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Himalrandia tetrasperma, ethanolic extracts preliminary phytochemical analysis, antibacterial and antifungal activities

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Key words: Himalrandia tetrasperma, Phytochemical analysis, Antibacterial, Antifungal activities, Disc diffusion method.

Abstract

The current piece of research was designed to determine the anti-microbial potential of root, stem and leaves of selected medicinal plant *Himalrandia tetrasperma* against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Salmonella typhi,* (gram-negative) *Staphylococcus aureus, Bacillis subtillis, Bacillis cereous* (gram-positive) bacteria and *Candida albicon* by disc diffusion method. Ethanol extracts of root, stem and leaves of *Himalrandia tetrasperma* were applied in concentration of 1mg and 2mg/disc in 6µl and 12 µl volumes. Antibiotics Azithromycin, Ciprofloxacin and Chlotrimazole were applied 6 µl/disc separately as positive controls for gram positive and gram negative bacteria and *Candida albicon*. Dimethylsulfoxide (DMSO) was applied 6 µl/disc separately as negative controls. These plates were then incubated at 37c° for 24 hours. The diameter of zone of inhibition was taken as a measure of antimicrobial effect of the extracts. All the parts of selected plants show sensitivity against selected microbial species. Phytochemical screening of the plants show that most of the active phytochemical, for which tests were carried out, were present in various parts of the plants. Analysis were carried out for the presence of alkaloids, tannins, anthraquinone, Saponins, terpenoids, flavonoids, phlobotannins, steroids, glycosides and reducing sugars, and the results were tabulated .The presence of phytoconstituents and antimicrobial activities shows that the selected plant could be used as a potential source of antimicrobial drugs.

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Introduction

Plants are used as remedies for the treatment of diseases since prehistoric period. Plants are important source for maintaining health and especially in the last few decades extensive study has been carried out for developing natural therapies. Traditional medicines are used in all parts of the world because they are economical than modern medicines (Jehan *et al*., 2011). It has been reported that more than 400,000 species of tropical plants possess medicinal properties (Yildrim *et al*., 2000). In twentieth century remarkable development in organic synthetic products has occurred. In industrialized countries more than 25% of prescribed medicines are directly derived from plants (Newman *et al*., 2000). Still the plants used in traditional medicines are poorly studied (Geoffrey *et al*., 1996). Almost all plants have one or other importance but herbs have enumerable application in human health. At present quarter of drugs are derived from medicinal plants. W.H.O. encourages the use of herbal medicines and 75-80% of the population use medicinal plants in whole or partly for health care. In Japan, India, China, Pakistan, Sri Lanka and Thailand the practice of traditional medicines is widespread. In China, about 40% medicines are traditional (Lucy *et al*., 1999).

In Pakistan Unani system is common and the use of ethno medicinal plants is also in practice in remote areas (Ahmad *et al*., 2003).Various bacterial strains (gram positive and gram negative bacteria) e.g. staphylococcus aurous and pseudomonas aeruginosa have created resistance against antimicrobial drugs (Eloff *et al*., 2005) which gives special emphasis by pharmacist and natural products chemists on the investigation of new affordable and easily available antibiotics from medicinal plants (Adekunle *et al*., 2009). The valuable medicinal effect of plants is due to the combined effect of secondary metabolites and therefore need investigation for identification of the phytochemicals (Briskin *et al*., 2009). Medicinal plants are the rich source of traditional medicines, pharmaceutical intermediates, modern medicines, food supplements and need further investigation for their biologically active compounds (Hammer *et al*., 1999). The practice of plants for the treatment of diseases is since the time immemorial. (Henrich *et al*., 2012).

Medicinal plants are safe natural resources that have been tested for hypoglycemic, antimicrobial and biological activities. They also play important role in modern medicines (Hassawi *et al*., 2006, Bhatt *et al*., 2009). It is obvious that most synthetic drugs have their origin from plants (Ríos *et al*., 2005). Long before the discovery of microorganism's existence, it was known that some plants possess healing potential. The antimicrobial property of plants is due to the presence of secondary metabolites that vary from plant to plant. The use of plants extracts to treat diseases is a therapeutic modality (Anwar *et al*., 2009).

Phytochemicals are divided in two main groups (Krishna *et al*., 2009) primary one which include proteins, sugars, amino acids and chlorophyll etc. and secondary one consists Saponins, flavonoids, tannins, terpenoids, alkaloids, essential oils and phenolic compounds etc. (Krishnaiah *et al*., 2007; Edeoga *et al*., 2005). Most of the phytochemicals have shown valuable therapeutic activities such as insecticidal (Kambu *et al*., 1982), antifungal, antibacterial, anticonstipative, spasmolytic, antiplasmodial and antioxidants activities etc. (Kambu *et al*., 1982; Lemos *et al*., 1990; Ferdous *et al*., 1992; Sontos *et al*., 1998; Benoitvical *et al*., 2001; Vardar-unlu *et al*., 2003). Terpenoids exhibit various important pharmacological activities i.e., anti-inflammatory, anti-cancer, anti-malarial, inhibition of cholesterol synthesis, ant-viral and anti-bacterial activities. Terpenoids are very important in attracting useful mites and consume the herbivorous insects. (Kappers *et al*., 2005) Alkaloids are used as anaesthetic agents and are found in medicinal plants. (Hérouart *et al*., 1998).

According to WHO report medicinal plants are the

best source of potent herbal drugs. About 80% peoples of the develop countries uses traditional medicines containing potent compounds derived from plants. These plants should be screened to obtained safe drugs against resistant microbes (Khan *et al*., 2012). It is also reported that 30% of worldwide medicines and their derivatives are extracted from medicinal plants directly. Due to the indiscriminate use of antibiotics, microorganisms have developed multiple resistances therefore discovery of new antimicrobial sources is necessary (Ahmed *et al*., 2011).

Himalrandia tetrasperma with synonym *Randia tetrasperma* and common name is *Torsataka* in Pashto belongs to family robiaceae.It is widely distributed in temperate and sub-tropical Himalayas, Salt range, Pakistan(Hazard), Kashmir, Nepal, Sikkim, Bhutan and India (Assam). Pulp of fruits is uses in dysentery, as anthelmintic and abortifacient. The plant is locally used as antiseptic Therefore the aim of current piece of research work was to determine qualitative analysis of phytonutrients from the leaves, stem and roots of ethanolic extract of locally collected *Himalrandia tetrasperma*. More ever this work was further proceeded to find out the antimicrobial activities of ethanolic extracts of this plant against some gram positive and gram negative bacterial as well some fungal strains.

Materials and methods

Collection of Plants

Himalrandia tetrasperma plants was collected from different areas of Dir (L) Khyber Pakhtunkhwa, Pakistan during the month of April and May 2012, different parts of the plants including root, stem and leaves were mechanically separated. These parts of the plants were thoroughly washed twice with flowing tape water. The plants materials were dried in shade in current of air for four weeks. The shade dried plants materials were processed in Medicinal Botanical Center of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex Peshawar.

Grinding of the plants materials

The plants materials were first cut into small pieces mechanically and then grinded by electrical grinding machine. The grinded powder materials of root stem and leaves were stored separately in polythene bags for further processing and extraction.

Crude extracts preparation

About 500 grams from each of the root, stem and leaves of *Himalrandia terasperma* were soaked in 1.5 liter distilled ethyl alcohol in 2 liter separating funnels for two days and shaken it occasionally. The dilute extract were tapped of after two days and concentrated on vacuum rotary evaporator under reduced pressure at temperature between 50-60^{o}C. The distilled ethanol was added to the separating funnels again and the process was repeated three times for each part of the plant. The crude residue was further dried on water bath at 60^{o}c.

Phytochemical Screening of various parts of the plants

For qualitative screening of ethanol extracts of various parts of *Himalrandia terasperma* standard procedures was used, as described by (Trease *et al*., 1978; Harborne JB *et al*., 1973).

Test for alkaloids

From each extract 0.2 g was taken in a test tube and warmed with 2% V/V H_2SO_4 for two minutes. The reaction mixture was filtered through Waxman filter paper and few drops of Dragon doff reagents (potassium, bismuth iodide solution) were added to it. The formation of orange red precipitate indicates the presence an alkaloids moiety in the extracts.

Test for tannins

A little amount of each extract was mixed with water and warmed on water bath. The mixture was filtered and few drops of $FeCl_3$ were added to each filtrate. Appearance of dark green coloration indicates the presence of tannins.

Test for anthraquinone

From each extract 0.5 g was boiled with 10% v/v solution of HCl and on the water bath for few minutes. The reaction mixture was filtered and cooled. Equal volume of $CHCl_3$was added to each filtrate. Few drops of 10% NH_3 were added and heated the formation of rose pink color indicates the presence of anthraquinone.

Test for Glycosides

Each extract was hydrolyzed with HCl and then neutralized with NaOH solution. Few drops of Feeling's solution A and B were added to each mixture, the formation red precipitate indicates the presence of glycosides.

Test for reducing sugars

Each extract was dissolved in distilled water by shaking it will and then filtered. The filtrates were boiled with Filling's solution A and B for few minutes' .The formation orange red precipitates indicates the presence of reducing sugars.

Test for Saponins

From each extracts 0.2 g was dissolved in 5ml distilled water by shaking. The appearance of creamy mass of small bubbles indicates the presence of saponin.

Test for flavonoids

From each extract 0.2 g was dissolved in diluted NaOH and few drops of HCl was added to it .The formation of yellow coloration that turned colorless within a few minutes indicates the presence of flavonoids.

Test for phlobotannins

From each extract 0.5 g was dissolved in distilled water and filtered. The filtrates were boiled with 2%HCl solution for few minutes; the formation of red precipitates indicates the presence phlobotannins.

Test for steroids (Liebermann- Buchards test)

Each extract was treated with $CHCl_3$ and filtered. The filtrates were treated with acetic anhydrides, boiled and cooled. Conc H_2SO_4 was added along the sides of the test tubes. The formation of brown ring at the junction indicates the presence of steroids.

Test for terpenoids

From each extract 0.2 g was mixed with $CHCl_3$ and 3m l of Conc H_2SO_4 was carefully added to form a layer. The formation of reddish brown coloration at the interface shows *the presence of terpenoids.*

Antimicrobial activities Culture media used

The culture media used for the growth of microorganisms include Nutrient agar (NA), Nutrient Broth (NB) and Muller Hinton Agar (MHA).

Nutrient agar media was used for culturing and growth of microorganisms used in current study. Nutrient broth was used for inoculation, shaking incubation and standardization of microorganisms under consideration in this study.

Preparation of Media

The required amount (2.8 gl^{-1}) of nutrient agar and (1.3 gl^{-1}) nutrient broth medium were prepared in distilled water and poured into conical flasks (20ml/flask). About 7-8 ml/test tube of the nutrient broth was poured into the test tubes. All the media, flasks and test tubes were plugged with cotton wool and sterilized in an autoclave at 1.5 pounds pressure and 121^{o}C Temperature for 15 minutes. The nutrient agar media after sterilization was poured in aseptic environment into sterilized Petri plates in laminar flow hood. In order to avoid contamination sterile environment was maintain during pouring of the agar medium. The media was allowed to solidify for about an hour, after solidification these Petri plates were placed in an incubator at 37 ^{o}C for twenty-four hours in inverted position to avoid the water loss from the medium. After 24 hours, the contaminated plates were discarded and the un-contaminated plates were used for culturing bacteria and fungi. The nutrient broth in the test tubes was used for standardization microbial culture while nutrient broths in the flasks were used for shaking incubation of microorganisms.

Preparation of stock solutions

Ethanol extracts of root, stem and leaves of *Himalrandia tetrsperma* and root, stem and leaves were evaluated for their antimicrobial activities. The crude extracts of various parts of both plants were diluted and adjusted to 1mg/ 6µl in DMSO (dimethyl sulfoxide) solvent.

Positive controls

Azithromycin 30µg/6µl: against Gram-positive bacteria.

Sulphmethaxazole 30 µg/6µl: against Gram-negative bacteria.

Chlotrimazole 30µg/6µl: against Candida albicon.

Microbial strains used in our study.

Four gram-negative and three gram-positive bacterial strains and one fungal strain were selected for the current study.

Disc diffusion susceptibility method

The following standard procedure was used for the determination of antimicrobial potential as described by Aida *et al* (2001).The bacterial cultures were adjusted to 0.5 McFarland turbidity standards and inoculated into nutrient agar in Petri plates. The *Candida albican* were adjusted to 10^8 cfu/ml.6 mm Whatmann-1 filter paper disc were put on each plate and extracts were applied in concentration of 1mg and 2mg/6µl and 12µ volumes previously seeded with bacteria and fungi standardized with 0.5Mc Farlandturbidity standard. Bacterial and Candida albicon cultures were then incubated at 37°C for 24 hours.

Results and discussion

Phytochemical screening of Himalrandia tetrasperma.

After a thorough study it was found that no such study was performed on locally grown plant *Himalrandia tetrasperma* and due to its wide range medicinal application by local practitioner it was necessary to find out the broad range importance of the mentioned plant and to disclose its further medicinal applications to local markets and pharmaceutical industries as well. Therefore the current study revealed the presence of various phytonutrients as a very important and active medicinal part of the plant extract. The root of *Himalrandia tetrasperma* show the presence of some important phytochemicals i.e. alkaloids, flavonoids, steroids and reducing sugars which gives positive results while tannins, anthraquinone, saponin, terpenoids, phlobotannins and glycosides gives negative results.

Table 1. Bacterial and fungal strains used in our study.

Specie	Type	Detail of microorganisms used
Escherichia coli	Gram-negative	ATCC # 2592
Pseudomonas aeruginosa	Gram-negative	ATCC#27853
Salmonella typhi	Gram-negative	Clinical isolates from microbiology lab QAU
Klebsiella pneumoniae	Gram-negative	ATCC# 25923
Staphylococcus aureus	Gram-positive	Clinical isolates from microbiology lab QAU
Bacillus subtillis	Gram-positive	Same source as above
Bacillus cereus	Gram-positive	Same source as above
Candida albicans	Fungus	Clinical isolates from HMC Peshawar

The stem show the presence of alkaloids, tannins, saponin, flavonoids, steroids, and reducing sugars while anthraquinone, terpenoids, phlobotannins and glycosides were found absent. The leaves show the presence of alkaloids, tannins, saponins, steroids and reducing sugars while anthraquinones, terpenoids,

flavonoids, phlobotannins and glycosides were found absent. The above discussions revealed that most of the medicinally important phytochemicals are presents in all parts of the plants while few are absent. The determination of percentage extractive value is an important parameter for standardization and quality assessment of medicinal plants, because it shows the quantity of the active phytochemicals of the plant. The polarity and nature of phytochemical of different plants are also different from each other.

Table 2. Qualitative screening results of leaves stem and stem of *Himalrandia tetrasperma*.

S.NO	Phytochemical constituents	Root	Stem	Leaves
1	Alkaloids	+ive	+ive	+ive
2	Tannins	-ive	+ive	+ive
3	Anthraquinones	-ive	-ive	-ive
4	Glycosides	-ive	-ive	-ive
5	Reducing sugars	+ive	+ive	+ive
6	Saponins	-ive	+ive	+ive
7	Phlobotannins	-ive	-ive	-ive
8	Steroids	+ive	+ive	+ive
9	Terpenoids	-ive	-ive	-ive
10	Flavonoids	+ive	+ive	-ive

Table 3. Antimicrobial activities of *Himalrandia tetrasperma* root extracts.

S.No	Microorganisms	1mg/6µl	2mg/6µl
1	*Escherichia coli*	9±0.22	12±0.14
2	*Pseudomonas aeruginosa*	14±0.05	19.5±0.08
3	*Salmonella typhi*	7.1±0.08	8±0.03
4	*Klebsiella pneumoniae*	11±0.14	16±0.25
5	*Staphylococcus aureus*	8±0.04	10±0.02
6	*Bacillus subtillis*	10±0.32	14±0.42
7	*Bacillus cereus*	10.5±0.12	12±0.03
8	*Candida albicans*	7±0.04	10±0.05

The same plant grown in different areas also differ in phytochemicals because of different environmental conditions. In current study percentage, extractive value of root, stem and leaves of *Himalrandia tetrasperma* were determined in ethanol and the results are shown in table 1. The important medicinal effects of plant materials typically come from the combinations of secondary metabolites i.e. phytonutrients present in the plant. That the medicinal actions of plants are unique to particular plant species or groups is consistent with this concept as the combinations of secondary products in a particular plant are often taxonomically distinct. This is in contrast to primary products, such as carbohydrates, lipids, proteins, heme, chlorophyll, and nucleic acids, which are common to all plants and are involved in the primary metabolic processes of building and maintaining plant cells.

Table 4. Antimicrobial activities of *Himalrandia tetrasperma* stem extracts.

S.No	Microorganisms	1mg/6µl	2mg/6µl
1	*Escherichia coli*	12±0.12	16±0.13
2	*Pseudomonas aeruginosa*	0.00	0.00
3	*Salmonella typhi*	7±0.05	9±0.06
4	*Klebsiella pneumoniae*	12±0.07	16±0.08
5	*Staphylococcus aureus*	9±0.09	10±0.13
6	*Bacillus subtillis*	8±0.05	9±0.06
7	*Bacillus cereus*	7±0.05	10±0.02
8	*Candida albicans*	10±0.12	14±0.22

Table 5. Antimicrobial activity of *Himalrandia tetrasperma* leaves extracts.

S.No	Microorganisms	1mg/6µl	2mg/6µl
1	*Escherichia coli*	9.5±0.15	16.5±0.16
2	*Pseudomonas aeruginosa*	10±0.06	16±0.03
3	*Salmonella typhi*	0.00	0.00
4	*Klebsiella pneumoniae*	0.00	0.00
5	*Staphylococcus aureus*	9±0.03	10±0.05
6	*Bacillus subtillis*	8±0.10	10±0.22
7	*Bacillus cereus*	0.00	0.00
8	*Candida albicans*	8±0.06	9±0.15

Antimicrobial activities of Himalrandia tetrasperma root stem and leave extracts

The ethanol extracts of root of *Himalrandia tetrasperma* exhibit sensitivity against all the selected microorganisms. It shows highest sensitivity against Pseudomonas aeruginosa (14 mm and 19.5 mm) followed by Klebsiella pneumonia (11mm and 16 mm), Bacillus subtillis (10 mm and 14 mm), Bacillis cereus (10.5 mm and 12 mm), Escherichia coli (9mm and 12 mm), Staphylococcus aureus (8mm and 10 mm), Salmonella typhi (7mm and 8 mm) and Candida albican (7mm and 10 mm).as shown in Table 3 and Fig 1.

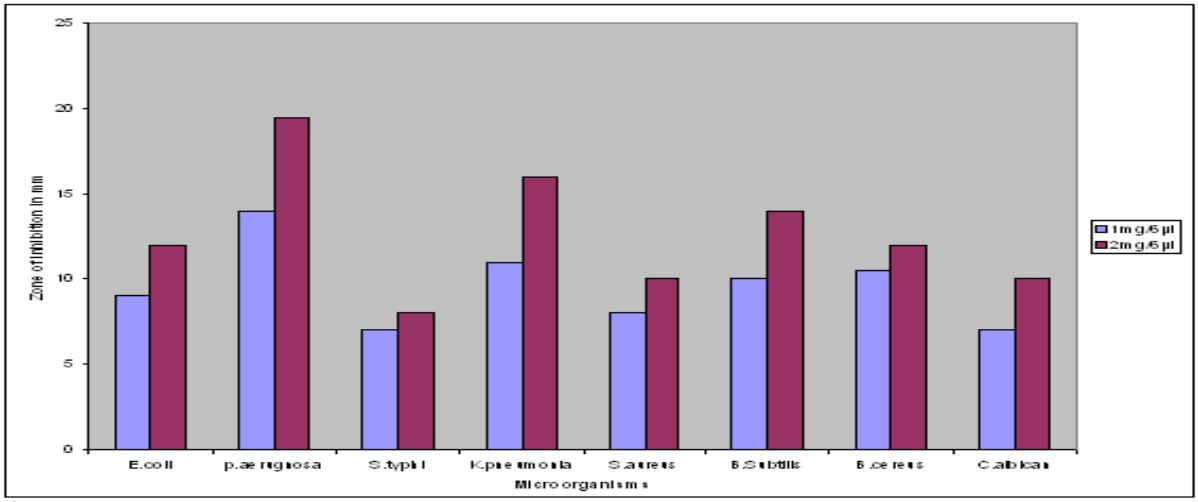

Fig. 1. Antimicrobial activities of *Himalrandia tetrasperma* root extracts.

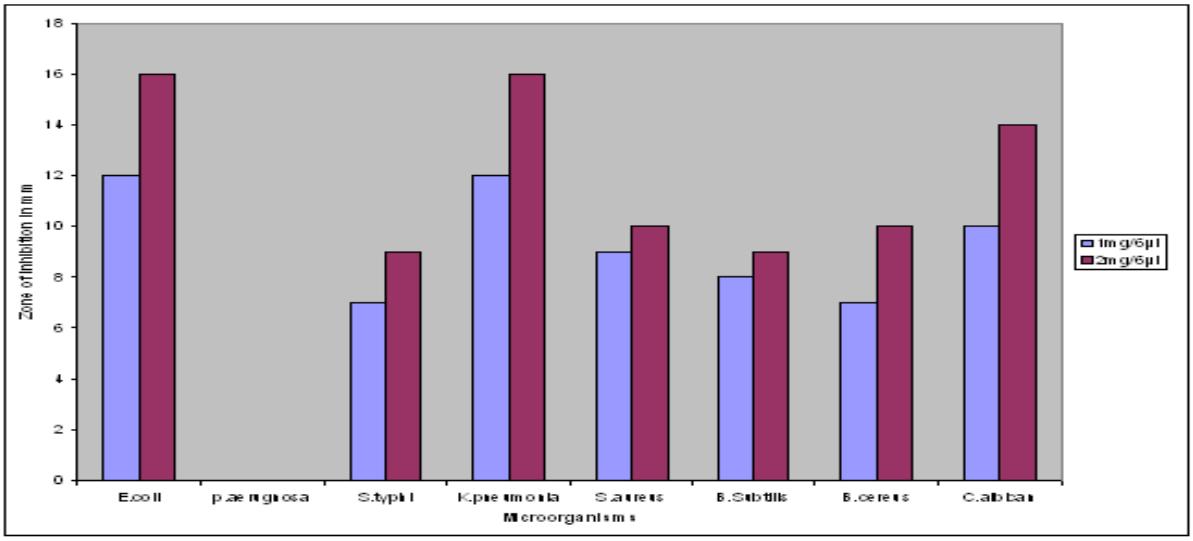

Fig. 2. Antimicrobial activities of *Himalrandia tetrasperma* stem extracts.

The ethanol extracts of stem of *Himalrandia tetrasperma* show sensitivity against all selected microorganisms except Pseudomonas aeruginosa as shown in Table 4 and fig 2. It show highest sensitivity against *Escherichia coli* and *Klebsiella pneumonia* (12 mm and 16 mm),followed by *Staphylococcus aureus* (9mm and 10 mm) *Bacillus subtillis* (8mm and 9mm),Bacillus cereus (7mm and 10 mm) and Candida albican (10mm and 14mm) zone diameter.

The ethanolic extracts of *Himalrandia tetrasperma* leaves show highest sensitivity against *Pseudomonas aeruginosa* (10 mm and 16 mm), Escherichia coli (9.5 mm and 16.5 mm), *Staphylococcus aureus* (9 mm and 10 mm), *Bacillis subtillis* (8 mm and 10 mm) and *Candida albicon* (8mm and 9mm) while resistant against *Salmonella typhi* and *Klebsiella pneumonia* and *Bacillus cereus*. Shown in table 5 and Fig 3.

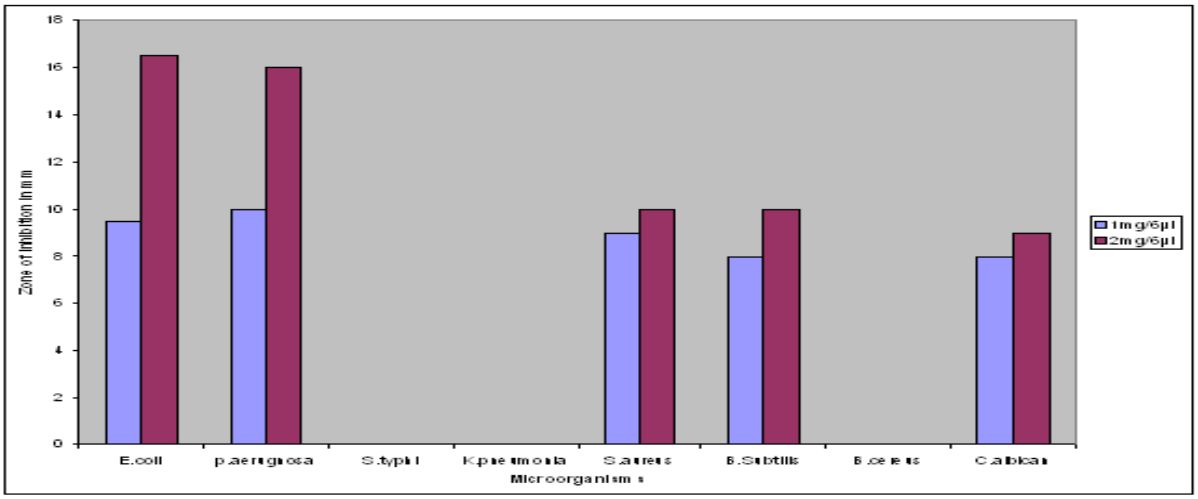

Fig. 3. An antimicrobial activity of *Himalrandia tetrasperma* leaves extracts.

Conclusion

Himalrandia tetrasperma is a rich source of many important secondary metabolites. Medicinal plants are used for discovering and screening of the phytochemical constituents which are very helpful for the manufacturing of new drugs. Similarly it was noted that phytochemical analysis of the medicinally important plant are of commercial interest in both research institutes and pharmaceutical industries for the synthesis and designing of new antibiotics and life saving drugs for the curement of various disease.

Moreover the anti-inflammatory, anti-bacterial. Anti-fungal, anti-viral and anti-malarial activities of plants are due to the presence of the mentioned secondary metabolites, therefore the ethanolic extract of leaves, stem and root of *Himalrandia tetrasperma* shows amazing activities against some gram positive, gram negative as well as some fungal strains. Thus we hope that important phytochemical extracted and antimicrobial properties identified of the *Himalrandia tetrasperma* plants obtained from district Dir. will help us in the long run for copping of various diseases of our region.

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