EVALUATION AND COMAPRISON OF ANTIOXIDANT ACTIVITY IN *DIPLAZIUM ESCULENTUM*

AND

EVALUATION OF ANTIOXIDANT ACTIVITY IN *RHODODENDRON ARBOREUM*

Project report submitted in fulfillment of the requirement for the degree of

B.TECH IN BIOTECHNOLOGY

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DECLARATION BY THE SCHOLAR

We hereby declare that the work in the B.tech thesis entitled **"Evaluation and comparison of antioxidant activity in *Diplazium esculentum* and Evaluation of antioxidant activity in *Rhododendron arboreum***" submitted at **Jaypee university of Information Technology, Waknaghat , Solan (H.P**) is an authentic record of our work, We have not submitted this work elsewhere for any other degree or diploma. We are fully responsible for the contents of our B.tech project.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the B.tech project entitled **"Evaluation and comparison of antioxidant activity in *Diplazium esculentum* and Evaluation of antioxidant activity in *Rhododendron arboreum*"** is a bonafide record of their original work carried out under my supervision, This work has not been submitted elsewhere for any other degree or diploma.

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CHAPTER 1

INTRODUCTION

1.1 Traditional use of plants for medicines

As traditional medicine, the pteridophytes which contains ferns and ferns allies, have been known to humans for more than 2000 years, and also been mentioned in ancient literature [1].Ferns are viewed as starvation sustenance and utilized by tribal in different regions of the world. Tribal and rural individuals in different parts of India rely heavily on herbal medicine to satisfy their health needs [2]. The World Health Organization (WHO) assesses that 80% of the world's population depend mostly upon conventional medications. The different bioactive molecule present in ferns makes them a good source for making the medicine. Ferns are the influential biochemical factories and have been part of phytomedicine since ancient times [3]. Indian literature also revealed that many of ferns have been used for treatment of various infections. Therefore, it becomes basic to start pressing ventures for screening of plants for secondary metabolites or phytochemichals. Photochemical are mostly recognized as secondary metabolites of which there are several classes containing alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids. These substances serve as molecules of plant defense against predation by microorganisms, insects and herbivores and at the same time also exhibit medicinal properties for treating several ailments. The plants phytochemicals help to remove the oxygen reactive species or free radicals that are produced in the body. The ROS readily react with organic molecule such as lipids, enzymes; proteins and cause tissue injury [6]. Due to unbalance of the ROS production and antioxidant defenses oxidative stress develops. Free radicals are produced due to the oxidative stress which may cause various diseases such as cancer atherosclerosis, diabetes, aging and neurodegenerative diseases [7]. Phenolic compounds have an attribute of donating hydrogen due to which these undergo redox reaction with free radicals. Free radicals take the proton and gets stabilized [8]. Antioxidants can be used to prevent the accumulation of the ROS species and can be used for treatment of these diseases [9]. Therefore, there has always been a particular interest in finding the natural antioxidant from the plant material. Due to the various bioactive molecules present in plants there has been a growing interest in exploiting the active principles of flora due to their natural origin, cost effectiveness and lesser side effects [5]. Infectious diseases are the world's driving reason for unexpected losses. Therefore, there is a constant need to discover new antimicrobial compounds with various chemical structures and mechanisms of action. Numbers of researchers are investigating plants for the antimicrobial activity, worldwide [10]. In recent

years the various plants are evaluated for the antibacterial activity for different diseases and it has also been proved that various plant extract possesses bacteriostatic and bactericidal effects. The presence of antimicrobial action in a specific part of a specific species might be because of the presence of at least one bioactive compound, for example, alkaloids, glycosides, flavonoids, steroids, saponins etc. In recent times, a number of plants have been reported for antimicrobial properties across the world [11].

1.2 Overview of Plants used in present study

This study is focused on fern and shrub that are easily available and widely used vegetable in Himachal Pradesh, India namely *Diplazium esculentum* and *Rhododendron arboreum*. *Diplazium esculentum* is a pteridophyte from the family Anthyriaceae. This is considered as high economic value fern. The dried rhizomes can be used as insecticides and decoction to aid cough and haemoptysis. Besides consuming as vegetable, *Diplazium esculentum* is used in traditional medicine in diseases like fever, dermatitis, indigestion, measles, glandular dwellings, dysentery, diarrhoea and various skin infections The fern has different phytochemicals like flavones, triterpenoids ,steroids, flavonoids phenols such as alpha tocopherol and myricetin[12][13]. Pharmacological properties that are shown by the plants in previous studies are laxative [18], antioxidant [15], anti-inflammatory[14] [15], anthelmintic[16] and antimicrobial[17].

Figure1.1: Young fronds of *Diplazium esculentum*

Rhododendron arboreum is one of the most beautiful and amazing *rhododendron* species. It is commonly known as burans or gurans in Nepal. The tastefully engaging blooms owe its religious hugeness; it is viewed as consecrated and offered in sanctuaries for beautification and decoration purposes. This plant has the Guinness Record for World's Largest *Rhododendron* and is broadly well known for its therapeutic benefits and monetary esteem. In hilly areas, *Rhododendron arboreum* flowers are used for producing jellies, jams as well as local brew. Flower of this plant are used for treatment of various diseases like diarrhoea, dysentery and dyspepsia [20].The fresh and dried corolla, are used to remove fish bones that get stuck in the gullet. The dried twigs and wood in the wake of squashing blended with tea and are utilized by the general population of Bikeybhanjan and Kalapokhri regions of Darjeeling slopes in treating perpetual fevers. When the flowers are eaten in abundance they are known to cause the intoxication. *R. arboreum* is further detailed to be hostile to tubercular properties [21] and is a genuine hazard to domesticated animals. The various parts of this shrub contain various phytochemicals like Quercetin-3-rhamnoside, glycosides, tannins, saponins, alkaloids etc. This plant shows various pharmacological properties like Anti-inflammatory [23] and Anti-nociceptive activity, Hepatic protective activity, Anti-diabetic activity, Anti-diarrhoeal activity [22], Antioxidant activity.

Figure1.2. Flower of Rhododendron arborium

1.3 Objectives of the work

1. Photochemical screening and evaluation of antioxidant and antimicrobial activity in *Diplazium esculentum* collected from two different regions of Himachal Pradesh i.e. Kotkhai and Karsog.
2. To compare the plant collected from two different regions in order to see the regional differences in the antioxidant potential and the nutritional component of the plant.
3. Phytochemical screening of *Rhododendron arboreum* collected from the Himachal Pradesh i.e. Chail and evaluation of antioxidant potential in flower and leaves of the *Rhododendron arboreum.*
4. Comparing the antioxidant potential in flower and leaves of the *Rhododendron arboreum.*

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

Human society has been using plants as a source of medicine since the medieval era. The earliest records show that around 1000 plants and plant-derived substances were used during 2600 BC as medicines in Mesopotamia [64]. India has a tradition of using spices and medicinal herbs. In ancient India there are records of usage of different plants in Veda and Ayurveda for treating various diseases. Although conventional medicinal practitioners have long been using medicinal plants to treat several diseases, but there has always been debate among scientist about the pharmacological properties. The vast majority of plants still need to be examined for phytochemicals and pharmacological effects. Many herbal medicines are said to have a positive impact that helps the body in maintaining health. It may therefore be sensible to assume that there is some immune response associated with this impact [25]. From early 1970s, researchers became more aware of the significance of structurally different compounds that were not directly involved in plant metabolism [26]. Phytochemicals, huge numbers of which get accumulated in high concentrations in plants were considered as waste products of plant metabolism. We currently realize that these compounds shield plants from consumers and pest, and they fill in as attractants for pollinators and seed scattering creatures, as allopathic agents, and as channels for UV radiation. They likewise help plants to recoup from damage, endure and adjust.

At high concentrations, secondary compounds can antagonistically influence the cellular and metabolic processes, diminish scavenge activity, and cause weight reduction and even death. Therefore, at the appropriate concentration, secondary compounds have valuable effects on herbivores. The dual action of phytochemicals i.e. toxin/medicine is entirely a matter of the dosage and result of animal tolerance and current physiological state.

2.2 Description of plants used in the present study

2.2.1 *Diplazium esculentum*: Distribution, Natural Occurrence and Brief morphological description

Diplazium esculentum is a palatable plant, pan tropical in circulation and happens generally and regularly all through India, China, Cambodia, Laos, Thailand, Vietnam and Malaysia. It develops in gregarious provinces in open damp regions, stream banks and waterways from ocean level to 2,300 m. The rhizome is erect, frequently framing a thin inclining

dark trunk to 1 m tall, flaky at the summit. Scales are 1 cm long, dim dark coloured, edges finely toothed, and zenith long-taper. Fronds 1– 2 m long, 0.5– 1 m wide, erect to arcuate. Stipe dark and layered at the base, paler above optional pinnae variable in size 1.5– 2.5 cm wide ordinarily, veins are basic or forked, 5– 8 cm long, edges in all respects shallowly lobed, Lamina 2– 3-pinnate0.5– 1 m wide, dull green, 0.5– 1.5 m long, basal projections longer than the rest, flaps are toothed, globous underneath, most reduced 3– 5 sets of neighbouring vein bunches. Sori spreading along most veins; indusium slender, dull dark colour, edges getting to be uneven with age.

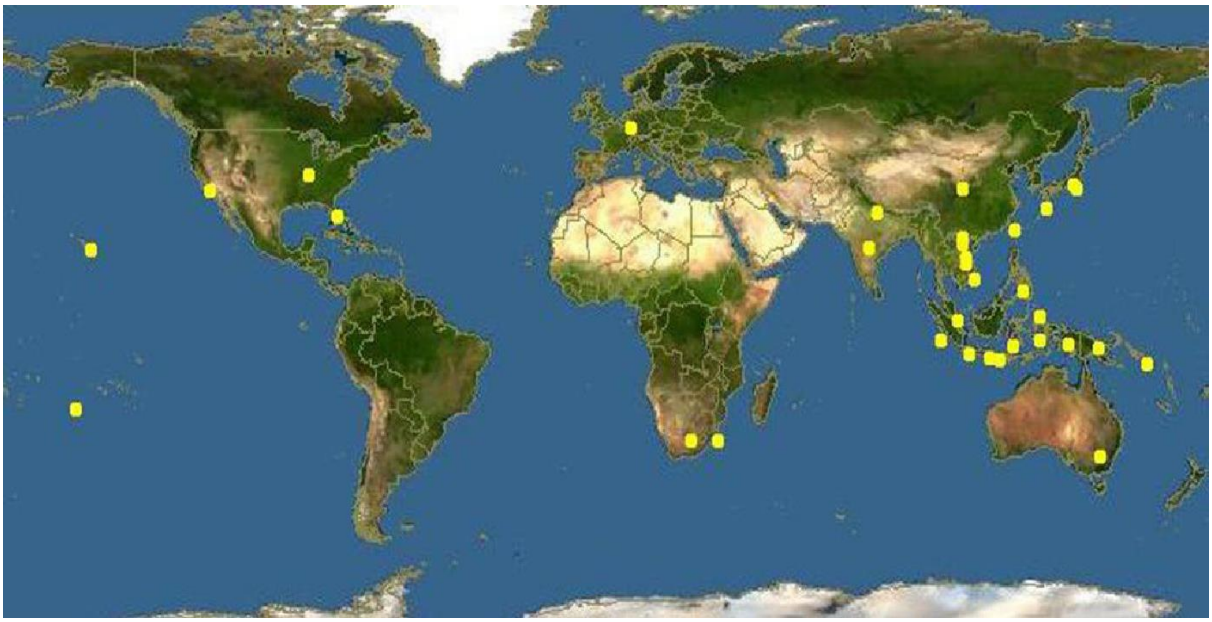

Figure 2.1 Distribution of *Diplazium esculentum* throughout the world (Image adapted from Discover Life, Designed by The Polistes Corporation)

Figure 2.2 *Diplazium esculentum* in its natural habitat and collected frond (the main edible part)

Table 2.1 Taxonomic classification of *Diplazium esculentum*

Kingdom	Plantae
Phylum	Pteridophyte
Class	Polypodiopsida
Order	Polypodiales
Family	Athyriaceae
Genus	*Diplazium*
Species	*Esculentum*

Common uses of *Diplazium esculentum*

1. **Food**

Diplazium esculentum is a standout amongst the most well-known fern and the most regularly fern all through Oceania and Asia. In India, new ferns of *Diplazium esculentum* are prevalently recognised as Lungru in Northern India, Lochanchand Rukjain North Eastern India and dhekisak in West Bengal, India. The freshly emerging ferns are eaten after cooking as a seasonal delicacy during season of monsoon which proceeds for right around five months. The most widely recognized formula utilizing *Diplazium esculentum* includes cooking the dried fronds in margarine or oil, utilizing them in a vegetable curry is less liked. In the north-eastern

India, particularly in Sikkim, and in the North-western and central Himalayan states, Uttarakhand and Himachal Pradesh, the people relish the pickles and vegetables from *Diplazium esculentum*. Locals consider these formulas compelling both to counteract constipation and as an appetizer, especially as "Achaar" (FAO, 2010).

2. Ethno-medicine

Diplazium esculentum frond is used as plant medicine for constipation and indigestion, and sometimes to cure skin ailments in the Apatani and Nyishi tribe of Arunachal Pradesh, India. Study conducted in the villages of the Parvati valley, Himachal, India revealed that, of 50 consumed wild edibles, *Diplazium esculentum* is used as a vegetable/pickle by an average of 66 per cent of the inhabitants.

3. Brief Description of the pharmacological reports of *Diplazium esculentum*

In spite of the fact that there are a few writings on the ethnobotanical and ethnomedicinal investigations of this edible fern, just couple of studies has been done on its pharmacological properties. The epidemiological investigations of this plant have not yet been endeavoured. The investigations done so far on this fern were for the beneficial effects either in vitro or in vivo in little laboratory animals. Not many scientists have concentrated on its health impacts.

4. Beneficial health effects of *Diplazium esculentum*

The dietary substance and phytochemical composition of *Diplazium esculentum* of Philippines have been examined previously [35]. They likewise evaluated the absolute phenolic and flavonoid substance to additionally evaluate the medical advantages of the plant species and its potential bioactivities. They demonstrated that *Diplazium esculentum* is high in inorganic minerals (fiery debris content). Moreover, the previous results demonstrated that *Diplazium esculentum* is high in fiber and protein substance. Researchers demonstrated that the chloroform and methanol concentrate of *Diplazium esculentum* shows antioxidants, antimicrobial and cytotoxic activity. Utilizing the Folin-Ciocalteu strategy, the total phenolic substance were 125.60 ± 13.44 and 11.65 ± 0.87 mg gallic acid equivalents per 100 g air-dried sample for the ethanolic and aqueous concentrates, separately. The comparing absolute flavonoid substance were 110.81 ± 11.16 and 16.21 ± 0.72 mg quercetin equivalents parts per 100 g air-dried example for the aqueous and ethanolic extracts, respectively [35]. Different dissolvable

concentrates of *Diplazium esculentum* have additionally been appeared to have anti-inflammatory activity which was screened by estimating the decrease in carrageenan instigated rear paw edema in rodents [36]. It has been demonstrated that the fluid leaf concentrate of *Diplazium esculentum* fundamentally expanded the locomotor action in mouse. *Diplazium esculentum* has been considered for its CNS stimulant movement and it was found that the aqueous concentrate of this plant animate the CNS work in mouse [36]. The DPPH radical searching limit was low contrasted with the standard. Study performed in Lag valley of Kullu district, Himachal Pradesh, India additionally revealed the presence of moderate level of quercetin in *Diplazium esculentum* samples [37].

The antimicrobial study was conducted by the researchers that revaled the antimicrobial susceptibity test shown by *Diplazium esculentum* towards different bacteria as *Vibrio cholerae* (10.67 mm)< *Staphylococcus aureus* (11.33mm)< *Escherichia coli* (12.33 mm)< *Shigella boydii* (14.67 mm)<*Klebsiella pneumoniae* (15.33 mm) < *Bacillus subtilis* (15.33 mm)< *Salmonella typhimurium* (16.33 mm) < *Sarcina lutea* (18.67 mm). Minimum Inhibitory Concentration estimations of DEM and DECH were between 1.6 - 12.5 mg/ml. The most reduced minimum inhibitory concentration esteem was 1.6 mg/ml appeared by DEM against *Bacillus subtillis* and *Salmonella typhimurium*. The biggest minimum inhibitory concentration esteem was 12.5 mg/ml appeared by the two concentrates against *Klebsiella pneumoniae*. The consequences of the examination recommend that the leaves of *Diplazium esculentum* have critical antioxidant, cytotoxic and antimicrobial properties [34].

5. Adverse effects of *Diplazium esculentum* on human and animal health.

Study unfolds that rodents and guinea pigs nourished for 30 days with solidified and shade dried *D.esculentum* demonstrated poor development, expanded unconstrained (vertical and flat) and diminished constrained motor movement [37]. Hematological examinations in rodents and guinea pigs indicated critical adjustments in estimations of blood glucose and leukocyte tally. Serum organic chemistry uncovered increment SGOT level in rodents just as in guinea pigs and lessening in other blood parameters in the two types of these creatures. Tissue biochemistry of visceral organs revealed increment in lipase and SDH [37]. Mortality was increased as 53% guinea pigs that had been nourished with freezed plant material were died. Huge changes were found in the weight of certain instinctive organs like mind, lungs and liver in these dead guinea

pigs. Freeze-and shade -dried samples of *Diplazium esculentum* indicated lack of plant toxin ptaquiloside but existence of 10.95 to 16.35 mg/kg pterosin B just in 2 of the freezed dehydrated samples by HPLC technique. The results demonstrate that *Diplazium esculentum* caused gentle pathologic impacts in rodents while sustaining of freezed *Diplazium esculentum* incited mortality and moderate kind of clinic pathological impacts in guinea pigs [37]. The young fronds of *Diplazium esculentum*, gathered from Harsil, Gangotri, and Uttarkashi of northern India were freeze dried and investigated for the presence of ptaquiloside (Pta) by LC-MS. The investigation revealed the presence of 19 mg/kg of Pta in these examples (Ptaquiloside).

2.2.2 *Rhododendron arboreum*: Distribution, Natural Occurrence and Brief morphological description

The plant remains green throughout the year and is spread in China, Bhutan, India, Pakistan and Nepal. In India *Rhododendron arboreum* is known as the state tree of Uttarakhand and flowers are state flower of Nagaland. It is Nepal's national flower. Another species of *Rhododendron* i.e. *Rhododendron campanulatum* is known as state flower of Himachal Pradesh. In the Himalayan regions of India, the distribution of this tree covers from Kashmir to Western Ghats and Nagaland. It grows at the height of 4550 to 10,550 feet (above sea level) [39]. The *R.aeboreum* can attain a height of 13 to 16 m but occasionally it can attain over 28 m of height. The *R.arboreum* tree has been honored in the Guinness Book of World Records as the reports suggested that it was the largest Rhododendron. *Rhododendron arboreum* is recognized for its flowers that are dark crimson/red which occur in the months of April to September. The flowers of *R. arboreum* are believed to be holy and are presented to the gods in temples. Several parts of the plants (Flowers, leaves and roots) are having medicinal attributes and are used by the locals in the treatment against several illnesses in modern and traditional systems of medication [43].

Table 2.2 Vernacular names of *Rhododendron arboreum*

Nepal	Laligurans
Sanskrit	Kurvak
Garhwal	Burans
Kumaoun	Eras
Punjab	Adrawal
Malyalam	Kuttupoovarasu
Tamil	Pu
Kannada	Billi

Table 2.3 Taxonomic classification of the *Rhododendron arboreum*

Kingdom	Plantae
Phylum	Magnolliophyta
Class	Angiospermae
Order	Ericales
Family	Ericaceae
Genus	Rhododendron
Species	R.arboreum

2.2.2.1 Different uses, phytochemisrty, pharmacological effect of Rhododendron arboreum

1. Traditional uses

Several parts of the *R. arboreum* are mentioned to have diverse therapeutic effects and are used in the recovery of numerous ailments as it has been declared in conventional way of drug treatments. According to Homeopathic Materia Medica the leaves had been customarily used for treatment of rheumatism and gout [55]. Ashoka Aristha, an Ayurveda composition contains *Rhododendron arboreum* that's reckoned to prevent action of estrogenic, prostaglandin

synthetase and oxytocic synthetase. The flowers of *R.arboreum* are efficient against blood dysentery as well as diarrhoea [38].

2. Phytochemistry

Some researchers had pronounced the presence of pectolinarigenin 7-Orutinoside,[1-6]-O-alpha-L-rhamnopyranoside 7, 2'-dimethoxy-four', five-methylenedioxyflavanonequercetin 3-O- beta –D-glucopyranosyl in leaves of *Rhododendron arboreum* [56]. Some researchers had quantified the quantity of rutin, coumaric acid and quercetin by usage of HPTLC in blooms of *Rhododendron arboreum* accumulated from the regions of Western Himalayas [54]. Methanolic leaves extract of *Rhododendron arboreum* accumulated from the regions of Western Himalayas have indicated the existence of syringic acid, quercitrin, epicatechin and quercetin-3-o-galactoside by means of the HPTLC. Researchers have recognized the presence of tannin, flavonoid, terpenoid and steroid in flower; anthraquinones, flavonoid, steroids terpenoids, and tannins in *Rhododendron arboreum* leaves; tannins, alkaloids, terpenoids, steroids and saponin reducing sugars in root, stem and bark of R. arboreum by way of initial phytochemical analysis. *Rhododendron arboreum* flowers amassed from (Western Ghats) have been recorded for the existence of proteins, saponins, carbohydrates, steroids, coumarins, xanthoproteins and tannins in distinct extracts [52] .The outcomes confirmed that saponins are there in chloroform , benzene, and ether- petroleum extract; proteins are found in ethanolic and chloroform extract; In ethanolic and benzene extract steroids were obtained; In ethanolic ,acetone and water extract tannins are obtained; xantho proteins exist alone in extract made in acetone; In chloroform, acetone and ether -petroleum extract coumarins were found, and in benzene, chloroform, ethanolic and ether-petroleum extract carbohydrates are found . Researchers also stated the presence of hyperin (0.18%) within the flower extract of *Rhododendron arboreum* by means of High-power thin layer chromatography evaluation. Some researchers organized wooden extracts of *Rhododendron arboreum* by way of two solvents ethanol: benzene (1:1) and acetone and similarly diagnosed several UFA and SFA in them. In ethanol: benzene (1:1) extract pentanoic acid, methyl butanoate, butanoic acid and methyl ester had been recognized as SFA, whereas 5-heptenoic acid,methyl ester and three-heptenocacid methyl ester were reported as UFA. The acetone and wood extract of *Rhododenedron arboreum* butanoic acid, methyl butanoate, pentanoic acid and methyl ester 4-methyl-methyl ester have been reported as SFA. 8-nonynoic acid methyl esters

and 4-heptenoic acid methyl ester have been recognized. Some researchers have reported the existence of quercetin in flower made extract of *Rhododendron arboreum* [33]. People have reported the existence of flavonoids, gallic acid, quercetin and phenols in the leaves of *Rhododendron arboreum* accumulated from Arunanchal Pradesh. Catcechol and Pyrogoll are recognized in bark, leaf and flower of *Rhododendron arboreum* by initial phytochemical study [54]. Researchers have recorded the existence of β-sitosterol, lupeol and urosolic acid in the flowers and leaves accumulated from Garhwal, Shillong and Assam region [46].

3. Pharmacology reports

1. Antioxidant activity

The antioxidant potential of aqueous-methanolic (1:1) leaf extract of *Rhododendron arboreum* was done with the help of oxidation of linoleic acid coupled reaction and beta carotene were calculated to be 54.2%. TFC came out to be 57.3 mg gallic acid equivalent per gram and 43.8 mg quercetin equivalent per gram. The 1,1-diphenyl-2-picrylhydrazyl assay show that radical scavenging activity (IC50-0.47) of leaf concentrate of *Rhododendron arboreum*. Researchers had contemplated that ethanolic concentrate of *Rhododendron arboreum* had adaptogenic property; the action of ethanolic extract is considered in mice model [43]. Antioxidant activities were assessed through superoxide radical scavenging assay, hydroxyl radical scavenging test and lipid peroxidation measure. The half maximal effective concentration (EC50) values for hydroxyl radical were examined to be 280µg/ml, 41.7µg/ml and 260µg/ml, individually for heated water, ethanolic extracts and cold water, separately. The EC_{50} estimations for superoxide radical searching measure were determined to be 239 µg/ml, 45.6µg/ml and 286.3µg/ml for high temp water, ethanolic flowers and cold-water separates, individually. The TFC was seen to be greatest in methanolic extract (0.0652 mg quercetin g-1 of dry plant material) trailed by hydro (0.0570 mg quercetin/g of dry plant material) and aqueous-ethanolic extracts (0.0449 mg quercetin/g of dry plant material). The maximum phenolic content was seen in aqueous extract (0.0964 mg tannic acid/g of dry plant material) that in by hydro-ethanolic (0.0961 mg tannic corrosive/g of dry plant material). Roy et al., (2014) [48] additionally assessed the in vivo adaptogenic action of different concentrates of leaves of *Rhododendron arboreum*. Researchers explored the radical scavenging activity of, methanolic, acetone, oil ether, and chloroform leaf concentrates of *Rhododendron arboreum*. In1, 1-diphenyl-2-picrylhydrazylassay

the highest antioxidant activity was seen in methanol (19.70%) at 500µg/ml of concentration pursued by acetone (78.98%), chloroform (42.83%) and oil ether (7.89%) extract concentration of 500µg/ml.

2. **Anti-diabetic activity**

Researchers found a detailed in vivo anti-diabetic action of the flower concentrate in hydro-methanol extract of *Rhododendron arboreum*. Concentrate has been found to restrain the α-glucisidase that is present in intestine of rat. Furthermore, aqueous-methanol extract fraction, water and ethyl acetate was tested against anti-diabetic activity which showed that the two parts may hinder the fraction of α-glucisidase yet ethyl acetate indicated higher action [53].

3. **Immunomodulatory action**

Researchers showed the immunomodulatory property of alcoholic leaf concentrate of *Rhododendron arboreum* in swiss pale skinned mice. The extract appeared to be critical immunomodulatory action at 100 mg /kg of fixation and the research recommended alcoholic concentrate of *Rhododendron arboreum* was viable, safe immune suppressant [33].

4. **Anti-diarrheal action**

Researchers found that ethyl acetate extract of *Rhododendron arboreum* flower has anti-diarrheal property. The action against diarrhoea was considered on an animal model and orally the extract was used at concentrations of 400 mg/kg, 200 mg/kg and 100 mg /kg body weight. The result showed that anti-diarrheal activity against diarrhoea induced by magnesium sulphate and castor oil was there in ethyl acetate extract of the flower.

2.3 Photochemical present in Plants

Phytochemicals are chemical compounds formed in the plant's primary metabolic pathways. These are often known as secondary metabolites of which there are a few groups including steroids, terpenoids, catecholamines, alkaloids, tannins, saponins, anthraquinones, coumarins, fats, flavonoids, glycosides, gums, iridoids, adhesives etc.

a) **Alkaloids**

A group of naturally occurring compounds that mostly contains the basic nitrogen atoms produced by bacteria, fungi, plants and animals and are part of natural products called secondary metabolites which display a variety of effects on animals. Archaeological information demonstrated the usage of alkaloids since 4000 BC. Antediluvian records demonstrated the utilization of poppy and opium by Sumerians, Persians, Egyptians, Greeks and Arabs for its qualities. Morphine (isolated from poppy), was recognized to be the first unrefined medication and nowadays is used as a pain relieving drug. Toxic activity is associated with many alkaloids that can cause death even in very little amounts. The examination for photochemical in genus *Kopsia* confers the disclosure of different category of alkaloids, conveying anti-tumour, anti-mitotic and anti leishmanial properties. Morphine, taxol, scopolamine and hyoscyamine are few significant instances of alkaloids that are known for their therapeutic qualities. Alkaloids are accounted for having anti-cancerous, anti-asthmatic, cholinomimetic, vasodilatory, pain relieving, anti-hyperglycemia activities [59].

b) **Terpenoids &Terpenes**

Terpenes and terpenoids are among the most common chemical group of naturally occurring compounds that are present in plants and are the largest group of secondary metabolites that contains approximately 22000 compounds. For example, these are the major component of essential oils, lactones, iridoids, sesquiterpenes, cardiotonicheterosides, saponins [61]. Geraniol, Caryophyllene, menthol and linalool belong to the terpene class and are recognised for their aromatic properties. Essential oil obtained from *L. Daucuscarota* was reported to hinder human enteropathogenesis [62]. Insecticidal, antibacterial, anti-inflammatory, anticancer and antioxidant properties of terpenoids have also been reported [63].

c) **Phenolics**

Phenolic is one of the most abundant and widely distributed classes in the flora kingdom. There are approximately 8,000 known phenolic structures, including approximately 4,000 flavonoids [66] [67]. They are produced through plant pathways of malonic acid and shikimic acid and consist of a wide spectrum of defense-associated components including tannins,

flavonoids, anthocyanins, phytoalexins, furanocoumarins and lignin. Major groups of phenolic include two groups: -

1) **Flavonoids-**They consist of 2 aromatic rings joined by a 15-carbon 3-carbon bridge. There are few of richest class of phenolics available in the leaf epidermis and outer layer of fruit in maximum quantities. Flavonoids have a sole function as a secondary metabolite. These carry out multiple actions in plants such as disease resistance, UV protection, nodules stimulation, nitrogen-fixing and pigmentation. Flavanoids also provide colors for the flowers and fruits and thus facilitate the attraction of insects by plants that further assist in their pollination and dispersal of seed. Quercetin, kaempferol, catechin, etc. are the most common flavonoids found in about 70% of plants. In addition, flavonoids are divided into flavones, flavanols, anthocyanidins, flavan-3-ols, isoflavones and flavanones [62].
2) **Phenolic acids: -**In all plants, phenolic acid is the major part of polyphenols / phenols. The group consists of a distinctive aromatic structure in phenyl propionic type with C6-C3 and in phenyl methyl type with C6-C1. The main component of phenolic acid is gallic acid, which is the basic compound of Gallo tannins and form ellagitannins along with hexahydroxydiphenoyl moieties. They are found abundantly in fruits, vegetables, seeds and grains, both free and bound. All phenolic members, particularly phenolic acid and flavonoid, have enormous antioxidant, antimicrobial, antiviral, anti-inflammatory, anti-carcinogenic and anticancer properties.

d) **Essential oils**

Due to volatile nature, essential oils are also called volatile oils. Essential oils are found in aromatic plants and are responsible for plant aroma production. They are synthesized and released either directly by the plants protoplasm or by some glycosides hydrolysis. Structures such as schizogenesis or lysogenic passages, glandular hairs, oil tubes or vittae, and modified parenchyma are found in plants for secretion of various essential oils. Qiang et al. (2011) [65] reported 208 compounds from various Rhododendron species, most of which are diterpenoids

and flavonoids. Hu and Xiao (1989) examined the leaves of 166 Rhododendrons and found that quercetin, kaempferol, farrerol, polystachoside, hyperoside, and quercitrin were present.

2.4 Impact of phytochemicals of plants on immune system and overall health

a. Impact of Tanins

Condensed tannins or proanthocyanidins are commonly found in herbal and legume plants [27]. Eating plants, high in tannins, is a way for herbivores to reduce internal parasites, and tannins alleviate bloat by binding to proteins in the rumen. Due to this protein bypass effect there is increase resistance to gastrointestinal nematodes and enhancement in the immune response. Due to the bypass of amino acids (glutamine, cysteine, arginine) there is improve immune response as these amino acids are directly involve with the B and T lymphocytes, macrophages, natural killer cells, lymphocyte multiplication and gene expression. Changes in the gastrointestinal tract populations of commensal bacteria may stimulate gut-associated myeloid tissues and thus modulate immune responses mediated by T or B cells. Additionally, the selective effects of tannins on bacteria in both the rumen and the intestines can be an important avenue for research.

b. Impact of flavonoids

Fruits and vegetables contain the phenolic compounds that are known as flavonoids. The main importance of flavonoids in immune response may be due to actions such as active site interference, protein binding, and antioxidant effects. Some flavonoids show targeted effect on the function of enzymes involved in generating inflammatory responses. The inflammatory responses are shown by the dietary flavonoids as they have inhibitory effects primarily on the T lymphocytes [40]. Few flavonoids modify immune responses which could be included in the immune-surveillance of tumours. Quercetin stifles antigenic incitement of cytotoxic T lymphocytes and also restrains regular executioner cell intervened cytolysis[40].

c. Impact of alkaloids

Alkaloids are class of compounds containing nitrogen which are present in about 20% plant species, derived mostly from amino acids. Some alkaloids have been tested for their effects on immune function. A few alkaloids have been accounted to improve the antibody-dependent

cell mediated cytotoxicity, activity of natural killer cell. The alkaloids have been utilized to treat numerous acute and chronic diseases. Alkaloids straight forwardly or in a roundabout way enact macrophages through T cell- associated effects [41]. Alkaloids may have immunosuppressant impacts and this may be because of the altered antioxidant status.

d. Impact of Terpenes

Terpenes are huge and a different class of hydrocarbons that are formed with the help of isoprene units. They are one of the most varied classes of secondary plant metabolites. Data on immunomodulatory impacts of this compound is rare. Terpenes have been accounted to improve the cytotoxic action of natural killer cell in vivo and in vitro[42]. These decrease the generation of some pro-inflammatory cytokines and work as immunosuppressive agents also have bactericidal and bacteriostatic properties. Terpenes also affect the ruminal bacteria [42].

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials used

3.1.1 Chemicals used

1-Diphenyl-2-picrylhydrazyl (DPPH), 2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ethanol, gallic acid, sodium carbonate, Folin-ciocalteu, NaOH, $NaNO_2$, $AlCl_3$, $FeSO_4$, EDTA, potassium persulfate, ascorbate, H_2O_2 , TCA, TBA, Distilled water, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, hydrochloric acid, FeCl3, Chloroform, methanol, concentrated H_2SO_4, Potassium ferricyanide, Glacial acetic acid, DMSO, quercetin, HCL, Acetate buffer

3.1.2 Equipment's used

Test tube, lyophilizer, water bath, spectrophotometer, beakers, eppendorf, tips, pipette, Rota evaporator, round bottom flask etc.

3.1.3 Reagent used

Mayer reagent, Dragendroff reagent, Folin–Ciocalteu reagent, Fehling A, Fehling B

3.2 Methodology

3.2.1 Collection of the plant materials

In this study, two different plant collected from two different times from Himachal Pradesh region i.e. *Diplazium esculentum* and *Rhododendron arboreum*. *Diplazium esculentum* was collected from the two different regions of Himachal Pradesh one from the Kotkhai (1881 m) District Shimla and other from Karsog (1404 m) District Mandi in the month of October 2018. *Rhododendron arboreum* was collected from the Chail (2250 m) District Shimla in the month of April 2019.

3.2.2 Preparation of the extract

The leaves, fronds of the *Diplazium esculentum* and flowers, leaves of Rhododendron arboreum was washed and shade dried in the sterilized environment. We obtained 40 g (approx.) of dried powder of each of the *Diplazium* collected from the two regions from approximately 300g of the original plant. Approximately 60g of dried flower and 50g of the dried leaves powder was obtained from the 300g of the fresh flowers and 200g of fresh leaves. Using solvent

extraction method, the methanolic extract of *Diplazium esculentum* and ethanolic extract of Rhododendron arboreum was prepared. The different samples were macerated with 100 ml methanol and 100 ml of ethanol for seven days with occasional stirring and after seven days the rotaevaporator was used to get the crude extract. The yield percentage of the extract was calculated with the help of given formula:

$$\% \text{ Yield} = E_X / E_Y \times 100$$

Where E_X = extract weight after lyophilisation; E_Y= total plant weight taken for extraction.

3.3 Photochemical Screening

Phytochemical analysis was performed on the methanolic and ethanolic extract of both plants. The concentration of various plant extracts used in this study was 1 mg/ml.

3.3.1 Phytochemical qualitative analysis

Colorimetric testing was used for qualitative phytochemical evaluation [50].

1. **Test for saponins:** 1ml of extract was stirred and warmed with 1 ml of distilled water in eppendorf and waited for the stable foam formation that indicates the presence of saponins.
2. **Test for flavonoids:** 1 ml of extract, 1 ml of 10 per cent $Pb(C_2H_3O_2)_2$ lead acetate solution has been added and seen for the formation of the yellow precipitate that indicate the presence of flavonoids.
3. **Test for steroids:** 1 ml of extract was mixed with 2 ml of chloroform and then 2 ml of concentrated H_2SO_4 was added and waited for the formation of red colour in the lower chloroform layer indicating steroid presence.
4. **Test for glycosides:** 1 ml of crude extract was mixed with 2 ml of chloroform and added 2 ml of concentrated H_2SO_4 then seen for reddish brown colour in the lower chloroform layer indicating Glycosides presence.
5. **Test for reducing sugars:** Mixed 1 ml extract with 1 ml solution of Fehling A and B in equal proportions. Then the solution was gently boiled and then waited for the formation of red precipitates in order to see sugars will be reduced or not.

6. **Test for polyphenols:** 1ml of extract was added to 1ml of 2% $FeCl_3$ and then seen for green blue colour that indicates that polyphenols are present.

7. **Test for alkaloids: :** 1ml raw extract was added to 2ml 1% Hydrochloric acid then gently heated and Mayer reagent was added(1 to 2 drops) and waited for the appearance of buff-coloured precipitates that indicates the presence of the alkaloids.

3.3.2 Quantitative analysis of phytochemicals

i. Determination of total flavonoid content.

The total flavonoid content of the crude extract was measured with the help of Aluminium chloride ($AlCl3_)$ colorimetric method.125 µl (2mg/ml) of extract was added to 75µl of 5%$NaNO_2$ in a test tube. The solution was incubated for 6 min at room temperature. 150 µl of 10 % $AlCl_3$ was added to the mixture. Then mixture was incubated for 5 min at room temperature.750 µl of 1M NaOH was added to the solution mixture. Then the final vol. of mixture was made up to 2500 µl with D.W. Incubated at 37 °C in the incubator for 15 min. Observed under spectrophotometer at 510 nm. The flavonoid content in different extract was calculated as quercetin equivalent per gram dried sample weight.

ii. Determination of Total Polyphenol content.

The total phenolic content present in the extract was measured by the help of Folin-Ciocalteu reagent assay [57] [58]. The extract was diluted with water to a concentration of 2mg/ml. 1ml Folin-Calcateaue reagent was added to sample and mixed well and incubated at room temperature for 5 min at room temperature. Then to the above mixed solution, 1 ml of 35% aqueous sodium carbonate was added and the final volume made up to 10ml with distilled water. Further, the mixture was incubated for 30 min at room temperature and the absorbance was recorded at 730 nm against the reagent blank. Results were calculated as Gallic acid equivalents (mg/g).

iii. Antioxidant activity assay by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

Firstly, the DPPH reagent was made after incubation of the 24 hours the OD of DPPH was set under 1 i.e. (0.9-0.8) with the help of 80% ethanol. After the OD was set extract of

2mg/ml concentration was made using 80% methanol as solvent. The reaction was set using 250µl of extract and 1250µl DPPH reagent was added to it. The total volume of 3 ml was made with distilled water. Content was mixed forcibly and incubated at room temperature for 25-30 minutes in light less area. After incubation, the absorbance was read at 517 nm against blank and assay was conducted in triplicates. Radical scavenging activity was measured as

$$\% \text{ Radical scavanging} = [(Ab_c - Ab_s)/(Ab)] \times 100$$

(Ab_c = absorbance of control; Ab_s = absorbance of sample.)

iv. Antioxidant activity assay by 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

Antioxidant capacity was also measured on ABTS radical scavenging capacity according to a method previously described. Firstly, the ABTS reagent was prepared using ABTS sol. that was combined with potassium per sulphate and incubated for 12-14 hours in shade to make ABTS cations. Then 50 µl extract (80% aqueous ethanolic extract in 1:10 dilution) was mixed with 1450 µl ABTS solution. In the control preparation 50 µl of 80% aqueous ethanol was used in place of sample. The contents were mixed immediately and incubated for 5 minutes in a dark place. The O.D was taken at 534nm in an Ultra-Violet-Vis spectrophotometer. ABTS scavenging activity was expressed in % radical scavenging activity.

v. Antioxidant activity by the ferric reducing antioxidant power assay (FRAP).

Ferric reducing antioxidant power- The total antioxidant activity can be measured by reducing antioxidant power assay. Different concentrations (2-10mg/ml) of methanolic or ethanolic extract were taken and added to 2.5 ml Sodium phosphate buffer(0.2M) and 2.5 ml potassium ferricyanide(1%) solution. The combination of the reaction was well vortexed and then incubated at 50 ^{0}C for 20 minutes. At the termination of the incubation, 10% trichloroacetic acid 2.5 ml was added to the combination and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was combined with 0.5 ml 0.1% ferric chloride and 2.5 ml deionized water. The colour solution was read with reference to the standard at 700 nm against the blank. The flavonoid content in different extract was calculated as quercetin equivalent per gram dried sample weight.

vi. Antimicrobial activity of *Diplazium esculentum* sample by Antimicrobial susceptibility test

The pure organism culture plate to be tested has been taken. With the help of micro pipette, colony was taken from the plate in the sterile environment and put into nutrient broth. Culture has been incubated overnight to revive the organism that was endeavoured. Next day secondary culture was prepared. Sterile nutrient agar (NA) plate was taken and secondary culture was spread over the plate. The plate was allowed to dry for 5 minutes after the spread is complete. Using the gel puncher three well were punched on the nutrient agar plate. 50µl of two different extract was poured into the two well. Plates were incubated for 24 hours at 37ºC. A metric ruler was used after incubation to measure the zone of inhibition diameter for each extract used. To determine sensitivity zone the diameters of the extract are compared with the standards.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Results of Diplazium esculentum collected from two different regions of Himachal Pradesh

4.1.1 Phytochemicals screening with the help of Qualitative assays.

The result of qualitative analysis performed for prevelance of different calss of phytochemichals in two different samples of *Diplazium esculentum* are presented in table 4.1. It is evident from the perusal of table 4.1 that both samples methanolic extract collected from different regions shows the presence of glycosides, saponins, terpenoids, phenolics, tannins, flavonoids and steroids. Whereas, carbohydrates were present in sample collected from Karsog but absent in sample collected from Kotkhai. There was no presence of alkaloids in both the extract.

Table 4.1 Qualitative analyses of the Diplazium Esculentum extract collected from two regions of Himachal Pradesh.

Serial no.	Phytochemicals	Extract 1	Extract 2
1.	Glycosides	Positive	Positive
2.	Tannins	Positive	Positive
3.	Saponins	Positive	Positive
4.	Alkaloids	Negative	Negative
5.	Flavonoids	Positive	Positive
6.	Carbohydrates	Positive	Negative
7.	Steroids	Positive	Positive

In this Extract 1 signifies the plant collected from Karsog (HP) and Extract 2 signifies the plant collected from the Kotkhai (HP).

Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4 shows the results of different qualitative phytochemical tests that were done in order to reveal the phytoconstituents of the plants collected from two different regions of Himachal Pradesh.

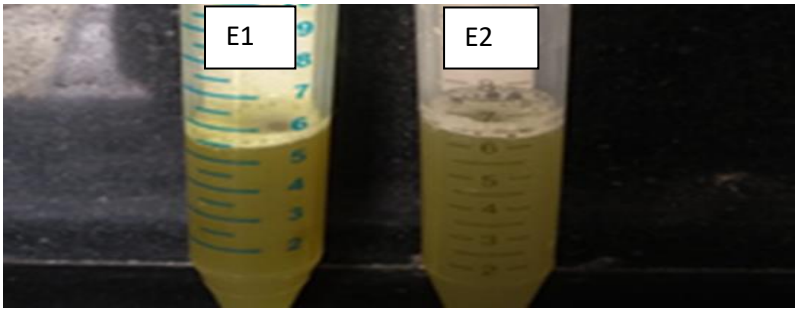

Figure 4.1 Positive results for saponin in both E1 and E2 as there is formation of stable foam

Figure 4.2 Reddish brown ring in the test tube showed the presence of glycosides in E1 and E2

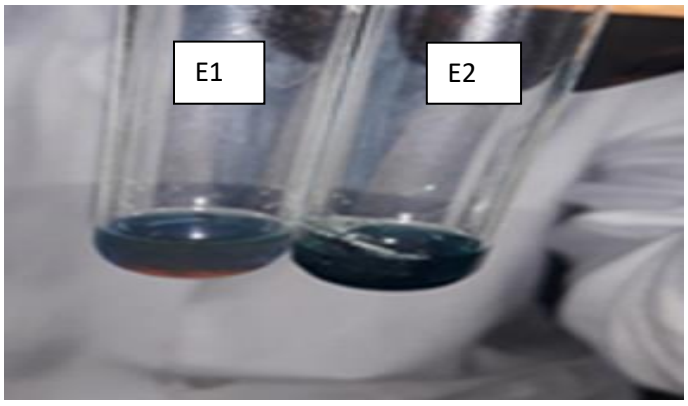

Figure 4.3 Orange red colour precipitates in the left test tube indicate the presence of carbohydrate in E1

Figure 4.4 Red colour produced in lower layer indicates the presence of steroids in both E1 and E2

4.1.2 Phytochemicals screening with the help of Quantitative assays.

1. Total phenol content

A standard calibration curve with the y = 0.705x+0.0683 and the regressive coefficient, R^2= 0.995, were drawn using different dilutions (Gallic acid) as shown in table 4.2. Total phenolic content was calculated using linear equation and expressed as Gallic acid equivalents (µg/mg). The total phenolic content of *Diplazium esculentum* collected from the regions of Karsog and Kotkhai are presented in Figure 4.6. The TPC values were found out to be 120 ± 0.160 and 50 ± 0.160 µg GAE /mg of the extract 1 and extract 2 respectively.

Table 4.2 Total phenolic values of standard [Gallic acid].

Concentration of Gallic acid (mg/ml)	Absorbance mean value ± SD
0	0
0.2	0.159±0.002
0.4	0.300±0.001
0.6	0.459±0.003
0.8	0.596±0.004
1	0.693±0.002

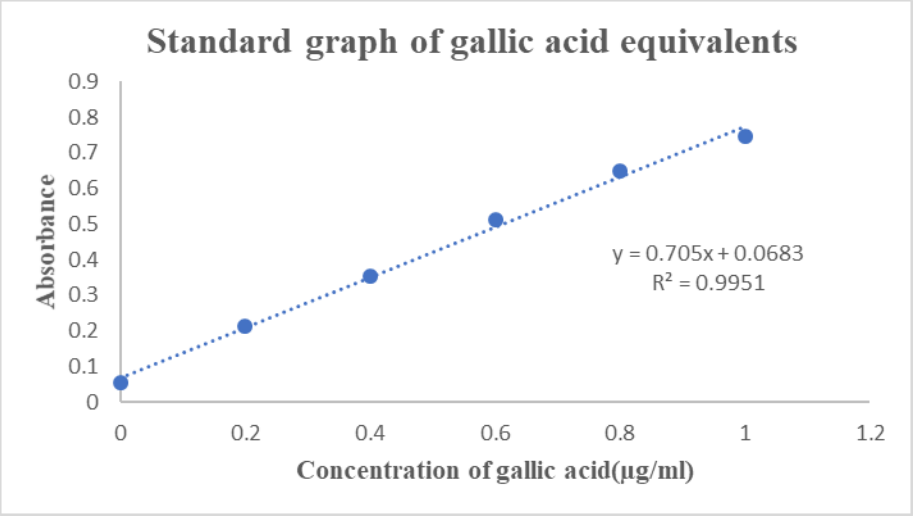

Figure 4.5 Total phenolic content Gallic acid calibration curve between Y-axis absorbance (765 nm) and X-axis Gallic acid concentration.

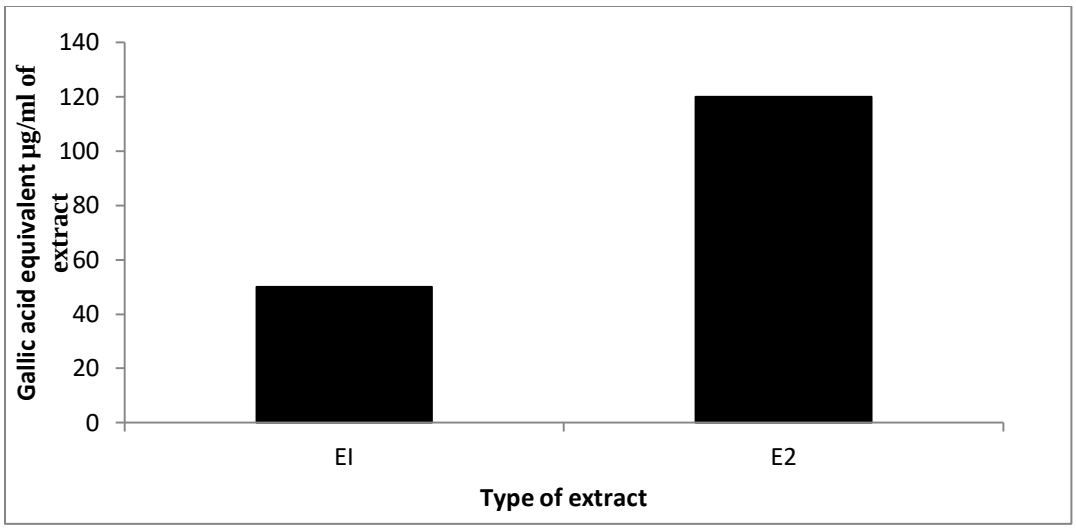

Figure 4.6 TPC (mg GAE g-1 of extracts) of *Diplazium esculentum* collected from two regions of Himachal Pradesh. In this figure E1 is extract from Kotkhai and E2 is extract from Karsog.

2. Antioxidant activity by DPPH assay

1, 1-diphenyl-2-picrylhydrazyl assay is widely used to estimate the scavenging of several natural compounds and crude mixtures. At the radical reduction in DPPH by antioxidant, the colour of the test solution is converted to yellow from violet and therefore absorption at a wavelength of 515 nm is reduced accordingly.

The percentage radical scavenging activity of the *Diplazium esculentum* collected from the two different regions i.e. Karsog and Kotkhai were found out to be 35.82 and 30.56 % respectively. This data show that the antioxidant activity of the sample collected from the Karsog has high % radical scavenging than the sample collected from Kotkhai as depicted in Figure 4.7.

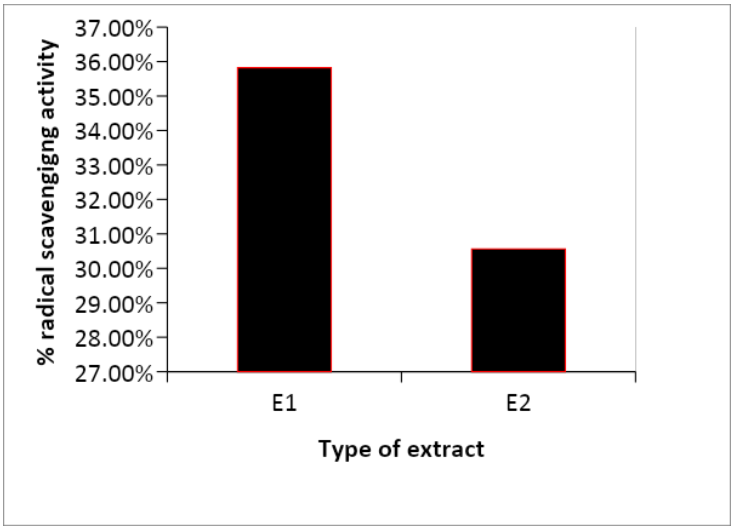

Figure 4.7 1, 1-diphenyl-2-picrylhydrazyl % radical scavenging activity for different extract collected from two region. E1 is extract collected from Karsog and E2 is collected from Kothkhai.

3. Antioxidant activity by ABTS assay.

2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) assay is widely used to estimate the scavenging of several natural compounds and crude mixtures. At the radical reduction in ABTS by antioxidant, the colour of the test solution is converted to yellow from violet and therefore absorption at a wavelength of 515 nm is reduced accordingly. The percentage radical scavenging activity of the Diplazium esculentum collected from the two different regions i.e. Karsog and Kotkhai were found out to be 13.62 % and 10.51 % respectively. This data show that the antioxidant activity of the sample collected from the Karsog has high % radical scavenging than the sample collected from Kotkhai that was shown in Figure 4.8.

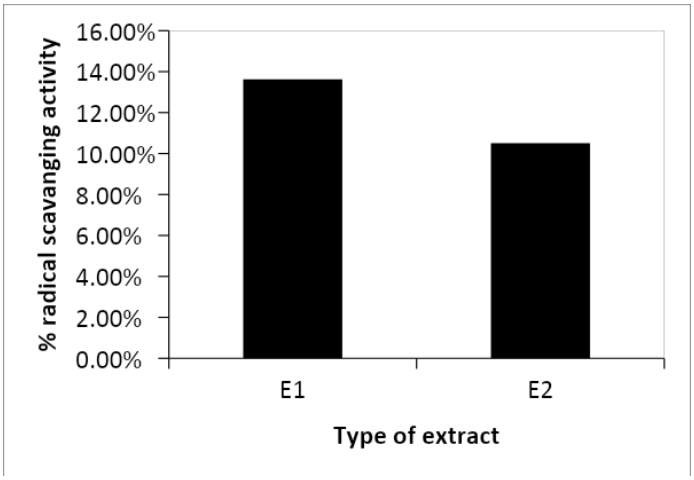

Figure 4.8. 2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) % radical scavenging activity for extract collected from two regions. E1 is extract collected from Karsog and E2 is collected from Kotkhai.

4. Ferric reducing antioxidant power activity assay

A standard calibration curve with the y = 0.0019x+0.0052 and the regressive coefficient, R2= 0.998, were drawn using different dilutions (Ascorbic acid) that are shown in Table 4.3. Ferric reducing antioxidant power was calculated using linear equation and expressed as µmol Fe (II)/mg DW. The ferric reducing antioxidant power activity of Diplazium esculentum collected from the regions of Karsog and Kotkhai were found out to be 70.93µmol Fe (II)/mg DW and 64.5µmol Fe (II)/mg DW respectively. The ferric reducing antioxidant power of *Diplazium esculentum* collected from the Karsog region is more that is depicted with help of figure 4.10.

Table 4.3 Values of standard (ascorbic acid) for FRAP

Sample	Concentrations (µmol)	Absorbance
Standard 1	31.25	0.061
Standard 2	62.5	0.128
Standard 3	125	0.236
Standard 4	250	0.488

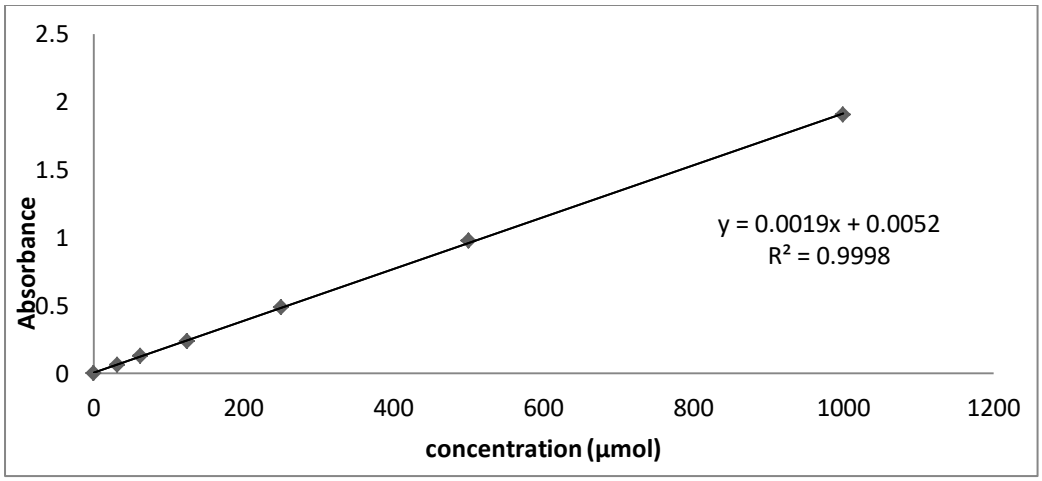

Figure 4.9 FRAP ascorbic acid calibration curve between Y-axis absorbance (700 nm) and X-axis ascorbic acid concentration.

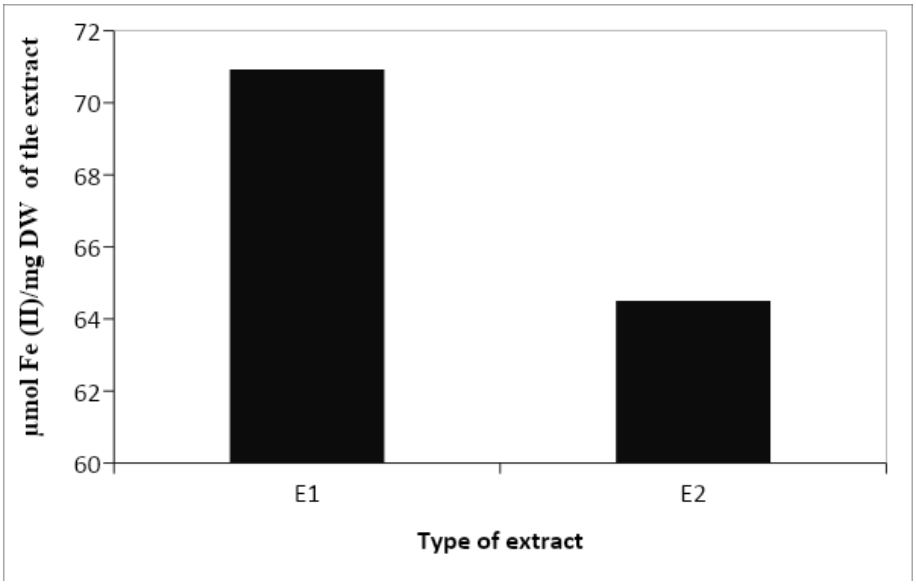

Figure 4.10 FRAP (µmol Fe (II)/mg DW of extracts) of *Diplazium esculentum* collected from two regions of Himachal Pradesh. In this figure E1 is extract from Kotkhai and E2 is extract from Karsog.

4.1.3 Analysis of extract for antimicrobial properties

Extracts of *Diplazium esculentum* collected from two regions were examined for antibacterial activity. Four strains of bacteria i.e. *Escherichia coli Dh 5ᵅ, Staphylococcus aureus, Salmonella typhi,* were used for checking antimicrobial activity of extracts with help of

antimicrobial susceptibility test. Figure 4.11, figure 4.12, Figure 4.13, Figure 4.14 depicts that no extract show the antimicrobial action that can be inferred by the following plates as there is no zone of inhibition in any of plates.

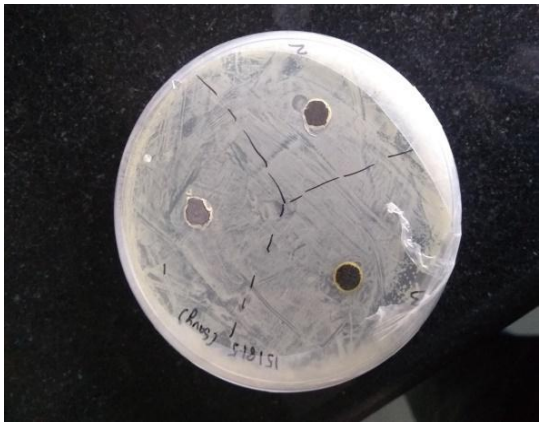

Figure 4.11 No zone of inhibition was there in case of ***Escherichia coli Dh 5α*** as shown above. 1, 2, 3 are control, extract 1, extract 2 respectively.

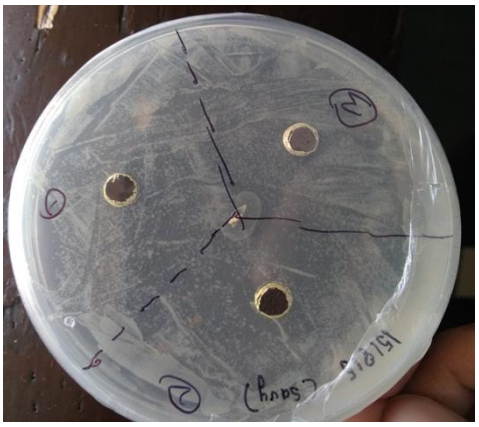

Figure 4.12 No zone of inhibition was there in case of ***Staphylococcus aureus*** as shown above. 3, 2, 1 are control, extract 1, extract 2 respectively.

Figure 4.13 No zone of inhibition was there in case of ***Escherichia coli*** as shown above. 1, 2, 3 are controls , extract 1 ,extract 2 respectively.

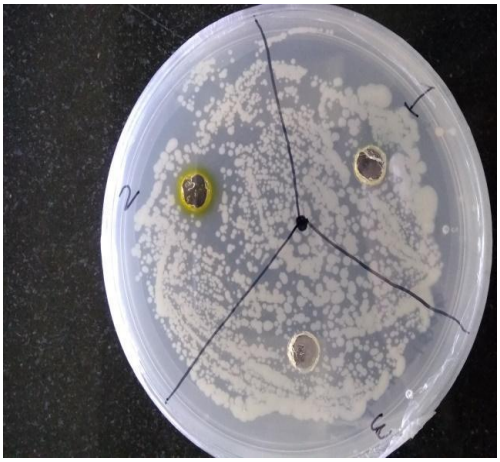

Figure 4.14 No zone of inhibition was there in case of ***Salmonella typhi*** as shown above. 1, 2, 3 are control, extract 1, extract 2 respectively.

4.2. Results of *Rhododendron arboreum* collected from Chail District Shimla, Himachal Pradesh

4.2.1 Phytochemicals screening with the help of Qualitative assays.

During the qualitative research the analysis of plant ethanolic extract collected from Chail HP showed the presence of glycosides, saponins, terpenoids, phenolics, tannins, flavonoids, carbohydrate and steroids. There was no presence of alkaloids in both the leave and flower extract. On the basis of the color of the precipitates or reaction the results of both the extract are tabulated in the table 4.4.

Table 4.4 Qualitative analyses of different parts of *Rhododendron arboreum* i.e. leaves extract [Extract L] and flower extract [Extract F] collected from Chail, Himachal Pradesh.

Serial no.	Phytochemicals	Extract F	Extract L
1.	Glycosides	Positive	Positive
2.	Tannins	Positive	Positive
3.	Saponins	Positive	Positive
4.	Alkaloids	Negative	Negative
5.	Flavonoids	Positive	Positive
6.	Carbohydrates	Positive	Positive
7.	Steroids	Positive	Positive

4.2.2 Phytochemicals screening with the help of Quantitative assays.

1. Total phenol content

A standard calibration curve with the $y = 0.7532x$ and the regressive coefficient, $R^2 = 0.9899$, were drawn using different dilutions (Gallic acid) that are shown in table 4.5. Total phenolic

content was calculated using linear equation and expressed as Gallic acid equivalents (µg/mg). The total phenolic content of *Rhododendron arboreum* leaves and flower collected from the region of Chail were found out to be 342 ± 0.004 and 262.8 ± 0.002 µg GAE /mg of the flower extract and leaves extract respectively that depicted in Figure 4.6.

Table 4.5 Total phenolic value of standard [Gallic acid].

Concentration of Gallic acid (mg/ml)	Absorbance mean value ± SD
0	0
0.2	0.179±0.001
0.4	0.320±0.004
0.6	0.479±0.002
0.8	0.616±0.001
1	0.713±0.001

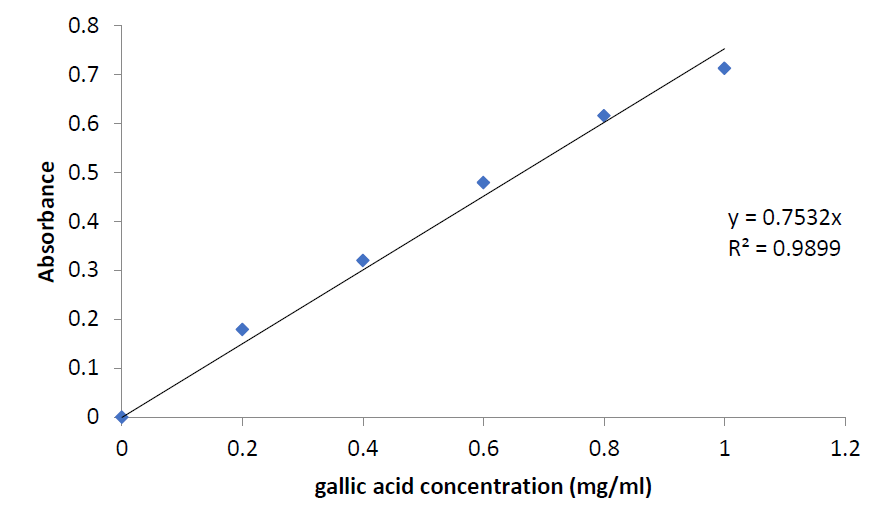

Figure 4.15 Total phenolic content Gallic acid calibration curve between Y-axis absorbance (765 nm) and X-axis Gallic acid concentration.

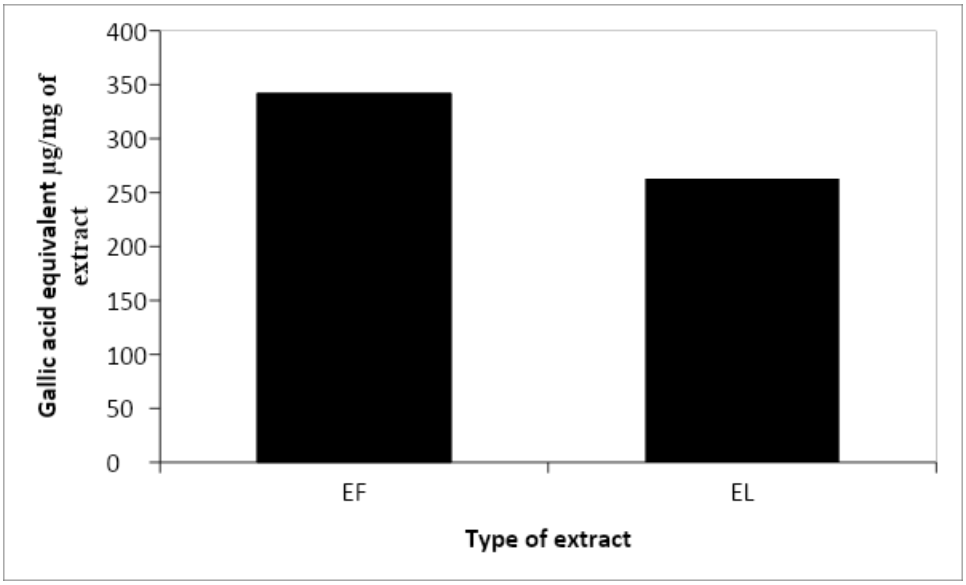

Figure 4.16 TPC (mg GAE g-1 of extracts) of *Rhododendron arboreum* collected from Himachal Pradesh. In this figure EF is extract of flower and EL is extract of leaves.

2. Total flavonoid content

A standard calibration curve with the y = 0.0009x and the regressive coefficient, R^2= 0.985, were drawn using different dilutions (quercietin) that are shown in Table 4.6. Total phenolic content was calculated using linear equation and expressed as quercietin equivalents (µg/mg). The total flavanoid content of *Rhododendron arboreum* leaves and flower collected from the region of Chail were found out to be 72.2 ± 0.001 and 58.6 ± 0.004 µg GAE /mg of the flower extract and leaves extract respectively which was depicted by Figure 4.18.

Table 4.6 Value of standard for total flavonoids content

Concentration of Quercietin (mg/ml)	Absorbance mean value ± SD
0	0
0.1	0.062±0.002
0.2	0.150±0.004
0.4	0.300±0.004
0.6	0.550±0.004
0.8	0.780±0.004
1	0.900±0.017

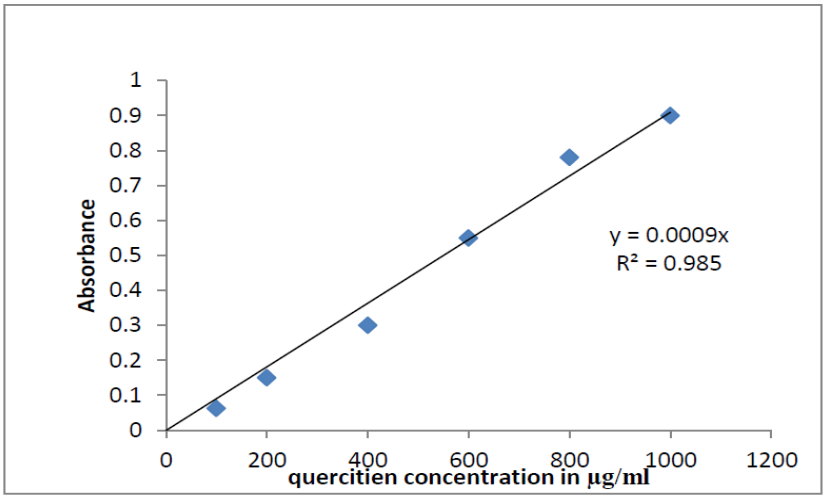

Figure 4.17 Total flavonoid content quercetin calibration curve between Y-axis absorbance (765 nm) and X-axis quercetin concentration.

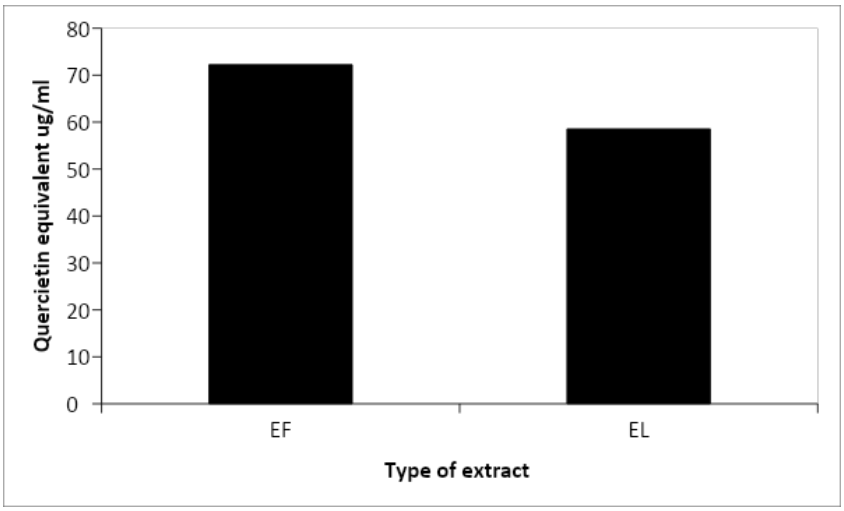

Figure 4.18 TFC (quercetin equivalent µg/mg of extracts) of *Rhododendron arboreum* collected from Himachal Pradesh. In this figure EF is flower extract and EL is leaves extract.

3. Antioxidant activity by DPPH assay

1, 1-diphenyl-2-picrylhydrazyl assay is widely used to estimate the scavenging of several natural compounds and crude mixtures. At the radical reduction in DPPH by antioxidant, the colour of the test solution is converted to yellow from violet and therefore absorption at a wavelength of 515 nm is reduced accordingly.

The percentage radical scavenging activity of the *Rhododendron arboreum* two different parts i.e. flowers and leaves were found out to be 80.73 and 71.99 % respectively. The Figure 4.19 shows that the antioxidant activity of the flower is more than the leaves.

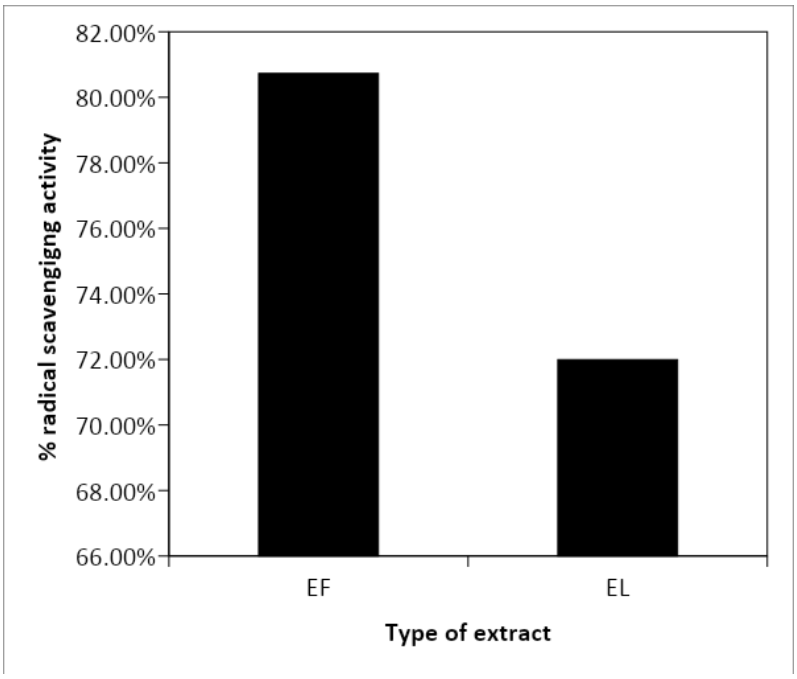

Figure 4.19 1, 1-diphenyl-2-picrylhydrazyl % radical scavenging activity for different parts of plant extract. EF is flowers extract and EL is leaves extract of *R.arboreum*.

4. Antioxidant activity by ABTS assay.

2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) assay is widely used to estimate the scavenging of several natural compounds and crude mixtures. At the radical reduction in ABTS by antioxidant, the colour of the test solution is converted to yellow from violet and therefore absorption at a wavelength of 734 nm is reduced accordingly. The percentage radical scavenging activity of the different parts of the Rhododendron arboreum collected from the Chail region HP were found out to be 84.70 % and 62.80% respectively. The figure 4.20 shows that the antioxidant activity of the sample collected from the Karsog has high % radical scavenging than the sample collected from Kotkhai.

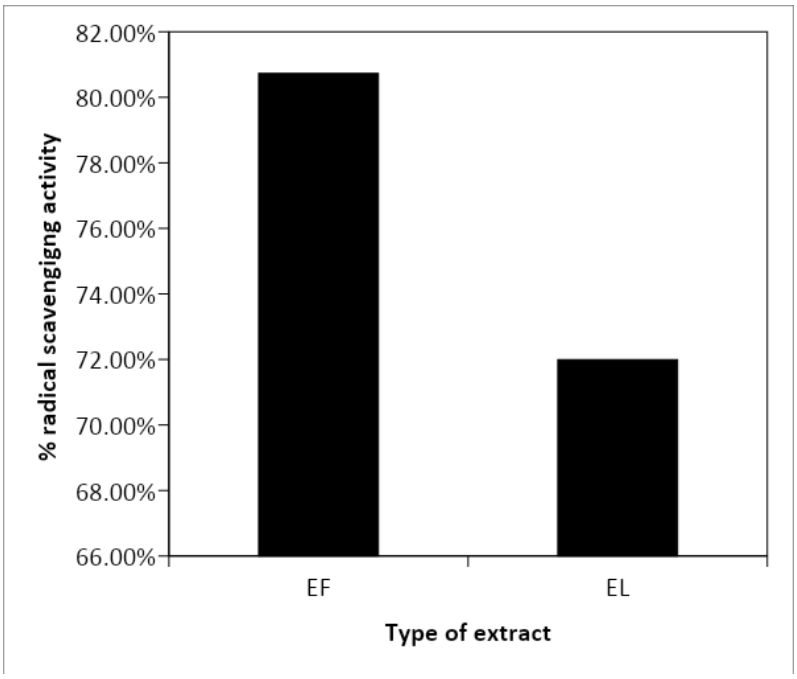

Figure 4.20 2, 2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) % radical scavenging activity for different parts of the plant. EF is flower extract and EL is leaves extract.

5. Ferric reducing antioxidant power activity assay

A standard calibration curve with the y = 0.0006x and the regressive coefficient, R^2= 0.9805, were drawn using different dilutions (Ascorbic acid) that are shown in Table 4.7. Ferric reducing antioxidant power was calculated using linear equation and expressed as µmol Fe (II)/mg DW. The ferric reducing antioxidant power activity of Rhododendron arboreum leaves and flowers collected from the Chail region were found out to be 221.66 µmol Fe (II)/mg DW and 130 µmol Fe (II)/mg DW respectively. The ferric reducing antioxidant power of flower is more than the leaves that is depicted in figure 4.22.

Table 4.7 Values of standard (ascorbic acid) for FRAP

Sample	Concentrations (µmol)	Absorbance
Standard 1	100	0.097±0.001
Standard 2	200	0.170±0.002
Standard 3	500	0.340±0.002
Standard 4	1000	0.690±0.002
Standard 5	1500	0.900±0.01

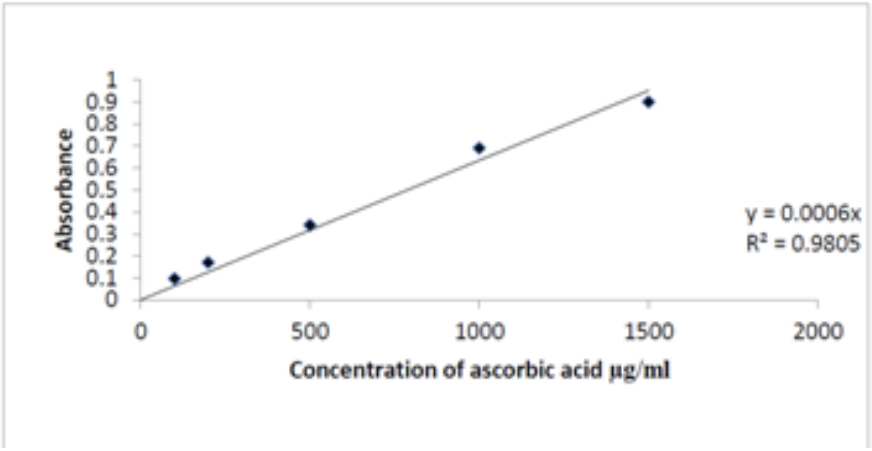

Figure 4.21 FRAP ascorbic acid calibration curve between Y-axis absorbance (700 nm) and X-axis ascorbic acid concentration.

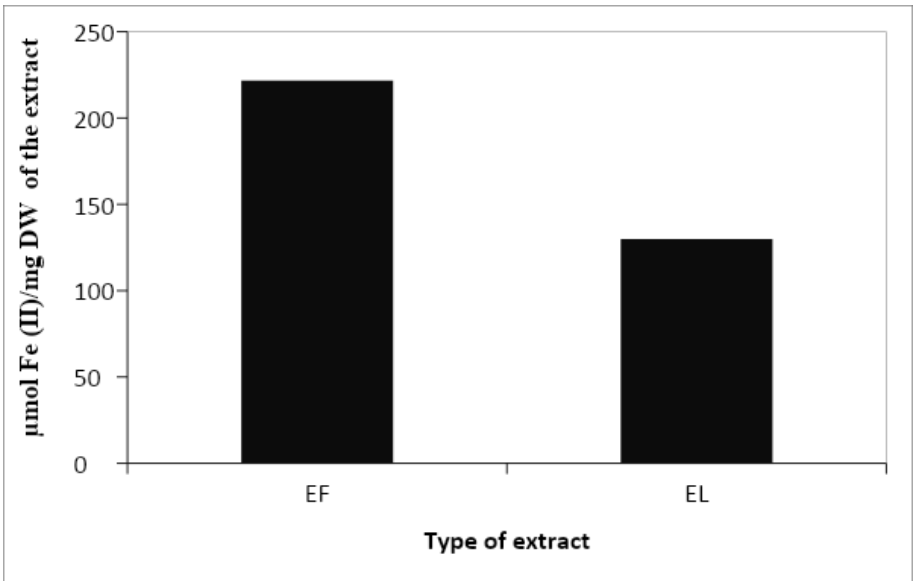

Figure 4.22 FRAP (µmol Fe (II)/mg DW of extracts) of *Rhododendron arboreum* collected from Chail Himachal Pradesh. In this figure EF is flower extract and EL is leaves extract.

4.3 Discussion

The medicinal plant therapeutic characteristics are generally attributed to the phenomenon of known antioxidants [69], such as natural phytochemichals (flavonoids, phenol, terpenoids, and so on). Free radicals are volatile reactive molecules that cause extensive intracellular damage. The most common objectives of such free radicals are DNA, lipids, and proteins [70]. Antioxidants are able to prevent oxidative stress through the removing of free radicals. Extract collected from two regions of Himachal Pradesh were examined for the antioxidant activity of *Diplazium esculentum* by three methods: FRAP, DPPH, ABTS radical scavenging. ABTS and DPPH are effective methods of evaluating various crude and natural compounds by evaluating the scavenging activities. DPPH is used to measure the scavenging activity of anti-oxidants after a given time frame in an extract or prospective compound with the DPPH solution and absorbance (515 nm). The ABTS test combines the extract with the ABTS radical solution, and antioxidant reduction of the radicals can be measured at a wavelength of 734 nm. Discoloration is caused by the decreasing amount of radical extracts that have radical scavenging properties and antioxidant properties of the solution. FRAP also have same principle that work on ability of antioxidant to convert ferric ions to ferrous ion. To estimate the redox potential (i.e. antioxidant property) of the extract the TPC and TFC were quantified.

The present learning shows that the radical scavenging by the *Diaplazium esculentum* of the two different regions i.e. Karsog and Kotkhai were found out to be 35.82% and 30.56% respectively when done with DPPH assay. The radical scavenging shown by both with ABTS assay was around 10-14%. Total phenol content of both the samples varied in good amount. Both the samples shows the notable difference and reducing activity when the FRAP results were analysed. In the early studies the researchers Akhtar et al. (2014) [68] demonstrated that the chloroform and methanol concentrate of *Diplazium esculentum* shows antioxidants, antimicrobial and cytotoxic activity. Utilizing the Folin-Ciocalteu strategy, the total phenolic substance were 125.60 ± 13.44 and 11.65 ± 0.87 mg gallic acid equivalents per 100 g air-dried sample for the ethanolic and aqueous concentrates, separately.

When compared to the earlier studies *Diaplazium esculentum* show the good antioxidant properties. The present study shows the regional difference in the concentrations of the phenols, flavones and other antioxidants due which there is difference in the % radical scavenging and conversion of the ferric to ferrous ions. The difference in samples may be due to the altitude difference or the area from where the plant is collected.

The present study revealed that *Rhododendron arboreum* collected from Chail region Himachal Pradesh contains significant amount of antioxidant properties. The present study shows that total phenol content of leaves and flowers of rhododendron was found out to be 342 ± 0.160 and 262.8 ± 0.160 µg GAE /mg of the extract respectively. The total flavonoid content of flowers and leaves in the same plant was found out to be 72.2 ± 0.001 and 58.6 ± 0.004 µg GAE /mg of the extract respectively. The percentage radical scavenging activity by DPPH assaay in *Rhododendron arboreum* two different parts i.e. flowers and leaves were found out to be 80.73 and 71.99 % respectively. The percentage radical scavenging activity by ABTS assay in *Rhododendron arboreum* leaves and flowers collected from the Chail region HP were found out to be 84.70 % and 62.80% respectively. The ferric reducing antioxidant power activity of *Rhododendron arboreum* leaves and flowers were found out to be 221.66 µmol Fe (II)/mg DW

and 130 µmol Fe (II)/mg DW respectively. The ferric reducing antioxidant power of flower is more than the leaves.

The TFC and TPC for the methanolic extract previously reported in the leaf extract of the *R. arboreum* were found out to be 62.26 and 17.5 GAE mg/g of extract [49]. The methanoilc extract of *R. campanulatum* revealed the TFC and TPC values 24.3 and 123.6 GAE mg/g of extract [49]. The high value of the TPC and TFC in present study is due to the plant collected from high altitude and the ethanolic extraction method used for the extraction of the metabolites. Prior examinations have proven antioxidant in the leaf concentrate of *R. arboreum*. The aqueous-methanolic leaf concentrates of *R. arboreum* cinn and *R. arboreum arb*. (Arranged through maceration) have demonstrated great cancer prevention agent properties, 1, 1-diphenyl-2-picrylhydrazyl by measure (IC50: 0.47 mg ml-1 and 0.34 mg ml-1, individually), and by Ferric reducing antioxidant power examine as far as ascorbic corrosive identical ml^{-1} (1.23ml^{-1} and 0.5 ml^{-1}) [49] . As compared to previous studies the extract shows good antioxidant properties.

Conclusion

The phytochemichal analyses of the *Diaplazium esculentum* shows the presence of the different phytochemichals like tannins, glycosides, steroids, saponins, phenolic and flavonoids in both the sample collected from two different regions i.e. Karsog and Kotkhai. It was found that there was no carbohydrate content present in Kotkhai sample but was present in the Karsog sample. The plant extracts exhibit significant levels of antioxidant activity as shown by the quantitative tests performed. The antioxidant activity in Kothkai sample was calculated to be 30.56% and 35.82% in Karsog sample from the results of DPPH tests. Lastly, we performed FRAP that showed the antioxidant power of Kothkai sample was 64.5 µmol Fe II\ mg DW and in the Karsog sample was 70.93 µmol Fe II\ mg DW. Therefore it can be concluded that the plant collected from Karsog region have high antioxidant potential and radical scavenging activity this may be due to the high content of the flavonoid and phenolic present in the extract which was determined with help of the total phenolic content. The high antioxidant may be due to the altitude difference and the habitat of the plant from where the plant is collected. There was no antimicrobial activity associated with both of the samples which was tested with the help of Antibiotic Susceptibility Tests.

The phytochemicals analysis of *Rhododendron arboreum* leaves and flowers collected from the Chail region district Shimla showed the presence of phytochemicals like tannins, glycosides, steroids, saponins, phenolic and flavonoids. The plant extracts exhibit significant levels of antioxidant activity as shown by the quantitative tests performed. The present study shows that total phenol content of leaves and flowers of *Rhododendron* was found out to be 342 ± 0.160 and 262.8 ± 0.160 µg GAE /mg of the extract respectively. The total flavonoid content of flowers and leaves in the same plant was found out to be 72.2 ± 0.001 and 58.6 ± 0.004 µg GAE /mg of the extract respectively. The percentage radical scavenging activity by DPPH assay in *Rhododendron arboreum* two different parts i.e. flowers and leaves were found out to be 80.73 and 71.99 % respectively. The percentage radical scavenging activity by ABTS assay in *Rhododendron arboreum* leaves and flowers were found out to be 84.70 % and 62.80% respectively. The ferric reducing antioxidant power activity of *Rhododendron arboreum* leaves and flowers were found out to be 221.66 µmol Fe (II)/mg DW and 130 µmol Fe (II)/mg DW respectively. The ferric reducing antioxidant power of flower is more than the leaves. The high

antioxidant potential of flowers is due to the high content of phenolic and flavonoids present in it which scavenge the free radicals show higher antioxidant activity.

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