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Research Article

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Evaluation of *in vitro* antioxidant potential of methanolic leaf and stem extracts of S*olena amplexicaulis* (Lam.) Gandhi.

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ABSTRACT

The antioxidant capability and total phenolic and flavonoid contents of methanol extract from leaves and stem of S.amplexicaulis (Lam.) Gandhi. were evaluated. Among the both extracts studied, leaf extract gave higher values of total phenolics (130.17µg GE/10mg), flavonoids (235.67µg QE/2mg), DPPH$^{\bullet}$ Scavenging activity (92.51% at 250µg/ml), ABTS$^{\bullet+}$ radical scavenging activity (3223.11µmol TE/g) and reducing power assay (0.753 absorbance at 700µg/ml) followed by stem extract. However, ferrous iron chelating activity was well established in methanol extract of stem (42.28% at 5000µg/ml), than that of the leaf extract. Thus, the results obtained in the present study indicate that this plant has the potential as natural source of antioxidants, capable of protecting against free radical mediated damage and may have applications in preventing and curing various diseases.

Key words: *Solena amplexicaulis*, total phenol, flavonoids, antioxidant, DPPH$^{\bullet}$, ABTS$^{\bullet+}$.

INTRODUCTION

A free radical is defined as any atom or molecule possessing unpaired electrons. In living systems these free radicals are produced by the body to aid in the normal metabolic processes such as digestion and the conversion of food into energy. They are actually quite helpful in many of body's natural functions [1]. However, the uncontrolled production of oxygen derived free radicals are associated with the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis, arteriosclerosis and degenerative processes associated with aging. The food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Scavenging of reactive oxygen species (ROS) is important in preventing potential damage to cellular components such as DNA, proteins and lipids [2]. Our bodies try to protect us from free radical damage by producing enzymes that neutralize them. However, they are not capable of handling this function without antioxidants provided by our diets. Antioxidants are protective molecules also, referred to as free radical scavengers and hence prevent and repair damage done by these free radicals [3]. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases [4]. Synthetic antioxidants have been used for industrial processing in recent years. These are such as butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) which are suspected to be highly carcinogenic and being harmful to liver [5,6]. Natural antioxidants such as flavonoids, phenolics, tannins, curcumin and terpenoids are found in various plants [7,8,9] which act as reducing agents, hydrogen donators, free radicals scavengers and singlet oxygen quenchers and therefore, as cell saviors [10]. Hence, now a days search for natural antioxidant source is gaining much importance.

Solena amplexicaulis (Lam.) Gandhi. (Syn. *Melothria amplexicaulis*) commonly known as creeping cucumber belonging to Cucurbitaceae family is widely distributed throughout tropical Asia. The whole plant is potential source of natural antioxidant [11]. The tubers, leaves and seeds of the plant are extensively used in traditional system for various ailments like hepatosplenomegaly, spermatorrhoea, thermogenic, appetizer, cardiotonic,

diuretics, haemorrhoids and invigorating [12]. The leaves of this plant have good anti-inflammatory activity also. Hence it is recommended for inflammation, skin lesions and skin diseases [13].

The objective of the present study was to investigate the antioxidant activity of leaves and stem of *S.amplexicaulis* using four *in vitro* models. Total phenolic and flavonoid contents of the same were also determined in order to evaluate a relationship between the antioxidant activity and the phytochemical constituents.

EXPERIMENTAL SECTION

1. Plant material

The leaves and stem of *Solena amplexicaulis* were collected from Madukarai, Coimbatore district, Tamil Nadu, India. Collected plant materials were washed thoroughly in tap water and then shade dried.

2. Preparation of extract

About 50g of coarsely powdered leaf and stem parts of *S.amplexicaulis* was extracted with 250ml of methanol through soxhlet apparatus separately for 8 to 10 hours. The extracts obtained were then concentrated and finally dried to a constant weight.

3. Chemicals

2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), dimethyl sulphoxide (DMSO), ferrous sulfate ($FeSo_4$), 3-(2-pyridyl) - 5,6-bis (4-phenyl-sulfonic acid) - 1,2,4 - triazine (Ferrozine), di-sodium hydrogen phosphate (Na_2HPO_4), Sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$) potassium ferric cyanide, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), 2,2´-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$), potassium persulfate ($K_2S_2O_8$), folin-ciocalteu's reagent (FCR), sodium bicarbonate ($NaHCO_3$), sodium nitrate ($NaNo_3$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), quercetin, gallic acid, ascorbic acid (vitamin C), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Ascorbic acid, butylated hydroxyl toluene (BHT), Ethylenediaminetetraacetic acid (EDTA).

4. Determination of total phenolics content

The total phenolic content of plant extracts was determined using folin-ciocalteu reagent (FCR) according to the procedure reported by Singleton *et al.* (1999) [14]. About 500 µl sample (20mg/ml) was added to 25ml double distilled water mixed with 1ml of folin-ciocalteu reagent (1:10). This mixture was kept at room temperature for 3 min after that added with 1.5ml of 2% $NaHCO_3$. Subsequently, the mixture was shaken on a shaker for 1 hour at room temperature and then its absorbance was measured at 760nm. Total phenolics were quantified by calibration curve obtained from measuring the absorbance of known concentrations of gallic acid standard. The total phenolic contents were expressed as gallic acid equivalence (GAE) in µg.

5. Determination of total flavonoids content

The aluminium chloride colorimetric assay was used for total flavonoid determination, as described by Zhishen *et al.* (1999) [15]. 500µl extract (4mg/ml) was mixed with 2.5ml of distilled water and 300µl of 5%$NaNo_3$. After 5 min, 300µl of 10% $AlCl_3$, 2ml of 1% NaOH and 1ml of distilled water were added to the mixture. The absorbance of the reaction mixture was measured at 512nm, along with standard quercetin. The total flavonoids content was determined as µg of quercetin equivalent by using the standard quercetin graph.

6. *In vitro* antioxidant activity

6.1. $DPPH^{\bullet}$ radical scavenging assay

The antioxidant activity of methanol extract was determined on the basis of their hydrogen donating or radical scavenging activity of the stable $DPPH^{\bullet}$ according to the method of Blois (1958) [16]. About 0.2mM solution of $DPPH^{\bullet}$ was prepared from this 100µl and was added to various concentration of extracts i.e. 50µg-250µg/ml and shaken vigorously. Absorbance at 517nm was determined after 30 minutes at room temperature and the scavenging activity was calculated as a percentage of the radical reduction. BHT was used as a reference compound. The graph was plotted with mean values. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula,

$$\text{Inhibition (\%)} = (\text{Control OD-Sample OD/Control OD})X100$$

6.2. $ABTS^{\bullet+}$ radical scavenging assay

The free radical scavenging activity was determined by $ABTS^{\bullet+}$ radical cation decolorization assay described by Re *et al.* (1999) [17]. $ABTS^{\bullet+}$ radical cation solution was prepared by reacting 7mM $ABTS^{\bullet+}$ with 2.45mM potassium persulfate on incubation the mixture at room temperature in dark for 12-16 hours. The radical was stable in this form

for more than two days when stored in the dark at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance at 734nm of 0.700±0.02. After that, 2ml of diluted $ABTS^{\bullet+}$ solution was added to 20µl/ml of sample. The absorbance was recorded after incubation at room temperature for 30min at 734nm and inhibition percentage was calculated. Trolox was used as reference standard. The unit of total antioxidant activity (TAA) is defined as the concentration of trolox having equivalent antioxidant activity expressed as µmol/g sample extract on dry matter.

6.3. Reducing power assay

Total reducing power was determined as described by Yildirim *et al.* (2001) [18]. Various concentrations of methanol extract (300µg-700µg/ml) were separately mixed with 1ml of 0.2M sodium phosphate buffer (pH=6.6) and 1ml of 1% potassium ferric cyanide, followed by incubation at 50ºC for 20 min. 1ml of 10% TCA was added to the mixture, which was then centrifuged at 3000rpm for 1min. Finally 2ml of the supernatant solution were mixed with equal volume of distilled water. Absorbance was measured at 700nm after the addition of 0.5ml of 1% $FeCl_3$. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as standard.

6.4. Ferrous ion chelating assay

The ferrous ion chelating activity was measured by the decrease in the absorbance at 562nm of iron(II)-ferrozine complex by the method of Singh and Rajini (2004) [19]. 100µl of 2mM $FeSo_4$ and 300µl of 5mM ferrozine were mixed with different concentration of samples (1000µg-5000µg/ml). The mixture was allowed to equilibrate for 10 min before measuring the absorbance. The ability of the sample to chelate ferrous ion was calculated by the formula of inhibition percentage as employed for $DPPH^{\bullet}$ radical scavenging activity.

7. Statistical analysis

All the analysis was done in triplicate and results were expressed as mean±SD. The data were subjected to one way analysis of variance (ANOVA) and the significance of the difference between mean was determined by Duncan's Multiple Range Test with significance level, $P<0.05$. ANOVA was performed using the statistical software SPSS (SPSS Inc. Chicago, USA).

RESULTS AND DISCUSSION

1. Recovery percent, total phenolic and flavonoid contents

The radical scavenging activity of the extracts could be related to the nature and amount of phenolics, flavonoids and their hydrogen donating ability (Shimada *et al.*, 1992) [20]. Table 1 presents the yield and the total phenolic and flavonoid contents of the extracts. In the Folin-Ciocalteu assay, gallic acid was used as a standard. The total phenolic contents of methanolic extract of leaf and stem of *S.amplexicaulis* were 130.17µg/10mg and 120.33µg/10mg respectively. The total flavonoid content was evaluated using quercetin as a standard. The total flavonoid contents of leaf and stem were 235.67µg/2mg and 35.33µg/2mg respectively.

Table 1: Extract yield percentage, total phenolic and flavonoid contents of the methanolic leaf and stem extracts of *Solena amplexicaulis*

Sample	Extraction yield (%)	Total phenolic content (µg of GAE/10mg DW)[a]	Total flavonoid content (µg of QE/2mg DW)[b]
Leaf	13.8	130.17±2.25	235.67±2.08
Stem	7.2	120.33±2.52	35.33±1.53

[a] Total phenolic content (µg of gallic acid equivalent per 10mg dry weight).
[b] Total flavonoid content (µg of quercetin equivalent per 2mg dry weight).
Values are expressed as mean±SD of three parallel measurements.
Values within a column followed by different letters are significantly different (P<0.05).

2. *In vitro* antioxidant activity

2.1. $DPPH^{\bullet}$ radical scavenging assay

$DPPH^{\bullet}$ is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [21], which does not absorb at 519nm [22]. The reduction capacity of DPPH radical was determined by the increasing in its percentage inhibition. It is visually noticeable as a change in color from purple to yellow.

The free radical scavenging activity of both leaf and stem parts of *S.amplexicaulis* was increased with the increase in concentration (Table 2). The leaf extract showed significantly higher activity (92.51% at 250µg/ml) than stem extract (90.85% at 250µg/ml). The DPPH radical scavenging activity was detected and compared with the standard, BHT.

Table 2: DPPH$^{\bullet}$ radical scavenging activity of methanol extracts of leaf and stem of *Solena amplexicaulis* and the standard, BHT

S.No.	Sample concentration (µg/ml)	Percentage Inhibition		
		Standard BHT	Leaf	Stem
1.	50	$36.24^{a} \pm 0.31$	$78.10^{a} \pm 0.57$	$13.72^{a} \pm 0.68$
2.	100	$42.21^{b} \pm 0.38$	$82.13^{b} \pm 0.67$	$59.21^{b} \pm 0.69$
3.	150	$49.39^{c} \pm 0.34$	$91.17^{c} \pm 0.89$	$62.68^{c} \pm 1.06$
4.	200	$52.16^{d} \pm 0.40$	$91.33^{c} \pm 0.46$	$87.51^{d} \pm 0.89$
5.	250	$57.15^{e} \pm 0.24$	$92.51^{d} \pm 0.53$	$90.85^{e} \pm 0.74$

Values are expressed as mean±SD of three parallel measurements.
Values within a column followed by different letters are significantly different (P<0.05).

2.2. ABTS$^{\bullet+}$ scavenging activity

ABTS$^{\bullet+}$, a protonated radical, has characteristic absorbance maxima at 734nm which decreases with the scavenging of the proton radicals. ABTS$^{\bullet+}$ was generated by incubating it with potassium persulfate. The presence of chemical compounds in the tested extracts that inhibit the potassium persulfate activity may reduce the production of ABTS$^{\bullet+}$. In the present investigation, the methanolic leaf extract of *S.amplexicaulis* registered the highest total antioxidant activity (3223.11µmol/g) than the stem extract (2946.36µmol/g).

Table 3: ABTS$^{\bullet+}$ radical scavenging activity of methanol extracts of leaf and stem of *Solena amplexicaulis.*

Sample	Total antioxidant activity (µmol of TE/g DW)
Leaf	3223.11±1.74
Stem	2946.36±2.54

Total antioxidant activity (µmol equivalent trolox performed by using ABTS$^{\bullet+}$ radical cation).

2.3. Reducing power assay

The presence of reductants (antioxidants) in the sample would result in reduction of the Fe^{3+}/ferric cyanide complex to the ferrous form. The Fe^{2+} could therefore, be monitored by measuring the formation of Perl's Prussian blue with absorbance at 700nm. The strengthening compounds of reducing power had a stronger peroxide reducing ability.

Table 4 shows the reductive capabilities of different concentrations of methanolic extracts of leaf and stem of *S.amplexicaulis* in comparison to the standard, ascorbic acid. It was found that the reducing power increased with increasing concentration of extract. In the present study, leaf extract showed the highest reducing ability (absorbance 0.753 at 700µg/ml) than the stem extract (absorbance 0.441 at 700µg/ml). However, the activity was lesser than the standard, ascorbic acid (absorbance 1.09 at 700µg/ml).

Table 4: Reducing power assay of methanol extracts of leaf and stem of *Solena amplexicaulis* and the standard, ascorbic acid.

S.No.	Sample concentration (µg/ml)	Ascorbic acid absorbance at 700nm	Leaf extract absorbance at 700nm	Stem extract absorbance at 700nm
1.	300	$0.55^{a} \pm 0.03$	$0.551^{a} \pm 0.003$	$0.032^{a} \pm 0.001$
2.	400	$0.64^{b} \pm 0.04$	$0.676^{b} \pm 0.004$	$0.061^{b} \pm 0.002$
3.	500	$0.86^{c} \pm 0.03$	$0.681^{b} \pm 0.004$	$0.088^{c} \pm 0.002$
4.	600	$0.98^{d} \pm 0.02$	$0.689^{c} \pm 0.003$	$0.417^{d} \pm 0.005$
5.	700	$1.09^{e} \pm 0.04$	$0.753^{d} \pm 0.003$	$0.441^{e} \pm 0.004$

Values are expressed as mean±SD of three parallel measurements.
Values within a column followed by different letters are significantly different (P<0.05).

2.4. Ferrous ion chelating assay

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. In complex systems, such as food and food preparations, various different mechanisms may contribute to oxidative processes, such as Fenton reaction, where transition metal ions play a vital role. Different reaction oxygen species might be generated and various target structures such as lipids, proteins and carbohydrates can be affected. Therefore, it is important to characterize the extracts by the variety of antioxidant assays [23].

The chelating effect on the ferrous ions by methanolic leaf and stem extract of *S.amplexicaulis* is presented in Table 5. Both the sample extracts exhibited the ability to chelate metal ions. Among the two extracts, stem showed higher activity (42.28% at 5000µg/ml) than the leaf extract at the same concentration (32.30%). Metal chelating capacity was significant as they reduced the concentration of catalyzing transition metal in lipid peroxidation [24].

Table 5: Ferrous iron chelating assay of methanol extracts of leaf and stem of *Solena amplexicaulis* and the standard, EDTA.

S.No.	Sample concentration (µg/ml)	Percentage Inhibition		
		Standard, EDTA	Leaf	Stem
1.	1000	$56.28^{a} \pm 0.19$	$24.63^{a} \pm 0.54$	$33.45^{a} \pm 0.56$
2.	2000	$71.55^{b} \pm 0.33$	$28.58^{b} \pm 0.52$	$34.65^{b} \pm 0.64$
3.	3000	$83.46^{c} \pm 0.20$	$31.12^{c} \pm 0.70$	$36.55^{c} \pm 0.54$
4.	4000	$93.19^{d} \pm 0.21$	$31.41^{cd} \pm 0.51$	$38.38^{d} \pm 0.44$
5.	5000	$96.69^{e} \pm 0.15$	$32.30^{d} \pm 0.65$	$42.28^{e} \pm 0.76$

Values are expressed as mean±SD of three parallel measurements.
Values within a column followed by different letters are significantly different (P<0.05).

CONCLUSION

The results of the present study indicate the methanolic leaf and stem extracts of *S.amplexicaulis* are high in phenolic and flavonoid contents and these extracts exhibit strong antioxidant activities. The scavenging activities noted against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, lead us to propose *S.amplexicaulis* leaf and stem parts as promising natural sources of antioxidants suitable for application in nutritional/pharmaceutical fields, in the prevention of free radical-mediated diseases. Further studies are needed to explore the potential phenolics compounds(s) from *S.amplexicaulis* and *in vivo* studies are needed for better understanding on their mechanism of action.

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