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Evaluation of antimicrobial and phytochemical screening of Fennel, Juniper and Kalonji essential oils against multi drug resistant clinical isolates

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ABSTRACT

Objective: The inhibitory effects of essential oils including fennel, juniper and kalonji from *Foeniculum Vulgare*, *Juniperus Osteosperma* and *Nigella Sativa* on multi drug resistant clinical isolates were investigated. All the oils have been evaluated for phytochemical constituents, antibacterial activity and TLC bioautography assay. **Methods:** Preliminary phytochemical analysis was performed. The antibacterial potential of essential oils from fennel, juniper and kalonji fennel, juniper and kalonji was evaluated by agar well diffusion method against multi drug resistant clinical isolates. The antibacterial effect was investigated using the TLC-bioautographic method. **Results:** Preliminary phytochemical analysis demonstrated the presence of most of the phytochemicals including saponins, cardiac glycosides, steroids, terpenoids, flavonoids and tannins. Antibacterial activity of essential oils was assessed on eight multi-drug resistant (MDR) clinical isolates from both Gram-positive and Gram-negative bacteria and two standard strains. All the oils tested showed significant to moderate antibacterial activity toward all tested strains except *Acinetobacter* sp and *Staphylococcus aureus* MRSA. The maximum zone of inhibition was found to be 25±0.12 mm for juniper oil followed by 21±0.085 mm for kalonji oil against *Staphylococcus aureus* 2. Thin layer chromatography and bioautography assay demonstrated well-defined growth inhibition zones against *Staphylococcus aureus* 2 and *E. coli* for juniper essential oil in correspondence with tannins observed at Rf values of 0.07 and 0.57. **Conclusions:** Based on the present study, the essential oils from juniper and kalonji possess antibacterial activity against several multi drug resistant pathogenic bacteria and thus can be used as a base for the development of new potent drugs and phytomedicine.

1. Introduction

Control of the spread of antibiotic-resistant bacteria and the treatment of infections caused by them is a major problem worldwide [1]. It is estimated that about 70 per cent of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. In India, antimicrobial resistance has been reported in for the most predominant pathogenic microorganisms including *Staphylococcus aureus*, Enterococcus faecalis, Mycobacterium tuberculosis and Pseudomonas. aeruginosa. Looking at this scenario there is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. The emergence of multi drug resistant bacteria has raised the needs for new antimicrobial drugs. Essential oils (also called volatile oils) are the concentrated, hydrophobic liquids containing volatile aromatic compounds from plants. They can be obtained by expression, fermentation or extraction but the method of steam distillation is most commonly used for commercial production. Essential oils are a rich source of biologically active compounds [2], their potential antimicrobial traits are due to compounds synthesized in the secondary metabolism of the plant.

Foeniculum vulgare Mill, commonly known as fennel, belongs to family Apiaceae), is a small genus of annual, biennial or perennial herbs cultivated for its aromatic fruits, which are used as culinary spices. Fennel and its preparations are used to cure various disorders, and also act as a carminative, digestive and diuretic agent [3]. Fennel increases lactation, act as a stimulant to the circulatory and digestive systems and act as an anti-aging agent. Earlier,

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the antimicrobial properties of the fennel essential oil have been studied by Gulfraz [4]. Chaouche *et al* [5] reported the phytochemical tests on the stem, roots, and seeds of the plant fennel. The presence of flavonoids, tannins, coumarines, saponins, sterols, essential oil and absence of anthocyanes and alkaloids was reported. *Juniperus Osteosperma*, commonly known as juniper is an evergreen tree or shrub growing up to 20 feet in height and has blue-green needles and small flowers. The berries of the juniper tree are initially green, maturing to black after the first year. The juniper berry oil has been used effectively with pain associated with joints and muscles along with cough reduction and respiratory issues. It is also found to be a great analgesic, a natural diuretic and anti-inflammatory oil. *Nigella Sativa* commonly named as Kalonji belongs to Ranunculaceae family is an herbaceous plant used for the treatment of various infectious diseases. The seeds of the plant possess several medicinal properties [6]. Antimicrobial activity of *Nigella Sativa* oil and crude extracts was reported against MDR bacteria from clinical isolates [7].

The present study aimed at evaluating the phytochemical screening, in vitro antibacterial activity and TLC bioautography assay of Fennel, Juniper, Kalonji oil against multi-drug resistant (MDR) Gram-positive (Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus aureus* and Enterococcus sp.) and Gram-negative (Klebsiella sp., Escherichia coli, Pseudomonas aeruginosa and *Acinetobacter* sp.) bacterial strains isolated from human infections.

2. Materials and methods

2.1. Acquisition of fennel, juniper, kalonji essential oil

Commercial brands of *Foeniculum Vulgare* seed oil (Fennel), *Juniperus Osteosperma* (Juniper) and *Nigella Sativa* (Kalonji) were purchased from Delhi, India. As per manufacturer's information, it was prepared by steam distillation. The oil was further distilled by rotary evaporator. The essential oil was dissolved in methanol (0.3 ml oil/ 2 ml methanol). The oil was transferred into sterile vials and stored at −200C until needed.

2.2. Bacterial strains and growth conditions

The pure cultures of the bacteria with their antibiotic resistance profiles were obtained from the Department of Microbiology, Rajiv Gandhi Cancer Research Institute, Delhi, India. Multi-drug resistant clinical isolates of *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), Escherichia coli, Kleibsella pneumoniae, Pseudomonas aeruginosa, Enterococcus sp. and *Acinetobacter* sp. were used. The cultures of bacteria were maintained in their appropriate agar slants at 4 ℃ throughout the study and sub-cultured on to nutrient broth for 24 h prior to testing. These bacteria served as test pathogens for antibacterial activity assay. Standard strains *Staphylococcus aureus* ATCC 25923 and Escherichia coli ATCC 25922 were used for quality control.

2.3. Phytochemical screening

The oils dissolved in methanol (0.3 ml oil/2 ml methanol) were evaluated for the presence of different phytochemicals to ascertain the presence of metabolites such as tannins, steroids, reducing sugars, alkaloids, anthraquinones, glycosides, flavonoids, saponins, triterpenoids and phlobatanins by using wet reactions [8, 9].

2.4. Antibacterial activity assay

The agar well diffusion method [10] was employed with minor modification to determine the antibacterial activities of oils tested. Nutrient agar plates were inoculated with 0.1 ml of each bacterial organism (1×10^8 CFU/ml) and spreaded well with sterile swabs. Subsequently, the surface of the agar was punched with an 8 mm diameter wells. Each well was filled with 50 μl of oil in methanol and allowed to diffuse at room temperature for about 2 h. The plates were incubated at 37 ℃ for 24 h. The control well containing the same volume of methanol while standard antibiotic discs of Imepenem (10 μg) and Vancomycin (30 μg) were used as the positive controls. After incubation, the zone of inhibition was measured and expressed in millimeter. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition with their standard deviation.

2.5. TLC bioautography assay

Juniper seed oil exhibiting significant antibacterial potential against *Staphylococcus aureus* 2 and *E. coli* as determined by agar well diffusion method was analyzed using TLC bioautography. About 10 μl of oil in methanol was applied on pre-coated aluminium silica gel G 25 plates. The plates were developed with toluene and ethyl acetate (93:7 v/v). The TLC plates were run in triplicate. Plate A, the reference chromatogram was used to determine the spots as visualized by UV light to see if the separated spots were UV active after which it was sprayed with vanillin sulphuric acid (2%) spray reagent, plate B was used for bioautography and the TLC plates which were used to identify spots with the various TLC reagents to detect the presence of tannins and saponins as described by Johanne *et al* [11] was denoted as plate *C. Individual* Rf for each spot was measured.

TLC bioautography was carried out using the selected strains of bacteria. The developed TLC plates were thinly overlaid with molten nutrient agar inoculated with an overnight culture of bacteria. The plates were incubated in a dark and humid chamber overnight at 37 ℃. Subsequently, the bioautogram was sprayed with an aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride and further incubated for at 37 ℃ for 4 h. Microbial growth inhibition appeared as clear zones against a pink background. The Rf values of the spots showing inhibition were determined. The Rf of the inhibition zones on plate B was compared with the Rf of

reference chromatogram (plate A) as well as Rf of the spots on plate C. The experiment was repeated twice.

3. Results

Pure cultures of the bacteria with their antibiotic resistance profiles (Table 1) were obtained from the Department of Microbiology, Rajiv Gandhi Cancer Research Institute, Delhi, India. Preliminary phytochemical screening of fennel, juniper and kalonji seed oil showed that the essential oils tested contain most of the phytochemicals (Table 2) including saponins, cardiac glycosides, steroids, terpenoids and tannins. However, anthraquinone, alkaloids and reducing sugars were not observed in any of the oil tested. Saponins and cardiac glycosides were found to be present in all the oils. Flavonoids are observed only in fennel oil and phlobatanins and tannins are found in juniper oil alone.

Table 1

Antibiotic resistance profile of various Gram-positive and Gram-negative bacterial isolates used

Antibiotics	Kp	Ec	Pa	Sa 1	Sa 2	Sa MRSA	Asp	Esp
AK	S	S	S	S	S	S	R	R
AC	R	R	R	S	S	R	R	R
CFX	R	R	R	R	R	R	R	R
CS	R	S	S	S	S	S	R	R
CE	R	R	R	S	S	R	R	R
CI	R	R	R	R	R	R	R	R
CF	R	R	S	R	R	R	R	R
G	S	R	S	S	S	R	R	R
I	S	S	S	S	S	S	R	R
LE	S	R	S	S	S	S	R	R
MR	S	S	S	S	S	R	R	R
OF	R	R	S	R	R	R	R	R
VA	–	–	–	S	S	S	S	S

AK: Amikacin, AC: Amoxycillin/Clavulanic acid, CFX: Cefixime, CS: Cefoperazone+ Sulbactum, CE: Cefotaxime, CI: Ceftriaxone, CF: Ciprofloxacin, G: Gentamicin, I: Imipenem, LE: Levofloxacin, MR: Meropenem, OF: Ofloxacin, VA: Vancomycin, R: Resistant, S: Sensitive, Kp: Klebsiella pneumoniae, Ec: Escherichia coli, Sa: *Staphylococcus aureus*, Pa: Pseudomonas aeruginosa, Asp: *Acinetobacter* sp, Esp: Enterococcus sp.

Table 2

Phytochemical analysis of Fennel, Juniper and Kalonji oil

Phytoconstituents	Fennel oil	Juniper oil	Kalonji oil
Alkaloids	–	–	–
Anthraquinone	–	–	–
Flavonoids	+	–	–
Cardiac glycosides	+	+	+
Saponins	+	+	+
Steroids	–	+	+
Reducing sugars	–	–	–
Tannins	–	+	–
Terpenoids	–	+	+
Phlobatanins	–	+	–

a) +: Positive, b) –: Negative

The three different essential oils were examined for the antibacterial activity against multi drug resistant clinical isolates and the results were given in Table 3. The results from the agar well diffusion method revealed that all the oils tested showed significant to moderate antibacterial activity toward all tested strains except *Acinetobacter* sp and *Staphylococcus aureus* MRSA. The maximum zone of inhibition was found to be 25±0.12 mm in diameter for juniper oil followed by 21±0.085 mm for kalonji oil against *Staphylococcus aureus* 2. The growth of S. aureus 1, S. aureus 2 and Pseudomonas aeruginosa was only inhibited by juniper and kalonji essential oil. Fennel seed oil exhibited the lowest inhibitory effect against all tested isolates. The control plate did not exhibit inhibition on the tested bacteria where as standard antibiotics Imepenem and Vancomycin produced significantly larger inhibition zones against Gram-negative and Gram-positive bacteria respectively.

TLC analysis revealed the presence of saponins in all the three oils tested whereas, flavonoid was observed in fennel oil and presence of tannins was observed in juniper oil (data not shown). TLC bioautography were performed for juniper essential oil against *Staphylococcus aureus* 2 and E. coli. Bioautography showed presence of four to five active compounds at different Rf values. The components of the juniper oil separated into two big spot and two or

Table 3

Antibacterial activity of Fennel, Juniper and Kalonji seed oil determined by agar well diffusion method

Test Microorganisms	Zone of Inhibition (in mm)		
	Fennel oil	Juniper oil	Kalonji oil
Staphylococcus aureus 1	–	12±0.10	12±0.085
Staphylococcus aureus 2	–	25±0.12	21±0.085
Staphylococcus aureus MRSA	–	–	–
Staphylococcus aureus ATCC 25923	14±0.09	19±0.11	18±0.13
Escherichia coli	9±0.12	19±0.08	9±0.10
Escherichia coli ATCC 25922	15±0.10	22±0.075	19±0.12
Enterococcus sp.	8±0.11	13±0.10	8±0.12
Klebsiella pneumoniae	8±0.10	9±0.12	14±0.09
Pseudomonas aeruginosa	–	16±0.12	15±0.10
Acinetobacter sp	–	–	–

Zone of inhibition is expressed as mean± standard deviation, –: no inhibition

more smaller spots. Of the number of spots observed, two compounds J1 and J2 at Rf values 0.07 and 0.57 were found to be active. Compound J1 was found to exhibit antibacterial activity against *Staphylococcus aureus* 2 and E.coli both while compound J2 showed zone of inhibition only for *Staphylococcus aureus* 2 (Fig. 1) and no visible inhibitory zone was observed against *E. coli* for the bigger spot with Rf value 0.57. Spot with Rf values of 0.07 and 0.57 corresponds to the spots representing tannins on spraying with 10% FeCl3 spray reagent. This result suggests that the antibacterial activity present in juniper oil may be due to the presence of tannins.

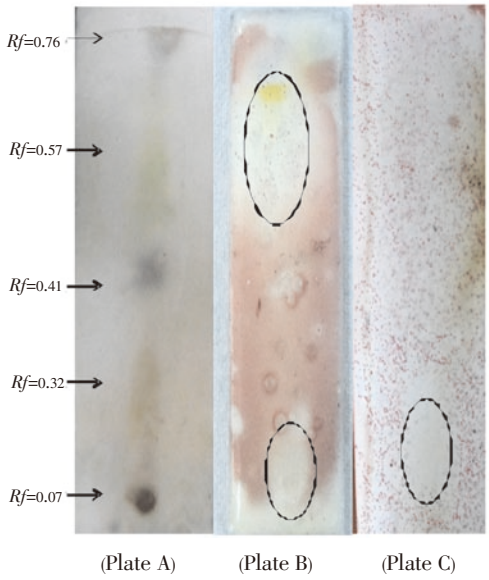

Figure 1. Chromatogram for (Plate A) and Bioautograms (Plates B and C) for Juniper seed oil against *Staphylococcus aureus* 2 and *E. coli*. Plate A: arrow indicates spots visualized when sprayed with 2% vanillin sulphuric acid reagent. Zones of inhibition (Plates B and C) are observed as clear spots against pink background. Mobile phase: Toluene/Ethyl acetate (93:7 v/v)

4. Discussion

Antimicrobial activity of plants has been known and studied for centuries. Recently, few scientists have reported that plant essential oils also possess antimicrobial potential. Essential oils may provide potential alternatives to active substances currently used for antimicrobial potential, since they are not only a fragrance and flavour source for food and beverages but are also being discovered as bioactive substances tanks. Therefore, the use of essential oils as antimicrobial agents can be an interesting field of investigation as the toxicity to mammals is mostly quite low, and their degree of volatility allows their use for fumigation in cold storage or for active packing and possessing application in food industry [12]. In the present investigation, three medicinally important plant essential oils have been screened for the presence of phytochemicals, antimicrobial potential and bioactive compound responsible for anti bacterial potential by TLC bioautographic analysis.

Phytochemical screening of fennel seed oil showed that the oil contain most of the phytoconstituents including flavonoids, saponins, cardiac glycosides, steroids, terpenoids and tannins. Our results were in agreement with Chaouche *et al* [5] who observed that flavonoids, tannins, coumarines, saponins and sterols were found present and total absence of alkaloids and anthocyanes in the stem, root and seed extracts of *Foeniculum vulgare* Mill.

In vitro antibacterial potential of the three essential oils was quantitatively assessed on the basis of zone of inhibition by agar well diffusion method. All the oils tested in the present study exhibited varying degree of inhibitory effect against the selected gram positive and gram negative MDR clinical isolates. Earlier studies on essential oil of fennel and kalonji revealed its antimicrobial potential [3, 7, 13]. The antimicrobial activity of *Foeniculum vulgare* against E. coli, S. aureus, Enterococcus sp and K. pneumonia has also been reported by Saviuc *et al* [3]. The results are consistent with the reports of previous investigators. However, Saviuc *et al* [3] reported antibacterial potential of fennel oil against Pseudomonas aeruginosa which was not observed in the present investigation. Juniper and kalonji oils were found more effective on Gram-positive bacteria than on Gram-negative isolates. The results obtained are in conformity with observations of previous researchers [7, 14]. It could be explained by the different cell wall structures of these bacteria. Gram-negative outer membrane comprising of phospholipids and lipopolysaccharides that acts as a barrier to the entrance and reaction of most antibiotics and/or antimicrobial agents through cell envelope [15, 16]. However, Kumar *et al* [17] reported similar antibacterial potential against Gram-positive and Gram-negative isolates tested.

The bioactive components were separated on TLC followed by TLC bioautography of juniper essential oil against potential inhibitors, *Staphylococcus aureus* 2 and E. coli. The potential antibacterial activity present in juniper oil may be due to the presence of tannins as confirmed by spraying with 10% FeCl3 spray reagent. These findings corroborated with the observations of Sibi *et al* [18] who reported the antibacterial efficacy of tannins against S. aureus. It is possible that the observed inhibition was likely due to one or more active compounds which overlap possibly due to the solvent system used for screening. No zone of inhibition was observed for spots with Rf values of 0.32, 0.41 and 0.76 in juniper on reference chromatogram. This could be attributed to evaporation of the active components, photo-oxidation or insufficient amount of the active component [19]. Synergism might play a major role in extracts that were active when the MIC of the mixture was determined, while the separated components showed no antimicrobial activity. Further investigation need to focus on the isolation and elucidation of active compounds detected by TLC bioautography by employing various developing solvent systems and using

different MRSA isolates. It is hoped that this study would lead to the establishment of some compounds that could be used to formulate new and more potent antimicrobial drugs for multi drug resistance clinical isolates.

Conflict of interest statement

We declare that we have no conflict of interest.

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