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Invitro free radical scavenging activity of ethanolic root extract of *Saccharum spontaneum* Linn

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ABSTRACT

Under most pathological conditions there is generation of reactive oxygen species and other free radicals. An increase in the antioxidant reserves of the organism can reduce oxidative stress and some of the plant-derived agents may help to reduce it. The aim of this study was to evaluate *in vitro* antioxidant activity inhibition of superoxide, nitric oxide, hydroxyl, reducing power assay and total antioxidant capacity. At (10-50mg/ml) concentration the *S.spontaneum of* 50mg/ml concentration exhibited high superoxide, nitric oxide and hydroxyl radical scavenging capacity using vitamin E rutin and ascorbic acid as positive control. In the concentration range (1-5mg/ml) investigated, the extract demonstrated that reducing power and total antioxidant capacity were increased linearly with concentration. Collectively, our results indicate that the ethanolic root extract of *S.spontaneum has* the potential to scavenge free radicals and act as a good antioxidant for treating various diseases.

Introduction

An enormous variety of medicinal plants are used worldwide by about 80% of the world population, although in most cases no scientific studies have been done to prove the efficacy of these medicinal plants. Considering that most of the present- day western medicines are based on the traditional medicinal plants of European, Mediterranean and Arabic origin, the variety of plants in use around the world may very well represent an enormous treasure for drug(Verpoorte, 2000).

Oxidation is a basic part of the aerobic life and our metabolism. During oxidation, many free radicals are produced which have an unpaired nascent electron. Atoms of oxygen or nitrogen having central unpaired electron are called reactive oxygen or nitrogen species(Finkel and Holbrook, 2000; Halliwell, 2000; Pietta, 2000 and Visioli *et al*., 2000).This may be harmful to the body and may cause peroxidation of membrane lipids, aggression of tissue membranes and proteins or damage to DNA and enzyme(Husain *et al*., 1987). These can be related to some pathology, such as arthritis, haemorrhagic shock, coronary artery diseases, cataract, cancer, AIDS as well as age-related degenerative brain diseases (Parr and Bolwell, 2000). The immune system is vulnerable to oxidative stress. Oxidative stress refers to an imbalance between the production of free radicals and the antioxidant defense system. Reactive oxygen species (ROS) are various forms of activated oxygen which causes oxidative damage. Mechanisms responsible for the ROS-mediated injuries mainly include lipid peroxidation, oxidative DNA damage and protein oxidation (Bahramikia *et al*., 2009; Sfahlan *et al*., 2009).

Antioxidants are compounds that detoxify ROS and prevent their damage through multi mechanisms. Synthetic antioxidants have been in use as food additives for a long time, but reports on their involvement in chronic diseases have restricted their use in foods. Therefore, international attention has been focused on natural antioxidants mainly from plant sources(Dehghan *et al*., 2007; Kai-Wei *et al*., 2009). During certain diseased state, as well as during aging, there is a need to boost the antioxidant abilities, thereby potentiating the immune mechanism(Devasagaya and Sainis, 2002). The antioxidants preserve and stimulate the function of immune cells against homeostatic disturbances(De La Fuente and Victor, 2000).

In the human body the free radicals are continuously produced due to the oxygen utilization by the cells of the body. This generates a series of reactive oxygen species (ROS) like super oxide anion ($O2^{\cdot-}$) and hydroxyl ($HO\cdot$) radicals and non-free radical species such as H_2O_2, singled oxygen (O_2) and nitric oxide (NO) (Vilasrao *et al*., 2010). The free radicals are known to be scavenged by synthetic antioxidants, but due to their adverse side effects leading to carcinogenicity; search for effective and natural antioxidants has become crucial(Rao *et al*., 2010). Free radical reactions have been implicated in the pathology of many human diseases like atherosclerosis, ischemic heart disease, diabetes and neurodegenerative disease etc. and disease conditions like aging process, inflammation, immuno suppression etc. A number of plants and plant isolates have been reported to protect free radical-induced damage in various experimental models.

Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and thus prevent disease. In the present study, activity guided fractionation was adapted to identify the active fraction of this drug responsible for the antioxidant activity(Scartezzini and Speroni, 2000). In recent times, focus on plant research has increased all over the world and a large body of evidences has collected to show immense potential of medicinal plants used in various traditional systems(Modi *et al*., 2010). Plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains and other metabolites, which are rich in antioxidant activities (Aiyegoro *et al*., 2010). A number of plants and plant isolates

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have been reported to protect free radical induced damage in variousexperimental models(Himakar *et al*., 2010).

Materials and Methods

Phytochemical studies of *Saccharum spontaneum* Linn.

Collection of plant material

Saccharum spontaneum Linn. was collected from Koorappalayam, Erode district, Tamil Nadu, India during the month of September to November, 2011. The plant was identified and authenticated by taxonomist Dr.K. Arumugasamy, Assistant Professor, Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamilnadu, India. Voucher specimen was deposited in herbarium centre, Department of Botany, Kongunadu Arts and Science College, Coimbatore.

Super oxide radical scavenging activity

Superoxide radical scavenging activity of root extract was assayed by the method of [20].The reaction mixture contained EDTA (6µM), with 3µg NACN, riboflavin (2µM), NBT (2µM), KH_2PO_4 – Na_2HPO_4 buffer (67 mM, pH 7.8) and various concentrations of the extracts in a final volume of 3.0ml. The tubes were illuminated under incandescent lamp for 15 minutes. The optical density at 560 nm was measured before and after illumination. The inhibition of superoxide radical was determined by comparing the absorbance values of the control with those of the treatments. Ascorbic acid was used as a standard.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of root extract was assayed by the method of [21]. The reaction mixture (6.0ml) containing sodium nitro prusside (4.0ml), phosphate buffer saline (PBS, 1.0ml) and extract (1.0ml) in DMSO was incubated at 25°C for 15 minutes after incubation, 0.5ml of the reaction mixture containing nitrite was removed, 1.0ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 minutes for completion of diazotization and 1.0ml of naphthyl ethylene diamine dihydrochloride was added, mixed well and allowed to stand for 30 minutes in diffused light. A pink coloured chromophore was formed. The absorbance of these solutions was measured at 540 nm against corresponding blank solutions. Ascorbic acid was used as a standard.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of root extract was assayed by the method of [22] . The reaction mixture contained deoxyribose (2.8mM), FeCl3 (0.1mM), EDTA (0.1mM), H_2O_2(1mM), ascorbate (0.1mM), KH_2PO_4 - KOH buffer (20mM pH 7.4) and various concentrations of the sample extracts in final volumes of 1.0ml. The reaction mixture was incubated for 1 hr at 37°C. The extent of dioxyribose degradation was measured by TBA method. 1.0ml of TBA 1% (w/v) and 1.0ml of TCA 2.8% (w/v) were added to the mixture and heated in a water bath for 100°C for 20 minutes. The absorbance of resulting solution was measured spectrophotometrically at 532 nm. The inhibition of degradation was calculated according to the equation I= Ao-A1/Ao x100, where Ao is the absorbance of control reaction, A1 is the absorbance of test compound. Ascorbic acid was used as a standard.

Reducing power assay

Reducing power assay by the method of[23]. The reaction mixture contained 2.5 ml of various concentrations of methanol extract of the sample, 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture was incubated at 50° C for 20 min. and was terminated by the addition of 2.5 ml of 10% (w/v) of trichloroacetic acid , followed by centrifugation at 3000 rpm for 10 min. 5.0 ml of the supernatant upper layer was mixed with 5.0 ml of deionized water and 1.0 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicates increased reducing power of the sample.

Total antioxidant capacity

Total antioxidant capacity was measured by spectrophotometric method. 0.1ml of the extract (10mg/ml) dissolved in water was combined in an eppendorf tube with 1.0ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95º C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695nm against blank. Ascorbic acid was used as standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

Statistical analysis

Results were expressed as mean ± SD of six animals in each group. Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test.

Results and Discussion

Superoxide radical scavenging activity

Free radicals have been implicated in many disease conditions, the important one being superoxide radical. Superoxide anion is a weak oxidant; still it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress[24] (Chandha and Dave, 2003). The superoxide anion derived from dissolved oxygen by phenazine methosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The decrease in the absorbance at 560nm with the root extract thus indicates the consumption of superoxide anion in the reaction mixture. In the present study, the ethanolic extract of *S.spontaneum root* was found to scavenge the superoxides generated by photoreduction of riboflavin. The superoxide radical scavenging activities of root extract and ascorbic acid are represented in the figure1.

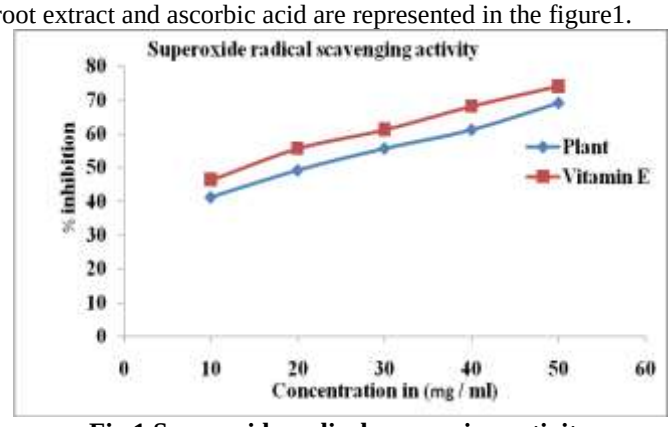

Fig.1 Superoxide radical scavenging activity

The activity was found to be increased in a dose-dependent manner from 41.29% to 69.15% for concentrations from 10 to 50 mg /ml. The extract exhibited an IC_{50} value of 22mg /ml. Therefore, the superoxide radical scavenging activity of ethanolic root extract of *S.spontaneum* indicates its ability to scavenge free radicals, thereby preventing lipid oxidation via a chain-breaking reaction. For the superoxide radical test, ascorbic acid scavenging activity increased with concentrations from 46.26 to 74.12 for concentrations from 10 to 50mg /ml. IC_{50} value was found to be 16 mg/ml. The scavenging effect also

increased in the dose dependent manner. The IC_{50} value indicates that vitamin E has better super oxide radical scavenging activity than that of the ethanolic root extract of *S.spontaneum*.

Our results were similar to that of Sreedhar *et al*.(2010) [25] who reported the *in vitro* studies on antioxidant and free radical scavenging activities of *Vitex trifoliateroot* extract was showed potential free-radical scavenging activity.

Nitric oxide radical scavenging activity

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases[26] (Ray and Hussain, 2002). It is well known that nitric oxide has an important role in various inflammatory processes. Sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis [27] (Bibhabasu *et al*.,2008). The toxicity of NO increases greatly when it reacts with super oxide radical, forming the highly reactive peroxy nitrite anion (ONOO-). The nitric oxide generated from sodium nitro prusside reacts with oxygen to form nitrite. The ethanolic extract *S.spontaneum* root inhibits the nitrite formation by directly competing with oxygen in the reaction with NO.[28] (Huie and Padmaja, 1993).

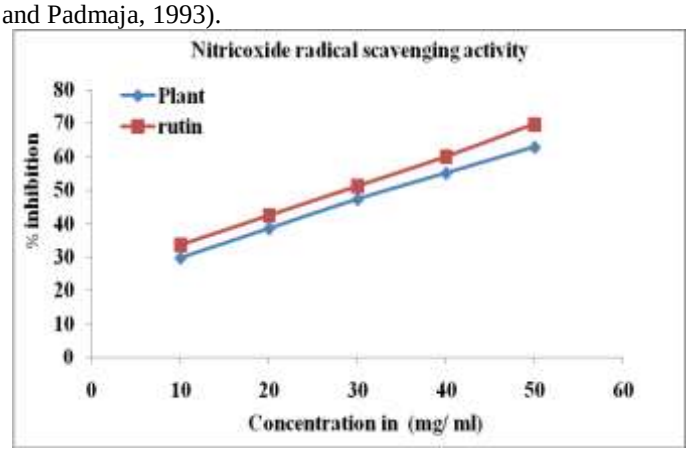

Fig.2 Nitric oxide radical scavenging activity

The scavenging activity was found to be increased in a dose-dependent manner from 29.68% to 62.89% at concentrations of 10 to 50 mg /ml. The extract exhibited an IC_{50} value of 34mg/ml. Therefore, the nitric oxide radical scavenging activity of ethanolic root extract of *S.spontaneum* indicates its ability to scavenge free radicals, thereby preventing lipid oxidation via a chain-breaking reaction. For the nitric oxide radical test, rutin is used as standard and its activitys increased with concentrations 33.56 % to 69.72% for concentration from 10-50mg /ml. IC_{50} values was found to be 28 mg/ml(Figure 2). The scavenging effect also increased in the dose dependent manner. The IC_{50} value indicates that rutin has better nitric oxide radical scavenging activity than that of the ethanolic root extract of *S.spontaneum*.

Our results are in agreement with the findings of Tyagi *et al*. (2010) [29] who reported *Flacourtia indica* leaves extract exhibitpotent free radical scavenging and antioxidant activity. The findings of the study suggest that *Flacourtia indica* leaves could be a potential source of natural antioxidant that could have great importance astherapeutic agents in preventing or slowing the progressof aging and age associated oxidative stress relateddegenerative diseases.

Hydroxyl radical scavenging activity

Hydroxyl radicals are very reactive and can be generated in biological cells through the Fenton reaction. Hydroxyl radical scavenging activity was quantified by measuring inhibition of the degradation of the deoxyribose by free radicals. Hydrogen peroxide itself is not very reactive; it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout the human systems [30] (Nabavi *et al*., 2008).

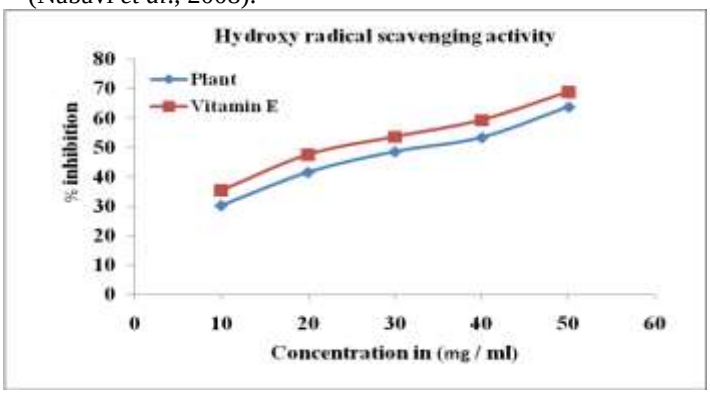

Fig.3 Hydroxyl radical scavenging activity

The activity was found to be increased in a dose-dependent manner from 30.26% to 63.82% at concentrations of 10-50 mg /ml. The extract exhibited an IC_{50} value of 35 mg /mL(Figure3). Therefore, the hydroxyl radical scavenging activity of ethanolic root extract of *S.spontaneum* indicates its ability to scavenge free radicals, thereby preventing lipid oxidation via a chain-breaking reaction. For the hydroxyl radical test, the activity of standard ascorbic acid increased with concentrations from 35.37%, to 68.96% scavenging activity for 10, 20, 30, 40, 50mg /ml. vitamin E. IC_{50} values was found to be 25mg/ml. The scavenging effect also increased in the dose dependent manner. The IC_{50} value indicates that vitamin E is better hydroxyl radical scavenging activity than that of the ethanolic root extract of *S.spontaneum*.

Our findings were similar to that of Surya *et al*. (2011)[31] who showed that the ethanolic extract of *Tabernaemontana coronaria* has the potential to scavenge free radicals and act as a good antioxidant for treating various diseases.

Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Li *et al*., 2008)[32].The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing ability is generally associated with the presence of reductones, which breaks the free radical chain by donating a hydrogen atom. The extract hadreductive ability which increased with increasing concentrations ofthe extract. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chaininitiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention ofcontinued hydrogen abstraction, reductive capacity and radical scavenging (Govind *et al*.,2011)[33].Figure 4 represents the reductive capabilities of the *S.spontaneum* root extract, which was compared with butylated hydroxyl toluene (BHT) standard. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of $Fe^{3+}$to $Fe^{2+}$by donating an electron. The results show that there was increase in absorbance and reducing of the

reaction mixture with increasing concentration of the root extract. The reducing power of a compound is related to its electron transferability and may serve as a significant indicator of its potential antioxidant activity. In this assay, the yellow color of the test solution changes to green and blue depending on the reducing power of test specimen. Greater absorbance at 700 nm indicated greater reducing power.

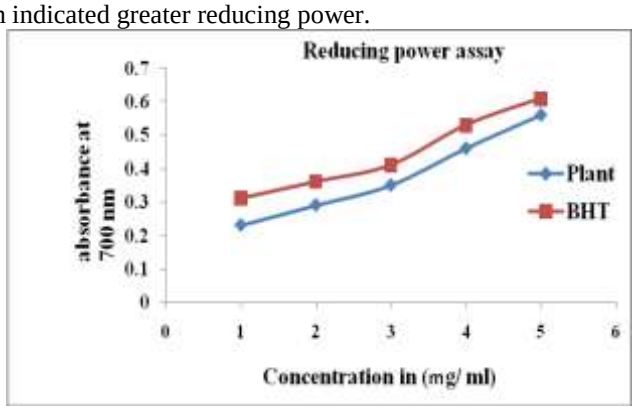

Fig.4 Reducing power assay

Figure 4 presents the reductive capabilitiesof the ethanolic extract root of *S.spontaneum*. In the concentration range investigated, the extract demonstrated reducing powerthat increased linearly with concentration. At concentration 1, 2, 3, 4 and 5 mg/ml, reducing power of ethanolic root extract was found to be 0.23, 0.29, 0.35, 0.46 and 0.56 nm respectively. Higher absorbance indicates high reducing power due to formation of reduced intermediate. Figure 4 shows the reductive capability of the plant extract compared to BHT For the reducing test, BHT was used as a standard were the activity was found to be 0.31 to 0.61 nm at a concentration of 1-5 mg /ml. From this present investigation, it can be concluded that *S.spontaneum* can be used as potent antioxidant for various oxidative stress induced disorders.

Total antioxidant capacity

The total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophtometrically at 695nm. The total antioxidant capacity is shown in the Figure5. The scavenging effect of the *S.spontaneum* root extract on the total antioxidant assay was 7.06 at 5mg /ml. Standard ascorbic acid showed the inhibition value of 7.89% at 5mg /ml. concentration.

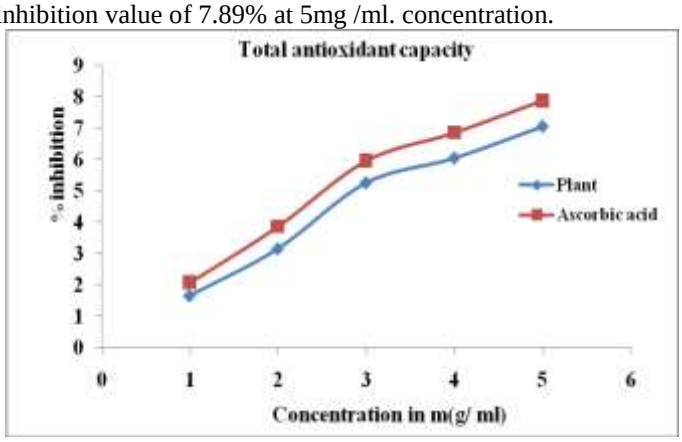

Fig.5 Total antioxidant capacity

The scavenging results reveal that the antioxidant activity of the extract exhibits increasing trend with the increasing concentration of the root extract. All the above scavenging activities of the ethanolic root extract showed the best antioxidant potential of the plant which is comparable to the standards. This might be helpful in preventing the progress of various oxidative stress related diseases.

Conclusion

This assay provided useful information on the reactivity of the compounds with stable free radicals, because of the odd number of electrons. The encouraging results of with the various *in vitro* antioxidant tests proved the *S.spontaneum* root as a reducing agent, its hydrogen donating ability and effectiveness as scavengers of superoxide, nitric oxide, hydroxyl radicals, reducing power assay and total antioxidant capacity. The results obtained in the present study indicate that *S.spontaneum* root extract exhibit potent free radical scavenging and antioxidant activity. This might be attributed to the presence of various phytoconstituents viz., alkaloids, flavonoids, tannins, steroids, terpenoids, glycosides and phenolic constituents. The findings of the present study suggest that *Saccharum spontaneum* root might be a potential source of natural antioxidant that could have great importance as therapeutic agents in disease prevention, health preservation and promotion of longevity promoter.

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