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INVESTIGATION OF PHYTOCHEMICALS AND ANTIOXIDANT POTENTIAL OF LEAF EXTRACT OF *Sesbania grandiflora* L. Pers

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ABSTRACT

Nowadays there is resurgence of interest in the potential health benefits of natural remedies like medicinal plants and their extracts. The presence of antioxidants such as phenolics and flavonoids in plants may provide protection against a number of diseases and have attracted much interest because of their ability to scavenge free radicals. Medicinal plants are therefore investigated for their antioxidant properties, and their demand is increasing worldwide. The present study was aimed to screen the phytochemicals as well as to evaluate the antioxidant potential of ethanolic extract of *Sesbania grandiflora* L. Pers leaves. Finely powdered leaves of *S.grandiflora* (white variety) were macerated using 95% ethanol. The ethanol extract was subjected to phytochemical screening and estimation of total phenol and flavonoid contents. *In vitro* antioxidant assays such as DPPH, Nitric oxide and Hydrogen peroxide radical scavenging assays were performed to determine the free radical scavenging activity. Qualitative phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, phenolics, tannins, steroids, anthraquinone glycosides, carbohydrates and proteins. Total flavonoid and phenol contents in 10 mg of extract were found to be 0.956 mg and 0.136 mg of standard quercetin and gallic acid respectively. The extract showed satisfactory scavenging in all the antioxidant assays with potent activity against hydrogen peroxide radical with an IC_{50} of 39.25 µg /ml. Antioxidant activity of the ethanol extract of *S.grandiflora* can be attributed to the presence of flavonoid and phenolic contents.

KEYWORDS: Free radicals, Reactive oxygen species, Phenolics, Flavonoids.

INTRODUCTION

Changing environmental conditions are giving rise to variety of free radicals, which plants have to deal, in order to survive. Reactive oxygen species, such as singlet oxygen, superoxide ion, hydroxyl ion and hydrogen peroxide, are highly reactive, toxic molecules, which are generated normally in cells during metabolism. An antioxidant can be defined as any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of molecules, by inhibiting the initiation or propagation of oxidizing chain reactions (Khatoon *et al*., 2013). The physiological role of antioxidants is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals (Young and Woodside, 2001). Free radical is constantly generated in all living cells as a part of normal cellular function, however, excess free radical originating from endogenous or exogenous sources are responsible for aging and causing various human diseases. A free radical is defined as any atom or molecule possessing unpaired electrons. Antioxidants are agents that scavenge the free radicals and prevent the damage caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS).Free radicals cause oxidative damage to different molecules, like lipids, proteins and nucleic acids and thus they are involved in the initiation phase of some degenerative diseases. Research has shown that free radical mediated oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, aging, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases and others. Antioxidants prevent free radicals from doing harm to our DNA, proteins and cells by donating electrons to stabilize and neutralize the harmful effects of the free radicals. This action helps in protecting the body from degenerative diseases. With that, the role of antioxidants has drawn much attention as a candidate to combat certain diseases and prevent the aging process. A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases (Kalita *et al*., 2013).

The plant, *Sesbania grandiflora* is a small growing, short-lived, white, soft-wooded tree belonging to the family *Leguminosae* and it is used for different treatment purposes. Tender leaves, flowers and pods are used as vegetable and considered as excellent source of vitamin C and calcium. *Sesbania* is a folk remedy for bruises, catarrh, dysentery, fever, headache, smallpox, sores, sore throat, and stomatitis. Also used for the treatment of anaemia,

bronchitis, ophthalmia, inflammation, leprosy, gout, and rheumatism. In addition, *Sesbania* is mentioned as a potent antidote for tobacco and smoking-related diseases (Ghani, 1998).

MATERIALS AND METHODS

CHEMICALS

DPPH (1, 1diphenyl-2-picryl hydrazyl radical) and Folin- Cio-calteau reagent were purchased from Sigma –Aldrich (Germany), Aluminium chloride were purchased from Merck (Germany). Gallic acid and ascorbic acid were purchased from Sigma–Aldrich (USA).

PLANT MATERIAL

Fresh healthy leaves of *S.grandiflora* L.Pers were collected from Cherthala, Alleppey district and were identified by Mr.Rojimon.P.Thomas, Department of Botany, CMS College, Kottayam .The voucher specimens of the plant were stored in Department of pharmaceutical sciences, Cheruvandoor. The leaves were washed and dried in solar drier under UV sheet at room temperature. The dried leaves were finely powdered and stored in air tight container in dark place for the extraction process.

100g of finely powdered, dried leaves of *Sesbania grandiflora* were soaked in 250 ml 95% ethanol and it is then allowed to stand at room temperature for 72 hours with frequent agitation. After three days, the mixture is strained by filtration, evaporated to dryness to a sticky mass at room temperature and stored in refrigerator for further use.

PRELIMINARY PHYTOCHEMICAL SCREENING

The preliminary phytochemical screening of the ethanolic extract of the leaves of *S.grandiflora* were carried out to determine qualitative contents of carbohydrates, alkaloids, saponins, flavonoids, tannins, sterols, proteins, terpenes, and anthraquinones. The experiments were performed according to method proposed by Khandelwal, 2007.

ESTIMATION OF TOTAL FLAVONOID CONTENT

Flavonoids are potent antioxidants and have aroused considerable interest recently because of their potential beneficial effects on human health in fighting diseases. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their free radical scavenging activities.

In this method, Quercetin was used to make the calibration curve. 10 mg of quercetin was dissolved in methanol and then diluted to 100, 200, 400, 800, and 1000µg/ml. A calibration

curve was made by measuring the absorbance of the dilutions at 415 nm (λ max of quercetin) with a UV spectrophotometer.

Preparation of test solution

10 mg of the extract was accurately weighed and made upto 1ml with DMSO (Stock solution).

0.5ml of extract stock solution, 1.5 ml methanol, 0.1 ml 10% aluminium chloride solution, 0.1 ml 1M potassium acetate solution and 2.8 ml distilled water were added and mixed well. Sample blank was prepared in similar way by replacing aluminium chloride with distilled water. All prepared solutions were filtered before measuring and their absorbance was measured at 415 nm. The experiment was carried out in triplicate and the total flavonoid content was estimated as mg equivalents quercetin per gram extract.

ESTIMATION OF TOTAL PHENOLIC CONTENT

Phenolic compounds are secondary plant metabolites that can counteract oxidative stress in the human body by maintaining a balance between oxidant and antioxidant substances. In the present study, total phenolic content of the extract was evaluated by Folin-Cio-calteau method using gallic acid as standard. Samples containing polyphenols are reduced by the Folin-Cio-calteau reagent by producing blue coloured complex. The experiment was carried out in triplicate and the total phenolic content was expressed in terms of gallic acid equivalent per gram dry weight of the extract.

For estimation, 10mg Gallic acid was weighed and made up to 1ml with methanol in a 10ml standard flask. From the stock solution (10mg/ml), solutions of concentration 100, 200, 400, 800 and 1000µg/ml were prepared. To the above solutions, 5ml of Folin Cio-calteau reagent was added and 4ml of 7.5% sodium carbonate solution was added after 5 minutes. It was stirred and incubated at room temperature for 2 hours. After 2 hours, absorbance of the solutions were measured at 750nm using uv-visible spectrophotometer. The absorbance values were plotted against concentration and standard graph was obtained.

Preparation of sample solution

100µl was pipetted out from extract solution and 5ml of Folin Cio-calteau reagent was added. After 5 minutes, 4ml of sodium carbonate solution was added and incubated at room temperature for 2 hours. Then, absorbance was measured at 750nm and the values obtained

were interpreted in the standard graph of Gallic acid to get the milligram equivalents of Gallic acid.

ANTIOXIDANT ASSAYS

DPPH RADICAL SCAVENGING ASSAY

The free radical scavenging activity of extract was determined by using DPPH assay according to Chang *et al.*, 2001. 1, 1 -diphenyl-2-picryl hydrazyl is a stable free radical with pink color which turns yellow when scavenged. The degree of discoloration indicates the free radical scavenging potential of the antioxidant compounds or extracts in terms of their hydrogen donating ability The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

DPPH + (H-A) → DPPH – H + (A)

In this assay, 0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml ethanol. Ascorbic acid (10mg/ml DMSO) was used as standard. Different volumes of extract (6.25 - 100µg/ml) from a stock concentration 10mg/ml were made up to a final volume of 20µl with DMSO and 1.48ml 0.1mM DPPH solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control. The same procedure was repeated with synthetic antioxidant, Ascorbic acid and free radical scavenging potential was calculated by the following equation.

Percentage inhibition = [(control absorbance - sample absorbance) / (control absorbance)] ×100

The concentration of sample providing 50% inhibition was calculated from the graph of percentage inhibition plotted against concentration of sample by using linear regression analysis.

NITRIC OXIDE RADICAL SCAVENGING ASSAY

Nitric oxide (NO) is an important bio regulatory molecule which has a number of physiological effects including control of B.P, neural signal transduction, platelet function, antimicrobial and antitumor activity in low concentrations. However during infections and inflammations, formation of NO is elevated and may bring some undesired deleterious effect. (Wink et al., 1993).

This assay is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions.

For estimation, Sodium nitroprusside ($5mmolL^{-1}$)in phosphate buffered saline pH 7.4,was mixed with different concentration of the extracts 20-100 µg /ml from a stock concentration of 10mg/ml and incubated at 25°C for 30minutes .A control without the test compound, but an equivalent amount of distilled water was taken. After 30minutes, 1.5mL of the incubated solution was removed and diluted with 1.5ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride).Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphthyl ethylene diamine dihydrochloride was measured at 546nm and the percentage scavenging activity was measured with reference to the standard.

Percentage inhibition = [(control absorbance - sample absorbance) / (control absorbance)] ×100

The tests were carried out in triplicates. The concentration of sample providing 50% inhibition was calculated from the graph of inhibition percentage plotted against concentration of sample.

HYDROGEN PEROXIDE RADICAL SCAVENGING ASSAY

Hydrogen peroxide is a non-radical reactive oxygen species having absorbance maxima at 230nm and it has the ability to penetrate cell membranes, inactivate enzymes by oxidation of thiol groups, and initiate lipid peroxidation (Zhang *et al.*, 2011).

In this assay, a solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). Different concentration of extracts 12.5 -200 µg/ml from a stock concentration of 10mg/ml was added to H_2O_2 solution (0.6 ml) and the absorbance was read at 230nm after 10 minutes.

Percentage inhibition = [(control absorbance - sample absorbance) / (control absorbance)] ×100

The tests were carried out in triplicates. The concentration of sample providing 50% inhibition (IC_{50}) was calculated from the graph of percentage inhibition plotted against concentration of sample.

RESULTS

- **Preliminary phytochemical screening**

Qualitative phytochemical analysis in the ethanolic extract of *S.grandiflora* leaves (EESG) shown the presence of various phytoconstituents like alkaloids, flavonoids, phenolic compounds, carbohydrates, proteins, anthraquinone glycosides, terpenoids, steroids and saponins. The results of phytochemical analysis were tabulated in table 1.

Table 1: Phytochemical screening of EESG.

Sl. No	Chemical category	Tests	Ethanol extract
1.	ALKALOIDS	Dragendorff's test Hager's test Wagner's test Mayer's test	- - + +
2.	GLYCOSIDES	Borntrager's test Modified Borntrager's test Baljet test Legal test	+++ +++ - -
3.	CARBOHYDRATES	Molisch's test Fehling's test Benedict's test Barfoed's test	+++ +++ - -
4.	FLAVONOIDS	Shinoda test Aqueous NaOH test	+++ +
5.	PHENOLICS & TANNINS	Lead acetate test Ferric chloride test	+ +
6.	PROTEINS AND AMINOACIDS	Millon's test Biuret test Ninhydrin test	++ + ++
7.	TERPENOIDS	Isoprenoid test	++
8.	STEROIDS	Salkowski test	++
9.	SAPONINS	Foam or froth test	++

+++ indicates presence of high amount of phytochemical, ++ indicates presence of moderate amount of phytochemical, + indicates presence of phytochemical and – indicates absence of phytochemical.

- **Estimation of total flavonoid content**

Total flavonoid content of ethanolic extract of *S.grandiflora* (EESG) was determined by Aluminium chloride colorimetric method using quercetin as standard. A standard graph was plotted between various concentrations and corresponding absorbances of standard quercetin. The absorbance values obtained for different concentrations of the standard quercetin are tabulated in table 2.

Table 2: Mean absorbance of different concentrations of the standard quercetin.

Concentration of quercetin (µg/ml)	Absorbance (415nm)
100	0.0275±0.002
200	0.0300±0.001
400	0.0720±0.001
800	0.1160 ±0.002
1000	0.1450 ±0.002

Absorbance expressed as Mean ± SD, n=3

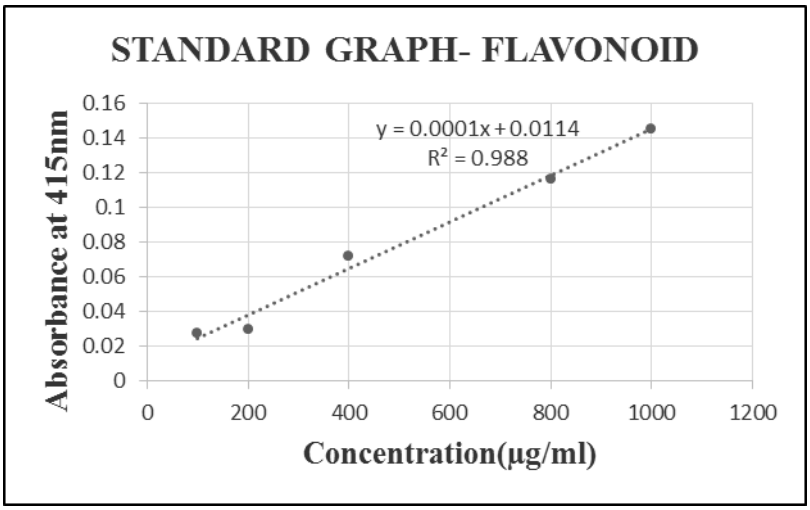


Fig 1: Calibration curve of quercetin.

Table 3: Estimation of total flavonoid content of EESG.

Absorbance of EESG(nm)	Gram equivalent of quercetin in 10mg sample
0.0685	0.956

The total flavonoid content in 10mg of EESG was found to be equivalent to 0.9560 mg of standard quercetin.

- **Estimation of total phenolic content**

Total phenolic content of EESG was determined by Folin Cio-calteau method using gallic acid as standard. A calibration curve for gallic acid was plotted using absorbance on the Y

axis and concentration on the X axis. Linear regression analysis were used to determine the concentration of EESG from the calibration curve The absorbance values obtained for different concentrations of standard are tabulated in table 4.

Table 4: Mean absorbance of different concentrations of the standard gallic acid.

Concentration of gallic acid (µg/ml)	Absorbance (750nm)
100	0.1801 ± 0.001
200	0.2712 ± 0.003
400	0.3500 ± 0.001
800	0.6806 ± 0.002
1000	0.8500 ± 0.001

Absorbance expressed as Mean ± SD, n=3

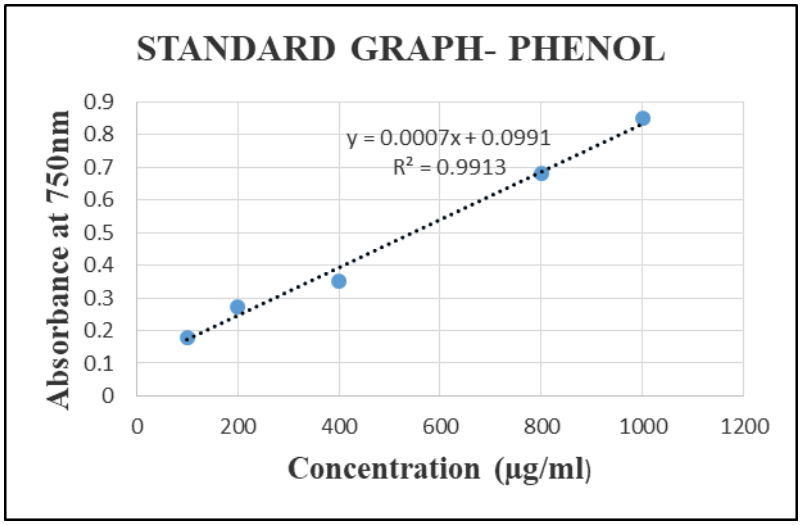


Fig 2: Calibration curve of gallic acid.

Table 5: Estimation of total phenolic content of EESG.

Absorbance of EESG(nm)	Gram equivalent of gallic acid per 10mg
0.0910	0.137mg

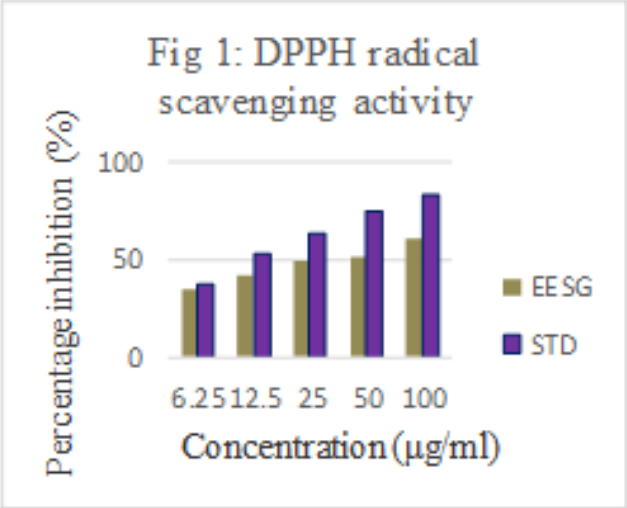
The total phenolic content in 10 mg of EESG was found to be equivalent to 0.137 mg of standard gallic acid.

- **Antioxidant Assays**

1. DPPH radical scavenging assay: The absorbance of different concentrations (6.25-100µg/ml) of standard (ascorbic acid) were measured at 517 nm and the percentage inhibition obtained were tabulated on table 6**.** A graph of concentration Vs percentage inhibition of DPPH free radical by EESG and standard ascorbic acid was plotted and shown in the fig 1.

Table 6: Mean % inhibition of standard ascorbic acid and EESG in DPPH assay.

Sample	Concentration µg/ml	Mean Absorbance ± SD	Percentage inhibition
	Control	0.813 ± 0.0001	
Standard: Ascorbic acid	6.25	0.513 ± 0.0001	36.9
	12.5	0.382 ± 0.0002	53.01
	25	0.297± 0.0001	63.46
	50	0.202± 0.0001	75.15
	100	0.139 ± 0.0001	82.9
IC_{50}	9.72 µg/ml		
	Control	0.827 ± 0.0002	
Ethanol extract of *S. grandiflora*	6.25	0.542 ± 0.0001	34.46
	12.5	0.476 ± 0.0001	42.44
	25	0.417± 0.0001	49.57
	50	0.405 ± 0.0001	51.02
	100	0.326 ± 0.0001	60.58
IC_{50}	48.88 µg/ml		

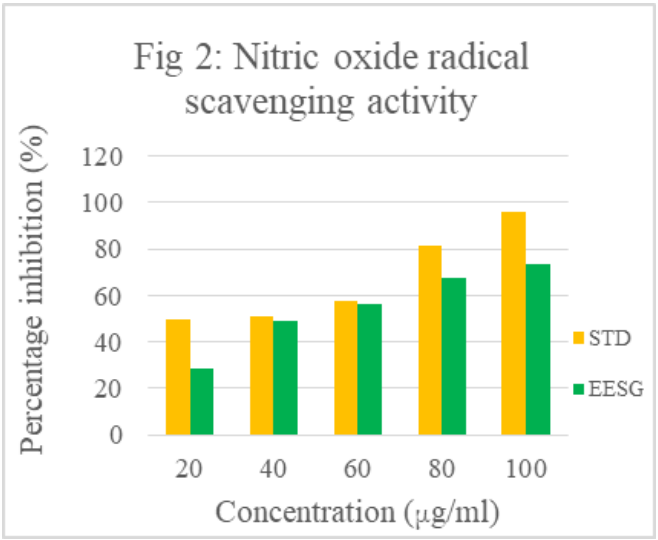


From the results of DPPH assay on EESG, a concentration dependent increase in percentage inhibition was observed. DPPH radical scavenging assay results an IC_{50} value of 48.88 µg/ml for EESG while standard ascorbic acid showed an IC_{50} of 9.72 µg/ml.

2. Nitric oxide radical scavenging assay - The absorbance of different concentrations (20–100µg/ml) of standard (gallic acid) were measured at 546 nm and the percentage inhibition obtained were tabulated on table 7. A graph of concentration Vs percentage inhibition of nitric oxide free radical by EESG and standard gallic acid was plotted and shown in the fig 2.

Table 7: Mean % inhibition of standard gallic acid and EESG in NO scavenging assay

Sample	Concentration µg/ml	Mean Absorbance ± SD	Percentage inhibition
	Control	0.819± 0.001	
Standard: Gallic acid	20	0.414 ± 0.0005	49.45
	40	0.399 ± 0.005	51.28
	60	0.346 ± 0.001	57.75
	80	0.154 ±0.0005	81.19
	100	0.031 ± 0.001	96.21
IC_{50}	32. 16 µg/ml		
	Control	0.815 ± 0.001	
Ethanol extract of *S. grandiflora*	20	0.582 ± 0.0001	28.5
	40	0.415± 0.0002	49.07
	60	0.357± 0.0001	56.19
	80	0.263 ± 0.0001	67.63
	100	0.214 ± 0.001	73.74
IC_{50}	50.78 µg/ml		

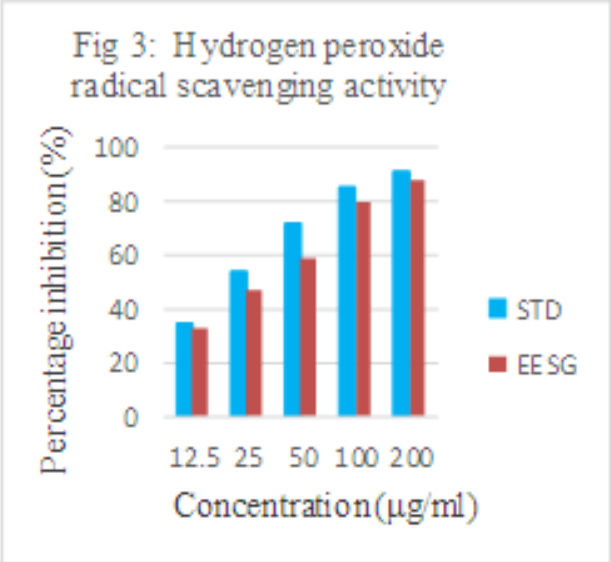


The results of NO free radical scavenging assay on ethanolic extract of *Sesbania grandiflora* L. leaves revealed a concentration dependent increase in percentage inhibition. NO free radical scavenging assay results an IC_{50} value 50.78 µg/ml for EESG while standard gallic acid shown an IC_{50} of 32.16 µg/ml.

3. Hydrogen Peroxide radical scavenging assay- The absorbance of different concentrations (12.5–200µg/ml) of standard (ascorbic acid) were measured at 230 nm and the percentage inhibition obtained were tabulated in table 8 .A graph of concentration Vs percentage inhibition of Hydrogen Peroxide free radical by EESG and standard ascorbic acid was plotted and shown in the fig 3.

Table 8: Mean % inhibition of standard ascorbic acid and EESG in H_2O_2 assay.

Sample	Concentration µg/ml	Mean Absorbance ± SD	Percentage inhibition
	Control	0.786 ± 0.0005	
Standard: Ascorbic acid	12.5	0.513 ± 0.0005	34.73
	25	0.362 ± 0.001	53.94
	50	0.224 ± 0.001	71.5
	100	0.117 ± 0.001	85.11
	200	0.068 ± 0.001	91.34
IC_{50}	10.90 µg/ml		
	Control	0.762 ± 0.001	
Ethanol extract of *S. grandiflora*	12.5	0.512 ± 0.0001	32.8
	25	0.403 ± 0.0002	47.11
	50	0.314 ± 0.0001	58.79
	100	0.194 ± 0.0001	74.54
	200	0.132 ± 0.001	82.67
IC_{50}	39.25 µg/ml		



From the results, a concentration dependent increase in percentage inhibition was observed. At 200 µg/ml, the extract showed maximum inhibition of 82.67 %. H_2O_2 free radical scavenging assay results an IC_{50} value 39.25 µg/ml for EESG while standard ascorbic acid shown an IC_{50} of 10.90 µg/ml.

DISCUSSION

Over the past decades, there has been considerable interest in the investigation of medicinal plants as a source of potential drug candidate, with lesser side effects.

Qualitative phytocompounds were screened in *S.grandiflora* leaves, according to the results alkaloids, flavonoids, terpenoids, phenolics, tannins, steroids, anthraquinone glycosides, carbohydrates and proteins were present.

As the results of preliminary phytochemical screening shown the presence of phenols and flavonoids, total flavonoid and phenolic content estimation was also performed in ethanolic extract of *S.grandiflora* leaves .The total flavonoid content of EESG was determined by using Aluminium chloride colorimetric method. The total flavonoid content in 10mg of EESG was found to be equivalent to 0.9560 mg of standard quercetin. The total phenolic content in EESG was determined by Folin Cio-calteau method and 10mg of EESG was found to be equivalent to 0.1365 mg of standard gallic acid. Phenolics are secondary plant metabolites which possess a wide range of therapeutic uses such as antioxidant, anti-mutagenic, anti-carcinogenic and free radical scavenging activities while flavonoids are a group of phenolic compounds which exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, antiulcer, anti-allergic, anti-viral, free radical scavenging and anticancer activities.

In this study, free radical scavenging activity of EESG was determined by various *in vitro* assay models such as DPPH free radical, NO free radical and H_20_2 free radical scavenging assays. Free radicals are unstable chemical species that cause damage to lipid cells, proteins and DNA as a result of imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzyme. Some examples of these free radicals are superoxide anions, hydroxyl, nitric oxide and hydrogen peroxide radicals. These free radicals may oxidize nucleic acids, proteins, lipids and DNA and can initiate many neuro degenerative diseases Antioxidants are compounds that are capable of either delaying or inhibiting the oxidation process which occurs under the influence of atmospheric oxygen / reactive oxygen species
DPPH assay is widely used as a preliminary test that provides information on the reactivity of test compound with a stable free radical since odd electron of DPPH gives strong absorption band at 517nm and when it is quenched by the extract, there is a decrease in absorbance. EESG showed anti-radical activity in scavenging DPPH radical with a maximum inhibition of about 60.58 % at a concentration of 100 µg/ml. IC_{50} values of standard ascorbic acid was found to be 9.72 µg/ml while that of EESG was found to be 48.88µg/ml.

NO is a free radical with a single unpaired electron. NO itself is not a reactive free radical, however overproduction of NO is involved in ischaemic perfusion, neurodegenerative and

chronic inflammatory diseases. In this assay, significant NO radical scavenging activity was shown by EESG in a concentration dependent manner with .highest inhibition of 73.74% at the concentration of 100 µg/ml. IC_{50} values of standard gallic acid was found to be 32.16 µg/ml and EESG was found to be 50.78 µg/ml.

Although H_2O_2 is not a highly reactive molecule, it can sometimes be toxic to cells because it may give rise to hydroxyl radicals and singlet oxygen by reacting with transition metal ions (Halliwell and Gutteridge, 1999). Scavenging of H_2O_2 by plant extracts may be attributed to their phenolics, that donate electrons to H_2O_2 thus reducing it to water. EESG showed potent H_2O_2 radical scavenging activity with a maximum inhibition of about 82.67 % (compared to standard ascorbic acid) at a concentration of 200 µg/ml. IC_{50} values of standard ascorbic acid was found to be 10.90 µg/ml and EESG was found to be 39.25µg/ml.

CONCLUSION

The medicinal properties of plants have been investigated in the recent scientific developments due to their potent therapeutic efficacy and antioxidant activities, lesser side effects and economic viability**.** The present study revealed that ethanolic extract of *S.grandiflora* leaves exhibited free radical scavenging effect in all the scavenging assays and use of this plant would exert several beneficial effects by virtue of its antioxidant activity. Antioxidant potential of the plant may be due to the presence of flavonoid and phenolic contents. Further works may be performed for the isolation and characterization of antioxidant component(s) in the plant.

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