Comparative evaluation of antioxidant properties of edible and non-edible leaves of *Anethum graveolens* Linn.

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The study was conducted to compare *in vitro* antioxidant activities of ethanol extracts of edible and non-edible leaves of *Anethum graveolens* Linn. The antioxidant activity was evaluated using nine different standard methods. The green leaves extract exhibited high percentage of inhibition in most of the methods, when compared to the non-edible yellow leaves extract. The HPTLC of the yellow leaves extract exhibited six compounds instead of four observed for green leaves extract transformation towards inactive compounds. The total phenol content of the yellow leaves extract was found to be high, indicating there was no relationship between the activity and the total phenol content. The study supports the traditional use of green leaves as vegetable and food flavouring agent.

**Keywords:** *Anethum graveolens*, Dill herb, Free radical scavenging, Antioxidant, Edible, Non-edible.

**IPC code; Int. cl.**—A61K 36/00, A61P 17/18.

**Introduction**

*Anethum graveolens* Linn (Family: Apiaceae), commonly known as Dill is traditionally being used in the treatment of gastric disturbances of infants and children¹. Both the herb and fruits are used for the extraction of volatile oil and in food preparations. Dill herb is reported to possess antidiabetic, antihyperlipidaemic and antihypercholesterolemic activities²-⁴. Anethoferon, carvone and limonene as potential cancer preventive agents were isolated from the dill oil and with maturity of the herb, carvone content of the oil increases and phellandrene and d-limonene contents decreases⁵. All these activities are related to antioxidant activity. Antioxidants are known to prevent a large number of diseases including atherosclerosis, cancer, Parkinson’s disease, diabetes, liver diseases, etc. The aqueous extract of dill fruits showed potent antioxidant activity in *in vitro* systems⁶. In India, the green leaves of dill are considered as edible and used as a vegetable and when the leaves become yellow, they are considered as non-edible and not used as a vegetable. The present study was planned to carry out a comparative evaluation of *in vitro* antioxidant activity of these two kinds of leaves of dill using standard methods. Polyphenols from fruits and vegetables are known to possess antioxidant activity. Hence, the total phenol and flavonol contents and HPTLC evaluation was also carried out.

**Materials and Methods**

**Collection and extraction of edible and non-edible leaves of A. graveolens**

The plant was collected from the local market, Ootacamund in May 2007 and authenticated by Dr. S. Rajan, Medicinal Plants and Collection Unit, Ootacamund, India. The fresh leaves were separated from the plant, crushed and subjected to single extraction (240g) in a Soxhlet extractor using ethanol (1000ml) for 24h. The extract was concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature. Yield of dark brown semisolid was 9.2g (3.8% w/w). During maturation, the colour of leaves was changed from green to yellow, they were separated and crushed. The paste (240g) was extracted with ethanol (1000 ml) and concentrated by following the above procedure.

**Chemicals**

DPPH (2, 2-diphenyl-1-picryl hydrazyl) and ABTS (2, 2’-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt were obtained from Sigma.
Aldrich Co, St Louis, USA. Rutin and p–NDA (p–nitroso dimethyl aniline) from Acros Organics, New Jersey, USA and NEDD (Naphthyl ethylene diamine dihydrochloride) from Roch–Light Ltd., Suffolk, UK. Ascorbic acid, NBT (nitro blue tetrazolium) and BHA (butylated hydroxy anisole) were supplied by S.D. Fine Chem, Ltd., Biosar, India. 2-Deoxy-D-ribose was purchased from Hi-media Laboratories Ltd., Mumbai, India. All chemicals used were of analytical grade.

Preparation of test and standard solutions

The extracts and the standard antioxidants ascorbic acid, rutin and BHA were dissolved in freshly distilled DMSO (Dimethyl Sulfoxide) separately and used for the in vitro antioxidant activity using different methods. For the hydrogen peroxide method, the extracts and the standards were dissolved in distilled methanol. For the total phenol content a solution of extracts in methanol was used. The stock solutions were serially diluted with the respective solvents to obtain the lower dilutions. Gallic acid monohydrate was dissolved in distilled water and used as a standard for total phenol estimation.

Estimation of total phenol content

In a series of test tubes, 0.4ml of the extract (1mg/ml to 0.1mg/ml) in methanol was taken, mixed with 2ml of Folin-Ciocalteu reagent in distilled water (1:10) and 1.6 ml of sodium carbonate. After shaking, it was kept for 2h reaction time. The absorbance was measured at 750 nm. Using gallic acid monohydrate, standard curve was prepared and linearity was obtained in the range of 1-10 µg/ml. The total phenol content was obtained and expressed as gallic acid equivalent in mg/g of the extract.

HPTLC studies of extracts

The edible and non edible leaves extract of A. graveolens were applied on HPTLC plate with bandwidth of 8 mm. Application rate was maintained at 10µl/min, this was applied using Linomate-IV applicator, (automatic TLC applicator, Camag, Switzerland). 10µl of sample was applied and the plate was developed in twin-trough chamber using the solvent system (ethyl acetate : methanol = 9:1). After developing, the plate was air-dried and observed under UV chamber (Camag, UV chamber, model No. 022.9120). The developed plate was scanned using densitometer at 254 nm (Camag TLC Scanner, model No. 027.6480).

In vitro antioxidant activity

All the extracts were tested for in vitro antioxidant activity using several standard methods. The final concentration of the extract and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81µg/ml. The absorbance was measured spectrophotometrically against the corresponding blank solution. The percentage inhibition was calculated by the following formula.

\[
\text{Radical scavenging activity(%) = } \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100
\]

IC_{50}, which is the concentration of the sample required to scavenge 50% of free radicals was calculated.

Scavenging of ABTS radical cation

ABTS assay is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixture such as plant extracts, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS* for the estimation of the antioxidant activity. To 0.2 ml of various concentrations of the extracts or standards, added 1.0 ml of distilled DMSO and 0.16 ml (2mM) of ABTS solution. Absorbance was measured spectrophotometrically, after 20 min at 734 nm.

DPPH radical scavenging method

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in colour and upon reaction with hydrogen donor’s changes to yellow in colour. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured. The extract or standard solution (10µl) was added to DPPH in methanol solution (200 µl, 100µM) in a 96-well microtitre plate [Tarsons Products (P) Ltd., Kolkata, India]. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 490 nm using an ELISA microtitre plate reader (Bio Rad Laboratories Inc, California, USA, Model 550). The remaining DPPH was calculated.

Scavenging of hydroxyl radical by P-NDA method

Hydroxyl radical is measured by the inhibition of p-nitrosodimethyl aniline (p-NDA) bleaching by
hydroxyl radical. Hydroxyl radical is generated through Fenton reaction. Hydroxyl radical scavenger shows scavenging activity by inhibition of bleaching and percentage of scavenging as absorbance is measured. To a solution mixture containing ferric chloride (0.1 mM, 0.5 ml), EDTA (0.1 mM, 0.5 ml), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and p-NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4), (20 mM), were added to various concentrations of extract or standard (0.5 ml), to give a final volume of 3 ml. Absorbance was measured at 440 nm.

Scavenging of hydroxyl radical by deoxyribose method

The sugar deoxyribose (2-deoxy-D-ribose) is degraded on exposure to hydroxyl radical generated by irradiation or by Fenton systems. If the resulting complex mixture of products is heated under acid conditions, malondialdehyde (MDA) is formed and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen. To the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer, pH 7.4 (20 mM), were added to 0.2 ml of various concentrations of extract or standard in DMSO to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37°C. After incubation, ice-cold trichloroacetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v), in 0.25 N hydrochloric acid were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and absorbance was measured at 532 nm.

Nitric oxide radical inhibition assay

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, can be estimated by the use of modified Griess Ilosvay reaction. Nitrite ions react with Griess reagent, which forms a purple azo dye. In the presence of test components (likely to be scavengers), the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, 1 ml) and extracts or standard solutions (1 ml) were incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was removed, 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) was mixed and allowed to stand for 5 min for completing diazotization and 1 ml of NEDD was added, mixed and allowed to stand for 30 min. A pink coloured chromophore was formed in diffused light. The absorbance was measured at 540 nm against the corresponding blank solutions in microtiter plates using ELISA reader.

Scavenging of hydrogen peroxide

In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically. Various concentrations of the extracts or standards in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution (20 mM) in PBS (pH 7.4). The absorbance was measured at 230 nm after 10 min.

Scavenging of superoxide radical by alkaline DMSO method

In alkaline DMSO method, superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature and that can be measured. Superoxide scavenger capable of reacting inhibits the formation of a red dye formazan. To the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml of water) and 0.3 ml of the extracts or standards at various concentrations, added 0.1 ml of NBT (1 mg/ml) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm.

Lipid peroxidation inhibitory activity

Lipid peroxidation can be initiated by ROS such as hydroxyl radicals by extracting a hydrogen atom from lipids and forming a conjugated lipid radical. This reacts rapidly with oxygen to form a lipid radical until the chain reaction is terminated. The lipid peroxidation adducts may induce the oxidation of biomolecules such as DNA, proteins and other lipids, resulting in cellular damage. The reaction mixture containing egg lectin (1 ml), ferric chloride (0.02 ml), ascorbic acid (0.02 ml) and extract or standard (0.1 ml) in DMSO at various concentrations was kept for incubation for 1 hour at 37°C. After incubation, 2 ml of 15% TCA and 2 ml of 0.37% TBA were added. Then the reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm.
Evaluation of total antioxidant capacity of the extracts

The total antioxidant capacity was determined by phosphomolybdenum method and is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex which has the maximal absorption at 695 nm. An aliquot of 0.1 ml of the sample solution containing a reducing species in DMSO was combined in an Eppendorff’s tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in water bath at 95°C for 90 min. The samples were cooled to room temperature and the absorbance of each solution was measured at 695 nm. The total antioxidant capacity was expressed as mM equivalent of ascorbic acid.

Results and Discussion

The polyphenol content of green and yellow leaves extracts of \textit{A. graveolens} when measured by the Folin-Ciocalteu method, were 10.60 and 14.30% w/w, respectively in terms of gallic acid equivalents. The total polyphenol content of yellow leaves extract was found to be more when compared to the green leaves extract. It indicates that more phenolic constituents were formed on maturation of dill leaves. HPTLC studies also confirmed the same. The HPTLC of the green leaves extract exhibited four compounds with \( R_f \) values of 0.10, 0.41, 0.81 and 0.91. The HPTLC of the yellow leaves extract showed six spots with \( R_f \) values of 0.10, 0.35, 0.45, 0.58, 0.66 and 0.92 (Fig. 1 & 2). The compounds with \( R_f \) values of 0.10, 0.41 and 0.81 were transformed to other compounds with low contents in the yellow leaves extract. The major compound in the green leaves extract with \( R_f \) value of 0.91 and percentage area 39.78 is again the major compound in the yellow leaves extract but with higher percentage area, 62.63.

In the \textit{in vitro} antioxidant activity using nine different methods, the green leaves extract was found to be more active in most of the methods tested. The percentage inhibition obtained for green leaves was found to be more than the yellow leaves extract in nitric oxide, lipid peroxidation, hydrogen peroxide, p-NDA and deoxyribose methods, when tested at 250, 500 and 1000 µg/ml concentrations. In the ABTS and alkaline DMSO methods both the extracts were found to be equally potent. However, in the DPPH method the yellow leaves extract was found to be more active (Table 1). The IC\(_{50}\) values of these extract and the standards are given in Table 2. The total antioxidant

![HPTLC spectrum of green leaves of Anethum graveolens](image)
**Table 1— In vitro antioxidant activity of ethanolic extracts of green and yellow leaves of *Anethum graveolens***

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>DPPH</th>
<th>ABTS</th>
<th>Nitric oxide</th>
<th>Lipid peroxidation</th>
<th>Hydrogen peroxide</th>
<th>p-NDA</th>
<th>Deoxy ribose</th>
<th>Alkaline DMSO</th>
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<tbody>
<tr>
<td>Green</td>
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<tr>
<td>1000</td>
<td>43.60</td>
<td>78.30</td>
<td>55.52</td>
<td>54.47</td>
<td>71.68</td>
<td>43.13</td>
<td>36.20</td>
<td>8.10±0.83</td>
</tr>
<tr>
<td>500</td>
<td>19.83</td>
<td>63.50</td>
<td>42.71</td>
<td>40.86</td>
<td>51.90</td>
<td>40.86</td>
<td>15.01</td>
<td>11.50</td>
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<tr>
<td>250</td>
<td>9.60</td>
<td>36.63</td>
<td>29.60</td>
<td>26.50</td>
<td>42.17</td>
<td>36.53</td>
<td>12.00</td>
<td>9.10±1.13</td>
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<tr>
<td>Yellow</td>
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</tbody>
</table>

*Average of three determinations.

**Table 2— IC₅₀ values of ethanol extracts of green and yellow leaves of *Anethum graveolens* by *in vitro* antioxidant methods**

<table>
<thead>
<tr>
<th>Standards</th>
<th>DPPH ± SEM (µg/ml)</th>
<th>ABTS ± SEM (µg/ml)</th>
<th>Nitric oxide ± SEM (µg/ml)</th>
<th>Lipid peroxidation ± SEM (µg/ml)</th>
<th>Hydrogen peroxide ± SEM (µg/ml)</th>
<th>p-NDA ± SEM (µg/ml)</th>
<th>Deoxy ribose ± SEM (µg/ml)</th>
<th>Alkaline DMSO ± SEM (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>&gt;1000</td>
<td>796.66 ± 24.03</td>
<td>453.33 ± 8.88</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>Yellow</td>
<td>496.66 ± 6.66</td>
<td>820.00 ± 1.28</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Rutin</td>
<td>3.91 ± 0.01</td>
<td>-</td>
<td>65.44 ± 2.56</td>
<td>-</td>
<td>36.66 ± 0.22</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Ascorbic acid</td>
<td>11.25 ± 0.49</td>
<td>2.69 ± 0.05</td>
<td>-</td>
<td>187.33 ± 3.93</td>
<td>&gt;1000</td>
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<td>-</td>
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<tr>
<td>BHA</td>
<td>-</td>
<td>-</td>
<td>112.66 ± 1.32</td>
<td>-</td>
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<td>86.16 ± 4.04</td>
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</table>

*Average of three determinations, mean ± SEM
capacity was found to be 0.165 ± 0.04 and 0.148 ± 0.03 mm of ascorbic acid per gram of green and yellow leaves extracts, respectively.

Conclusion

The green leaves extract of A. graveolens was found to be more active in most of the methods tested. However, high antioxidant activity was not observed for the yellow leaves extract. This indicates that the phenolic compounds formed after maturation does not possess any antioxidant activity. More phenolic toxic compounds are known to form in plants after maturation of leaves and flowers. The present study supports the use of only green leaves as vegetable or flavouring green spice, which may be due to its antioxidant and other properties.

References