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In vitro angiotensin converting enzyme inhibitory and antioxidant activities of seed extract of *Apium graveolens* Linn.

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ABSTRACT

The present study was carried out to investigate the in vitro angiotensin converting enzyme (ACE) inhibitory and antioxidant activities of the methanolic seed extract of Apium graveolens Linn. belonging to the family Apiaceae. Traditionally the seeds of A. graveolens L. were used as diuretic, anti-inflammatory and aphrodisiac. The ability to inhibit ACE was evaluated by quantifying the decrease in N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) peak area at 345 nm using reverse phase high performance liquid chromatography (RP-HPLC). The antioxidant activities of the extracts were determined using various in vitro assays. The ACE inhibitory potency of the seed extract (IC_{50} = 666.26 ± 1.32µg/ml) was found to be significant ($P<0.01$) when compared with the standard lisinopril (IC_{50} = 0.19 ± 0.02ng/ml). The extract showed a strong antioxidant activity when compared with the standard. The results of this study suggest that the methanolic seed extract of A. graveolens has significant antioxidant and ACE inhibitory effect.

Keywords: Angiotensin converting enzyme, Apium graveolens, Cardiovascular diseases, Free radicals.

INTRODUCTION

The renin-angiotensin system participates extensively in the pathophysiology of myocardial infarction, diabetic nephropathy and congestive heart failure. This realization has led to a thorough exploration of the renin-angiotensin system and the development of new ways for inhibiting its actions [1]. Angiotensin converting enzyme (ACE), a crucial enzyme in the regulation of the renin angiotensin system, is a zinc-containing peptidyl dipeptide hydrolase whose active site consists of three parts: a carboxylate binding moiety such as the guanidinium group of arginine, a pocket that accommodates a lipophilic side chain of amino acid residues at C-terminal and a Zn ion. The Zn ion binds to the carbonyl group of the penultimate peptide bond

of the substrate, thereby making the carbonyl group polarized and subjecting it to a nucleophilic attack [2].
Currently available ACE inhibitors are synthetic pharmacological drugs and their use in healthy or low-risk populations is not advisable because of their adverse effects like dry cough, skin rashes and angioneuroticedema [3]. Certain compounds derived from plant extracts like hydrolysable tannins [4], phenylpropanes [5], proanthocyanidins [6], flavonoids [7], terpenoids [8] and peptide amino acids [9] have been reported for their in vitro ACE inhibitory activity. These findings opens up the possibility of finding newer plant derived compounds which mimic synthetic ACE inhibitors and provide health benefits without any adverse side effects.

The reactive oxygen species (ROS) include free radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydroperoxyl ($HOO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and non-radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($O_2^{\bullet}$). Similarly, reactive nitrogen species (RNS) include nitric oxide ($NO^{\bullet}$), peroxy nitrite ($ONOO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3). Oxidative stress is a condition in which there is an increased production of oxygen species and diminished levels of antioxidant system resulting in cell damage leading to the pathogenesis of a variety of diseases. Antioxidants prevent the oxidative reactions that occur naturally in tissues by scavenging the free radicals, chelating metal ions and acting as electron donors [10].

The oxidative stress in cardiac and vascular myocytes caused by ROS has been noted to induce cardiovascular tissue injury and to sustain homeostasis of the vascular wall, a balance between the endogenous transmitter's angiotensin II, nitric oxide, and ROS is of great value. It has been clearly noted that hypertension caused by chronically increased levels of angiotensin II is mediated in part by superoxide anions [11]. The cardiovascular diseases caused by increased levels of angiotensin II are found to be mediated by vasoconstriction and thus decreased concentration of vascular nitric oxide seems to promote the angiotensin II dependent cardiovascular diseases [12].

Apium graveolens Linn. (Apiaceae), basically known as celery is commonly available throughout the world. Seeds of celery are used traditionally as a diuretic, aphrodisiac and tonic [13]. Literatures suggest that seeds of A. graveolens L. possess hepatoprotective [14, 15] and anti-microbial [16] activity. However, no such report is available in the literature to our knowledge regarding in vitro ACE inhibitory and antioxidant activities.

Hence, the main objective of the present study was to evaluate the ACE inhibitory and antioxidant activities of the methanolic seed extract of A. graveolens using in vitro models.

MATERIALS AND METHODS

Plant material

The seeds of A. graveolens were collected from Nilgiris district, Tamilnadu, India during the month of June 2010. The plant was identified and authenticated by Mr. G.V.S. Murthy, Joint Director Scientist, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference no BSI/SC/5/08-09/Tech-730.

Extraction

The seeds of A. graveolens L. were air dried, powdered mechanically, sieved through No. 20 mesh sieve and defatted with petroleum ether (60º-80ºC) for 30 minutes. About 100g of defatted powdered seeds was extracted with 500 ml of methanol using a Soxhlet apparatus for 48 hrs.

After extraction, the methanolic extract was concentrated at 60°C [14]. The percentage yield of methanolic seed extract was 9.8% w/w.

Chemicals and instruments

Angiotensin converting enzyme from rabbit lung (EC.3.4.15.1), Furnacryloyl-l-phenylalanylglycylglycine (FAPGG) and Lisinopril wer purchased from Sigma Aldrich, USA. 2-deoxy-2-ribose, (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethane sulphonic acid (HEPES) buffer, pyrocatechol and quercetin were purchased from Himedia Labs., Pvt. Ltd, Mumbai, India. All other drugs and chemicals used in the study were purchased commercially and were of analytical grade. HPLC instrument with L-4000 UV detector, L-6200 Intelligent pump and LiChrosorb RP-18 column (50 × 7 mm) from HITACHI with Data Ace workstation was used for the analytical studies (HITACHI Ltd, Tokyo, Japan).

Phytochemical screening of the extract

Chemical tests were carried out for the methanolic seed extract of A. graveolens for the presence of phytochemical constituents like phenols, tannins, saponins, flavonoids, terpenoids, alkaloids, glycosides and steroids [17].

Angiotensin converting enzyme inhibition assay

The angiotensin converting enzyme inhibitory activity was carried out by using n-furnacryloyl-l-phenylalanylglycylglycine (FAPGG) as the substrate [18]. The extract and the standard drug Lisinopril (1 mg/ml) were prepared by dissolving in reaction buffer (HEPES 25mM, NaCl 293mM, pH 8.3). The assay mixture (750µl) consisting of 530µl of FAPGG (3mM in reaction buffer) and 200 µl of extract at different concentrations (100-800 µg/ml) was incubated for 3 min at 37°C. The reaction was initiated by adding 20 µl of ACE solution (0.05U/ml) to the test reaction and the samples were incubated for one hour at 37ºC. The reaction was then stopped by adding 80 µl of 5% trifluoroacetic acid solution and samples were centrifuged at 9000 rpm for 5 minutes at room temperature. The enzymatic activity was calculated by quantifying the decrease in FAPGG concentration by recording the decrease in absorbance at 345 nm using RP-18 column (50 mm × 7 mm, 3 µm pore size) with isocratic elution using acetonitrile and 1.1% trifluoroacetic acid in Milli-Q in the ratio of 75:25 v/v; it was filtered through 0.45 µL filter (Sartorius, Germany) and using an ultrasonic bath was degassed before use. The column temperature was ambient and the total running time was 10 min using a flow rate of 1.5 ml/min with retention time of 2.7 min for FAPGG, the injection volume was 20µl and the detection wavelength was 345 nm. Percentage enzyme inhibition was calculated by comparing the enzymatic activity with, and without inhibitor using the following formula,

$$\%\text{ACE inhibition} = (1-a) \times 100$$

where a is the activity with inhibitor and activity without inhibitor [2, 19].

Antioxidant activity

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extract is determined by its ability to scavenge the hydroxyl radicals produced by the EDTA-Fe^{3+}-H_2O_2-ascorbic acid system by the Fenton reaction [20]. The reaction mixture consists a final volume of 1.0 ml which contains 100 µl of 2-deoxy2-ribose (28 mM) in phosphate buffer solution (20 mM, pH 7.4), 500 µl of the extract at various concentrations (5-80 µg/ml) in buffer solution, 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1v/v), 100 µl of H_2O_2 (1.0 mM) and 100 µl of ascorbic acid (1.0 mM). Test samples were incubated at 37°C for 1 h. The free radical damage on the substrate, deoxyribose was assessed

with the thiobarbituric acid test. The positive control used for this assay was quercetin (5-80 µg/ml). The percentage inhibition of the extract and standard was calculated [21].

Superoxide radical scavenging activity

Superoxide radical generation using the phenazine methosulfate(PMS)-b-nicotinamide adenine dinucleotide (NADH) system. The superoxide radicals are generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 78 mM NADH, 50 mM NBT, 10 mM PMS and samples to be tested at different concentrations (25-400 µg/ml). The color reaction between superoxide radicals and NBT was detected at 560 nm using a UV-spectrophotometer and the percentage inhibition calculated. Ascorbic acid was used as positive control [22].

Nitric oxide radical scavenging activity

Various concentrations of the extracts (50-800 µg/ml) and sodium nitroprusside (5mM) in phosphate buffer saline (0.025 M, pH 7.4) in a total volume of 3 ml was incubated at room temperature for a period of 150 min. After which, 0.5 ml of the incubated solution and 0.5 ml Griess' reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) were added togather and allowed to react for 30 min. Control samples without the test compounds but with equal volume of buffer was prepared in a similar manner as done for the test. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and successive coupling with naphthyethylene diamine dihydrochloride was measured at 546 nm. The experiment was carried out using curcumin (50-800 µg/ml) as positive control [23]. The percentage inhibition of the extract and standard was calculated.

Ferrous chelating ability

In the Fe^{2+} chelating assay, Fe^{2+} level in the assay mixture is determined by measuring the formation of the Fe^{2+}-ferrozine complex. The reaction mixture containing various concentrations (50-800 µg/ml) of the extract was added to 2mM ferric chloride (0.1 ml) and 5mM ferrozine (0.2 ml) to begin the reaction and the resulting mixture was shaken and left to stand for 10 min at 25º C. The absorbance of the assay solution was measured at 562nm. The experiment was carried out using ascorbic acid (50-800µg/ml) as positive control. The percentage chelating effect of ferrozine-Fe^{2+} complex formation was calculated [24].

Reducing power ability

Reducing power ability was measured by mixing 1 ml extract of various concentrations (50-800 µg/ml) prepared with distilled water to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 30 min. Next an aliquot of 2.5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged for 10 min at 3000 rpm, 2.5 ml from the upper part was diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1% ferric chloride. The absorbance was measured at 700 nm. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. Butylated hydroxyltoluene (BHT) was used as positive control [25].

Estimation of total phenolic content

Total phenolics present in the extract can be estimated using folin-ciocalteu reagent and pyrocatechol as the standard. An aliquot of 1 ml of extract solution in a test tube was added to 0.2 ml of Folin-Ciocalteu reagent (1:2 in distilled water) and, after 20 min, 2 ml of purified water and 1 ml of sodium carbonate (15%) was added. After 30 min, the absorbance was measured at

765 nm. The total phenolic compounds present in the extract were determined as µg pyrocatechol equivalent (PCE) with the use of the standard pyrocatechol graph [26].

Estimation of total flavonoid content

Total flavonoids present in the extract can be determined by treating it with aluminium nitrate and then comparing it to that of the standard quercetin. One mg of the extract was first added to 1ml of ethanol (80%) and from which 0.5 ml was added to the test tubes containing 0.1 ml of aluminium nitrate (10%), 0.1 ml of 1 M potassium acetate and 4.3 ml of ethanol (80%). After a period of 40 min the absorbance of the supernatant solution was measured at 415 nm using UV spectrophotometer [27].

Calculation of 50% inhibitory concentration (IC_{50})

The concentration (µg/mL) of the extract required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extracts. Percentage inhibition (I%) was calculated using the formula:

$I\% = A_c - A_t / A_c \times 100$

where A_c is the absorbance of the control and A_t is the absorbance of the sample.

Statistical analysis

All the experiments were carried out in triplicate and results expressed as mean ± SEM. Significant differences among means of samples were evaluated by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Phytochemical screening of the extract

Phytochemical screening showed the presence of tannins, phenolics, flavonoids glycosides and steroids in the extract.

Table 1. In vitro ACE inhibitory activity of ASME and standard

Extract / Standard	Concentration	% Inhibition	IC_{50} µg/ml
ASME (µg/ml)	100	11.10 ± 0.38	666.26 ± 1.32*
	200	24.90 ± 0.33	
	400	46.10 ± 0.49	
	800	52.15 ± 0.54	
Lisinopril (ng/ml)	0.1	43.12 ± 0.23	0.19 ± 0.02
	0.2	50.38 ± 0.32	
	0.4	77.92 ± 0.45	
	0.8	81.98 ± 0.56	

ASME- Apium graveolens seed methanolic extract
Values are expressed as mean ± SEM of three parallel measurements.
**P < 0.01 when compared with standard.*

Angiotensin converting enzyme inhibition assay

The ACE inhibitory activity of Apium graveolens L. seeds were represented as percentage ACE inhibition by the extracts. The seeds of A. graveolens demonstrated ACE inhibitory activity at a concentration of 800µg/ml, showing an inhibition greater than 50%. The IC_{50} value seed extract was 666.26 ± 1.32µg/ml and that of standard, lisinopril , was 0.19 ± 0.02ng/ml (Table 1). The extract inhibited ACE in a concentration dependent manner (Table 1). Flavonoids are a group of polyphenolics compounds, which have been reported to possess ACE inhibitory activity [7] and the activity of flavonoids and other polyphenols may be due to the formation of chelate complexes with the zinc atom within the active centre of zinc-dependent metallopeptidases or

possibly by the formation of hydrogen bridges between the inhibitor and amino acids near at the active site [2]. Thus, the presence of phenolic and flavonoid content in the extract would have contributed towards ACE inhibition.

Antioxidant assays

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by assessing the inhibition of deoxyribose degradation by hydroxyl radicals produced by Fenton reaction [20]. The extract and the standard (quercetin) inhibited the formation of hydroxyl radical. The IC_{50} value of the seed extract was 33.90 ± 0.24µg/ml and the standard, quercetin, was 16.90 ± 0.18µg/ml (Table 2). The hydroxyl radical is an exceedingly reactive free radical which has been implicated as an extremely damaging species of ROS in free radical pathology, capable of damaging almost all molecules present in living cells. It can join nucleotides in DNA as well as cause strand breakage which ultimately lead to carcinogenesis, mutagenesis and cytotoxicity. The hydroxyl radical scavenging capacity of an extract gives a direct relation to its antioxidant activity. The ability of the extract to inhibit hydroxyl radical mediated deoxyribose damage was determined by means of the Fe^{2+}-dependent deoxyribose assay. Malondialdehyde, a degradation product of deoxyribose produces a pink chromogen with thiobarbituric acid [21]. The plant extract scavenged the hydroxyl radicals present in the reaction mixture and the degradation of deoxyribose was prevented.

Table 2. In vitro antioxidant activity of ASME and standard

Extract / Standard	IC_{50} µg/ml			
	$OH^{\cdot}$	$O_2^{\cdot-}$	NO	Fe^{2+} chelating
RSEE	33.90 ± 0.24*	171.42 ± 0.14*	533.33 ± 0.23*	458.66 ± 0.36*
Quercetin	16.90 ± 0.18	-	-	-
Ascorbic acid	-	61.71 ± 0.85	-	173.42 ± 0.15
Curcumin	-	-	89.28 ± 0.14	-

ASME- Apium graveolens seed methanolic extract
Values are expressed as mean ± SEM of three parallel measurements.
**P < 0.01 when compared with standard.*

Superoxide radical scavenging activity

The superoxide anion radical scavenging activity of the extract was assayed by the PMS-NADH system where the system produces superoxide radicals which reduce the NBT to form a chromophore (diformazan) that absorbs at 560 nm [22]. The seed extract was found to be an efficient scavenger of superoxide anion radical generated from PMS-NADH system in vitro and the activity was comparable to that of positive control, ascorbic acid. Inhibition of NBT reduction by superoxide in the presence of the test preparation increased with increasing concentrations. The IC_{50} value of seed extract was 171.42±0.14µg/ml and that of standard, ascorbic acid, was 61.71±0.85µg/ml (Table 2). The decrease of absorbance with seed extract indicates the consumption of superoxide anion radicals in the reaction mixture and thus its antioxidant activity.

Nitric oxide radical scavenging assay

The extract exhibited a concentration-dependent scavenging effect on the nitric oxide radicals and effectively reduced the generation of nitric oxide radicals. The IC_{50} value of the seed extract was 533.33 ± 0.23µg/ml and that of the standard curcumin was 171.42 ± 0.15µg/ml (Table 2). NO is a potent pleiotropic agent involved in various physiological processes like smooth muscle relaxation, inhibition of platelet aggregation and regulation of cell mediated toxicity. However, over production of NO manifest in various pathological conditions basically by formation of peroxynitrites. In the nitric oxide scavenging assay, sodium nitroprusside upon interaction with the oxygen present in the saline buffer solution produce nitrite ions that is estimated by Griess

reagent [23]. The plant extract was found to decrease the quantity of nitrite ions in vitro which can be attributed to the antioxidant constituents present in the extract.

Ferrous chealating ability

The formation of the Fe^{2+} - ferrozine complex was interrupted in the presence of extract, indicating that the extract has the ability to chelate the iron. The absorbance of the extracts decreased with increasing concentration (from 50 to 800µg). The IC_{50} value of the seed extract was 458.66 ± 0.36µg/ml and that of the standard, curcumin was 89.28 ± 0.14 µg/ml (Table 2). Ferrozine on reaction with ferrous ions develops a red coloured complex and in the existence of an agent that can chelate, the complex formation is interrupted and thus formation of complex is hindered. Thus metal chelating ability of the extracts was denoted by the extent of ferrous-ion-ferrozine complex formation. It was reported that the chelating agents that can form σ bond with a metal, are most efficient as secondary antioxidants because they decrease the redox potential and thus steady the oxidized form of the metal ion [28]. The current study shows that the extract has iron binding ability and thus signifying its antioxidant activity.

Reducing power ability

Table 3 shows the reductive capabilities of the methanolic seed extract when compared with the standard, BHT. The reducing power increased with increasing concentration of the extract. However, the activity of the extract was lesser than that of the standard. The reducing power ability of the plant extract was found out by measuring the transformation of Fe^{3+} into Fe^{2+}. The reducing power ability of a compound usually depends on the existence of reductones, which mainly act by breaking the free radical chain reaction by donating a proton [29].

Table 3. Reducing power ability of ASME and standard

Extract / Standard	Concentration µg/ml	Absorbance nm
ASME	50	0.0279 ± 0.0018
	100	0.0946 ± 0.0014
	200	0.1440 ± 0.0027
	400	0.1971 ± 0.0014
	800	0.2524 ± 0.0020
BHT	50	0.6842 ± 0.013
	100	0.7933 ± 0.005
	200	0.8865 ± 0.005
	400	0.9536 ± 0.014
	800	1.0653 ± 0.024

ASME- Apium graveolens seed methanolic extract

Values are expressed as mean ± SEM of three parallel measurements.

The antioxidant principles present in the plant extracts caused the reduction of ferric complex into its ferrous form, and thus accounting for the reducing power ability.

Total phenolic content

Total phenolic assay using folin-ciocalteu reagent is an easy, suitable and reproducible method and the phenolic content is calculated from the standard pyrocatechol graph and expressed as µg pyrocatechol equivalents (PCE). It is employed regularly in studying phenolic antioxidants. The seed extract was found to contain 13.44 ± 0.052µg PCE/mg of phenolic compounds. Phenolics are secondary metabolite in plant kingdom found in great abundance. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator [30]. It has been reported that the antioxidant activity of phenol is principally due to their redox potentials, hydrogen donors and singlet oxygen quenchers.

Total flavonoid content

The total flavonoid content in the extract was determined as µg quercetin equivalent (QE) by means of the standard quercetin graph. The total flavonoids content in the seed extract was found to be 4.08 ± 0.123µg QE/mg. Flavonoids are water soluble pigments present in the cytosol or stored in the vacuole of the plant cell and represent the largest group of phenolic compounds in plants. They interact with the cardiovascular system in several ways like, reducing reactive oxygen species, increasing nitric oxide concentration and also by inhibiting ACE mainly by binding to the Zn^{2+} at the active site. Flavonoids due to the presence of their phenolic hydroxyl groups are highly capable of scavenging ROS and are known to be potent antioxidants [27].

CONCLUSION

The current study has demonstrated that the seed extract of Apium graveolens is capable of inhibiting angiotensin converting enzyme, quenching free radicals and acting as reducing and chelating agents. Further in vivo studies are necessary to identify a potential chemical entity for clinical use in the treatment of hypertension and other related cardiovascular disorders.

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