Myricaria germanica* extracts contain Flavonoids, Tannins and Saponins in good amount, and no Alkaloids were detected. In aqueous solution the saponins formed

abundant foam which might be due to lipophilic portion in its chemical structure, called aglycone or sapogenin, and a hydrophilic portion, formed by one or more sugars that provide detergent properties to it. The flavonoids and tannins are responsible for broad pharmacological profile, including anti-inflammation, anti-oxidant, anti-bacterial, and anti-histamine activity. Therefore the methanolic extracts of the *Myricaric germanica* were screened for their possible antimicrobial and cytotoxic activities.

3.3.2 Antimicrobial activities

The results of antimicrobial activites are depicted in the form of table 3.2. The antibacterial spectrum of *M.germanica* seems closer to reference antibiotic Kanamycin. The demonstration of broad spectrum of antibacterial activity of extracts of *M.germanica* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease, chemotherapy and control. Results of these studies indicate that further searches and characterizations of *Myricaria germanica* for antimicrobial compounds are warranted. In addition, research on synergistic combinations of extracts with broad spectrum or a high degree of inhibition against a particular micro organism would seem worthwhile. As the search for new antimicrobial agents intensifies, plant extracts may provide attractive alternate sources of molecules for consideration. Drug resistance becomes an increasing problem and consumer demand for products with natural preservative grows on each passing day, therefore *Myricaria germanica* like plants can form the basis of future antimicrobial research efforts.

Table -3.2 Antimicrobial activity of *Myricaria germanica.*

	Zones of Inhibition (in millimeters)		
Materials/ Microrganisms	**Methanolic extract**	**Standard***	**Control****
P.aeruginosa	17	30	-
P.vulgaris	-	30	-
S.aureus	25	30	-
E.coli	-	30	-
C.albicans	18	30	-
B.subtilis	27	30	-
S.epidermis	20	30	-

**Standarol:Kanamycin; **Control : Methanol; (-) : No inhibition*

3.3.3 Cytotoxic Assay

Sulpharhodamine-B assay was performed against five human cancer cell lines namely THP-1 (Leukemia), A-549 (Lung), HCT-15 (Colon), Cervix (Hela) and Prostrate (PC-3), which revealed increase in growth of inhibition during 48 hour incubation at the concentration range of 100µg of the sample. DMSO control was set up separately to cancel out the cell death occurred by DMSO, which was used as a solvent for dissolving samples homogeneously. The results (table 3.3) depicted that the inhibition of different human cancer cell lines of varying tissue origin with 100µg imparted significant cellular cytotoxic effects on all the cell lines that were tested. However the most promising results were obtained against Leukemia (THP-1), Colon (HCT-15) and Lung (A549) cancer cell lines.

Table 3.3: In-vitro Cytotoxic activity of *Myricaria germanica.*

Tissue type	Leukemia	Lung	Colon	Cervix	Prostate

Cell line type		THP-1	A549	HCT-15	Hela	PC-3
Material	Conc.(µg)					
M.germanica (Root)	100	16	13	**68**	-3	15
M.germanica (Aerial)	100	**83**	**40**	30	22	24
Paclitaxel	$1x10^{-6}$	13	**61**	17	6	7
Mitomycin-C	$1x10^{-6}$	23	43	21	4	**67**

3.4 EXPERIMENTAL

3.4.1 Plant Material

The *Myricaria germanica* plant material was collected from district Pulwama, Jammu & Kashmir, India. The localities were the plant material was collected are usually situated between 2400-4600m. Voucher specimen of *M.germanica* bearing specimen no.27913, was deposited at KASH herbarium in centre of plant taxonomy, Kashmir University, Srinagar.

3.4.2 Extraction and Isolation:

Dried and powdered plant material was subjected to soxhlet extraction with organic solvents in increasing order of their polarity (Petroleum ether, Chloroform and Methanol). The extracts thus obtained were concentrated by vacuum evaporation using rota-vapor.

3.4.3 Test Micro Organisms

Clinical isolates gram positive bacteria and gram negative bacteria were grown in nutrient broth medium and incubated at 37^0C for 24 hrs, followed by frequent sub

culturing (every 24hr) to refresh medium. Bacterial strains were maintained on Muller Hinton Agar Medium.

3.4.3.1 Preparation of Microbial Cultures

The bacterial strains procured form MTCC- Chandigarh, were inoculated in to nutrient broth for 24hrs. In the Agar well diffusion method, sterile Muller Hinton Agar for bacteria was inoculated with the test and incubated at 35^0C for 24 hrs. At the end of the period, zones of inhibition were measured in millimeter (table 3.2).

3.4.4 Cytotoxic activity

In order to understand the effects of *Myricaria germanica* extracts on human cancer cell lines, experiments were out carried using cultured THP-1 (Leukemia), A-549 (Lung), HCT-15(Colon), Hela (Cervix) and PC-3 (Prostrate) cell lines by SRB assay. Cell lines were exposed to concentration of 100µg for 48 hours, which reduced the viability of these cell lines. The extract was active against all the tested cancer cell lines. Results are depicted in table-3.3.

3.4.4.1 Human cell lines and culture

The optimum density of seeded cell suspension were introduced to each well of 96-well plates (Iwaki) and exposed to indicated range of concentration of the plant extract (samples). In the cultured RPMI-1640 medium, supplemented with known cytotoxic agent Paclitaxel and Mitomycin-C (Sigma-Aldrich, Fluka, UK) as positive controls. The cells were incubated with sample for 48 hours incubation. Fixed the cells with ice cold TCA for 1 hour at 40^0C. The plates were washed with distilled water and allowed to dry in the air. Sulpharhodamine-B (SRB) solution (0.4%) was added to each well of dry 96-well plates and allowed staining at room temperature for 30-minutes.The unbound SRB solution was removed by washing the plates quickly with 1% (v/v) acetic acid. The bound SRB dye was solubilised by adding 100µl of

10mM unbuffered tris base (PH=10.5) to each well and shaking was performed for 5-minutes on shaker platform. The plates were read in a 96-well plate reader at 540nm. The results showed that the extract of *M. germanica* inhibited different human cancer cell lines of varying tissue origin. However the best results were obtained against Leukemia (THP-1), Colon (HCT-15) and Lung (A549) cancer cell lines.

3.4.5 Phytochemical Screening of Crude Extracts

The phyotchemical screening of the plant extracts was carried out according to standard procedure of Farnesworth [22] as follows.

Alkaloids

The methanolic extract (30ml) was evaporated to dryness in an evaporating dish on water bath. Five ml of 2 N HCl were added and stirred while heating on the water bath for 10min., cooled, filtered, and the filtrate was treated with few drops of Mayer's reagent. The samples were than observed for the presence of turbidity or precipitate.

Flavonoids

The alcoholic plant extract (75 ml) was evaporated to dryness on a water bath, cooled and the residue was defatted using petroleum ether. The defatted residue was dissolved in 30ml 80% ethanol and filtered. The filtrate was treated with a few drops of concentrated HCl and Magnesium turnings (0.5g). The presence of flavonoids was indicated with a pink or magneta red color developing within 3 min.

Tannins

The alcoholic extract (25ml) was evaporated to dryness on a water bath. The residue was extracted several times with n-hexane & filtered, the insoluble residue was stirred with 10ml of hot saline solution, the mixture was cooled, filtered and the volume of filtrate was adjusted to 10ml with more saline solution. To 5 ml of this

solution, few drops of ferric chloride test reagent were added. An intense green blue or black colour was taken as an evidence for the presence of tannins.

Saponins

1 gm of ethanol extract was dissolved in 10ml of distilled water in a test tube and shaked vigorously for 1-2 min. The presence of saponins was indicated by characteristic honeycomb froath at least 1cm in height, which persisted for 30 min.

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Chapter 4

Phytochemical Investigation of *Caltha palustris*

(a) **(b)**

THE FIGURE SHOWS:

(a) ***Caltha palustris*** **alba (plant)**

(b) ***Haemonchus contortus*** **of sheep**

4.1 INTRODUCTION

Caltha belongs to family *Ranunculaceae*. It is a genus of rhizomatous perennial plants, native to wet areas in temperate and cold regions of both northern and southern hemispheres. It is commonly found in wet lands in temperate regions of the Northern hemisphere and is well known for its toxicity to livestock.

Livestock production is the principal activity of farming community in the hills of Jammu and Kashmir which plays an important role in the rural economy and earn substantial amount of foreign currency by exporting skins and other by-products. Livestock is an important source of income and employment for millions of landless and small landholders particularly in the less favored environments. However, productivity is constrained by parasitic infections [1, 2]. Gastrointestinal parasites cause major economic losses in sheep production throughout the world. The cost of decreased reproductive efficiency, decreased meat and wool production. High parasite loads pose a serious health threat and a limitation to the productivity of small ruminants due to the associated morbidity, mortality, cost of treatment and control measures [3]. Nematode parasites of small ruminants result in low productivity due to stunted growth, poor weight gain and poor feed utilization [4]. *Haemonchus contortus*, found in the abomasum of sheep and goats, causes blood loss resulting in decrease in erythrocytes, lymphocytes, haemoglobin, packed cell volume, body weight and wool growth [5]. A decrease in profitability up to 15% and weight loss up to 50% due to gastrointestinal parasites have been reported by Hussain [6] in 1985.

A number of control measures to combat the helminth infections of ruminants are available nowadays. The main methods for control of nematodes are prophylactic treatment with synthetic anthelmintics. The perceivable drug residues in animal products, the increasing prevalence of anthelmintic-resistant strains of helminthes and

high cost of conventional anthelmintics is alarming. For the sheep industry, this situation has now reached a crisis point in some countries [7]. There is an urgent need for the development of sustainable alternatives to anthelmintics in ruminant production systems [8]. Considerable research has shown that some plants not only affect the nutrition of animals, but also have antiparasitic effects [9]. The vast majority of studies investigating the effects of plant extract on helminth parasites, either in experimental or in grazing conditions, have been conducted using sheep [10-23]. Various plant species have been screened for anthelmintic efficacy against different helminth parasites of ruminants. Further, the increasing prevalence of anthelmintic resistant strains of helminthes, drug residues in animal products and high cost of conventional anthelmintics has created an interest in studying medicinal plants as an alternative source of anthelmintics.

Keeping this in view, the present study was carried out with the objective of evaluating the anthelmintic efficacy of *Caltha palustris* alba against gastrointestinal nematodes of sheep under in-vitro and in-vivo conditions. In the present studies, we report for the first time the most promising results of anthelmintic activity of the crude extracts of *Caltha Palustris* alba via in-vitro and in-vivo procedures. However appreciable antimicrobial and cytotoxic activities of the said plant extract has also been reported for the first time in this chapter.

4.2 REVIEW OF LITERATURE

In literature the following 10 species are reported from different parts of the world.

Caltha palustris

Caltha appendiculata

Caltha dionaeifolia

Caltha introloba

Caltha leptosepala

Caltha natans

Caltha novae-zelandiae

Caltha obtusa

Caltha sagittata

Caltha scaposa

Caltha palustris alba. (*Ranunculaceae*) or white marsh-marigold, a perennial herb commonly distributed in wet lands in temperate regions of the Northern hemisphere, is well known for its toxicity in cattle, horses, and man. In India, this plant frequently occurs in the marshes, wet meadows and in the water pools of mountain areas. The plant is used as an antispasmodic and sedative [24]. Systematic research has been carried out on the karyotype [25] serology [26]. and the presence of phenol, alkaloids, cyanogenic compounds and acrid principles [27, 28]. Previous studies have lead to isolation of two new 24-norlupane lactones from alcoholic extract of Caltha palustris.[29] The structure of a new triterpene lactone, palustrolide, has been elucidated as 3β, 23-dihydroxylupan-13β -28 lactone on the basis of physico-chemical studies. In addition, sitosterol, its glucoside, hederagenin, 16, 17-dihydroxykauran-19-oic acid and hederagenic acid have been characterized [30]. However terpenoids linalool oxide (pyranoid) and α-muurolene has also been reported from *C. palustris* [31]. The toxicity of marsh-marigold is often attributed to its protoanemonin content. Protoanemonin is widespread in Ranunculaceae [32-34], and its greater quantity is poisonous for humans and livestock [35]. In addition, this compound shows insecticidal activity against *Drosophila melanogaster* Meigen and *Tribolium castaneum* Herbst [36], and a broad spectrum antimicrobial activity [37-39].

4.2 RESULTS AND DISCUSSIONS

4.2.1 In-vivo Anthelmintic activity

The in-vivo anthelmintic activity (in terms of reduction of nematode egg output) of Methanolic extracts of *Caltha palustris* alba in sheep naturally infected with mixed species of GI nematodes demonstrated significant anthelmintic activity of extracts tested. In-vivo anthelmintic activity of Crude Methanolic Extract (CME) of *Caltha palustris* alba demonstrated a maximum faecal egg count reduction (FECR) of 73.69% in sheep treated on day 15 post treatment (PT), (table 4.1). A progressive decline in the faecal egg count in all treated animals with extracts was observed from day 5 to day 15 PT.

Table 4.1. Mean faecal egg counts and percentage reduction in egg counts for extract-treated sheep compared with untreated controls.

Parasite	**Mean ± SEM of eggs per gram of faeces pre- and post-treatment**			
	Pre-treatment	**Post-treatment**		
	Day 0	**Day 5**	**Day 10**	**Day 15**
Group I M. E. *Haemonchus contortus*	918.4 ± 5.03	770.8±22.37 (14.22)	501.6 ±20.46 (44.94)	261.6 ± 1.43 (73.69)
Group II Untreated (Control) *Haemonchus contortus*	742.6 ±3.93	705.0 ±2.79 (5.06)	697.6 ±2.92 (6.05)	682.6 ±4.95 (8.07)

Figures in parentheses indicate faecal egg count percentage reduction (FECR %).

4.2.2 In-vitro Anthelmintic activity

The crude methanolic extracts (CME) of *Caltha palustris* alba demonstrated the time dependent anthelmintic activity against *H. contortus* as revealed from the inhibition of motility and/or death of the worms after treatment (Table 4.2).The CME of *Caltha palustris* alba resulted in mean percentage worm motility inhibition (%WMI) of 93.44%, as observed after the worms were put in lukewarm PBS for 30 min after exposure to different treatments. The mean mortality index (MI) of the CME was 0.95. The lethal concentration 50 (LC_{50}) for the CME was 0.11mg/ml. Thus from the above tested parameters, we found that CME showed good activity and this could be due to the presence of a higher concentration of the protoanemonin or other alcohol-soluble active molecules in *Caltha palustris* alba. The worms that were exposed to albendazole were found to be paralysed and/or dead at 5 hours (100% mortality or paralysis).

Table 4.2. **In-vitro anthelmintic efficacy of crude methanolic extracts of Caltha palustris alba on *Haemonchus contortus* of sheep.**

Treatment	**Mean ± SEM of number of *Haemonchus contortus* worms showing motility up to 8 hours exposure**					
	0 hour	**1 hour**	**2 hours**	**5 hours**	**8 hours**	**Fresh**PBS **for 30 min**
Albendazole at 0.55mg/ml	20±0.00	8± 0.57	3 ± 0.57	0±0.00	0 ±0.00	0 ± 0.00
C. palustris at 25 mg/ml	20±0.00	17± 0.57	15 ± 0.00	8± 0.57	2.6± 0.33	2 ± 0.57
PBS	20± .00	20± 0.00	20 ± 0.00	20 ± 0.00	18 ± 0.00	18 ± 0.00

SEM, standard error of mean; PBS, phosphate-buffered saline.

4.2.3 Antimicrobial Activity

The methanolic extract of *Caltha palustris* alba showed strong antimicrobial effects against the all test microorganisms used for screening. The maximum zones of inhibition were measured in *S. epidermidis and P. vulgaris* where they showed highest inhibition zones of 24mm and 23mm respectively. Kanamycin was treated as positive control which showed 30mm zone of inhibition. However no activity was observed with methanol which acted as negative control during the whole process. Therefore the antibacterial activity of *C. Palustris* alba seems closer to reference antibiotics (Table 4.3). The demonstration of broad spectrum of antibacterial activity of the methanolic extract of *C. palustris* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease, chemotherapy and control. Results of these studies indicate that further searches and characterizations of *Caltha palustris* for antimicrobial compounds are warranted. In addition, researches on synergistic combinations of extracts with broad spectrum or a high degree of inhibition against a particular micro organism would seem worthwhile. As the search for new antimicrobial agents intensifies, these plant extracts may provide attractive alternate sources of molecules for consideration.

Table -4.3 Antibacterial activity of *C. palustris* alba.

Zones of inhibition (in mm)

Materials /microorganisms	**Methanolic extract**	**Standard***	**Control****
P. aeruginosa	14	30	-
P.vulgaris	23	30	-
S.aureus	19	30	-
B.Subtilis	21	30	-
S. epidermidis	24	30	-

**Standarol : Kanamycin;* ***Control : Methanol;* *(-) : No inhibition*

4.2.4 Cytotoxic Activity.

In order to understand the effects of *C.palustris* on human cancer cell lines, experiments were carried using cultured THP-1(leukemia),A-549(lung), HCT-15(Colon), Cervix(Hela) and PC-3 (Prostrate) cell lines by SRB assay. Cell lines were exposed to concentration of 100µg for 48 hours, which reduced the viability of these cell lines. As shown in Table 4.4, the extract was active mainly against A-549(lung) cancer lines.

Table-4.4 In-vitro *Cytotoxic activity of C. palustris alba.*

Tissue type		**Leukemia**	**Lung**	**Colon**	**Cervix**	**Prostrate**
Cell line type		**THP-1**	**A546**	**HCT15**	**Hela**	**PC-3**
Material	Conc.(µg)					
C.palustris	100	8	**37**	3	22	14
Paclitaxel	$1x10^{-6}$	13	**61**	17	6	7
Mitomycin-C	$1x10^{-6}$	23	43	21	4	**67**

4.4 EXPERIMENTAL

4.4.1 Plant Material

The *Caltha palustris* alba plant material was collected from district Pulwama of Jammu & Kashmir, India. The localities were the plant material was collected are usually situated between 2400-4600m. Voucher specimen of *Caltha palustris* alba bearing specimen No. 27912, was deposited at KASH herbarium in centre of plant taxonomy, University of Kashmir, Srinagar, J & K, India.

4.4.2 Extraction and Isolation

Shade dried and powdered plant material was subjected to soxhlet extraction with organic solvents in increasing order of their polarity (Petroleum ether, Chloroform and Methanol).The extracts thus obtained were concentrated by vacuum evaporation using rota-vapor.

4.4.3 In-vitro Experiment

For the evaluation of anthelmintic activity of the crude methanolic extract (CME) under in-vitro conditions against adult *Haemonchus contortus*, the worm motility inhibition assay was adopted. Mature *H. contortus* worms were collected from the abomasa of freshly slaughtered sheep. The worms were washed and finally suspended in phosphate buffered saline (PBS) and transported to the laboratory. CME in 0.5% dimethyl sulphoxide (DMSO) were tested at 25 mg ml^{-1}. DMSO (0.5%) was the negative control. Twenty worms were exposed to each of the treatments at controlled temperature (37±1 ^{o}C). Three replicates were performed for each treatment. Inhibition of worm motility was the rationale for anthelmintic activity. The time required for paralysis or complete inactivity and mortality was recorded at 0, 1, 2, 5 and 8 hours intervals. After 8 hours the extracts were washed away and parasites resuspended in lukewarm PBS 30 min to test the revival of the worm motility. Percent worm motility inhibition (% WMI) was determined according to Rabel *et al*. (1994) by the following formula:

%WMI=151[(number of mobile worms in negative control Petri dish-number of mobile worms in treatment Petridish)/(number of mobile worms in negative control Petridish)]100

The mortality index was calculated by the following formula:

Mortality Index (MI) = [(Total number of immobile worms (Death)/ (Total number of worms per Petridish)] x 100

4.4.4 In-vivo experiment

4.4.4.1 Animals and experimental design

The in-vivo studies were performed using the faecal egg count reduction (ECR) assay in sheep harbouring a naturally acquired GI nematode infection. The animals were pre-adapted to the pen conditions for 18 days prior to the start of the study. Water, hay and feed were provided regularly to the study animals. The study continued for a period of 15 days post-treatments. Before the start of the study, the animals were confirmed positive with an infection of mixed GI nematodes by faecal examination using the standard parasitological procedures applicable to detection of nematode eggs in sheep faeces [40]. The animals used for the study were randomly divided into two treatment groups of two animals each and assigned to different treatments which were administered orally using a syringe. Group 1 received a single dose of CME at 1.0 g kg^{-1}body weight (bw). Group 2 received no treatment and served as negative control. Each group was isolated from other groups and no physical contact was possible between animals from different treatment groups.

4.4.4.2 Laboratory procedure

To determine the faecal egg count reductions of GI nematodes in sheep, faecal samples of each animal in the respective treatment groups were collected in the morning, starting from day 0 and at days 5, 10 and 15 post-treatment (PT). The faecal samples were homogenized so that the eggs were uniformly distributed throughout the faeces prior to counting. The total numbers of nematode eggs (faecal egg counts) were determined using Stoll's technique [40]; with each egg count representing 50 eggs per

gram of faeces. Faecal egg count percent reduction (FECR %) was calculated using the following formula.

FECR% = 198[(Pretreatment egg count per gram-Posttreatment egg count per gram)/ (Pretreatment egg count per gram)]100

4.4.5 Antimicrobial Assay

The effect of plant extracts on microorganisms have been studied by a very large number of researchers in different parts of the world [41-44]. The aim of the present study was to evaluate the antimicrobial potential of *Caltha palustris* alba against different bacterial strains.

4.4.5.1 Microbial Strains and Culture Media

Gram positive and gram negative bacterial strains were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The bacterial strains used were *Pseudomonas aeruginosa* MTCC 1688, *Proteus vulgaris* MTCC 426, *Bacillus subtilis* MTCC 441, *Staphylococcus epidermidis* MTCC 435, *Staphylococcus aureus* MTCC 96, *Penicillium chrysogenum* MTCC 947 and *Aspergillus niger* MTCC 1344. Bacterial strains were grown on nutrient agar plates at 37^0C and maintained on nutrient agar slants at 25^0C.

4.4.5.2 Agar Well Diffusion Method

The antibacterial susceptibility tests were carried out using the agar well diffusion assay. The bacterial cultures were developed for 24 hours and were later transferred into boiling tubes containing 20ml of liquid nutrient agar. The contents of the tubes were transferred to petri plates. After 10 minutes of solidification of the agar, petri plates were punched in the form of wells. Later these agar wells were filled with 20µl of the plant extract (100mg/ml) dissolved in methanol. The incubation was

carried out for 24 hours at 37 ^{0}C. After the incubation period, the antimicrobial activity was evaluated by measuring the width of the zones of inhibition. Kenamycin (10µg per disc) was used as positive control and pure methanol was used as negative control.

4.4.6 Cytotoxic Studies

4.4.6.1 Human Cell Lines and Culture

The optimum density of seeded cell suspension were introduced to each well of 96-well plates (Iwaki) and exposed to particular concentration of the plant extract. In the cultured RPMI-1640 medium, supplemented with known cytotoxic agent Paclitaxel and Mitomycin-C (Sigma-Aldrich, Fluka, UK) as positive controls. The cells were incubated with sample for 48 hours incubation. Fixed the cells in ice cold TCA for 1 hour at 40^{o}C. The plates were washed with distilled water and allowed to dry in the air. Sulpharhodamine-B (SRB) solution (0.4%) was added to each well of dry 96-well plates and allowed staining at room temperature for 30-minutes.The unbound SRB solution was removed by washing the plates quickly with 1% (v/v) acetic acid. The bound SRB dye was solubilised by adding 100µl of 10mM unbuffered Tris base (PH=10.5) to each well and shaken for 5-minutes on shaker platform. The plates were read in a 96-well plate reader at 540nm.

4.4.6.2 Cytotoxic Assay

Sulpharhodamine-B assay was performed against five human cancer cell lines namely THP-1(Leukemia), A-549 (Lung), HCT-15 (Colon), PC-3 (Prostrate) and Hela (Cervix), which revealed increase in growth inhibition during 48 hour incubation at the concentration of 100µg of the sample. DMSO control was set up separately to cancel out the cell death occurred by DMSO, which was used as a solvent for dissolving samples homogeneously. The results depicted that the inhibition of

different human cancer cell lines of varying tissue origin with 100µg imparted cellular cytotoxic effects on all the cell lines that were tested.

Conclusion

This study attempts to highlighten the anthelmintic and antibacterial potential of *C. palustris* alba. Significant anthelmintic effects of crude methanolic extract on live adult *Haemonchus contortus* worms were observed in terms of the paralysis and/or death of the worms at different hours of post treatment. The administration of the extract in sheep was associated with significant reduction in faecal egg output by the GI nematodes. The extract is approximately as effective as the reference drug-albendazole. The results of the present study suggest that *C. palustris* alba are a promising alternative to the commercially available anthelmintics for the treatment of GI nematodes of sheep. Therefore this plant can hopefully be considered in future for more clinical evaluations and possible applications and as adjuvant to current medications. We should maintain our efforts in considering and valorizing our natural patrimony as well as conducting more research in *C. palustris* alba and its pharmacological aspects

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(4) **Sofi Mubashir**, Wajaht A. Shah, M. Yousuf Dar, Seema Akbar, Kuratull Ain, M. Younis. Antimicrobial Activities of *Myricaria germanica* extracts, 6th JK Science Congress, J&K, India, (2010).

(5) **Sofi Mubashir,** Wajaht A. Shah, Ajaz Ahmed, Seema Akbar, M. Younis. In-vitro and In-vivo Anthelmintic Activities of *Caltha palustris* against GI Nematodes of Sheep, 7th JK Science Congress, J&K, India, (2011).

(6) M.Yousuf Dar, Kuratull Ain, **Sofi Mubashir**, Showkat Rashid, Wajaht A. Shah. Antioxidant and Cytotoxic Activities from *Cymbopogon jawarancusa* Essential Oil, 6th JK Science Congress, J&K, India, (2010).

(7) Ajaz Ahmed, Wajaht A. Shah, **Sofi Mubashir,** Seema Akbar, M. Younis. Antioxidant and Anti-inflammatory activities of ethyl acetate extracts of *Salix capraea*. Department of Botany, University of Kashmir, J&K, India, (2011).

(8) Sofi Mubashir, Wajaht A. Shah, Ajaz Ahmed, Seema Akbar, M. Younis. Antimicrobial Activities of Methanolic extracts of *Caltha palustris var alba.* Department of Botany, University of Kashmir, J&K, India, (2011).

(9) Ajaz Ahmed, Wajaht A. Shah, Seema Akbar, **Sofi Mubashir,** M. Younis. Anti-inflammatory activities of extracts of *Salix capraea*. , 7th JK Science Congress, J&K, India, (2011).

CONFERENCES / WORKSHOPS ATTENDED

(1) Participated in **2nd Biennial International Conference** on **'**New Developments in Drug Discovery from Natural Products and Traditional Medicines' held from 20-24 November, 2010 at NIPER, Chandigarh, India.

(2) Participated in **International Conference on Chemistry**: Frontiers and Challenges, held during 5-6 March, 2011 at AMU, Aligarh, India.

(3) Participated in 6th JK Science Congress, organized by University of Kashmir, during 2-4 December, 2010.

(4) Participated in 7th JK Science Congress, organized by University of Jammu, during 13-15 October, 2011.

(5) Participated in the workshop organized by department of Botany, University of Kashmir during October, 2011.