CHAPTER ONE

INTRODUCTION

1.1 General Background

Nepal is a country of diverse topography and climatic zones. It shares an asset of various medicinal and aromatic plants (MAPs). There is a great possibility of utilizing these natural flora in developing various valuable herbal drugs, aroma chemicals and processed industrial products. The traditional healers have practiced various types of crude drugs since long time. The number of plant species used in medicinal purpose is 1624 out of which 1515 species are angiosperms (Shrestha *et. al* 2001). However, plants that are actually traded or used in commercial medicine represent some 70-100 species (Sharma, 2003). Medicinal and aromatic plants (MAPs) not only have medicinal and aromatic value but also the religious and economic value. In Nepal, 150 species of medicinal plants are used for commercial trade value (Edward, 1996).

Country's richness in medicinal and aromatic plant is also revealed by the other various sources of information. Malla and Shakya (1999) compiled a list of 630 species of MAPs from Nepal; out of which 120 species are either exotic or indigenous that are cultivated since long. However about 700 species are used in traditional medicinal practice which represent about 20% of total estimated flora of Nepal. Out of the estimated 2000 drugs which have been used in the Indian sub-continent, more than 1500 are of plant origin and out of these about 700 species are used in the Nepalese traditional system of medical treatment (Singh, 1997). Out of 246 endangered species of Nepal 101 species are under endangered and eight species are under feared extinct groups (Shrestha and Joshi 2001). The aromatic plants constitute various elements of which essential oils are the important constituents used in medicine and perfumery.

1.2 *Valeriana jatamansii* Jones.

1.2.1 Description of the plant

The plant *Valeriana jatamansii* Jones. is one of the important medicinal plants of Nepal. It is found mostly at an altitude of 1200-3000 m from the sea level (Press *et. al* 2000).

Valeriana jatamansii Jones. is one of the important members of family Valerianaceae. The plants within the family Valerianaceae have been practiced as herbal medicines since long time. *Valeriana jatamansii* Jones, *V. officinalis* and *Nardostychus jatamansii* DC. have been widely used in India, China and Nepal since ancient time. This plant is important for its essential oil and valepotriates obtained from the sub teranian rhizome (Anonymus 1993).

This plant is locally known as nakalli jatamansii and commonly known as Indian valerian. It is perennial herb growing in moist and shady places with creeping rhizome. The height of plant is up to 45 cm tall with thick, horizontal, nodular and aromatic rootstock and tufted stem. Leaves are both cauline and radically. Radical leaves are more or less opposite, petiolate, heart shaped, ovate, glaborous and 3.5-5 cm by 2.5-3 cm in size. Flower is liliac and small white in color. Flowering season is March to April. The ordinary soil well supplied with moisture and farmyard manure is best preferred by the plant. Under the forest act (1993) this plant has been banned for export. However, by obtaining permission from government, its processed material can be exported (Joshi and Joshi 2000).

The cultivation of this plant for commercial purpose is less practiced. However the available literatures reveal that this plant is usually propagated by planting division of old plants. The vegetative propagation is the best over propagation by seeds. The collection of seed is done during March-April and seeds of this plant are very small. The bed should be prepared by making the soil well grinded and moist enough but water lodging should be avoided. It takes about 20-25 days for the germination of seed.

1.2.2 Properties and uses

The therapeutic uses of the roots and rhizomes of *Valeriana jatamansii* Jones. are stimulant, carminative, and antispasmodic, useful in hysteria, epilepsy and neurosis. The plant is used in hypochondriacs, nervous unrest and similar emotional state. It has remarkable influence on the cerebrospinal system. The drug decreases pain and promotes sleep. It does not possess any effects produced by narcotics, so it is especially useful for those who are suffering from nervous unrest and overstrain. It prevents the serious results of nervous strain if valerian with other simple ingredients is taken in a single dose or repeated according to the need.

It is commonly administered as tinctura valerianal ammoniata and often in association with the alkali bromides. Sometimes it is given in combination with quinine, the tonic powers of

which, are appreciably increased. Valepotrates obtained from the rhizomes have cytotoxic and antitumer activities (Becker *et al.* 1981).

Oil of valeriana is used as cholera drops to be safe from cholera. It is also used in soap and perfumery. The energentene made from the juice of fresh roots of valeriana has been recommended as a narcotic in insomania and as an anti-convulsant in epilepsy. It also has slight influence over blood circulation, slowing the heart and increasing its force. It has been used in the treatment of cardiac palpitations. Fabius Calumna for the first time in 1592 noticed valerian as a specific drug for epilepsy. In India, the dried rhizomes are used in perfumes and hair preparations and as incense (Annonymus, 2000).

1.2.3 Chemical Composition

The irridoid glycoside, valerosidatum and valepotriates etc. are the major compounds found in valerian extract. From the powdered roots and rhizomes an essential oil is extracted. The essential oil of *Valeriana jatamansii* contains L-bornyl isovalerate, valeranone, valerenal etc. as the major constituents.

Generally, a yellowish-green to brownish-yellow oil is present in the dried root. The percentage yield of essential oil is about 0.5-2% though an average yield rarely exceeds 0.8%. The variation in quantity of oil is partly due to the influence of locality. A dry, stony soil, yield a root richer in oil than one that is moist and fertile (Fierster *et al*. 1984). Oil of valerian is of complex composition, containing valerinic acid, formic acids and acetic acids. The characteristically unpleasant odour of valerian is due to the presence of isovalericnic acid. It is gradually liberated during the process of drying, being yielded by the decomposition of the chief constituent bornyl-isovalerate due to fermentation (Guenther, 1960).

The root also contains two alkalods-chatarine and valerianine, which are still under investigation

1.3 Justification of Present Study

Medicinal and aromatic plants (MAPs) are the most important and valuable assets of our country. It is due to the varied climatic conditions and geographical settings. Since the time immemorial these plants and plant products have been used for various purposes. Such plant

products are more in population but less in their biomass. For the collection of less amount of raw material, numbers of plants are to be uprooted. So, their population is depleting rapidly. Many important medicinal plants have become threatened and even some are extinct due to their habitat loss and human encroachments.

Plants have been the rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved for chemical defenses against predation or infections. Most of the plants possess one or more of the medicinal properties, viz. antibacterial, antiviral, antifungal, anticancer, laxative, sedative, cardio-tonic, diuretic and others (Parajuli *et al.,* 1998). The quality, quantity and controlled production of desired chemical substances have been possible through tissue culture technology. In a single species, there has been proven by number of evidences of the differences in the content of chemicals between the *in vitro* developed plants and *in-vivo* plants. Similarly, the chemical constituents in callus, hairy roots, leaf primordial, tender stem, rhizomes, etc. vary within the same plant species cultured *in vitro*. Thus, it is important to investigate the different stages and aspects of an *in vitro* plants and chemical constituents present in them.

The plant considered for the present investigation is *Valeriana jatamansii* Jones. The importance of this plant is due to the presence of valepotriates, epoxy iridoid esters. Valepotriate, the combination of four active components is mainly responsible for the sedating and tranquilizing effects. They include valtrate, acevaltrate, dihydrovaltrate and isovaleroxy hydroxyl isovaltrate (Fierster *et. al*., 1984). The plant is also important for religious purpose. It is used in aurvedic medicine by the traditional healers. The use of medicinal plants in traditional healing system is done without knowing the concentration of active constituents. Sometimes the crude extracts may not have right composition of drug, causing low dose or overdose problems. This unscientific use of the plant product can cause some serious health hazards and great economic loss. On the other hand this plant in many places is depleted due to the lack of proper knowledge.

Use of medicinal plants in pharmaceutical industry demands greater amount of crude material, which is very difficult to obtain naturally. Small herbs like *Valeriana jatamansii* have to suffer from over exploitation from their natural habitat. So tissue culture, an alternative ways of their mass production should be encouraged. *In vitro* multiplication of the plant parts and the hairy root production help to fulfill the demand of the plant material. In order to preserve the genetic heritage of such important medicinal plant and for rapid

production of important chemical constituents (alkaloids and secondary metabolites) the tissue culture technique has become an important tool. *In vitro* developed plants are useful for obtaining superior quality of secondary metabolites that are essential for manufacturing medicines, cosmetics etc. Such practices can be developed in Nepal too in future. Transformed hairy roots of V*aleriana officinalis var. sambucifolia* are found to contain higher concentration of the valepotriates than in normal roots. The valepotriate content in hairy root is found about four times the amount detected in the normal control roots (Granicher *et al.* 1995). The amount of valepotriates and the other secondary metabolites can be increased in the callus, cell suspension and transformed root cultures of the plant. So far, the *in vitro* propagation of Nepalese valeriana has been done by the different researchers but the chemical constituents present in successfully acclimatized plant materials and the hairy roots produced *in vitro* have not been compared with that of the plant materials collected from natural habitat. So the phytochemical screening, biological screening and the variation in essential oil composition of such materials are thought to be significant for the present study.

1.4 Objectives of the Study

Keeping the above background in consideration the following objectives have been selected for the present study.

i. To compare the phytochemical constituents present in the plant materials obtained *in-vivo* rhizomes, rhizomes of *in vitro* grown plants and the hairy roots *valeriana jatamansii* Jones.
ii. To compare the percentage yield and chemical constituents found in the essential oil from *in-vivo* rhizomes and from hairy roots produced in MS-medium supplemented with 0.5 mg. /l NAA.
iii. To study the biological screening (brine shrimp assay) of the different extracts of *V. jatamansii* Jones.

CHAPTER TWO

LITERATURE REVIEW

Stahl *et al.* (1971) made micro analytical investigation of roots and rhizomes of more than 40 species of different genera of family valereneaceae and showed that the valepotraties (iridoids) (valtrate, didrovaltrate, acevaltrate and IVHD-valtrate) are characteristic for tribe valerianeae. Valtrate is in general the main compound among the valepotriates. In the tribe patrinieae, no valepotriate could be identified.

Evan *et al.* (1979) Production of valepotrates by hairy root cultures of *Valeriana officinalis* var. sambucifolia. (Wallichi). The total valepotrate concentration in comparison with the roots of non- transformed plants, cultures in half strength of B5 medium supplemented with 2% sucrose showed the highest valepotriate content. (10.3% dry wt.). This is about 4 times the amount detected in the normal condition. The hairy roots cultured in media with low sucrose concentration showed generally larger valepotrate contents.

Choudhary (1983) worked on somatic callus tissues of *Dioscorea floribunda* and concluded that shoot tip and tuber derieved callus tissues from medium suplimented with 1 and 2 mg/l 2, 4-D can induce diosgenin production.

Furvya *et al.* (1983) reported that the combination of indole 3-butyric acid and kinetin was found to be the most effective hormonal condition for saponin production in cell suspersion culture of *Panaxginseng*.

Becker *et al.* (1984) studied the production of new valepotriates from colchicin-treated tissue culture of *Valeriana wallichii*. They isolated nine genuine valepotrates and two degradation products and their structure were elucidated by means of their 13 C-NMR spectra.

Crespo *et al.* (1988) studied the essential oil constituents of *Thymus serplloides* sps. *gradorensis*. The essential oil showed its major (y-torpinene and p- cymene).

Eugenois (1988) studied the essential oil constituents of *Riziphora taurica subsp cleoniolides* by GC-MS analysis. He found that the bulk of oil (73%) was monoterpenic ketones with major components pullegione (62.72%) and isomenthone (8.00%). 12.5% of the oil consists of hydrocarbons (7% aliphatic, 5.3% monoterpenic and 0.16% aromatic).

Jain *et al.* (1990) studied *in vitro* production of essential oil from proliferating shoots of *Rosmarinus officinalis*. The 40-day-old *in vitro* proliferating shoots of *Romarinus officinalis* L. var. *Genuine formaerectus* produced an appreciable quantity of essential oil, i.e., 1.8% fw, which was similar in its constituents to that obtained from 1-year-old plants whether naturally grown or *in vitro*-raised potted plants. The essential oil content of 1-year-old naturally grown plants was 2.4% fw, while it was 2.38% fw in the *in vitro*-raised potted plants of the same age.

Tanka and Matsumoto (1993) had obtained a clone of Ri-transformed regenerated plant of *Ajuga reptans var. tropurpurea*. The plant was derived from a hairy root line selected from more than 120 hairy root clones. It produces high growth rate in culture as well as more than four times as much 20-hydroxyedsone as the original wild grown Ajuga plant.

Boss *et al.* (1993) while working on seasonal variation of essential oil, valerenic acid derivatives and valepotriates in *V. officinalis* root found the maximum content of essential oil in September-October (<2% v/w) that of the valerinic acid in March (0.9%) and that of the valepotriates in April-May (1.3%). The lowest content were found in Dec-Jan viz. 1.3% essential oil, 0.3% valerenic acid derivatives and 0.2% valepotriates. They also concluded that for the best quality parameter for the drugs the plants should be harvested in March.

Pande *et al.* (1994) developed a simple isocratic reverse phase method for the determination of 4 methology 8-pentyl-1-naphoic acid, methyl eleosanoate, acevaltrate, isovaletoxyhydroxy didovaltrate and didovaltrate in the rhizomes of *Valeriana wallichii*. The sensitivity was found to be linear in the range of 0.2-5mg. to the best of their knowledge this is the first HPLC report for the determination of naptholic acid derivative and methyl eleosanoate in this plant.

Granicher *et al.* (1995) studied and compared the steam-distilled essential oil from the roots of 9- month. Old-field, grown *Valeriana officinalis* var. *sambucifolia* with that from Agrobacterium-mediated transformed roots of the same species. Capillary GC and GC-MS studies revealed, that the normal oil contained bornyl acetate (13.3%) and valerenal (12.4%) and the transfermed oil kessane derivatives tentatively identified as kessyl alcohol (10.5%) and kessyl acetate (10.4%), as the main constituents.

Tanka *et al.* (1995) had successfully extracted a chemical named vincamine from the multiple shoot culture derived from hairy roots, Vm-101, proliferated rapidly in *in vitro,* which displayed a high degree of lateral branching and rapid shoot elongation and had a growth index 2.5 times that of an untransformed plant.

Kaur *et al.* (1999) studied the *in vitro* propagation of *Valeriana jatamansii* Jones. The sterilized explants, shoot buds were established on solid media supplemented with benzyl amino purine (BAP) alone or in combination with IAA or NAA. The buds cultured on media supplemented with BA and IAA or NAA formed shoots which after 3-4 weeks produce roots on the same medium.

Niraula and Saiju (2000) obtained multiple shoots from young shoot tips of *Valeriana jatamansii* in MS medium supplemented with BAP (1mg/l) and NAA (0.1mg/l). 90% micro-shoots rooted on non-sterile sand and these plants were successfully established in the field.

Yoshikawa *et al.* (2000) studied about the production by transgenic tissue, i.e. hairy root cultures, which were induced by the transfer into plant cell or RI plasmid DNA in *Agrobacterium rhizogenes*. This has been expected as an effective method to produce the useful secondary metabolites, which has not been produced by the usual culture methods using callus or cell culture. The pure saponin content is produced by hairy root. The culture was almost the same as that of native root in a fresh weight and four times higher in dry weight. The effects of saponin extracted from hairy root culture were better than those of the native roots because the cultured class were purer and rich in the saponin content per dry material than those of the field cultivated root. This may contain agricultural chemicals.

Amanzaden *et al* (2002) isolated two new valeporiates from the roots of *Valeriana sisymbriifolia*. A dichloromethane extract of the roots of valeriana sisymbriifolia vahl. afforded a new valepotriate and a new valepotriatehydrine acetoxydeiso-valeroxy-1-2-acetoxy-1-2-acetoxy-isovaleroxy isvaltratehydrine together with a known compound, valtrate.

Boldyreva *et al.* (2002) while working on *Catharanthus roses* L. Don described that an ptomization of the saccharose concentration in the nutrient medium leads in enhancing the biosynthesis of alkaloids.

Fokialakis *et al.* (2002) studied essential oil constituents of *Valeriana italica* and *Valeriana tuberosa* by GC/MS analysis. Seventy-three and forty-one constituents were identified from each plant respectively. The major constituent of the oil obtained from the roots of *V. italica* was isolated and identified as 15- acetoxyvaleranone. The oil obtained from *V. tuberosa* completely lacked the characteristic *Valerane kessane* sesquiterpenes.

Janusz *et al.* (2002) isolated sequiterpene lactones from hairy root culture of *Cichorium intybus*. A transformed root culture of *Cichorium intybus* L. (Asteraceae) was found to produce sesquiterpene lactones of guainane and germacrane type. Lactucopicrin, 8-deoxylactucin and three sesquiterpene lactone glycosides; crepidiaside B, sonchuside A and ixerisoside D were isolated from the roots. The yield of 8-deoxylactucin was reported up to 0.03 g/l at the early stationary phase of the culture.

Rajyalakshmi *et al.* (2002) studied essential oil composition of four medicinal plants of family Astaraceae. They found that the major essential oil components identified broadly belong to monoterpenoids and sesquiterpenoids while sesquiterpenoids predominate these oils. Caryophylene was found to be common constituents of all the investigated species.

Tang *et al.* (2002) isolated five new iridoids from the rhizomes and roots of *Valeriana jatamansii* Jones. Structural elucidation of these iridoids based on spectroscopic data interpretation showed 1-homoaceraltrate, 1-homoisoacevaltrate, 11-homohydroxyl-dihydrovaltrate, 10-acetoxy- 1-homovaltrate hydrin and 10-acetoxy-1-acevaltrate hydrin along with ten known analogs.

Laghate and Grampurohit (2003) studied on pharmacognostical and phytochemical constituents on the roots of Butea monosperma (Lam) Taub was able to detect the presence of alkaloid, anthraquinones, glycosides, tannins, saponin, flavonoids etc.

Joel *et al.* (2003), investigated that the unique secretary tissue that were composed of closely packed unicellular hairs and located in the gaps between placenta along the fruit cavity in vanilla orchid secrete the most important flavoring agent vanillin. These cells contain enzymes that are involved in vanillin biosynthesis and seem to be responsible for vanillin biosynthesis.

Singh (2003) isolated the four compounds (i) isovaltrates (ii) didrovaltrate (iii) valtrate and (iv) acevaltrate from rhizomes of *Valeriana jatamansii* Jones. These compounds were identified by comparing with authentic samples using thin layer chromatography and acevaltrate was also identified by nuclear magnetic resonance.

Ali *et al.* (2004) studied the antimicrobial activity, essential oil composition and micromorphology of the trichomes of *Satureja laxiflora* C. Koch from Iran. The essential oil obtained from leaves during flowering period by hydrodistillation and analysed by GC-MS. Thirty three compounds representing 99.1% of the total oil were characterized. The major compounds were thymyl (63.9%) and Y-terpinene (11.9%) followed by carvacrol (4.8%), p-cymene (3.9%), geraniol (3.2%) and geranylacetate (3.1%).

Fernandze *et al.* (2004) reported the presence of a anxiolytic flavone 6-methylapigenin (MA) and of the sedative and sleep enchancing flavonone glycoside 25 (-) hesperidin (HN) in *Valeriana officianalis* and *Valeriana wallichii*. MA in turn was able to potentiate the sleep-induced properties of HN. The paper reported the identification of *V. officinalis* of the flavone glycoside linarin (LN) and the discovery that it has, like HN sedative and sleep enhancing properties that are potentiated by simultaneous administration of valerinic acid (VA).

Pavlovic *et al.* (2004) obtained an essential oil by hydrodistillation from underground parts of *Valeriana officinalis* L. growing wild on Tara Mountain (Western Serbia) and analysed by GC-MS. Fifty-three components, representing 90.7% of the oil, were identified. The main characteristic of this valerian essential oil was the presence of valerianol (57.3%) and bornyl acetate (11.3%).

Srivastav (2004) obtained 1.5% (v/w) aromatic oil with a sweet spicy characteristic odor in Karkar Singhi; a gall like structure, caused by an insect on the leaves and petioles of *Pistacia integerrima*. Chromatographic study of the oil showed that the oil is rich in α- pinene and other components; β-pinene, phalandrene, lemonene were also found.

Weadeneh *et al.* (2004) studied the essential oil content and the composition of subterranean parts of two valerian (*Valeriana officinalis*, L) cultivars select and Anthose, from certified commercial organic fields, were determined by hydrodistillation, followed by gas chromartography GC and GC-MS analysis. Eight and fourteen month old cv. select had 0.67 and 0.87% essential oil, while similar aged cv. anthose contained 0.97 and 1.1% essential oil.

Forty three and fifty three components from cv. select and cv. anthose oils were detected, respectively. The oil composition significantly varied due to the cultivar type, plant age and harvesting time. The major components for cv. select were valerenal, bornyl acetate, 15-acetoxy valeranone, valerenic acid and camphene, while cv. anthose had valerenal, (-)-bornly aceate, 2- humulene, camphene, 15-acetoxy valeranone, and valerenic acid. With further ageing of the plants, the valerenal, valerenic acid and 2- humulene contents increased.

Zhang *et al.* (2004) used engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures for scopolamine (a tropane alkaloid). They concluded that the biosynthetic pathway is an efficient approach for large-scale commercial production of scopolamine by hairy root culture system as bioreactor.

Chen *et al.* (2005) isolated 11-methoxyviburtinal a new irodold from *Valeriana jatamansii*. Five compounds of iriodids, lignan and phenyl propanoid glycosides were isolated from the roots of *Valeriana jatamansii* by column chromatography. Their structures were elucidated as (1) 11-methoxyviburtinal (2) baldrinal (3) prinsepiol-40O-B-D glucoside (4) confiero and (5) hexacosanic acid by spectroscopic analysis.

Mathela *et al.* (2005) studied the major constituents of the oils from roots and rhizomes of *Valeriana wallichii* DC. They found two types of chemotype in the chemical composition. The type I was characterized by the preserve of maaliol (64.3%), viridiflorol (7.2% and sesquiterpene hydrocarbons (19.2%). The type II contained patchouli alcohol (40.2%), viridiflorol (5.2%), 8-acetoxy-patchouli alcohol (4.5%) and sesquiterpene hydrocarbons (34.5%). Viridiflorol and 8-acetoxy-patchouli alcohol have been isolated from *V. wallichii* for the first time.

Pullela *et al.* (2005) investigated a new acylated cloinasterol glycoside from *Valeriana officinalis*. The chloroform extract of *Valeriana officinalis* led to the isolation of clionosterol -3-O-B-D glucopyronoside and a mixture of 8E, 11E - octadeadienoyl and 14-methyl pentadecanoyl by alkaline hydrolysis followed by GC-MS analysis.

Han. Mi-Kyeong (2006) studied that the plant extracts derived from *Nardostychus chinensis* rhizome exhibited complete antefreedaunt activity at 1-3 mg/cm over 30 days period against the *Attagenus unicolor japonicus*.

Larsen *et al.* (2006) studied the impact of replanting or regeneration of the medicinal plant *Nardostychus grandiflora* DC (valerianaceae). An experiment with 209 2×2 in plots on both south and north facing slopes of the valley in Gorkha district was conducted over two years. Harvesting 100% of the plants in plots followed by replanting of the tipper plant parts and 2cm of the rhizome provided the fastest regeneration and rhizome production.

Maurmann *et al.* (2006) studied the quality of valepotriates in callus, suspended cells and untransformed root culture of *Valeriana glechomifolia*. By using the different concentrations of phytohormones like 2-4 D, Kinetin and B_5 as basal medium increase the rate of valepotriate synthesis in all of its organs and parts.

Navarrete *et al.* (2006) studied the chemical finger printing of *valeriana species*; simultaneous determination of valerenic acids, flavonoids and phenylpropanoids using liquid chromatography with ultraviolet detection. Different valerian species and commercial products showed remarkable quantitative variations. Chlorogenic acid (0.2-1.2%), 3 lighans, Linarin (0.02-0.24%) and valepotrates were detected in all the valerian species analysed. Highest amount of valerenic acids were detected in *V. officinalis* L. and trace amount in *V. sitchensis*.

Yuanqiang *et al.* (2006) isolated a new iridoid glycoside, 10-150 valeryl kanokoside C (1), and a new sesquiterpene (2) together with two known compounds from the rhizomes and roots of *Valeriana fauriei*. Their structures were elucidated on the basis of spectroscopic analysis.

Pant *et al*. (2005) developed a protocol for micro propagation of *Valeriana jatamansii* Jones. by tissue culture. The shoots were acclimatized and hardened in three different media: coco pit, sand and mixture of sand and soil (1:1) by volume. The highest percentage of survival in vivo (91.6%) was achieved in coco pit flowed by sand (83.3%). The size of rhizomes seemed bigger in those plants, which were hardened in sand and then transferred into the soil. He also found that the MS medium supplemented with 0.5 mg/l NAA was the best combination for proliferation of hairy roots.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The materials used for the present investigation were rhizomes of *in vitro* and *in vivo* grown *Valeriana jatamansii* Jones. and *in vitro* hairy roots maintained in tissue culture laboratory of Central Department of Botany (CDB), Tribhuvan University (TU) Kathmandu. The rhizomes of *in vitro* grown *Valeriana jatamansii* were developed by successful acclimatization for one year in the garden of (CDB) TU. The rhizomes of *in vivo* plants were collected from Pyuthan and Gulmi districts during September 2005 and from Lalitpur district during October 2005.

3.2 Methodology

For the present investigation, Murashige and Skoog (1962) solid medium (MS) supplemented by the phytohormone (NAA - 0.5 mg/l) was used for the hairy root production.

3.2.1 Sterilization of Glass wares and Metal Instruments.

The required glass wares were dipped in detergent rich water for 24 hours and then cleaned them with bottlebrush. The glass wares were washed thoroughly with tap water and finally rinsed with distilled water. They were sterilized in hot-air oven at 160^0C for two to three hours. Forceps, knife, scalpel and gloves were wrapped in aluminum foil and then autoclaved them at 121^0 C for 30 minute at 15 lb/sq. inch pressure.

3.2.2 Preparation of stock solution for MS medium.

The different stock solutions were prepared in the given concentration by first weighing the required ingredients in an electric balance and then dissolving them completely in distilled water in the sequence of ingredients as established in the protocol. Each stock solution was kept in sterilized brown bottles with label indicating the name of preparations and date. The stock solutions were preserved in refrigerator at 4^0C.

Macronutrients: - Stock A

Chemicals	**10X (gm/liter)**

Potassium nitrate (KNO_3)	19.00
Ammonium nitrate (NH_4NO_3)	16.50
Magnesium sulphate ($MgSO_4.7H_2O$)	3.70
Potassium dihydrogen phosphate (KH_2PO_4)	1.7
Calcium chloride ($CaCl_2.2H_2O$)	4.40

Mironutrients – stock B	**1000X (mg/100ml)**
Chemicals	
Boric acid (H_3BO_3)	620
Manganese sulphate ($MnSO_4.\ 4H_2O$)	2230
Zinc sulphate ($ZnSO_4.7H_2O$)	860
Sodium molybdate ($Na_2M_0O_4.2H_2O$)	25
Copper sulphate ($CuSO_4.5H_2O$)	2.5
Cobalt chloride ($CoCl_2.6H_2O$)	2.5
Potassium iodide (KI)	83

* KI was prepared and stored separately.

Iron EDTA - Stock C

Chemicals	**100X (mg/100ml)**
Sodium ethylene diamine tetracetate (Na_2EDTA)	373
Ferrous sulphate (Feso4.$7H_2O$)	278

Vitamins - stock C

Vitamins	**1000X (mg/100ml)**
Glycine	200
Nicotinic acid	50
Pyridoxine HCl	50
Thiamin HCl	10
Myo- inositol	100

Carbon source

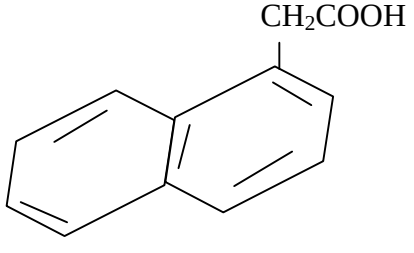
Sucrose	30 gm/liter

Solidifying agent:

Difco bacto agar	8 gm/liter

3.2.3 Hormone used for investigation

Auxin: Napthalene acetic acid. (NAA)



NAA

3.2.4 Preparation of desired hormone (NAA)

10 mg of NAA was first dissolved in 2 ml of 95 percent ethyl alcohol. Then 98 ml sterile water was added in the dissolved NAA in 2 ml ethyl alcohol resulting to 100ml final volume.

- 1 mg NAA dissolved in 1000 ml water becomes 1 mg/l NAA
- 10 mg NAA dissolved in 1000 ml water becomes 10 mg/l NAA.

Therefore, 10 mg NAA dissolved in 100 ml water became 100 mg/l NAA stock solution. It was labeled and kept in refrigerator as a stock and used when required.

3.2.5 Preparation of MS media (as the basal media)

For the preparation of one liter of MS Medium, 400ml of distilled water was taken in a clean, dry and sterilized conical flask. The water filled flask was kept on the magnetic stirrer. Then from the stock solution 100ml of macronutrient (stock -A), 1ml of micronutrient (stock B), 10ml of Iron EDTA (stock C) and 1ml of vitamins were added into the flask respectively until each of the solutions dissolved completely before adding the next in the order. Then 30gm of sucrose (3%) was dissolved little by little with the motion set by magnetic stirrer. The solution was maintained at pH 5.8 by adding 0.1N NaOH or 0.1N HCl when required. Finally additional volume of sterile water was added and the final volume was made 1000 ml. MS medium or the basal medium was made ready. As per the requirement (i.e. 0.5 mg/l NAA) the concentration of Phytohormone was prepared by using the following formula (Gamborg and Philips, 1995).

$$(v_1 = \frac{S_2 \mid V_2}{S_1})$$

Where,

V_1= Volume of hormone to be added in the given medium from the stock solution.

S_1= Concentration of hormone in the stock solution.

V_2= Volume of medium desired for investigation.

S_2= Concentration of hormone required in medium.

The prepared hormone was added in the medium and the agar power (Difco bacto agar), at the rate 8 (gm/1000 ml) solutions was dissolved in hot media which was kept in the electric heater to enhance the faster solubility of agar in the solution. The medium was dispensed in the clean and freshly sterilized culture jars (10-12 cm height and 7-9 cm diameter). They

were capped with aluminum foil and tied with durable rubber bands in order to maintain aseptic environment in the medium. The medium was labeled specifically and autoclaved at 121^0C for 15-20 min at15 psi. (Bhattarai,2000). The autoclave was allowed to cool to normal atmospheric pressure and the culture jars were taken out and kept in chamber of tissue culture laboratory. The media was observed for a week. If there is no sign of microbial contamination, the media is perfectly sterilized and thus it is used for sub-culturing the material.

3.2.6 Preparation of inoculation chamber

To ensure absolute aseptic condition of the inoculation chamber, the laminar airflow cabinet or clean bench was thoroughly cleaned with 95% ethanol. The culture tubes with media, sterilizing box containing autoclaved glass wares were cleaned by spraying 95% ethanol externally. All the necessary materials with their containers viz. spirit, ethanol, sodium hypoclorite solution, sterile distilled water, rubber bands, aluminum foils, match box, marker pen and gloves were exposed to ultra violet light for 45 min. Then the air blower was kept on during the inoculation. In the mean time, hands were washed with liquid detergent, dried and disinfected with 75 percent ethanol. Gloves were also sterilized with ethanol. Then the inoculation of explants was carried out with the continuous flow of air in the chamber. After the inoculation was completed, the culture jars were kept in the aseptic growth chamber of tissue culture laboratory.

3.2.7 Sub culture of hairy roots of *Valeriana jatamansii* Jones. on 0.5 mg/l NAA

In this culture condition MS (with 0.5 mg/l NAA) is the most ideal condition for the proliferation of hairy roots. So for the sub-culture of hairy roots on MS-medium supplemented with 0.5 mg/l NAA, the nodes of 0.5cm-1cm were obtained from in *in vitro* developed shoots. These nodes were sub-cultured on the medium in the clean bench.

3.3 Acclimatization and collection of rhizomes

The plant materials grown aseptically under *in vitro* condition were transplanted for acclimatization. As plants grown in *in vitro* condition are physiologically less developed and unable to adapt to the external environmental conditions, they need to be transferred with great care. The gradual lowering of humidity and development of functional roots in such

plant materials are the key steps to be done during acclimatization. Taking this in consideration the acclimatization of *Valeriana jatamansii* Jones was done in this study. The plant materials were taken out from the culture room and the lid of jar was opened and left for a week on the same jar. After a week the plant materials were transferred to the Petri dish, with the help of forceps, Agar was washed, the *in vitro* roots were removed and the leafy shoots were cut into small pieces of about 4-6 centimeter. These shoots of the plant were dipped in a hormone; Indole-3 acetic acid (500 mg/l) for about 1 minute. The hormone treated leafy shoots were planted on coco pit bed by making small holes and covered by white transparent plastic box. For the maintenance of humidity the plants were watered in a regular manner for eight weeks.

3.4 Transplantation of rooted plants to sand and soil mixture

After eight weeks the rooted plants were transferred to the pots having the mixture of sand and soil (1:1) and kept inside a plastic box. Initially the plants were exposed to a room atmosphere for whole day and covered by plastic box for night. The regular spraying of water and exposal of plants to room atmosphere was done for two months that made plants hardened. After hardening the plants were shifted to the garden and exposed completely to the open environment. When the plants were completely shifted to the open environment, average height of the *in vitro* grown plant was 5 cm with two shoots.

3.5 Collection of rhizomes of *in vitro* grown plants

After acclimatization for about one year, the plants developed rhizomes in the different sizes. Though they need longer period for the development of rhizomes due to the limitation of time they were collected after one-year air dried at room temperature ($\backsim 25^0$ C) for a week and powdered. The powdered material was used for phytochemical screening and partial fractionation on different polar solvents.

3.6 Collection of hairy roots

Hairy roots were produced in sterilized culture jars. When the mass of hairy roots grew well occupying almost all the media and started turning brownish from creamy white colour, the hairy roots were collected in filter paper. Then it was washed with distilled water so as to

remove media and it was air dried at room temperature ($\leq 25\ ^0C$) for a week. Then it was powdered and used for various tests.

3.7 Collection of rhizomes of *in vivo* plants

Rhizomes of *Valeriana jatamansii* Jones were collected in September 2005 from Pyuthan and Gulmi districts from their natural habitat. The plants were collected from margins and northern facing walls of croplands. Similarly, the rhizomes of the same plant were collected from the roadside of Godawari to Phulchowki hill, Lalitpur during October 2005.

3.8 Extraction of essential oil

The essential oil was extracted by hydro distillation in Clevenger's apparatus. Different samples from different locality (100 gm fw) were hydro-distilled separately in a Clevenger's apparatus using distilled water (500 ml) for 12 hours and the essential oil was collected with diethyl ether. The ether was distilled off. The essential oil thus obtained was dehydrated over anhydrous sodium sulphate. Then the essential oil was examined by gas chromatography and mass spectroscopy (GC-MS) analysis in the Institute of Engineering and Chemical Science, Singapore. For the extraction of essential oil from in-vitro hairy roots (5.8gm) powdered material was used in separate small sized Clevenger's apparatus.

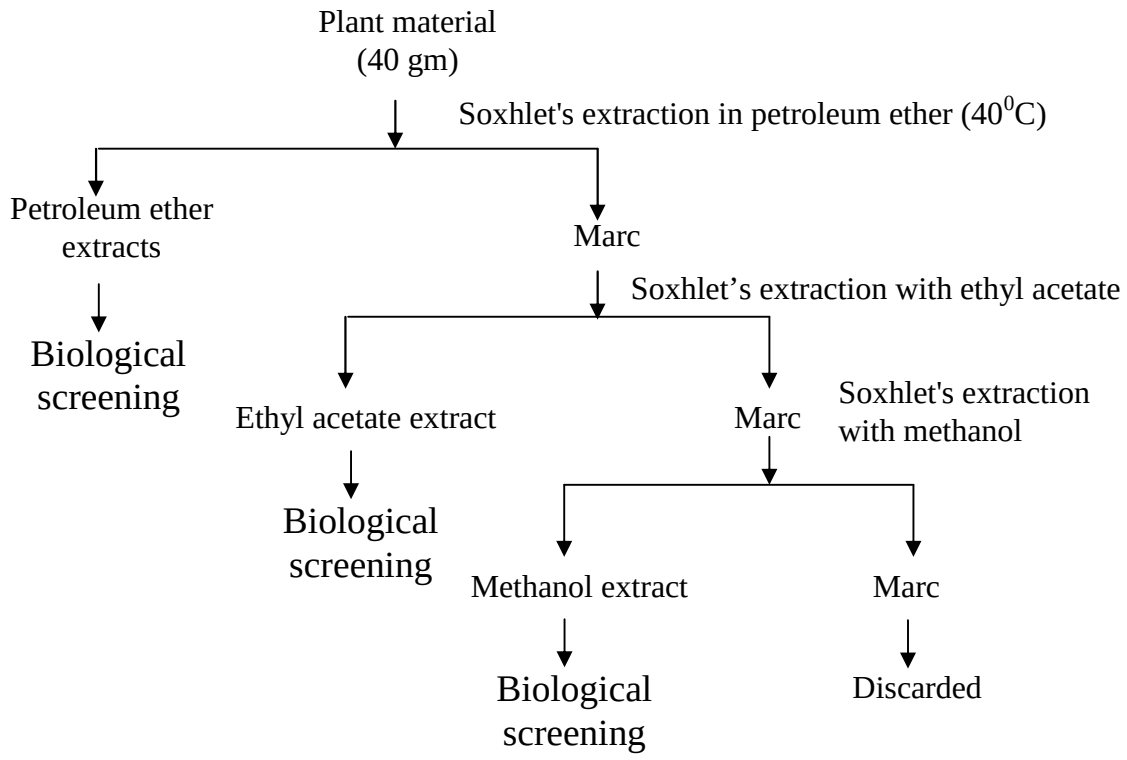
3.9 Condition for GC- MS analysis

GC-MS analysis was carried out by HUISHI method in Acq condition. Helium gas was used as carrier.and the samples were subjected for 17.15 minutes.

3.10 Partial fractionation of sample with different polar solvents

Soxhlet's extraction of the sample was done in the different solvent system in increasing order of their polarity. 40 gm of powdered material was used for extraction. The extracts of different solvents were obtained by heating the solvent. When the solvent in arm of Soxhlet's apparatus seemed transparent then heating mantle was turned off and the extracts were concentrated by distillation. Then the extracts thus obtained were used for the study of biological activities (i.e. Brine shrimp assay).

Scheme I



(20 gm-powdered materials was used for the *in vitro* grown rhizomes)

3.11 Determination of some physical parameters of essential oil

3.11.1 Specific gravity determination.

For the determination of specific gravity, an ignition tube previously cleaned and dried was weighed and its weight was determined to be 'w'. The tube was filled with oil and was weighed as W_1. The same procedure was performed using the same tube containing water and its weight was noted as W_2. Then specific gravity was calculated by using the following equation.

$$dt\,X\frac{W_1\,ZW}{W_2\,ZW}$$ At particular temperature.

3.11.2 Refractive index

Refractive index of the oil was measured by using Abbe's refractometer.

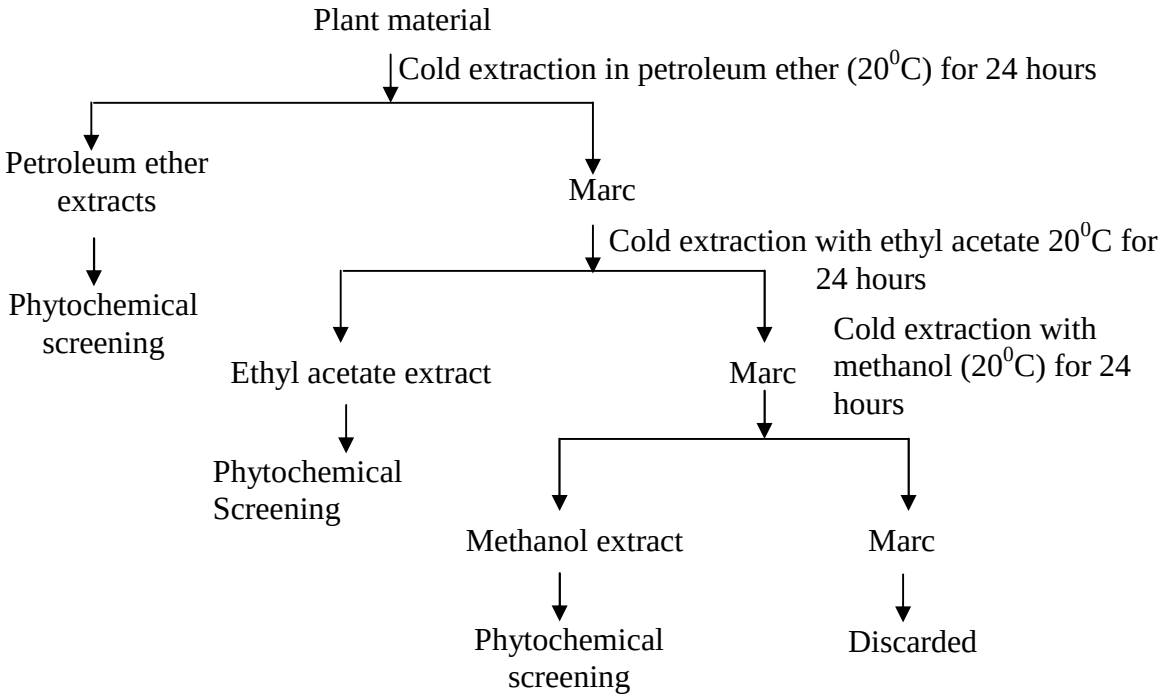
3.11.3 The organoleptic characteristics like appearance, color and aroma of essential oil were noticed after the extraction of essential oil.

3.12 Phytochemical screening

Phytochemical screening

The method employed for the phytochemical screening was mainly based on the protocol developed by Manandhar (2001). The plant materials were extracted selectively and successfully with the solvent of increasing polarity. The presence of main group of constituents in these different extractive solutions was then analyzed by using different specific reagents. Phytochemical screening was performed on *in*-vivo rhizomes collected from Lalitpur in November 2005, hairy roots cultured for one year in 0.5mg/l NAA at pH 5.8 and rhizomes of *in vitro* developed plants after acclimatization for one year.

Scheme II



Preparation of petroleum ether extract

Air-dried powdered plant material (20 gm) was extracted with petroleum ether (200 ml) in a conical flask by cold percolation for 24 hours. This extract was concentrated by simple

distillation over water both until the volume was reduced to about 20 ml. Thus concentrated petroleum extract was subjected to screening tests.

3.12.1 Screening test for petroleum ether extract

1. Test for volatile oils (spot test)

The petroleum ether extract (4 ml) was concentrated to yield a residue. To this residue methanol (1 ml) was added and shaken vigorously then filtered. Few drops of filtrate were spotted on a filter paper. A yellow spot was persistent even after evaporation indicating the presence of volatile oils.

2. Test for basic alkaloids

The petroleum ether extract (10 ml) was concentrated to yield a residue, which was dissolved in 2% (v/v) hydrochloric acid. This solution was equally divided into two test tubes.

A. Maeyer's test: The first test solution was treated with Maeyer's reagent (3 drops). No white precipitate was observed indicating the absence of basic alkaloids.

B. Dragendroff's test: The second test solution was treated with Dragendorff's reagent (3 drops). No yellow or orange color was observed thus indicating the absence of alkaloids.

C. The concentrated solution was directly spotted on TLC plate and sprayed with Dragendorff's reagent but no orange or yellow color was observed thus indicating the absence of alkaloids.

Saponification of petroleum extract

The remaining extract was re-extracted thrice with 5% (w/v) potassium hydroxide solution (5 ml) using separating funnel. The combined upper petroleum layer (A) was equally divided into two test tubes for the test numbers 3 and 4. The lower alkaline layer was acidified with concentrated hydrochloric acid until pH 3 then was re-extracted thrice with solvent ether (10 ml). Thus obtained combined upper etheric extract (B) was used for screening test numbers 5, 6, 7, 8 and 9. The lower acidic layer was then discarded.

3. Test for carotenoids

The petroleum solution A was concentrated and then treated with concentrated sulfuric acid (1 ml). Orange yellow color was developed and on long standing the solution turned into red indicating the presence of carotenoids.

4. Test for sterols and triterpenes (Liebermann-Burchard test)

The petroleum ether solution A was concentrated to yield a residue, which was dissolved in acetic anhydride (1 ml) and chloroform (1 ml). To this, concentrated sulfuric acid (1 ml) was added from the side of test tube without disturbing the solution. A violet ring at the junction of two liquids was observed and the upper layer was green in color indicating the presence of sterols.

5. Test for fatty acids

The etheric solution (B) (2 ml) was concentrated and then few drops of concentrated solution were spotted on a filter paper. A yellow spot was persistent even after evaporation indicating the presence of fatty acids.

6. Test for coumarins

The etheric solution (B) (4 ml) was concentrated to yield a residue which was dissolve din hot water (4 ml). After cooling, the solution was divided into two test tubes. The first test tube was used as control. To the second test tube 10% (v/v) ammonium hydroxide solution was added drop by drop until pH 8 and was then observed under UV light. Greenish yellow fluorescence was observed indicating the presence of coumarins.

7. Test for flavonic aglycones

The etheric solution (B) (10 ml) was concentrated to yield a residue, which was dissolved in methanol (4 ml). The methanolic solution was equally divided into two test tubes.

A. Shinoda's test: The first test solution was treated with one small spatula of magnesium powder in presence of cone hydrochloric acid (5 drops). The violet color was developed indicating the presence of flavonic aglycones.

B. Shibata's test: The second test solution was treated with one small spatula of zinc dust in presence of hydrochloric acid (5 drops). The violet color was developed indicating the presence of flavonic aglycone.

8. Test for emodins (Borntiager's test)

The etheric solution (B) (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for few minutes to separate to layers. Neither the upper etheric layer was decolorized nor the lower alkaline layer gained red color indicating the absence of emodins.

9. Test for quinones

To the etheric solution (B) (2 ml) freshly prepared ferrous sulfate solution (1 ml) and few crystals of ammonium thiocyanate were added and treated with concentrated sulfuric acid drop by drop. The deep red color was persistent indicating the presence of quinines.

3.12.2 Screening test of the methanolic extract

10. Test for polyphenols (Ferric chloride test)

The methanolic extract (1 ml) was mixed with water (1 ml). To this solution, 1% (w/v) ferric chloride solution (3 drops) was added. A greenish blue color was developed indicating the presence of polyphenols.

11. Test for reducing compounds (Fehling's test)

The methanolic extract (1 ml) was diluted with water (1 ml). To this solution, Fehling's reagent 1: 1 mixture of fehling's reagent A and B (1 ml) each was added and then the mixture was warmed over water bath for 30 minutes. A brick red precipitate was produced indicating the presence of reducing compounds.

12. Test for alkaloid salts

The methanolic extract (10 ml) was concentrated to yield a residue. To this residue 2% (v/v) hydrochloric acid (4 ml) was added and shaken vigorously then filtered. The filtrate was treated with 10% (v/v) ammonium hydroxid solution until pH 8. The solution was extracted thrice with chloroform. The upper alkaline layer was discarded. The lower combined chloroform layer was concentrated over water bath in fume hood and then 2% (v/v) hydrochloric acid (5 ml) was added. Thus obtained solution was equally divided into two test tubes.

A. Maeyer's test: The first test solution was treated with Maeyer's reagent (3 drops). No white precipitate was observed indicting the absence of alkaloid salts.

B. Dragendorff's test: The second test solution was treated with dragendorff's reagent (3 drops). No orange or yellow precipitate was obtained indicating the absence of alkaloid salts.

C. The concentrated solution was directly spotted on the TLC plate and sprayed Dragendorff's reagent. Orange color was observed on the spot indicating presence of alkaloid salts.

13. Test for glycosides

The methanolic extract (8 ml) was concentrated to half the original volume and divided into two test tubes.

A. The first solution (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (2 ml) and was shaken vigorously. A cherry red color was not developed indicating the absence of anthraquinone glycosides.

B. Molisch's test: The second test solution was treated with Molisch's reagent (5 drops) and concentrated sulphuric tube without disturbing the solution. A violet ring at the junction of the two liquids was observed and on shaking, the solution turned violet completely indicating the presence of glycosides.

14. Test for quinones

To the methanolic extract (2 ml) freshly prepared ferrous sulfate solution 1 ml and ammonium thiocyanate (few crystals) were added and treated with concentrated sulfuric acid drop wise. A persistent deep red color was observed indicating the presence of quinones.

Hydrolysis of the methanolic extract

The remaining methanolic extracts were hydrolyzed by refluxing with equal volume of 10% (v/v) hydrochloric acid for 30 minutes. After cooling the hydrolyzed extract was re-extracted thrice with solvent ether (10 ml). The lower acidic layer was used for the screening test number 15. The upper combined layer was dried over anhydrous sodium sulfate and filtered. The filtrate etheric solution was used for the screening test number 16, 17, 18 and 19.

15. Test for anthocyanosides

The red acidic layer (4 ml) was basified with sodium carbonate until basic to the litmus paper. A godrej, special gray color developed gradually but no green or blue color indicating the absence of anthocyanosides.

16. Test for anthracenosides

The etheric solution (2 ml) was treated with 25% (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for few minutes to separate two layers. A greenish yellow color in the lower alkaline layer was not observed indicating the absence of anthracenosides.

17. Test for coumarin derivatives

The etheric solution (4 ml) was concentrated to yield residue, which was dissolved in hot water (4 ml). After cooling, the solution was divided into two test tubes. The first test tube was used as control. To the second test tube, 10% (v/v) ammonium hydroxide solution was added drop by drop until pH 8 and was then observed under UV light. Yellow fluorescence in the second test tube was observed indicating the presence of coumarin derivatives.

18. Test for flavonic glycosides

The etheric solution (10 ml) was concentrated to yield a residue, which was dissolved in methanol (4 ml). The methanolic solution was equally divided into two test tubes.

A. Shinoda's test: The first test solution was treated with one small spatula of magnecium powder in presence of concentrated hydrochloric acid (5 drops). No orange or yellow color developed indicating the absence of flavonic glycosides.

B. Shibata's test: The second test solution was treated with one small spatula of zinc dust in presence of concentrated hydrochloric acid (5 drops). No yellow color was observed indicating the absence of flavonic glycosides.

19. Test for cardiac glycosides (Kedde's test)

The etheric solution (4 ml) was concentrated to yield a residue, which was dissolved in methanol (2 ml). To this solution 1% (w/v) methanolic potassium hydroxide (1 ml) and 1% (w/v) methanolic solution of 3, 5 - dinitrobenzoic acid (3 drops) were added. The mixture was warmed gently. A reddish brown color was observed instead of violet indicating the absence of cardiac glycosides.

3.12.3 Screening tests for ethyl acetate extracts

20. Test for volatile oils (Spots test)

The petroleum extract (4 ml) was concentrated to yield a residue, which was dissolved in (1 ml) methanol and shaken vigorously then filtered. Few drops of filtrate were spotted on a filter paper. A yellow spot was persistent even after evaporation indicating the presence of volatile oils.

21. Test for basic alkaloids

The petroleum extract (10 ml) was concentrated to yield a residue which was dissolved in 2% (v/v) hydrochloric acid. This solution was equally divided into two test tubes.

A. Maeyer's test: The first test solution was treated with 3 drops Maeyer's reagent. No white precipitate was observed indicating the absence of basic alkaloids.

B. Dragendorff's reagent: The second test solution was treated with 3 drops Dragendorff's reagent. No orange or yellow precipitate was observed indicating the absence of basic alkaloids.

C. The solution was directly spotted on a TLC plate and sprayed with Dragendorff's reagent. Orange a yellow spot was observed indicating the presence of basic alkalodis.

Saponification of ethyl acetate extract

The remaining petroleum extract was re-extracted thrice 5% (w/v) potassium hydroxide solution (5 ml) in a separating funnel. The combined upper petroleum layer A was equally divided into two test tubes for the screening test no. 22 and 23. The lower alkaline layer was acidified with concentrated hydrochloric acid until pH 3 then was re-extracted thrice with solvent ether 10 ml. Thus obtained combined upper etheric layer (B) was used for screening test numbers 24, 25, 26, 27 and 28. The lower acidic layer was then discarded.

22. Test for carotenoids

The petroleum solution A was concentrated and then treated with concentrated sulfuric acid (1 ml). An orange yellow color similar to the extract solution was developed which in long standing turned to red indicating the presence of carotenoids.

23. Test for sterols and triterpenes (Liebermann-Burchard test)

The petroleum solution A was concentrated to yield a residue, which was dissolved in acetic anhydride (1 ml) and chloroform (1 ml). To this solution, concentrated sulfuric acid (2 ml) was added from the side of the test tube without disturbing the solution. A violet ring at the junction of two liquids was observed and the upper layer was green in color indicating the presence of sterols.

24. Test for fatty acids

The etheric solution B (2 ml) was concentrated and then few drops of concentrated solution were spotted on a filter paper. A yellow spot was persistent even after evaporation indicating the presence of fatty acids.

25. Test for coumarins

The etheric solution B (2 ml) was concentrated to yield a residue, which was dissolved in hot water (4 ml). After cooling, the solution was divided into two test tubes. The first test tube was used as a control. To the second test tube 10% (v/v) ammonium hydroxide solution was added drop by drop until pH 8 and was then observed under UV light. Greenish yellow florescence was observed iondicating the presence of coumarins.

26. Test for flavone aglycones

The etheric solution B (10 ml) was concentrated to yield a residue, which was dissolved in methanol (4 ml). The methanolic solution was equally divided into two test tubes.

A. Shinoda's test: The first test solution was treated with one small spatula of magnesium powder in presence of concentrated hydrochloric acid (5 drops). No red, pink a violet color was developed indicating the absence of flavone aglycones.

B. Shibata's test: The second test solution was treated with one small spatula of zinc dust in presence of concentrated hydrochloric acid (5 drops). No characteristic color was developed indicating the absence of flavone aglycones.

27. Test for emodins (Borntiager's test)

The etheric solution B (2 ml) was treated with 25 % (v/v) ammonium hydroxide solution (1 ml) and was shaken vigorously. The test tube was allowed to stand for few minutes to separate two layers. Neither the upper etheric layer was decolorized nor the lower alkaline layer gained red color indicating the absence of emodins.

28. Test for quinones

To the etheric solution B (2 ml) freshly prepared ferrous sulfate solution (1 ml) and ammonium thiocyanate few crystals were added and treated with concentrated sulfuric acid drop by drop. The deep red color was persistent indicating the presence of quinines.

3.13 Biological screening

Although, phytochemistry is related to isolation and separation of chemical constituent of plant materials but modern phytochemistry should be complimented with its biological activities as well. The main aim of present investigation is to isolate biologically active constituents of plant material. Thus the biological screenings of the plant materials have been carried out so as to establish possible correlation between traditional uses and their biological activities.

3.13.1 Brine-shrimp bioassay

The method adopted for brine-shrimp bioassay is based on procedure given by Mayer *et al*. (1982). In this method the newly hatched brine-shrimp nauplii are exposed to the plant extracts. The pharmacological activities of the plant extracts are evaluated on the basis of their toxicity towards these nauplii. In this method, cytotoxicity is expressed in LC_{50}-value. Plant materials having LC_{50}-value less than 1000 are supposed to be biologically active.

3.13.2 Preparation of artificial sea water

For the preparation of artificial seawater first of all the required glass wares were cleaned and dried. Then the chemical ingredients as tabulated below were weighted carefully and dissolved in sterile distilled water simultaneously.

Chemicals	Amount to be dissolved (gm/1000 ml)
NaCl	23
Na_2SO_4	4.0
KCl	0.68
H_3PO_4	0.026
$MgCl_2.2H_2O$	1.47
$NaHCO_3$	0.196
Na_4EDTA	0.0003

3.13.3 Hatching of the shrimp

10 mg of eggs of brine-shirmp (i.e. *Artemia salina*) were kept in a breaker containing artificial seawater. After filling baker with artificial seawater and the eggs of brine shrimp the set was kept undisturbed and illuminated with table lamp for 24 hours.

3.13.4 Preparation of samples

20 mg. of the samples to be tested was dissolved in 2 ml acetone. This solution was taken as stock solution. Then 500⇑l, 50⇑l and 5⇑l samples of the plant stock solutions were transferred to total nine test tubes, three for each dose level and the final concentration was made 1000 mg/l, 100 mg/l and 10 mg/l respectively. The solvent was evaporated by standing overnight.

3.13.5 Bioassay

After complete evaporation of the solvent, few drops of dimethyl sulphoxide (DMSO) were used to dissolve the material and added 10ml of seawater. For each test levels one vial was taken as controlled vial i.e. having only 10ml seawater. Then 10 matured nauplii were transferred to the test vials and left for 24 hours. After 24 hours, by the help of dispensable pipette, the number of survivors were counted and recorded.

3.13.6 Data analysis

LC_{50}-value is log concentration for 50% i.e. dosage required to kill 50% of nauplii. It can be determined as following.

Thus,

$$\wp = \frac{xy\,Z \quad x. \quad y/n}{x^2\,Z\int \quad x\text{Å}/n}$$

Where,

x = Log z

z = Concentration of the solution in mg/l scale

y = Average survivors in three replicates

Where, n = Number of replicates

$$\Im = \frac{1}{n}\int \quad y\,Z\beta \quad x$$

Then, $\overline{X}X\ \frac{Y\,Z\alpha}{\beta}$

Where, 'Y' is constant having value of 5 for calculating LC_{50}-value.

Thus, LC_{50} = Antilog $\overline{X}$

CHAPTER FOUR

RESULTS

4.1 Extraction and quantification of essential oil

The essential oils present in the dried rhizomes and dried hairy roots of *Valeriana jatamansii* were obtained by hydro distillation method by using Clevenger's apparatus. The percentage amount of oils obtained from dried powder of rhizomes of different locality and that from hairy root were as follows:

Table 4.1: Amount of essential oil obtained

S.N.	Sample	Locality	Amount of essential oil (%)
1.	P/76	Pyuthan	0.52
2.	R/77	Gulmi	0.69
3.	Q/78	Godawari	0.57
4.	S/79	*In vitro* Hairy roots	0.80

4.2 Organoleptic properties of essential oil

The essential oils extracted from dried rhizomes of different locality and from *in vitro* grown hairy roots of *Valeriana jatamansii* Jones. had the following organoleptic properties.

Table 4.2: Organoleptic properties of essential oil

S.N.	Sample	Appearance	Color	Aroma
1.	P/76	Slightly viscous	Faint yellow	Heavy, sweet woody, spicy odour
2.	R/77	Slightly viscous	Brownish yellow	
3.	Q/78	Slightly viscous	Brownish yellow	
4.	S/79	Slightly viscous	Brownish yellow	

4.3 Physical parameters

4.3.1 Specific gravity

The specific gravity of essential oil from sample P (76) was 0.959 at 26 ^{0}C.

4.3.2 Refractive index

Refractive index of essential oil obtained from sample P (76) was 1.457 at 28 ^{0}C.

4.4 Amount of Soxhlet's extracts from different solvent

The sample used for Soxhlet's extraction P (76) (Dry powdered rhizomes from Pyuthan); 40 gm and powdered materials of rhizomes of *in vitro* developed plant T (80); 20 gm have the following percentage yield in different solvent system.

Table 4.3: Percentage yield of Soxhlet's extract

Sample	Petroleum ether extract %	Ethyl acetate extract %	Methanol extract %
P (76)	2.7	4	14
T (80)	3	4	13

4.5 Phytochemical screening of petroleum ether extracts

Phytochemical screening of the different samples in the order of increasing polarity showed that petroleum ether was appropriate solvent for the extraction of volatile oil, sterol, triterpenes, fatty acids, glycosides and quinones. Basic alkaloids were found to be absence in petroleum ether extract. The comparative study of phytochemical constituents of rhizomes collected from Pyuthan; P (76), rhizomes of *in vitro* grown plants T (80) and *in vitro* grown hairy roots S (79) showed that most of the phytochemical constituents which were present in the different extracts of sample P (76) were also present in sample T (80) and sample S (79).

Table 4.4: Phytochemical screening of petroleum ether extracts of plant collected from Pyuthan, rhizomes of in vitro grown plants and hairy roots

S.N.	Family of natural constituent	Name of test	Remark		
			Sample		
			P (76)	T (80)	S (79)
1.	Volatile oil	Spot test	+ve	+ve	+ve
2.	Basic alkaloid	a. Maeyer's test b. Dragendorff's test	-ve -ve	-ve -ve	-ve -ve
3.	Carotenoids	Sulphuric acid test	-ve	-ve	-ve
4.	Polyphenols	Ferric chloride test	-ve	-ve	-ve
5.	Sterol and triterpenes	Liebermann-Burchard's	+ve	+ve	+ve
6.	Reducing sugar	Fehling's test	+ve	+ve	+ve
7.	Glycosides	Molish test	+ve	+ve	+ve
8.	Flavone aglycones	a. Shinoda's test b. Shibata's test	-ve -ve	-ve -ve	-ve -ve
9.	Fatty acid	Spot test	+ve	+ve	+ve
10.	Emodines	Borntiager's test	-ve	-ve	-ve
11.	Quinones	Ammonium hydroxide test	+ve	+ve	+ve
12.	Coumarins	UV methods	+ve	+ve	+ve

4.6 Phytochemical screening of ethyl acetate extract.

The phytochemical screening of the ethyl acetate extractive solution showed the presence of volatile oil, carotenoid's and sterols and triterpenes respectively. Similarly the constituents like reducing sugar, glycosides, quiniones and coumarins were also detected in the ethyl acetate extracts. The basic alkaloids were not detected by Maeyer's and Dragendorff's test but when the sample was concentrated and loaded on the TLC paper and sprayed with Dragendoff's reagent, the spot appeared dark red in color indicating the presence of basic alkaloid.

Table 4.5: Phytochemical screening of ethyl acetate extracts of plants collected from Pyuthan, *in vitro* grown rhizome and hairy roots

S.N.	Family of natural constituent	Name of test	Remark		
			Sample		
			P (76)	T (80)	S (79)
1.	Volatile oil	Spot test	+ve	+ve	+ve
2.	Basic alkaloid	a. Maeyer's test b. Dragendorff's test	-ve +ve	-ve +ve	-ve +ve
3.	Carotenoids	Sulphuric acid test	+ve	+ve	+ve
4.	Polyphenols	Ferric chloride test	-ve	-ve	-ve
5.	Sterol and triterpenes	Liebermann-Burchard's	+ve	+ve	+ve
6.	Reducing sugar	Fehling's test	+ve	+ve	-ve
7.	Glycosides	Molish test	+ve	+ve	-ve
8.	Flavone aglycones	a. Shinoda's test b. Shibata's test	+ve +ve	+ve +ve	-ve -ve
9.	Fatty acid	Spot test	+ve	+ve	-ve
10.	Emodines	Borntiager's test	-ve	-ve	-ve
11.	Quinones	Ammonium hydroxide test	+ve	+ve	+ve
12.	Coumarins	UV methods	+ve	+ve	+ve

4.7 Phytochemical screening of methanol extracts

On increasing the polarity of solvent i.e. methanolic extract gave positive Fehling's test showing presence of the reducing sugars. The methanolic extract gave positive test with Dragendorff's reagent showing presence of alkaloids. Similarly glycosides, carotenoids, sterol and triterpenes and fatty acids were detected. The methanolic extracts of samples P (76) and T (80) gave positive Shinoda's and Shibata's test indicating the presence of flavone aglycones but it was not detected in sample S (79).

Table 4.6: Phytochemical Screening of Methanolic extract of plant collected from Pyuthan, rhizomes of *in vitro* grown plants and hairy roots.

S.N.	Family of natural constituent	Name of test	Remark		
			Sample		
			P (76)	T (80)	S (79)
1.	Volatile oil	Spot test	+ve	+ve	-ve
2.	Basic alkaloid	a. Maeyer's test b. Dragendorff's test	-ve +ve	-ve +ve	-ve +ve
3.	Carotenoids	Sulphuric acid test	+ve	+ve	+ve
4.	Polyphenols	Ferric chloride test	-ve	-ve	-ve
5.	Sterol and triterpenes	Liebermann-Burchard's	+ve	+ve	+ve
6.	Reducing sugar	Fehling's test	+ve	+ve	+ve
7.	Glycosides	Molish test	+ve	+ve	+ve
8.	Flavone aglycones	a. Shinoda's test b. Shibata's test	+ve +ve	-ve -ve	-ve -ve
9.	Fatty acid	Spot test	+ve	+ve	+ve

4.8 Biological screening

Biological screening (brine-shrimp bioassay) to ethyl acetate and methanolic extracts of rhizomes collected from Pyuthan P (76) and rhizomes of *in vitro* grown plants T (80) were carried out and showed the LC_{50} as shown in the table below.

Table 4.7: Biological screening of rhizomes collected from Pyuthan

Extracts	**'z'**	**Logz=x**	**y**	**xy**	**x^2**	**LC_{50}**
Ethyl acetate extract	10	1	8	8	1	69.18
	100	2	3.6	7.2	4	
	1000	3	2	6	9	
Methanolic extract	10	1	8	8	1	198
	100	2	7.6	15.2	4	
	1000	3	2	6	9	

Table 4.8: Biological screening of rhizomes *of In vitro* grown plants

Extracts	**'z'**	**Logz=x**	**y**	**xy**	**x^2**	**LC_{50}**
Ethyl acetate extract	10	1	3.5	3.5	1	0.36
	100	2	3	6	4	
	1000	3	1.5	4.5	9	
Methanolic extract	10	1	4.5	4.5	1	0.66
	100	2	2	4	4	
	1000	3	0.5	1.5	9	

From the above study it was found that the ethyl acetate extracts of both the samples have less LC_{50} value indicating more cytotoxic than the methanolic extracts. LC_{50} value of ethyl acetate extract of T (80) was found 0.36 showing more cytotoxic activity than other samples.

4.9 Chemical constituents present in the essential oil.

The GC-MS analysis of essential oils of Nepalese variety of *Valeriana jatamansii* Jones. was carried out. Four samples subjected for GC-MS analysis were the essential oil obtained from rhizomes of *Valeriana jatamansii* Jones collected from Pyuthan; sample P (76), the essential oil obtained from rhizome of *V. jatamansii* Jones collected from Godawari Lalitpur; sample R (77), essential oil obtained from the rhizomes collected from Gulmi Q (78), and the essential oil obtained from the hairy roots produced *in vitro on* Murashige and Skoog medium (1962) Supplemented with 0.5 mg/l of NAA; S (79). The name of compounds present in all four samples with their retention time are tabulated in table no 4.9.

Table 4.9: Chemical constituents present in all samples of essential oil of *Valeriana jatamansii* Jones.

S.N	Retention time (min)	Name of identified compounds
1	4-5	3- Methyl butanoic acid
2	5.4-5.43	3- Methyl pentanoic acid
3	8.20	Borneol acetate
4	9.01	β- Patchoulene
5	9.29	α- Guaene
6	9.37	α- Gurjunene
7	9.49	α- Panasinsen
8	9.54	α- Patchoulene
9	9.73	α- Bulnesen
10	10.89	Patchoulialcohol

It was found that the compound carotol with its retention time 10.10 min.was present only in sample P (76) but absent in other samples. The compound matricarin with retention time 11min.was foind in sample R (77) and in Q (78) but it was absent in sample P (76) and S (79).Similarly the compound jatamonsone with retention time 12.74 min. was found in sample R (77), Q (78) and S (79) but absent in sample P (76). The compounds patchoulinone, 1, 2-butyl octyl ester of benzene dicarboxylic acid and bis (2-ethyl hexyl) phthalate with their retention time 11.26 min and 11.72 min. respectively were present only in sample S (79).

Table 4.10: Comparative study of number of number of peaks observed and number of compounds identified

Sample	P (76)	R (77)	Q (78)	S (79)
No. of peaks observed	26	26	29	24
No. of compounds identified	12	13	13	15

4.10 Structure of identified compounds

3-methyl butanoic acid

3- methyl pentanoic acid

Borneol acetate

β-patchoulene

α-guaene

α-gurjunene

α-panasinsen

α-patchoulene

α-bulnesen

Carotol

Panchouli alcohol

Matricarin

Patchoulenone

Jatamansone

1,2- butyl octyl ester of benzene dicarboxylic acid

Bis(2-ethyl hexyl) phthalate

CHAPTER FIVE

DISCUSSION

Findings of present investigation carried on *Valeriana jatamansii* Jones. are based on the Nepalese variety of the plant species. Three parameters were considered for the investigation and the data have been discussed with the relevant information and the similar works carried out by the different investigators. Very few works has been done in Nepalese variety of *Valerians* but several works have been reported by many foreign researchers.

The quantification of essential oil showed that the highest percentage of essential oil was found in the hairy roots produced on MS-medium supplemented with 0.5 mg/l NAA for one year with repeated subculture. The percentage of oil extracted from hairy root was 0.8 % and it was followed by the rhizomes collected from Gulmi 0.69 %. The yield of essential oil by the rhizomes collected from Lalitpur was 0.57 % and that from Pyuthan was 0.52 %.

The percentage yield of essential oil was almost similar in all the samples, which was within the range of 0.5 to 0.8 percentages. Fierster *et al.* (1984) reported that the essential oil of *Valeriana jatamansii* ranges between 0.5 to 2 percentages but an average yield rarely exceeds 0.8 %. They explained that the variation in the quantity of essential oil is due to the influence of locality. A dry, stony soil yields a rhizome richer in the oil content than the one in moist and fertile soil. The percentage yield of essential oil by the different samples was very strongly supported by the findings of Fierster *et al.* (1984).

The organoleptic properties of the essential oil obtained from the different samples were slightly viscous in appearance, faint yellow to brownish yellow in color and heavy, sweet, woody and spicy in aroma. The heavy woody and the typical odor of the plant is due to the presence of isovaleric acid (3- methyl butanoic acid) which is gradually liberated during the process of drying. It is being yielded by the decomposition of chief constituent bornyl-isovalerate by fermentation (Annonymus, 2000).

The physical properties like specific gravity and refractive index were examined in essential oil obtained from the sample collected from Pyuthan; P (76). The result showed that the specific gravity of the essential oil at 26 ^{0}C was 0.959 and the refractive index at 28 ^{0}C was 1.457. These results are found similar to the result reported for the essential oil obtained from the rhizomes of *Valeriana officinalis* by Granicher *et al.* (1995). They reported that the

specific gravity at 15 ^{0}C ranges from 0.931 to 0.960 and the refractive index at 20 ^{0}C ranges from 1.4733 to 1.4975.

In order to analyze the appropriate solvent for the extraction of different class of naturally occurring compounds in the chosen species, the plant material was extracted in the solvent with low polarity followed by high polarity i.e. petroleum ether followed by ethyl acetate and finally by methanol. The quantification of extracts using Soxhlet's extractor showed the methanol extracts of P (76) and T (80) were 14% and 13% respectively. Similarly the percentage yield of ethyl acetate was 4 % in each. The yield of petroleum ether extract was 2.7 % for P (76) and 3 % for T (80). It showed that petroleum ether soluble i.e. non polar constituents were very low whereas more polar constituents i.e. soluble in ethyl acetate and methanol were high.

In the present investigation the glycosides were found present in all extracts. The presence of glycosides in Valeriana family was reported by the different researchers previously. Pullela *et al.* (2005) reported a new acylated glycoside from chloroform extractive of *Valeriana officinalis*. Similarly the phenyl glycosides were isolated from the roots of *Valeriana jatamansii* Jones by Chen *et al.* (2005).

Methanolic extracts from cell suspension cultures, calli and *in-vitro* regenerated organs of *Hypericum perforatum* were studied by Pasqua *et al.* (2003). They concluded that methanol would be good solvents for extraction of active metabolites such as flavonoids and xanthone.

In the present investigation the sample P (76) and sample S (79) gave positive test with Dragendoff's reagent showing presence of alkaloids. Though, the comparison was not made with and without using phytohormones in the basal medium, it can be predicated that the biosynthesis of basic alkaloids in *in-vitro* rhizomes and hairy roots produce in 0.5 mg/l NAA on basal medium was enhanced by the phytohormones used during *in-vitro* growth.

Furvya *et al.* (1983) while working on saponin production in cell suspension culture of *Panax ginseng* found that saponin production could be possible by the combination of IBA and kinetin.

Sedat *et al.* (1983) isolated seven forms of flavonoids in Digitalis species by extensive column and preparative layer chromatography of ethanolic extractive and identified them by comparing MP and IR with authentic samples. However, the present investigation could not be carried out to identify the different forms of flavonoids, but presence of flavonoid was recorded in all the samples P (76), sample T (80) and sample S (79).

Biological screening of the ethyl acetate and methanolic extracts of rhizomes collected from Pyuthan P (76) and the rhizomes of in-vitro grown plants T (80) showed that the ethyl acetate extracts are more cytotoxic and have less LC_{50} value than methanolic extracts. It was alsso found that the ethyl acetate extract of rhizomes of *in vitro* grown plant T (80) was more cytotoxic than ethyl acetate extract of rhizomes collected from Pyuthan P (76).

GC-MS analysis of essential oil obtained from the rhizomes of *V. jatamansii* collected from Pyuthan, Lalitpur,Gulmi and hairy roots showed the different number of peaks.the numberof peaks observed in the chromatogram and the number of compounds identified were listed in table 4.10. The chemical constituents present in the essential oil of all four samples i.e., P (76), R (77), Q (78) and S (79) have been tabulated in table 4.9

The amount of essential oil extracted from hairy roots of *Valeriana jatamansii* in the present investigation was higher in comparison to the rhizomes collected from the different locality. Jain *et al.* (1990) studied *in-vitro* production of essential oil from proliferating shoots of *Rosmarinus officinalis*. The forty-days-old *in-vitro* proliferating shoots of *Rosmarinus officinalis* produced an appreciable quantity of essential oil i.e. 1.8% f.w. which was similar its constituents to that obtained from one year-old plant whether naturally grown or *in-vitro* raised potted plants.

The study of the chemical constituents present in the essential oil present in different samples showed that 3-methyl butanoic acid, 3-methyl pentanoic acid, borneol acetate, ℘patchoulene, ℑ-guaene, ℑ-gurjunene, ℑ-panasinsen, ℑ-patchoulene, ℑ-bulnesen and patchoulialcohol in all four samples analyzed. The compound jatamonsone was found in samples R (77), Q (78) and S (79) but it was absent in sample P (76). The compound carotol was found only in the sample P (76) but it was absent in other samples.Similarly matricarin was present in samples R (77) and Q (78) but absent in samples P (76) and S (79).The compounds like patchoulinone and 1, 2-butyl octyl ester of benzene dicarboxylic acid and bis (2-ethyl hexyl) phthalate were present only in sample S (79). The compounds were identified by GC-MS analysis followed by mass library search.

It was found from the above study that twenty six peaks were observed in the chromatogram out of which twelve compounds could be identified in sample P (76). The number of peaks observed in the chromatogram of R (77) was twenty six of which thirteen compounds could be identified. Similarly out of twenty nine peaks of Q (78) thirteen compounds could be

identified and out of twenty four peaks of sample S (79), fifteen compounds could be identified.

Pavlovic *et al.* (2004) detected fifty-three components of essential oil from underground parts of *Valeriana officinalis*, which represented valerianol (57.3%) and bornylacetate (11.3%). Similarly patchouli alcohol (8.6-12.9 %) was obtained from the root and leaf oils of *Valeriana pyrlaefolia* decne by Sati *et al.* (2006).

The result obtained in the present investigation could also be compared with the result obtained by Granicher *et al.* (1995) in *Valeriana officinalis* roots oil. They found that the essential oil of *V. officinalis* contained bornyl acetate (13.3 %), valerenal (12.4%) and transferred oil kessane derivatives tentatively identified as kessyl alcohol (10.5%) and kessyl acetate (10.4 %), as the main constituents.

CHAPTER SIX

CONCLUSION

The following conclusions can be drawn from the findings of the present investigation.

1) Phytochemical screening of the different extracts of *in-vitro* and *in-vivo* rhizomes and hairy root extracts showed almost similar group of compounds. The petroleum ether was appropriate solvent for the extraction of volatile oil, sterol and triterpenes, fatty acids and glycosides. Similarly the ethyl acetate was found appropriate solvent to detect the group of compounds like volatile oil, sterol, triterpenses, reducing sugars, glycosides, quinones and coumarin derivatives. On increasing the polarity of the solvent i.e. methanol, it was found that the solvent was appropriate for the extraction of sterol, triterpenses, glycosides, quinones and coumarins. Thus the above-mentioned group of compounds could be detected from the different solvent extracts.
2) Biological screening of the ethyl acetate and methanolic extracts showed that ethyl acetate extract of rhizomes of *in-vitro* grown plants was more cytotoxic than other extracts to brine shrimp nauplii indicating more biological activities.
3) The comparative study of essential oil obtained from the *in-vitro* hairy roots and rhizomes collected from different localities showed the amount of essential oil was greater i.e. 0.8 % in the hairy roots and it was followed by the rhizomes collected from Gulmi 0.69 %. The amount of essential oil was minimum i.e. 0.52 % in the rhizomes collected from Pyuthan.
4) GC-MS analysis of the essential oil of different samples of *Valeriana jatamansii* showed almost similar chemical constituents. Among the identified compounds 3-methyl butanoic acid (isovaleric acid), 3-methyl pentanoic acid, borneol acetate, ℘ patchoulene, ℑ-patchoulene, ℑ-gurjunene, ℑ-panasinsen, ℑ-pachoulene, ℑ-guaene, ℑ-gurjunene, ℑ-bulnesen and patchoulialcohol were the common constituents of essential oil of *Valeriana jatamansii* Jones. Patchoulinone, 1, 2-butyl octyl ester of benzene dicarboxylic acid and bis (2-ethyl hexyl) phthalate were present only in the essential oil obtained from hairy roots.

CHAPTER SEVEN

RECOMMENDATION

The following recommendations can be drawn from the above study.

1. As the plant used for the present investigation is threatened herb and its rhizomes are over exploited in nature, mass propagation and the suitable cultural conditions should be developed.
2. As the plant materials collected from little dry and stony soil yield more amount of essential oil than the plant collected from moist and soft soil; it is necessary to select the plant from its natural habitat if a work from biochemical perspective needs to be carried out.
3. To supply the sufficient amount of raw materials for the industry and production of secondary metabolites using tissue culture is expected to give promising results. So it is recommended to carry out complete phytochemical screening of the hairy roots, and rhizomes to monitor the biosynthesis of desired constituents.
4. Biosynthesis of secondary metabolites is influenced by the different plant hormones used during tissue culture. So further study should be carried out to select the most appropriate combination of hormones.
5. As ethyl acetate extract of rhizomes of *in vitro* grown plants is more toxic to brine shrimp nauplii, the biological activity of *in vitro* grown plants should be studied in detail.

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PHOTO PLATES

Fig 01: Plant in its natural habitat

Fig 02: Fully grow *in vitro* plant material

Fig 03: Plant material ready for acclimatization

Fig 04: Rooting of in vitro plant material in coco pit bed

Fig 05: Rooted plantlet in coco pit

Fig 06 :*In vitro* acclimatized plan

Fig 07: Rhizome of acclimatized plant.

Fig 08: Successfully acclimatized plant with inflorescence.

Fig 09:Hairy roots produced in 0.5mg/l NAA.

Fig 10: Extraction of essential oil Clevenger's apparatus (Hydro distillation process).

Fig 11: Essential oils extracted from different samples.

Fig 12: Extraction of the plant material by Soxh let's extractor.

Fig 13:Concentration of the Soxh let's extractives by Rotatory Evaporator.

Fig 14: Biological screening of plant material in brine shrimp nauplii.

Fig 01

Fig 02

FIG 03

FIG 04

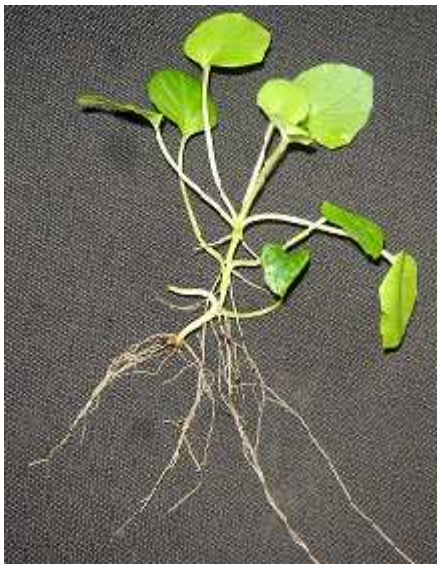

Fig 05

Fig 06

FIG 07 **FIG 08**

FIG 09

Fig 10

Fig 11

Fig12

FIG 13

FIG 14