

EFFICACY OF MEDICINAL PLANTS AGAINST HUMAN PATHOGENS ISOLATED FROM WESTERN HIMALAYAS OF HIMACHAL PRADESH

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ABSTRACT

Objective: The present study was aimed to evaluate the antibacterial effect of Himalayan medicinal plants on several human pathogens.

Methods: The leaves of *Mesua ferrea*, *Cannabis sativa*, and *Urtica dioica*, bark of *Juglans regia*, *Glycyrrhiza glabra*, fruit of *Terminalia chebula*, and seed of *Piper nigrum* were dried and powdered before methanolic extraction (ME). Antibacterial assay of MEs was performed against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* using agar well diffusion method. The minimum inhibitory concentrations of these extracts were assessed using microdilution method. The phytochemical analysis of these extracts was carried out to know the bioactive compounds and was fractionated with various solvents such as chloroform, butanol, ethyl acetate, and hexane to isolate the bioactive compound.

Results: The ME of *C. sativa* was found highly active, followed by *T. chebula*, *J. regia*, *G. glabra*, and *M. ferrea* against these pathogens. While the extracts of *U. dioica* and *P. nigrum* found less effective. MIC values of *J. regia* extract were found much more significant (0.219 g/ml). The antibacterial assay of fractions revealed that the chloroform fraction was highly active against all the pathogens than those of ethyl acetate, butanol, and hexane.

Conclusion: It was concluded from the present study that methanol was a good solvent for isolating compounds from medicinal plants and also favors the traditional uses of medicinal plants as antibacterial agents and as a potential source for the new drug discovery. It could also be possible that the compound responsible for antibacterial activity must be present in chloroform fraction of *J. regia*.

Keywords: Antibacterial activity, Medicinal plants, Methanolic extracts, Human pathogens, Phytochemicals, Fractionation.

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INTRODUCTION

Plants have been used for centuries as a remedy for human diseases as they contain components of therapeutic values. They are natural sources of antimicrobial agents primarily due to large biodiversity and relatively large quantity of metabolites that can be extracted from them [1,2]. The traditional values of these plants in medicines led researchers to make them an alternative form of health care to investigate the antimicrobial therapy [3,4]. The natural products of plants, animals, and minerals have been the basis of treatment of diseases from time immemorial. The history of medicine includes many ludicrous therapies; ancient wisdom has been the basis of modern medicine and will remain as one of the important sources of future medicine and therapeutics. The 80% of world's population are relied on medicinal plants. In India, the medicinal plants as therapeutics remain the most important component of the traditional medicinal system [5,6]. A lot of work has been reported on medicinal plants as constituents of antimicrobial and phytochemicals. The treatment of microbial infections is considered as possible alternatives over chemically synthesized drugs to whom resistant have been developed by several infectious microorganisms [7,8]. The antimicrobial activity of plant extracts is due to their various phytoconstituents.

These compounds usually include the secondary metabolites, which function as phytoprotectants and respond to environmental changes in plants. However, in humans, the compounds have more beneficial effects [9,10]. On the basis of literature found on the beneficial aspects of medicinal plants, the present study is aimed to evaluate the antimicrobial effect of some medicinal plants used in Ayurveda and traditional medicinal system for the treatment of manifestations caused by microorganisms. Therefore, methanolic extracts (MEs) of the following 7 medicinal plants from different families were tested for

their potential activity against microbial pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*).

METHODS

Sample collection

The bacterial strains *E. coli* (MTCC-739), *K. pneumoniae* (MTCC-39), *S. aureus* (MTCC-737), *P. aeruginosa* (MTCC-741), and *B. subtilis* (MTCC-441) were collected from IMTECH, Chandigarh. The microorganisms are subcultured in nutrient broth and incubated at 37°C for 24 hrs before the experiment. A total of 7 authenticated medicinal plants, i.e., *Juglans regia*, *Mesua ferrea*, *Glycyrrhiza glabra*, *Urtica dioica*, *Pepper nigrum*, and *Terminalia chebula* were collected from different areas of Himachal Pradesh. The fresh leaves of *J. regia*, *M. ferrea*, *Cannabis sativa*, *U. dioica*, and the fresh seeds, fruits, and roots of *P. nigrum*, *T. chebula*, and *G. glabra*, respectively, were collected in the month of March from Solan district of Himachal Pradesh located at the altitude of 800 m.

Preparation and extraction of the plant material

The plant parts were collected and surface sterilized with distilled water and then with mercury chloride (HgCl₂). The parts were dried at room temperature and grounded into powder using pestle and mortar. The powders were weighed and stored. The dried powdered of the plant was extracted with methanol by extraction process using orbital shaker for 3d (72 hrs). The crude extract was concentrated and dried using rotary vacuum evaporator.

Evaluation of antimicrobial activity of plant extract

Antimicrobial activity of various plant extracts was assessed by well-diffusion method [11]. The turbidity of the subcultured microorganisms

was adjusted with sterile distilled water using 0.5 McFarland as standard ($\sim 1.5 \times 10^8$ cells/ml). Mueller Hinton Agar (HiMedia) was prepared by dissolving ready-made agar powder in distilled water. Agar plates were prepared and inoculated with the test microorganisms by spread plate method. The plates were left undisturbed for 30 min at room temperature. The dried MEs were weighed and dissolved in dimethyl sulfoxide (DMSO) and used in triplicates. Then, the prepared extracts were added in the wells in a constant concentration of 50 μ l/well. The standard was also prepared for the antibiotic as a positive control ciprofloxacin (HiMedia). DMSO was also added in a well as negative control to make sure that the solvent used for dissolving the extracts do not have antimicrobial activity. Then, the plates were incubated at 37°C for 24 hrs in an upright position. After incubation, the zone of inhibition was measured and compared with the standard antibiotic zone.

Minimum inhibitory concentration (MIC)

The MIC assay was performed for those plants whose extract was found active by well diffusion susceptibility assay (inhibition zone >10 mm) [12,13]. The MIC values were determined by microdilution method using 96 well plates. The 96-well plates were prepared by dispensing 100 μ l of nutrient broth into each well. 100 μ l was taken from the stock solution of tested extracts (concentration of 5 mg/ml) and added into the first well of the plate. Then, two-fold serial dilutions were performed using a micropipette. The obtained concentration range was from 5 mg/ml, and then added 50 μ l of inoculum to each well except negative control. The positive control of antibiotic (ciprofloxacin), negative control (nutrient broth), broth alone, and the inoculum alone were also put in the experiment. The test plates were incubated at 37°C for 24 hrs. The lowest sample concentration showing clear well and inhibited complete growth was taken as MIC value [14].

Phytochemical screening

Phytochemical analysis of methanol leaf extracts of *J. regia*, *U. dioica*, *M. ferrea*, and *C. sativa* was carried out qualitatively to test for the presence of carbohydrates, proteins, tannins, phenols, flavonoids, cardiac glycosides, phytosterol, saponins, and alkaloids [15].

Fractionation of the plant extracts

Fractionation was done to separate the active components of the plants. MEs were taken in a beaker and diluted with 5% HCl. After complete

dissolution, separated liquid layer and repeated till solution appeared colorless. Filtered HCl treated layer and treated with 10 ml chloroform. Two layer was formed, i.e., chloroform layer and aqueous layer, the chloroform layer was separated in a beaker and filtered through a funnel containing Whatman filter paper and anhydrous sodium sulfate to hold the H₂O molecules and repeated 2-3 times and evaporated. Basified aqueous solution with ammonia solution and again treated with chloroform, ethyl acetate, n-butanol, and hexane, respectively. The layers were separated and evaporated in the same manner as chloroform. The different fractions were then collected in separate vials and analyzed for antimicrobial sensitivity assay using agar well diffusion method.

Antibacterial susceptibility assay of fractions

Antimicrobial activities of the fractions were performed using agar well diffusion method. The fractions (i.e., butanol, chloroform, ethyl acetate, and basified chloroform) were dissolved in 1000 μ l of the DMSO in an Eppendorf and mixed well. Same protocol was followed for the antimicrobial assay and MIC of fractions as described earlier for the susceptibility assay of plant extracts. The data were analyzed by mean and standard (mean \pm standard deviation) deviation using GraphPad prism.

RESULTS

Bacterial isolates

A total of five laboratories adopted bacterial isolates two Gram-positive (*B. subtilis* and *S. aureus*) and three Gram-negative (*E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa*) as shown in Fig. 1 were used for the study, which were obtained from IMTECH and characterized at molecular and Immuno-Parasitology Research Laboratory, and cultured on nutrient agar plates.

Antimicrobial activity of ME

Leaf, root, fruit, and bark were selected as experimental materials to screen their potential antimicrobial activity. MEs were investigated for the antimicrobial activity (Table 1) against the bacterial species mentioned. The antibacterial activity of different plant extracts against the pathogenic bacteria showed varied levels of inhibition. As shown in Table 1, the present study revealed that out of seven medicinal plants only 5 showed potential antibacterial activity

Table 1: Antibacterial effect of the ME against several bacterial strains

Bacterial strains	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>K. pneumoniae</i>	<i>B. subtilis</i>	-C	+C
Plants ME	Zones of inhibition in mm						
<i>J. regia</i>	11.6 \pm 1.1	12.3 \pm 0.5	14.3 \pm 1.5	10.6 \pm 1.1	11.6 \pm 1.5	-	23 \pm 0
<i>M. ferrea</i>	11.0 \pm 0.0	-	10.5 \pm 0.7	11.3 \pm 1.5	11.0 \pm 1.0	-	24 \pm 0
<i>G. glabra</i>	13.6 \pm 1.5	12 \pm 2.0	11.6 \pm 2.1	13.0 \pm 3.0	11.0 \pm 1.0	-	22 \pm 0
<i>T. chebula</i>	16.0 \pm 1.7	15.6 \pm 1.1	15.6 \pm 1.1	22.3 \pm 1.5	24.0 \pm 0.0	-	18 \pm 0
<i>C. sativa</i>	21.6 \pm 0.5	21.6 \pm 0.5	24.0 \pm 1.0	18.0 \pm 1.0	23.6 \pm 0.5	-	24 \pm 0
<i>P. nigrum</i>	-	-	-	-	-	-	19 \pm 0
<i>U. dioica</i>	-	-	-	-	-	-	25 \pm 0

ME: Methanolic extract, mm: Millimeter, +C: Positive control, -C: Negative control, -: No zone of inhibition, *E. coli*: *Escherichia coli*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *K. pneumoniae*: *Klebsiella pneumoniae*, *B. subtilis*: *Bacillus subtilis*, *S. aureus*: *Staphylococcus aureus*, *C. sativa*: *Cannabis sativa*, *J. regia*: *Juglans regia*, *G. glabra*: *Glycyrrhiza glabra*, *M. ferrea*: *Mesua ferrea*, *U. dioica*: *Urtica dioica*, *P. nigrum*: *Piper nigrum*

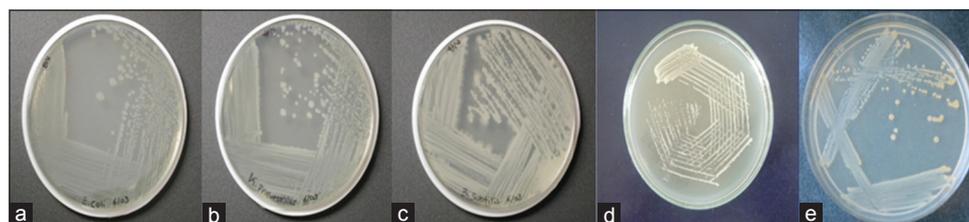


Fig. 1: Growth of microorganism on nutrient agar (a) *Escherichia coli* (b) *Klebsiella pneumoniae* (c) *Bacillus subtilis* (d) *Pseudomonas aeruginosa* and (e) *Staphylococcus aureus*

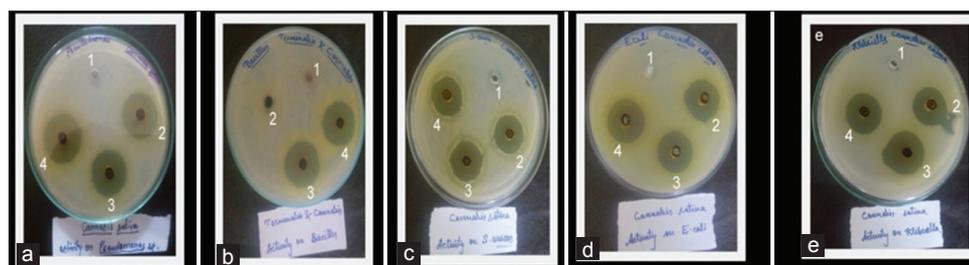


Fig. 2: Antibacterial activity of methanolic leaf extract of *Cannabis sativa* against the test bacteria (a) *Pseudomonas aeruginosa*, (b) *Bacillus subtilis* (c) *Staphylococcus aureus*, and (d) *Escherichia coli* (e) *Klebsiella pneumoniae*

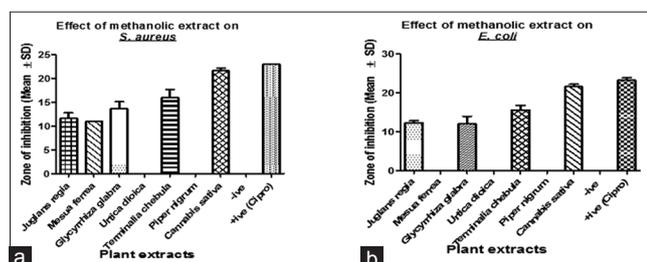


Fig. 3: (a and b) Graphical representation of plant extracts showing varied degree of sensitivity against *S. aureus* and *Escherichia coli*

against *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *B. subtilis*, and *E. coli*. Although, *U. dioica* and *P. nigrum* do not show any activity against any pathogen.

The results obtained by measurement of zone of inhibition were presented in Table 1 and Figs. 2-4, the MEs of *C. sativa* showed a maximum zone of inhibition against *P. aeruginosa*, followed by *B. subtilis*, *E. coli*, *S. aureus*, and *K. pneumoniae*, respectively.

The MEs of *T. chebula* showed the highest antimicrobial effect on *B. subtilis* followed by *K. pneumoniae*, *S. aureus*, and *E. coli*. Whereas the *J. regia*, *M. ferrea*, and *G. glabra* have showed near about similar effect against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *K. pneumoniae*, respectively (Table 1, Figs. 2-4).

Determination of MIC

Broth dilution method was used to determine the MIC values of MEs of *M. ferrea*, *J. regia*, *G. glabra*, and *C. sativa*. Microdilution method was used to determine the lowest plant extracts concentration that was inhibiting the growth of the bacteria and found effective in the evaluation of best activity of medicinal plants.

MIC values of MEs of *Mesua ferrea*, *J. regia*, and *G. glabra* revealed that the MIC of *J. regia* was much more significant as compare to the other plants, i.e., 0.219 as shown in Table 2.

Phytochemical screening

MEs which had shown good antibacterial activities (i.e., *J. regia*, *G. glabra*, *M. ferrea*, and *C. sativa*) were screened for the presence of phytochemical compounds. Investigation of the phytochemical screening summarized in Table 3. Phytochemical screening on the crude methanol extracts of four medicinal plants was performed in the test tube. The observations (Table 3) revealed the presence of secondary metabolites such as saponins, tannins, alkaloids, phytosterol, flavonoids, terpenoid, glycosides, phlobatannins, triterpenoids, and cardiac glycosides. The commonly identified components of importance in the four plant extracts include flavonoids, cardiac glycosides, and saponins.

In addition, tannins were present in *J. regia* and *C. sativa*, phlobatannins were present in *J. regia*, *M. ferrea*, and *C. sativa*, and carbohydrates and saponins were present in all the four plant extracts. Alkaloids

Table 2: MIC (MIC in mg/ml) of *J. regia*, *G. glabra*, *M. ferrea*, and *C. sativa* ME against bacterial strains

Extracts/ bacterial strains	<i>C. sativa</i>	<i>J. regia</i>	<i>G. glabra</i>	<i>M. ferrea</i>
<i>E. coli</i>	0.438	0.438	0.875	1.750
<i>P. aeruginosa</i>	0.219	0.219	0.875	1.750
<i>K. pneumoniae</i>	0.875	0.219	0.875	2.500
<i>B. subtilis</i>	0.438	0.219	0.438	0.875
<i>S. aureus</i>	0.219	0.438	0.875	0.875

E. coli: *Escherichia coli*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *K. pneumoniae*: *Klebsiella pneumoniae*, *B. subtilis*: *Bacillus subtilis*, *S. aureus*: *Staphylococcus aureus*, *C. sativa*: *Cannabis sativa*, *J. regia*: *Juglans regia*, *G. glabra*: *Glycyrrhiza glabra*, *M. ferrea*: *Mesua ferrea*, MIC: Minimum inhibitory concentrations

Table 3: Phytochemical constituents of ME of *J. regia*, *G. glabra*, *M. ferrea*, and *C. sativa*

Phytochemical constituents	ME			
	<i>J. regia</i>	<i>M. ferrea</i>	<i>G. glabra</i>	<i>C. sativa</i>
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Alkaloids	+	+	+	+
Phlobatannins	+	+	-	-
Fixed oils and fats	-	-	-	-
Cardiac glycosides	+	+	+	+
Saponins	+	+	-	-
Amino acids	-	-	-	-
Carbohydrates	-	-	-	-
Terpenoids	+	+	+	+
Steroid	-	-	-	-

+: Represents presence of the phytoconstituent, -: Represents absence of the phytoconstituent. *J. regia*: *Juglans regia*, *M. ferrea*: *Mesua ferrea*, *G. glabra*: *Glycyrrhiza glabra*, *C. sativa*: *Cannabis sativa*

Table 4: Antimicrobial activity of fraction against *S. aureus*, *E. coli*, *P. aeruginosa*, *Klebsiella sp.*, and *Bacillus sp.*

Bacterial strains	<i>J. regia</i> fractions		
	Zones of inhibition in mm		
	Butanol	Chloroform	Ethyl acetate
<i>E. coli</i>	8±0.001	20±0.001	9±0.002
<i>B. subtilis</i>	10±0.002	21±0.001	7±0.001
<i>P. aeruginosa</i>	10±0.002	21±0.002	10±0.001
<i>K. pneumoniae</i>	10±0.001	24±0.001	10±0.002
<i>S. aureus</i>	9±0.002	19±0.001	10±0.001

S. aureus: *Staphylococcus aureus*, *E. coli*: *Escherichia coli*, *J. regia*: *Juglans regia*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *B. subtilis*: *Bacillus subtilis* and *K. pneumoniae*: *Klebsiella pneumoniae*

were also found in all four plant extracts (Table 3). The antimicrobial (antibacterial and antifungal) activity of the plant extracts is due to the

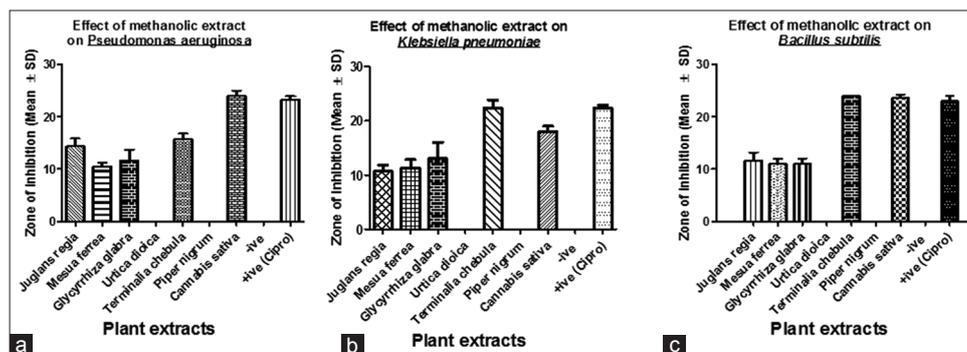


Fig. 4: (a-c) Graphical representation of plant extracts showing a varied degree of sensitivity against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Bacillus subtilis*

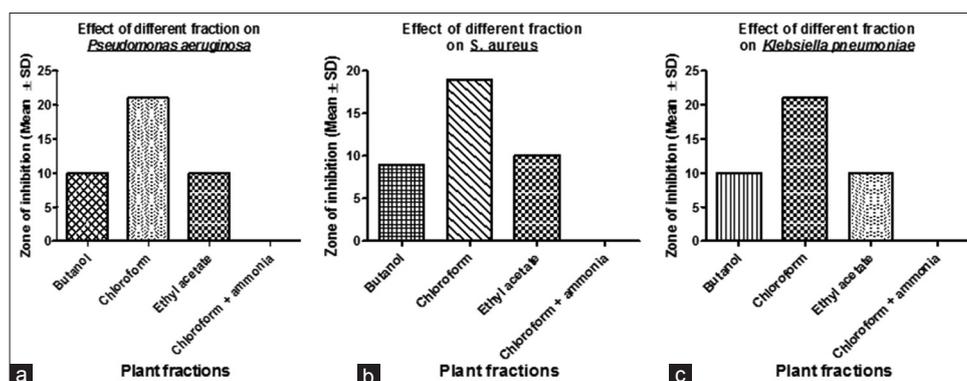


Fig. 5: (a-c) Graphical representation of methanolic extract fraction showing varied degree of sensitivity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*

presence of these active compounds which may be acting independently or synergistically with other compounds.

Fractionation of the most active plant extracts

The antimicrobial activity of fractions

MEs of *J. regia* were fractionated using LLE method. Fraction of butanol, chloroform, basified chloroform, and ethyl acetate was tested against human pathogen. The antimicrobial activity of these plants was tested against these bacterial strains. The chloroform fraction showed the highest zone of inhibition against *K. pneumoniae*, followed *P. aeruginosa* and *B. subtilis* (Table 4 and Figs. 5 and 6).

The chloroform fraction was found highly active against *E. coli* (20 mm zone) followed by *S. aureus* (19 mm; Table 4). The least active fractions were ethyl acetate and butanol. On the other hand, basified chloroform does not show any result against all of five bacteria.

DISCUSSION

Many naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against pathogens [16,17]. Therefore, the development of new antimicrobial agents for the treatment of bacterial infections is of increasing interest. The main objective of the present study was to evaluate the ability of selected medicinal plants extract to inhibit the growth of pathogenic bacteria. In our study, agar well diffusion method was used to assess the activity of plants extracts, and significant zones of inhibitions were observed. The ME of *C. sativa* was found highly active followed by *T. chebula*, *J. regia*, *G. glabra*, and *M. ferrea*, respectively, against these pathogens. A fairly similar study was done by Naveed *et al.*, on the leave extract of *C. sativa* [18] which exerted pronounced antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli*, whereas the extracts of *U. dioica* and *P. nigrum* did not show any significant inhibitory effects.

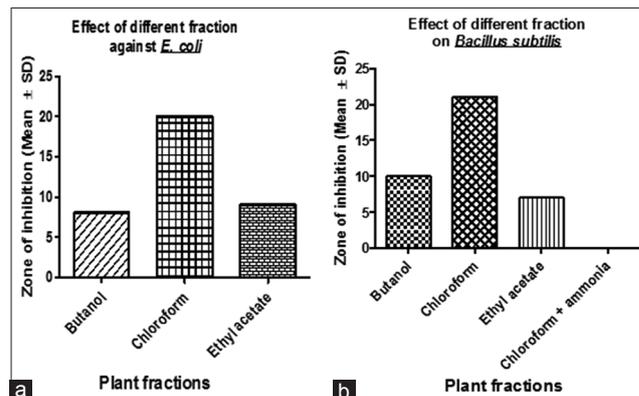


Fig. 6: (a and b) Graphical representation of various plant fractions showing varied degree of sensitivity against *Escherichia coli* and *Bacillus subtilis*

Microplate dilution method determines the MIC values of MEs of *M. ferrea*, *J. regia*, and *G. glabra*. The study revealed that the MIC values of *J. regia* were much more significant as compare to the other plants. *J. regia* bark is used in Iranian folk medicine as antimicrobial medicine [19,20]. This study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. The result of phytochemicals showed that the plant contains more or less same components such as alkaloid, phlobatannins, saponins, terpenoids, steroids, glycosides, carbohydrates, flavonoids, and tannins. The alkaloids have been investigated for many pharmacological properties including antiprotozoal, cytotoxic, antidiabetic [21], and anti-inflammatory [22] properties, but there are only a few reports of antimicrobial properties. Baravalia *et al.* have shown the importance of polyphenols as antimicrobial compounds [23]. Polyphenols and

flavonoids exhibit a wide range of biological effects and antibacterial activity [24-27]. Tannins have amazing stringent properties. They are known to hasten the healing of wounds and inflamed mucous membranes [28]. Tannins are also known for their antimicrobial activity [29,30]. Prasad *et al.* have reported the growth inhibition of many fungi, bacteria, and viruses by tannins [31].

ME was fractionated using LLE method. The chloroform fraction showed the highest zone of inhibition as compared to the other fractions. Asha *et al.* similarly reported that the chloroform and ethyl acetate fractions of *J. regia* were much more effective on these pathogenic strains [32]. A similar observation from the study done by Raj *et al.* reveals that chloroform extract fractions had highest antibacterial potential against all pathogenic microbes studied [33]. Hence, we can discuss from the above result that the compound responsible for the antibacterial activity must be present in chloroform that had potent antimicrobial activity.

CONCLUSION

It can be concluded that the fraction of medicinal plants showed potent antimicrobial activities against the tested bacterial strains. The antimicrobial activities may be due to strong occurrence of active compounds, i.e., saponins, tannins, alkaloids, steroids, phenols, and flavonoids. However, these medicinal plant species may be subjected to detailed phytochemicals and pharmacological studies to find out new drugs against pathogenic strains. Further studies are needed with these herbs to isolate, characterize, and elucidate the structure of the bioactive compounds of the herbs which are responsible for the antimicrobial activity and other therapeutic values.

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