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Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC-AQUEOUS EXTRACT POWDER (ROOT AND STEM) OF *SALACIA OBLONGA*

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ABSTRACT

Objective: *Salacia oblonga* Wall. (SO) is a woody climber, mainly distributed in Sri Lanka and Southern India. In spite of its antidiabetic and antioxidant potential, an incomplete data exists in relation to standardization of this plant in comparison to other *Salacia* species. The present study characterized the Methanolic-Aqueous (roots and stems) extract powder of SO and further evaluated the *in vitro* antioxidant potential.

Methods: The *in vitro* antioxidant activity of the extract was analyzed through Reducing Power assay, H_2O_2 Scavenging activity, Superoxide Radical Scavenging activity, and Nitric Oxide Radical Scavenging activity assays.

Results: The Phenolic, Flavonoid, Flavonol and Tannin content of SO were found to be 65.8 mg Gallic acid equivalent (GAE)/g, 8.89 mg GAE/g, 5.78 mg GAE/g and 83.6 mg GAE/g dry weight of sample.

Conclusion: Statistical analysis of the data revealed that, the total Phenolic and Tannin content of SO significantly enhanced it's Reducing Power, while the total Flavonoid content contributed significantly to the H_2O_2 and $O_2^{\cdot-}$ Scavenging activity. The Phenolic compounds of SO brought about significant scavenging of $NO^{\cdot}$ radical. The present study thus, proves the Methanolic-Aqueous extract of SO to be a good radical scavenger which might be considered as a natural source of antioxidants for medicinal and commercial uses.

Keywords: *Salacia oblonga*, Phytochemicals, Flavonoids, Tannins, Methanolic-Aqueous extract, Fluorescence

INTRODUCTION

Salacia oblonga (Family: Celastraceae) is very well known for its Antidiabetic nature. Hypoglycemic nature, antioxidant activity *etc*. of this plant have been worked upon a lot but, the characterization part i.e. the type of phytocompounds responsible for the antioxidant activity as well as, the *in vitro* antioxidant potential of a combination of roots and stems of SO in scavenging free radicals has not been reported yet. Moreover, literature shows that, the roots of *Salacia reticulata* and *Salacia oblonga* have been explored for its mechanism of action but, still the data remains inadequate. Other species of *Salacia* like *S. chinensis* has been comparatively well characterized and documented.

Antioxidant behavior of any compound depends upon neutralizing the oxidant behavior of any chemical or environment. Molecular oxygen in the ground state is a bi-radical in a triplet state and it provides energy to every aerobic organism [1]. During this process, excitation of one of the two unpaired electrons changes its spin and results in formation of singlet oxygen. The latter is a powerful oxidant as the two electrons with opposing spins can react with other pairs of electrons, especially double bonds [1]. The free radicals are thus, highly unstable and reactive atoms or molecules that have one or more unpaired electrons, thereby forming Reactive Oxygen Species (ROS) like superoxide ($\cdot O_2^-$), hydroxyl ($\cdot OH$), *etc*. and Reactive Nitrogen Species (RNS) like nitric oxide ($\cdot NO$), peroxynitrite ($ONOO^-$) *etc* [2]. An uncontrolled increase in the steady-state concentration of these oxidants generates the vicious free radical-mediated chain reactions, thereby causing 'oxidative stress' in biological systems [1]. Plant derived antioxidants (mainly the Phenolics and Flavonoids) have proved to be beneficial in imparting human health and prevents this oxidative stress associated with the etiopathogenesis of several diseases. It is important to note that a mixture of phenolic compounds may provide better antioxidant activity in comparison to a single phenol, as they may bring about a cooperative and modified biological activity.

The genus *Salacia* (Family: Celastraceae) comprises of several medicinally important species (*S. oblonga, S. reticulata, S. chinensis, etc*.) and is known as 'Saptrangi' in Ayurvedic medicine [3]. This herb has been reported in Ayurveda as a treatment for Madhumeha (ancient name of Diabetes) and is distributed in Sri Lanka, India, China, Malaysia and other countries [4]. But certain reports have red listed *Salacia oblonga* (SO) and *Salacia reticulata* as endangered species [5-6]. The root bark is used by either boiling in oil as a decoction or as powder for the treatment of Rheumatism, Itches, Asthma and Ear diseases along with Diabetes and Obesity [7]. Since 1960s, scientists have tried to explore the hypoglycemic potential of various extracts of roots, root bark, and stems of SO in diabetic models [8-10]. SO also possesses hypolipidemic, anti-inflammatory, anti-oxidant, and pancreatic lipase inhibitory activities [3,11]. Several bioactive compounds like Mangiferin, Salacinol, Kotalanol, Sesquiterpene alkaloids, Tannins like Epicatechin, and Pentacyclic triterpenes have already been isolated from *Salacia* species [11-12]. Besides these compounds, much interest is focused into the antidiabetic activity of Mangiferin, the polyphenolic constituent of *Salacia*. The antioxidant potential of Aqueous-Methanolic extract of stems of *Salacia chinensis*, Petroleum Ether extract of roots of SO, and Mangiferin from roots of *Salacia reticulata* have already been reported [13].

Our study is thus, an attempt to contribute to a selective analysis of this aspect of Methanolic-Aqueous (root and stem) extract of *Salacia oblonga* through various *in vitro* antioxidant activity assays. We have already carried out the preliminary phytochemical analysis, using various biochemical tests and techniques like TLC and HPTLC on the Methanolic, Ethanolic, Ethyl acetate, Chloroform, Petroleum ether, and Aqueous extracts of *Salacia oblonga* (accepted for publication). The study showed the presence of significant amount of marker compound Mangiferin as well as, several other polyphenolic phytocompounds in the Methanolic and Ethanolic extracts of SO. The Methanolic extract of SO showed a maximum yield of Mangiferin whereas, the Aqueous extract showed the presence of Mangiferin and a highly non-polar compound that might have a significant effect on the antioxidant property of SO.

MATERIALS AND METHODS

All the analytical grade reagents *viz*. Folin-ciocalteu reagent, Sodium carbonate (Na_2CO_3), Gallic acid, Sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), Sodium acetate, Ferric chloride ($FeCl_3$), Ethylenediaminetetraacetic acid (EDTA), and Riboflavin were purchased from Central Drug House (P) Ltd., New Delhi. Other reagents like Aluminium chloride ($AlCl_3$), Potassium ferricyanide {$K_3Fe(CN)_6$}, Trichloroacetic acid, and Hydrogen peroxide (H_2O_2) were purchased from Qualigens Fine Chemicals, Mumbai. The

Nitroblue tetrazolium (NBT) and Ascorbic acid were procured from Himedia Laboratories, Mumbai; Sodium nitroprusside from SRL Limited, New Delhi; and Griess reagent from Sigma-Aldrich Co., India.

Plant material

The SO powder was purchased from Natural Remedies Private Limited, Bangalore, India. It contains a mixture of roots and stems of *Salacia oblonga* that were extracted with methanol and water in the ratio of 10 : 1, and obtained as a brown powder.

Quantitative phytochemical analysis of Methanolic extract of SO

The HPTLC analysis of six extracts (Methanolic, Ethanolic, Ethyl acetate, Chloroform, Petroleum ether, and Aqueous) of *Salacia oblonga* in comparison to the marker compound Mangiferin, proved the Methanolic and Ethanolic extracts to possess a good number of phytocompounds that might be of significance as antioxidants in the living system. Scientists have shown that 80% Aqueous-Methanolic extract of *Salacia chinensis* exerts hypoglycemic effects, gastroprotective effects, α-glucosidase and aldose reductase inhibitory activities, nitric oxide production inhibitory effects, and antioxidative activity [14]. The Water extract of *Salacia oblonga* roots have shown strong α-glucosidase inhibitory activity *in vitro* [15].

In view of such medicinal value of *Salacia* species, we have attempted to investigate the amount of Phenolic, Flavonoid and Tannin content as well as, the antioxidative property of the Methanolic-Aqueous extract of SO (roots and stems) through several *in vitro* antioxidant activity assays. The SO powder (roots and stems) was suspended in Methanol and the extract (SOE) obtained was used for all experimental procedures.

Determination of total Phenolic content

The total Phenolics in SOE were determined by the spectrophotometric Folin Ciocalteu method [16-17]. In this assay, the extract (10 - 300 µg/ml) was mixed with Folin-ciocalteu reagent (1 : 10) followed by addition of Na_2CO_3 (0.7 M). The tubes were vortexed for 15 s and incubated at room temperature for 1 h for development of blue colour. Absorbance was measured at 765 nm. The quantification of total Phenolics was done using a calibration curve obtained from measurement of absorbance of Gallic acid in the concentration range of 10 - 300 µg/ml in methanol.

Determination of total Flavonoid content

The total Flavonoid content of SOE was measured by the Aluminium Chloride colorimetric method [18-19]. The extract of SO (100 - 1000 µg/ml) was mixed with 5% $NaNO_2$ and incubated for 5 min at 25ºC. The reaction mixture was then mixed with 10% ethanolic solution of $AlCl_3$ and incubated for another 5 min at room temperature. Finally, 1 mM NaOH and distilled water was added and absorbance of the reaction mixture was measured at 510 nm. The calibration curve was prepared using Gallic acid in the concentration range of 100 - 1000 µg/ml in methanol.

Determination of total Flavonols

The total Flavonols in SOE were estimated using the method of Kumaran and Karunakaran [17,20]. 100 - 1000 µg/ml of SOE was mixed with 2% $AlCl_3$ (ethanol solution) and sodium acetate (50 mg/ml). The reaction mixture was incubated at 20ºC for 1 h for development of yellow colour, and the absorbance was read at 440 nm. The amount of Flavonols present in the extract was calculated using the same formula as that of flavonoids.

Determination of total Tannin content

Initially, the Methanolic-Aqueous extract powder of SO was made to react with 1 drop of 5% $FeCl_3$ solution. The appearance of greenish black colour indicated the presence of condensed form of Tannins in the plant extract. Further, the quantitative estimation of total Tannins in SOE was done by the method of Price and Butler [21]. The extract (0.25 - 2.5 µg/ml) was mixed with 1% $K_3Fe(CN)_6$ and 1% $FeCl_3$. The reaction mixture was vortexed, kept for 5 min at room temperature for colour development, and absorbance was read at 720 nm. The calibration curve was prepared using Gallic acid in the concentration range of 0.25 - 2.5 µg/ml and the total Tannin content was expressed as mg Gallic acid equivalent/g dry weight of plant extract.

In vitro antioxidant activity assays

Reducing power assay

The Ferric Reducing Power of SOE was determined using the FRAP method [22-23]. Different concentrations of the plant extract (5 - 200 µg/ml) were mixed with phosphate buffer (0.2 M, pH 6.6) and 1% $K_3Fe(CN)_6$. The reaction mixture was incubated for 20 min at 50ºC followed by rapid cooling of the solution. 10% trichloroacetic acid was added to this mixture to stop the reaction. This was followed by addition of distilled water and 0.1% $FeCl_3$ and the reaction mixture was allowed to stand for 30 min at room temperature. The absorbance was measured at 700 nm and the activity was reported as O. D. values in comparison to the reference standard Gallic acid.

H_2O_2 Radical Scavenging activity

The Hydrogen Peroxide Radical Scavenging activity of SOE was determined by the method of Ruch *et al* with slight modification [16,22]. The plant extract in the concentration range of 50 - 800 µg/ml was made to react with H_2O_2 (30 mM) solution for 10 min at room temperature. Absorbance of hydrogen peroxide left out in the reaction mixture was measured at 230 nm. The percentage of H_2O_2 scavenged by SOE and the standard Ascorbic acid was calculated using the following formula:

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{\text{Abs (control)} - \text{Abs (test)} \times 100}{\text{Abs (control)}}$$

Superoxide Radical Scavenging activity

The Superoxide Radical Scavenging activity of Methanolic extract of SO was determined by using the riboflavin-light-NBT system as reported in several studies, but with slight modifications [23-24]. The reaction mixture initially contained SOE (50 - 300 µg/ml), phosphate buffer (20 mM, pH 7.6), EDTA (0.1 M), 9 µl nitroblue tetrazolium (NBT), followed by mixing and incubation for 10 min in dark at room temperature. The superoxide anions were generated in the samples by the photochemical reduction of riboflavin for 15 min. Finally, the reduction of NBT to blue formazan (NBT^{2+}) was measured at 570 nm. Ascorbic acid was used as standard. The Superoxide Anion Radical Scavenging activity of the plant extract was calculated using the formula:

$$\text{\% inhibition} = \frac{\text{Abs (control)} - \text{Abs (test)} \times 100}{\text{Abs (control)}}$$

Decreased absorbance of the reaction mixture indicates increased Superoxide Anion Radical Scavenging activity.

NO^- Radical Scavenging activity

The Nitric Oxide Radical Scavenging activity of SOE was determined by the method of Garrat, 1964 [22,25]. The assay involves spontaneous generation of nitric oxide from sodium nitroprusside in aqueous solution at physiological pH, which interacts with oxygen to produce nitrite ions. These ions further undergo diazotization through the Griess Illosvoy reaction to give a pink colour. The reaction is started by addition of SOE in the concentration range of 5 - 100 µg/ml, PBS (20 mM) and sodium nitroprusside, mixing and incubation for 2 h at 27ºC. From the incubated mixture, 100 µl was added to Griess reagent (N-1-napthylethylenediamine dihydrochloride, H_3PO_4, and sulfanilamide diozonium salt) and incubated at room temperature for 20 min. Ascorbic acid was used as positive control. The absorbance of the chromophore was read at 540 nm and the percentage NO^- scavenging activity by SOE and standard was calculated using the formula:

$$\text{\% } NO^- \text{ scavenging activity} = \frac{\text{Abs (control)} - \text{Abs (test)} \times 100}{\text{Abs (control)}}$$

The NO^- scavenging activity of SO was compared to that of Ascorbic acid in terms of IC_{50} value.

RESULTS AND DISCUSSION

Several *in vivo* models have shown SO to have very good antioxidant potential [26] but its instinct antioxidant potential due to merely its own chemical entities, has not been characterized so far. The preliminary phytochemical analysis performed previously by us, showed that the Methanolic, Ethanolic, and Ethyl acetate extract of SO possesses considerable amount of active compound Mangiferin, as shown in Table 1.

Table 1: The percentage yield of crude extract and the active compound Mangiferin in six different extracts of *Salacia oblonga* (roots)

Extraction solvent											
Methanol		**Ethanol**		**Ethyl acetate**		**Chloroform**		**Petroleum ether**		**Water**	
Crude ext.	Mang.	Crude ext.	Mang.	Crude ext.	Mang.	Crude ext.	Mang.	Crude ext.	Mang.	Crude ext.	Mang.
8.6	1.2	9.6	1.18	11.2	1.28	6.2	0.62	26.3	0.02	14.15	0.38

Mang. = Mangiferin

As reported in case of *Salacia chinensis* [27], and the HPTLC analysis performed by us on six different extracts of SO, the Methanolic-Aqueous extract of SO was chosen and subjected to different quantitative analysis and antioxidant activity assays. A combination of the two plant parts (roots and stems) of SO was chosen, thereby expecting a better pharmacological effect, and also taking the issue of plant conservation into consideration.

Statistical Analysis

The data were analyzed using correlation coefficient and one way ANOVA analysis functions.

Total Phenolic content

Phenolics offer a wide distribution in the plant kingdom and form the most abundant secondary metabolite of plants [28]. The plant phenolics comprises of phenolic acids like Gallic acid (a derivative of benzoic acid), Flavonoids, Tannins, Stilbenes and Lignans. The polyphenols modulate the activity of a wide range of enzymes and cell receptors, thus interfering with basic cellular functions like cell cycle, apoptosis etc. [28]. The phenolics have hydroxyl groups that serve as hydrogen donors. They exhibit their powerful antioxidant activity by scavenging radical species such as ROS/RNS, suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical formation, and up-regulating or protecting antioxidant defense system [28]. The daily intake of about 1 g of polyphenolic compounds in diet rich in fruits and vegetables is presumed to provide good antioxidant potential to the body [16]. The total Phenolic content of *Salacia oblonga* (root and stem) was 65.8 ± 0.01 mg Gallic acid equivalents/g dry weight of plant extract.

Total Flavonoid content

This group of phytocompound forms the most abundant polyphenolic section of our diets. The flavonoid structure contains a flavan nucleus with 15 carbon atoms arranged in three rings (C6-C3-C6) and are classified into six subgroups based on oxidation state of the central C ring [28]. The total Flavonoid content of *Salacia oblonga* (root and stem) was 8.89 ± 0.02 mg Gallic acid equivalents/g dry weight of plant extract.

Total Flavonol content

The Flavonols belong to the polyphenolic group of phytocompounds present in plant extracts and food samples. The total Flavonol content of SO was 5.78 ± 0.02 mg Gallic acid equivalents/g dry weight of plant extract.

Total Tannin content

The Tannins form a major group of polyphenols in our diets and are classified into two groups namely hydrolysable tannins and condensed tannins [28]. On treating SOE with a drop of 5% $FeCl_3$, the reaction mixture turned into a greenish black colour indicating the presence of condensed tannins in this plant species. The total Tannin content of SOE was 83.6 ± 0.01 mg Gallic acid equivalents/g dry weight of plant extract.

The total phenolic, total flavonoid, total flavonol, and total tannin content of Methanolic-Aqueous extract power of SO (roots and stems) is summarized in Table 2.

Table 2: Total phenolic, total flavonoid, total flavonol, and total tannin content of Methanolic-Aqueous extract of *Salacia oblonga* (roots and stems)

Total phenolics (mg/g)	Total flavonoids (mg/g)	Total flavonols (mg/g)	Total tannins (mg/g)
65.8 ± 0.01	8.89 ± 0.02	5.78 ± 0.02	83.6 ± 0.01

Results expressed as mg Gallic acid equivalents/g dry weight of plant extract.

Values are the average of triplicate readings, and expressed as mean ± SD

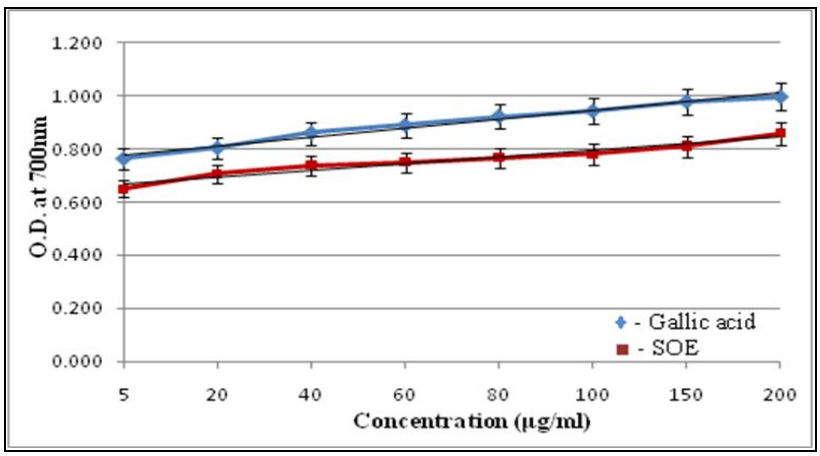

Fig. 1: The reductive ability of *Salacia oblonga* extract and the standard Gallic acid

Reducing power assay (FRAP method)

The reducing capacity of a compound (such as those present in phyto-extracts) serves as a significant indicator of its potential antioxidant activity. The FRAP (ferric reducing antioxidant power) assay is an electron transfer-based assay that measures the change in colour due to reduction of an oxidant probe by an antioxidant in the sample, to the ferrous form. The amount of Fe^{2+} complex formed can be measured according to the colour change (Perl's Prussian blue colour) which is directly proportional to the antioxidant concentration in acidic pH like pH 3.6 [28]. Statistical analysis showed strong correlation between the total reduction abilities of both Gallic acid and SOE. The increasing absorbance of the reaction mixture at 700 nm (Figure 1) exhibited a dose dependent increase in their activity. The total phenolic (R^2 = 0.957, $p < 0.001$) and total tannin (R^2 = 0.949, $p \leq 0.001$) content of SOE were found to significantly contribute to the Reducing Power of the plant.

H_2O_2 Scavenging activity

Hydrogen peroxide is itself not a very reactive compound, but on being oxidized inside the cells, gets converted to highly reactive hydroxyl radicals. In this assay, the IC_{50} value of the standard Ascorbic acid (195 µg/ml) was less than SOE (380 µg/ml) (Figure 2) thus indicating SOE to be a moderate scavenger of H_2O_2. Moreover, statistical analysis showed that the total Flavonoid content (R^2 = 0.949, $p \leq 0.001$) of SOE significantly contributed to its Hydrogen Peroxide Scavenging activity.

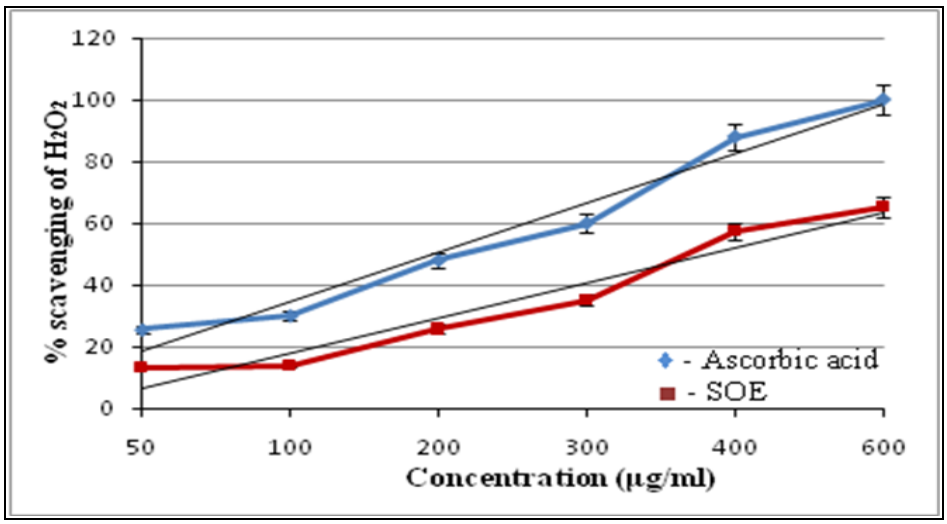

Fig. 2: H_2O_2 scavenging activity of *Salacia oblonga* extract and the standard Ascorbic acid

Superoxide Radical Scavenging activity

The purine nucleotides and bases obtained as a result of DNA and RNA degradation as well as hypoxanthine are metabolized by the flavoenzyme xanthine oxidase that converts hypoxanthine to uric acid through a superoxide radical generating enzymatic step [29]. These anion radicals serve as a good source of reactive oxygen species (ROS) in the endogenous system. In this assay, the O_2^{-} generated by the photochemically reduced riboflavin, further reduced NBT to blue formazan. As a result, the decrease in absorbance at 570 nm with the antioxidants in the sample indicates the consumption of O_2^{-} in the reaction mixture. This indicates a dose-dependent increase in superoxide radical scavenging activity of SOE. SOE (IC_{50} value of 186 µg/ml) showed a comparative Superoxide Radical Scavenging activity with respect to the standard Ascorbic acid (IC_{50} value of 117 µg/ml) (Figure 3). Statistical analysis proved the total flavonoids to significantly (R^2 = 0.887, $p \leq 0.05$) contribute to the Superoxide Radical Scavenging activity of SOE.

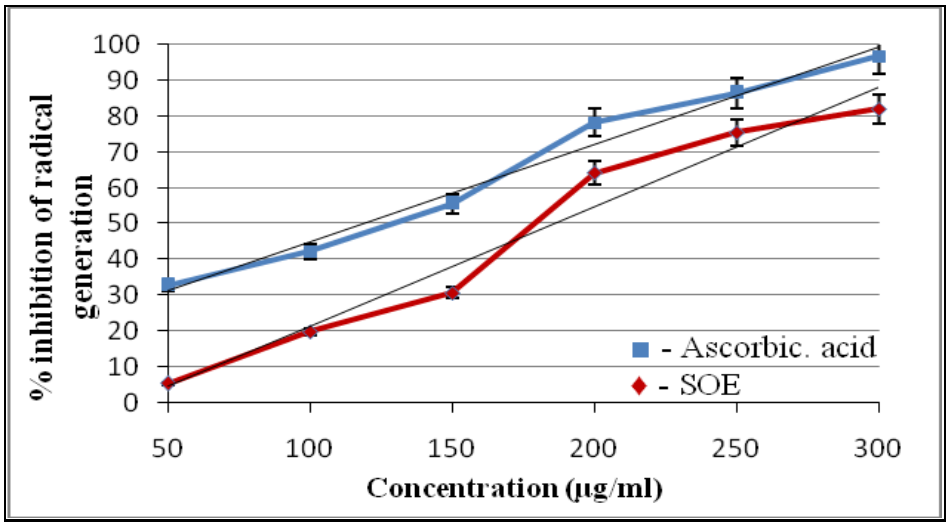

Fig. 3: Superoxide Radical Scavenging activity of *Salacia oblonga* extract and the standard Ascorbic acid

NO^- Radical Scavenging activity

Nitric oxide (NO) is an unstable species under aerobic condition and is a potent pleiotropic mediator in physiological processes. It is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes like NO synthases (NOS) [30]. NO reacts with superoxide anion to form peroxynitrite ($ONOO^-$) whose protonated form is a strong oxidant. It can pass in and out of cells during pathological conditions and cause damage to cells through nitration or hydroxylation of aromatic compounds. The peroxynitrite also forms an adduct with CO_2 dissolved in body fluid, thereby causing damage to body proteins [16]. SOE showed a concentration dependent NO scavenging activity that reached a peak at 80.76% at 1000 µg/ml and was almost near to that of standard Ascorbic acid. The extract did not show better ability to scavenge nitric oxide (IC_{50} value of 690 µg/ml) as compared to the reference compound Ascorbic acid (IC_{50} value of 250 µg/ml) (Figure 4). But, the total Phenolic content of SOE provided statistically significant ($R^2 = 0.971$, $p < 0.001$) contribution towards Nitric Oxide Scavenging activity of the plant.

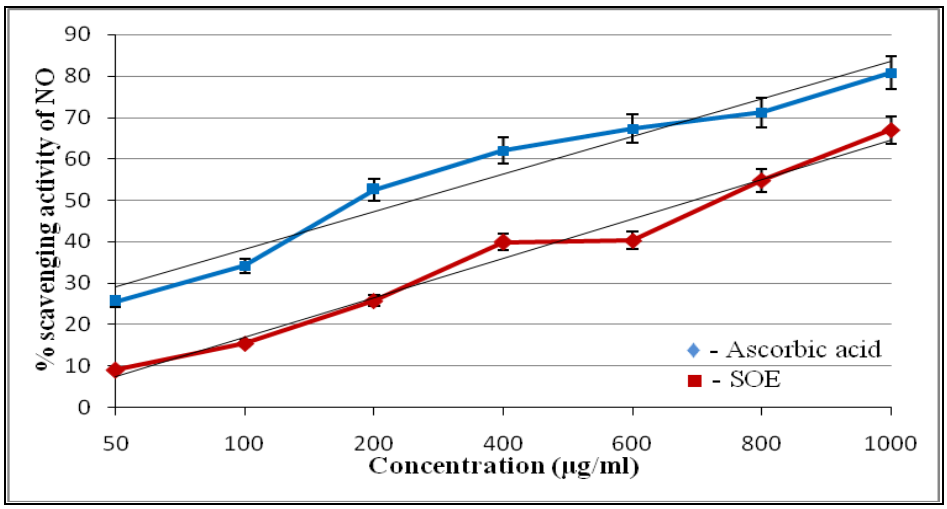

Fig. 4: Nitric Oxide Radical Scavenging activity of *Salacia oblonga* extract and the standard Ascorbic acid

The overall results of % inhibition of the radical scavenging assays with respect to IC_{50} values and regression r^2 (values are a mean of data set n = 3) values are shown in Table 3.

Table 3: Antioxidant potential of a combination of roots and stems of *Salacia oblonga*

Assays	Samples	Equation	r^2 values	IC_{50} values (µg/ml)
Reducing Power assay	Standard Gallic acid	y = 0.033x + 0.744	0.979	-
	SOE	y = 0.032x + 0.618	0.938	-
H_2O_2 Scavenging activity	Standard Ascorbic acid	y = 15.95x + 2.733	0.967	195
	SOE	y = 11.41x - 4.781	0.943	380
Superoxide Radical Scavenging activity	Standard Ascorbic acid	y = 13.58x + 17.79	0.966	117
	SOE	y = 16.69x - 12.18	0.943	186
NO^- Radical Scavenging activity	Standard Ascorbic acid	y = 9.083x + 19.88	0.847	250
	SOE	y = 9.536x – 2.100	0.954	690

CONCLUSION

The results from this study indicate that a combination of the roots and stems of *Salacia oblonga* possess considerable amount of Phenolic compounds along with other phytocompounds like Tannins and Flavonoids. They are found to significantly enhance the antioxidant potential of SO in fighting against ROS and RNS. It provides further evidence that, even though the plant has lesser amount of flavonoids in comparison to the polyphenolic compounds as a whole, yet both of them have contributed almost equally to the free radical scavenging activity as per statistical data analysis. The *in vitro* assays thus, indicate that the Aqueous-Methanolic *Salacia oblonga* extract powder is a valuable source of natural antioxidants, which can be used in preventing oxidative stress in biological systems. This emphasizes the future exploration of the active phytoconstituents of the roots and stems of this plant species towards scavenging of free radicals, prevention of oxidative stress, or enhancement of antioxidant levels in physiological conditions. Further evaluation of the antioxidant potential of the plant through *in vitro* as well as *in vivo* studies, is a must in order to know more about its mechanism of action. This might help in considering *Salacia oblonga* as a plant of choice for functional foods as well as, pharmaceutical plant-based products.

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