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2 Evaluation of total antioxidant and free radical scavenging 3 activities of Callistemon *citrinus* (Curtis) Skeels extracts 4 by biochemical and Electron Paramagnetic Resonance 5 analyses

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7 Sowndarya Sampath [1], Balasaraswathi Kalimuthu [1], Vidhya
8 Veeramani [1], Sridevi Janardhanam [2], Mandal Asit Baran [3],
9 Rose Chellan[1*]

10

11

12 1. *Biotechnology division – CSIR-Central Leather Research Institute,*
13 *Chennai, India.*
14 2. *Chemical Physics laboratory – CSIR-Central Leather Research Institute,*
15 *Chennai, India.*
16 3. *Chemical laboratory – CSIR-Central Leather Research Institute,*
17 *Chennai, India.*

18

19 ***Corresponding Author:** Dr. C. Rose
20 Chief Scientist & Chairman-Cluster Biology
21 Department of biotechnology
22 CSIR - Central Leather Research Institute
23 Adyar, Chennai – 600 020.

24

25 *Email: rose@clri.res.in*
26 *chellanrose@yahoo.co.uk*

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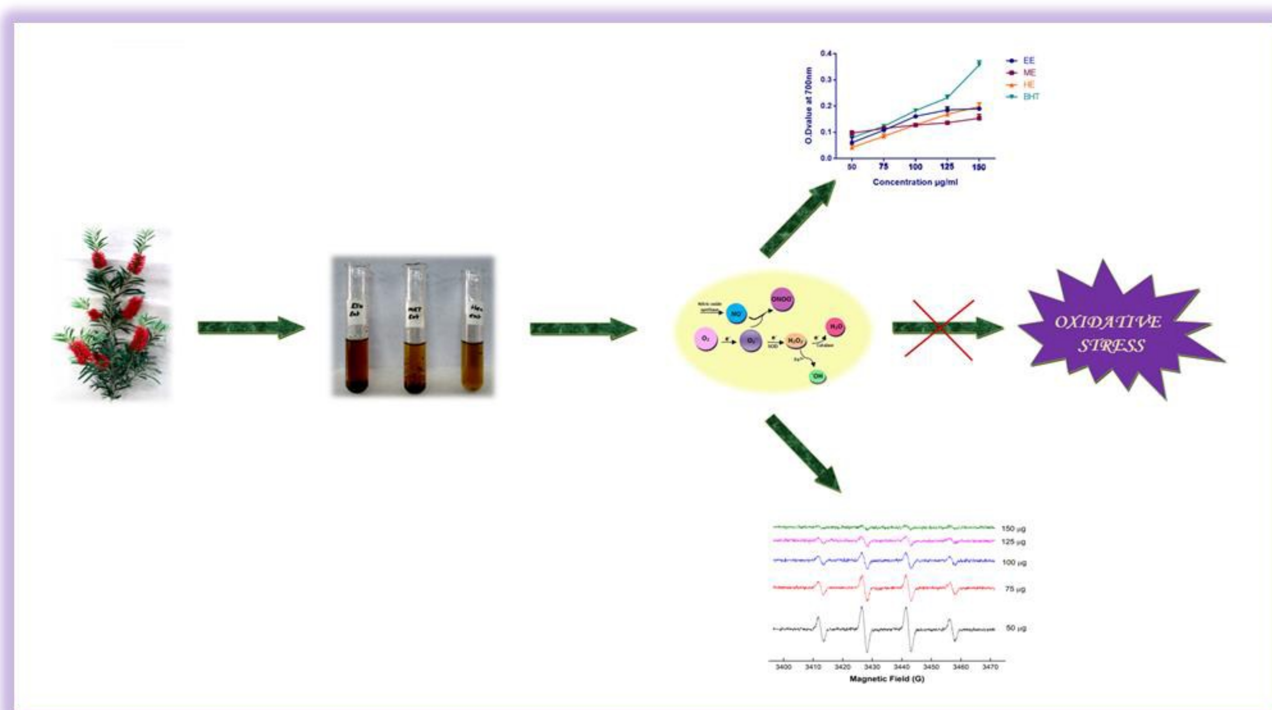
GRAPHICAL ABSTRACT



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

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40 **ABSTRACT**

41 Plants are known to contain a variety of compounds exhibiting antioxidant and free
42 radical scavenging activities. The present study focuses on the preparation of crude extracts
43 from Callistemon *citrinus* (CC) plant leaf using different solvents (such as, Ethanol,
44 Methanol and n-hexane), to assess their antioxidant and free radical scavenging ability. The
45 extracts of ethanol (EE), methanol (ME) and n-hexane (HE) were used separately to measure
46 their individual free radical quenching efficiency against 1,1-diphenyl-2-picrylhydrazyl
47 (DPPH), $ABTS^{\cdot +}$, Superoxide ($O_2^{\cdot -}$), hydroxyl ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) and hydrogen
48 peroxide (H_2O_2). The reducing power as well as the phenolic and flavonoid contents of the
49 extract was also assayed. Concentration and time dependent $HO^{\cdot}$ radical scavenging
50 potentials of these extracts were monitored by Electron Paramagnetic Resonance (EPR)
51 spectroscopy. The results of all these studies suggested that the EE had highest free radical
52 scavenging property followed by ME and HE. This activity increased with increase in extract
53 concentration in situ. The observed potential antioxidant and free radical scavenging
54 activities of the EE of CC leaves could be used for therapeutic purpose in the treatment of
55 oxidative stress induced disorders.

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

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60 **1. INTRODUCTION**

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

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

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

87 In recent years, search for new effective natural antioxidants has increased, especially
88 from herbal sources. Callistemon *citrinus* is an ornamental plant belonging to the family
89 Myrtaceae, also known as bottlebrush, is widely distributed in east and southeast of
90 Australia.[6] In China, Callistemon species have been reported to be used as traditional
91 medicine for the treatment of haemorrhoids.[7,8] It has several medicinal properties which
92 includes antibacterial,[9] antifungal [10] and anthelmintic activity.[11]

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

100 **2. MATERIALS AND METHODS**

101 **2.1 Chemicals and Reagents**

102 1,1-diphenyl-2-picrylhydrazyl (DPPH), Quercetin, 6-hydroxy-2,5,7,8-
103 tetramethylchroman-2-carboxylic acid (TROLOX), Sodium nitroprusside (SNP), α-
104 Tocopherol, 2,2-Azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Ammonium
105 persulphate (APS), Nitroblue tetrazolium (NBT), Aluminium chloride, 2-Deoxyribose,
106 Butylated hydroxytoluene (BHT), and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were
107 obtained from Sigma-Aldrich (USA). Ethylenediamine -tetra acetic acid (EDTA), Curcumin,
108 Tannic acid, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Potassium ferricyanide,

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

111 **2.2 Plant material, identification and preparation of solvent extracts**

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

120 **2.3 Determination of total phenolic content**

121 The total phenolic content in the respective solvent extract was determined
122 calorimetrically by following Folin-Ciocalteu procedure as described by Siddhuraju *et al.*[12]
123 About 0.5 mL of each extract (EE, ME or HE) was mixed separately with 250 µL of Folin-
124 Ciocalteu reagent (diluted with water 1:1). After the mixture was allowed to stand for 5 min,
125 1.25 mL of sodium carbonate (25% w/v) solution was added. The absorbance was then read
126 at λ=765 nm against a control without crude plant extract. The phenolic content was
127 calculated from a tannic acid standard curve.

128 **2.4 Determination of total flavonoid content**

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

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132 5% $NaNO_2$ solution and incubated for 5 min at 25°C. Then added about 0.03 mL of 10%
133 $AlCl_3$ and the mixture was allowed to stand for 5 min followed by which the reaction mixture
134 was treated with 0.2 mL of 1 mM NaOH. Finally the mixture was diluted with 1 mL of
135 distilled water and the absorbance was read at λ=510 nm against a control without crude plant
136 extract. The flavonoid content was calculated from a quercetin standard curve.

137 **2.5 DPPH radical scavenging assay**

138 DPPH radical scavenging activity was performed according to the method described
139 by Arife *et al.*[14] The principle of the assay is based on the color change of the DPPH solution
140 from purple to yellow, due to the quenching of the radical by the plant antioxidant. Briefly,
141 1 mL of 0.1 mM DPPH in methanol was mixed with 3 mL aliquot of plant extracts (EE, ME
142 or HE) of varying concentrations (50–150 µg). The samples were vortexed and kept in dark
143 for 30 min at room temperature and then the decrease in absorbance at λ=517 nm was
144 recorded against a control without crude extract. The radical scavenging activity of individual
145 extract at different concentration was compared with Vitamin-E which was used as a standard
146 antioxidant. The extent of decolourization as an index of scavenging activity was calculated
147 using the formula:

148 $\% \text{ Scavenged} = [(A_0 - A_1) / A_0] \times 100.$

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

151 **2.6 $ABTS^{\bullet+}$ scavenging assay**

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

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157 final volume was made up to 1 mL with ethanol and the absorbance was read at λ=734 nm
158 against a control in the absence of the respective crude extract. The activities were compared
159 with Vitamin - E which was used as a standard antioxidant. The percentage inhibition the
160 oxidation of $ABTS^{\bullet +}$ was calculated using the formula:
161 % Scavenged = $[(A_0 - A_1) / A_0] \times 100$.
162 Where A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant
163 extract or standard.

164 **2.7 Hydrogen peroxide scavenging activity**

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

176 **2.8 Nitric oxide radical ($NO^{\bullet}$) scavenging assay**

177 Assay of nitric oxide radical ($NO^{\bullet}$) scavenging activity was performed according to
178 the method of Sreejayan *et al.*[17] In this method, 5 µM sodium nitroprusside in 0.2 M
179 phosphate buffer (pH 7.4), was incubated with different concentrations (50-150 µg) of EE,
180 ME or HE at 25°C for 5 h. An aliquot of 0.5 mL of incubated solution was then diluted with
181 0.5 mL Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2%

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182 phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The
183 absorbance of chromophore formed during diazotization of nitrite with sulphanilamide and its
184 subsequent coupling with napthyl ethylenediamine was read at λ=546 nm against a control.
185 The activity was compared with Curcumin which was used as a standard antioxidant. The
186 extent of diazotization and reduced production of nitrite ions was calculated using the
187 formula: % Scavenged = $[(A_0 - A_1) / A_0] \times 100$.
188 Where A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant
189 extract or standard.

190 **2.9 Hydroxyl radical ($HO^{\bullet}$) scavenging activity**

191 The hydroxyl radical assay was performed according to the method described by
192 Halliwell *et al.*[18] with a slight modification. The assay is based on the quantification of the
193 degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was
194 generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction
195 mixture contained, in a final volume of 1 mL, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH
196 buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid
197 (100 µM) and varying concentrations (50–150 µg) of the test sample or reference compound.
198 After incubation for 1 h at 37°C, 0.5 mL of the reaction mixture was added to 1 mL 2.8%
199 TCA, followed by 1 mL 1% aqueous TBA. The mixture was then incubated at 90°C for 15
200 min to develop the color. After cooling to room temperature, the absorbance was measured at
201 λ=532 nm against a control without crude plant extract. The $HO^{\bullet}$ scavenging activity of each
202 solvent extract was compared with L-ascorbic acid which was used as a standard antioxidant.
203 The percentage inhibition of $HO^{\bullet}$ was calculated using the formula:
204 % Scavenged = $[(A_0 - A_1) / A_0] \times 100$.
205 Where, A_0 was the absorbance of the control, and A_1 was the absorbance of the crude plant
206 extract or standard.

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207 **2.10 Superoxide radical ($O_2^{\bullet -}$) scavenging assay**

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

220 **2.11 Reducing power assay**

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

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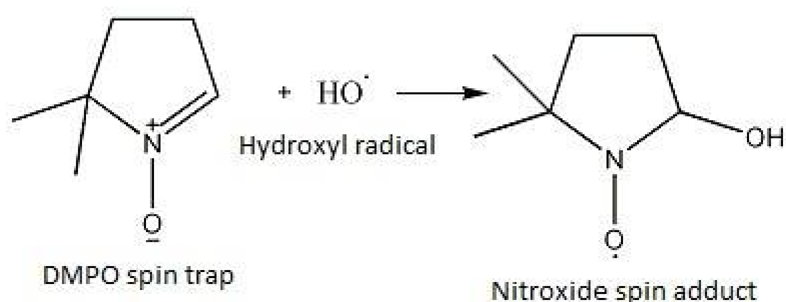
2.12 Total Antioxidant activity

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

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^{-}$$



In this method DMPO, the nitrone 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

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252 and 1.3 mM $FeSO_4$. The samples of EE and ME were dried separately in a rotor vapor and
253 dissolved in distilled water to make a stock solution of 5 mg/ml. From this varying volumes
254 (10-30 μL) of solutions were appropriately diluted with water to give a final concentration in
255 the range of 50-150 μg in 40 μL. The contents were mixed well for 7 min and were
256 transferred into an RT-aqueous flat cell (Wilmad Labglass), and then experiment was carried
257 out on Bruker EMX-EPR Spectrometer, Germany, at an operating frequency of 9.67 GHz,
258 with centre field set at 3480 G, modulation frequency set as 100 KHz, modulation amplitude
259 0.10 G. Acquisition was carried out for 4 scans.

260 **2.14 Data analysis**

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

265 **3. RESULTS AND DISCUSSION**

266 **3.1 Total phenolic and flavonoid content**

267 Phenolic compounds are secondary metabolites, associated with flavour and colour
268 characteristics of fruits and vegetables. Flavonoids are the natural polyphenols, widely
269 present in the plants, fruits and food products. Polyphenol compounds are effective hydrogen
270 donors, exhibiting inhibitory effect of mutagenesis and carcinogenesis in human, attributing
271 as good antioxidants.[22,23] The amount of phenols and flavonoids obtained in the crude
272 extracts of ethanol (EE) were found to be 0.37±0.28 and 0.39±0.52 mg/g, methanol (ME)
273 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
274 same are presented in (Table 1). The results are expressed as mg of tannic acid or quercetin

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

282 **3.2. DPPH radical scavenging activity**

283 The solvent extracts of CC leaf showed a concentration-dependent anti-radical
284 activity by inhibiting DPPH radical with an increasing concentrations of 50-150 µg. DPPH is
285 usually used as a substrate to evaluate anti-oxidative activity of antioxidants.[4] The method is
286 based on the reduction of purple coloured methanolic DPPH solution to yellow, in the
287 presence of a hydrogen donating antioxidant. In this study, the different solvent extracts of
288 CC leaf serve as a source of H-donor and act as antioxidants.[24] The disappearance of purple
289 colour, as an index of FR scavenging ability increased with increasing concentration of the
290 extract (Table 2). The EE, ME and HE extracts showed maximum activity of 93.74%,
291 91.47% and 52.71% respectively at 150 µg mL^{-1}concentration. The absorbance of DPPH was
292 more rapidly decreased at λ=517 nm in the presence of EE followed by ME and then HE at
293 an IC_{50} concentration of 62, 76 and 145 µg mL^{-1} respectively, as against Vitamin-E the
294 reference antioxidant which showed an IC_{50} of 11 µg mL^{-1}. This indicates that EE possesses
295 more antioxidant activity in terms of hydrogen atom donating capacity. The decreased levels
296 of radical scavenging activities of ME and HE may be either due to reduced levels of H-
297 donors or increased levels pro-oxidants in them, more particularly in HE. A separate study is
298 proposed to conduct in the next phase to assess the pro-oxidant characteristics of HE.

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299 **3.3. ABTS$^{•+}$ scavenging activity**

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

312 **3.4. Hydrogen peroxide scavenging activity**

313 Scavenging of H_2O_2 by the extracts are attributed to their phenolic content, which can
314 donate electrons to H_2O_2, thus neutralizing it to water.[26] The ability of the extracts to
315 effectively scavenge hydrogen peroxide was compared with that of Ascorbic acid as standard
316 antioxidant and the results are presented in Table 4. The extracts were capable of scavenging
317 hydrogen peroxide and this efficiency was directly proportional to the concentration. The EE,
318 ME and HE extracts showed an IC_{50} value of 90, 127 and 150 μg mL^{-1} respectively, against 5
319 μg mL^{-1} concentration of Vitamin E, the reference antioxidant. H_2O_2, generally is not very
320 reactive at very low concentration; but sometimes, it can cause cytotoxicity by giving rise to
321 the formation of hydroxyl radical in the cell. Thus, elimination of H_2O_2 is also important to
322 maintain stress free biological environment.

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323 **3.5. Nitric oxide scavenging activity**

324 In the $NO^{•}$ scavenging study, the nitrous oxide produced by SNP reacts with oxygen
325 to form stable nitrite and nitrate ions. The plant extract containing free radical scavenger,
326 competes with oxygen leading to the suppression of nitrite formation. The nitrite ion in the
327 aqueous solution further reacts with sulphanilamide present in the Griess' reagent to produce
328 diazotised molecule, that was measured spectrophotometrically.[27] The $NO^{•}$ scavenging ability
329 of all the three extracts is given in Table 5. Accordingly to the results obtained, the decrease
330 in the concentration of nitrite in the presence of extracts is attributed to the prevention of
331 nitrite formation from $NO^{•}$. The EE showed a 92.42% $NO^{•}$ scavenging activity followed by
332 89.75% for ME and 71.53% for HE at 150 μg mL^{-1} concentration, where the IC_{50} values for
333 these extracts are 90, 90 and 100 μg mL^{-1} as against curcumin inhibition at 25 μg mL^{-1}
334 concentration. It is therefore clear that some of the active components of individual extracts
335 compete with O_2 to react with nitrous oxide and prevent the formation of stable nitrite/nitrate,
336 thereby decreasing the formation of diazotised coloured molecule.[28]

337 **3.6 Hydroxyl radical scavenging activity**

338 The results of the $HO^{•}$ scavenging power of the plant extracts are provided in Table 6.
339 The $HO^{•}$ generated via Fenton reaction degrades de-oxyribose using Fe^{2+} as catalytic
340 component. The presence of radical scavenging molecules of the extract neutralizes the
341 reactivity of the radical to prevent the degradation of de-oxyribose.[29] The results of $HO^{•}$
342 scavenging by the plant extracts are given in Table-6. The scavenging activity increased with
343 increasing concentration of each extract. The activity was also dependent on the dose of the
344 individual extract. The EE, ME and HE extracts showed maximum scavenging of 89.74%,
345 94.03% and 53.63% respectively, at 150 μg mL^{-1} with an IC_{50} value of 85, 90 and 132 μg

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346 mL^{-1} as against 10 μg mL^{-1} of vitamin C, the reference antioxidant. The variation in the
347 scavenging skill may be due to the diversification in the phytocomposition of the extracts.

348 **3.7 Super oxide radical scavenging activity**

349 The superoxide radical formed by the reaction of riboflavin and NBT is suppressed by
350 suitable scavenging molecule. In this reaction NBT is oxidised to form formazan which is
351 measured spectrophotometrically.[13] In the present study, the superoxide radical scavenging
352 molecules present in the plant extracts viz. EE, ME and HE were shown to inhibit the
353 formazan formation by NBT oxidation (Table 7). The extracts at different concentration, even
354 after 1 h of incubation time did not produce a purple colour formazan. This observation
355 revealed that the crude extracts of all the three types were able to fight the superoxide radicals
356 and inhibit the NBT oxidation. These extracts showed a maximum free radical scavenging
357 activity 93.80%, 91.5% and 69.6% respectively for EE, ME and HE at 150 μg mL^{-1}
358 concentrations with an IC_{50} value of 79, 85 and 105 μg mL^{-1} as against 7 μg mL^{-1} of vitamin
359 E, the reference antioxidant.

360 **3.8. Reducing power of CC extracts**

361 The reducing power of a herbal extract is associated mainly with its phenolic
362 antioxidant activity. The presence of antioxidants in the CC plant extract resulted in the
363 reduction of potassium ferricyanide to potassium ferrocyanide. The initial yellow colour of
364 the reaction mixture of this assay changed to green and blue shades, due to the formation of
365 Fe^{2+} complex[30], depending upon the reducing power of the extracts on dose dependent
366 manner. Among the three different extracts studied, EE exhibited better activity, compared to
367 ME and HE, at 100 μg mL^{-1}concentration. At concentration below 100 μg mL^{-1} the activity of
368 EE is on par with the reference BHT. At concentration above 100 μg mL^{-1} (i.e., at 150 μg
369 mL^{-1}) both EE and HE displayed nearly same antioxidant activity; whereas, the ME showed a

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

373 **3.9 Total antioxidant activity**

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

387 **3.10 EPR spectroscopic investigations**

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

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393 contain the extracts possessing antioxidant activity. Parallel to this, the $HO^{\bullet}$ scavenging
394 ability and capacity limit the DMPO - $HO^{\bullet}$ adduct formation by the extracts were examined.

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

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

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417 ## 4. CONCLUSION

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

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438 **Conflict of interest**

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

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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)

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

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

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

Table 4 H_2O_2 scavenging activity of solvent extracts of CC with reference to Vitamin C as standard

Sample	Concentration (µg/ml)	% H_2O_2 Scavenged by			Standard Vitamin-C (µg/ml)
		EE	ME	HE	
CC crude extract	50	24.31±2.49	12.99±1.38	10.39±0.64	
	75	36.03±2.42	27.48±1.15	13.07±1.57	
	100	55.27±3.13	40.63±2.91	24.28±2.24	
	125	72.91±1.85	48.18±2.06	35.99±2.70	
	150	82.16±1.62	58.77±1.20	48.40±1.42	
IC_{50}		90	127	150	5

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

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

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

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

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

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

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

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

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

Results are expressed as percentage scavenging and are mean ± 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 µg mL^{-1}), Each value represents a mean ± SD (n=3)

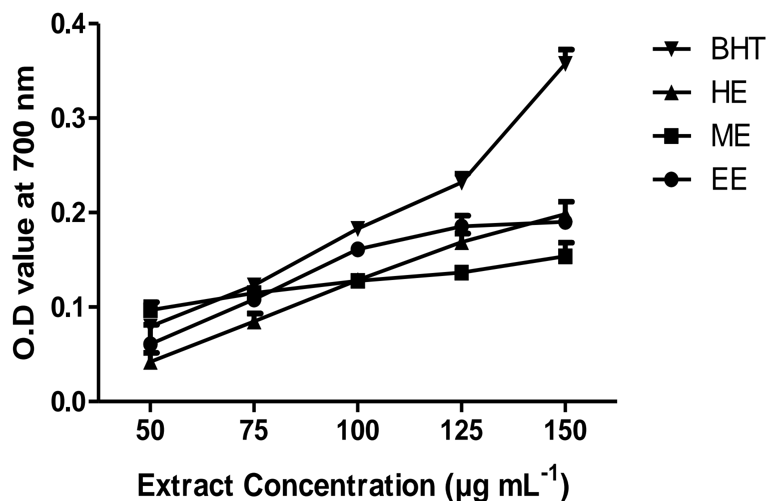

Fig. 2 Total antioxidant capacity of CC extracts at varying concentration were calculated and compared with TROLOX equivalent expressed as mmol/L, Each value represents a mean ±SD (n=3)

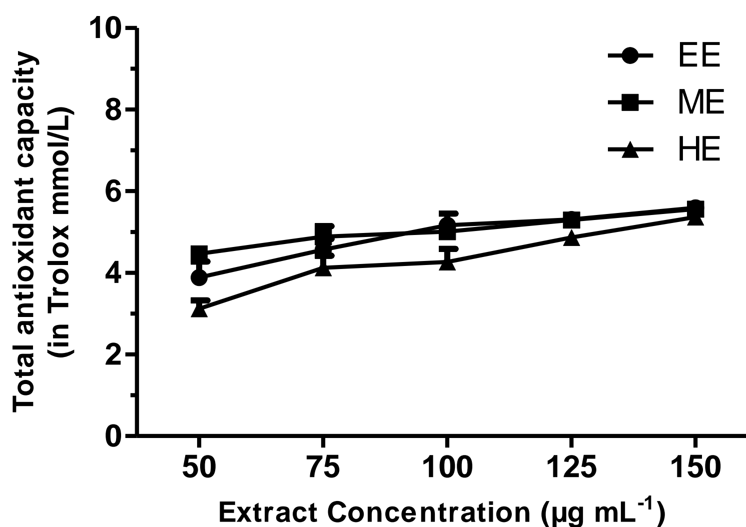

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Fig. 3 EPR Spectra of DMPO - HO• spin adduct formed via Fenton Reaction (Control)

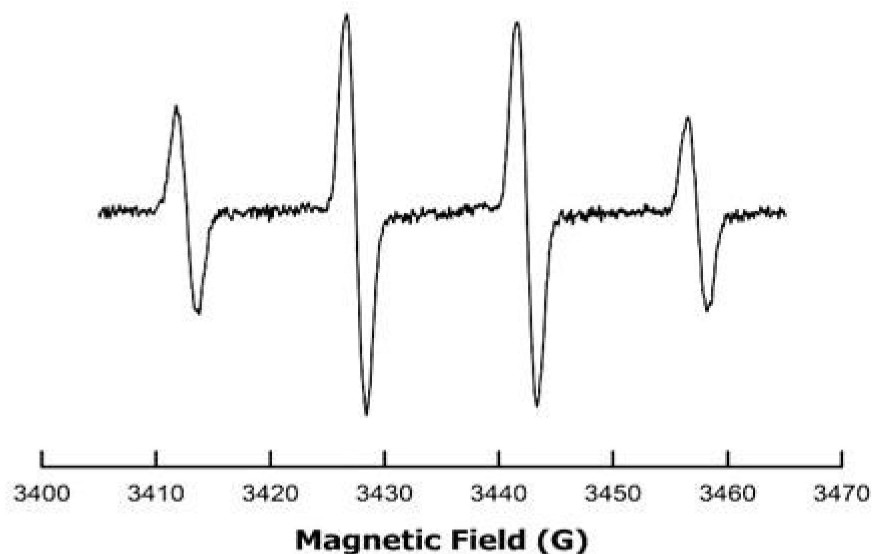
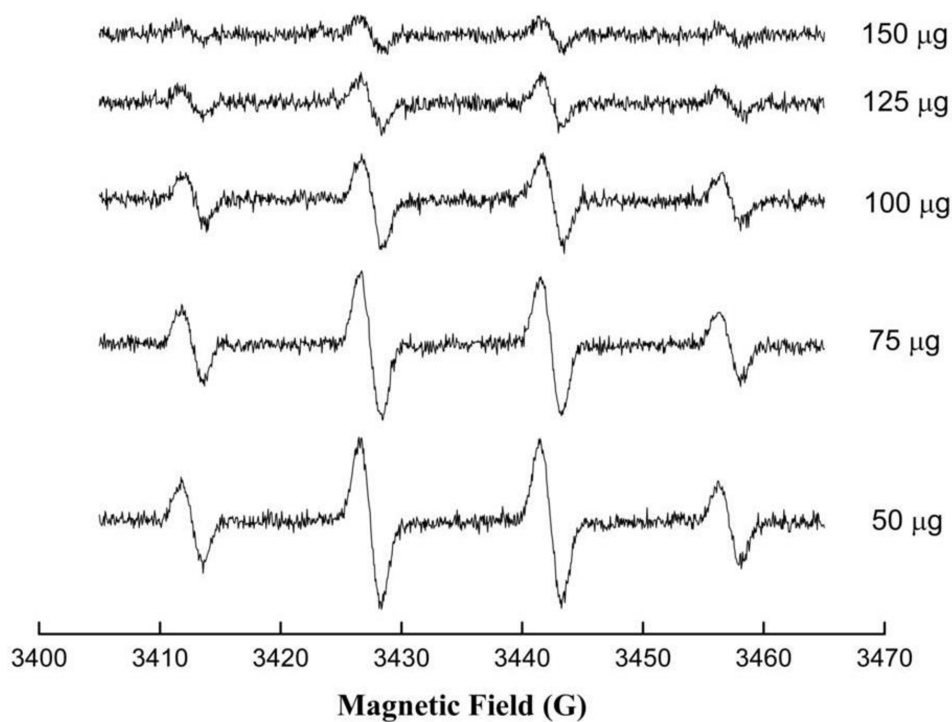


Fig. 4 X-band EPR Spectra: Effect of EE on HO• scavenging and retardation of DMPO - HO• adduct formation



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

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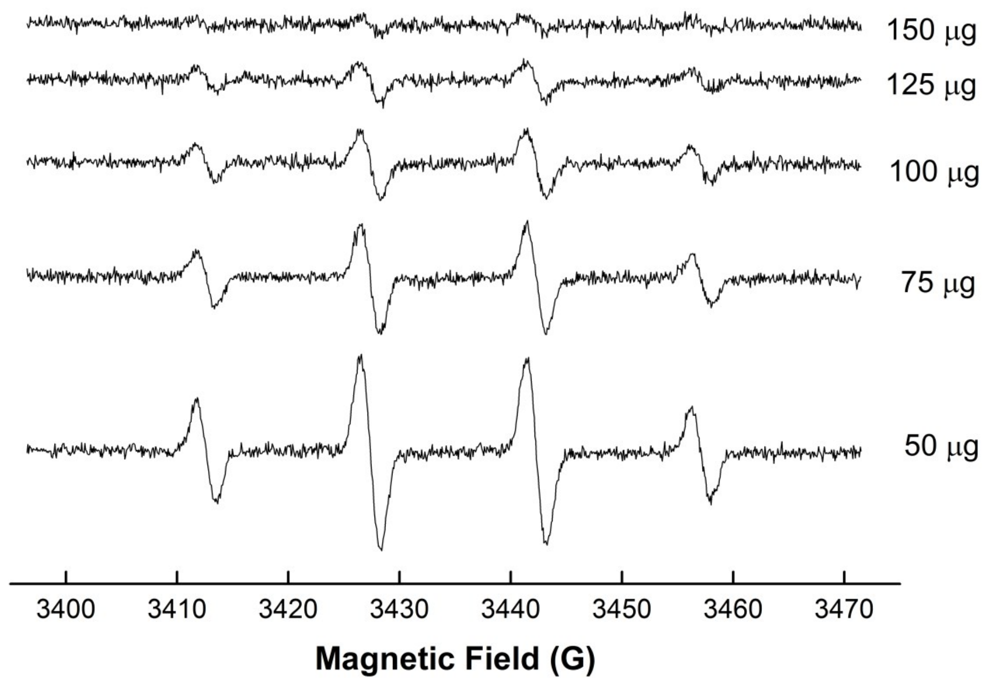
Fig. 5 X-band EPR Spectra: Effect of ME on HO$^{•}$ scavenging and retardation of DMPO - HO$^{•}$ adduct formation



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

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