

QUALITATIVE AND QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS AND *IN VITRO* ANTIOXIDANT ASSAYS IN THE TUBER OF *SOLENA AMPLEXICAULIS* (LAM.) GANDHI. (CUCURBITACEAE)

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ABSTRACT

Most of the traditional medicinal plants in India are not scientifically validated. Scientific evaluation of medicinal property along with traditional knowledge is essential to obtain effective drugs for commercial purpose. *Solena amplexicaulis* belongs to the family, Cucurbitaceae, a traditional medicinal plant species of Tamil Nadu, India is being prescribed to cure various diseases. In this study, the objective was to investigate the qualitative and quantitative determinations of certain phytochemicals and *in vitro* antioxidant capabilities of various alcoholic and aqueous tuber extracts of *S. amplexicaulis* by adapting standard procedures. In all the assays methanolic tuber extract registered significantly high amount of secondary metabolites and also it effectively scavenge the free radicals in a concentration dependent manner than the other extracts. These results were compared with synthetic (BHA and BHT) as well as natural antioxidants (rutin and quercetin). The outcome of the study revealed most valuable information and also supports the continued sustainable use of this species in traditional systems of medicine.

Keywords: *Solena amplexicaulis*, Cucurbitaceae, phytochemical analysis, antioxidant properties.

1. INTRODUCTION

Herbal medicines are in huge demand in the developing countries for primary health care because of their effectiveness, safety and lesser side effects. Now traditional medicine is being reevaluated by extensive research on different plant species and their therapeutic principles. The phytochemical compounds play a significant role in biological functions. There is growing interest in correlating the bioactive components of a medicinal plant with its pharmacological activity. Based on accumulative evidence, in recent decades tremendous interest has considerably increased in finding out the natural substances especially from plants (Dezfuli *et al.*, 2014; Servili *et al.*, 2014). Antioxidant compounds in food plays an important role as a health protecting factor. The main characteristic of an antioxidant is its ability to trap free radicals. These free radicals may oxidize nucleic acids, proteins, lipids and DNA that can initiate degenerative diseases (Carlsen *et al.*, 2010).

Solena amplexicaulis is commonly called as creeping cucumber, belongs to the family, Cucurbitaceae mainly distributed in the dry deciduous forests of southern India (Matthew, 1983; Paulsamy and Karthika, 2014). The traditional healers are prescribing the tubers of this species as astringent, appetizer, carminative, cardiogenic, digestive, diuretic, expectorant, invigorating, purgative, stimulant, sour and thermogenic (Dhananjay, 2006). The whole plant is a potential

source of natural antioxidant (Venkateshwaralu *et al.*, 2011; Karthika *et al.*, 2012) and anti-inflammatory agent (Arun *et al.*, 2011). It is recognized as CNS active, diuretic, febrifuge and hypothermic (Dhananjay, 2006). Crude leaf juice is used to cure jaundice (Mohammed *et al.*, 2011). Raw unripe fruits are eaten to strengthen the body (Jeyaprakash *et al.*, 2011). The decoction of the root is administered orally to cure stomachache (Abdolbaset *et al.*, 2011). The seeds are used as purgative (Jeyaprakash *et al.*, 2011).

However, no study on antioxidant properties has been available for the tuber of this species. To address this lacuna, an attempt has been made to investigate the qualitative and quantitative phytochemical analysis and certain *in vitro* antioxidant activities of successive extracts (hexane, benzene, chloroform, methanol and water) from the tuber of *S. amplexicaulis*. These antioxidant values were compared with commercially available synthetic as well as natural antioxidants.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH[•]), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), diosgenin (DE), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ferric chloride (FeCl₃), ferrous ammonium sulphate (Fe(NH₄)₂(SO₄)₂·6H₂O),

Folin-Ciocalteu reagent, gallic acid, polyvinyl polypyrrolidone (PVPP), potassium ferricyanide ($K_3Fe(CN)_6$), potassium persulfate ($K_2S_2O_8$), quercetin, sodium carbonate (Na_2CO_3), sodium nitroprusside ($Na_2[Fe(CN)_5NO]$), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), L-ascorbic acid (vitamin C) and β -carotene were purchased from Himedia (Mumbai, India). All other reagents and solvents used were of analytical grade.

2.2. Plant material

The fresh tuber parts of *S. amplexicaulis* were collected from the thorny scrub jungles of Madukkarai, Coimbatore district, Tamil Nadu, India during the month of April, 2014. The authenticity of the plant was confirmed by comparing with the reference specimen (Vide No: CPS 313) preserved at Botanical Survey of India, Southern Circle, Coimbatore. The samples were cleaned, washed with copious amount of water, shade dried and coarsely powdered in a Willy Mill to 60 mesh size (Nippon Electricals, Chennai, India) for extraction.

2.3. Preparation of extracts

About 50g of powdered plant material was extracted (50g/250mL) in a soxhlet extractor for 8 to 10 h, sequentially with the alcoholic solvents viz., hexane, benzene, chloroform and methanol and aqueous. Then the extracts were evaporated to dryness and stored at 4°C in storage vials for experimental use.

2.4. Qualitative estimations

All the extracts were subjected to preliminary phytochemical analysis followed by the methods of Harborne (1998) and Trease and Evans (2002).

2.5. Quantitative estimations

Major non-enzymic antioxidants of the plant extracts were determined by using standard quantitative methods. The alkaloid content was gravimetrically determined by the method of Harborne (1998). The total phenolic and tannin contents were estimated and expressed as mg gallic acid equivalent (GAE)/g extract according to the method described by Siddhuraju and Becker (2003). The total flavonoids content was determined spectrophotometrically using a standard curve rutin as per the method of Zhishen *et al.* (1999) and expressed as mg rutin equivalent (RE)/g extract. Content of ascorbic acid was calculated on the basis of calibration curve of authentic L-ascorbic acid and the results were expressed as mg ascorbic acid equivalent (AAE) /g extract, proposed by Klein and Perry (1982). Total saponin content was determined

by the method described by Makkar *et al.* (2007) with some modifications. The values were expressed as mg diosgenin equivalents (DE)/g extract.

2.6. Determination of in vitro antioxidant activity

2.6.1. Reducing power assay

The Fe^{3+} reducing power of the extract was determined according to the method suggested by Oyaizu (1986). The plant extracts (300-700 μ g/mL) were mixed with 5.0 mL of 0.2 M phosphate buffer of pH 6.6 and 5.0 mL of 1% $K_3Fe(CN)_6$ and the mixtures were incubated at 50°C for 20 min. The reaction was terminated by adding 5.0 mL of 10% TCA (w/v), and the mixture was centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5.0 mL) was mixed with 5.0 mL of distilled water and 1.0 mL of 0.1% (w/v) $FeCl_3$ and the absorbance was read at 700 nm. Rutin, quercetin, BHA and BHT served as the reference materials. Increased absorbance indicates increased reductive capability.

2.6.2. DPPH radical scavenging activity

The hydrogen donating capacity was assessed using the stable DPPH \cdot method (Blois, 1958). Briefly, a solution of 0.1mM DPPH \cdot was prepared using methanol. The samples (50–250 μ g/mL) were mixed with 5.0 mL of DPPH \cdot solution. Reaction mixture was shaken, incubated at 27°C for 20 min and the absorbance was measured at 517 nm. Results were compared with the activity of rutin, quercetin, BHA and BHT. Per cent DPPH \cdot discolouration of the samples was calculated using the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Control OD} - \text{Sample OD}) / \text{Control OD}] \times 100.}$$

Antioxidant activities of the extracts were expressed as IC_{50} , these values were calculated from the linear regression of the percentage antioxidant activity versus concentration of the extracts. A lower IC_{50} value indicates greater antioxidant activity.

2.6.3. Total antioxidant activity

Total antioxidant activity was performed using an improved ABTS $\cdot+$ method proposed by Siddhuraju and Manian (2007). The ABTS radical cation (ABTS $\cdot+$) was generated by a reaction of 7 mM ABTS $\cdot+$ and 2.45 mM potassium persulphate and the mixture was incubated for 12–16 h at room temperature in dark. Prior to assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated to obtain an absorbance of 0.700 ± 0.02 at 734 nm. 10 μ L/mL of sample was added to 1.0 mL of diluted ABTS $\cdot+$ solution. After 30 min of incubation,

absorbance was read at 734 nm. Trolox was used as a reference material.

2.6.4. Inhibition of β – carotene bleaching

The antioxidant capacity of the extract was evaluated using β -carotene-linoleate model system (Taga *et al.*, 1984). 1mg of β – carotene was dissolved in 10 mL of chloroform and mixed with 20 μ L of linoleic acid and 200 mg of Tween – 40 emulsifier mixture. Chloroform was completely evaporated using rotary vacuum evaporator at 45°C. 50mL of oxygenated distilled water was added to the flask with vigorous shaking, to form an emulsion. 5mL of emulsion was added to 100 μ L of sample from each tube, the zero-time absorbance was measured at 470 nm. Subsequent absorbance readings were recorded at 15 min intervals by keeping the sample tubes in a water bath at 50°C until the colour of the control sample disappeared (about 120 min). A blank, devoid of β – carotene, was prepared for background subtraction. Rutin, quercetin, BHA and BHT were used as standards. β – carotene bleaching activity was calculated as:

$$AA (\%) = [1 - (A_s^0 - A_s^{120}) / (A_c^0 - A_c^{120})] \times 100$$

Where, A_s^0 - absorbance of sample at 0 min, A_s^{120} - absorbance of sample at 120 min, A_c^0 - absorbance of control at 0 min, and A_c^{120} - absorbance of control at 120 min.

2.6.5. Antihemolytic activity

Antihemolytic activity was performed according to the method set forth by Naim *et al.* (1976). The erythrocytes from cow blood were separated by centrifugation (2000 rpm for 10 min) and washed with saline phosphate buffer (pH 7.4) until the supernatant become colourless. The erythrocytes were then diluted with saline phosphate buffer to give 4% (v/v) suspension. 500 μ g of extract/mL of saline phosphate buffer were added to 2.0 mL of erythrocytes suspension and made up to 5.0 mL with saline phosphate buffer. This mixture was pre-incubated for 5 min and then 0.5 mL of H₂O₂ solution of appropriate concentration in saline buffer was added. The concentration of H₂O₂ in the reaction mixture was adjusted so as to bring about 90% haemolysis of blood cells after 240 min. After the incubation time, the reaction mixture was centrifuged at 1500 rpm for 10 min and the extent of haemolysis was determined by measurement of the absorbance (at 540 nm) corresponding to haemoglobin liberation. Natural and synthetic standards at the same concentration as sample extract were used for comparison.

The percent haemolysis inhibition was calculated using the formula:

$$\text{Inhibition percentage} = [A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}] \times 100$$

Where, A_{control} - absorbance of control and

A_{sample} - absorbance of sample.

2.7. Statistical analysis

All the values were expressed as mean \pm standard deviation (SD) of three determinations and subjected to one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test using SPSS (version 9, SPSS Inc., Chicago, USA). $P < 0.05$ was chosen as the criterion for statistical significance.

3. RESULTS

The study revealed that the percent yield of methanolic extract was higher (9.60%) followed by water extract (4.0%). The other solvents *viz.*, hexane, benzene and chloroform yielded very less quantity of residue only (Table 1).

3.1. Qualitative estimations

The major secondary metabolites present in the extracts were varied across the solvents used (Table 1). The methanolic extract of *S. amplexicaulis* tuber containing more number of secondary metabolites than the other extracts studied. Cardiac glycosides were present in all the alcoholic and aqueous extracts of tuber but resins, steroids, terpenoids and triterpenoids were totally absent in all the extracts. However, the degree of precipitation of phytochemicals varies in all the extracts.

3.2. Quantitative estimations

The quantity of phytochemicals estimated were varied among the extracts tested (Table 2). Among the six components, alkaloids (92.02mg/g dry powder) and saponins (39.4-135.8mg DE/g extract) contents were significantly higher and the tannins (0.01-1.57 mg GAE/g extract) content was very low when compared to the other compounds studied. Further, the degree of precipitation of secondary metabolites varies according to the extractive power of the solvents. Mostly the methanol extract contained high amount of secondary metabolites than the other solvents studied.

3.3. In vitro antioxidant activities

In the reducing power assay, the presence of antioxidants in the samples would result in the reduction of Fe³⁺ to Fe²⁺ by donating an electron which can be monitored by spectrophotometrically at 700nm. The reductive abilities displayed an

apparent linear relationship with concentration. The chloroform extract showed higher reductive capability than the other extracts studied. The activity increases exponentially with the increase in concentration of sample. These values were compared with two natural (rutin and quercetin) and two synthetic (BHA and BHT) antioxidants (Fig. 1a and 1b).

DPPH•, a stable organic radical, widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. It was visually noticeable by a colour change from purple to yellow. It possess concentration dependent scavenging activity. The IC₅₀ values of the samples were ranging between 150.91 and 345.75 µg/mL and compared with standards as shown in Table 3.

In the evaluation of total antioxidant capacity by measuring ABTS•+ method, it is known that all the sample extracts were able to quench ABTS radical more effectively and the values were ranged between 2055.4 and 6226.8 µmol Trolox equivalent/g extract. Among the samples investigated, hexane, methanol and water extracts showed maximum values 6129.0, 6172.8 and 6226.8 µmol Trolox equivalent/g extract respectively. On the other hand, the benzene and chloroform extracts registered markedly low ABTS radical scavenging activity (Table 3).

In β carotene/linoleic acid bleaching assay it is known that all the extracts are having potential to inhibit the peroxidation of linoleic acid and subsequent bleaching to β carotene in various

degrees. Apparently, the most effective extracts were benzene (97.85%) and water (95.41%). Furthermore, these values were comparatively higher than those of natural and synthetic antioxidants tested as shown in Fig. 2.

The protective effect of *S. amplexicaulis* tuber extracts and positive standards against H₂O₂ mediated haemolysis were investigated and presented in Fig. 3. In general, all the sample extracts contributed satisfactory antihaemolytic activity ranged between 34 and 53%. Interestingly these values surpassed the efficiency of synthetic antioxidants, BHA (5%) and BHT (7%).

4. DISCUSSION

Many phytochemicals are now studied extensively for their potential ability of curing diseases. Herbal preparations are effectively and extensively used for their medicinal properties and have become increasingly popular worldwide. Standardization of crude drug is an integral part of establishing its correct identity. Qualitative and quantitative phytochemical screening of *S. amplexicaulis* tuber revealed that alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, saponins, vitamin C and tannins were present in this plant. All the active compounds were excessively present in methanol extract (Table 1). Different solvents have been reported to have different capacity to extract phytoconstituents according to their solubility and polarity and most of the compounds dissolve well in high polar solvents (Karthika *et al.*, 2014).

Table 1. Preliminary phytochemical screening of various extracts of *Solena amplexicaulis* tuber.

Solvent	Yield (%)	Phytoconstituents*										
		A	CG	F	G	P	R	S	St	T	Te	Tr
Hexane	0.60	-	-	-	-	-	-	+++	-	-	-	-
Benzene	0.40	-	-	-	-	-	-	++	-	-	-	-
Chloroform	0.17	-	-	-	-	-	-	++	-	-	-	-
Methanol	9.60	+++	+++	+++	++	+++	-	+	-	+++	-	-
Water	4.00	+	+++	+	+++	-	-	-	-	-	-	-

*A – Alkaloids; CG - Cardiac glycosides; F - Flavonoids; G - Glycosides; P - Phenols; R - Resins; S - Saponins; St – Steroids; T - Tannins; Te - Terpenoids; Tr - Triterpenoids. +++ : highly present; ++ : moderately present; + : low, - : absent.

Table 2. Extractive yield, alkaloids, total phenolics, tannins, total flavonoids, vitamin C and saponins contents of different solvent extracts of tuber of *Solena amplexicaulis* extracts.

Solvent	Total phenolics (mg GAE/g extract)	Tannins (mg GAE/g extract)	Total flavonoids (mg RE/g extract)	Vitamin C (mg AAE/g extract)	Saponins (mg DE/g extract)
Hexane	0.55±0.02 ^a	0.20±0.03 ^b	Not detected	0.069±0.46 ^a	135.8±0.56 ^b
Benzene	0.21±0.01 ^a	0.02±0.01 ^a	Not detected	0.032±0.25 ^a	121.5±0.27 ^b
Chloroform	0.17±0.01 ^a	0.01±0.02 ^a	Not detected	0.014±0.07 ^a	122.4±0.16 ^b
Methanol	6.32±0.18 ^c	1.57±0.47 ^d	8.94±0.21 ^b	0.666±0.76 ^c	55.6±0.13 ^a
Water	2.51±0.12 ^b	0.79±0.06 ^c	4.59±0.25 ^a	0.293±0.82 ^b	39.4±0.14 ^a

Values are mean ± standard deviation (SD) of three independent experiments.

Values not sharing a common letter in a column are significantly different (*P* < 0.05).

Table 3. DPPH• scavenging and total antioxidant activities of different solvent extracts of *Solena amplexicaulis* tuber.

Solvents/Standards	IC ₅₀ values (µg / mL)	Total antioxidant activity (µmol of TE/g dry weight)
	DPPH• scavenging activity	
Hexane	345.75±0.14 ^e	6129.01±21.31 ^b
Benzene	264.23±0.25 ^d	2311.93±17.25 ^a
Chloroform	214.12±0.11 ^c	2055.45±15.42 ^a
Methanol	150.91±0.18 ^b	6172.81±29.36 ^b
Water	238.15±0.29 ^c	6226.87±23.51 ^c
Rutin	15.75±0.01 ^a	-
Quercetin	20.71±0.04 ^a	-
BHA	21.41±0.11 ^a	-
BHT	34.74±0.26 ^a	-

Values are mean ± standard deviation (SD) of three independent experiments. Values not sharing a common letter in a column are significantly different ($P < 0.05$).

The reducing power of a compound may serve as a significant indicator for potential antioxidant activity. Reducing properties are generally associated with the presence of reductones. The presence of reductones in sample extracts might cause the reduction of the Fe³⁺/ferric cyanide complex to Fe²⁺/ferrous form which can be monitored by measuring the formation of Perl's Prussian blue with absorbance at 700nm. The study revealed that the methanolic extract of *S. amplexicaulis* tuber due to the presence of reductones might significantly contribute the antioxidant activity (Singhal *et al.*, 2011).

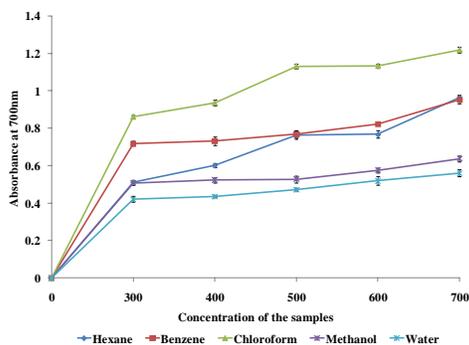


Fig. 1a. Reducing ability of different extracts of *Solena amplexicaulis* tuber. Values are mean ± standard deviation (SD) of three independent experiments.

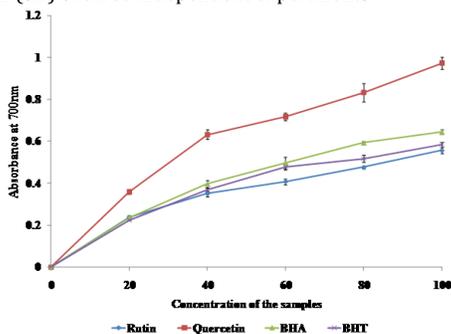


Fig. 1b. Reducing ability of natural and synthetic antioxidants. Values are mean ± standard deviation (SD) of three independent experiments.

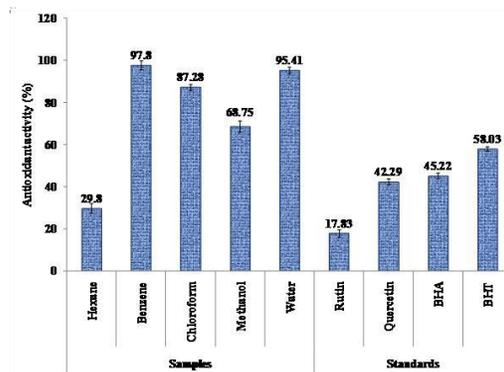


Fig. 2. Lipid peroxidation preventive property of tuber extracts of *Solena amplexicaulis* with certain standards in β - carotene linoleic acid system. Values are mean ± standard deviation (SD) of three independent experiments.

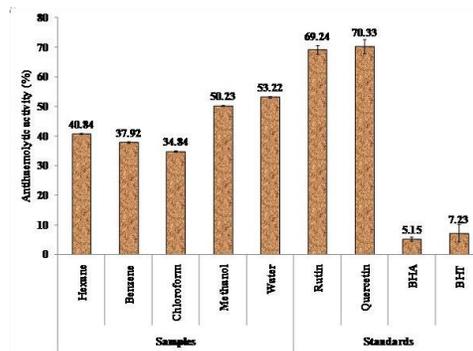


Fig. 3. Antihemolytic property of tuber extracts of *Solena amplexicaulis* compared with certain standards. Values are mean ± standard deviation (SD) of three independent experiments.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable molecule. Antioxidants in the sample on interaction with DPPH•, transfer electron to DPPH• and thus neutralizing its free radical character and convert it to 1-1 diphenyl-2-picryl hydrazine and the degree of discoloration (purple to yellow) indicates the scavenging activity of the drug (Apak *et al.*, 2013).

The results of DPPH• scavenging assay indicate that the methanolic plant extract possess high antioxidant activity (Table 3). The extracts showed a significant correlation with total phenolics, tannins and total flavonoids content ($R^2=0.972$, $R^2=0.952$ and $R^2=0.953$ respectively) and partially correlation with alkaloids and saponins ($R^2=0.822$ and $R^2=0.890$ respectively). This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups, in addition to synergistic effects of the compounds present in this plant species (Noguer *et al.*, 2014).

ABTS^{•+} (protonated radical) is a blue chromophore produced by the reaction between ABTS and potassium persulphate (Ruby *et al.*, 2009). The characteristic absorbance maxima is at 734nm. The presence of bioactive chemical compounds in the tested extracts that inhibit the potassium persulphate activity may reduce the production of ABTS^{•+}. From the results, the aqueous extract of *S. amplexicaulis* tuber possessed the highest ABTS^{•+} scavenging activity. Thus, it might be speculated that the antioxidant activity of the extract may be possibly attributed to the phytochemicals present in it. Further the extract also demonstrated a high correlation with total phenolics and vitamin C content ($R^2=0.991$ and $R^2=0.986$ respectively).

In the β carotene bleaching assay, the oxidation of linoleic acid generates peroxy free radicals due to the abstraction of a hydrogen atom from diallylic methylene groups of linoleic acid. The free radicals will then oxidize the highly unsaturated β carotene. The presence of antioxidants in the sample will minimize the oxidation of β carotene by hydroperoxides. Hydroperoxide formed in this system will be neutralized by the antioxidants from the samples. Thus the degradation rate of β carotene depends on the antioxidant activity of the sample (Chakraborty and Verma, 2010). In the present study all the extracts inhibited peroxidation of linoleic acid and subsequent bleaching to β carotene in various degrees. The extracts showed a significant correlation with total phenolics ($R^2=0.999$), tannins ($R^2=0.993$) and vitamin C ($R^2=0.988$) contents. Therefore, it can be explained that the β carotene bleaching assay of the studied plant extracts may be attributed to the presence of phenolics, tannins and vitamin C in them.

Erythrocytes are considered as major targets for the free radicals which are potent promoters of activated oxygen species. The red blood cells were treated with hydrogen peroxide (H_2O_2) the haemolysis was done. This could be attributed to the oxidizing nature of H_2O_2 . In the

present study lipid oxidation of cow blood erythrocyte membrane mediated by H_2O_2 induces membrane damage and subsequently haemolysis (Dai *et al.*, 2006). Among the plant samples investigated, benzene and water extracts have higher antihaemolytic activity. Dai *et al.* (2006) also recorded that flavonols and their glycosides are competent antioxidants which are capable of protecting human red blood cells against oxidative haemolysis stimulated by free radical. The extracts demonstrated a high correlation with total phenolics ($R^2=0.980$), tannins ($R^2=0.967$), flavonoids ($R^2=0.862$) and vitamin C ($R^2=0.932$) content. The statistical analysis using the Pearson tests indicated a positive linear correlation between the secondary metabolites and antioxidant assays, in agrees with other reports (Ebrahimzadeh *et al.*, 2014; Ghasemi *et al.*, 2014). The analyses were statistically significant ($P<0.05$), showing correlation coefficients greater than 0.748 in this test.

5. CONCLUSION

Based on the active profile exposed through various assays, it can be concluded that major secondary metabolites identified in *S. amplexicaulis* tuber are playing pivotal role in the scavenging of radicals and hence the better antioxidant activity. Hence it is a promising natural source of antioxidant can be used in nutritional or pharmaceutical fields for the prevention of free radical mediated diseases. However, pharmacognostical studies are suggested to confirm the antioxidant ability before going for commercialization.

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