# Evaluation of phytochemical constituents and antibacterial potential of *Anethum graveolens*: Comparative illustration of the effect of ethanolic extracts of callus and leaf

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***Abstract***
*The purpose of the present study was to analyze the antibacterial activity of plant extract of Anethum graveolens against different bacterial strains. The increasing multidrug-resistant strains of pathogenic bacteria and decreasing spectrum of antibiotics against different pathogens have developed renewed interest in plant based treatments, therefore, the production of important plant secondary metabolites through in vitro plant cell cultures becomes necessary. The effectiveness of in vitro callus and in vivo leaf extracts of the plant was assessed against bacterial strains (Klebsiella pneumonia, Staphylococcus aureus, Escherichia coli, Salmonella typhi and Proteus mirabilis).*

*The antibacterial susceptibility examination of the plant extract showed maximum zone of inhibition by in vivo leaf extracts 9.267±0.176 mm for Staphylococcus aureus and 11.73±0.033 mm for Klebsiella pneumonia. Phytochemical screening of ethanolic plant extract showed the presence of different secondary metabolites. The effectiveness of the plant extracts to inhibit bacterial growth validates the medicinal properties of the plant. Hence, the plant could efficiently be used for commercial production of pharmaceuticals.*

**Keywords**: *Anethum graveolens*, ethanolic extract, phytochemical analysis, antibacterial susceptibility, zone of inhibition.

## Introduction

Medicinal plants are a rich source of pharmaceutically important bioactive compounds. Currently, there is an increasing awareness on the plant derived active components to treat a variety of diseases affecting humans. *Anethum graveolens* (Dill), an annual aromatic herb belonging to the family Apiaceae is already in use as a dietary herb and as conventional remedy for treatment of various infections. [11]

Recent researches on the plant have shown that Dill oil is useful in treatment of cancer, microbial infection, gastric irritation and inflammation[5] and is also known for its antihyperlipidaemic[23] and antihypercholesterolaemic activity.[20] These properties are attributed to the presence of many active metabolites including saponins, steroids, flavonoids, terpenoids, phenols, carotenoids, saponins, coumarins and so on. The presence of phytochemicals has also been validated using different analytical techniques. [12,19] The use of naturally occurring plant based antimicrobial compounds possesses the potential to be efficiently used in food and drug system to control the diseases caused by microorganism without any side effects.

Herbs are the safest when used as medicines because of natural antimicrobial components and antioxidant properties.[7, 9] Till date, a lot of investigations have been carried out on antimicrobial compounds present in herbs, volatile oils and plant extracts.[4,6,22] Antibacterial activity of different plant extracts has been reported in previous studies using various organic solvents such as ethanol, methanol and petroleum ether. [18] Several comparative studies have also demonstrated the effect of *in vitro* and *in vivo* plant extracts.[15]

Present study analyzes the components determined to be present in the plant along with assessment of the antimicrobial action of *in vitro* and *in vivo* extracts and comparison of these extracts with the standard antibiotics on bacterial growth. The study was conducted in three stages. In the first stage, the extraction from *in vitro* developing callus cultures with ethanol was carried out through Soxhlet extraction method followed by phytochemical analysis of the plant extracts. In the final step, antimicrobial examination of the extracts against different bacteria was studied.

## Material and Methods

**Collection of plant material**: Hardened plants of *Anethum graveolens* growing in green house of Botany Department, Jodhpur were used for generation of callus.[5] MS medium fortified with plant growth hormones, specifically a combination of BAP (1mg $L^{-1}$) and NAA (0.5mg $L^{-1}$) was found to be suitable for appropriate growth of callus. The callus was sub cultured after every three weeks. To analyze the potency of in vitro cultures against various bacteria, the callus extract was compared with *in vivo* grown leaves. Ethanol without any plant extract served as control.

**Preparation of MH medium and bacterial strain collection**: 38g of Muller Hinton agar medium (Himedia) was suspended in 1 liter of distilled water. The medium was

boiled and the pH was adjusted to 7.3±0.1 prior to autoclaving at 121psi 36.2℃ for 15 minutes and allowed to cool at room temperature. The medium was poured in Petri dishes under laminar air flow hood.

The bacterial isolates of *Klebsiella pneumonia*, *Staphylococcus aureus, Escherichia coli, Salmonella typhi* and *Proteus mirabilis* were obtained from Department of Microbiology, Dr. S.N. Medical College and Associated hospitals, Jodhpur.

**Isolation of plant extract**: The extraction was achieved by the method given by Ahmed et al[1] with some minor modifications. Air dried samples were extracted thrice with ethanol in Soxhlet apparatus. After filtration, the samples were dried over anhydrous sodium sulfite; the extracts were dissolved in ethanol (2ml) and stored at -4℃ for further analysis.

**Phytochemical analysis of *Anethum graveolens***: For the preliminary screening, different tests for phytochemical constituents such as tannins, terpenoids, saponins, flavonoids, anthraquinones, phenols and alkaloids were carried out on the *Anethum graveolens in vitro* callus and *in vivo* leaf extracts. The presence or absence of the phytochemical constituents of material was analyzed using the following standard methods[10, 21]

**Analysis for Tannins**: To 5 ml of aqueous extract, bromine water (10 ml) was added; discoloration of bromine water indicated the presence of tannins.

**Test for Terpenoids**: To about 1ml of plant extract, 2ml of chloroform and conc. $H_2SO_4$ were added carefully through the walls of tube. Appearance of a reddish brown color at the interface showed the presence of terpenes.

**Estimation of saponins**: Saponin content of plant extract was estimated by dissolving 1ml of extract to 10 ml of hot distilled water. The development of persistent foam indicated presence of saponins.

**Estimation of flavonoids**: One ml of each extract was dissolved in 3 ml of methanol and then treated with a drop of hydrochloric acid and 0.5 g of magnesium chips. Three minutes later, a pink or red coloration indicates the presence of flavonoids.

**Test for Steroids**: Development of a green color on addition of 2 ml of the aqueous extract to chloroform (2 ml) followed by treatment with concentrated sulphuric acid and acetic acid indicates the presence of steroids.

**Test for Anthraquinones**: Approximately 1ml of each extract was boiled with hydrochloric acid (10%) for 2-3 minutes in water bath. The mixture was filtered and set aside to cool. An equal volume of 10% ammonia solution was added to it and heated. Development of rose-pink color indicates the presence of Anthraquinones.

**Estimation of Phenols**: To 5ml of distilled water, 1ml extract was added followed by addition of a few drops of neutral 5% ferric chloride solution. A dark green color formation indicated the existence of phenolic compounds.

**Test for Alkaloids**: To 2-3ml of extract, 1ml of dil. HCl and Wagner's reagent were added and shaked well. Formation of reddish-brown precipitate showed the presence of alkaloids.

**Estimation of antibacterial activity by disc diffusion method**: The molten muller hinton agar medium was poured into sterile Petri plates. After solidification, peptone water consisting of a loop full of bacterial isolates was poured over solidified medium. Individual plant extracts along with control were introduced into sterile discs of filter paper. The discs saturated with extracts were placed on agar plates after drying. Ethanol alone served as negative control. The standard antibiotic discs (containing 30 ug of the antibiotic on each disc) of Chloramphenicol, Meropenem and Vancomycin (for *Staphylococcus aureus*), Ampicillin, Penicillin and Meropenem (*Klebsiella pneumoniae*) served as positive control for determination of antibacterial susceptibility for each microorganism. The inoculated plates with discs and antibiotics were left overnight for incubation at 36.2℃. The zone of inhibition was determined with the help of Vernier caliper scale.

**Statistical analysis**: The data is represented as Means ± SE. The data means were calculated through Sigma Plot software (Version. 12). Statistical analysis of the data was conducted using one way analysis of variance (ANOVA) using SPSS ver. 17 for windows (SPSS Inc., USA) followed by separation of significantly different means using Duncan multiple range test (P = 0.05).

## Results and Discussion

**Qualitative assay of *in vitro* extracts of *Anethum graveolens***: The preliminary screening of phytoconstituents laid an empirical foundation for the use of plant derived compounds in traditional medicines. The preliminary phytochemical investigation of ethanolic extract of *Anethum graveolens* indicated the presence of some important phytoconstituents such as alkaloids, tannins, phenolic compounds, phytosterols, terpenoids, saponins and flavonoids (Table 1).

These phytochemicals possess significant pharmacological activities like anti mutagenic, anti inflammatory and antibacterial properties for which the extracts could be further utilized.[8]

**Antibacterial susceptibility Test**: Different concentrations of plant extracts along with control were compared with standard antibiotics specific for bacterial growth and the growth inhibition activity was recorded. All the bacterial

strains demonstrated some degree of sensitivity to the plant extracts examined (Fig. 1). The results presented in table 2 are in accordance with previous reports.[16] Amongst all the microorganisms assessed, *Staphylococcus aureus* and *Klebsiella pneumonia* showed maximum response against the plant extracts. For *Staphylococcus aureus*, the maximum zone of inhibition among antibiotics 12.867±0.133 mm was developed by Meropenem. The zone activity by *in vivo* leaf extracts 9.267±0.176 mm was found to be more than callus extracts 7.1±0.1 mm whereas control did not show bacterial growth inhibition. Comparative to zone inhibition developed by other antibiotics, Vancomycin showed a sharp zone of 8.067±0.014 mm.

**Table 1**
**Preliminary Screening of *Anethum graveolens* plant extracts**

| S.N. | Chemical constituent | Phytochemical Test | *In vitro* callus extract | Leaf extract |
|---|---|---|---|---|
| 1. | Tannins | Braymer's test | + | + |
| 2. | Terpenoids | Salkowski's test | + | + |
| 3. | Saponins | Foam test | + | + |
| 4. | Flavonoids | Alkaline reagent test | + | + |
| 5. | Steroids | Lieberman Bruchard test | + | + |
| 6. | Anthroquinones | Borntrager's test | - | - |
| 7. | Phenols | Ferric chloride test | + | + |
| 8. | Alkaloids | Wagner's test | + | + |

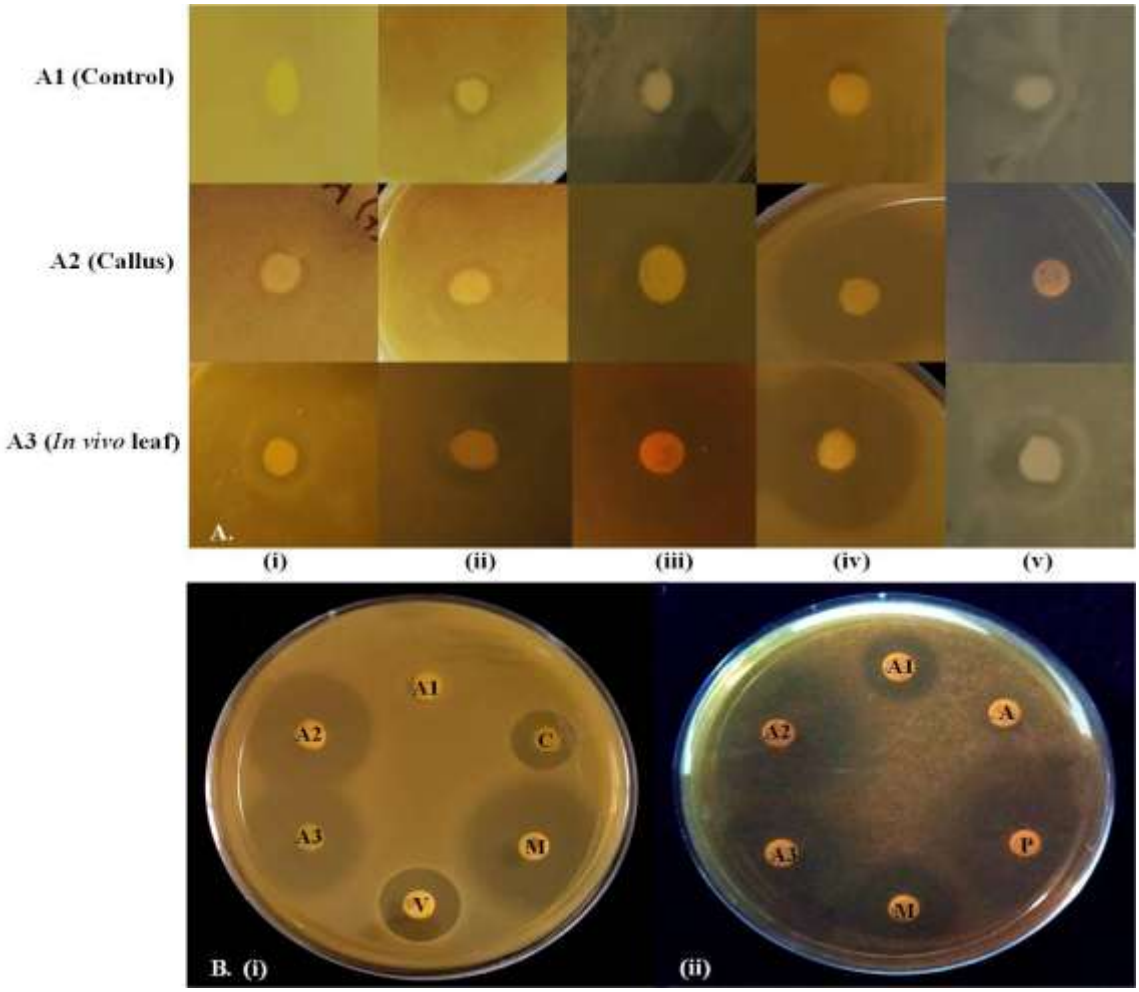


**Fig. 1: Antibacterial activities of *in vitro* and *in vivo* plant extracts (through disk diffusion method) a. zone of inhibition (mm) of bacterial isolates; (i) *Escherichia coli* (ii) *Salmonella typhi* (iii) *Proteus mirabilis* (iv) *Staphylococcus aureus* (v) *Klebsiella pneumonia* b. Antibacterial activity of plant extract and standard antibiotic against (i) *Staphylococcus aureus* A1 (control); A2 (callus); A3 (*in vivo* leaf); V (Vancomycin); M (Meropenem); C (Chloramphenicol) (ii) *Klebsiella pneumonia* A1(Control); A2 (callus); A3 (*in vivo* leaf); M (Meropenem); P (Penicillin); A (Ampicillin)**

**Table 2**
**Antibacterial activity (depicted as zone diameter) of plant extracts against human bacterial pathogens**

| S.N. | Bacteria | Antibiotic/Sample | Zone of inhibition (mm) | Zone edge |
|---|---|---|---|---|
| A. | ***Staphylococcus aureus*** | Chloramphenicol | 7.867 ± 0.033 | Sharp |
| | | Meropenem | 12.867 ± 0.133 | Diffuse |
| | | Vancomycin | 8.067 ± 0.014 | Sharp |
| | | Control (A1) | - | Diffuse |
| | | Callus extract (A2) | 9.267 ± 0.176 | Diffuse |
| | | *In vivo* leaf extract (A3) | 7.1 ± 0.1 | Diffuse |
| B. | ***Klebsiella pneumoniae*** | Ampicillin | - | - |
| | | Penicillin | 13.1 ± 0.1 | Diffuse |
| | | Meropenem | 8.617 ± 0.088 | Diffuse |
| | | Control (A1) | 6.967 ± 0.186 | Sharp |
| | | Callus extract (A2) | 11.73 ± 0.033 | Diffuse |
| | | *In vivo* leaf extract (A3) | 9.2 ± 0.2 | Diffuse |

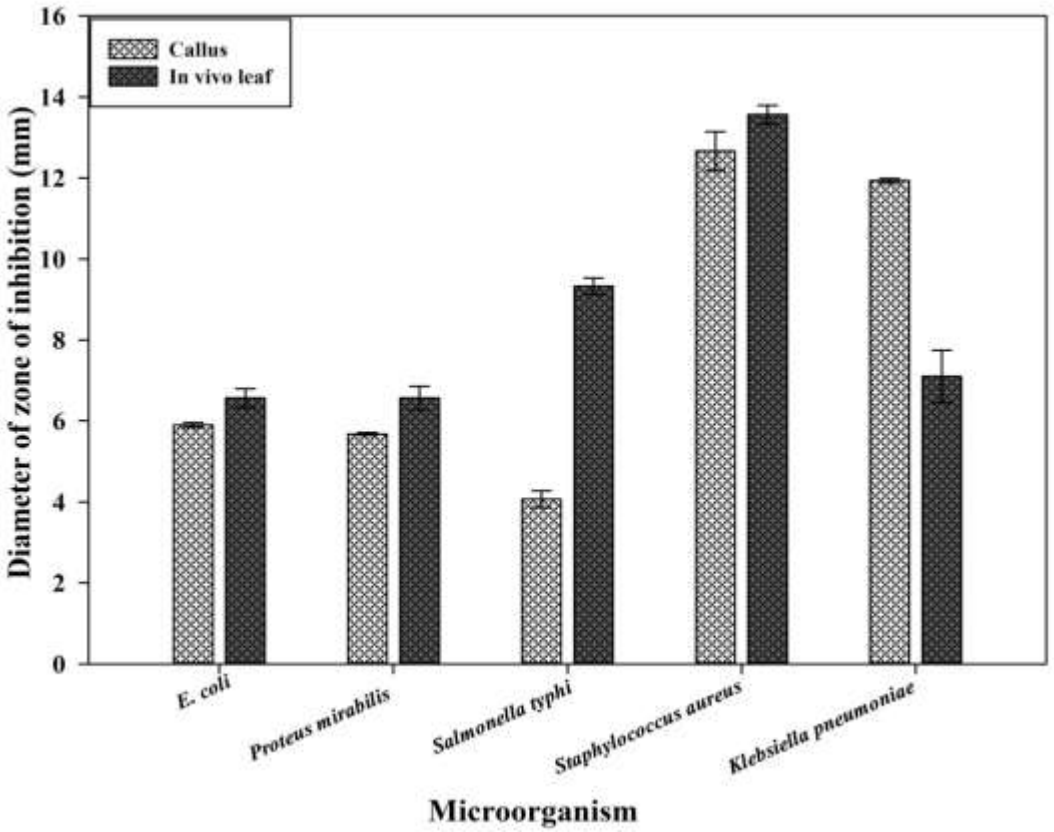


**Fig. 2: Graphical representation of diameter of zone of inhibition (mm) of *in vitro* callus and *in vivo* leaf extracts against different bacterial strains**

For *Klebsiella pneumonia*, maximum zone of inhibition 13.1±0.1 mm was observed for Penicillin among antibiotics. On the other hand, ampicillin did not respond for bacterial growth. A sharp zone of 6.967±0.186 mm was recorded for control (Fig. 2). The zone diameter for *in vivo* leaf extracts 11.73±0.033 mm was more that of callus extract (9.2±0.2 mm).

Among all bacterial populations, the susceptibility response to the plant extracts followed the order:

*Klebsiella pneumonia* > *Staphylococcus aureus* > *Escherichia coli* > *Salmonella typhi* > *Proteus mirabilis*

The results of the study are corroborated by the experiments conducted by Jana and Shekhawat[11] who reported the effect of ethanolic extracts of callus and leaf with maximum zone of inhibition of 15.4±0.08 mm against *Escherichia coli*. Liouane et al[13] sated that the petroleum ether extract of *Cotula coronopifolia* showed significant activity against *Staphylococcus aureus* and *Escherichia coli*.

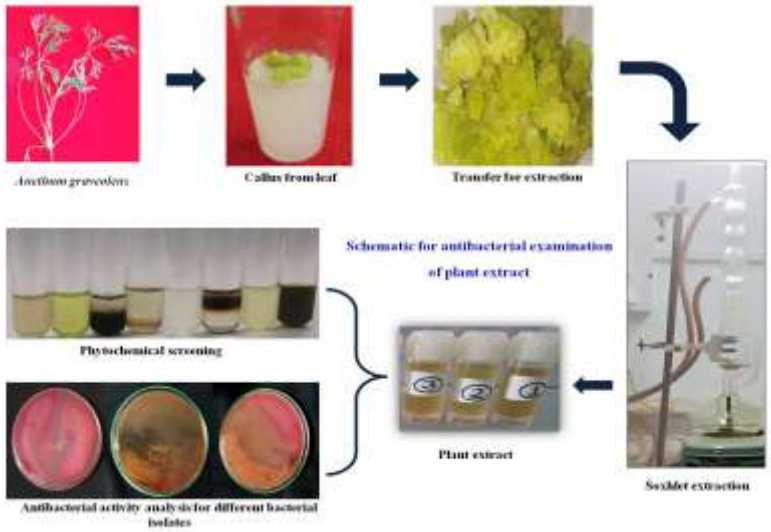


**Fig. 3: Schematic representation of methodology followed in the present study for phytochemical screening and evaluation of antibacterial activity**

Similar results were reported by Suganya and Jothi[17] using acetone and ethanolic extracts of the plant *Commulina nudiflora* against similar microbial profile. It has also been demonstrated that the ethyl acetate extract of *Cotula cinerea* exhibited an antibacterial effect against *Pseudomonas sp*. and *Bacillus sp*. It also stated that butanolic extract of this plant was very effective in particular against *Pseudomonas fluorescens* and *Bacillus sp*.[3,14]

Previous studies have reported that the ability of plant extracts to inhibit bacterial growth depends upon the solubility of the bioactive constituents.[2] Results of the present investigation elucidated that the crude extract of *Anethum graveolens* proved its efficiency to be used as a source of antibacterial compounds when compared with some standard antibiotics due to its inhibitory effects on microbial strains.

## Conclusion

The study showed that there existed a significant variation between the antibacterial activity of *in vitro* callus extract and *in vivo* grown leaf extract, although the phytochemical occurrence remains the same. It could be concluded that plant extracts conferred significant antibacterial activity against broad spectrum bacterial population as compared to callus extracts.

The extracts responded considerably for *Klebsiella pneumonia* and *Staphylococcus aureus* among all microbial strains under study (Fig. 3). The comparative investigation with standard antibiotics showed that *Anethum graveolens* possesses the potential to be used for medicinal purpose.

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