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ANTIOXIDANT AND PRELIMINARY PHYTOCHEMICAL STUDIES OF *ANETHUM GRAVEOLENS* LEAVES

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ABSTRACT

Plants being rich in polyphenols are an important source of natural antioxidants which can neutralize free radicals generated in the system responsible for causing oxidative stress during diabetes. The present study deals with phytochemical screening and in vitro assessment of antioxidant potential of aqueous leaves extract of *Anethum graveolens*. The phytochemical analysis of the extract revealed the presence of tannins, saponins, carbohydrates, glycosides, alkaloids, flavonoids and proteins. The total phenolics and flavonoids contents present in the *A. graveolens* leaves extract were 66.5 mg of Gallic Acid Equivalent (GAE)/g and 29.8 mg of Quercetin Equivalent (QE) /g respectively. Significant antioxidant efficacy of *A. graveolens* leaves was further confirmed by percent inhibition of $DPPH^{\cdot}$, $NO^{\cdot}$, O^{2-} radicals, OH radicals, $ABTS^{+}$ radicals and Metal Chelating efficacies as it was found to be 87.57, 68.29, 82.32, 74.02, 97.05 and 61.51% inhibition respectively. Therefore, the data suggest that the aqueous extract of *A. graveolens* leaves could be developed not only as an anti-aging agent but also as an agent for managing oxidative stress due to diabetic complications.

KEYWORDS: ***Anethum graveolens leaves, Phytochemicals, Antioxidant, Diabetic complications, Antiaging Agent, Polyphenols.***

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INTRODUCTION

Medicinal plants act as an indigenous source of phytochemicals possessing a therapeutic value which can be used in drug development. Phytochemicals as secondary metabolites are directly responsible for bioactivities of medicinal plants viz. antidiabetic, antioxidant, antimicrobial, anticancer and anti-inflammatory etc. Hence, bioactive secondary metabolites serve as important leads for the development of much cost-effective and less toxic pharmaceutical drugs and form the backbone of the modern system of medicine[1,2]. Thus, correlation between the phytochemicals and the bioactivity of the plant is desirable to know for developing the safe drugs with specific activities to treat various diseases[3]. Antioxidants are gaining much importance as anti-aging agents since they protect the human system from oxidative stress causing cell damage. Though, the production of free radicals in the human system helps in many cellular processes but sometimes due to over-production of these reactive oxygen species (ROS), resulting from an imbalance between their formation and neutralization causes oxidative stress. This failure of antioxidant defense system damages the cells and hence increases the risk of assaults of different diseases[4]. Both experimental and clinical studies suggest that oxidative stress induced by ROS also plays a major role in the pathogenesis of diabetes mellitus. Abnormally high levels of free radicals and the simultaneous decline of the antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, an increase of lipid peroxidation, and development of insulin resistance as well. These consequences of oxidative stress can promote the development of diabetic complications. Thus, the search for antioxidants from natural resources has paved the way for exploration of new compounds with better efficacy and lower toxicity. These are safe and easily accessible at lower cost in comparison to synthetic antioxidants. Natural antioxidants present in medicinal plants work mostly as free radical scavengers and therefore, are known to protect the system against ROS-mediated oxidative stress[5]. The use of various medicinal plants rich in antioxidant constituents has already been proposed by our research group as an effective therapeutic approach for controlling diabetes as well as diabetes-induced oxidative stress[6,7]. *Anethum graveolens L*. (Apiaceae), commonly known in English as 'dill' and in Hindi as 'Soya', is an annual herb found in Mediterranean region, Europe, and Central and South Asia. *A. graveolens* is used as an aromatic herb as well as medicine due to its high therapeutic value[8]. It is used in Iranian folk medicine as an anti-hypercholesterolaemic plant[9]. It has traditionally been used for gastrointestinal ailments[10]. It is well known for its pharmacological effects, such as anti-microbial[11-13], antioxidant[14], anti-spasmodic, antisecretory, and mucosal protective effects[10]. It also has significant lipid lowering effect being a promising cardioprotective agent[15]. Various different compounds have also been isolated from its seeds, leaves, inflorescence and fruits[16]. The presence of flavonoids, phenolic compounds and essential oil in the seeds of *A. graveolens* make it an important component for the preparation of gripe water[17]. Therefore, the present study deals with the evaluation of the bioactive potential of *A. graveolens* with special reference to antioxidant potential in addition to their phytochemical analysis.

MATERIAL AND METHODS

Plant Material

Fresh leaf of *Anethum graveolens* (500g) were collected from the local area of Allahabad-U.P. (India) and authenticated by Professor Satya Narayan, Taxonomist Department of Botany, University of Allahabad, India (MRL/AG/03). The authenticated fresh leaves were washed and dried under shade. The shade dried leaves (180g) were mechanically crushed, powdered and preserved until further use.

Chemicals

Folin-Ciocalteau reagent, Griess reagent, gallic acid, potassium ferricyanide, TCA (Tricarboxylic Acid), PMS (Phenazine Methosulphate), NADH (Nicotinamide Adenine Dinucleotide), quercetin, BHT (Butylated Hydroxy Toluene), ascorbic acid, sodium carbonate, $FeCl_3$, DPPH (1, 1-Diphenyl-2-picrylhydrazyl, H_2O_2, KNO_2, NaOH, MnO_2, $AlCl_3$, TPTZ (2, 4, 6-tripyridyl-*s*-triazine), $FeSO_4$, HCl, SNP (Sodium Nitroprusside), NBT (Nitroblue Tetrazolium), Ferrozine, sodium salicylate, acetic acid, sodium acetate were purchased from Merck Chemicals, New Delhi. All other chemicals and reagents were of analytical grade.

Preparation of Extract

The dried leaf powder (180g) was refluxed repeatedly thrice with double distilled water, for 2 hour each time and filtrates were collected, combined and concentrated in rotary evaporator at 45°C±5°C under reduced pressure, to obtain semisolid material, which was then lyophilized to get dark brown powder (yield ~15.5% w/w).

Phytochemical Analysis

Screening for Phytochemicals was carried out based on the following analytical tests[18-22]as given in Table 1.

Table-1
Analytical Tests for Phytoconstituents

Phytoconstituents	Colour Tests	Observation
Alkaloids *(Hager's Test)*	2ml extract + few drops of Hager's reagent	Yellow precipitate
(Wagner's Test)	2 ml extract+ 2 drops HCl (1.5%) + 3(reagent	Brown precipitate
Anthraquinones *(Borntrager's Test)*	3ml extract + 3ml Benzene + 5ml NH_3 (10%)	Pink, Violet or Red colouration
Coumarins	2ml extract + 3ml NaOH (10%)	Yellow colouration

Carbohydrate *(Molisch's Test)*	2ml extract (EtOH) + 10ml H_2O + 2 drops ethanolic α-naphthol (20%) + 2 ml conc.H_2SO_4	Reddish violet ring at the junction
Emodins	2ml extract + 2ml NH_4OH + 3ml Benzene	Red colouration
Flavonoids	1ml extract + 1ml $Pb(OAc)_4$ (10%)	Yellow precipitate
Glycosides *(Liebermann's Test)*	2ml extract + 2ml $CHCl_3$ + 2ml CH_3COOH	Violet to Blue to Green colouration
Phlobatannins *(Precipitate Test)*	2ml extract + 2ml HCl (1%) + boil	Red precipitate
Proteins *(Xanthoproteic test)*	1ml extract + 1ml conc. H_2SO_4	White precipitate turned Yellow on boiling
Saponins *(Foam Test)*	5ml extract + 5ml H_2O + heat	Froth appearance
Steroids *(Salkowski's Test)*	2ml extract + 2ml $CHCl_3$ + 2ml conc. H_2SO_4	Reddish Brown colouration at interface
Tannin *(Braymer's Test)*	2ml extract + 2ml H_2O + few drops of $FeCl_3$ (5%)	Green colouration
Terpenoids	2ml extract + EtOH+2ml $CHCl_3$+ ∆(2 mint.) 3 drops conc. H_2SO_4	Deep red colouration

Antioxidant Assays- in vitro

Antioxidant potential of *A. graveolens leaf extracts* (AGLE) was evaluated using different *in vitro* assays such as estimation of total phenolics, total flavonoids, and evaluation of free radical scavenging activity for $DPPH^{\cdot}$, $NO^{\cdot}$, superoxide and ABTS radicals. All the assays were carried out in triplicates and their average values were taken into consideration.

Estimation of Total Phenolics

Total phenolic contents were estimated by the modified Folin-Ciocalteau method[23]. An aliquot (100 µl) of a range of different concentration, from 100 to 500 µg/ml, of AGLE was mixed with 2.5 ml Folin-Ciocalteau reagent (diluted with water 1:10 v/v) and 2.0 ml (75 g/l) of sodium carbonate. The tube was vortexed for 15 s and allowed to stand in dark at room temperature. The absorbance was measured at 750 nm using Shimadzu UV-VIS spectrophotometer after half an hour. The content of phenolics in extracts was expressed in terms of mg of Gallic Acid Equivalents (GAE) per g of dried extract.

Estimation of Total Flavonoids

Total flavonoid contents were estimated using the method of Ordon et al. [24] Solutions of different concentrations from 100 to 500µg/ml of AGLE were prepared separately in methanol. A volume of 0.5 ml of 2% of $AlCl_3$ solution was added to 0.5 ml of different concentrations of each sample. The absorbance was measured at 420 nm after one hour. The content of flavonoids in extracts was expressed in terms of mg of Quercetin Equivalent (QE) per g of dried extract.

DPPH radical Scavenging assay

The effect of AGLE on DPPH radical scavenging was determined using the method of Liyana-Pathiranan and Shahidi.[25] About 1ml of sample in varied concentrations ranging from 100 to 500 µg/ml dissolved in deionised water was mixed with 1.0 ml of 0.135 mM DPPH. The mixture was vortexed thoroughly and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a control. Ascorbic acid (AA) was used as standard. The ability to scavenge DPPH radical was calculated by the following equation

$$\text{DPPH radical scavenging activity (\%)} = \{ A_C - A_S / Ac \} \times 100$$

where, A_C is the absorbance of control and A_S is the absorbance of sample.

ABTS radical Scavenging assay

The ability of AGLE, to scavenge ABTS Cation radical was determined by Re et al. [26] The ABTS+ radicals were pregenerated by mixing 7mM ABTS stock solution and 2.45 mM potassium persulfate. The solution was incubated for 12 hours in the dark at room temperature until the reaction was complete and the absorbance was stable. 1 ml of the above solution was mixed with 1 ml of varied concentration of sample ranging from 25 to125 µg/ml. After 6 minutes of incubation, absorbance was measured at 734 nm. Ascorbic acid (AA) was used as standard. The ability to scavenge ABTS+ radical was calculated by the following formula:

$$\text{ABTS}^{+} \text{ radical scavenging activity (\%)} = \{ A_C - A_S / Ac \} \times 100$$

where, A_C is the absorbance of Control and A_S is the absorbance of Sample.

Nitrosyl radical Scavenging assay

Scavenging of nitrosyl radical by AGLE was determined by the method of Green et al. [27] Reaction started by incubating 0.5 ml SNP solution (10mM in Phosphate Buffered Saline) with varied concentration ranging from 100 to 500 µg/ml of sample at 25°C. After 150 minutes, 0.25 ml of sulphanilamide and 0.25 ml of NED were added. The mixture was allowed to stand for 30 minutes. Ascorbic acid (AA) was used as standard. The absorbance of sample was measured at 540 nm. The ability of sample to scavenge NO radical was calculated by the following formula:

$$\text{NO radical scavenging activity (\%)} = \{A_C - A_S / Ac\} \times 100$$

where, A_C is the absorbance of control and A_S is the absorbance of Sample.

Hydroxyl radical Scavenging assay

Hydroxyl radical scavenging activity of AGLE was measured according to a modified method of Smirnoff and Cumbes. [28] The OH radicals were generated from the mixture of $FeSO_4$ and H_2O_2. The reaction mixture contained 1 ml $FeSO_4$ (1.5mM), 0.7ml H_2O_2 (6mM), 0.3 ml sodium salicylate (20mM) and 1ml of varied concentrations of sample ranging from 100 to 500µg/ml. After incubation, for 1 h at 37°C, the absorbance of the complex was measured at 562 nm against control. Ascorbic acid (AA) was used as standard. The ability to scavenge OH radical was calculated by the following equation:

$$\text{OH radical scavenging activity (\%)} = [1-\{A_S - A_B / Ac\}] \times 100$$

where, A_C is the absorbance of control and A_S is the sample, A_B is the absorbance of blank (without sodium salicylate).

Superoxide Anion radical Scavenging assay

Superoxide anion radicals were generated by the PMS/ NADH system according to the method of Kakkar et al.[29]. The reaction mixture was composed of 1 ml of NBT (156 µM), 1 ml NADH (468 µM) and 100 µl of AGLE ranging from 20- 100 ug/ml. The reaction was started by addition of 100 µl of PMS (60 µM) to the mixture. After 5 min incubation at 25ºC, absorbance was measured at 560 nm. Ascorbic acid (AA) was used as standard. The ability to scavenge O^{2-} radical was calculated by the following equation:

$$O^{2-} \text{ radical scavenging activity (\%)} = \{A_C - A_S / Ac\} \times 100$$

where, A_C is the absorbance of control and A_S is the absorbance of sample.

Metal Chelating Assay

The chelation of ferrous ions by AGLE was determined by the method of Dinis et al.[30] A solution of 2mM $FeCl_2$ (0.05 ml) was added to varied concentration from 100 to 500µg/ml of sample. The reaction was initiated by the addition of 5mM ferrozine (0.2 ml) and the mixture was shaken vigorously and kept at room temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm against control. Ascorbic acid (AA) was used as standard. The ability to chelate ferrous ions was calculated by the following equation

$$\text{Metal chelating activity (\%)} = \{A_C - A_S / Ac\} \times 100$$

where, A_C is the absorbance of control and A_S is the absorbance of Sample.

STATISTICAL ANALYSIS

Data was statistically evaluated using two-way ANOVA, followed by a post hoc Scheffe's test using the statistical package PRISM 3.0 version. The values were considered significant when $P<0.05$.

RESULTS AND DISCUSSIONS

Phytochemical Screening

Table 2, reveals the results of phytochemical screening of *Anethum graveolens* leaf which indicates the presence of high amount of Carbohydrates, glycosides flavonoids and tannins. Though, alkaloids, proteins and saponins were also present but in lesser extent. On the contrary phytochemical screening also revealed the complete absence of terpenoids, coumarins, anthocyanins, anthraquinones, emodins, leucoanthocyanins, phlobatanins, and steroids. Thus the medicinal values of the plant leaves may be due to these specific classes of phytochemicals present in it. Plants containing carbohydrates and glycosides are known to exert a beneficial action on immune system by increasing body strength and hence are valuable as dietary supplements[42]. Glycosides also have vast therapeutic efficacy as they are found in almost every medicinal plant. Flavonoids and other phenolics compounds also acts as a potent water-soluble antioxidant and free radical scavenger, which prevent oxidative cell damage[31-33]. It also helps in managing diabetes induced oxidative stress[43]. Tannins are known to be useful in the treatment of inflamed or ulcerated tissues and they have remarkable anticancer property[44]. Moreover, alkaloids represent a class which affects the central nervous system, reduces appetite and behaves as diuretic[45]. Proteins are the building blocks of life. The body needs protein to repair and maintain itself. Numerous studies have confirmed that saponins possess the unique property of precipitating and coagulating red blood cells[34,35]. Thus, the AGLE has a range of phytochemical compounds having therapeutic efficacy with special reference of antioxidant which can effectively protect from oxidative damage caused by free radicals.

Table 2
Phytochemical Screening of Aqueous Extract Of *A. Graveolens* Leaf

Phytoconstiuents Tested	Results Observed
Tannins	++
Carbohydrates	++
Glycosides	++
Flavonoids	++
Alkaloids (Wagner's Test); (Hager's Test)	+ +
Proteins	+
Saponins	+
Terpenoids	-
Steroids	-
Phlobatannins	-
Coumarins	-
Anthraquinones	-
Anthocyanins	-
Leucoanthocyanins	-
Emodins	-

(++) = Significant Presence, (+) = Presence, (-) = Absence

Antioxidant assay - in vitro
Total Phenolics

Table 3, shows the results of total phenolic content of *A. graveolens* leaf extracts expressed as Gallic acid Equivalents (GAE). Standard curve equation of Gallic acid was calculated with y = 0.0002x + 0.029, R^2 = 0.995. Total phenolic content was estimated 65.5 mg/g gallic acid equivalent. Thus, *A. graveolens* leaves were found to be a potential source of phenolics and hence could be explored as natural antioxidant. Phenolics are the well-known group of secondary metabolites and comprise a large group of biologically active compounds[36]. The antioxidant activities of phenols were credited to their redox properties, which permit them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, as well as their metal chelating abilities.

Total Flavonoids

Table 3, also shows the results of total flavonoid content of *A. graveolens* leaf extracts, expressed as Quercetin Equivalent (QE). Standard curve equation of Quercetin was calculated with with y= 0.0536x – 0.0038, R^2= 0.968. Total flavonoid content was estimated 29.8mg/g quercetin equivalent. Flavonoids are important secondary metabolites present in plants which modulate lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It is well established that pharmacological effect of flavonoids is associated with antioxidant activities[37].

Table 3
Quantitative Estimation Of Total Phenolics And Flavonoids Of *A. Graveolens Leaf*

Total Phenolic contents (mg/g of GAE)	Total Flavonoid contents (mg/g of QE)
65.5±1.23	29.8± 0.32

Free Radical Scavenging Activity
DPPH Radical

Fig. 1 clearly reveals the results of scavenging activity of AGLE and AA was found to exhibit 87.57 and 96.47% inhibition, respectively at the highest evaluated concentration of 400µg/ml. Moreover, Fig. 7 gives an account of IC_{50} values of AGLE and AA which were found to be 122.4 and 100.24µg/ml, respectively. The data validates the primary antioxidant nature of *A. graveolens* leaves which could be due to hydrogen donating ability of its phenolic constituents.

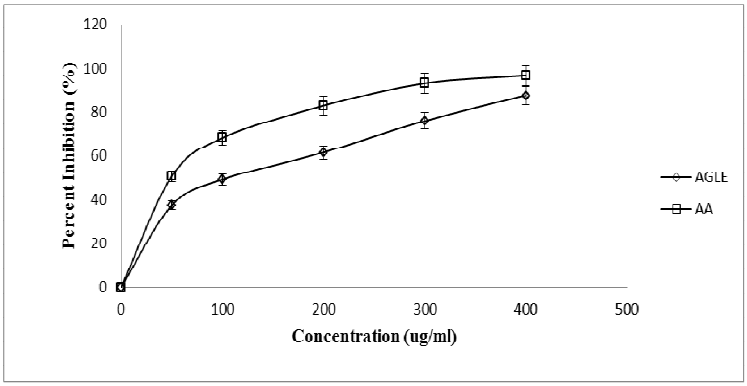

Figure 1
DPPH radical scavenging activity of *A. graveolens* leaf extract and ascorbic acid

Nitric oxide Radical Scavenging

Fig. 2 show the results of NO radicals scavenging activity of AGLE and AA was found to exhibit 68.29 and 83.43% inhibition, respectively at the highest evaluated concentration of 400µg/ml. Moreover, Fig. 7 gives an account of IC_{50} values of AGLE and AA which were found to be 244.3 and 114.61 µg/ml, respectively. The data is confirming *A. graveolens leaf* could be considered as better antioxidant due to their $NO^{\bullet}$ scavenging potential, which is widely used as a measure of antioxidant activity of plant extract. Nitric oxide radical ($NO^{\bullet}$) is known to be a ubiquitous free radical distributed in tissues or organ systems and have a vital role in neuromodulation or neurotransmitter in the central nervous system. In this assay, SNP gets decomposed in the aqueous solution at physiological pH 7.4 and produced NO radical. This NO radical reacts with oxygen to produce nitrate and nitrite, which was determined by using Griess reagent[38].

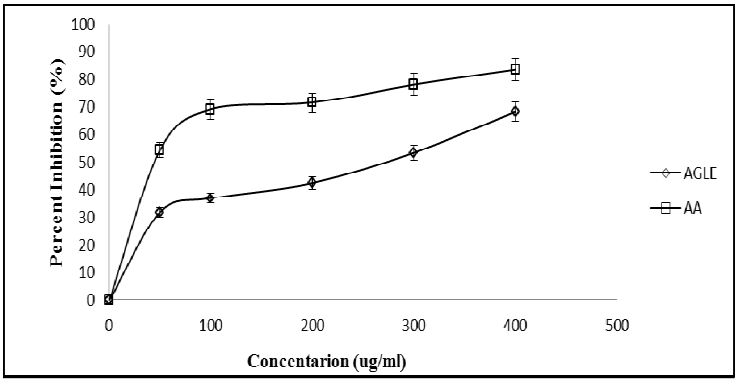

Figure 2
NO radical Scavenging activity of *A. graveolens* leaf extract and ascorbic acid

Superoxide Radical

Fig.3, depicts the results of Superoxide anion radical scavenging activity of both, the Extract and the Standard at varied range of concentration. Results depicts that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 400µg/ml with inhibition of 82.32% in case of AGLE and 92.46% in case of Standard, validating thereby the significant antioxidant potential of *A. graveolens* leaf extract. The IC_{50} values of the Extract and the Standard were found to be 173µg/ml and 117.22µg/ml respectively, which further confirmed that *A. graveolens* leaves could be developed as an effective antioxidant agent.

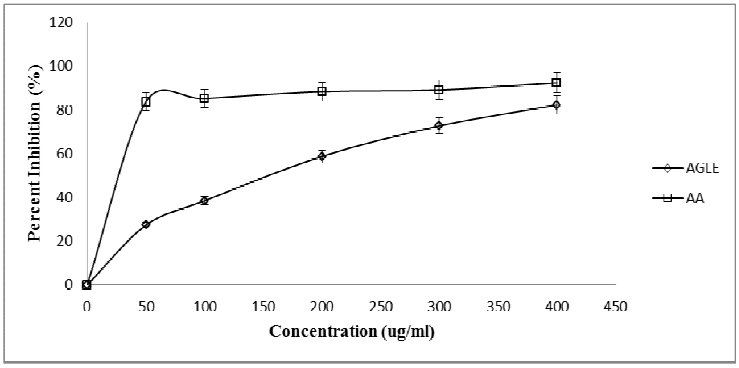

Figure 3
Superoxide radical scavenging activity of *A. graveolens* leaf extracts and ascorbic acid

ABTS cation Radical

Fig.4, exhibits the results of ABTS cation radical scavenging activity of both, the Extract and the Standard at varied range of concentration. Results depicts that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 150µg/ml with inhibition of 97.05% in case of AGLE and 99.12% in case of Standard, validating thereby the significant antioxidant potential of *A. graveolens* leaf extract. The IC_{50} values of the Extract and the Standard were found to be 33.45µg/ml and 66.61µg/ml respectively, which further confirmed that *A. graveolens* leaves could be developed as an effective antioxidant agent. The data shows appreciably higher scavenging activity of *A. graveolens* leaf extract against ABTS+• radical. This implies that the extract may be useful for treating radical related pathological damage. Proton radical scavenging is an important attribute of antioxidants. It is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The stability of ABTS+• for more than 2 days over a wide pH range raised the interest in the use of ABTS+• for the estimation of antioxidant activity.

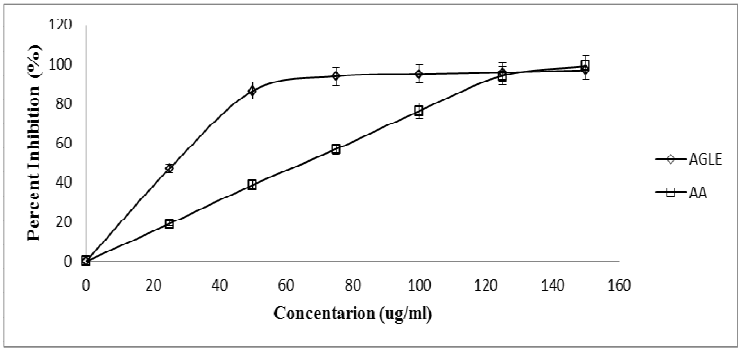

Figure 4
ABTS radical scavenging activity of *A. graveolens* leaf extracts and ascorbic acid

Hydroxyl Radical

Fig.5, exhibits the results of hydroxyl radical scavenging activity of both, the Extract and the Standard at varied range of concentration. Results depicts that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 400µg/ml with inhibition of 74.02% in case of AGLE and 92.22% in case of Standard, validating thereby the significant antioxidant potential of *A. graveolens* leaf extract. The IC_{50} values of the Extract and the Standard were found to be 116.42µg/ml and 20.54 µg/ml respectively, which further confirmed that *A. graveolens* leaf could be developed as an effective antioxidant agent. Hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and well known for initiating cell damage in vivo[39] and causing lipid peroxidation[40]. The evaluation of radical scavenging activity was based on the generation of OH radical by Fenton reaction[46].

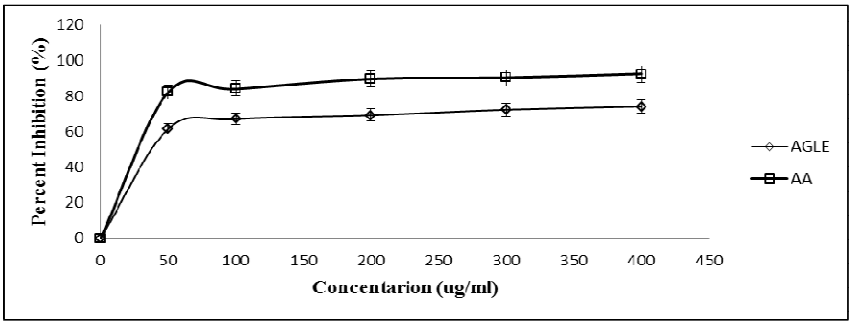

Figure 5
Hydroxyl radical scavenging activity of A. graveolens leaf extract and ascorbic acid

Metal Chelating

Fig.6, depicts the results of metal chelating activity of both, the Extract and the Standard at varied range of concentration. Results depicts that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 400µg/ml with inhibition of 61.51% in case of AGLE and 85.18% in case of Standard, validating thereby the significant antioxidant potential of *A. graveolens* leaf extract. The IC_{50} values of the Extract and the Standard were found to be 267.89µg/ml and 51.30µg/ml respectively, which further confirmed that *A. graveolens* leaves could be developed as an effective antioxidant agent. Metal chelating assay is also a simple yet an important assay as ferrozine can quantitatively form complexes with Fe^{2+} producing a intense violet Fe^{2+}/ferrozine complex. The chelating activity of antioxidant was measured by monitoring the decrease in the intensity of violet colour of the complex[41]. Chelating agents may inhibit lipid oxidation by stabilising transition metals. In this assay, the ability of antioxidants to disrupt the formation of the complexes or to prevent interaction between metal and lipid was measured in terms of their iron-chelating capacity.

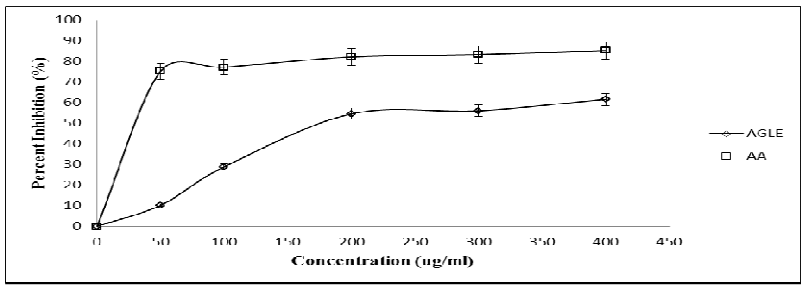

Figure 6
Metal Chelating activity of A. graveolens leaf extract and ascorbic acid

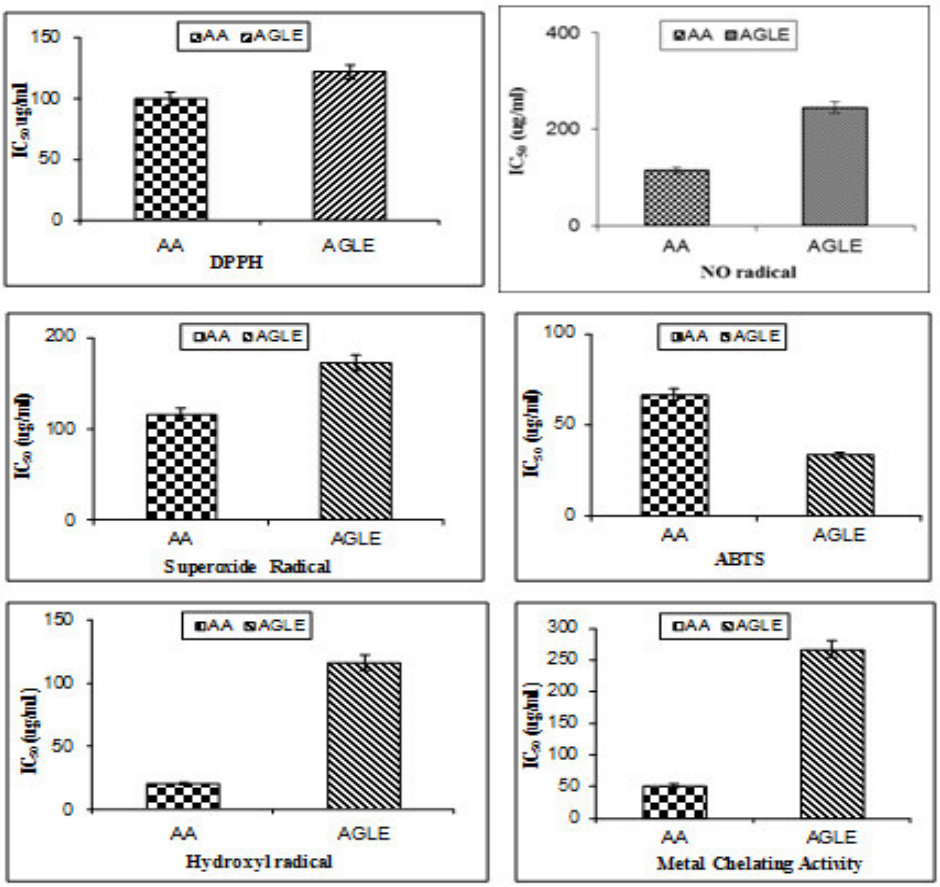

***Figure* 7**
***Free radicals Scavenging potential and Metal chelating activity of AGLE and AA in terms of IC_{50}* (each value represents a mean ± SD (n=3)**

CONCLUSION

Conclusively it could be stated that *A. graveolens* leaf can be explored further in order to develop a novel antioxidant agent with high therapeutic efficacy due to high content of their polyphenolics including tannins, carbohydrates and glycosides in addition to flavanoids. The highly significant antioxidant efficacy of *A. graveolens* leaf was also evident from its high free radical scavenging potential and low IC_{50} values in comparison to the standard drug, ascorbic acid, taken as reference.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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