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Evaluation of total antioxidant and free radical scavenging activities of *Callistemon citrinus* (Curtis) Skeels extracts by biochemical and Electron Paramagnetic Resonance analyses

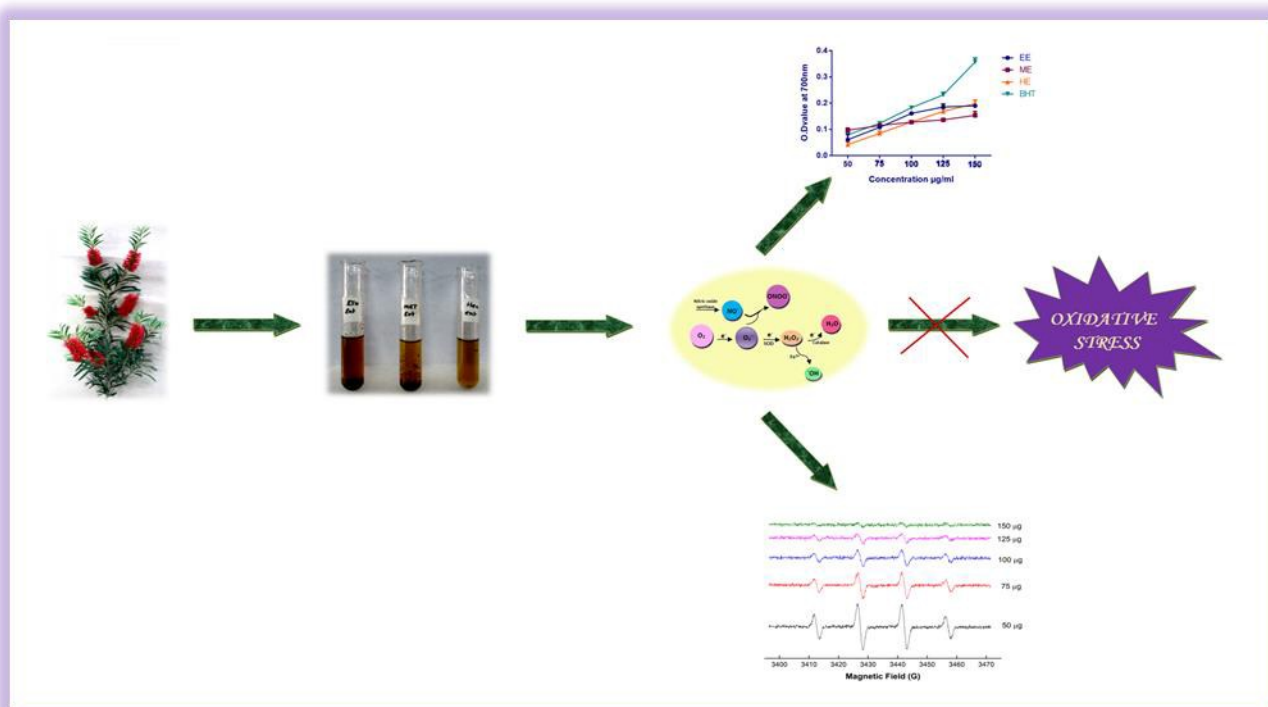
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GRAPHICAL ABSTRACT



Color Graphic Text: Free radical scavenging activity of *Callistemon citrinus* (Curtis) Skeels extracts analysed using biochemical and Electron Paramagnetic Resonance (EPR) studies.

ABSTRACT

Plants are known to contain a variety of compounds exhibiting antioxidant and free radical scavenging activities. The present study focuses on the preparation of crude extracts from *Callistemon citrinus* (CC) plant leaf using different solvents (such as, Ethanol, Methanol and n-hexane), to assess their antioxidant and free radical scavenging ability. The extracts of ethanol (EE), methanol (ME) and n-hexane (HE) were used separately to measure their individual free radical quenching efficiency against 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS^{•+}, Superoxide (O₂^{•-}), hydroxyl (HO[•]), nitric oxide (NO[•]) and hydrogen peroxide (H₂O₂). The reducing power as well as the phenolic and flavonoid contents of the extract was also assayed. Concentration and time dependent HO[•] radical scavenging potentials of these extracts were monitored by Electron Paramagnetic Resonance (EPR) spectroscopy. The results of all these studies suggested that the EE had highest free radical scavenging property followed by ME and HE. This activity increased with increase in extract concentration in situ. The observed potential antioxidant and free radical scavenging activities of the EE of CC leaves could be used for therapeutic purpose in the treatment of oxidative stress induced disorders.

Keywords: *Callistemon citrinus*, Free radical scavenging activity, EPR spectroscopy and DMPO spin trap.

1. INTRODUCTION

Safer antioxidants from plant origin are essential to prevent the progression of free radical mediated disorders. Free radicals (FR) are created when cells use oxygen to generate energy. An oxidation process, occurs naturally in the human body, involves electron transfer from one atom to another. Since oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP, oxidation is an essential part of aerobic life and human metabolism. But the problem arises when electron flow from oxidation process become unpaired and then subsequently generates free radicals, known as reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), peroxy ($ROO^{\cdot}$), hydroxyl ($HO^{\cdot}$), Hydrogen peroxide (H_2O_2) and Nitric oxide ($NO^{\cdot}$).¹ When generation of these reactive oxygen species (ROS) overtake the antioxidant defence capability of the cells, the FRs start attacking cellular macromolecules, hence resulting in degenerative diseases. Human body has several mechanisms to counteract oxidative stress by producing antioxidants, which bring interruption in ROS attack, by scavenging reactive metabolites or by converting them into less reactive molecules.^{2,3} Some of these known antioxidants are Vitamin C, Vitamin E, carotenoids, β -carotene and few plant-derived antioxidants, obtained mainly from diet, are capable of inhibiting the oxidation of other molecules.⁴

These antioxidants were discovered from different sources of plant origin and plant parts. Plants play an important role in the human life as the main source of food, medicine, wood, oxygen producer and many more. Plant-derived drugs can be defined as biologically active substances which serve as an important source of therapeutics from which 25% of the pharmaceuticals in current use have been derived.⁵ Several medicinal plants are traditionally noted for their bio-medicinal properties, often exhibiting a wide range of biological and pharmacological activities such as anti-inflammatory, anti-bacterial and anti-fungal properties. The active constituents contributing to these protective effects are the naturally

occurring phytochemicals, vitamins and minerals which give plants their unique colour and distinctive flavour.

In recent years, search for new effective natural antioxidants has increased, especially from herbal sources. *Callistemon citrinus* is an ornamental plant belonging to the family Myrtaceae, also known as bottlebrush, is widely distributed in east and southeast of Australia.⁶ In China, *Callistemon* species have been reported to be used as traditional medicine for the treatment of haemorrhoids.^{7,8} It has several medicinal properties which includes antibacterial,⁹ antifungal¹⁰ and anthelmintic activity.¹¹

In the present study, we have attempted to examine all the free radical scavenging activity and antioxidant potency of extracts obtained using Ethanol (EE), Methanol (ME) and n-hexane (HE). The total amount of phenols and flavonoids were also estimated as these compounds contribute significantly to the free radical scavenging ability. Additionally, the scavenging property of HO[•] radical, the most reactive among ROS and the major inducer of oxidative stress in biological system, was also estimated by using conventional EPR spectroscopy techniques.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), Quercetin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), Sodium nitroprusside (SNP), α -Tocopherol, 2,2-Azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Ammonium persulphate (APS), Nitroblue tetrazolium (NBT), Aluminium chloride, 2-Deoxyribose, Butylated hydroxytoluene (BHT), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were obtained from Sigma-Aldrich (USA). Ethylenediamine -tetra acetic acid (EDTA), Curcumin, Tannic acid, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Potassium ferricyanide,

Ascorbic acid, Riboflavin were purchased from Hi-media (India). All other reagents and organic solvents used were of analytical grade.

2.2 Plant material, identification and preparation of solvent extracts

The plant specimen of this investigation was identified as *Callistemon citrinus* (Curtis) Skeels (Basionym: *Metrosideros citrina* Curtis) and authenticated by Dr. G.V.S. Murthy, Botanical Survey of India, Southern Regional Centre, Coimbatore, India. A specimen was preserved in the herbarium (No.: BSI/SRC/5/23/2015/Tech/777). The healthy leaves of this plant were washed, dried in shadow and powdered. This powder was extracted separately using different solvents (ethanol, methanol or n-hexane) for several hours. After completion, the extract in solvent medium was concentrated in a rotary evaporator at 40-50°C and then stored at 4°C, after labelling as EE, ME and HE, for further analysis.

2.3 Determination of total phenolic content

The total phenolic content in the respective solvent extract was determined calorimetrically by following Folin-Ciocalteu procedure as described by Siddhuraju *et al.*¹² About 0.5 mL of each extract (EE, ME or HE) was mixed separately with 250 µL of Folin-Ciocalteu reagent (diluted with water 1:1). After the mixture was allowed to stand for 5 min, 1.25 mL of sodium carbonate (25% w/v) solution was added. The absorbance was then read at $\lambda=765$ nm against a control without crude plant extract. The phenolic content was calculated from a tannic acid standard curve.

2.4 Determination of total flavonoid content

The total flavonoid content in the solvent extracts of plant was determined according to the method described by Zhishen *et al.*¹³, using quercetin as a standard. Briefly, 0.1 mL of plant extract (EE, ME or HE) was added to 0.3 mL of distilled water followed by 0.03 mL of

5% NaNO₂ solution and incubated for 5 min at 25°C. Then added about 0.03 mL of 10% AlCl₃ and the mixture was allowed to stand for 5 min followed by which the reaction mixture was treated with 0.2 mL of 1 mM NaOH. Finally the mixture was diluted with 1 mL of distilled water and the absorbance was read at $\lambda=510$ nm against a control without crude plant extract. The flavonoid content was calculated from a quercetin standard curve.

2.5 DPPH radical scavenging assay

DPPH radical scavenging activity was performed according to the method described by Arife *et al.*¹⁴ The principle of the assay is based on the color change of the DPPH solution from purple to yellow, due to the quenching of the radical by the plant antioxidant. Briefly, 1 mL of 0.1 mM DPPH in methanol was mixed with 3 mL aliquot of plant extracts (EE, ME or HE) of varying concentrations (50–150 μ g). The samples were vortexed and kept in dark for 30 min at room temperature and then the decrease in absorbance at $\lambda=517$ nm was recorded against a control without crude extract. The radical scavenging activity of individual extract at different concentration was compared with Vitamin-E which was used as a standard antioxidant. The extent of decolourization as an index of scavenging activity was calculated using the formula:

$$\% \text{ Scavenged} = [(A_0 - A_1) / A_0] \times 100.$$

Where, A₀ was the absorbance of the control, and A₁ was the absorbance for the crude plant extract or standard.

2.6 ABTS^{•+} scavenging assay

The ABTS radical cation scavenging capacity of each extract was evaluated by studying its ability to bleach the radical (ABTS^{•+}) as described by Re *et al.*¹⁵ ABTS radical cation was produced by mixing 7 mM ABTS solution with 2.45 mM ammonium persulfate, followed by incubation in dark at room temperature for 12–16 h before use. In brief, different concentrations (50–150 μ g) EE, ME or HE was added to 0.3 mL of ABTS solution and the

final volume was made up to 1 mL with ethanol and the absorbance was read at $\lambda=734$ nm against a control in the absence of the respective crude extract. The activities were compared with Vitamin - E which was used as a standard antioxidant. The percentage inhibition the oxidation of ABTS^{•+} was calculated using the formula:

$$\% \text{ Scavenged} = [(A_0 - A_1) / A_0] \times 100.$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant extract or standard.

2.7 Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging capacity of each solvent extract was measured spectrophotometrically by monitoring the concentration of un-reacted H_2O_2 , by the method of Wettasinghe *et al.*¹⁶ In brief, 0.4 mL of different concentrations (50-150 μ g) of crude plant extract (EE, ME or HE) was added to 0.6 mL of H_2O_2 solution (40 mM). Final volume was made up to 2 mL with 50 mM sodium phosphate buffer (pH 7.4), and then the reaction mixture was incubated at 30°C for 40 min, followed by which the absorbance was recorded at $\lambda=230$ nm against a control. The activity was compared with L-ascorbic acid which was used as standard antioxidant. The percentage inhibition of H_2O_2 was calculated by using the formula: $\% \text{ Scavenged} = [(A_0 - A_1) / A_0] \times 100$.

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the extract or standard treated sample.

2.8 Nitric oxide radical (NO[•]) scavenging assay

Assay of nitric oxide radical (NO[•]) scavenging activity was performed according to the method of Sreejayan *et al.*¹⁷ In this method, 5 μ M sodium nitroprusside in 0.2 M phosphate buffer (pH 7.4), was incubated with different concentrations (50-150 μ g) of EE, ME or HE at 25°C for 5 h. An aliquot of 0.5 mL of incubated solution was then diluted with 0.5 mL Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2%

phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylenediamine was read at $\lambda=546$ nm against a control. The activity was compared with Curcumin which was used as a standard antioxidant. The extent of diazotization and reduced production of nitrite ions was calculated using the formula: % Scavenged = $[(A_0 - A_1) / A_0] \times 100$.

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant extract or standard.

2.9 Hydroxyl radical (HO[•]) scavenging activity

The hydroxyl radical assay was performed according to the method described by Halliwell *et al.*¹⁸ with a slight modification. The assay is based on the quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe³⁺-ascorbate-EDTA-H₂O₂ system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 mL, 2-deoxy-2-ribose (2.8 mM); KH₂PO₄-KOH buffer (20 mM, pH 7.4); FeCl₃ (100 μ M); EDTA (100 μ M); H₂O₂ (1.0 mM); ascorbic acid (100 μ M) and varying concentrations (50–150 μ g) of the test sample or reference compound. After incubation for 1 h at 37°C, 0.5 mL of the reaction mixture was added to 1 mL 2.8% TCA, followed by 1 mL 1% aqueous TBA. The mixture was then incubated at 90°C for 15 min to develop the color. After cooling to room temperature, the absorbance was measured at $\lambda=532$ nm against a control without crude plant extract. The HO[•] scavenging activity of each solvent extract was compared with L-ascorbic acid which was used as a standard antioxidant. The percentage inhibition of HO[•] was calculated using the formula:

$$\% \text{ Scavenged} = [(A_0 - A_1) / A_0] \times 100.$$

Where, A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant extract or standard.

2.10 Superoxide radical ($O_2^{\cdot-}$) scavenging assay

The superoxide scavenging activity of plant extracts was assayed according to the method performed by Martinez *et al.*¹⁹ In this assay, the photo-chemically reduced riboflavin generate $O_2^{\cdot-}$, which reduce NBT to form blue formazan. Briefly, the 1 mL reaction mixture containing 50 mM phosphate buffer (pH 7.4), 10 μ M riboflavin, 56 μ M NBT, 12 μ M EDTA and various concentrations (50–150 μ g) of sample solution was incubated for 5 min at RT. Followed by this, the reaction mixture was illuminated by fluorescent lamp for 2 min and the absorbance was measured at $\lambda=590$ nm, against an appropriate blank to determine the quantity of formazan generated. The activity was compared with Vitamin-E which was used as a standard antioxidant. The percentage inhibition of superoxide anion generation was calculated using the formula: % Scavenged = $[(A_0 - A_1) / A_0] \times 100$.

Where A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant extract or standard.

2.11 Reducing power assay

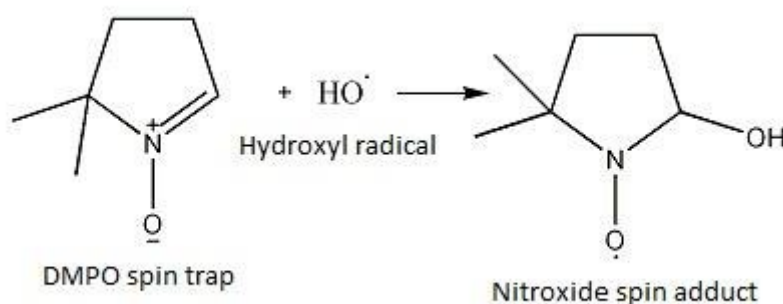
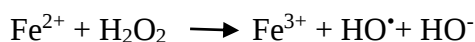
The Fe^{3+} reducing power of the extract was determined by the method as described by Oyaizu *et al.*²⁰ The extract samples of various concentrations (50-150 μ g mL^{-1}) were mixed with phosphate buffer (0.2 M, pH 6.6) and added potassium ferricyanide (1% w/v) followed by incubation at 50° C in a water bath for 20 min. The reaction was stopped by adding 10% TCA solution, and then centrifuged at 3,000 rpm for 10 min. The upper layer was mixed with equal volume of distilled water and added 0.5 mL of $FeCl_3$ solution (0.1% w/v), and the absorbance was recorded at $\lambda=700$ nm after vortexing. Increase in absorbance of the reaction mixture is the index of increase in reducing power. The activity was compared with BHT which was used as the standard antioxidant.

2.12 Total Antioxidant activity

The total antioxidant content of the solvent extracts of CC plant was estimated by following the manufacturer's directions provided along with the antioxidant assay kit from Sigma-Aldrich (USA). The assays were performed in 96 well plates. The reaction mixture contained 10 μL of test sample of various concentrations (50-150 μg), 20 μL of Myoglobin working solution; then 150 μL of the ABTS substrate working solution was added to each well and Incubated for 5 min at room temperature. Followed by this, 100 μL of stop solution was added to each well and the endpoint absorbance was read at $\lambda=405$ nm using a micro plate reader (Thermo Scientific Multiskan Ascent, USA). The activity was compared with TROLOX equivalent which was used as the standard.

2.13 Electron paramagnetic resonance (EPR) Spectroscopic analysis

The hydroxyl radical ($\text{HO}^\bullet$) scavenging efficacy of the solvent extracts of CC plant was studied by using Fenton reaction described by Harbour *et al.*²¹ as given below:



In this method DMPO, the nitron spin trap, was pretreated with activated charcoal to remove paramagnetic impurities before being used as working standard. The reaction mixture consisting of 30 μL DMPO (60 mM), 40 μL FeSO_4 (10 mM), 30 μL H_2O_2 (10 mM) in the presence or absence of various concentrations of dried EE or ME in water (40 μL) was made up to 300 μL using distilled water to give a final concentration 6 mM DMPO, 1 mM H_2O_2

and 1.3 mM FeSO₄. The samples of EE and ME were dried separately in a rotor vapor and dissolved in distilled water to make a stock solution of 5 mg/ml. From this varying volumes (10-30 µL) of solutions were appropriately diluted with water to give a final concentration in the range of 50-150 µg in 40 µL. The contents were mixed well for 7 min and were transferred into an RT-aqueous flat cell (Wilmad Labglass), and then experiment was carried out on Bruker EMX-EPR Spectrometer, Germany, at an operating frequency of 9.67 GHz, with centre field set at 3480 G, modulation frequency set as 100 KHz, modulation amplitude 0.10 G. Acquisition was carried out for 4 scans.

2.14 Data analysis

All the biochemical assays were performed in triplicates. The statistical significance were calculated from one way ANOVA analysis and the level was set to ($p < 0.05$) and the means were separated by using Prism Graph pad version 6.0; values of all parameters are expressed as mean $\pm$ SD of three independent measurements.

3. RESULTS AND DISCUSSION

3.1 Total phenolic and flavonoid content

Phenolic compounds are secondary metabolites, associated with flavour and colour characteristics of fruits and vegetables. Flavonoids are the natural polyphenols, widely present in the plants, fruits and food products. Polyphenol compounds are effective hydrogen donors, exhibiting inhibitory effect of mutagenesis and carcinogenesis in human, attributing as good antioxidants.^{22,23} The amount of phenols and flavonoids obtained in the crude extracts of ethanol (EE) were found to be 0.37 ± 0.28 and 0.39 ± 0.52 mg/g, methanol (ME) 0.21 ± 0.92 and 0.41 ± 0.27 mg/g, and hexane (HE) 0.53 ± 0.41 and 0.58 ± 0.09 mg/g, and the same are presented in (Table 1). The results are expressed as mg of tannic acid or quercetin

equivalents, per gram of dry extract. As is seen from the results, the HE displayed the highest levels of phenolic and flavonoid content, leading to a speculation that the solvent of cyclic hydrocarbon is able to solubilize more amounts of these compounds than methanol or ethanol. But it is uncertain whether the entire phenolic compounds contribute to free radical scavenging or they function as antioxidants. This ambiguity can be cleared only after checking the free radical scavenging activity of the individual solvent extracts, in the remaining part of this study.

3.2. DPPH radical scavenging activity

The solvent extracts of CC leaf showed a concentration-dependent anti-radical activity by inhibiting DPPH radical with an increasing concentrations of 50-150 μg . DPPH is usually used as a substrate to evaluate anti-oxidative activity of antioxidants.⁴ The method is based on the reduction of purple coloured methanolic DPPH solution to yellow, in the presence of a hydrogen donating antioxidant. In this study, the different solvent extracts of CC leaf serve as a source of H-donor and act as antioxidants.²⁴ The disappearance of purple colour, as an index of FR scavenging ability increased with increasing concentration of the extract (Table 2). The EE, ME and HE extracts showed maximum activity of 93.74%, 91.47% and 52.71% respectively at 150 $\mu\text{g mL}^{-1}$ concentration. The absorbance of DPPH was more rapidly decreased at $\lambda=517$ nm in the presence of EE followed by ME and then HE at an IC_{50} concentration of 62, 76 and 145 $\mu\text{g mL}^{-1}$ respectively, as against Vitamin-E the reference antioxidant which showed an IC_{50} of 11 $\mu\text{g mL}^{-1}$. This indicates that EE possesses more antioxidant activity in terms of hydrogen atom donating capacity. The decreased levels of radical scavenging activities of ME and HE may be either due to reduced levels of H-donors or increased levels pro-oxidants in them, more particularly in HE. A separate study is proposed to conduct in the next phase to assess the pro-oxidant characteristics of HE.

3.3. ABTS^{•+} scavenging activity

The antioxidant activity of various concentrations of EE, ME and HE of CC leaf, was determined by measuring the decolourization of the ABTS^{•+}. The reduction of the radical cation is expressed as the percentage of scavenging by the phenolic and flavonoid compounds of the extracts. The decolourization of radical cation was measured at $\lambda=734$ nm with no participation of any intermediary radical. The results presented in Table 3 indicate the potential scavenging activity of the extracts by inhibiting the formation of the ABTS^{•+}, because both the inhibiting and scavenging properties of antioxidants towards ABTS^{•+} have been already well documented.²⁵ The data of Table-3 also signify that the crude extracts of all the three different solvents exhibited free radical scavenging activity on dose dependent manner with maximum activity of 97.41%, 91.17% and 61.91% at 150 $\mu\text{g mL}^{-1}$ for EE, ME and HE respectively, with an IC₅₀ value of 50, 65 and 110 $\mu\text{g mL}^{-1}$ against 7 $\mu\text{g mL}^{-1}$ for Vitamin E.

3.4. Hydrogen peroxide scavenging activity

Scavenging of H₂O₂ by the extracts are attributed to their phenolic content, which can donate electrons to H₂O₂, thus neutralizing it to water.²⁶ The ability of the extracts to effectively scavenge hydrogen peroxide was compared with that of Ascorbic acid as standard antioxidant and the results are presented in Table 4. The extracts were capable of scavenging hydrogen peroxide and this efficiency was directly proportional to the concentration. The EE, ME and HE extracts showed an IC₅₀ value of 90, 127 and 150 $\mu\text{g mL}^{-1}$ respectively, against 5 $\mu\text{g mL}^{-1}$ concentration of Vitamin E, the reference antioxidant. H₂O₂, generally is not very reactive at very low concentration; but sometimes, it can cause cytotoxicity by giving rise to the formation of hydroxyl radical in the cell. Thus, elimination of H₂O₂ is also important to maintain stress free biological environment.

3.5. Nitric oxide scavenging activity

In the NO[•] scavenging study, the nitrous oxide produced by SNP reacts with oxygen to form stable nitrite and nitrate ions. The plant extract containing free radical scavenger, competes with oxygen leading to the suppression of nitrite formation. The nitrite ion in the aqueous solution further reacts with sulphanilamide present in the Griess' reagent to produce diazotised molecule, that was measured spectrophotometrically.²⁷ The NO[•] scavenging ability of all the three extracts is given in Table 5. Accordingly to the results obtained, the decrease in the concentration of nitrite in the presence of extracts is attributed to the prevention of nitrite formation from NO[•]. The EE showed a 92.42% NO[•] scavenging activity followed by 89.75% for ME and 71.53% for HE at 150 µg mL⁻¹ concentration, where the IC₅₀ values for these extracts are 90, 90 and 100 µg mL⁻¹ as against curcumin inhibition at 25 µg mL⁻¹ concentration. It is therefore clear that some of the active components of individual extracts compete with O₂ to react with nitrous oxide and prevent the formation of stable nitrite/nitrate, thereby decreasing the formation of diazotised coloured molecule.²⁸

3.6 Hydroxyl radical scavenging activity

The results of the HO[•] scavenging power of the plant extracts are provided in Table 6. The HO[•] generated via Fenton reaction degrades de-oxyribose using Fe²⁺ as catalytic component. The presence of radical scavenging molecules of the extract neutralizes the reactivity of the radical to prevent the degradation of de-oxyribose.²⁹ The results of HO[•] scavenging by the plant extracts are given in Table-6. The scavenging activity increased with increasing concentration of each extract. The activity was also dependent on the dose of the individual extract. The EE, ME and HE extracts showed maximum scavenging of 89.74%, 94.03% and 53.63% respectively, at 150 µg mL⁻¹ with an IC₅₀ value of 85, 90 and 132 µg

mL⁻¹ as against 10 µg mL⁻¹ of vitamin C, the reference antioxidant. The variation in the scavenging skill may be due to the diversification in the phytochemical composition of the extracts.

3.7 Super oxide radical scavenging activity

The superoxide radical formed by the reaction of riboflavin and NBT is suppressed by suitable scavenging molecule. In this reaction NBT is oxidised to form formazan which is measured spectrophotometrically.¹³ In the present study, the superoxide radical scavenging molecules present in the plant extracts viz. EE, ME and HE were shown to inhibit the formazan formation by NBT oxidation (Table 7). The extracts at different concentration, even after 1 h of incubation time did not produce a purple colour formazan. This observation revealed that the crude extracts of all the three types were able to fight the superoxide radicals and inhibit the NBT oxidation. These extracts showed a maximum free radical scavenging activity 93.80%, 91.5% and 69.6% respectively for EE, ME and HE at 150 µg mL⁻¹ concentrations with an IC₅₀ value of 79, 85 and 105 µg mL⁻¹ as against 7 µg mL⁻¹ of vitamin E, the reference antioxidant.

3.8. Reducing power of CC extracts

The reducing power of a herbal extract is associated mainly with its phenolic antioxidant activity. The presence of antioxidants in the CC plant extract resulted in the reduction of potassium ferricyanide to potassium ferrocyanide. The initial yellow colour of the reaction mixture of this assay changed to green and blue shades, due to the formation of Fe²⁺ complex³⁰, depending upon the reducing power of the extracts on dose dependent manner. Among the three different extracts studied, EE exhibited better activity, compared to ME and HE, at 100 µg mL⁻¹ concentration. At concentration below 100 µg mL⁻¹ the activity of EE is on par with the reference BHT. At concentration above 100 µg mL⁻¹ (i.e., at 150 µg mL⁻¹) both EE and HE displayed nearly same antioxidant activity; whereas, the ME showed a

significant decrease (Fig. 1) in its reducing power. The inconspicuous results of the extracts are attributed to the mixture of phytochemicals with different chemical properties, present in the crude extract.

3.9 Total antioxidant activity

Plants have a large number of extractable secondary metabolites. These molecules play the role of antioxidants, to prevent the oxidative stress in any reaction system. Therefore, quantitative measurement of the cumulative antioxidant capacity of any extracted material may provide important biological information. The total antioxidant capacity of the plant extracts of this study measured by TROLOX equivalent antioxidant capacity (TEAC) are depicted in Fig 2. The principle of the assay method, as reported by Miller and Rice-Evans *et al.*³¹ involves the formation of a ferryl myoglobin radical from metamyoglobin and hydrogen peroxide, which oxidises the ABTS to produce ABTS^{•+}, a green colour soluble chromogen that can be determined spectrophotometrically at 405 nm. It was observed from Fig. 2 that the CC plant extracts were able to suppress the production of the radical cation in a dose dependent manner with a proportionate decrease in the intensity of colour. However, the HE displayed a significantly ($p < 0.05$) decreased antioxidant activity compared to EE and ME. Trolox, a water-soluble vitamin E analog was used as a standard reference.

3.10 EPR spectroscopic investigations

The hydroxyl radicals generated via the Fenton reaction and its scavenging by ethanol or methanol extracts of CC plant was monitored using electron paramagnetic resonance (EPR) spectroscopy by a spin trapping method with DMPO as spin trap. Initially, the formation of DMPO-HO[•] spin adduct, resulted from DMPO trapping of HO[•] generated from Fenton reaction, was established through EPR spectrum (Fig. 3). This control run did not

contain the extracts possessing antioxidant activity. Parallel to this, the HO[•] scavenging ability and capacity limit the DMPO - HO[•] adduct formation by the extracts were examined.

EPR analysis carried out exactly 7 min after homogeneous mixing of the reaction mixture in room temperature condition resulted in the formation of DMPO-HO[•] spin adduct. As could be seen from the results presented in Fig. 4 (for EE) and Fig. 5 (for ME), both the EE and ME extracts were able to suppress the observed EPR signal from the DMPO-HO[•] adduct (Fig. 3), but to varying degrees. As observed from the results (Fig. 4 and 5), the suppression of DMPO- HO[•] adduct formation by ME is almost same as that of EE of CC leaf. However, there was slight difference in the HO[•] scavenging activity between these two extracts (Table-6), where the de-oxyribose degradation was significantly reduced ($p < 0.05$) in the presence of ME compare to that of EE. This two different solvent extracts have shown a dose dependent inhibition of the EPR signal, where the concentration of extracts was in the range of 50-150 $\mu\text{g mL}^{-1}$. The EE exhibited a low intensity signal at a maximum concentration used (150 μg), while the ME showed barely detectable signal at this concentration. The difference in the scavenging ability of two different extracts of the same plant may be due to the presence of varying amount of polyphenols having direct scavenging activities against hydroxyl radicals.^{16,32,33}

It was also noted that the EPR signal in this study was stable for nearly 20 min followed by a complete degeneration of the quartet signal, confirming the formation of stable DMPO-HO[•] adduct. The decrease or loss in the intensity of this signal is due to competing ability of the individual extract for accepting the reactive HO[•] species. EPR analysis for HO[•] scavenging activity by HE was not carried out because of its poor solubility in water, as it contained oily substance. However, the results of HO[•] scavenging activity mentioned in section 3.6 may serve as an index.

4. CONCLUSION

The solvent extracts had varying levels of antioxidant capacity contributed possibly by different antioxidant compounds, as measured by comparing with the abilities of known standards such as Vitamin E, Vitamin C, Curcumin, BHT, TROLOX and Quercetin. The variation could also be related to the extractability of the individual solvent. The free radical scavenging activity of EE, ME and HE (Tables 2-7) extracts showed a dose responsive scavenging ability. Compared to the other extracts, HE showed remarkably lower activity, despite its increased phenolic and flavonoid levels, and not contributing to antioxidant activity. The decreased free radical scavenging and antioxidant properties of HE was confirmed by its increased cytotoxicity (data not shown), and further work is in progress in this direction. Hexane, being a non-polar solvent, generally extracts compounds such as alkaloids and terpenoids (which are of pro-oxidant in nature), while the ethanol and methanol extract antioxidant metabolites such as phenolics and flavonoids from CC leaf. This study, therefore reveals that the *Callistemon citrinus* leaf has important biologically active phytochemicals of medical importance of both pro- and antioxidants. True benefits of these compounds could be understood after separating the individual phytochemical by employing appropriate separation techniques.

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Conflict of interest

The Author(s) declare no conflicts of interest to disclose.

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- 498

499 **Table 1** Total Phenolic and Flavonoid content of the solvent extracts of CC

Samples	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
EE	0.37±0.28	0.39±0.52
ME	0.21±0.92	0.41±0.27
HE	0.53±0.41	0.58±0.09

Note: Tannic acid and Quercetin were used as reference standards for phenolic and flavonoid respectively.

Table 2 DPPH radical scavenging activity of solvent extracts of CC with reference to Vitamin E as standard

Sample	Concentration (µg/ml)	% DPPH radical Scavenged by			Standard Vitamin-E (µg/ml)
		EE	ME	HE	
CC crude extract	50	29.49±2.64	30.97±0.60	23.87±1.92	
	75	74.93±4.58	46.56±2.97	32.12±2.96	
	100	91.0±1.54	74.47±4.08	31.78±1.38	
	125	96.34±0.33	87.38±3.42	38.69±2.83	
	150	93.74±0.12	91.47±3.23	52.71±3.03	
IC ₅₀		62	76	145	11

Results are expressed as percentage scavenging and are mean ± SD of three independent values (n=3)

Table 3 ABTS radical cation scavenging activity of solvent extracts of CC with reference to Vitamin E as standard

Sample	Concentration ($\mu\text{g/ml}$)	% ABTS ^{•+} Scavenged by			Standard Vitamin-E ($\mu\text{g/ml}$)
		EE	ME	HE	
CC crude extract	50	49.16 $\pm$ 1.86	43.85 $\pm$ 2.70	26.40 $\pm$ 3.09	
	75	73.56 $\pm$ 1.45	52.24 $\pm$ 3.97	37.61 $\pm$ 1.69	
	100	87.30 $\pm$ 1.34	60.46 $\pm$ 3.56	42.32 $\pm$ 1.57	
	125	93.26 $\pm$ 1.56	83.16 $\pm$ 3.21	54.76 $\pm$ 2.09	
	150	97.41 $\pm$ 0.35	91.17 $\pm$ 3.17	61.91 $\pm$ 1.71	
IC ₅₀		50	65	110	7

Results are expressed as percentage scavenging and are mean $\pm$ SD of three independent values (n=3)

Table 4 H₂O₂ scavenging activity of solvent extracts of CC with reference to Vitamin C as standard

Sample	Concentration ($\mu\text{g/ml}$)	% H ₂ O ₂ Scavenged by			Standard Vitamin-C ($\mu\text{g/ml}$)
		EE	ME	HE	
CC crude extract	50	24.31 $\pm$ 2.49	12.99 $\pm$ 1.38	10.39 $\pm$ 0.64	
	75	36.03 $\pm$ 2.42	27.48 $\pm$ 1.15	13.07 $\pm$ 1.57	
	100	55.27 $\pm$ 3.13	40.63 $\pm$ 2.91	24.28 $\pm$ 2.24	
	125	72.91 $\pm$ 1.85	48.18 $\pm$ 2.06	35.99 $\pm$ 2.70	
	150	82.16 $\pm$ 1.62	58.77 $\pm$ 1.20	48.40 $\pm$ 1.42	
IC ₅₀		90	127	150	5

Results are expressed as percentage scavenging and are mean $\pm$ SD of three independent values (n=3)

Table 5 Nitric oxide radical scavenging activity of solvent extracts of CC with reference to Curcumin as standard

Sample	Concentration ($\mu\text{g/ml}$)	% Nitric Oxide Scavenged by			Standard Curcumin ($\mu\text{g/ml}$)
		EE	ME	HE	
CC crude extract	50	34.17 $\pm$ 2.46	32.69 $\pm$ 1.12	29.22 $\pm$ 3.31	
	75	40.48 $\pm$ 1.86	38.80 $\pm$ 1.70	38.08 $\pm$ 2.67	
	100	53.98 $\pm$ 2.61	50.30 $\pm$ 2.85	54.63 $\pm$ 3.06	
	125	73.51 $\pm$ 3.53	71.43 $\pm$ 2.83	62.58 $\pm$ 1.78	
	150	92.42 $\pm$ 2.01	89.75 $\pm$ 1.03	71.53 $\pm$ 0.54	
IC ₅₀		90	90	100	25

Results are expressed as percentage scavenging and are mean $\pm$ SD of three independent values (n=3)

Table 6 Hydroxyl ($\text{HO}^\bullet$) radical scavenging activity of solvent extracts of CC with reference to Vitamin C as standard

Sample	Concentration ($\mu\text{g/ml}$)	% $\text{HO}^\bullet$ Scavenged by			Standard Vitamin-C ($\mu\text{g/ml}$)
		EE	ME	HE	
CC crude extract	50	23.76 $\pm$ 1.66	21.83 $\pm$ 1.77	18.70 $\pm$ 2.00	
	75	38.60 $\pm$ 0.93	36.00 $\pm$ 2.60	26.24 $\pm$ 2.05	
	100	56.40 $\pm$ 2.53	55.28 $\pm$ 2.82	34.66 $\pm$ 1.40	
	125	70.79 $\pm$ 0.89	78.28 $\pm$ 1.17	46.56 $\pm$ 1.33	
	150	89.74 $\pm$ 1.47	94.03 $\pm$ 1.45	53.63 $\pm$ 2.02	
IC ₅₀		85	90	132	10

Results are expressed as percentage scavenging and are mean $\pm$ SD of three independent values (n=3)

Table 7 Superoxide ($O_2^{\cdot -}$) radical scavenging activity of solvent extracts of CC with reference to Vitamin E as standard

Sample	Concentration ($\mu\text{g/ml}$)	% Super Oxide Scavenged by			Standard Vitamin-E ($\mu\text{g/ml}$)
		EE	ME	HE	
CC crude extract	50	26.97 $\pm$ 1.64	28.86 $\pm$ 2.05	24.02 $\pm$ 1.78	
	75	45.92 $\pm$ 3.18	40.22 $\pm$ 2.48	34.18 $\pm$ 1.22	
	100	68.56 $\pm$ 2.27	58.86 $\pm$ 1.36	48.56 $\pm$ 1.22	
	125	79.05 $\pm$ 1.09	77.44 $\pm$ 2.98	60.98 $\pm$ 1.46	
	150	93.80 $\pm$ 1.87	91.51 $\pm$ 1.51	69.63 $\pm$ 0.91	
IC ₅₀		79	85	105	7

Results are expressed as percentage scavenging and are mean $\pm$ SD of three independent values (n=3)

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503 **Fig. 1** Reducing power of CC extracts in comparison with BHT at different concentrations
504 (50-150 $\mu\text{g mL}^{-1}$), Each value represents a mean $\pm$ SD (n=3)

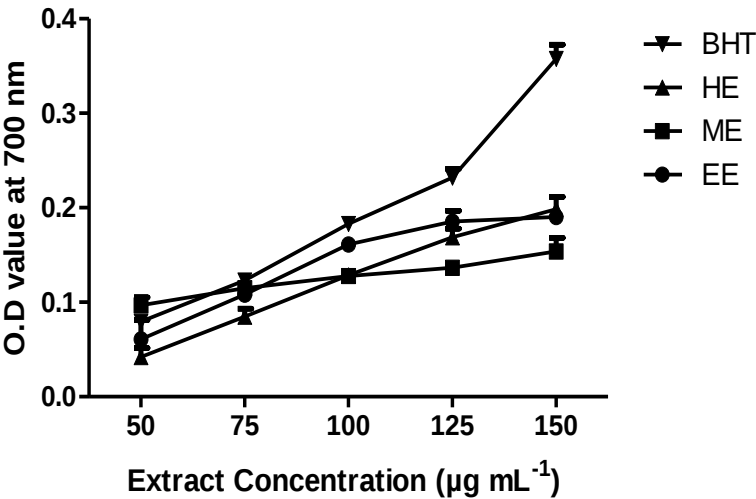


Fig. 2 Total antioxidant capacity of CC extracts at varying concentration were calculated and compared with TROLOX equivalent expressed as mmol/L, Each value represents a mean $\pm$ SD (n=3)

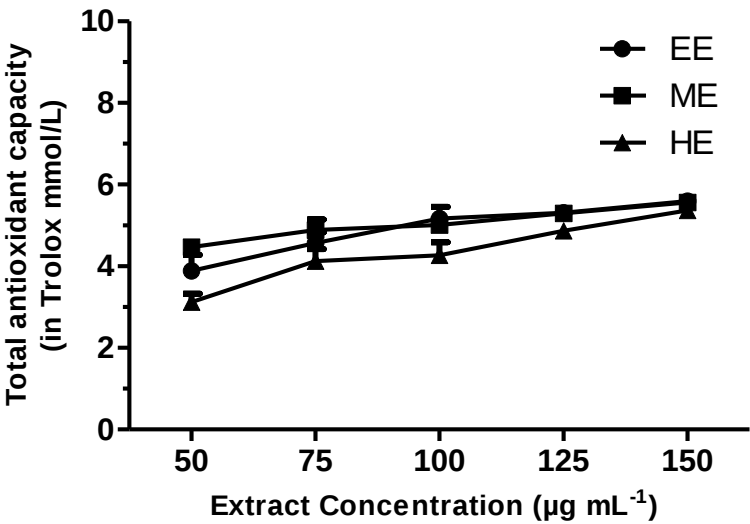


Fig. 3 EPR Spectra of DMPO - HO[•] spin adduct formed via Fenton Reaction (Control)

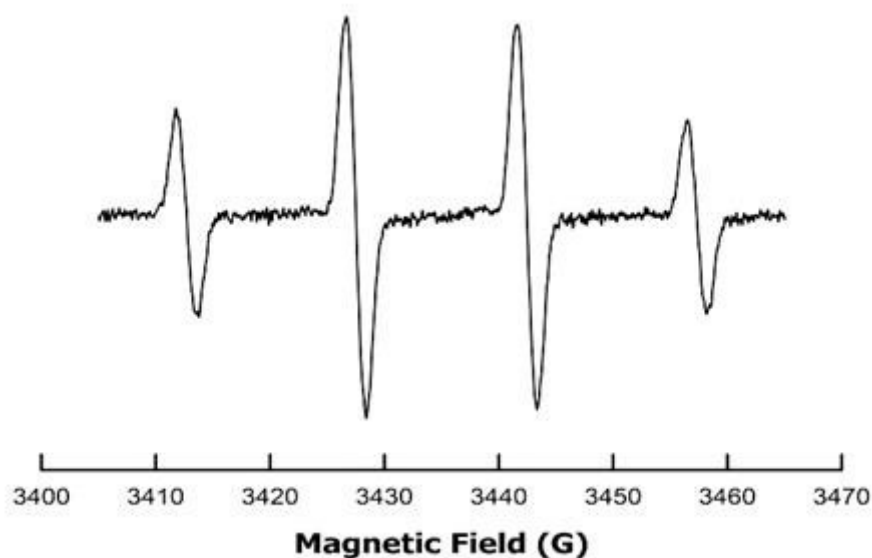
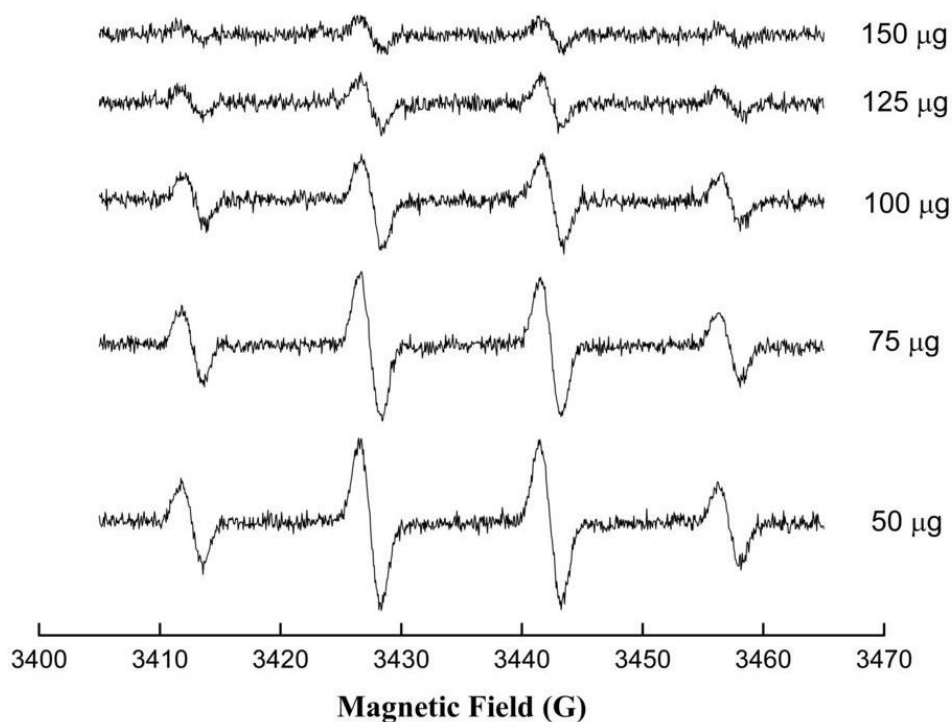
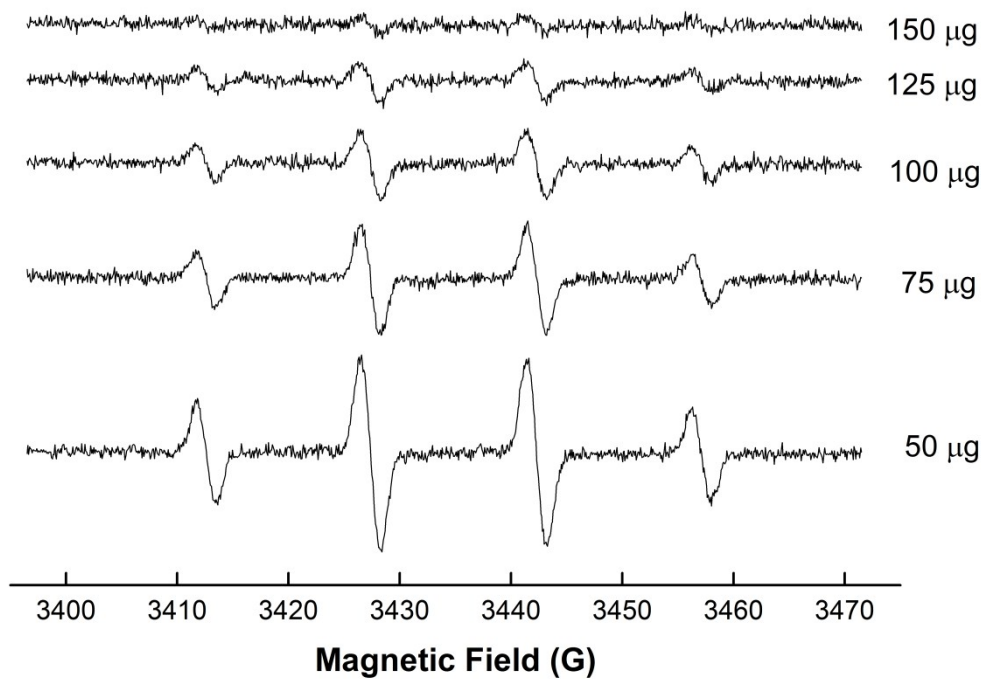


Fig. 4 X-band EPR Spectra: Effect of EE on HO[•] scavenging and retardation of DMPO - HO[•] adduct formation



Note: DMPO spin trap in aqueous solution was used in ambient condition.

Fig. 5 X-band EPR Spectra: Effect of ME on HO[•] scavenging and retardation of DMPO - HO[•] adduct formation



Note: DMPO spin trap in aqueous solution was used in ambient condition.