Academic Sciences Asian Journal of Pharmaceutical and Clinical Research
Vol 5, Issue 2, 2012 ISSN - 0974-2441

Research Article

PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL EFFICACY OF DILL SEED OIL AGAINST MULTI-DRUG RESISTANT CLINICAL ISOLATES

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Received: 9 Novemberr 2011, Revised and Accepted: 27 January 2012

ABSTRACT

Dill (*Anethum graveolens*) seed oil has been evaluated for phytochemical constituents, antibacterial activity (agar well diffusion) and TLC bioautography assay. Phytochemical analysis demonstrated the presence of tannins, glycosides, saponins, steroids, terpenoids and reducing sugars. Antibacterial activity of Dill seed oil was assessed on eight multi-drug resistant (MDR) clinical isolates from both Gram-positive and Gram-negative bacteria and two standard strains. It showed broad antibacterial activity against both Gram-positive bacteria such as *Staphylococcus aureus, S. aureus* MRSA*, Enterococcus* sp. and Gram-negative bacteria *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*. The highest *in vitro* inhibitory activity was observed for MDR *Enterococcus* sp. with wide inhibition zone diameters (15±0.11 mm) followed by standard *S. aureus* ATCC 25923 (14±0.12 mm). Thin layer bioautography assay demonstrated a single large well-defined growth inhibition zone against *Enterococcus* sp. and *S. aureus* MRSA observed at *Rf* value of 0.74. This established a good support to the use of this essential oil in herbal medicine and as a base for the development of novel potent drugs and phytomedicine.

Key words: *Anethum graveolens*, dill seed oil, agar well diffusion, multi-drug resistant, TLC bioautography

INTRODUCTION

Dill (*Anethum graveolens*), also known as Shapt or dill-weed, belongs to family *Umbelliferae*, is an annual herb growing to a height of 1.5 m. Dill originates from Mediterranean and West Asia. Its leaves are commonly used in salads and tea while its seeds are used in tea, breads, soups, salads and preserves. It is cultivated for use as a vegetable and also as a source of essential oil. Its medicinal uses are as an antispasmodic, carminative, diuretic, stimulant and stomachic [1]. The main constituents of dill oil which is pale yellow in color, darkens on keeping, with the odor of the fruit and a hot, acrid taste are a mixture of a paraffin hydrocarbon and 40 to 60% of d-carvone (23.1%) with d-limonene (45%). It also consists of α-phellandrene, eugenol, anethole, flavonoids, coumarins, triterpenes, phenolic acids and umbelliferones [2]. Some of the earlier studies had shown the antimicrobial activity of *A. graveolens* against *Saccharomyces cerevisia* and *Listeria monocytogenes* [3]. The antioxidant activity of the aqueous extracts of dill is comparable with ascorbic acid, alpha-tocopherol and quercetin in *in-vitro* systems [4, 5]. The essential oil produced from the seed of dill is found to be effective against vulvovaginal candidiasis in immunosuppressed mice [6]. Furthermore, the dill essential oil has hypolipidemic activity and could be used as a cardioprotective agent [7].The quantity and chemical composition of dill essential oil varies depending on the plant parts and the developing stage of the plant at harvest time [8].

Development of bacterial resistance to synthetic antimicrobial agents and the side effects associated with their use favour essential oils for alternative or complementary use. In India, antimicrobial resistance has been reported in for the most predominant pathogenic microorganisms including *Staphylococcus aureus*, *Enterococcus faecalis*, *Mycobacterium tuberculosis* and *Pseudomonas. aeruginosa.* Essential oils (also called volatile oils) are aromatic oily liquids obtained from plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). 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[9]. Hence, there has been an increased interest in looking at antimicrobial properties of extracts from aromatic plants particularly essential oils.

The present study relates to phytochemical screening, antibacterial activity and TLC bioautography assay against multi-drug resistant (MDR) Gram-positive (Methicillin-resistant *Staphylococcus aureus* and *Enterococcus* sp.) and Gram-negative (*Klebsiella* sp., *Escherichia coli*, *Pseudomonas aeruginosa, Proteus mirabilis* and *Acinetobacter* sp.) bacterial strains isolated from human infections.

MATERIALS AND METHODS

Acquisition of oil of *Anethum graveolens*

Commercial brand of *Anethum graveolens* seed oil (Dill seed oil) was 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 -20^{0} C until needed.

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 [Table 1]. Multi-drug resistant clinical isolates of *Staphylococcus aureus,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Kleibsella pneumoniae, Pseudomonas aeruginosa*, *Enterococcus* sp., *Acinetobacter* sp. and *Proteus mirabilis* were used. The cultures of bacteria were maintained in their appropriate agar slants at 4°C 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.

Table 1: Antibiotic resistance profile of various Gram-positive and Gram-negative bacterial isolates used

Antibiotics					*Sa* MRSA			*Pm*
	Kp		*Pa*	*Sa*		*Asp*	*Esp*	
AK	S	S	S	S	S	R	R	S
AC	R	R	R	S	R	R	R	R
CFX	R	R	R	R	R	R	R	R
CS	R	S	S	S	S	R	R	S
CE	R	R	R	S	R	R	R	S
CI	R	R	R	R	R	R	R	S
CF	R	R	S	R	R	R	R	S
G	S	R	S	S	R	R	R	S
I	S	S	S	S	S	R	R	S
LE	S	R	S	S	S	R	R	S
MR	S	S	S	S	R	R	R	S
OF	R	R	S	R	R	R	R	S
VA	-	-	-	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., *Pm*: *Proteus mirabilis

Phytochemical screening

Dill seed oil dissolved in methanol (0.3 ml oil/2 ml methanol) was evaluated for the presence of different phytochemicals to ascertain the presence of metabolites such as reducing sugars, alkaloids, anthraquinones, glycosides, flavonoids, tannins, steroids, saponins, triterpenoids and phlobatanins by using wet reactions following the procedures described by Sofowora [10] and Trease and Evans [11].

Antibacterial activity assay

The antibacterial activity of Dill seed oil was done using agar well diffusion method with minor modifications[12]. Nutrient agar plates were inoculated with 0.1 ml of each bacterial organism (1×10^8 CFU/ml) and spreaded well with sterile swabs. Wells of 8 mm size were made into the agar set plates containing the bacterial culture and the lower portion was sealed with a little molten agar media. Subsequently, wells were filled with 50 µl of oil in methanol and allowed to diffuse at room temperature for about 2 h. The plates were incubated at 37ºC 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.

TLC bioautography assay

Dill seed oil exhibiting significant antibacterial potential against *S. aureus* MRSA and *Enterococcus* sp. as determined by agar well diffusion method [Table 3] was analyzed using TLC bioautography assay. 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 duplicate. One of the strips was visualized under UV light to see if the separated spots were UV active after which it was sprayed with 2% vanillin sulphuric acid reagent, the second strip was used for bioautography assay. 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°C. Subsequently, the bioautogram was sprayed with an aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride and further incubated for at 37°C for 4 h. Microbial growth inhibition appeared as clear zones against a pink background. The *Rf* values of the spots showing inhibition were determined.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical analysis of dill seed oil showed that the essential oil contain most of the phytochemicals [Table 2] including tannins, glycosides, saponins, steroids, terpenoids and reducing sugars However, anthraquinone and phlobatanins were not observed in dill oil. Similarly, Jana and Shekhawat [13] reported the presence of saponins, tannins, steroids, flavonoids, glycosides and terpenoids in leaves, stems, roots, *in vitro* callus and regenerated leaves of *Anethum.*

Antibacterial activity assay

The antibacterial activity of Dill essential oil against selected bacterial strains was assessed (Table 3). The results from the agar well diffusion method revealed that the oil showed significant to moderate antibacterial activity toward all tested strains except *P. mirabilis* and *Acenitobacter* sp. The maximum zone of inhibition was found to be 15±0.11 mm in diameter against *Enterococcus* sp. followed by standard *S. aureus* ATCC 25923 (14±0.12 mm). Earlier studies on essential oil of *A. graveolens* revealed its antimicrobial potential [3, 14]. The antimicrobial activity of *A. graveolens* against *E. coli*, *Salmonella typhi*, *Bacillus subtilis* and *S. aureus*, has also been reported by Badar *et al* [15]. Our results are consistent with the reports of previous investigators. 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.

Table 2: **Phytochemical analysis of *Anethum graveolens* oil (Dill seed oil)**

Phytoconstituents	Dill seed oil
Reducing Sugar	+
Tannins	+
Glycosides	+
Saponins	+
Flavonoids	+
Steroids	+
Anthraquinone	-
Terpenoids	+
Phlobatanins	-
Flavonosides	+

a) +: Positive, b) –: Negative

Table 3: Antibacterial activity of Dill seed oil determined by agar well diffusion method

Test Bacteria	Zone of Inhibition (in mm)
Staphylococcus aureus	13.5±0.08
Staphylococcus aureus MRSA	11±0.10
S. aureus ATCC 25923	14±0.12
Escherichia coli	12.2±0.09
E. coli ATCC 25922	13±0.13
Enterococcus sp.	15±0.11
Pseudomonas aeruginosa	10.5±0.085
Klebsiella pneumoniae	10±0.10
Proteus mirabilis	-
Acinetobacter sp.	-

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

TLC bioautography assay

Bioautographic assay are usually used to screen for antimicrobial activity by separating components onto the surface of chromatographic plates and overlaying the TLC plate with molten bacterial agar. The essential oil of Dill revealed a significant antibacterial activity against *Enterococcus* sp. and *S. aureus* MRSA as was characterized by both TLC-bioautography and agar well diffusion methods. One large inhibitory zone with *Rf* value 0.74 was observed against the growth of both *Enterococcus* sp. and *S. aureus* MRSA on the TLC plates B and C as white spot on pink background when sprayed with aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride [Figure 1].

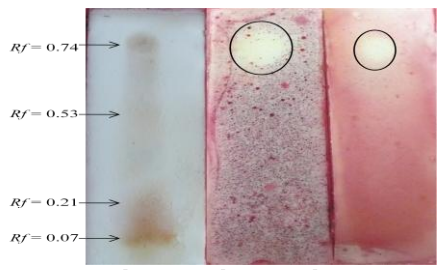

Plate A Plate B Plate C

Figure 1: Chromatogram for (Plate A) and Bioautograms (Plates B and C) for Dill seed oil against *Enterococcus* sp. and *Staphylococcus aureus* MRSA. 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)

Acknowledgements

We acknowledge profound gratitude to Dr. Charu Aggrawal, Department of Microbiology, Rajiv Gandhi Cancer Research Institute, Delhi, India for providing clinical isolates of MDR bacteria. The authors are also thankful to Amity Institute of Biotechnology, Amity University, Noida, U.P, India for offering facilities to carry out this study.

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