PREPARATION OF PLANT EXTRACT OF *EUPHORBIA PROSTRATA*, ITS PHYTOCHEMICAL SCREENING & EVALUATION OF ANTIBACTERIAL & ANTIOXIDANT ACTIVITY

Submitted in fulfillment of the requirement for the degree of
Bachelor of Technology in Biotechnology

Submitted by

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CERTIFICATE

This is to certify that Ms Soumya Bhatnagar has carried out the undergraduate project work on **"PREPARATION OF PLANT EXTRACT OF *EUPHORBIA PROSTRATA*, ITS PHYTOCHEMICAL SCREENING & EVALUATION OF ANTIBACTERIAL & ANTIOXIDANT ACTIVITY"** under my supervision from July 2018 to May 2019. The work presented in this project report is original and has not been submitted anywhere else for any other degree.

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DECLARATION OF SCHOLAR

I hereby declare that the project titled **"PREPARATION OF PLANT EXTRACT OF *EUPHORBIA PROSTRATA*, ITS PHYTOCHEMICAL SCREENING & EVALUATION OF ANTIBACTERIAL & ANTIOXIDANT ACTIVITY"** submitted towards fulfilment for the award of degree of Bachelor of Technology in Biotechnology from Jaypee University Of Information Technology is based on the results of studies carried out under the supervision of Dr.Gopal Singh Bisht. This work, in part or in whole, has not been submitted anywhere else for award of any degree or diploma. I am responsible for the contents of this report.

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List of abbreviations

Abbreviation	Full Form
NA	Nutrient Agar
LB	Luria- Bertani Broth
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
NAT	Nutrient Agar Tween
LBGT	Luria-Bertani Broth Glycerol Tween
˚C	Degree Celsius
TEMP	Temperature
CONC	Concentration
HR	Hours
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
E. prostrata	*Euphorbia* family
LAB	Laboratory
NH_3	Ammonia
HCl	Hydrochloric Acid
NaOH	Sodium Hydroxide
$CHCl_3$	Chloroform

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ABSTRACT

In Himachal, the study of medicinal plant is very precise & mainly focuses on inventory, although many studies are there addressing the distribution patterns, diversity, nativity, and priority of conservation of medicinal plants. There are total of 643 medicinal plants in Himachal Pradesh, in which 269 are native, 374 non-natives. Out of which 12 are critically endangered and 21 are endangered. In Ayurveda & other traditional medicine system the medicinal plants were the main base & approximately 90% of the medicinal plant species are extracted from the wild. Among this wild variety of medicinal plants, *Euphorbia Prostrata* is from the spurge species and is wildly known as the road side weed. All the parts of *Euphorbia Prostrata* including roots, seeds, stem barks and entire plant is used for the treatment of migraine, warts, etc due to the healing properties of Euphorbias because of the presence of different chemicals found in these plants as secondary metabolites. Plants in the *Euphorbiaceae* family are well recognized for their chemical richness in isoprenoid constituents. Due to their diverse structures and therapeutic importance, *Euphorbia* has been given increasing public exposure. It has been found that macrocyclic diterpenes of this species *Euphorbia* have antitumor, antimicrobial, anti-candidal, antidiarheogic, analgesic & anti- inflammatory properties. Due to the different compounds present in this genus makes it rich in many biological activities. So the plant *Euphobia prostrata* of Himanchal Pradesh region is studied to find the presence of such secondary metabolites responsible for various activities.

CHAPTER 1

INTRODUCTION

Himachal Pradesh, in the Indian Himalayan region (IHR), has a variety of natural resources and a rich biological diversity spreaded in an area of 591,000 square kilometres [1] and extends from Jammu & Kashmir in the North-West to Arunachal Pradesh in the East. It covers about 18% of India and is approximately 2,800 km long and 220 to 300 km vast, with elevations from 200-8000 m [2]. The flora contains more than 8,000 angiosperms species (≈40% common), 44 gymnosperms species (≈15.91% common), 600 pteridophytes species(≈25% common),1,737 bryophytes species (≈32.53% common), 1,159 lichens species (≈11.22% common) & 6,900 fungi species (≈27.39% common) [3,4]. There are over 816 species of tree and 675 species of edibles. 118 Medicinal plant species yield essential oils, 279 fodder species, and 155 sacred plants [5]. The resident of Himalayan region relies on these plants to stay healthy and extend their lives worth. According to estimates by the World Health Organization (WHO), 3.5 billion populations in developed countries depend on plant-based primary health care medicine. About 6,500 plant species are used as household-based medicines in Asia [6].

With the increasing concern of using the natural products for the skin and the healing properties, leads to the new research site of the medicinal plants which were traditionally used by the people living in this region. According to the study conducted, there were traditionally more than 2,500 species of plant in India having the therapeutic purposes [7]. Though the survival of these natural resources is now threatened by vastly expanding human population and rapidly occurring concurrent environmental degradation [8-10]. These Himalayas are house of some of the most endangered and native species in the world, as well as some of the poverty- stricken people who depends on biological resources for the treatment of various ailments. In regular healthcare practices, the value of medicinal plants provides information to the latest research fields, and conservation of biodiversity is now gleaming [11].

In Himachal, the study of medicinal plant is very precise & mainly focuses on inventory, although many studies are there addressing the distribution patterns, diversity, nativity, and priority of conservation of medicinal plants [12]. There are total of 643 medicinal plants in Himachal Pradesh, in which 269 are native, 374 non-natives. Out of which 12 are critically endangered and 21 are endangered [13-16]. When the study was conducted in Himachal Pradesh, the North Western Himalaya, it was recorded that total of 357 species of medicinal plant were used by the local rural population & these plants belongs to 98 families and 237

genera. There are 27 trees, 269 herbs, 54 shrubs, 02 climber and 05 ferns in these medicinal plants. Of the 193 medicinal plants in total, 03 were native, and 43 were almost endemic [3].

In Ayurveda, other traditional medicine systems and plant-based pharmaceutical industries, medicinal plants are worn. The Tibetan medical system also depends on the species of the Himalayas [17-20]. Approximately 90% of the medicinal plant species are extracted from the wild forests [21]. Among this wild variety of medicinal plants, *Euphorbia Prostrata* is from the spurge species and is wildly known as the road side weed.

Euphorbia Prostrata is Caribbean-born and some parts of South America. Though the availabity of this plant is in abundance as it is present up to the altitude of 2000m, where it can be found in different types of habitat and grows as a weed on the roadside in many areas. This road-side weed belongs to the *"Euphorbiaceae"* family and Genus *"Euphorbia"*.

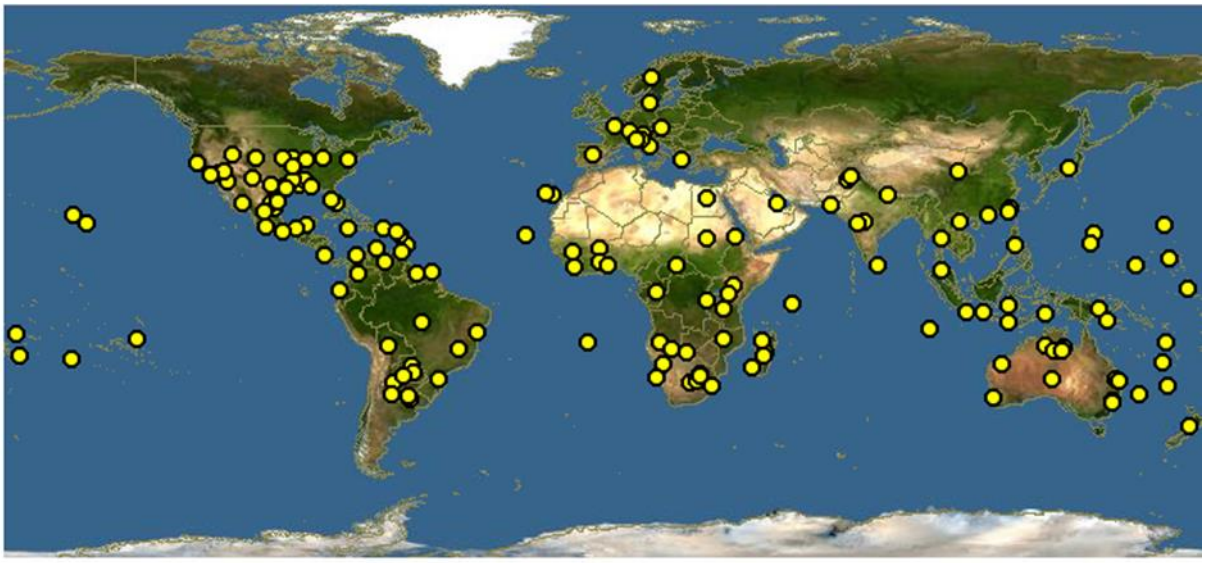

Fig1.2: Representation of availability of *E. prostrata* worldwide [22].

The *Euphorbia* genus is the widest in the spurge family *(Euphorbiaceae)* with over 2000 species. Some species of the *Euphorbia* genus have been used as medicinal plants to treat skin diseases, migraine and intestinal parasites as well as wart cures. This was possible because of the wealth of information about traditional and folk medicine due to its rich cultural heritage and relatively rich flora. All the parts of *Euphorbia Prostrata* including roots, seeds, stem barks and entire plant is used for the treatment of migraine, warts, etc due

to the healing properties of *Euphorbias* because of the presence of different chemicals found in these plants as secondary metabolites.

Plants in the *Euphorbiaceae* family are well recognized for their chemical richness in isoprenoid constituents. Due to their diverse structures and therapeutic importance, *Euphorbia* has been given increasing public exposure. It has been found that macrocyclic diterpenes of this species Euphorbia have antitumor, antimicrobial, anti-diarrheagenic, cytotoxic, anti-HIV activities & many other analgesic properties [23]. This genus contains a wide variety of terpenoids, from mono-, and diterpenes to triterpenoids and steroids. Many of these molecules have been examined for potential healing activity, and some have been used as medicinal products since prehistoric times. Due to the different compounds present in this genus makes it rich in many biological activities. So the plant *Euphobia prostrata* is studied to find the presence of such secondary metabolites responsible for various activities.

E. prostrata is a yearly herb that produces thin prostrate stems up to about 0.2 m (7.9 inches) long, sometimes purple-tinted in colour. The oval-shaped leaves are up to 0.01m (0.39 inches) long with fine dented edges. There are four male and one female flower, the latter one to two mm wide (0.039 to 0.079 in) develops into a lobed, hairy fruit.

The similarity of *E. prostrata* with other *Euphorbia species*:

E. prostrata shows similarity with *E. maculata* and *E. serpens*, but is mostly hairy on the leaves and stems part, whereas the latter two species are often smooth with less amount of leaves and stems. It is different from *E. maculata* by its less dense leaves. *E. serpens* differs as it has more rounded leaves than *E. prostrata* with larger and more amount of flowers. The *E. prostrata* flowers don’t show the white "petals" as present in *E. serpens* or *E. maculata* and thus makes it harder to differentiate between the former species.

Fig1.3: Red-tinted *E. prostrata* growing in the natural habitat.

Fig1.4: Large & dense leaf structure of *E. maculata.*

Fig1.5: Rounded broad leaves of *E. serpens.*

CHAPTER 2

OBJECTIVES

2. OBJECTIVES:

The following are the objectives of this project work:

2.1 GENERAL OBJECTIVE

To perform phytochemical screening, analyse antioxidant property & to check the antibacterial potency of *E. prostrata* extract.

2.2 SPECIFIC OBJECTIVE

- To collect & authenticate the plant *E. prostrata.*
- To obtain the crude extract of the plant using two different extraction technique.
- To perform initial phytochemical screening of the prepared extract.
- To evaluate the antibacterial & antioxidant activity of the respective extracts.

CHAPTER 3

REVIEW OF LITERATURE

. REVIEW OF LITERATURE:

This is the overview of the basic knowledge collected throughout my project work. This study includes the Botanical description, classification of the plant *E. prostrata*, antimicrobial properties, antioxidant properties, different extraction methods and its bioactive compounds responsible for all the properties.

3.1 SCIENTIFIC CLASSIFICATION:

Table 3.1.1: Scientific classification of the plant *"E. prostrata"*.

Kingdom	Plantae
Clade	Angiosperms
Clade	Eudicots
Clade	Rosids
Order	Malpighiales
Family	*Euphorbiaceae*
Genus	*Euphorbia*
Species	*E. prostrata*

Scientific name – *Euphorbia prostrata*

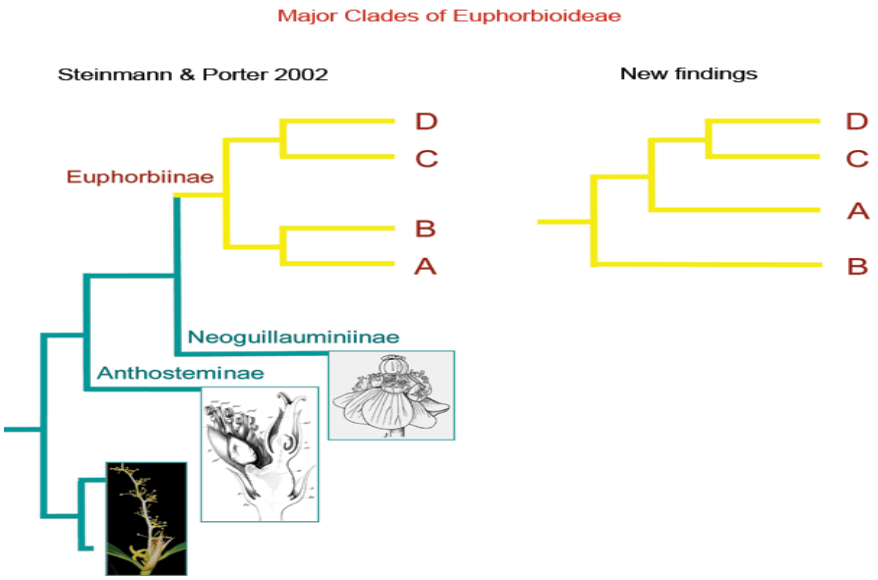

Fig 3.1: This flow chart explains the new findings of the four clade of *Euphorbiaeace* (A, B, C & D), in which *E. prostrata* belongs to clade D [33].

A (Subgenus *Rhizanthium*)

E. meloformis

'medusoid' *Euphorbia*

E. clava

Clade B (Subgenus *Esula*)

E. segetalis

E. regis-jubae

E. characias

Clade C (Subgenus *Euphorbia*)

E. officinarum subsp. *officinarum*

E. speciosa

E. duranii

Clade D (Subgenus *Chamaesyce*)

E. prostrata

E. leucocephala

E. cotinifolia

3.2 INTRODUCTION TO FAMILY – *EUPHORBIACEAE*

The spurge family, the *Euphorbiaceae*, is a fifth largest family of floral plants. They are sometimes commonly referred to as *euphorbias* in English, [24] which is also the name of a family genus. Mostly members of this family are herbs, but some are shrubs or trees, especially in the tropical region. But the species of this family are not only specific to the tropical region, some of the species are also available in non-tropical region.

The spurge family consist of three subfamilies, 37 tribes, and approximately 300 genera [25] having 7,500 species in it [26]. The leaves of this genus are mostly symmetrical in shape and are alternately aligned with stipulations, rarely the opposite. Stipulations can be limited to glands, spines or hairs as well as are often absent in luscious species. Mostly the unisexual plants have radially symmetrical flowers, normally having the female and male flowers on the same individual plant. As *Euphorbiaceae* contains the wide variety of species so mostly the structure of the flowers also differs, as well as can be found synoicous and dioecian. The family includes a wide variety of toxins (plant-produced toxic substances), mostly esters of diterpene, glycosides, alkaloids & toxins of the ricin-type. In most of the species of this genus the presence of Diterpenes were mostly found and various studies were performed based on the core framework for eg., Lathyranes [27], Jatrophanes [28], Myrsinols [29], Ingenanes [30], Tiglianes [31], etc.

What features makes *Euphorbia* different?

The feature that makes the flowers of family *Euphorbia* different from the other flowers is the presence of “cynathium” [32]. As in *Euphorbia*, the flowers are segregated into very small parts and then are distributed into a bunch of flowers or inflorescence, which is called as “cynathium”. This feature makes stunning modifications in different species of this Genus.

Cyathium's main defining feature is the envelope, & the uniqueness of the envelope surrounding every group of flower petals. The involucre mostly have one or more unique glands connected to it, but mostly connected at the top, and the shape and size of these glands and their body parts vary significantly.

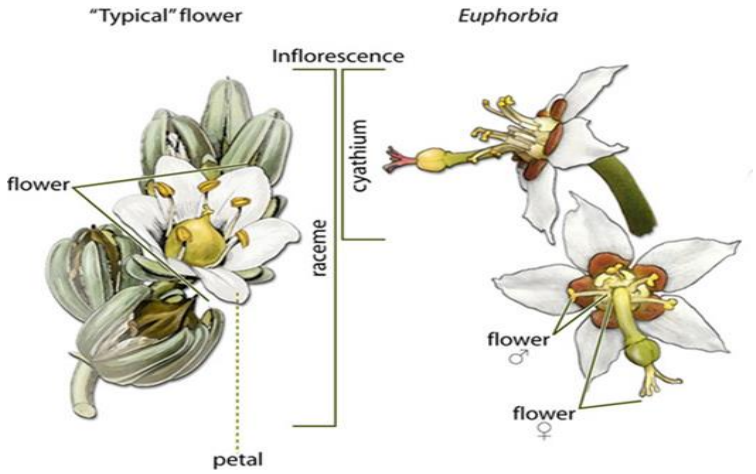

Fig 3.2: The structural difference between the typical flower and the flower of the Genus *Euphorbia* [33].

Inside the membranous envelope of the flower, there is usually a male flower containing one anther, pedicel & the filament, with the female flower in the centre holding the three-parted ovary and one pedicel, without any petals linked to it [32].

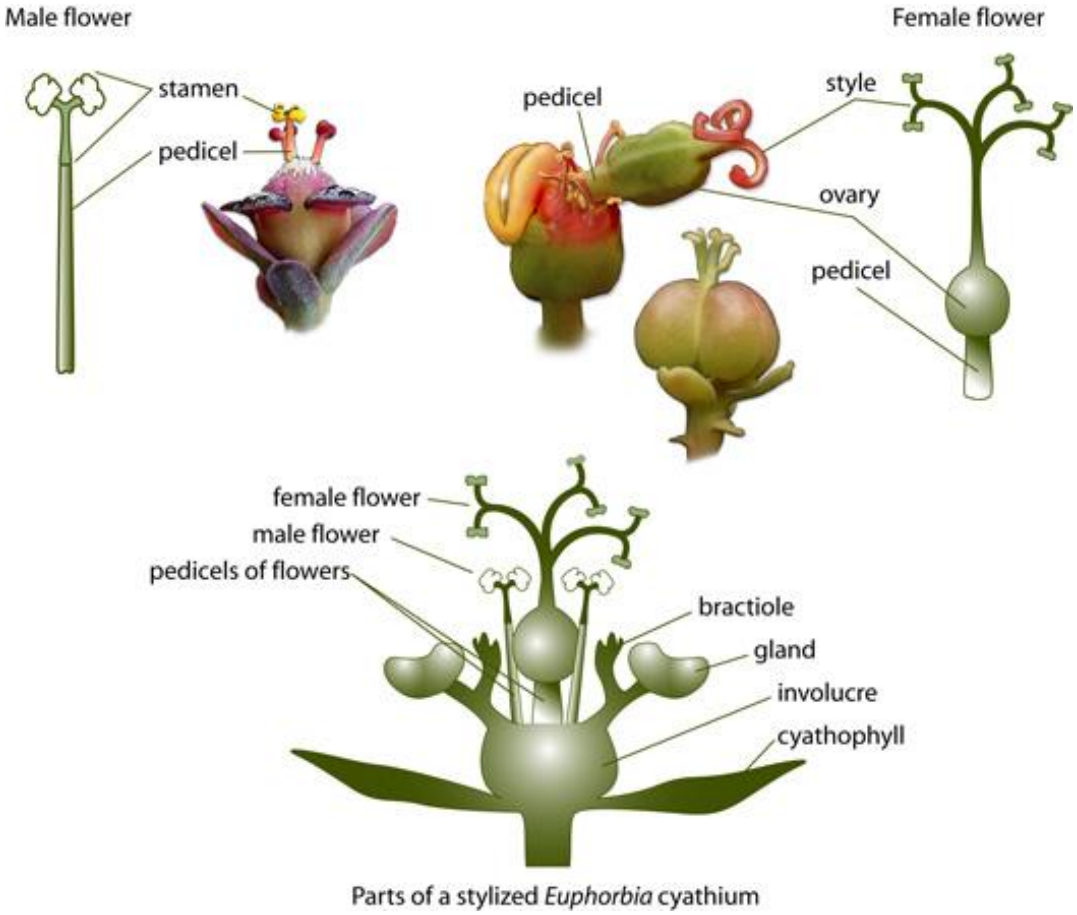

Fig 3.3: The parts of *Euphorbia* cyathium [33]

Fig 3.4: Male and female flowers (female in the front, male at the back with yellow pollen) [34].

Fig 3.5: close-up of the male flowers [34].

The plants of this family have a common characteristic property that is the presence of the milky fluid in their stems, known as “latex”. This characteristic is also present in the subfamilies of *Euphorbiaceae* , i.e, *Acalyphoideae, Crotonoideae, & Euphorbioideae*. In other subfamilies like *crotonoideae*, this milky fluid is harmless but in *euphorbioideae*, it is very toxic [35]. The toxicity level of the latex is so high that earlier it was applied on the head of the arrow as an arrow poison [36]. But on the other hand the spurge’s latex is also used as purgatives/laxatives, which are used for the treatment and prevention of constipation [37].

3.3 EARLIER REPORTED BIOLOGICAL PROPERTIES OF SPECIES *EUPHORBIA*.

Table 3.3.1: Reported biological properties of species *"Euphorbia"*

S. No	Medicinal property	Plant species tested	References
1	Acetyl cholinesterase inhibiting activity	*E. hirta*	Anuradha et al., 2010 [38]
2	Aniline hydroxylase inhibition activity	*E. tirucalli*	Patil and Magdum, 2012 [39]
3	Antibacterial activity	*E. carcasana* *E. cotinifolia* *E. fusiformis* *E. hirta*	Rojas et al., 2008 [40] Rojas et al., 2008 [40] Natarajan et al., 2005 [41] Basma et al., 2010 [42]
4	Antidiabetic activity	*E. hirta*	Kumar et al., 2010 [43]
5	Antidiarrhoeic activity	*E. hirta*	Galvez et al., 1993 [44]
6	Antifungal activity	*E. granulate* *E. hirta.*	Ahmad et al., 2012 [45] Basma et al., 2010 [42]
7	Antihelminthic activity	*E. thymifolia*	Kane et al., 2009b [46]
8	Antihyperglycemic activity	*E. thymifolia*	Mohammed Rahamatullah et al., 2012 [47]
9	Anti-inflammtory activity	*E. prostrata*	Singla and Pathak, 1990 [48]
10	Antimicrobial activity	*E. balsamifera* *E. macrorrhiza* *E. prostrata* *E. tirucalli*	Kamba and Hassan, 2012 [49] Lin et al., 2012 [50] Ahmad et al., 2011 [45] Upadhyay et al., 2010 [51]
11	Antinociceptive activity	*E. thymifolia*	Mohammed Rahamatullah et al., 2012 [47]
12	Antioxidant activity	*E. hirta,* *E. prostrata*	Basma et al., 2010 [42] Ahmad et al., 2011 [52]
13	Antispasmodic activity	*E. granulate*	Ahmad et al., 2012 [45]

S. No	Medicinal property	Plant species tested	References
14	Antitumor activity	E. macrorrhiza	Lin et al., 2012 [50]
15	Antiviral activity	*E. arenaria* *E. hirta*	Betancur-Galvis et al., 2002 [53] Gyuris et al., 2009 [54]
16	Cytotoxic activity	*E.arenaria* *E. hirta* *E. kansui* *E. macroclada* *E. pulcherrima* *E. tirucalli*	Betancur-Galvis et al., 2002 [53] Raja Sidambaram et al., 2011 [55] Farong Yu et al., 2008 [56] Sadeghi-Aliabadi et al., 2009 [57] Betancur-Galvis et al., 2002 [53] Betancur-Galvis et al., 2002 [53]
17	Diuretic activity, Diuretic & laxative activity	*E. fusiformis* *E. thymifolia*	Ashok et al., 2011 [58] Kane et al., 2009a [59]
18	Hepatoprotectivity	*E. fusiformis*	Anusuya et al., 2010 [60]
19	Immuno-modulatory activity	*E. hirta*	Ramesh and Padmavathi, 2010 [61]
20	Insecticidal activity	*E. antiquorum*	De Silva et al., 2008 [62]
21	Larvicidal activity	*E. tirucalli*	Yadav et al., 2002 [63]
22	Microbicidal activity	*E. tirucalli*	Prasad et al., 2011 [64]

Among the large family of *Euphorbiaceae* (more than 4,500 species), the mostly studied plant species is *E. hirta* and showed various activities like Antimicrobial activity against the wide spectrum of microbes i.e. *E. coli, P. aeruginosa, P. vulgaris, and S. aureus* [65]. Previous work, however, focused on the small section of this family. Even up to date all the species are not studied for the bioactive components. Among all the species *E. prostrata* is

the one which is less explored scientifically in Himachal Pradesh and on which environmental changes are not observed experimentally.

3.4 INTRODUCTION & COMMON NAMES OF *EUPHORBIA PROSTRATA*:

The availability of the plant is in abundance as it is an annual herb, so there is no need of any specific cultivation method and grows throughout the year if sufficient water availability is there. It mostly grows on the sandy soils, gardens, disturbed places, roadside, from the cracks and grows up to the altitude of 2000 m [66-67]. After germination the plant only takes 12-14 weeks for flowering and production of fruits. This is possible because it has a prolific reproductive capacity for seeds and grows rapidly during the rainy season [68]. As it is a naturalized weed and found throughout India for its local medical use in different languages. Here is the list of local names of *E. prostrata* which are called throughout India.

Table 3.4.1: Local names of *E. prostrata* [69]

Languages	Local names
Sanskrit	Goraksadugdhi, Ksirini, Laghudugdhika, Nagarjuni, svaduparni
Hindi	Dudhi, Duddhi, Dudhdee, Chhotidudhi
English	Roadside weed, red sandmat, prostrate sandmat, ground spurge
Punjabi	Dodhak, Baradodk, Hajardana, Hazardana
Gujarati	Naagalaa dudhelee, Raati Dudhelee
Bengali	Bara, Dudiya, Kerai, Kharui, Shwet Keruee
Marathi	Lahaandudhi, Lahaan naaytee, Naayeti,
Malayalam	Nilappal
Tamil	Sittirappaladi
Telugu	Peddivari manubaala
Kannada	Kempu nene hakki
Urdu	Dudhi

Synonyms by which they are known throughout the world, as well as recognized by ITIS taxonomy hierarchy & NCBI.

Table 3.4.2: Synonyms for *E. prostrata* recognized by ITIS taxonomy hierarchy & NCBI. [70]

Synonyms
1. Anisophyllum prostratum (Aiton) Haw.
2. Aplarina prostrata (Aiton) Raf.
3. Chamaesyce boliviana (Rusby) Croizat
4. Chamaesyce malaca Small
5. Chamaesyce prostrata
6. Chamaesyce prostrata (Aiton) Small
7. Chamaesyce villosior (Greenm.) Millsp.
8. Euphorbia boliviana Rusby
9. Euphorbia callitrichoides Kunth
10. Euphorbia chamaesyce
11. Euphorbia malaca (Small) Little
12. Euphorbia perforata Guss.
13. Euphorbia prostrata Aiton
14. Euphorbia prostrata var. caudirhiza Fosberg
15. Euphorbia prostrata var. vestita Engelm. ex Boiss.
16. Euphorbia ramosa var. villosior Greenm.
17. Euphorbia tenella Kunth
18. Euphorbia trichogona Bertol.
19. Tithymalus prostratus (Aiton) Samp.

3.5 THERAPEUTIC USES OF THE PLANT *"EUPHORBIA PROSTRATA"*:

The whole plant of *E. prostrata* was used as the medicinal plant traditionally, so here are some of the traditional uses listed below [66]:

- In Togo, the conc. Liquor resulting from heating or boiling of the leaf was drunk to treat the risky abortion.
- In Benin to treat the irregular mensuration, the aerial parts of the plant were consumed with pounded shells.
- The leaves soaked water was given to the mother to prevent difficult childbirth.
- To treat amoebic dysentery, the raw crushed leaves were consumed.
- In Nigeria, the plant's conc. Liquor was taken out because of its anthelmintic and astringent properties.
- To cure the fungal infections, the leaves conc. Liquor was consumed orally.
- In Kenya, the raw plant is consumed to cure gonorrhoea.
- To prevent miscarriage, the mixture of boiled shoots with sesame was given to the pregnant women.
- To cure stomach-ache & diarrhoea, the mixture of other plants with this plant is consumed.
- The bath with this plant was taken to heal measles and other skin diseases.
- As the first aid for wasp and scorpion sting, the leaves of this plant was rubbed and wrapped around the sting area.
- To treat the oral sores, the leaf soaked water was used as the mouthwash and for the gargle purposes.
- To induce labour, the plant juice was taken.
- The whole plant conc. Liquor was used as the eye wash against conjunctivitis.
- To cure headache, the palm oil was mixed with the powdered plant and then applied on the head.
- To treat the fungal infection and insect bite in DR Congo, the leaves were warmed on the fire and then the juice of the plant was squeezed over the infected area.
- To treat the painful mensuration and female sterility, tiny spherical balls of the plant was inserted inside the genitalia.
- Whole plant was crushed, spreaded & tied with the cloth to reduce the pain & swelling of the broken arms.

- To treat warts and ulcers, the milky fluid of the plant known as latex was applied over the infected area.

3.6 DIFFERENT TYPE OF EXTRACTION TECHNIQUES:

The different types of extraction method used to obtain the desired extract are listed below [71]**:**

I. **Tissue homogenization:** In this extraction technique the plant in any state either dried or fresh, is grinded in to fine powder & soaked in the solvent for 24 hrs or vigorously shaken for atleast 10 min & filtered. After collecting the filtrate, it is dried using the rotary evaporator to get the crude extract & is dissolved again in the solvent to find out the concentration. This technique is broadly used by the researchers [72].

II. **Exhaustive extraction (fractionation):** It is the other commonly used technique for obtaining the extract. In this method the dried plant sample is successively soaked in various solvents having the higher polarity then the previous solvent used. This procedure is followed to ensure that the compound from the wide range of polarity could be collected [72]

III. **Soxhlet extraction:** This method is only used when a particular compound is not very soluble in the solvent, whereas the impurities are completely insoluble in that particular solvent. This method is only preferred because less amount of solvent is used as the same batch of solvent can be used. If the compound is highly soluble in the solvent then simple filtration method can be used. Due to the long duration of heating, however it is not recommended for the thermolabile constituents as it may degrade the compound [73].

IV. **Cold maceration:** This method is mostly used for thermolabile compounds, as in this method the solvent with the powdered sample is stored in a closed container for a specific time period with occasional stirring after every 24 hr [74].

V. **Decoction:** In this method the powered plant sample is soaked in the water for 2-4 hr and then boiled for 15-20 min to make a drug like consistence after which a sufficient amount of cold water was added to the mixture to make-up the volume as required. This method can be used for both heat stable and water soluble compound [75]

VI. **Infusion:** It is used for the highly soluble constituents of the powdered plant sample. It can be prepared freshly by macerating the powder for a short span of time in the cold or hot boiling water [75].

VII. **Digestion:** this is the other kind of maceration in which the mixture of sample soaked in water was supplied some gentle heat to accelerate the solvent efficiency. This procedure is only used when slight increase in the temp. isn't objectionable [75].

VIII. **Percolation:** this method is used to obtain the liquid extract. For obtaining the liquid extract the instrument named as percolator is used which is conical in shape with both opening ends. In this the powdered sample was soaked in the desired amount of solvent & left undisturbed for 4 hr, after this the residue was packed in the percolator and the top was closed. The solvent is added to make a thin layer of solvent over the residue. This mixture was closed in the percolator & allowed to macerate for 24 hrs. Then the collected liquid is allowed to slowly drip in to the container. The solvent was again added to the mass until the collected liquid from the percolator measures the three-quarter volume of the required product. The sufficient amount of solvent is added to make-up the required volume & finally the liquid is purified by decanting or by simple filtration [76].

IX. **Sonication:** this method is useful in only some cases, for eg. to collect the extract from rauwolfia root, it is not commonly used because of its higher cost. This is helpful to increase the penetration power of the cell walls & produces voids in the structure, as it uses the ultrasound waves ranging from 20,000 Hz to 2,000,000 Hz. The major disadvantage of this method is the use of higher energy ultrasound wave that may cause the damage to the bioactive compounds present in the medicinal plants by forming the free radicals & consequently changing the drug molecule undesirably [76].

3.7 IMPORTANCE OF BIOACTIVE CONSTITUENTS:

3.7.1 TANNIS: This active component belongs to the class of astringent that is a polyphenolic biomolecules that binds to various organic compounds like amino acids, proteins, alkaloids, etc & forms the precipitate. Hydrolyzable tannins, phlorotannins & phlobatannins are the major classes.

i. Hydrolyzable tannins: These are the form of tannins which produces ellagic or gallic acids, when heated with sulphuric or hydrochloric acids. The major source of these tannins is plants & the base unit is Gallic acid.

ii. Phloroglucinol: These are the form of tannins majorly found in brown algae, and are generally oligomers of the compound phloroglucinol, which is the base unit of these types of tannins.

iii. Phlobatannins: These are the water- insoluble tannins but are soluble in alcohols and are mostly found in plants. For these types of tannins the base unit is “flavan-3-ol’s scaffold”.

Tannins, which are mostly polyphenols but some are also antioxidants both of these types plays a major role in our body as it has multiple healing properties that are mentioned below:

- Antioxidants, as they reduce the amount of free radicals in our body thus protect us from heart diseases.
- As the main cause of cancer is the abnormal cell division which causes the destruction of body tissue, but due to its antioxidant properties it prevent the cell damage.
- Due to the easy consumption of resveratrol & catechin found in grapes & black/ green tea it shows various health benefits.

3.7.2 FLAVONOIDS: This active compound belongs to the class of fungus & plant secondary metabolites. These compounds are further divided in 3 main classes that are bioflavonoids, isoflavonoids & neoflavonoids. The major role of flavonoids in the plants is to provide the pigmentation. The dark colour of the vegetable, fruit, leaves, etc is directly proportional to the antioxidant property of the plant. There are many health benefits due to the presence of flavonoids which are:

- The free radical scavenging property prevents cancer.
- Secrets the anti-inflammatory mediators, for eg- procyanidins.
- Delays the aging mechanism.
- Reduce the blood pressure thus reduce the risk of hypertension.
- Various flavonoids show the antibacterial activity against the bacterial infection**.**

3.7.3 ALKALOIDS: This active compound belongs to the class of organic compounds that contains nitrogen atoms. These compounds are further divided into 5 major groups:

i. True alkaloids - these are basically originated from amino acids & contains the basic nitrogen atom in the form of heterocycle.

ii. Protoalkaloids – this is similar to that of true alkaloids but it is not in the form of heterocycle.

iii. Polyamine alkaloids – these are the form of derivates of "spermine, putrescine & spermine".

iv. Cyclopeptide & peptide alkaloids

v. Pseudoalkaloids- these type of alkaloids are not originated from amino acids.

3.7.4 GLYCOSIDE: This is a molecule made up of the sugar bounding the other functional group which is linked through a glycosidic bond. Using the enzyme hydrolysis the inactivated glycosides, which are stored by the plants can be activated. This hydrolysis process breaks the sugar part & makes the chemical which is ready for use. These forms of chemicals or the plant glycosides are used for medications.

Example: Salicin ($C_{13}H_{18}O_7$) was the earlier known origin of Aspirin & is also related to it chemically.

CHAPTER 4

MATERIAL & METHODS

4.1 COLLECTION & AUTHENTICATION OF PLANT:

The whole plant of *E. prostrata* was collected from the college premises of Jaypee University of Information Technology, Waknaghat at the elevation of 1,544m (Lat, 31.02°N; long, 77.08°E) district Solan, Himachal Pradesh, India. The herbarium of the collected plant was authenticated from, Department of Forest Products, Dr Y.S Parmar University of Horticulture and Forestry, Nauni, district Solan, Himachal Pradesh, India.

Fig 4.1.1: Herbarium of the whole plant of *E.prostrata.*

The collected plant was washed with the regular tap water, and then surface sterilized with 70% ethanol that includes 0.1% of a non-toxic emulsifier i.e tween20 solution to prevent contamination of any microbes. The sample was then finally rinsed with the distilled water and allowed to shade dry for 2 days and then grinded into powder using electric blender.

4.2 EXTRACT PREPARATION:

i. **Using cold maceration extraction technique:**

Twenty grams of powdered plant sample was fractionated into polar (methanol) and non-polar (hexane) solvents. Up to 10gm of the powdered sample was soaked in 100ml of methanol and hexane solvent for 1 week each with the brick 30 min shaking after every 24 hrs. After one week the samples were first filtered with normal filter paper then followed by Whatmann filter paper No.1 and concentrated under vacuum by use of rotary evaporator to yield the respective crude extracts.

ii. **Using decoction extraction technique:**

Ten grams of powdered plant sample was soaked in 100ml of distilled water for 6 hr in the normal room temp and then kept overnight in the refrigerator to prevent the growth of fungi. After 24 hr, the plant soaked water was boiled for 1 hr and then filtered with the normal filter paper, followed by Whatmann filter paper No.1. Filtrate was collected in the round bottom flask & then kept in the -80˚c refrigerator to deep freeze the filtrate. After deep freezing, sample was lypholized overnight to get the powdered extract.

Both the obtained crude extracts were stored at 4˚c in a microcentrifuge tube until further use.

4.3 GENERAL LABORATORY PRACTICES:

The general lab guidelines for the cleaning of the glassware, preparation the inoculation, media, maintenance of chemical and culture, etc, are listed below:

4.3.1 CLEANING OF THE GLASSWARE:

Before starting the experimentation, all the glassware was kept in the solution of Aqua regia (hydrochloric acid mixed with nitric acid in 3:1 molar ratio). After this, all glassware was thoroughly washed with the mild detergent solution and then finally rinsed with distilled water. The washed glassware was kept on the normal filter paper in the dust free area to remove the excess of water and then dried in the oven at 100˚c for 1 hr.

4.3.2 STERILIZATION:

Test tubes (covered with cotton plugs), pipette tip box, distilled water, eppendrof tubes (microcentrifuge tubes), discs, broth and media were autoclaved at 15 psi of pressure for 30 mins. Bunsen burner was used to sterilize the inoculating loop, tip of the forceps, mouth of the culture tubes, & tips to puncture the media plate. To sterilize working platform of the laminar hood Ethanol was used.

4.3.3 CHEMICALS AND MEDIA:

All the chemicals, solvents & reagents used in the project were of analytical grade. Most of the chemicals and solvents were obtained from Merck, India and Sigma-Aldrich Chemical Co. The media used was from Hi- media Ltd., Mumbai.

4.4 PHYTOCHEMICAL SCREENING:

The phytochemical screening of the obtained crude extracts of *E. prostrata* was done to detect the bioactive constituents present in the plant. The expression of the desired therapeutic efficact is total based on these bioactive constituents. So for the identification of these compounds in the aqueous and methanol crude extract, the following chemical test were performed using the standard procedure [77]:

I. **Test for tannins:** Water was mixed with 2gm of extract. The solution of the extract was heated using the water bath & filtered afterwards. The dark green colour of the filtrate after the addition of 3-4 drops of ferric chloride indicated the presence of tannins.

II. **Test for anthraquinones:** 10% HCl was mixed and boiled with 1gm of each extract for a couple of minutes. The mixture was allowed to cool down and then filtered. The filtrate was then mixed with equal vol. of $CHCl_3$. The mixture was then heated again with 3-4 drops of 10% NH_3. The anthraquinones were indicated due to the appearance of rose- pink color.

III. **Test for flavonoids:** the mixture of 10% NaOH & 2M HCl in 500mg of each extract was prepared. The presence of flavonoid is detected, if the yellowish color of the solution changes to colourless.

IV. **Test for steroids:** The crude extract was mixed with 2ml $CHCl_3$ and conc. H_2SO_4. The formation of red- coloured layer below the chloroform layer indicated the existence of steroids.

V. **Test for terpenoids (salkowishki test):** the mixture of 500mg of extract with 2ml chloroform followed by 3ml of conc. Sulphuric acid was prepared. The reddish coloured layer formation between the solutions indicated the presence of terpenoids.

VI. **Test for diterpenes:** Water was mixed with 2g of extract followed by addition of few drops of $Cu(CH_3COO)_2$. The appearance of emerald green colour indicated the presence of diterpenes.

VII. **Test for alkaloids:** 2 grams of the extract was heated with 2% of sulphuric acid for few mins. The formation of orange ppt in the filtrate, after the addition of few drops of dragendrof's reagent shows the existence of alkaloids.

VIII. **Test for saponins:** The aq. solution of the crude extract was prepared by adding 5ml of distilled water. Then the test tube was shaken vigorously for about 1-2 min. After 30 mins, the presence of stable honeycomb like structured froth indicated the presence of saponins.

4.5 ANTIMICROBIAL SUSCEPTIBILITY TEST:

4.5.1 USING WELL DIFFUSION METHOD:

The well diffusion method was used to determine the antimicrobial activity of the *E. prostrata* crude extract against various bacterial strains such as *E. coli DH5α, E. coli ATCC 25922, S. typhi, S. aureus, B. subtilis & M. spegetis.* The inoculum tube containing each strain was prepared using the Luria Broth obtained from Hi-media laboratories Pvt Ltd. After the incubation of the inoculum tubes for 24 hr, 100µl of the pure culture was spread over the Luria agar petri plates using Luria agar or Nutrient agar obtained from Hi-media laboratories Pvt Ltd. 200µl of 5% DMSO & two different concentrations of crude extract (10mg/ml & 50mg/ml conc.) was transferred in each of the punched wells. The plates were observed after 24 hrs of incubation at 37˚C for the presence of zone of inhibition means the clear zone around the wells which were free from bacterial growth. The whole experiment was performed under the Laminar Hood.

4.5.2 USING BAUER KIRBY METHOD:

The Bauer Kirby (disc diffusion) method was used to determine the antimicrobial activity of the *E. prostrata* crude extract against various bacterial strains such as *E. coli DH5α & E. coli ATCC 25922* using the antibiotic as the standard strain. The inoculum tube containing each strain was prepared using the Mueller- Hinton Broth (MHB) obtained from Hi-media laboratories Pvt Ltd. After the incubation of the inoculum tubes for 24 hr, 100µl of the pure culture was spread over the MHA petri plates using MHA obtained from Hi-media laboratories Pvt Ltd. 10µl of 5% DMSO & 3 different concentrations of crude extract (1mg/ml,10mg/ml & 50mg/ml conc.) was transferred in each of the discs. The plates were observed after 24 hrs of incubation at 37˚C for the presence of zone of inhibition means the clear zone around the discs which were free from bacterial growth. The whole experiment was performed under the Laminar Hood.

4.6 ANTIOXIDANT ACTIVITY:

The ABTS & DPPH free radical scavenging assays were evaluated to check the antioxidant activity of *E. prostrata* crude extracts.

4.6.1 ABTS RADICAL SCAVENGING ASSAY:

7mM of ABTS solution (2, 2-Azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) was reacted with 2.45mM of potassium persulfate solution, both obtained from Sigma-Aldrich Chemical co. & Merck India respectively, in the ratio of 1:1 volume by volume (v/v). 80% pure ethanol was used to prepare the solutions of ABTS and Potassium persulfate. Both the solutions were mixed & kept overnight, allowing the mixture to form the $ABTS^{+}$ radical cation, which shows max. Absorbance at 734nm & appears dark blue in color. In 1450µl of above freshly prepared reagent, 50µl of each Methanol extract, two-fold diluted Methanol extract, Aq. extract & two-fold diluted Aq. Extract was added & left undisturbed in the dark area for 30 mins. The antioxidant potential of the samples was indicated by the decrease in the OD at 734nm due to the decrease in the $ABTS^{+}$ free radical cation which leads to the scavenging property of the samples.

The formula used for calculating the "Percentage Antioxidant activity is as follows:

$$\text{Antioxidant activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100$$

Where, Absorbance $_{blank}$ = OD_{734nm} of the reagent without sample at 0mins.

Absorbance $_{sample}$ = OD_{734nm} of the reagent containing sample at 30mins.

4.6.2 DPPH RADICAL SCAVENGING ASSAY:

DPPH stands for 2, 2-diphenyl-1-picrylhydrazyl reagent, which is a crystalline powder containing stable free-radical entities. The powder is dark in colour & changes to deep purple colour after the dissolution. 11.8mg of DPPH powder (from Sigma-Aldrich Chemical Co.) was mixed with 100ml of 80% ethanol. The deep purplish solution prepared after the dissolution gives the maximum absorption at 517nm. 250µl of each aqueous extract & methanolic extract was added to 1250µl of freshly prepared solution & finally left undisturbed in the dark area for 30 mins. After 30mins of incubation, the antioxidant potential of the samples was observed at 517nm. The decrease in the OD at 517nm indicates the decrease in the DPPH free radicals present in the solution which is directly proportional to the scavenging property of the samples. The formula used for calculating the "Percentage Antioxidant activity is as follows:

$$\text{Antioxidant activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100$$

Where, Absorbance $_{blank}$ = OD_{517nm} of the reagent without sample at 0mins.

Absorbance $_{sample}$ = OD_{517nm} of the reagent containing sample at 30mins.

CHAPTER 5

RESULTS

5.1 PHYTOCHEMICAL SCREENING OF METHANOL & AQ EXTRACT:

Table 5.1.1: Phyto-constituents present/ absent in both the extracts.

Phytochemicals	Cold macerated methanol extract	Decoction for aqueous extract
Tannins	+	+
Anthraquinones	-	-
Flavonoids	++	+
Terpenoids	-	-
Diterpenes	-	-
Alkaloids	+	-
Steroids	-/+	+
Glycosides	+	-
Saponins	+	+

I. PHYTOCONSTITUENTS PRESENT IN METHANOL EXTRACT:

Fig 5.1.1: tannins present

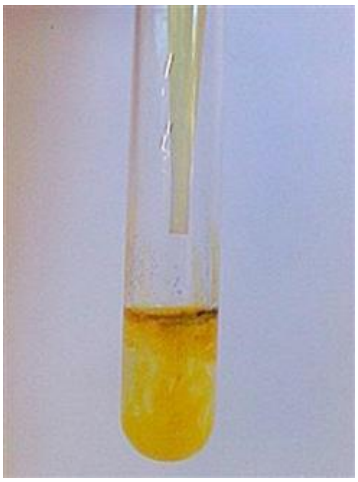

Fig 5.1.2: flavonoid present

Fig 5.1.3: alkaloids present

Fig 5.1.4: saponin present

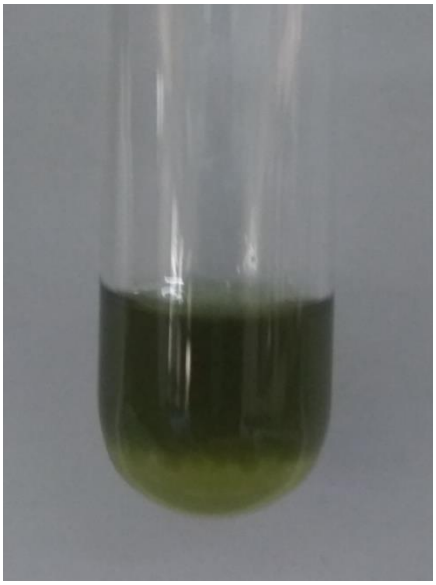

Fig 5.1.5: steroid present

II. PHYTOCONSTITUENTS PRESENT IN AQUEOUS EXTRACT:

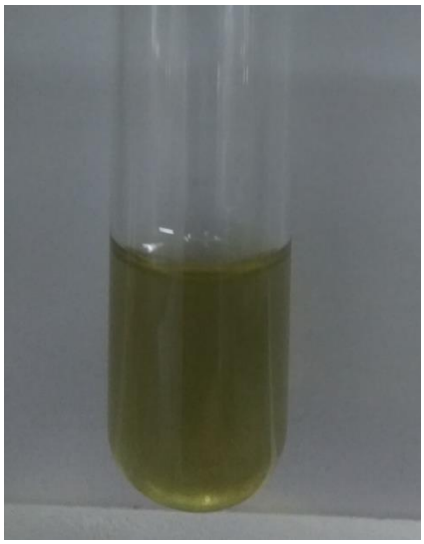

Fig 5.1.6: tannins present

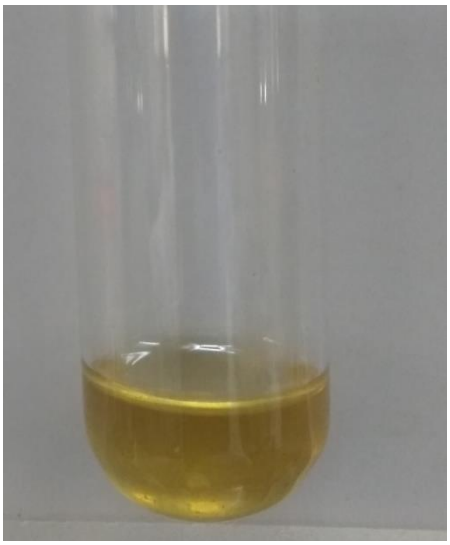

Fig 5.1.7: flavnoid present

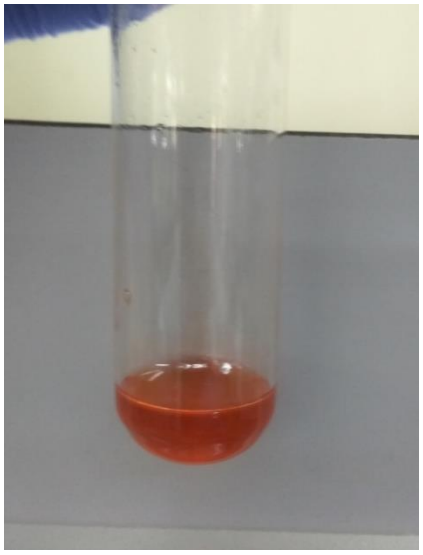

Fig 5.1.8: alkaloids present

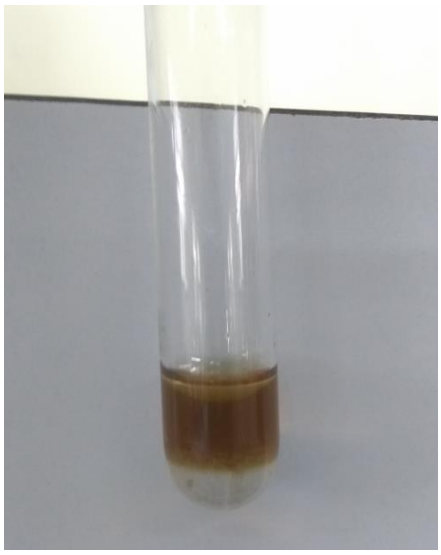

Fig 5.1.9: steroids present

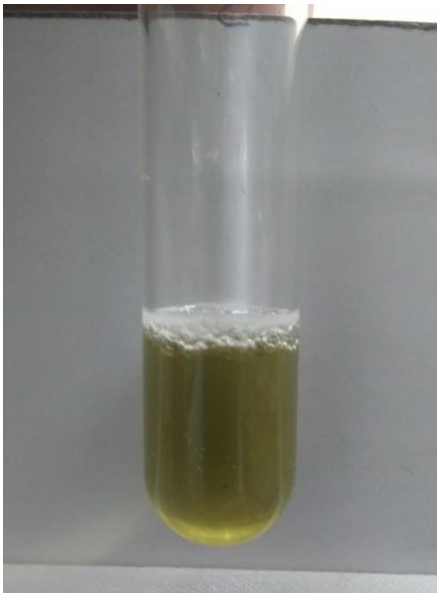

Fig 5.1.10: saponins present

4.2 ANTIBACTERIAL ACTIVITY RESULTS:

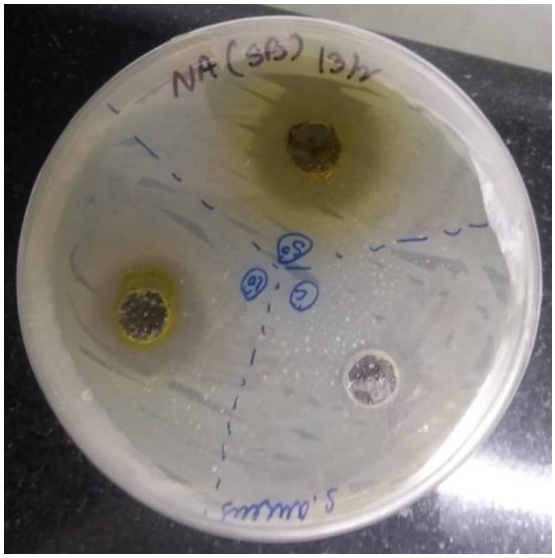

Fig 5.2.1: AST against *S. aureus*

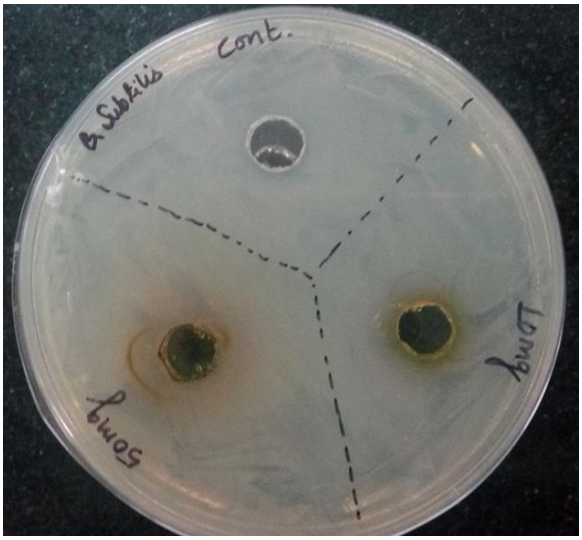

Fig 5.2.2: AST against *B. subtilis*

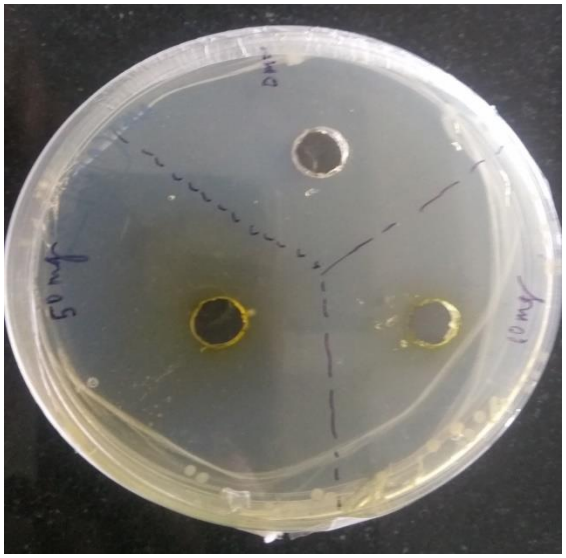

Fig 5.2.3: AST against *M. smegmatis.*

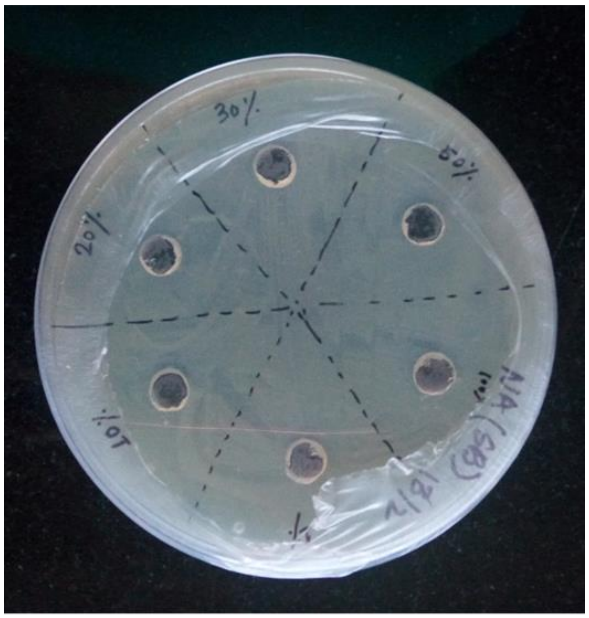

Fig 5.2.4: AST against *E. coli DH5α*

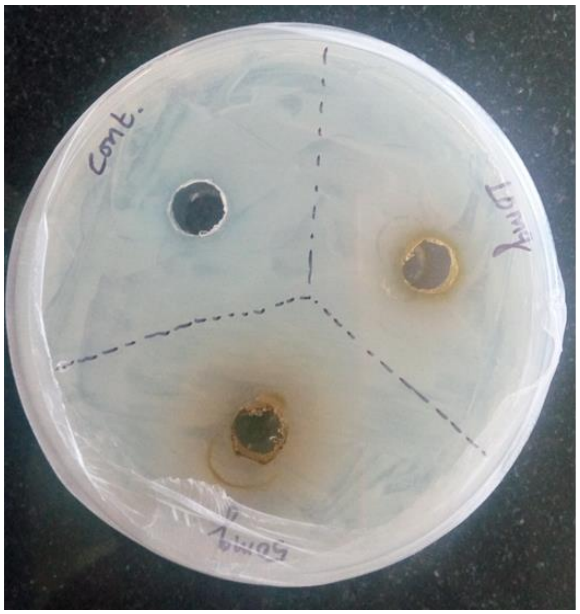

Fig 5.2.5: AST against *E. coli ATCC 25922*

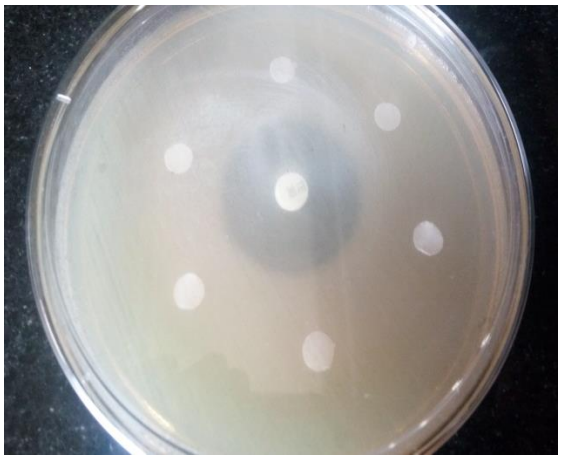

Fig 5.2.6: AST against *E. coli 87$^{5}i^{LF}$*

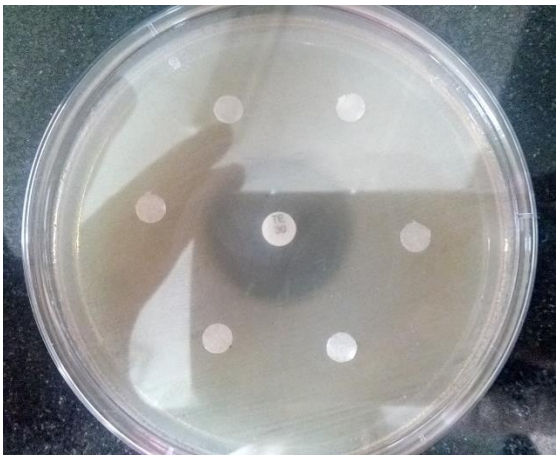

Fig 5.2.7 : AST against *E. coil 84$^{5}ii^{LF}$*

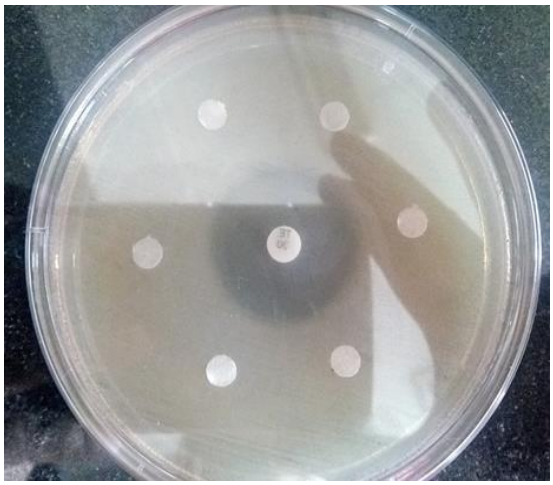

Fig 5.2.8: AST against *E. coli5Iii.*

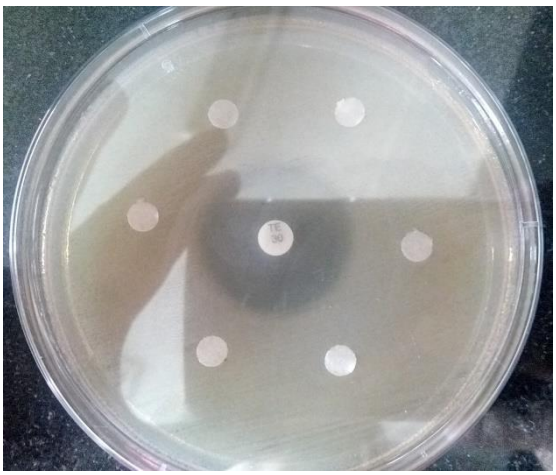

Fig 5.2.9: AST against *E. coli12dII.*

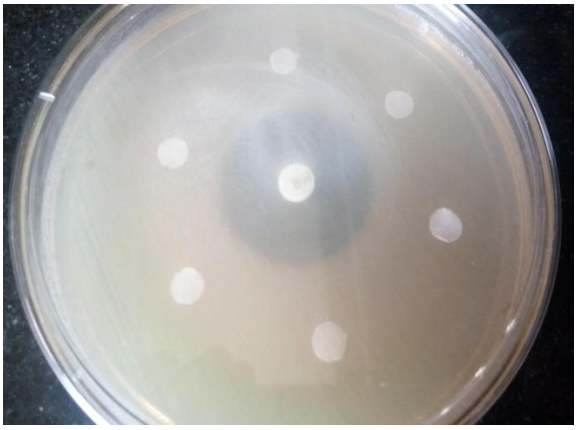

Fig 5.2.10: AST against *S. typhi*

5.3 ANTIOXIDANT ACTIVITY RESULTS:

5.3.1 ABTS FREE RADICAL SCAVENGING ASSAY:

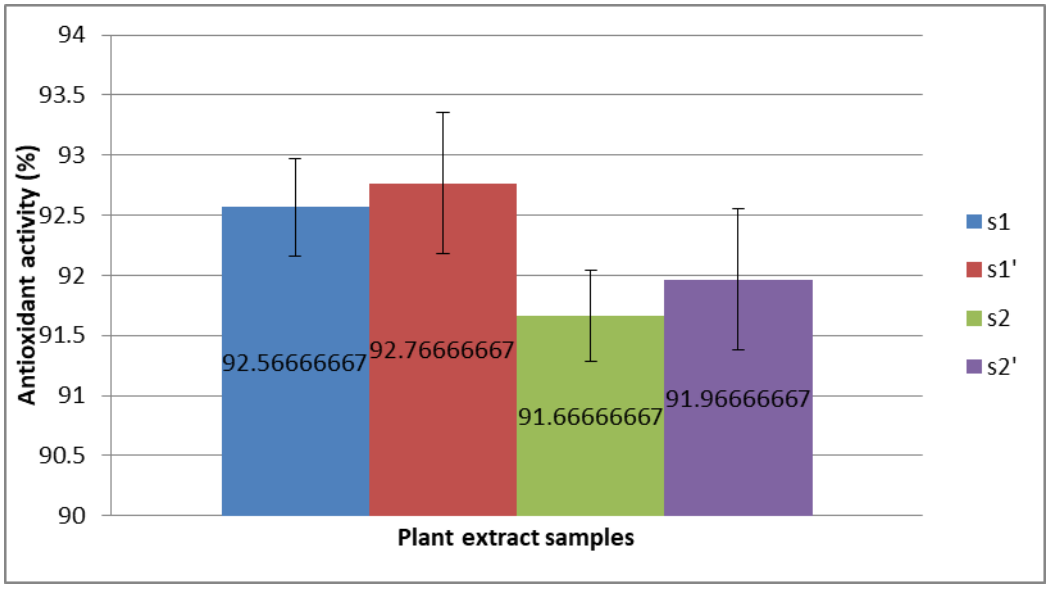

Fig 5.3.1: "percentage antioxidant activity $_{ABTS}$" for the samples s1, s1', s2 & s2' at OD $_{734nm}$

Where, s1= methanol extract of *E. prostrata* obtained from cold maceration technique.

S1'= 2-fold diluted methanol extract of *E. prostrata* obtained from cold maceration technique.

S2= aqueous extract of *E. prostrata* obtained from decoction ext. method.

S2'= 2-fold diluted aqueous extract of *E.prostrata* obtained from ext. method.

5.3.2 DPPH FREE RADICAL SCAVENGING ASSAY:

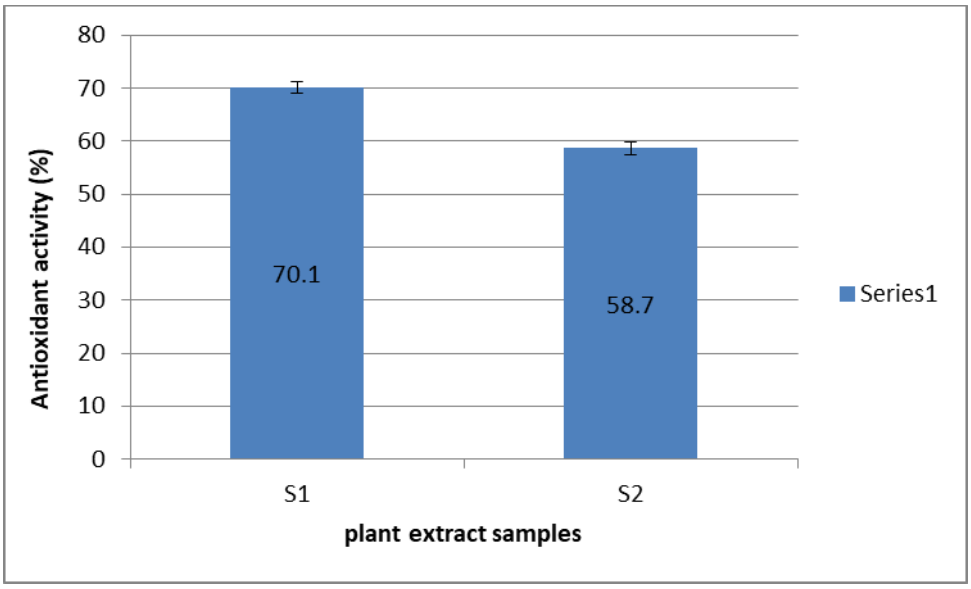

Fig 5.3.2: "percentage antioxidant activity $_{DPPH}$" for the samples S1 & S2 at $OD_{517nm.}$

Where, S1= methanol extract of *E. prostrata* obtained from cold maceration technique.

S2= aqueous extract of *E. prostrata* obtained from decoction extraction technique.

CHAPTER 6

DISCUSSION & CONCLUSION

The plant *E. prostrata* was collected from the college premises of JUIT, Waknaghat, distt. Shimla, (H.P.). Aqueous & methanol extract were prepared by decoction extraction method & cold maceration technique respectively. From the initial phytochemical screening we found that methanol extract contains tannins, flavonoids, alkaloids, steroids & saponins and aq. Extract contains tannins, flavonoids, steroids, glycosides, & saponins. This could be attributed to the difference in polarities of the solvents used (methanol & water) & also due to the different extraction techniques used.

Further the antibacterial activities of this plant's extracts was carried out against *S. aureus, B. subtilis & M. smegmatis* - 3 gram positive bacterial strains & *E. coli DH5α, 12dII, E. coli ATCC 25922, S. typhi, 87^5i^{LF}, 84^5ii^{LF}& 5Iii* -7 gram negative bacterial strains. Mushtaq ahmad et al. [78], have reported that *E. prostrata* plant extract is active against both Gram positive & gram negative bacterial strains. However from the results of antibacterial activity of this study, we found that *Euphorbia prostrata* is more active against the gram positive strains. The possible reason behind this difference in activity could be due to the fact that we have reported *E. prostrata* from the Himachal Pradesh region while the study by Mushtaq Ahmad et al., [78] has reported the *E. prostrata* plant from district Bannu, Pakistan region. Moreover, types of methods used for the preparation of plant extracts were also different in both the studies.

The antioxidant study done shows that the methanol extract was more antioxidant than the aqueous extract as the percentage antioxidant activity calculated from ABTS & DPPH was 92.7% & 70.1% respectively.

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