Research Journal of

Medicinal Plant

ISSN 1819-3455

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Medicinal Plant 4 (4): 206-212, 2010
ISSN 1819-3455
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Phytochemical Analysis and Antibacterial Screening of *in vivo* and *in vitro* Extracts of Indian Medicinal Herb: *Anethum graveolens*

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Abstract: The antimicrobial potential of the aqueous and ethanolic extracts of seeds, leaves, roots, callus and *in vitro* regenerated plantlets leaves of *Anethum graveolens* have been evaluated against important bacterial strains, *Escherichia coli* HB101 (MTCC-82), *Bacillus subtilis* (MTCC-441), *Bacillus cereus* (MTCC-1306), *Micrococcus luteus* (MTCC-2452). The antimicrobial activity was determined in aqueous and ethanol extracts using both agar disc diffusion and agar well diffusion method. The ethanolic extracts were found to be more potent than aqueous extracts of all parts of plant studied. The ethanolic extracts of seeds showed strong activity against all bacterial strains. In comparison to *in vivo*, *in vitro* plant extracts depicted reduced activity. The phytochemical screening of the plant parts showed that leaves, stems, roots, *in vitro* callus and regenerated leaves were rich in tannins, terpenoids, cardiac glycosides and flavonoids. Though, the seeds of Anethum have been used traditionally as decoctions or infusions prepared in water to treat various ailments, due to the presence of active component, hence, they show maximum activity. During the present study, we have tested effectiveness of *in vivo* plant parts as well as *in vitro* grown callus and leaves, on selected strains of bacteria. These phytochemicals and secondary metabolites could be responsible for the antibacterial activities exhibited by the extract and hence, justify the medicinal uses of *A. graveolens* by common folks.

Key words: *Anethum graveolens*, antibacterial activity, aqueous extract, ethanol extract, phytochemical screening, herb

INTRODUCTION

Medicinal plants have been used for the treatment of various human ailments since long. A revolution came in the medicinal world with the discovery of antibiotics, for treatment of various bacterial infections. However, their indiscriminate use has led to an alarming increase in antibiotic resistance among microorganisms, giving rise to multiresistant strains, which has become a global concern (Shariff, 2001). Thus, there is a renewed interest in exploring natural resources for such compounds. The need of the hour is to screen a number of new medicinal plants for promising biological activity and there *in vitro* propagation to conserve the biodiversity (Mathur *et al*., 2008; Shekhawat *et al*., 2009, 2002). Various plants have been documented in the development of novel drugs, by evaluating their antimicrobial activity, studying active phytochemical constituents and bioactive compounds by various modern

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analytical techniques. It is believed that crude extract from plants are more effective than isolated components due to their synergistic effect. From the safety point of view, spices and medicinal herbs are mostly targeted to meet the therapeutic demands. Since then efficacy of many medicinal plants in the treatment of many diseases have been put to test in many laboratories (Shajahan and Ramesh, 2004).

Anethum graveolens belongs to family Apiaceae, commonly known as sowa. The genus name *Anethum* is derived from Greek word aneeson or aneeton, which means strong smelling. Its common use in ayurvedic medicine is in abdominal discomfort, colic and for promoting digestion (Pullaiah, 2002). Its volatile seed oil is of utmost importance and interest. The seed is bitter, stomachic, antipyretic, carminative and antihelminthic and used in ulcers, abdominal pain, flatulence and preparations of gripe water (Sharma, 2004). Carvone and limonene are monoterpenes, which are present as main constituent of oil from seeds (Santos *et al*., 2002). The α-phellandrene, dill ether and myristicin are the compounds, which form the important odor of dill herb (Bonnlander and Winterhalter, 2000; Blank *et al*., 1992). Its activity against a range of bacteria and fungi has been the subject of many studies (Arora and Kaur, 2007; Nair and Chanda, 2007; Singh *et al*., 2005). But to the best of our knowledge, there are no reports available on roots, *in vitro* callus and regenerated leaves against antibacterial activities of its aqueous and ethanol extracts of this important plant. The objective of the present study was conducted to evaluate the antimicrobial activity of aqueous and ethanolic extracts of plant parts and *in vitro* grown callus and regenerated leaves and also the preliminary screening for phytochemicals in the plant parts. *In vitro* callus and regenerated plantlets antimicrobial activity have not been reported yet.

MATERIALS AND METHODS

Plant Material

Seeds were procured from Jawaharlal Nehru Krishi Vishwa Vidyalaya (JNKVV) Jabalpur in September 2008 and plants were raised from seeds in Botanical Garden of Banasthali University, Rajasthan, India. The identification and authentication of plant material was confirmed at the Department of Botany, Rajasthan University, Jaipur. Fresh plant material were washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles at 4°C. *In vitro* callus and regenerated leaves raised from leaf as explants were shade dried and then stored in powdered form.

Phytochemical Screening

Qualitative phytochemical analysis of the crude powder of plant parts collected were determined as reported by Aiyelaagbe and Osamudiamen (2009) and Egwaikhide *et al.* (2007) are as follows:

Tannins

Small quantity of extract was mixed with distilled water and heated on H_2O bath. It was filtered and Ferric chloride was added to the filtrate. A dark green color indicates presence of tannins.

Flavonoids

About 0.2 g was dissolved in diluted NaOH and HCl was added. A yellow solution that turns colorless indicates the presence of flavonoids.

Saponins

About 0.2 g of plant extract was taken and 5 mL of distilled water was added and then boiled. Frothing persistence shows presence of saponins.

Steroids (Libermann-Burchard Reaction)

Two hundred milligram plant material in 10 mL chloroform, filtered. Two hundred milliliter of acetic anhydride was added to 2 mL filtrate with 2 mL H_2SO_4. The color changes from violet to blue or green in some samples indicating the presence of steroids.

Phlobatanins

About 0.5 g of plant extract was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl solution. Red precipitate shows the presence of phlobatanins.

Terpenoids (Salkowski Method)

About 0.5 g of each extract in 2 mL of chloroform. Concentrated H_2SO_4 carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids.

Cardiac Glycoside

About 0.5g of each was treated with 2 mL of glacial acetic acid containing a drop of $FeCl_3$ solution. This was underlayered with 1 mL of conc. H_2SO_4. A brown ring obtained at the interface indicated the presence of de-oxy sugar characteristics of cardenolides.

Anthraquinone (Borntrger's Test)

About 0.5 g of the extract was taken into a dry test tube and 5 mL of chloroform was added and shaken for 5 min. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red color in the ammonical layer indicates presence of anthraquinone.

Preparation of Plant Extract

For aqueous extraction, 10 g of air-dried powder was added to distilled water and boiled on slow heat for 2 h. It was then filtered through 8 layers of muslin cloth and centrifuged at 5000 g for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 h, the supernatant collected at an interval of every 2 h, was pooled together and concentrated to make the final volume one-fourth of the original volume (Parekh and Chanda, 2007a, b). It was then autoclaved at 121°C temperature and at 15 lbs pressure and stored at 4°C.

For solvent extraction, 10 g of air dried powder was taken in 100 mL of organic solvent (ethanol) in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 h. After 24 h, the supernatant was collected and the solvent evaporated to make the final volume one-fourth of original volume (Parekh *et al*., 2005) and stored at 4°C in airtight bottles.

Bacterial Strains

The bacterial strains are procured from the Microbial Type Culture Collection (Institute of Microbial Technology, Chandigarh, India). Bacterial strains *Escherichia coliHB101* (MTCC-82), *Bacillus subtilis* (MTCC-441), *Bacillus cereus* (MTCC-1306) and *Micrococcus luteus* (MTCC-2452) were selected for present study. They were cultured on nutrient agar broth (HiMedia) and stored at 4°C.

Antibacterial Activity

The antibacterial activities of different plant part extracts were evaluated by agar disc diffusion method (Bauer *et al*., 1966; Garg and Jain, 1988) and agar well diffusion method (Parekh and Chanda, 2007a, b) using nutrient agar plates previously inoculated with 24 h old broth cultures of the test organisms. Active cultures for experiments were prepared by inoculating a loopful of cells from stock cultures to flask of nutrient agar broth and incubated on rotary shaker. The molten Mueller Hinton agar (Hi Media) was inoculated with 100 μL of the inoculum (1×10^8 cfu mL^{-1}) and poured into the sterile Petri plates (HiMedia). For agar disc diffusion method, a well was prepared in the seeded agar plate with the help of cork-borer (0.85 cm). The test compound (50 μL) was introduced in the well and the plates were incubated at 37°C for 24 h. For agar disc diffusion method, the test compound (50 μL) was introduced on the sterile disc (0.7 cm) (HiMedia) and then allowed to dry. Then, the disc was impregnated on the seeded agar plate. Equal volumes of distilled water and ethanol were assayed along to act as negative controls. The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. Microbial growth was determined by measuring the diameter of zone of inhibition in millimeters. The result was obtained by measuring the size of zone of inhibition and was expressed in terms of average diameter of zone of inhibition. The results were compared with the standard antibiotics Streptomycin (10 μg mL^{-1}).

Statistical Procedures

Antimicrobial activity experiments were repeated thrice in triplicates each time and the average values with Standard Deviation (SD) are presented in Table 2 and 3.

RESULTS

The phytochemical screening of the plant parts showed that leaves, stems, roots and *in vitro* callus and regenerated leaves were rich in tannins, terpenoids, cardiac glycosides and flavonoids (Table 1). Of aqueous extracts, seeds showed the maximum activity in comparison to leaves and roots extract, whereas aqueous extracts of *in vitro* grown callus and leaves has shown no activity against bacterial strains (Table 2, 3). However, none of the aqueous extracts showed any activity against *M. luteus*. Ethanolic extracts of leaves, roots, *in vitro* grown callus and regenerated leaves showed considerable activity against all the strains but maximum activity was shown by ethanolic extract of seeds against all the strains. Well diffusion method revealed that the maximum antimicrobial activity was showed by ethanolic extracts of seeds against *E. coli* followed by *B. cereus*>*B. subtilis*>*M. luteus*. Maximum activity against *E. coli* by ethanolic seed extract was 15.4 mm, against *B. cereus* zone of inhibition was 13.2 mm, against *B. subtilis*, it was 11.13 mm and zone of inhibition

Table 1: Qualitative analysis of the phytochemicals of *Anethum graveolens* L. seeds, leaves, roots, callus, *in vitro* regenerated leaves

Metabolites	*In vivo*	Callus	*In vitro* leaves
Tannins	+	+	+
Terpenoid	+	+	+
Saponins	+	+	-
Steroid	+	+	+
Flavonoid	+	+	+
Phlobatannin	-	-	-
Cardiac- Glycoside	+	+	+
Anthraquinone	-	-	-

+: Present, - : Absent

Table 2: Antibacterial activity of *Anethum graveolens* L. aqueous and ethanolic extracts against bacterial strains by well diffusion method

Plant samples	*E. coli*	*B. cereus*	*B. subtilis*	*M. luteus*
In vivo				
Aqueous seeds	3.80±0.04	3.70±0.00	1.20±0.04	-
Ethanolic seeds	15.40±0.08	13.20±0.08	11.13±0.04	8.90±0.09
Aqueous leaves	-	-	-	-
Ethanolic leaves	8.10±0.08	8.03±0.04	7.53±0.04	5.13±0.04
Aqueous roots	-	-	-	-
Ethanolic roots	3.40±0.04	4.43±0.04	1.60±0.04	1.20±0.04
In vitro				
Aqueous callus	-	-	-	-
Ethanolic callus	3.16±0.04	3.33±0.04	2.06±0.04	-
Aqueous leaves	-	-	-	-
Ethanolic leaves	2.26±0.04	2.43±0.04	2.10±0.00	1.10±0.00
Streptomycin	20.20±0.80	25.10±0.50	19.20±0.20	18.50±0.04

Table 3: Antibacterial activity of *Anethum graveolens* L. aqueous and ethanolic extracts against bacterial strains by disc method

Plant samples	*E. coli*	*B. cereus*	*B. subtilis*	*M. luteus*
In vivo				
Aqueous seeds	3.00±0.000	2.50±0.00	1.20±0.00	-
Ethanolic seeds	11.66±0.470	13.16±0.12	8.80±0.12	6.80±0.21
Aqueous leaves	1.86±0.040	1.16±0.47	-	-
Ethanolic leaves	8.83±0.040	8.53±0.23	6.06±0.09	3.06±0.75
Aqueous roots	2.40±0.081	1.16±0.07	1.86±0.04	-
Ethanolic roots	3.70±0.047	4.13±0.39	2.70±0.08	-
In vitro				
Aqueous callus	-	-	-	-
Ethanolic callus	3.46±0.000	3.40±0.74	2.10±0.08	-
Aqueous leaves	-	-	-	-
Ethanolic leaves	3.33±0.040	3.32±0.65	3.06±0.70	1.23±0.68
Streptomycin	22.36±0.030	25.95±0.37	20.08±0.06	19.31±0.62

against *M. luteus* was 8.9 mm (Table 2). *Micrococcus luteus* was most resistant among other strains as it showed minimal inhibitions. Agar disc method also revealed similar observations. Against *E. coli*, maximum activity was shown by ethanolic seeds (11.6 mm) and then ethanolic leaves *in vivo* (8.83 mm). Against *B. cereus* maximum activity was shown by ethanolic extract of seeds and inhibition zone was 13.16 m. Similarly, against *B. subtilis* maximum activity was shown by ethanolic seeds (8.86 mm). Against *M. luteus* only ethanolic extract of seeds and leaves showed activity, inhibition zone was 6.8 and 3.2 mm obtained subsequently. Thus, the trend with agar disc method was *B. cereus* > *E. coli* > *B. subtilis* and then *M. luteus* (Table 3). Ethanolic extracts of roots, callus and *in vitro* regenerated leaves has also shown very minimal antibacterial activity against the strains. It is quite possible that due to the optimum *in vitro* environmental conditions callus and regenerated leaves accumulate lesser amount of active components, in comparison to *in vivo* plant parts and hence, shows reduced activity.

DISCUSSION

The traditional healers use primarily water as the solvent but we found in this study, the extracts prepared by ethanol provided more consistent antimicrobial activity compared to those extracted by water. This might have resulted from the lack of solubility of the active constituents in aqueous solutions while ethanol extract showed some degree of antibacterial activity, as also reported in methanol extract of some medicinal plants by Parekh *et al*. (2005). Further trials using solvents of various polarities will explore the effects of solvent

composition on extract efficacy (Romero *et al*., 2005). Similarly, acetone extract of *Anethum graveolens* and its seed oil has been reported to show statistically significant antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhii*, *Pseudomonas aeruginosa*, *Bacillus subtilis* as well as antifungal activities against *Aspergillus niger*, *Fusarium moniliforme*, *Penicilium citrinum* (Singh *et al*., 2005). *Anethum graveolens* have been in use for many years as decoctions or infusions prepared in water to treat ailments. It has been reported that aqueous extracts of *Anethum graveolens* showed a broad-spectrum antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurim*, *Shigella flexneri* and *Salmonella typhii* (Arora and Kaur, 2007). As depicted from the results of antibacterial activity against all strains, ethanolic seed extract and aqueous extract have shown maximum activities. Hence, seeds have more potential than other plant parts in terms of microbial activity. It may be due to the constituents present in the seeds of *Anethum graveolens,* as it is rich in carvone (55.2%), limonene (16.6%), dill-apiole (43.2%), linoleic acid (23.1%) and anethole (11%) (Singh *et al*., 2005). The higher activity of extract can be explained on the basis of the chemical structure of their major constituents such as dill-apiole and anethole, which have aromatic nucleus containing polar functional group that is known to form hydrogen bonds with active sites of the target enzyme (Farag *et al*., 1989). It is reported that its seed oil has a little activity against gram-negative bacteria, which may be due to the differences in composition related to variety; agronomic practice and processing which also influence concentrations of active ingredients, hence, effecting antimicrobial properties (Delaquis *et al*., 2002).

In conclusion, seeds of the plant have strong antimicrobial potential but results also revealed that apart from seeds of *Anethum* other plant parts like leaves, roots, callus and *in vitro* regenerated leaves also have the potential to show antimicrobial activity. This plant can be further subjected to enhancement and isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation.

ACKNOWLEDGMENT

G.S. Shekhawat is thankful to Prof. Aditya Shastri, Vice Chancellor, Banasthali University, for providing all the necessary requirements to successfully carry out this research.

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