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Research Article

Satyrium nepalense: A RARE MEDICINAL ORCHID OF WESTERN HIMALAYA (INDIA); PHYTOCHEMICAL SCREENING, ANTIMICROBIAL EVALUATION AND CONSERVATION STUDIES

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ABSTRACT

Satyrium nepalense is an endangered medicinal herb found at the higher altitude of 2400-5000m. Local inhabitant used tubers of *Satyrium nepalense* as an energetic tonic and as an important medicine to cure different type of fever in traditional health care system of Uttarakhand (INDIA). Present study was carried out to evaluate the phytochemical, antibacterial screening against four (*Streptococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae*) microorganisms in methanolic extracts and habitat studies for conservation of *S. nepalense*. Tubers of *Satyrium nepalense* were extracted separately with methanol by hot extraction process using soxhlet apparatus. The extracts were subjected to Lyophilization to get dry extract and preserved in aseptic condition. The different group reagents used for phytochemical screening as dragendorffs's for alkaloid, molisch's test for carbohydrates, shinoda test for flavonoids etc. Antibacterial study was carried out by disc diffusion method. The highest zone of inhibition was recorded as 15.0±0.00 mm against *Klebsiella pneumonia* and 15.0±0.82 mm against *Staphylococcus aureus*. Phytochemical screening shows the presence of alkaloids, carbohydrates/glycosides, flavonoids and unsaturated sterols/ triterpenes in *Satyrium nepalense.* The result indicates that methanolic extract of *Satyrium nepalense* shows potent antibacterial activity against all four bacterial strains.

Key words: Uttarakhand, *Satyrium nepalense*, medicinal orchid, anti-bacterial, phytochemicals

INTRODUCTION

India is home to a great variety of ethno-medicinally important plant species and is ranked sixth among 12 mega diversity countries of the world. The Himalaya has one of the global varieties of plant hotspots, where ecological, phyto-geographical and evolutionary factors favour high species diversity. It supports about 18,440 species of plants, of which 25.3% is endemic to the region (Singh and Hajra 1996). The rich plant diversity of the Indian Himalaya is utilized by the native communities in various forms, including food and medicine. During the past several years, some important contributions have been made on Ethnobotanical knowledge and medicinal plants of Central Himalayas (Samant *et al.,* 1998; Gaur 1977; Gaur 1983; Gaur 1999; Pundeer and Singh 2002; Singh and Pundeer 2004).

Uttarakhand is a storehouse for a rich variety of herbs, medicinal and aromatic plant species where several medicinal herbs are red listed on which very less study have been carried out. One of them belongs to the orchid, occupy a wide range of habitats and exhibit highly specialized morphological, structural and physiological characteristics. Terrestrial orchids usually grow on the ground where sufficient moisture and shade are available and most of them generally appear during the rainy season. In the state of Uttarakhand, the distribution of the orchids is extremely patchy. The state has 237 species of orchids, of these 12 are medicinally important. Mostly the tubers of these orchids are used in medicine. Many of these orchids face the extreme danger of extinction due to over-exploitation and habitat destruction. Orchids require a special kind of environment and habitat. They are not only

habitat specific but within a habitat, also require unique micro-climatic conditions to survive and perpetuate. To formulate the conservation plan for a particular area and to understand the ecology of the species, studies on quantitative information play a vital role. At the same time it is also important to identify the habitat types preferred by orchids so that habitat wise conservation strategies can be applied (Jalal and Rawat, 2009).

The present study was aimed to investigate phytochemical profile, antibacterial properties and to identify the habitat types of a rare medicinal orchid of Western Himalaya (INDIA).

Satyrium nepalense [family Orchidaceae] (Kharkwal 2009) locally known as Salang mishri is a terrestrial herb having height of 25-60cm which is usually found in the region of 2400-5000m. It is commonly used by native people of Uttarakhand (INDIA) as folk remedy against various complaints. Decoction of tubers, roots and stems of the plant has been used in various infectious diseases and also as a nutritional supplement since ancient time. It is also used as a food, tonic, in diarrhoea, malaria and dysentery (Saklani *et al.,* 2011).

METHODOLOGY

Plant material and microbial collection

The plant was collected from the higher altitude of Garhwal Himalaya i.e. from the Chamoli district of Uttarakhand (INDIA) in the region of 2400-4000m and identified by the Department of Botany, H.N.B. Garhwal (A Central) University, Srinagar, Uttarakhand.

Microorganisms were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH) Sector 39-A, Chandigarh-160036 (India).

Study area

The study was conducted in the state of Uttarakhand (28'44" N to 31'28" N latitude and 77'35" E to 81'01" E longitude) of India. The altitudinal ranges vary from 2000 m to > 7000 m asl. The climate is influenced by the monsoon pattern of rainfall. Generally, the average annual rainfall ranges between 200 and 250 cm. There are six forest types known to occur in the state which include tropical moist deciduous forests, tropical dry deciduous forests, sub-tropical pine forests, Himalayan moist temperate forests, Himalayan dry temperate forests, sub alpine and alpine forests (Champion and Seth, 1968).

Data collection

Sampling process was carried out between the months of July and October, 2010 to 2011 covering an altitudinal range 2000 - 7000 m. A rapid survey was done in different localities *i.e.* Rudraprayag and Chamoli districts of Uttarakhand. The seven natural homes were identified which were used by medicinal orchids (Table I). Since the number of samples were not uniform in each of the natural home. Population study such as percentage frequency (%F) and density (D/m^2) was calculated according to the formulae given by Curtis and McIntosh (1950) (Table II). Jaccard's index was also calculated to see the similarity between different habitats. The similarity values were obtained by means of the formula ISj = a/a + b + c, where ISj is the index of similarity, a the total number of orchid species common between the habitats, b the total numbers of species unique to the first habitat and c the total number of species unique to the second habitat.

Extraction method

Five hundred gram tubers of *Satyrium nepalense* were chopped into small pieces and extracted separately by hot soxhlet extraction method using methanol as a solvent. After extraction the extracts were subjected to Lyophilization to get dry extract & preserved in aseptic condition. The dried extracts were subjected to various phytochemical and antibacterial screening.

Preliminary phytochemical screening *(Singh et al., 2009, Bateman et al., 2003)*

Carbohydrates tests

Molisch's Test (General test) The test is positive for soluble as well as insoluble carbohydrates.To 2-3 mL of aqueous extract add few drops of Molisch's reagent (alpha-naphthol in alcohol) and concentrated sulphuric acid along sides of test tube – Voilet colour ring at the junction of two liquids.

Table I. Types of Natural Homes with some important comments.

Types of Natural Homes	Remark
Alpine grassy slopes (AGS)	Dominated by *Danthonia cachmyriana* grass
Herbaceous meadows (HM)	Gentle and moist slopes in the sub-alpine region dominated by many alpine herbs species
Mixed oak (MO)	*Q. leucotrichophora* and *Q. floribunda*
Banj-oak (BO)	*Quercus leucotrichophora*
Banj grassy slopes (BGS)	*Q. leucotrichophora* forest between grassy patches
Grassy slopes (GS)	Lower elevational grassy slopes
Oak-pine (OP)	*Q. leucotrichophora* and *Pinus roxburghii*

Table II. Density and frequency of *Satyrium nepalense* in different habitats.

Habitat Types	*Satyrium nepalense*	
	Density (d/m^2)	Frequency (%)
Alpine grassy slopes (AGS)	Nil	Nil
Banj grassy slopes (BGS)	0.5	20
Banj-oak (BO)	0.5	25
Grassy slopes (GS)	0.7	30
Herbaceous meadows (HM)	Nil	Nil
Mixed oak (MO)	0.7	24
Oak-pine (OP)	0.3	10

Test for reducing sugars: Fehling's Test: Mix 1mL of T.S.+ 1mL of Fehling's solution A + 1mL of Fehling's solution B and boil on water bath for 5- 10 min – First a yellow, then brick red ppt is observed. Benedict's Test: Mix equal volume of Benedict's reagent and test solution in test tubes and heat on boiling water bath for 5 min. Solution appears green, yellow or red depending on amount of reducing sugar present in test solution. Test for monosaccharides: Barfoed's Test: Mix equal volume of Barfoed's reagent and test solution. Heat on boiling water bath for 1-2 min and cool. Red colour ppt is observed. Test for non-reducing sugars: Test solution does not give response to Fehling's and Benedict's tests.

Alkaloids tests

Evaporated the alcoholic extract and to the residue added dilute HCl. Shaked well and filtered, performed the following tests with filtrate. Mayer's Test: 2-3 mL of filtrate + few drops of Mayer's reagent – Creamy white ppt.. Wagner's Test: 2-3 mL of filtrate + few drops of Wagner's reagent – Reddish brown colored ppt. Dragendorffs's Test: To 2-3 mL of filtrate add few drops of Dragendorffs's reagent- Orange red colored ppt. were obtained. Hager's Test: 2-3 mL of filtrate + few drops of Hager's reagent – Yellow colored ppt.

Glycosides tests

Keller Killiani Test (for deoxysugars): To 2 mL of extract add few drops of glacial acetic acid, 1 drop of 5% FeCl3 solution and conc. H_2SO_4. Reddish brown color appears at the junction of two liquid layers and upper layer appears bluish green. Legal's Test (test for cardenoloids): To aqueous or alcoholic extract, add 1 mL of pyridine and 1mL of sodium nitroprusside solution. Pink to red color appears.

Flavonoid tests

Shinoda Test (Mg/HCl): 2-3 mL of test solution + pinch of Mg powder + 2-3 drops of conc. HCl – Deep red or magenta colour. Few mL of alcoholic extract + 1-2 drops of concentration H2SO4 + magnesium turnings- Deep cherry red colour. To 1 mL of aq. NaOH adds 1 mL of test solution – Yellow colour, which decolorizes after addition of acid.

Table III. Phytochemical screening of *Satyrium nepalense*. (+) Present, (-) Absent,

Test	Satyrium nepalense
Carbohydrates/ glycosides	
(1) Molish test	(+)
(2) Fehling test	(+)
(3) Benedict test	(-)
Alkaloid	
(1) Mayer's test	(-)
(2) Dragondroff test	(+)
Flavonoids	(+)
Saponins	(-)
Tannins	(-)
(1) Pyrogoll & catechol	(-)
(2) Gallic acid	
Unsaturated sterol/triterpenes	
(1) Liebermann Burchard test	(+)
(2) Salkowiskis test	(-)
Resin	(-)

Table IV. Anti-microbial activity of Methanolic extract of *Satyrium nepalense*.

Micro-organisms	Zone of inhibition in mm					Kanamycin
	1 mg/ 100µL	2 mg/ 100µL	4 mg/ 100µL	6 mg/ 100µL	8 mg/ 100µL	30 µg/ 100µL
Streptococcus mutans	Nil	Nil	Nil	5±0.0	14±0.00	20±0.94
Pseudomonas aeruginosa	Nil	Nil	Nil	6±0.0	12±0.82	18±0.90
Staphylococcus aureus	Nil	Nil	Nil	4±0.0	15±0.82	22±0.89
Klebsiella pneumoniae	Nil	Nil	Nil	5±0.0	15±0.00	26±0.99

Addition of lead acetate solution to small quantities of residue gives yellow coloured precipitated.

Saponins

Foam Test: Extract is shaken vigorously with distilled water in test tubes Honey comb like foam is produced.

Tannins

Tannins exhibits some chemical reactions; Gelatin test: Extract is dissolved in 2% gelatin + 10% NaCl – White precipitate. Extract + alcoholic vanillin solution + dilute HCl – Pink colour. Extract + Lime water – Brown colour. Tannins are precipitated by salts of copper, tin and lead. Tannins show colour reactions with iron salts. Ferric chloride gives bluish black or bluish green colour and potassium ferricyanide with ammonia gives deep red colour.

Antibacterial activity (Jonsen *et al.*, 1987, Mehlika *et al.*, 2007)

The compounds were tested *in vitro* for their antibacterial activity against four microorganism's viz. *Staphylococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus* and *Klebsiella pneumoniae* which are food pathogenic for human beings. The method was disc diffusion method using nutrient agar. Material used were nutrient broth (Himedia); Nutrient agar (Himedia); 18-24 hours growth culture in nutrient agar; Sterile petridis hes; Sterile micropipettes; Sterile cotton swabs; Sterile cork borer; Sterile test tubes.

Preparation of Nutrient broth

Nutrient broth - 3.8 g; Distilled water - 100 mL. Above components were dissolved in 100 mL distilled water and pH was adjusted to 7.2. This solution was sterilized by autoclaving at 15 psi for 20 min.

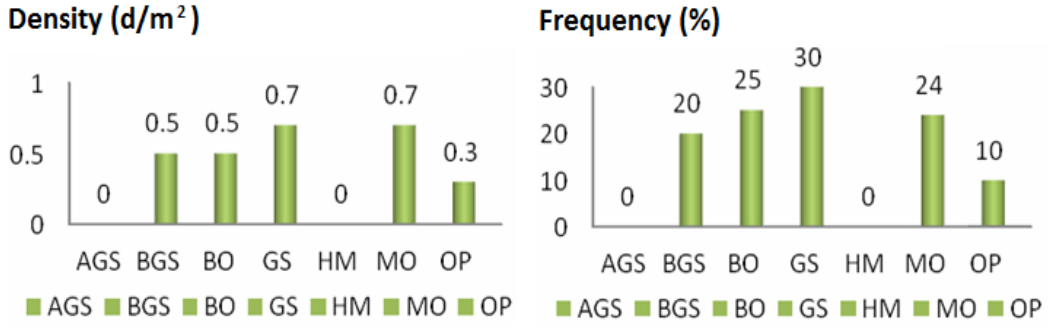

Figure 1. Bar diagram showing density and frequency of *Satyrium nepalense* in different habitats

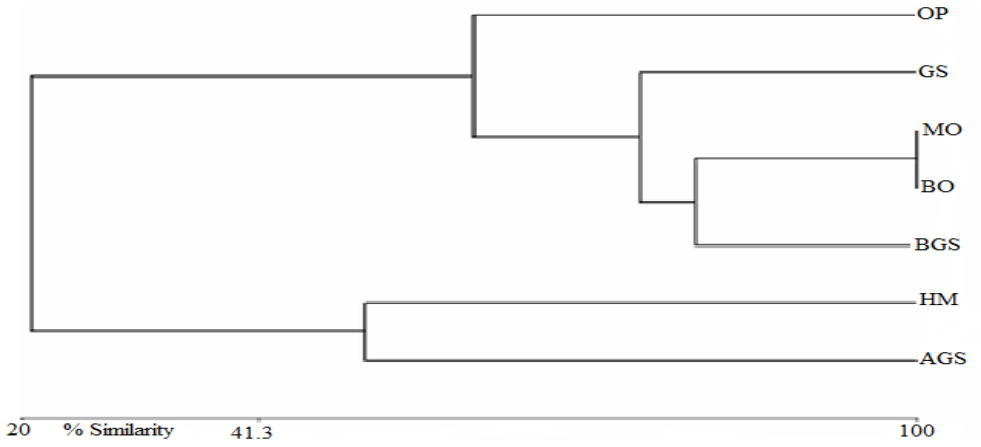

Figure 2. Phenogram derived from Jaccard's similarity values for different habitats.

Preparation of Inoculums

One day prior to these testing, inoculations of the above bacterial cultures were made in the nutrient broth and incubated at 37°C for 18 –24 hours.

Preparation of medium (Nutrient agar)

Muller hinton agar - 2.8 g; Distilled water - 100 mL. The agar was dissolved in to distilled water and pH was adjusted to 7.4+0.2. It was sterilized by autoclaving at 15 psi for 20 minutes.

Preparation of test solutions

The test compound of methanolic extract (1 mg, 2 mg, 4 mg, 6 mg and 8 mg) was dissolved in dimethyl sulfoxide (1 mL) separately to give stock solution of concentration 1mg/100µL, 2mg/100µL, 4mg/100µL, 6mg/100µL and 8mg/100µL.

Method of testing

Agar plates were prepared by pouring 15 – 20 mL of the medium in to each sterilized petri dish and were allowed to set at room temperature. The cell suspensionwas standardized to the density of 530 nm using spectrophotometer and was inoculated over the surface of agar medium using sterile cotton swab. A single disc was dipped into 1mg/100µL stock solution of methanolic plant extracts and placed in each plate. Repeat the same process for 2mg/100µL, 4mg/100µL, 6mg/100µL and 8mg/100µL stock solutions of methanolic extracts.

Then all plates were incubated at 37°C for 48 hours. The zone of inhibition was measured in mm for each organism.

RESULTS AND DISCUSSION

In different environment of plants, there are six medicinal orchid species belonging to four genera viz *D.hatagirea* (D. Don) Soo, *M.muscifera* (Jeewak), *M.acuminata* (Rishbhak), *H.intermedia* (Ridhi), *H.edgeworthi* (Virdhi) and *S.nepalense* (Salang Mishri). IUCN (International Union for Conservation of Nature) categorized *H.intermedia* as an endangered species (EN) and *D.hatagirea* has been categorized as critically. Other species are rare in the state. In habitat GS and MO, *S.nepalense* (0.7 d/m^2) showed the higher density and frequency (30% and 24% respectively) whereas in habitat OP it had least density (0.3 d/m^2) and frequency (10%) (Figure 1). In Alpine Grassy Slopes AGS and HM habitat *S.nepalense* had not found. To understand how close each habitat type was, Jaccard's similarity index was computed. The similarity values were used to generate the phenogram (Figure 1). Taking the index of similarity of ISj = 41.3 as a baseline, the habitats were grouped into two clusters. The analysis shows that the Mixed-oak and the Banj-oak habitats are closely related with the orchid species and the vegetation composition as well as highlights the fact that between them, maximum numbers of species (4) are shared. On the other hand, it was interesting to note that the habitat pairs viz. (AGS) and herbaceous meadows (HM) were somewhat isolated from the remaining habitat pairs. These habitats occur at higher elevations and are different, on the basis of vegetation as well as topography.

Phytochemical screening of *Satyrium nepalense*

Satyrium nepalense plant extract was subjected to preliminary qualitative chemical test which shows the presence of carbo-hydrates/glycosides, alkaloids, flavonoids and unsaturated sterols/triterpenes (Table III).

Antibacterial activity

of Methanolic extract of *S.nepalense* with concentration of 8mg/100µL has showed very good antibacterial activity against both gram positive and gram negative food pathogenic bacteria's namely as *S.mutans, P.aerugi-nosa, S.aureus* and *K.pneumonia.* 1mg/100µL, 2mg/100µL and 4mg/100µL concentration had not showed the antibacterial effect against all microorganisms whereas 6mg/100µL concentration has only showed the minimal effect against all four microorganisms. Methanolic extract of *S.nepalense* having the concentration of 8mg/100µL gave 15±0.00mm a high standard zone of inhibition against *K.pneumonia*, 15±0.82mm inhibitory effect against *S.aureus*, 14±0.00mm inhibition against *S.mutans* and 12±0.82mm lowest inhibition against *P.aeruginosa*, (Table IV) (Figure 3, 4). The zone of inhibition was compared with the activity of the positive control, kanamycin (30µg/100µL).

Orchids are those species which grow in the variety of environments and need a specific microhabitat for their growth and development. Microhabitat conditions vary in different habitats. The occurrence of specific mycorrhizal fungus in the microclimate might also influence the habitat of the orchids (Hegde SN 1982). The environmental factors that categorize orchid habitats are: soil requirements, freedom from competition, mycorrhiza, acidity, soil temperature and solar exposure. Seven habitat types were identified for medicinal orchids. Oak habitats (BO, MO and BGS) had the maximum diversity to help the growth of medicinal orchids. Species such as *H.edgeworthii* and *H.intermedia* generally prefer a canopy, which has less than 30% exposure to sun. *S.nepalense was* seen frequently at the edge of the forest and sometimes in open sunny meadows with moderate slopes. *M.acuminata* forms colonies in shady places, moist ground and in the areas that are wet and mossy. *M.muscifera* too prefer moist localities, but this species generally grows in a scattered way. It is clear that orchids need special microhabitat conditions and that these microhabitat features are responsible for their distribution. *D.hatageria* is only restricted to the Alpine Grassy Slopes (AGS) habitat and Herbaceous Meadows (HM) and from the conservation point of view, such species are more important. Species with specific habitat requirements have greater possibilities of extinction than the species with a broad habitat range.

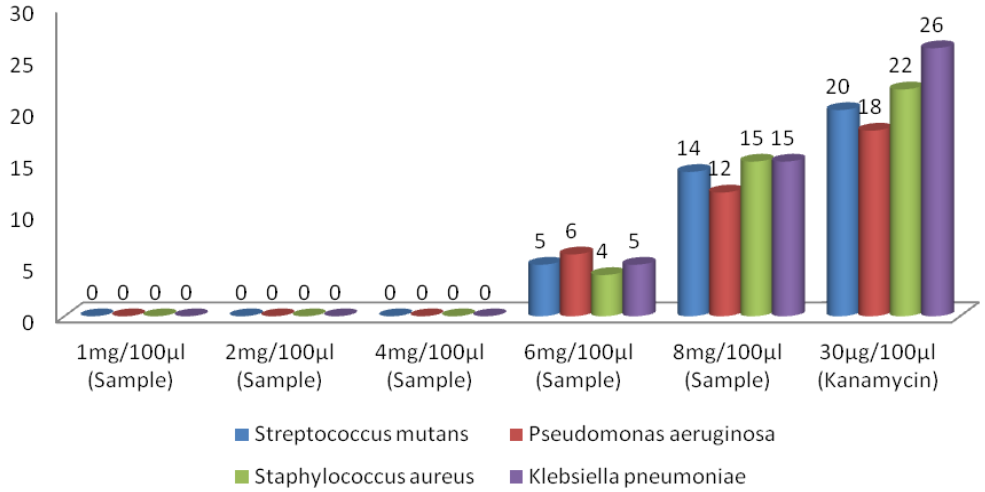

Figure 3. Anti-microbial activity of Methanolic extract of *Satyrium nepalense* at different dose level against Kanamycin

The abundance of many orchid species is believed to have fallen to critical levels in recent years. Orchids are subjected to high levels of threats, through both natural and anthropogenic causes (Kull *et al.*, 2006). It has been studied at various times that many known brands of herbal medicines use substitutes for some medicinal orchids due to their unavailability and one such example is that of *E.dabia.* It is so rare today that it has been substituted by *S.nepalense.* This is due to the depletion of the population of these medicinal orchids in the state. The economic potential of these medicinal orchids can be accessed on the basis of their high market demand. The annual demand of the species *D. hatageria* is 5000 tons (Kala *et al.,* 2004) and for the species *H. intermedia* it was 9995.5 kg during the year 2004 - 2005 in some localities of the state **(**Ahuja 2003). To get preliminary idea about the active constituents present in the methanolic extract different chemical tests were performed and found the presence of carbo-hydrates/glycosides, alkaloids, flavonoids, unsaturated sterols/triterpenes except saponins, tannins and resins. Antibacterial activity was tested by using the disc diffusion method. Disc diffusion method is widely acceptable for the preliminary screening of antibacterial activity. It is essentially a qualitative or semi qualitative test indicating the sensitivity or resistance of microorganisms to the test materials (Ronald, 1982). The antibacterial activity was assessed against four food pathogenic bacterial strains (both gram positive and gram negative) at the different dose (1mg/100µL, 2mg/100µL, 4mg/100µL, 6mg/100µL and 8mg/100µL) level and the results were compared with the standard drug kanamycin (30µg/100µL). The extract was found active against both gram positive and gram negative bacteria. The zone of inhibition varies within the ranges of 04±0.00-06±0.00mm and 12±0.82-15±0.0mm at the dose of 06 and 08 mg/100µL respectively. The highest zone of inhibition was found against *K.pneumonia* (15 mm) and showed moderate activity against *S.aureus, S.mutans* and *P.aeruginosa.* The results support the traditional use of this plant as a remedy of infectious diseases like diarrhoea, dysentery and gastrointestinal disturbances.

CONCLUSION

During the study a total of seven habitat types were identified where medicinal orchids were found. Among seven habitats Banj-oak habitat was found the most suitable environment for the orchids followed by Mixed-oak and Banj Grassy Slopes. The phytochemical fingerprint and the antibacterial

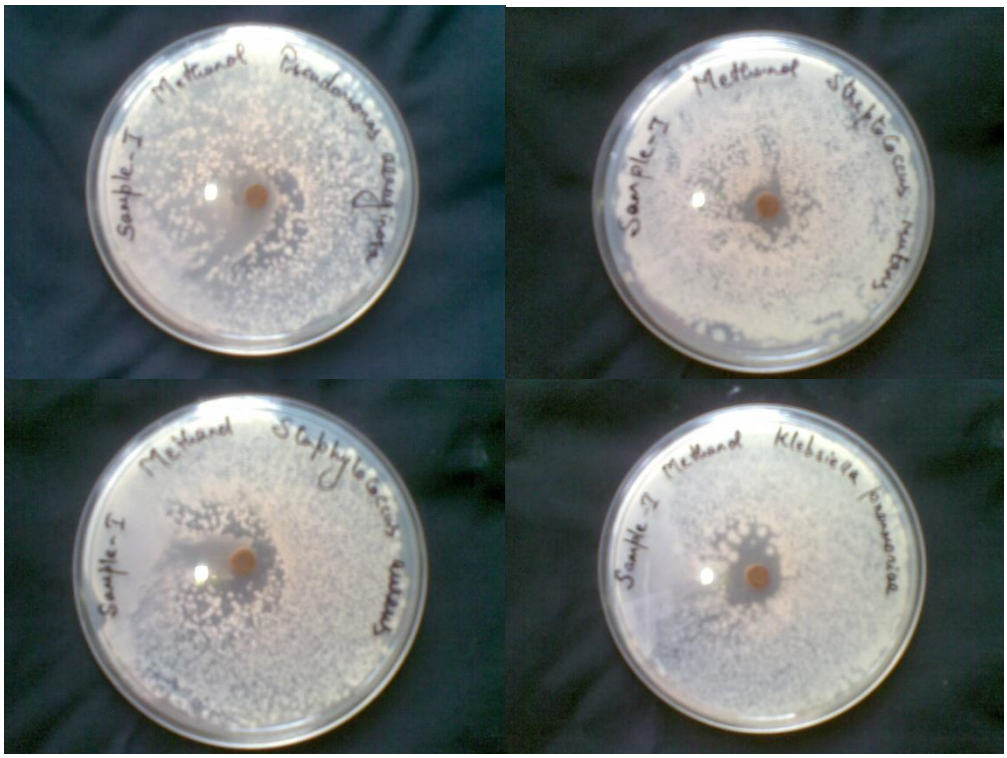

Figure 4. Photodocumentation of Antimicrobial activities of *Satyrium nepalense* methanolic extract against four bacterial strains. Disc size-3mm, Inhibitory zone size ±1mm, mm-millimetres

profile of *S.nepalense* was deeply investigated in triplicate. The results, confirmed the antibacterial effect of *S.nepalense* and it was clearly evidenced that the most potential extract in terms of microbial properties is the methanol one. Hence, results justify that, methanolic extract of *S.nepalense* has excellent antibacterial activity against both gram$^+$ as well as gram-ve bacteria and contains important chemical constituent's viz. carbohydrates, alkaloids, flavonoids and unsaturated sterols/triterpenes that confer upon it as a medicinal orchid.

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