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ASSESSMENT OF HIMALAYAN JUNIPER, *JUNIPERUS SQUAMATA* BUCH– HAM EX D. DON FOR PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL POTENTIAL AGAINST SOME INFECTION CAUSING PATHOGENS

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ABSTRACT

Leaves of *Juniperus squamata* Buch. – Ham ex D. Don was screened for phytochemicals and *in vitro* antibacterial activity. The shade dried leaves were extracted with methanol and ethanol. Ethanol and methanol extracts of *J. squamata* were assessed for their antibacterial potential and phytochemical screening against five pathogenic bacteria; *Agrobacterium tumefaciens, Bacillus subtilis, Escherichia coli, Erwinia chrysanthemi* and *Xanthomonas phaseoli*, employing disc-diffusion method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were further observed by employing serial dilution technique. Both extracts of *J. squamata* were found effective by showing a mark zone of inhibition (ZOI). The maximum inhibitory potential was recorded in ethanol extract (ZOI,15-21 mm). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanolic extract were also determined as 31.25 to 500 µg/ml and 125 to 500 µg/ml, respectively. The lowest MIC (31.25µg/ml) was observed in ethanol extract against *E. chrysanthemi* while lowest MBC (125µg/ml) was recorded against *E. chrysanthemi* and *B. subtilis*, in the same extract. Solvents treated discs were used as negative control and discs of standard antibiotics served as positive control in the experiment. The qualitative phytochemical analysis showed the presence of alkaloids, flavonoids, carbohydrates, glycosides, protein, saponin, terpenoids, phenols, starch, and resins, in both used extracts. The results showed that both leaves extracts of *J. squamata* possess a broad spectrum activity against a panel of bacterial strains responsible for the most common plant and animal diseases.

KEYWORDS: *Juniperus squamata*, phytochemical screening, antibacterial activity and gymnosperm plant extracts.

INTRODUCTION

Medicinal plants are the rich resource for drugs as well as food supplements, traditional medicine, modern medicine, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al., 2008). World Health Organization also suggested that medicinal plant would be the best source for variety of drugs. Therefore, there plants should be investigated to better understand their properties, protection and competency (Arunkumar and Muthuselvam, 2009).

Many gymnospermous plants extracts are used as pharmaceuticals, agrochemicals, flavours and fragrance, ingredients, food additives and pesticides (Balandrin and Klocke, 1988; Sati and Joshi, 2011). Unfortunately, a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is quite limited (Sati et al., 2015). Thus any phytochemical investigation of a given plant will reveal only a very narrow spectrum of its constituents. Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also disclosing new compound as tannins, oils, gums, precursors for the synthesis of complex chemical substances.

Juniperus squamata Buh.-Ham. ex D. Don a gymnosperm, vernacular name 'Dhupi' belonging to the family Cupressaceae (common name-Himalayan juniper), is a high altitude shrub which occurs at 2700 to 4200 m in Himalaya. It is well known for its traditional medicinal value. An essential oil is obtained from this by steam distillation of dried or partially dried ripe berries (fruits), leaves and twigs. In Ayurvedic medicine, juniper berry is one of the best diuretics for 'Vata' constitution, dispelling excess 'Vata' and improving digestion (Clutton, 1972), may be applied externally for arthritic pain and swelling (Kirtikar and Basu, 1935), also recommended in cough, infantile tuberculosis and diabetes (Zaman et al., 1970), whereas, ash of the bark is used for certain skin diseases (Baquar, 1989).

Himalayan juniper is used medicinally for urinary infections, respiratory problems as well as gastro-intestinal infections and worms. It has also been used in rubificant as well as antiseptic, antispasmodic, antitoxic, aphrodisiac, astringent, carminative, cicatrizant, depurative, diuretic, ammanagogue, nervine, parasiticide, sedative, stomachic, sudorific, tonic, vulnerary (Takacsova et al., 1995).

In addition, the knowledge of the chemical constituents of medicinally important plants would further be useful in discovering the actual value of folkloric remedies (Mojab, et al., 2003; Sati, et al., 2014). Since many microorganisms have developed resistance to many antibiotics (Cowan, 1999), therefore it is a regular need to develop alternative antimicrobial drugs. One approach is to screen local medicinal plants, which represent a rich source of novel antimicrobial agents (Khulbe and Sati, 2009). The present study was carried out to investigate the antimicrobial potential as well as phytochemical analysis of *Juniperus squamata*, a gymnospermic plant from Garhwal Himalaya hitherto unknown.

MATERIAL AND METHODS

Collection of plant material

The green leaves of *Juniperus squamata* Buch. – Ham ex D. Don (Cupressaceae) were collected in the month of October from Auli (District Chamoli in Uttrakhand), Gharwal Himalaya, India and authenticated by the Department of Botany, Kumaun University, Nainital. A voucher specimen was deposited in the herbarium of the department.

Extraction procedure

Leaves of the plant were thoroughly washed and dried at the room temperature (20±2°C). The dried material was powdered in an electric grinder. To prepare stock solution 50g of this powder was added to 200ml of solvents (w/v, 50g/200ml). Solvents used for extraction were methanol and ethanol. Both extracts were shaken for at least 6 h and after that extracts were passed through Whatman filter paper no.1 and the final filtrate as 25% crude extract thus concentrated on a rotary evaporator under vacuum at 20° C.

Microorganisms used

Five (Gram +ve and -ve) bacteria (*Bacillus subtilis* MTCC No. 121, *Escherichia coli* MTCC No.40, *Agrobacterium tumefaciens* MTCC No.609, procured from Institute of Microbial Technology, Chandigarh, India and *Xanthomonas phaseoli* and *Erwinia chrysanthemi* obtained from Plant Pathology Department, G. B. Pant University, Pantnagar, India) were used in this investigation.

Qualitative phytochemical analysis

Phytochemical analysis of ethanol and methanol leaves extracts of *J. squamata* subjected to following methodology of Harborne (1998) shown in table 1. for the presence of the active ingredients in the test sample.

1. Tests for Carbohydrate

Molisch's test

1ml of extract was treated with few drops of Molisch's reagent (α-naphthol, 20% in ethyl alcohol). Then about 1ml of concentrated sulfuric acid was added belatedly along the sides of the tube. Formation of violet color indicates the presence of carbohydrates.

Fehling's test

1ml of Fehling's A (Copper sulphate in distilled water) and 1ml of Fehling's B (Potassium tartarate and sodium hydroxide in distilled water) reagents were mixed and boiled for minute. Then equal volume of test solution was added to the above mixture. The solution was heated in a boiling water bath. Brick red precipitate was observed, indicating the presence of carbohydrates.

Iodine test

Crude extract was mixed with 2ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

2. Test for proteins

Millon's test

Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Xanthoproteic Test

The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

3. Tests for glycosides

Keller- Killiani test

1ml of glacial acetic acid containing traces of ferric chloride and 1ml of concentrated sulfuric acid, 1ml of extract was added carefully. Appearance of brown ring at the interface shows the presence of glycosides. A violet ring may also appear below the brown ring.

Legal's Test

Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Borntrager's test

To 1ml of extract, 1ml of benzene and 0.5ml dilute ammonia solution were added. A reddish pink color indicates the presence of glycosides.

Baljet test

To 1ml of extract, 1ml of sodium picrate is added. Appearance of yellow to orange color detects the presence of glycosides.

4. Tests for saponins

Foam test

1ml of extract was shaken vigorously with 20ml of distilled water for 5- 10 minutes in graduated cylinders. Formation of one centimeter layer of foam indicated the presence of saponins.

5. Test for terpenoids

Salkowski test

5 ml of each extract was mixed in 2 mL of chloroform, and concentrated sulphuric acid (3 mL) was carefully added to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Trichloroacetic acid test

To 1ml of extract, 2ml of trichloroacetic acid was added. Formation of colored precipitate showed the presence of terpenoids.

6. Tests for phenolic compounds

Ferric Chloride Test

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols and tannin.

Lead acetate test

On addition of lead acetate solution to the extract white precipitate appeared.

Dilute HNO3 test

On addition of dilute HNO3 solution to the extract reddish color appeared.

7. Test for alkaloids

Mayer's Test

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's test

To 1ml of extract, 2ml of Dragendroff's reagent is added. Orange red precipitate is formed indicating the presence of alkaloids.

8. Test for flavonoids

Alkaline reagent test

Small quantity of each extract sample was taken and added with lead acetate solution. After few minutes appearance of yellow colour precipitates which indicated the presence of flavonoids.

Shinoda test

To 1ml of extract, few drops of concentrated HCl were added. To this solution 0.5 gram of magnesium turnings were added. Observance of pink coloration indicated the presence of flavonoids.

Lead acetate test

To the 1ml of extract, lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoids.

Ferric chloride test

To 1ml of extract, 1ml of ferric chloride (5% in water) was added. Formation of brown color confirmed the presence of flavonoids.

9. Test for starch

To 1 mL of aqueous extract was added 10 mL of NaCl saturated solution. After heating, starch reagent was added a blue-purplish colour is a positive test for the presence of starch.

10. Test for volatile oils

NaOH-HCl test

2ml of extract solution was shaken with 0.1ml of dilute sodium hydroxide and a small quantity of dilute HCl. A white precipitate was formed with volatile oils.

11. Test for phlobatannins

1ml of extract was boiled with 1% aqueous HCl. The formation of red precipitate indicated the presence of phlobatannins.

12. Tests for resins

Turbidity test

Distilled water (5ml) was added to the extract. The occurrence of turbidity showed the presence of resins.

13. Test for quinones

Few drops of concentrated Sulphuric acid was added to 1 ml of extract appearance of red colour indicate the presence of quinons.

14. Test for Tannins

In 1 ml of extract add few drops of 5% FeCl3. Appearance of green colour indicates presence of gallotannin, and brown colour indicates the presence of tannins.

15. Test for fat

Few drops of sudan-III added in to 1 ml of extract, red colour appears with oil droplets.

16. Test for anthraquinones

Few drops of concentrated Sulphuric acid were added to 5 ml of extract. To this solution 1 ml of ammonia was added. Appearance of rose-pink colour indicates presence of anthraquinones.

Screening of antibacterial activity

Antibacterial tests of selected microorganisms were carried out using disc-diffusion method (Bauer et al., 1966). Nutrient agar plates (90mm size) were prepared and cooled down at room temperature (20±2°C). A small sterile cotton swab was dipped into the 24h old culture of bacteria and was inoculated by streaking the swab over the entire agar surface. This

process was repeated by streaking the swab 2 or more times rotating the plates approximately 60° each time to ensure even distribution of inoculums.

After inoculation the plates were allowed to dry at room temperature (20±2°C) for 15min in laminar chamber for settle down of inoculum. The filter paper discs (5 mm) loaded with 40µl of extract were placed on the surface of the bacteria seeded agar plates and it was allowed to diffuse for 5min then these plates were incubated at 37±1°C for 18h. Gentamycin (30 mcg), was placed into agar plates used as positive control and respective solvent discs (air-dried) were also used as negative control (Plate. 1). After 18h of incubation, the diameter was observed for inhibition zone (measured in mm including disc size). All tests were performed in triplicates (3 discs/plate) and observed values of ZOI are expressed as mean value with standard error of means (SEM).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

All the fractions were tested for the minimum inhibitory concentration to find out the lowest concentration of an extract that inhibits the visible growth of test microorganisms and same test was used to determine the minimum bactericidal concentration. MIC was performed at five concentrations of extracts (500, 250, 125, 62.3, 31.25 µg/ml) following two fold serial dilution technique. All the tests which did show no visible growth of microorganisms at MIC test were subcultured and incubated at 37±1°C overnight. The highest dilution showing 100% inhibition was recorded as MBC.

RESULTS

The results of phytochemical analysis are presented in table.1, which shows the presence of carbohydrate, glycosides, protein, saponin, terpenoids, phenol, alkaloids, flavonoids, starch, resins, in both methanol and ethanol extracts of *J. squamata*. Out of 16 test conducted for preliminary chemical analysis 13 phytochemicals were found in the methanol extract while ethanol extract comprises of only 11 phytochemicals. Phlobatannin and anthrqunones were absent in both the extracts. Tannins, volatile oils and fat are present only in methanol extract, on the other hand quinones was present in ethanol extract but absent in methanol extract. Different active phytochemicals present are found to possess a wide range of activities, which may help in the protection against incurable diseases.

Table 1: Phytochemical evaluation of *J. squamata* leaves extracts

Plant Metabolites	Observation	
	Ethanol extract	Methanol extract
Carbohydrates	+	+
Glycoside	+	+
Protein	+	+
Saponins	+	+
Terpenoids	+	+
Tannins	-	+
Phenol	+	+
Alkaloids	+	+
Flavonoids	+	+
Starch	+	+
Volatile oils	-	+
Phlobatannins	-	-
Resins	+	+
Quinones	+	-
Fat	-	+
Anthraquinone	-	-

+ = present; - = absent

Table 2: Antibacterial activity of *J. squamata* leaves extracts

Microorganisms	Diameter of Inhibition Zone (mm)*			
	E	**M**	**G**	**NC**
A. tumefaciens	15±0.6	11±0.6	24±0.9	na
B. subtilis	19±0.6	12±0.7	23±1.6	na
E. chrysanthemi	21±0.6	16±0.3	25±0.7	na
E. coli	17±0.9	13±0.7	26±0.6	na
X. phaseoli	18±1.2	14±0.3	25±0.6	na

* All the values are mean ± Standard Error of Mean (SEM) of three determinations **E-** Ethanol**, M-** Methanol, **G –** Gentamycin (+control), **NC-** Negative control, **na-** Not active

The antibacterial activities of *J. squamata* leaves extracts against 5 test microorganisms are presented in Table 2. The MIC and MBC for both fractions are presented in Table 3. As evident from Table 2, the ethanol extract showed the highest inhibitory activity against *E. chrysanthemi* having ZOI 21 mm, followed by *B. subtilis* (19mm), *E. coli* (ZOI, 17 mm), *X. phaseoli* (18 mm). But on the other hand the methanol extract showed a maximum activity against *E. chrysanthemi* (16mm) followed by *X. phaseoli* (14mm).

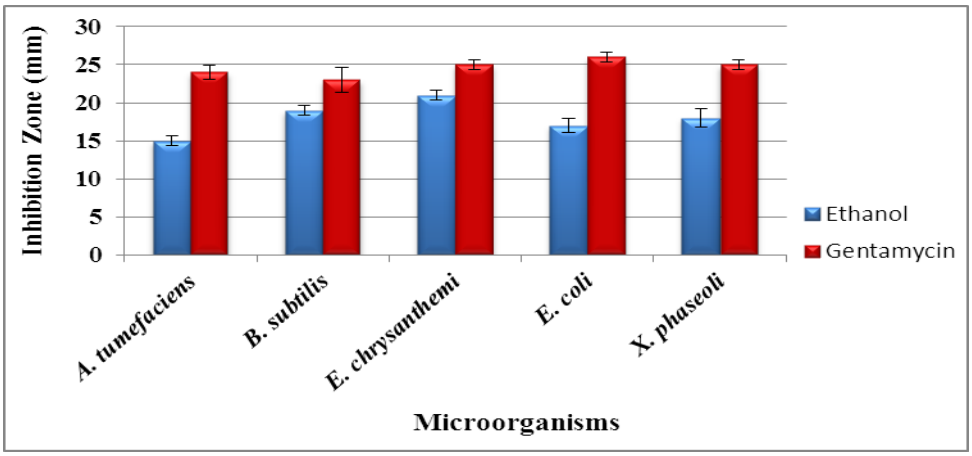

Fig. 1.1: Graphical representation of antibacterial activity of *J. squamata* leaves ethanol extract

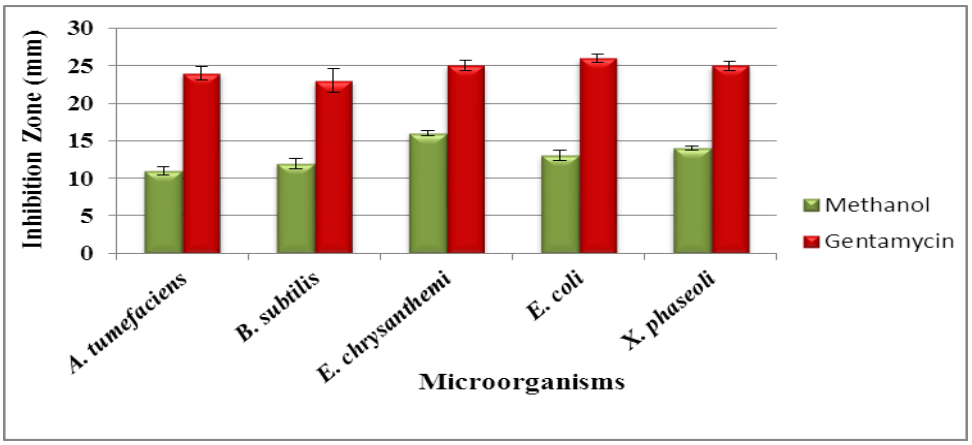

Fig. 1.2: Graphical representation of antibacterial activity of *J. squamata* leaves methanol extract

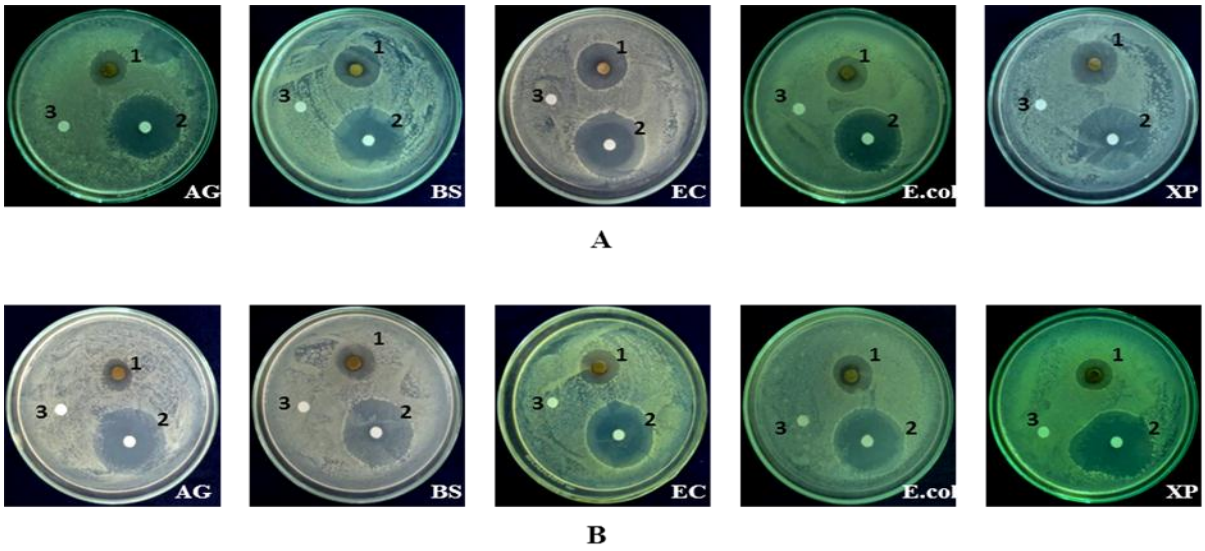

Plate 1. Antibacterial activity of *Juniperus squamata* leaves extracts against some pathogenic bacteria. **(A)** - Ethanol extract **(B)** - Methanol extract, **AG-** *Agrobacterium tumefaciens*,

E.col- *Escherichia coli*, **EC-** *Erwinia chrysanthemi*, **XP-** *Xanthomonas phaseoli*, **BS-** *Bacillus subtilis.*1- Extract, 2,- positive controls (Gentamycin,), 3- negative control (solvent)

MIC and MBC values for the microorganisms sensitive to the ethanol extract were in the range of 31.25 to 250 µg/ml and 125 to 500 µg/ml, respectively. In the present investigation, lowest MIC and MBC values were recorded against *E. chrysanthemi* in the ethanol fraction 31.25 µg/ml and 125 µg/ml, respectively (Table 3).

Table 3: MIC and MBC evaluation for *J. squamata* leaves extracts

Microorganisms	Concentration (µg/ml)			
	Ethanol		Methanol	
	MIC	MBC	MIC	MBC
A. tumefaciens	250	500	500	na
B. subtilis	62.5	125	250	na
E. chrysanthemi	31.25	125	250	500
E. coli	125	500	250	na
X. phaseoli	125	250	250	500

na: not active

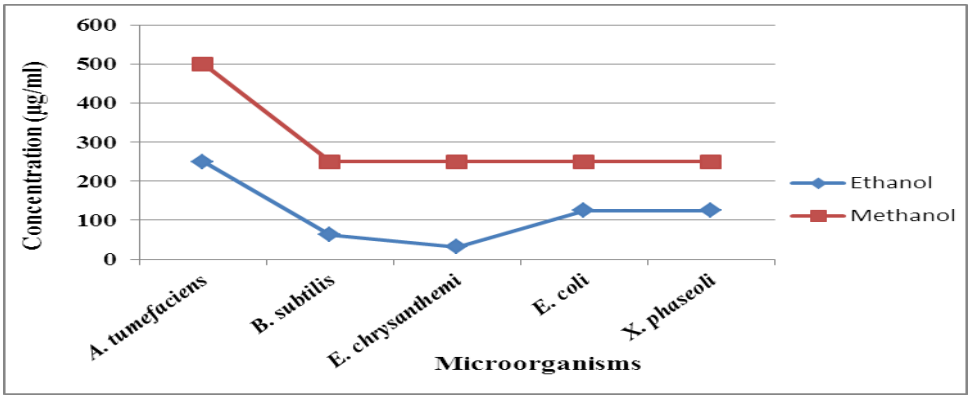

Fig. 2.1. Minimum inhibitory concentration (MIC) of *J. squamata* leaves extracts against some pathogenic bacteria.

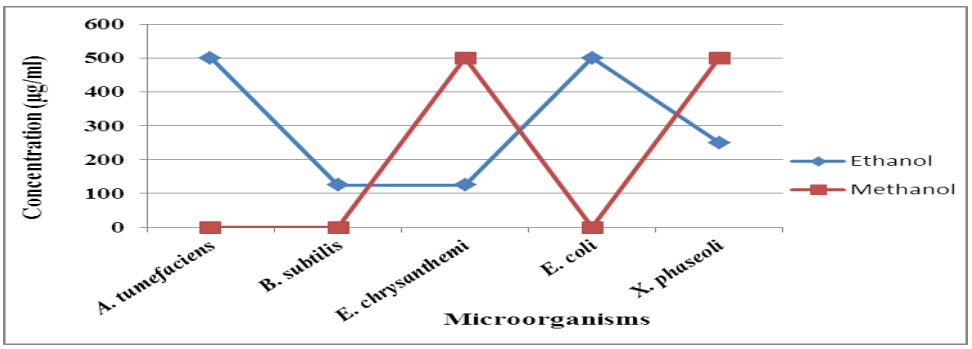

Fig. 2.2. Minimum bactericidal concentration (MBC) of *J. squamata* leaves extracts against some pathogenic bacteria.

DISCUSSION

The great Himalayan region harbours a high biodiversity of medicinal plants, used for different therapeutic and medicinal purposes. *Juniperus* is also a medicinal value plant of high altitude but not focused for its antimicrobial potential as well as for phytochemical screening (Sati and Joshi, 2010). *J. squamata* oil has been tested for its antifungal potential by Adams, et al., (1996). Acetoxycedrol derivative of cedranediol isolated from essential oil of *J. squamata* which is used as antiplatelet and vasorelaxing (Teng, et al., 1994). Although Mazari et al., (2010) studied the antimicrobial activity of the essential oils of *Juniperus phoenicea* against five bacteria (3 Gram-positive and 2 Gram-negative), as well as 3 fungi and found inhibition in growth of tested microbes. Similarly, Sati and Joshi (2010) studied five pathogenic bacteria namely; *Bacillus subtilis*, *Erwinia chrysanthemi, Escherichia coli*, *Agrobacterium tumefaciens* and *Xanthomonas phaseoli* against the crude leaf extracts in organic solvents (methanol, ethanol, chloroform and hexane) of Kumaun Himalayan *J. communis* and recorded positive results. Wedge et al., (2009) investigated chemical composition and antifungal activity of the essential oil of *J. saltuaria* and *J. squamata*. The essential oil of these species was found effective for the inactivation of *Colletotrichum aculatum, C. fragariae* and *C. gloeosporioides*. In the present study, results are also found positive and it is interesting to note that in comparison to gentamycin (antibacterial standard drug), ethanol extract has shown good activity for all the tested microbes (Fig. 1.1). Findings of MIC are quite interesting and rang between 500 - 31.25 µg/ml (Fig.2.1).

As evident from phytochemical analysis the present species of *Juniperus* produces chemical compounds such as sugar and fats as primary metabolites. However, alkaloids, tannins, glycosides, essential oils, terpenes, saponins, flavanoids, steroids and Phenolic compounds are present as secondary metabolites having broad and narrow spectrum which produce specific physiological action in human body. These phytochemicals have also antibacterial, antifungal, antiviral and antihelminthic properties and they serve as defence agent against invading microorganisms (Balandrin et al., 1985; Song et al., 2009; Benziane et al., 2012).

Relying upon the finding of present investigation, it can be concluded that *J. squamata* leaves extracts possess a broad spectrum activity against a panel of pathogenic bacteria, responsible for the most of human and plant diseases. The presence of a series of phytochemcial indicates the high medicinal value of the studied plant and demands for their commercial exploitation in pharmaceutical sectors. Thus it is clear that the Garhwal Himalayan gymnosperm *J.*

squamata possess a good antimicrobial potential as well as medicinally important phytochemicals which may be used to minimize animal and plant diseases. The present investigation is perhaps first attempt to explore the phytochemical analysis and antibacterial potentiality of high altitude Garhwal Himalayan gymnospermous plant *J. squamata*.

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