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Research Article

ANTI-OXIDANT ACTIVITY OF METHANOLIC EXTRACT OF C. SALIGNUS LEAVES BY DIFFERENT IN-VITRO ASSAYS

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ABSTRACT

In the present study free radical scavenging activity of methanolic leaf extract of *Callistemon salignus* was determined by five different in-vitro models, such as DPPH free radical scavenging activity, reducing activity , hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity and superoxide anion scavenging activity. For all the models Ascorbic acid was used as reference standard. Preliminary phytochemical study reveals the presence of glycosides, tannin, carbohydrate, volatile oil, flavonoid and steroid. Alterations of the absorbance due to free radicals were tested in control, test (*C salignus* extract), & also standard (stock solution of ascorbic acid) groups. Our findings suggested that, methanolic extract of *C salignus* leaves possessed highest antioxidant activity in dose dependant manner.

Key words: *Callistemon salignus* , Free radical, Anti-oxidant, *In-vitro* assays.

INTRODUCTION

Although oxygen is essential for the survival of all on this earth, molecular oxygen is toxic to virtually all forms of life and this toxicity become obvious on exposure to concentrations significantly greater than the atmospheric concentration and thus, it may produce threat to life. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Free radicals which are produced by oxidation can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are basically reducing agents such as thiols, ascorbic acid, or polyphenols[1].

Callistemon is a genus of around several species in the family Myrtaceae. Callistemon are commonly known as "bottlebrushes" because of the cylindrical, brush-like shape of the flower spike. Since this plant belongs to the same myrtaceae family as clove, eucalyptus, it is expected that it might also be a store- house of many chemicals of medicinal and pharmacological interest. Several research works on the various parts of the plant have been reported for their anti-thrombin [2], anti mycobacterium tuberculosis properties [3]. The literature review does not show any such investigations have been carried out in detail on the phytochemical and free radical scavenging nature of the leaves. That's why the aim of the present study is to carry out the free radical scavenging activity of the above said plant.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Callistemon salignus* were collected from Kolkata, West Bengal and identified by the Botanical Survey of India, Howrah.

Reagents and Chemicals

All the reagents and chemical were from S.D Fine Chemicals and Loba Chemie Pvt. Ltd. Mumbai, India and were analytical grade.

Preparation of Extract and Photochemical Study

The leaves were shade dried and the powdered leaves were macerated in methanol in a beaker for 48hrs. Occasional shaking and stirring was done. Then it was filtered through muslin cloth for the methanol extract. Then filtrate was concentrated to dryness under the vacuum. The percentage yield extractive was calculated with references to the air-dried drug. The extract was named as MECS (Methanolic Extract of *Callistemon salignus*) Several chemical tests like Alkaloids, Carbohydrates ,Glycosides; Fixed oils and Fats, Gums and Mucilage, Phenolic compounds and Tannins, Proteins and free Amino acids; Saponin, Sterols; Volatile oils etc were done to detect several and different group of compounds present in the extractions [4].

Antioxidant assay

The antioxidant activity of MECS was determined by five different *in vitro* methods such as DPPH free radical scavenging activity, reducing activity, hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity and superoxide anion scavenging activity. All the assays were carried out in triplicate and average values were considered. Statistical Analysis was done by t –Test: paired Two Samples for Mean.

DPPH radical scavenging activity method [5]

The free radical scavenging activity of the extracts was determined using DPPH. DPPH solution (0.1 mM) was prepared in 95% methanol. Methanol extract of *C salignus* (MECS) was mixed with 95% methanol to prepare the stock solution (0.5 mg/mL). Freshly prepared 1 mL of DPPH solution (0.1 mM) was added to 3 mL of various concentrations (10- 100 µg/mL) of MECS. After 30 min, absorbance was measured at 517 nm. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Ascorbic acid was used as a reference compound. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extracts) using the following formula - absorbance of the control minus absorbance of the test sample divided by absorbance of the control multiplied by 100 [6].

Reducing Activity [7]

1 mL of different concentrations of the extracts (MECS; 10-100 µg/mL) were mixed with potassium ferricyanide (2.5 mL, 1%) and 2.5 mL of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. 2.5 mL TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 mL of supernatant was taken out and to this 2.5 mL water and 0.5 mL FeCl3 (0.1%) were added and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Hydroxyl radical scavenging activity [8]

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and different extracts in respective solvents for hydroxyl radical generated by Fe3+Ascorbate–EDTA–H_2O_2 system (Fenton reaction). The reaction mixture contained in a final volume of 1.0 mL, 100 µL of 28 mM 2-deoxy-2-ribose in 20 mM KH_2PO_4 - KOH buffer of pH 7.4, 500 µL of the selected concentrations various extracts (MECS; 10-100 µg/mL) in KH_2PO_4-KOH buffer (20 mM, pH 7.4),100 µL of 1.04 mM EDTA, 100 µL 200 mM FeCl3, 100 µL of 1.0 mM H_2O_2 and 100 µL of 1.0 mM ascorbic acid was incubated at 37 ºC for 1 hour. Then 1.0 mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8 %) were added to

the test tubes and were incubated at 100 ºC for 20 min. After cooling, absorbance was measured at 532 nm against control containing deoxyribose and buffer (not treated with drug). Ascorbic acid was used as a positive control. The percentage inhibition was determined by comparing the results of the test and control compounds. The hydroxyl radical scavenging activity of the extract is calculated as % inhibition of deoxyribose degradation and is calculated according to formula used in DPPH method.

Hydrogen Peroxide Scavenging Activity[9]

A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4).Hydrogen peroxide concentration was determined spectrophotometrically. MECS (10,25,50,100µg/ml) in distilled water was added to hydrogen peroxide solution(0.6 ml,40mM).Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of both MECS and standard compound was calculated as described previously.

Superoxide anion scavenging activity[10]

Measurement of superoxide anion scavenging activity was performed with NBT/NADH/PMS system .About 1 ml of nitroblue tetrazolium (NBT) solution, 1 ml NADH solution and 0.1 ml of sample solution,. The reaction was started by adding 100 µM of test extracts and the standard solution quercetin were mixed phenazine methosulphate(PMS) solution in phosphate buffer, pH 7.4 to the mixture. The reaction mixture was incubated at 25 ^{0} C for 5 min, and the absorbance at 560 nm was measured against blank sample. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

RESULTS AND DISSCUSSION

DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in Absorbance [11]. Comparison of the antioxidant activity of different concentrations of extracts and ascorbic acid is shown result Table 1 and also in the Figure 1. This activity was increased by increasing the concentration of the sample extract. The methanolic extract of *C salignus* exhibited a significant antioxidant activity in dose dependent manner.

For the measurement of the reducing ability, we investigated the Ferric to ferrous transformations in the presence of C. *salignus a* methanolic extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reductive ability of a compound generally depends on the presence of reductants which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom. The presence of reductants (i.e.antioxidants) in *C salignus* extracts causes the reduction of the Fe^{3+} ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Figure 2 shows the comparison of the reductive capability of various concentration of extract of *C salignus* leaves with ascorbic acid. The reducing power of *C salignus* extracts was increased with the concentration of extracts, only 50 µg/ml concentration of extract shows somewhat lower percentage of antioxidant activity than the 10, 25 µg/ ml concentrated extract. The data reveals maximum reducing ability was at 100µg/ml of methanolic extract and the results also revealed that the leaf extract show significant reductive ability, which is quite similar to that of vitamin C (ascorbic acid) in dose dependent manner.

Hydroxyl radicals are reactive biological molecules and their scavenging property may provide an important therapeutic approach against oxidative stress induced

ailments. It is well established in the absence of EDTA, Fe^{3+} directly bind with deoxyribose sugar and causes its site specific degradation due to hydroxyl radicals which are found immediately at the vicinity of the ions binding site. Hydroxyl radicals are the most reactive radicals which are produced via the Fenton`s reaction in living system. Hydroxyl radicals scavenging activity was quantified by measuring the inhibition of the degradation of the deoxyribose by free radicals [12]. Deoxyribose levels were determined by reaction with thiobarbituric acid. Figure 3 shows comparison among the different extracts of *C salignus* and ascorbic acid in a dose dependent and significant manner.

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [13]. It has been seen that the maximum inhibitory effect was at 100 µg/ml of this methanolic extract in table 4 which is also comparable with the standard ascorbic acid in significant way.

Super oxide is biologically important as it can form singlet oxygen and hydroxyl radical. Overproduction of super oxide anion radical contributes to redox imbalance and associated with harmful physiological consequences. Super oxide anion are generated in PMS-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan. Table 5 and Figure 5 illustrates increase scavenging of superoxide radicals in dose dependent manner due to the scavenging ability of the *C salignus* methanolic extract.

CONCLUSION

It is extremely important to point out there is a correlation between antioxidant activity and phyto-chemical screened. Flavonoid compounds are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. It is found that the plant *C salignus* contains flavonoid component [14]. So this specific activity may be due to this flavonoid compound.

So, from this above study, it has been observed that MECS exhibits better scavenging activity against free radicals and having considerable reduction capability. All the methods have proven the effectiveness of the methanolic extract compared to the reference standard ascorbic acid and in certain concentrations is very much comparable to standard reference ascorbic acid.

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Table 1: DPPH Scavenging Activity

Treatment	Concentration (µg/ ml)	Absorbance (Mean ± S.E)	% Antioxidant Activity
Control	-	0.073 ± 0.005	-
Ascorbic Acid	10	0.041 ± 0.0028	43.83
Ascorbic Acid	25	0.031 ± 0.0005	57.53
Ascorbic Acid	50	0.027 ± 0.0002	63.01
Ascorbic Acid	100	0.025 ± 0.0005	65.75
MECS	10	0.056 ± 0.0008	23.28
MECS	25	0.036 ± 0.0003	50.68
MECS	50	0.030 ± 0.0005	58.90
MECS	100	0.028 ± 0.0007	61.64

Here calculated t- value (2.2) was less than the table values at 5 % as well as 2.5 % level of significance. So it can be concluded that there is no statistical significant difference exists between MECS and

ascorbic acid. P value = 0.05

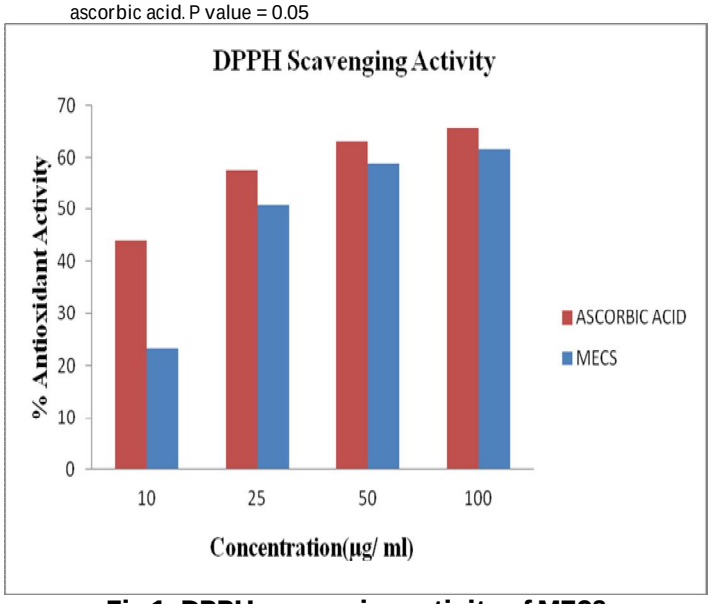

Fig 1: DPPH scavenging activity of MECS

Table 2: Reducing Activity

Treatment	Concentration (µg/ ml)	Absorbance (Mean ± S.E)	% Antioxidant Activity
Control	-	0.06 ± 0.02	-
Ascorbic Acid	10	0.542 ± 0.005	88.92
Ascorbic Acid	25	0.596 ± 0.005	89.26
Ascorbic Acid	50	0.624 ± 0.0013	90.38
Ascorbic Acid	100	0.802 ± 0.028	92.51
MECS	10	0.516 ± 0.028	88.37
MECS	25	0.543 ± 0.003	88.95
MECS	50	0.393 ± 0.0013	84.73
MECS	100	0.548 ± 0.022	89.05

Here calculated t- value (2.38) was less than the table values at 2.5 % level of significance. So it can be concluded that there is no statistical significant difference exists between MECS and ascorbic acid at that level. P value = 0.048.

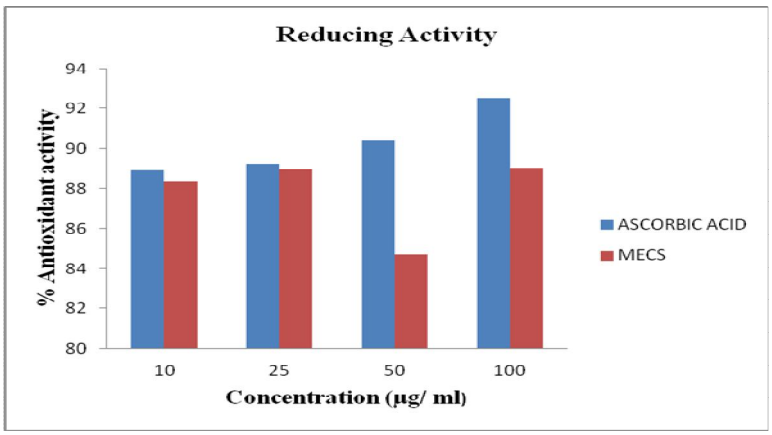

Fig. 2: Reducing Activity of MECS

Table 3: Hydroxyl Radical Scavenging Activity

Treatment	Concentration (µg/ ml)	Absorbance (Mean ± S.E)	% Antioxidant Activity
Control	-	0.030 ± 0.0012	-
Ascorbic Acid	10	0.156 ± 0.0008	80.76
Ascorbic Acid	25	0.237 ± 0.0002	87.34
Ascorbic Acid	50	0.293 ± 0.0017	89.76
Ascorbic Acid	100	0.312 ± 0.001	90.38
MECS	10	0.133 ± 0.008	77.44
MECS	25	0.213 ± 0.002	85.91
MECS	50	0.232 ± 0.001	87.06
MECS	100	0.287 ± 0.003	89.54

Here calculated t- value (3.59) was less than the table values at 1 % level of significance. So it can be concluded that there is no statistical significant difference exists between MECS and ascorbic acid at that level. P value = 0.018.

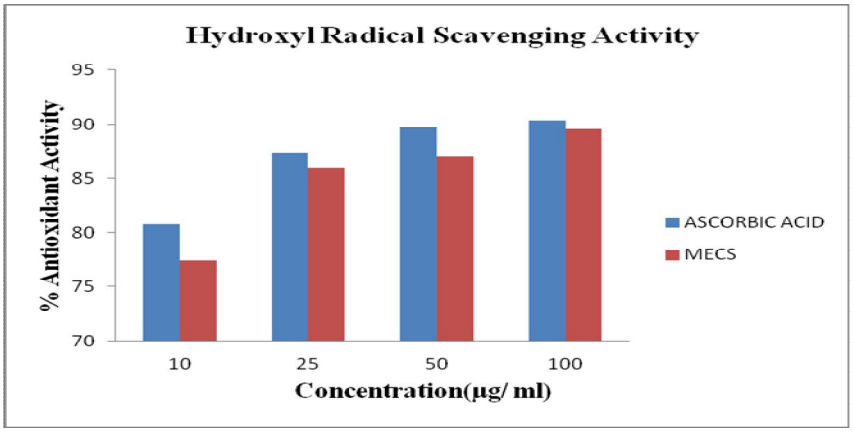

Fig. 3: Hydroxyl radical Activity of MECS

Table 4: Hydrogen Peroxide Scavenging Activity

Treatment	Concentration (µg/ ml)	Absorbance (Mean ± S.E)	% Antioxidant Activity
Control	-	0.030 ± 0.0012	-
Ascorbic Acid	10	0.079 ± 0.0005	62.02
Ascorbic Acid	25	0.085 ± 0.007	64.70
Ascorbic Acid	50	0.093 ± 0.0091	67.74
Ascorbic Acid	100	0.096 ± 0.002	70.96
MECS	10	0.043 ± 0.0002	30.23
MECS	25	0.072 ± 0.0003	58.23
MECS	50	0.087 ± 0.007	65.51
MECS	100	0.089 ± 0.005	66.29

Here calculated t- value (2.2) was less than the table values at 5 % as well as 2.5 % level of significance. So it can be concluded that there is no statistical significant difference exists between MECS and ascorbic acid. P value = 0.05

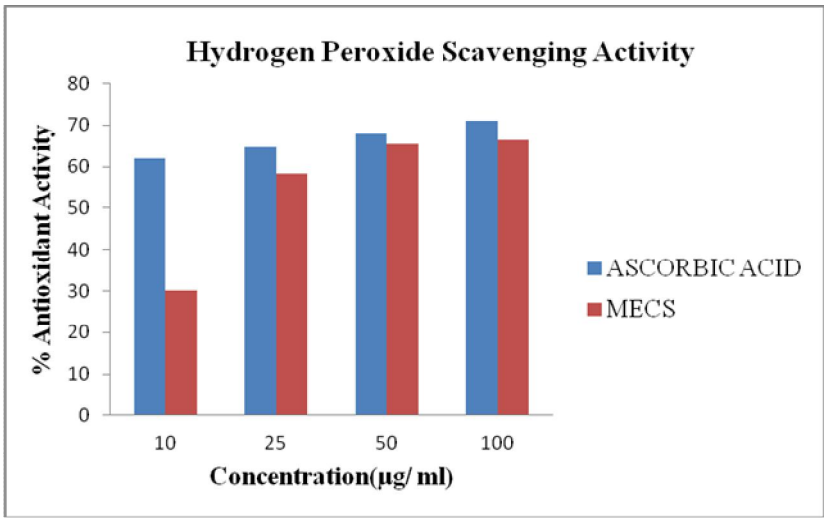

Fig. 4: Hydrogen Peroxide Scavenging Activity

Table 5: Superoxide Anion Scavenging Activity

Treatment	Concentration (µg/ ml)	Absorbance (Mean ± S.E)	% Antioxidant Activity
Control	-	0.09 ± 0.002	-
Ascorbic Acid	10	0.523 ± 0.005	82.79
Ascorbic Acid	25	0.739 ± 0.005	87.82
Ascorbic Acid	50	0.873 ± 0.001	89.69
Ascorbic Acid	100	0.929 ± 0.003	90.31
MECS	10	0.311 ± 0.005	78.20
MECS	25	0.387 ± 0.002	82.98
MECS	50	0.643 ± 0.002	85.27
MECS	100	0.706 ± 0.003	89.84

Here calculated t- value (2.38) was less than the table values at 2.5 % level of significance. So it can be concluded that there is no statistical significant difference exists between MECS and ascorbic acid at that level. P value = 0.048.

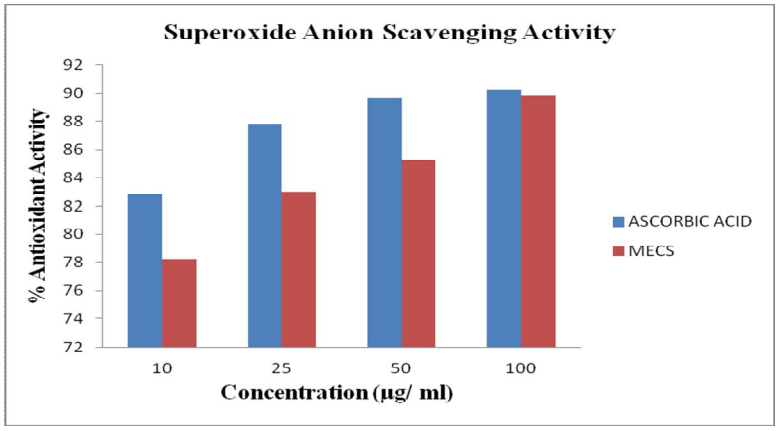

Fig. 5: Superoxide Anion Scavenging Activity of MECS

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