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Antibacterial Screening of Aqueous, Alcoholic and Hydroalcoholic extracts of a Unani drug Abhal (Fruits of *Juniperus communis*)

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ABSTRACT

Abhal is the dried ripe fruit of *Juniperus communis* Linn. It has long been using in Unani Medicine to treat many ailments including genitourinary infectious diseases. In the present study, the aqueous, alcoholic and hydroalcoholic extracts of Abhal berries were screened for their antimicrobial activity against both gram positive (*Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Corynebacterium xerosis* and *Bacillus cereus*) and gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus vulgaris*) bacterial strains compared with the standard Drug: Ciprofloxacin (SD060) 5µg/disk for gram positive bacterial strains and Gentamicin (SD016) 10µg/disk for gram negative bacterial strains using Zone of Inhibition (ZOI) with the help of Agar well method and Minimum Inhibitory concentration (MIC) & Minimum Bactericidal Concentration (MBC) with the help of Nutrient Broth method. The data was analysed using Gpad INSTAT software, one way ANOVA and post-test named Bonferroni. Alcoholic and hydroalcoholic extract showed significant antibacterial activity than the aqueous extract but not up to the mark as compared to standard group indicating that the alcoholic and hydroalcoholic extract has the capability of extracting more phytochemicals than aqueous extract which are responsible for their antimicrobial activity. It could be concluded that the present drug possesses antimicrobial property.

Keywords: Antimicrobial, Abhal, *Juniperus communis*, MIC, MBC.

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Introduction

Abhal is the dried ripe fruit of *Juniperus communis* Linn. The berries are used for medicinal and religious purposes since ancient time. In "Papyrus of Ani" it is mentioned as remedy to treat the tapeworm, this shows that human beings knew it since 550 BC. In ancient Egypt it was buried in temple as a part of purification ceremony. Medical man in 15th century burns the berries to guard the community against plague (Afaq *et al.,* 2006). In British Herbal Pharmacopoiea (1983, 1990, vol. 1) the berries are also mentioned as antiseptic. Due to its antiseptic properties its use in gonorrhoea, leucorrhoea and subcutaneous diseases appears justified. The fruit of some species of Juniper is, however, used by Hippocrates in certain disorders of the womb, and Dioscorides mentioned its diuretic and digestive properties, and used in cough and pectoral affections. Ibn Sina closely follows Dioscorides and gives no additional information concerning the plant. In Unani System of Medicine the drug is used for its diuretic, carminative and stimulant properties (Dymock *et al., 1893;* Afaq *et al.,* 2006). It is found in Himalayas from Kumaon Westwards at an altitude of 12500–14000 ft. to Temperate and Subarctic Europe, Asia, North Africa and North America regions. Irvine mentions that they are imported into Patna from Nepal, and used in the treatment of gonorrhoea (Kirtikar and Basu, 1996). A dense, more or less procumbent shrub, rarely a small tree (Anonymous, 2001). The berry like fruit takes 2 years to ripen. Eventually becoming a deep purple colour and having a bluish-grey bloom. On drying, the berries become somewhat darker and shrivel slightly; they are about 3-10 mm in diameter. The apex shows a tri radiate mark and depression indicating the suture of the three fleshy scales. At the base there are usually six, small, pointed bracts arranged in two whorls, but occasionally three or four such whorls are found. A transverse section of the fruit shows a thin outer skin or epicarp, a yellowish-brown, pulpy mesocarp and three seeds. The drug has a pleasant, somewhat terbinthinate odour, and a sweetish taste. They are employed for flavouring gin and food products; they are sometimes used as an article of food (Trease and Evans,

2009). The fruit contains volatile oil (about 0.5-1.5%), fermentable sugars (33%), resin (8%), juniperin (probably a mixture of tannin and sugar, 0.36%), fixed oil, proteins, wax, gum, pectin, organic acids (formic, acetic, malic, oxalic, and glycolic) and potassium salts. They are good source of ascorbic acid (c. 35mg/100g.) (Rastogi and Mehrotra, 1990; Anonymous, 2001).

Prior to the antibacterial screening, preliminary phytochemical tests of the fruits of *Juniperus communis* revealed the presence of secondary metabolites such as alkaloids, carbohydrates, glycosides, tannins, phenols, and sterol/terpenes (Akhtar *et al.,* 2019).

To validate the claims of Unani physicians being effectiveness of Abhal in infectious diseases, the present study was aimed to test the antibacterial potential of test drug, which has long been used in the treatment of various infectious diseases by using Agar well and Disc diffusion method.

Materials and Methods

Collection and Authentication of drug

The berries of *Juniperus communis* were procured from the local market of Aligarh city in the month of April 2017. The identity was confirmed with the help of literatures available and Pharmacognosy Section, Department of Ilmul Advia, Ajmal Khan Tibbiya College, Aligarh Muslim University, Aligarh. The sample was further authenticated by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (NISCAIR /RHMD/ Consult/ 2017/ 3089/ 38-3). The specimen of the test drug was submitted to Mawalid-e-Salasa Museum of the Department for future reference with the voucher Number of (SC-0217/17).

Figure 1: Market sample of Abhal (*Juniperus communis*)

Preparation of Plant Extracts

Coarsely powdered drug Abhal (25gm in 250ml solvent) were extracted from Double Distilled Water, ethanol (95%) and hydro-alcoholic mixture (50:50) as a solvent for aqueous, alcoholic and hydro-alcoholic extracts respectively using Soxhlet apparatus for 6 hours. The extract obtained was filtered over Whatman No. 1 filter paper and then subjected to dryness at 55º C on Water Bath and kept at 4º C till further use and was reconstituted with Dimethyl Sulphoxide (DMSO) used as a solvent to make stock solution at varying concentration for working with test drug. Aqueous extract was dissolved in Double Distilled Water.

Bacterial Strains

Six gram positive (*Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Corynebacterium xerosis* and *Bacillus cereus*) and four gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus vulgaris*) bacterial strains were used to assess the antimicrobial properties of the test drug. All the gram positive and gram negative strains were clinical isolates that were obtained from Microbiology Lab of the Department of Ilmul Advia, AMU, Aligarh. Bacterial strains were maintained on 1 % semisolid nutrient agar at 4°C and sub cultured every two weeks in Microbiology lab.

Concentration of test drug and Standard used

The stock solutions of aqueous, alcoholic and hydro-alcoholic extracts were prepared at a concentration of 5µgm/µl, 10µgm/µl and 20µgm/µl. 50µl/well was used from each stock solution for the study. Standard Drugs were Ciprofloxacin (SD060) 5µg/disk for gram positive bacterial strains and Gentamicin (SD016) 10µg/disk for gram negative bacterial strains.

Antibacterial Screening

The antibacterial screening of aqueous, alcoholic and hydroalcoholic extracts of Abhal was done as per Clinical and Laboratory Standard Institute (CLSI) guidelines against bacterial strains. Results were analyzed on the basis of Zone of Inhibition (ZOI), Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) by Kirby Bauer's disc diffusion method and Agar Well Method.

Media Preparation

Nutrient Agar No. 2 (HiMedia M1269S-500G) was prepared as per company instruction and autoclaved, and then 25-30 ml was poured into sterile disposable (10 cm in diameter) petri dishes on a surface level to give a uniform depth of 4 mm.

Agar Well Method

A sterilized swab (PW041, HiMedia Labs, Mumbai, India) was dipped into the inoculums, suspension (10^6cfu/ml) was rotated several times and pressed on the inner side of the test tube to remove excess inoculation from it. The dried surface of the nutrient plate (pH 7.2-7.4) was then streaked with the swab three times, turning the plate 60^0 angles between each streaking with the bacterium and finally the rim of the plate, so as to ensure even distribution over the entire surface of the plate (Anonymous, 1999). The inoculums were allowed to dry for 5-15 minutes with lid in plate but not more than 15 minutes, so as to allow for any excess surface moisture to be absorbed before applying the drugs. The wells of the equivalent size were then prepared with the help of a cork borer (6mm in diameter) in the plate at the previously marked sites, as the process also tear the bottom of the agar, the problem was solved by filling it with few µl of the autoclaved molten agar to avoid diffusion of the drug only at the base (Bell and Grundy, 1968). The wells so prepared were filled by the drug sample (50 µl) in their respective site with the help of a micropipette (Ananthanarayan and Paniker, 2009). The standard antibiotic discs (HiMedia Labs, Mumbai, India) were placed on the prepared plates with sterile forceps and pressed properly to make complete contact with the surface of the medium. Plates were incubated at 37º C for 24 hours placing them at an inverted position (Arora, 2007).

Interpretation

The results were observed after 24 hours, by measuring the diameters of clear halos around the discs (standard drug) and wells of test drug including the diameter of discs and wells of test drug, or the inhibition zones using a

HiAntibiotic Zone Scale™ –C (PW297-HiMedia Laboratories Pvt. Limited, Mumbai). The larger the area is, the greater the germ is sensitive.

Minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration where no visible turbidity is observed in the test tube (Bacteriostasis concentration) (Leelaprakash and Dass, 2011). MIC is considered as the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used as a method of precise assessment to determine the MIC of the test drugs to the organisms concerned. MIC of various extracts against tested organisms was determined by Broth Dilution Method (Andrews, 2001). The Broth Dilution Method was used, as it has an added advantage that the same wells from 96-well microtiter plates can be taken for Minimum bacterial concentration tests also. Among three concentrations (5µgm/µl, 10µgm/µl and 20µgm/µl) of the test drug the intermediate concentration i.e. 10µgm/µl was used for the determination of MIC and MBC as the highest antibacterial activity was observed at this concentration. Different dilutions of drug extracts were selected such that the concentration that allowed determination of MIC break-point defining susceptible and resistant values was included. The stock dilutions of the drug sample were prepared so that concentrations ranging from 166.6 to 0.02µg/µl were obtained from the original stock solution (10µg/µl).

Broth Dilution Method

In a sterile microtiter plates (96-u-shaped wells) 50µl of the sterile nutrient broth was poured Upto 9th well in three rows, from a fresh inoculums so formed (10^6cfu/ml diluted with 100µl Nutrient broth to have 10^6cfu/ml), 50 µl of the suspension was poured in each well in the first and third row, second row was again filled with 50µl of Nutrient broth, finally the drug sample of 50µl was added in the first well of first row diluting uniformly from 166.6 to 0.02 µg/µl till the 9th well. The first row was positive control (nutrient Broth + bacterial culture + test drug), the second row was plain control (nutrient broth) and the third row was used as negative control (nutrient broth + bacterial culture).

Minimum bactericidal concentrations (MBC)

The MBC is defined as the lowest concentration where no bacterial growth is observed (Bacteriocidal concentration) (Leelaprakash and Dass, 2011). MBC was determined from the broth dilution resulting from the MIC tubes by sub culturing to antimicrobial free agar. In this technique, the contents of the test tubes resulting from MIC was streaked using a sterile swab (PW041, HiMedia Labs, Mumbai, India) on agar plate free of bacteria and incubated at 37°C for 18 hours.

Interpretation of MIC and MBC

The MIC values were interpreted as the lowest concentration of the drug sample (in other words highest dilution of the sample) which showed clear fluids with no development of turbidity. The lowest concentration of the extract which showed no bacterial growth was noted and recorded as the MBC.

Statistical Analysis

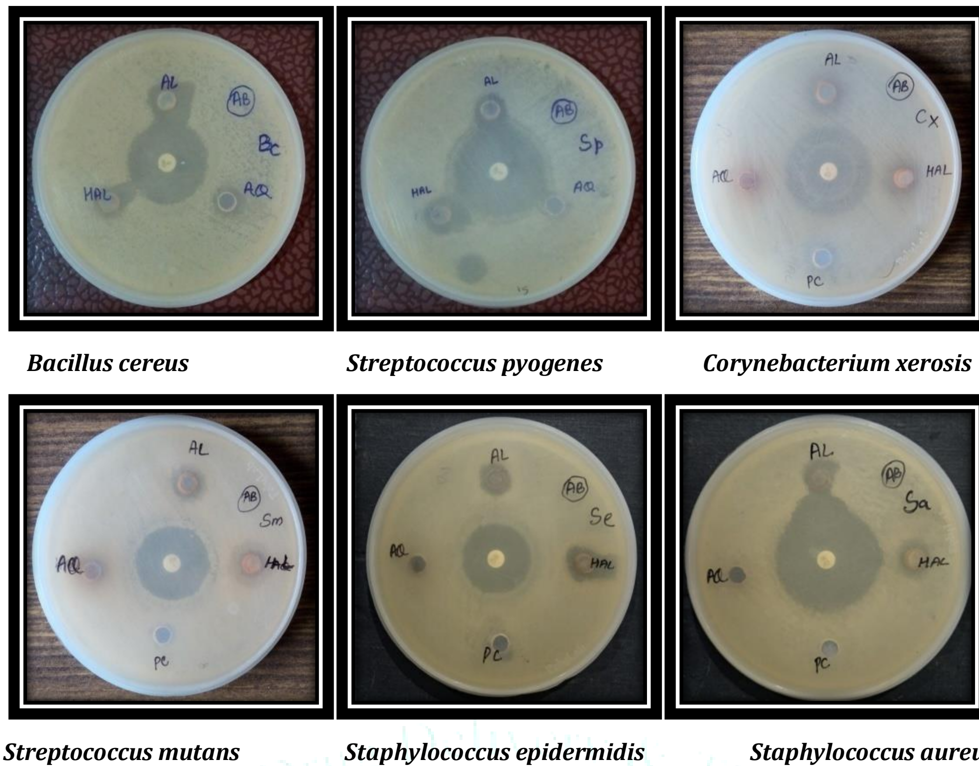
All the statistical analysis was done using Gpad (INSTAT) software, one way ANOVA and post test named Bonferroni. Selected pairs of column with multiple comparison were performed with p<0.05.

Observations

Table 1: Antibacterial Activity of Abhal (Fruits of *Juniperus communis*) against Gram positive bacterial strains

S.NO.	Test Strains (Clinical isolates)	Zone of Inhibition (in mm) expressed as Mean±SEM Probability of error										
		Aqueous extract (50µl/well)			Alcoholic extract (50µl/well)			Hydro-alcoholic extract (50µl/well)			Standard Ciprofloxacin (SD060) 5µgm/disc	Plain control DMSO (50µl)
		5µgm/µl	10 µgm/µl	20 µgm/µl	5 µgm/µl	10 µgm/µl	20 µgm/µl	5 µgm/µl	10 µgm/µl	20 µgm/µl		
1.	*Streptococcus mutans* (SM)	6.33±0.33	6.66±0.33	6.33±0.33	6.33±0.33	15.33±0.33***	15.33±0.33***	6.33±0.33	13.66±0.66***	17.33±0.66***	44.66±0.33	6.33±0.33
2.	*Staphylococccus aureus* (SA)	6.33±0.33	6.66±0.33	6.33±0.33	12.33±0.33***	12.33±0.33***	12.66±0.33***	12.33±0.33***	14.33±0.33***	14.33±0.33***	40.66±0.66	6.66±0.33
3.	*Staphylococccus epidermidis* (SE)	6.33±0.33	6.66±0.33	6.33±0.33	14.33±0.33***	12.33±0.33***	12.66±0.33***	12.33±0.33***	20.66±0.66***	15.33±0.33***	30.33±0.33	6.33±0.33
4.	*Streptococcus pyogenes* (SP)	6.33±0.33	6.33±0.33	6.33±0.33	12.33±0.33***	11.33±0.33***	14.33±0.33***	14.33±0.33***	10.33±0.33***	12.33±0.66***	32.33±0.66	6.33±0.33
5.	*Corynebacterium xerosis* (CX)	6.33±0.33	6.33±0.33	6.33±0.33	12.33±0.33***	17.33±0.33***	13.33±0.33***	12.33±0.33***	14.33±0.33***	15.66±0.33***	30.33±0.33	6.66±0.33
6.	*Bacillus Cereus* (BC)	10.66±0.33***	6.33±0.33	6.33±0.33	12.33±0.33***	15.33±0.33***	11.33±0.33***	12.33±0.33***	27.66±0.33***	16.66±0.66***	29.66±0.33	6.33±0.33

Significance: ***=p<0.001; **=p<0.01; =p<0.05; NS=Not Significant



Bacillus cereus *Streptococcus pyogenes* *Corynebacterium xerosis*

Streptococcus mutans *Staphylococcus epidermidis* *Staphylococcus aureus*

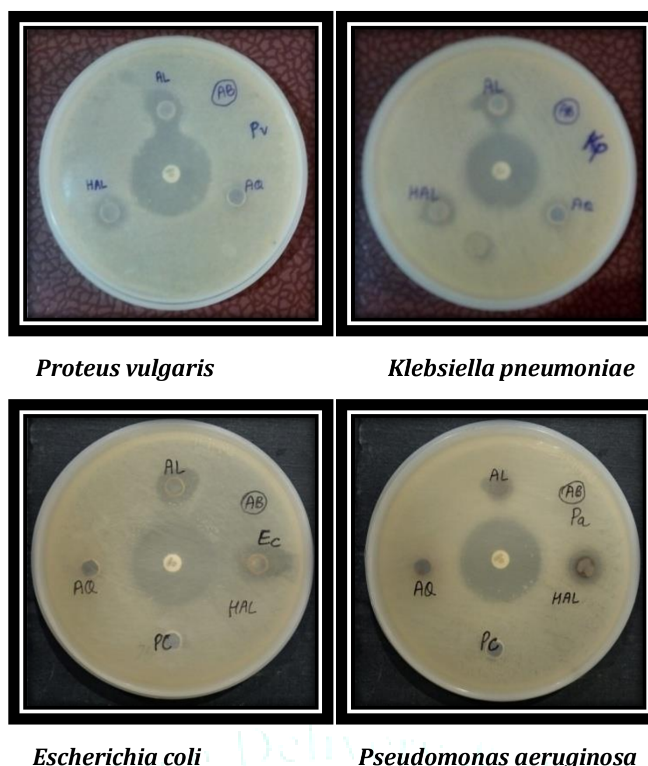
AQ = Aqueous; AL= Alcoholic; HAL = Hydroalcoholic; PC = Plain control (DMSO)

Fig. 2: Antimicrobial activity of aqueous, alcoholic and hydroalcoholic extract of Abhal, plain control (DMSO) and standard (Ciprofloxacin) against gram positive bacterial strains

Table 2: Antibacterial Activity of Abhal (Fruits of *Juniperus communis*) against Gram negative bacterial strains

S.NO.	Test Strains	Zone of Inhibition (in mm) expressed as Mean±SEM Probability of error										
		Aqueous extract (50µl/well)			Alcoholic extract (50µl/well)			Hydro-alcoholic extract (50µl/well)			Standard Gentamicin (SD016) 10µgm/disc	Plain control DMSO (50µl)
		5 µgm/µl	10 µgm/µl	20 µgm/µl	5 µgm/µl	10 µgm/µl	20 µgm/µl	5 µgm/µl	10 µgm/µl	20 µgm/µl		
1.	*Escherichia coli*	6.66±0.33	6.66±0.33	6.33±0.33	12.66±0.33***	17.33±0.33***	10.33±0.33***	12.33±0.33***	24.33±0.33***	10.33±0.33***	30.66±0.33	6.33±0.33
2.	*Klebsiella pneumoniae*	10.66±0.33	6.33±0.33	6.33±0.33	12.66±0.33***	20.33±0.33***	12.66±0.33***	12.33±0.33***	14.33±0.33***	15.66±0.33***	27.66±0.33	6.33±0.33
3.	*Pseudomonas aeruginosa*	6.66±0.33	6.33±0.33	6.33±0.33	12.66±0.66***	13.33±0.33***	12.33±0.33***	6.33±0.33	11.66±0.33***	11.66±0.33***	34.66±0.33	6.33±0.33
4.	*Proteus vulgaris*	6.33±0.33	6.33±0.33	6.33±0.33	13.33±0.33***	10.33±0.33***	12.33±0.33***	12.33±0.33***	19.33±0.33***	10.33±0.33***	30.66±0.33	6.33±0.33

Significance: ***=p<0.001; **=p<0.01; =p<0.05; NS=Not Significant



Proteus vulgaris ***Klebsiella pneumoniae***

Escherichia coli ***Pseudomonas aeruginosa***

Figure 3: Antimicrobial activity of aqueous, alcoholic and hydroalcoholic extract of Abhal, plain control (DMSO) and standard (Gentamicin) against gram negative bacterial strains

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in µgm/100 µl of the test drugs against various Gram Positive and Gram negative Bacterial strains

S.No.	Test Strains (Clinical strains)	ABHAL (Fruits of *Juniperus communis*)					
		Alcoholic Extract		Hydro-Alcoholic Extract		Aqueous Extract	
		MIC (µgm/100µl)	MBC (µgm/100µl)	MIC (µgm/100µl)	MBC (µgm/100µl)	MIC (µgm/100µl)	MBC (µgm/100µl)
1.	*Streptococcus mutans*	55.5	>166.6	18.5	>166.6	55.5	>166.6
2.	*Staphylococcus aureus*	55.5	>166.6	18.5	>166.6	55.5	>166.6
3.	*Staphylococcus epidermidis*	55.5	>166.6	55.5	>166.6	55.5	>166.6
4.	*Streptococcus pyogenes*	18.5	>166.6	18.5	>166.6	55.5	>166.6
5.	*Corynebacterium xerosis*	55.5	>166.6	18.5	>166.6	55.5	>166.6
6.	*Bacillus Cereus*	18.5	>166.6	18.5	>166.6	55.5	>166.6
7.	*Escherichia coli*	55.5	166.6	55.5	166.6	55.5	>166.6
8.	*Klebsiella pneumoniae*	55.5	>166.6	55.5	>166.6	55.5	>166.6
9.	*Pseudomonas aeruginosa*	55.5	>166.6	55.5	>166.6	55.5	>166.6
10.	*Proteus vulgaris*	55.5	166.6	18.5	166.6	55.5	>166.6

Discussion

Phytochemical constituents present in the drugs may vary, not only from plant to plant but also among different samples of same species. Preliminary phytochemical tests of the fruits of *Juniperus communis* revealed the presence of secondary metabolites such as alkaloids, carbohydrates, glycosides, tannins, phenols, and sterol/terpenes. The results of the present study on the antimicrobial activity of *Juniperus communis* against gram positive *Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Corynebacterium xerosis* and *Bacillus cereus* and gram negative *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus vulgaris* bacterial strains have shown that the aqueous, alcoholic and hydroalcoholic extracts of the seeds inhibited the growth of majority of the isolates. Hydroalcoholic and alcoholic extracts showed moderate to significant antimicrobial activity against all the micro-organisms but to a lesser extent as compared to the standard in all test strains observed by ZOI and the aqueous extract showed mild to moderate activity against both gram positive and gram negative bacterial strains. The study indicates that hydroalcoholic and alcoholic extracts have capabilities of extracting more phytochemicals in comparison to aqueous extract. However, it was the observed that among three concentrations (5µgm/µl, 10µgm/µl and 20µgm/µl) of the drug extracts, inhibitory effects were exhibited more at the intermediate concentration i.e. at 10µgm/µl. The antimicrobial properties showed by the extracts may be associated with the presence of these secondary metabolites through different mechanisms. Some phytochemicals like alkaloids, quinones, terpenoids, flavonoids and tannins have property of precipitating proteins (Tiwari *et al.,* 2011). The proposed antibacterial mechanism of alkaloids may be by inhibiting nucleic acid synthesis, as they inhibit the enzyme dihydrofolate reductase in cell free assays, by perturbing the Z-ring and inhibiting cell division, by compromising outer membrane and cytoplasmic integrity (Cushnie *et al.,* 2014). Similarly it has been observed that sterols either decrease the activity of *Staphylococcus aureus, Escherichia coli, Proteus vulgaris*, and *Pseudomonas pyocyanea* or have no effect in case of *Klebsiella* and *Shigella dysenteriae* (Anuradha and Goyal, 1995). The possible antibacterial mechanism of glycosides may be by inhibiting the RNA nucleic acid synthesis (Soulef *et al.,* 2014). The present study is an indication of usefulness of Abhal as a potent antibacterial drug that could be successfully used to treat the infectious disorders, however to make the study more comprehensive it is suggested that further screening may be done to elucidate the exact mechanism of action the drug.

Conclusion

The present study confirms that the alcoholic and hydroalcoholic extracts of the Abhal (fruits of *Juniperus communis*) possess significant antibacterial activity against both gram positive and gram negative bacterial strains as compared to aqueous extract. These activities may be due to many phytochemicals present in the drug or some unknown reasons. It is concluded that the test drug has potent antimicrobial activity and may be used as an antimicrobial agent to cure various infectious diseases.

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