**INTRODUCTION**

Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community since epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer. These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids, and carotenoids. Fruits contain many different antioxidant compounds i.e., vitamin C and E, carotenoids and phenolic compounds, that serve as radical scavengers. Recent studies have demonstrated the antioxidant activities and health benefits of the phenolic compounds occurring in fruits and vegetables. This lends support to the hypothesis that fruits and vegetables high in antioxidant potentially exert a protective effect against degenerative diseases.

**MATERIALS AND METHODS**

**Chemicals**

The solvents used in the present work were purchased from Qualigenes. Folin-Ciocalteu reagent, DPPH, Gallic acid and quercetin were procured from Sigma, USA. Ammonium molybdate tetrahydrate, aluminium chloride, TCA were acquired from E. Merck (INDIA) Limited. Mumbai, India. Nitro blue tetrazolium (NBT), Ferrozine were purchased from HI-MEDIA, Pvt. Ltd, India. All other reagents were of analytical grade.
Fruits
The fully ripened fruits of *M. calabura* were collected from Erode District, Tamil Nadu during May 2008 and June 2008. The fruits were pooled and were kept in cold (−4°C) dark storage until further analysis. Voucher specimens were prepared and deposited at the herbarium of Kongu Arts and Science College, Erode (T.N), India.

Preparation of fruit extracts
Firstly, the fruits (100 g) were removed from bunch and homogenized in a blender with 500 ml of hexane, chloroform, ethyl acetate, butanol and methanol, and extracted exhaustively. Then, the extracts were centrifuged thrice (3000 g, 15 min) and the clear supernatants were collected separately from the corresponding solvents and filtered over Whatman No. 1 filter paper. The extracts were then condensed to dryness by rotary flash evaporator (Buchi type Rotavapor). Various concentrations of the extracts were prepared from the resultant extracts to determine in vitro antioxidant activity.

Determination of total phenolics
Total phenolic contents in the extracts were determined by Folin-Ciocalteu method. An aliquot of the extracts was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml of NaCO₃ (7.5 g/l). The tubes were allowed to stand for 15 min and the total phenols were determined using UV/VIS Spectrophotometer (Elico SL 164 Double Beam, Elico Ltd.) at 765 nm. Total phenol content was expressed in terms of gallic acid equivalent in mg/100 g of fresh material (FM). All experiments were performed in triplicate.

Assessment of Free Radical Scavenging and Antioxidant Activity

**DPPH radical scavenging activity**
The quenching effect of extracts on DPPH radical was determined using the method of Blois. Briefly, to 1 ml of DPPH radical reagent (previously diluted with water 1:10 v/v) and 4 ml of NaCO₃ (7.5 g/l). The tubes were allowed to stand for 15 min and the total phenols were determined using UV/VIS Spectrophotometer (Elico SL 164 Double Beam, Elico Ltd.) at 765 nm. Total phenol content was expressed in terms of gallic acid equivalent in mg/100 g of fresh material (FM). All experiments were performed in triplicate.

**Reductive ability**
Total reducing power was determined as described previously. One ml of sample solution at different concentration was mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of potassium ferrocyanide (1%). The mixture was incubated at 50°C for 20 min. To this, 2.5 ml of trichloroacetic acid (TCA, 10%) was added and centrifuged at 3000 g for 10 min. The supernatant (5 ml) was mixed with 1 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

**Superoxide anion scavenging activity**
Measurement of superoxide anion scavenging activity was based on the method described previously. Superoxide radicals were generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In brief, 3 ml of sample solutions at different concentrations were mixed with 1 ml of NBT (156 µM) and 1 ml of NADH (468 µM). The reaction started by adding 0.1 ml of phenazine metho sulphate (PMS) solution (60 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples. The percentage inhibition of superoxide anion generation was calculated using the following formula: Inhibition of superoxide generation (%) = \[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100. \]

**Scavenging capacity towards hydroxyl ion (•OH) radicals**
The •OH scavenging activity of the fruit extracts was determined according to the method described previously. Different concentrations of extracts were added with 1.0 ml of ferrous ammonium sulfate (0.13% w/v) and 0.26% EDTA, 0.5 ml of EDTA solution (0.018%), and 1.0 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. The reaction was then terminated by the addition of 1.0 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm against reagent blank. BHT was considered as the reference standard. The hydroxyl radical scavenging activity is calculated by the following formula: HRSA (%) = \[ 1 - \frac{\text{difference in absorbance of sample}}{\text{difference in absorbance of blank}} \times 100. \]

**Scavenging activity against nitric oxide**
Nitric oxide interacts with oxygen to produce stable products, nitrite and nitrate. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite. The concentration of nitrite in aqueous solution was assayed spectrophotometrically by using the Greiss reagent, with which nitrite reacts to give a stable product absorbing at 546 nm. Nitric oxide radicals were generated from sodium.
nitroprusside (SNP) solution at physiological pH. Sodium nitroprusside (1 ml of 10 mM) was mixed with 1 ml of various concentrations of sample extracts in phosphate buffer (pH 7.4). The mixture was incubated at 25°C for 150 min. To 1 ml of the incubated solution, 1 ml of greiss reagent (1% sulphanilamide, 2% ortho phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm and percentage inhibition was calculated using the formula: inhibition (%) = \[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100. \]

Iron chelating activity
The iron chelating activity was measured by the decrease in absorbance at 562 nm of the iron (II) – ferrozine complex.[21] The reaction mixture contained 0.5 ml of various concentrations of the fruit extracts, 0.1 ml of ferric chloride (0.6 mM) and 900 µl of methanol. The mixture was shaken and left at room temperature for 10 min. To this, 0.1 ml of ferrozine (5 mM) in methanol were added, mixed and left for 5 min to complex the residual Fe²⁺. The absorbance of the resulting solution was measured at 562 nm. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula: Chelating effect (%) = \[ \frac{(Abs_{\text{control}} - Abs_{\text{sample}})}{Abs_{\text{control}}} \times 100. \]

Reduction of lipid peroxidation
Inhibition of lipid peroxidation (LPO) in rat liver homogenate was determined in terms of formation of thiobarbituric acid reactive substances (TBARS) with minor changes.[22] In brief, different concentrations of fruit extracts and standard were individually added to 0.2 ml of liver homogenate (10%) extracted with KCl (15%). To the above mixture, 0.1 ml of FeSO₄ (10 mM) solution was added to initiate LPO. The volume of the mixtures was finally made up to 2 ml with phosphate buffer (0.1 mM, pH 7) and incubated at 37°C for 30 min. At the end of the incubation period, reaction mixture (0.3 ml) was added with 1 ml of TBA (0.8%, w/v) and 0.1 ml of TCA (20%) solution. The mixture was then heated on a water bath at 100°C for 60 min. After cooling, n – butanol (4 ml) was added in each tube and centrifuged at 3000 × g for 10 min. The absorbance of the organic upper layer was read at 532 nm. BHT was used for comparison. The percentage reduction of LPO was calculated as follows:

\[ \text{Reduction of TBARS} (\%) = \frac{1 - \text{Sample}_{532nm}}{\text{Control}_{532nm}} \times 100 \]

**Table 1: Total phenolics content of M. calabura fruit**

<table>
<thead>
<tr>
<th>M. calabura</th>
<th>Total phenolics (mg/100g)⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>358 ± 0.020</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>447 ± 0.025</td>
</tr>
<tr>
<td>EA</td>
<td>1140 ± 0.02</td>
</tr>
<tr>
<td>BuOH</td>
<td>940 ± 0.03</td>
</tr>
<tr>
<td>MeOH</td>
<td>1486 ± 0.028</td>
</tr>
</tbody>
</table>

Each value is presented as Mean ± Standard Deviation (n = 3). ⁴mg GAE/100g FW

**Table 2: Antioxidant capacities of different extracts of M. calabura fruit**

<table>
<thead>
<tr>
<th>IC₅₀ (g/mL)</th>
<th>DPPH⁻</th>
<th>O₂⁻</th>
<th>OH⁻</th>
<th>NO</th>
<th>Metal chelating</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>98 ± 0.62</td>
<td>310.2 ± 0.04</td>
<td>79.46 ± 0.08</td>
<td>207 ± 0.02</td>
<td>480.6 ± 0.02</td>
<td>240.2 ± 0.04</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>245.42 ± 0.22</td>
<td>378.2 ± 0.08</td>
<td>280.4 ± 0.08</td>
<td>250 ± 0.08</td>
<td>91.2 ± 0.04</td>
<td>490.23 ± 0.24</td>
</tr>
<tr>
<td>EA</td>
<td>100.24 ± 0.24</td>
<td>240.5 ± 0.2</td>
<td>198.2 ± 0.02</td>
<td>497.2 ± 0.08</td>
<td>81.4 ± 0.04</td>
<td>190.2 ± 0.62</td>
</tr>
<tr>
<td>BuOH</td>
<td>350.12 ± 0.88</td>
<td>250.5 ± 0.48</td>
<td>52 ± 0.4</td>
<td>189 ± 0.26</td>
<td>290.2 ± 0.24</td>
<td>540.1 ± 0.02</td>
</tr>
<tr>
<td>MeOH</td>
<td>90 ± 0.04</td>
<td>79.2 ± 0.04</td>
<td>49.98 ± 0.2</td>
<td>187 ± 0.04</td>
<td>80.26 ± 0.08</td>
<td>110.4 ± 0.64</td>
</tr>
<tr>
<td>BHT</td>
<td>26.0 ± 0.65</td>
<td>83.0 ± 2.35</td>
<td>41.5±2.23</td>
<td>33.5 ± 2.12</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The data are presented as mean ± standard deviation SD (n = 3). Values with different letters in same column were significantly different at P > 0.05. ND – Not Determined. PE – Petroleum ether, CHCl₃ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxy Toluene.
MeOH extract rendered highest phenolic content (1486 ± 0.028 mg GAE/100g FW) followed by EA extract (1140 ± 0.02 mg/100g), BuOH extract (940 ± 0.03 mg/100g), CHCl₃ fraction (447 ± 0.025 mg/g GAE). The PE extract has the lowest content of phenolics (358 ± 0.020 mg/100g). The result of the study implied that total phenolics were in MeOH extract, and MeOH extract was expected to be the most active one.

**DPPH scavenging activity**

The dose-response DPPH radical-scavenging activity of various extracts studied in this study is shown in Figure 1. With regard to the estimated IC₅₀ values, all the extracts of *M. calabura* displayed significant DPPH radical quenching property. Among the extracts studied, the most active extract was found to be MeOH (IC₅₀ = 90 ± 0.04 µg/mL). The MeOH extract significantly quenched DPPH radical though it showed lesser activity than the standard BHT. The BuOH extract showed the weakest quenching capacity with an IC₅₀ value of 350.12 ± 0.88 µg/mL. The effectiveness of antioxidants as DPPH radical scavengers ranged in the following descending order: MeOH (90 ± 0.04 µg/mL) > PE (98 ± 0.62 µg/mL) > EA extract (100.24 ± 0.24 µg/mL) > CHCl₃ (245.42 ± 0.22 µg/mL) > BuOH (350.12 ± 0.88 µg/mL).

**Reducing power**

The reducing power of the crude extracts of the sample was examined as a function of their concentration. The concentration dependent reducing capacity for investigated extracts of *M. calabura* is illustrated in Figure 2. Out of five extracts, the EA displayed the highest reductive capacity (OD of 0.732 ± 0.04 at 1000 µg/mL) followed by BuOH, MeOH, PE and CHCl₃ extracts. The reference standard BHT showed 1.634 ± 0.02 at 1000 µg/mL. For the determination of reducing capacity, “Fe³⁺ – Fe²⁺ transportation” in the presence of extract was observed. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. The reducing power of the extracts might be due to the di and mono hydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities.

**Superoxide scavenging activity**

The O₂⁻ radical is one of the most dangerous free radicals in humans and also the source of hydroxyl radical (OH⁻).[24] The fruit extracts from *M. calabura* were screened for their O₂⁻ scavenging activity using PMS-NADH-NBT assay. In the PMS/NADH-NBT system O₂⁻ derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The effect of extracts (PE, CHCl₃, EA, BuOH and MeOH) on superoxide anion radicals in PMS-NADH/NBT system are shown in Fig. 3. The effect of MeOH and EA extracts was the highest with IC₅₀ value of 79.2 ± 0.04 µg/mL and 240.5 ± 0.2 µg/mL respectively. The PE and BuOH extracts exhibited moderate superoxide anion radical scavenging activity with IC₅₀ values of 310.2 ± 0.04 and 250.5 ± 0.48 µg/mL respectively and the CHCl₃ extract showed least activity (IC₅₀ 378 µg/mL).

**OH scavenging activity**

Hydroxyl radical (·OH) which is the most reactive free radical, has the capacity to conjugate with nucleotides in

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**Figure 1:** DPPH· scavenging activity of extracts from *M. calabura.*

The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCl₃ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.
DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity. The scavenging activity of the fruit extracts on OH is presented in the figure 4. The extracts from the fruit displayed dose dependent scavenging activity against the OH species, of which, MeOH and BuOH extracts were the most effective (IC50 49.98 ± 0.2 μg/mL and 52 ± 0.4 μg/mL respectively). The PE (IC50 79.46 ± 0.08 μg/mL), CHCl3 (IC50 280.4 ± 0.8 μg/mL), and EA (IC50 198.2 ± 0.02 μg/mL) extracts displayed moderate scavenging capacity.

Figure 2: Reductive ability of extracts from M. calabura.
The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCL3 – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

Figure 3: O2− scavenging capacity of extracts from M. calabura.
The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCL3 – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

Nitric oxide radical scavenging activity
In addition to reactive oxygen species, nitric oxide (NO) is also implicated in inflammation, cancer and other pathological conditions. The NO generated from sodium nitroprusside reacts with oxygen to form nitrite. From the results obtained, the extracts dose-dependently inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The nitric oxide (NO) scavenging activity of the extracts of fruit of M. calabura is depicted in figure 5. NO scavenging activity of MeOH extract from
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Figure 4: °OH scavenging capacity of extracts from *M. calabura*. The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCL₃ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

Figure 5: NO radical scavenging capacity of extracts from *M. calabura*. The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCL₃ – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxyl Toluene.

the fruits was higher (IC₅₀ 187 ± 0.04 µg/mL) compared to other extracts. The NO scavenging activity ranged in the following descending order: MeOH (IC₅₀ 187 ± 0.6 µg/mL) > BuOH (IC₅₀ 189 ± 0.26 µg/mL) > PE (IC₅₀ 207 ± 0.02 µg/mL) > CHCl₃ (IC₅₀ 250 ± 0.08 µg/mL) > EA extract (IC₅₀ 497.2 ± 0.08 µg/mL).

**Ferrous ions chelating activity**

Metal chelating activity is significant since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. Figure 6 shows concentration dependent chelating effects of fruit extracts from *M. calabura* on the Fe²⁺ - ferrozine complex. The MeOH extract displayed the highest chelating activity with an IC₅₀ value of 80.26 ± 0.08 µg/mL, followed by the EA (IC₅₀ 81.4 ± 0.04 µg/mL), CHCl₃ (IC₅₀ 91.2 ± 0.64 µg/mL), BuOH (IC₅₀ 290.2 ± 0.24 µg/mL) and PE (IC₅₀ 480.6 ± 0.02 µg/mL) extracts. Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of metal ion. Accordingly, it is suggested
that the low to moderate ferrous ions chelating effects of these fractions would be somewhat beneficial to protect against oxidation damage.

**Inhibition of lipid peroxidation**

Lipid peroxidation plays an important role in causing oxidative damage to biological systems and its by-product malondialdehyde (MDA) induces damage to other biomolecules.\(^{[27]}\) The inhibitory effect of fruit extracts on lipid peroxidation (LPO) is shown in Figure 7. In this study, a variation of inhibitory effect of the extracts on LPO was observed (Table 2). The MeOH (IC\(_{50}\) 110.4 ± 0.64 µg/mL) extract was able to inhibit the generation of LPO efficiently. The EA and PE extracts exhibited moderate LPO inhibitory activity with the IC\(_{50}\) values of 190.2 ± 0.62 µg/mL and 240.2 ± 0.04 µg/mL, respectively. CHCl\(_3\) and BuOH extracts showed least activity (IC\(_{50}\) 490.23 ± 0.24 µg/mL and IC\(_{50}\) 540.1 ± 0.02 µg/mL, respectively).

![Graph of Metal chelating capacity of extracts from M. calabura. The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCL\(_3\) – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxy Toluene.]

![Graph of LPO inhibitory activity of extracts from M. calabura. The data are presented as mean value ± standard deviation SD (n = 3). PE – Petroleum ether, CHCL\(_3\) – Chloroform, EA – Ethyl acetate, BuOH – Butanol, MeOH – Methanol, BHT – Butyl Hydroxy Toluene.]

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CONCLUSION

Recent years have seen an exponential increase in research antioxidant properties of fruits and vegetables. If it is accepted that higher intakes of natural antioxidants containing phenolics are associated with long-term health benefits, then the results presented in this paper offer possible avenues toward health promotion by identifying those compounds. The health promoting properties of fruits of *M. calabura* may be due to its antioxidant properties and is also attributed to its multitherapeutic characteristics. Thus, *M. calabura* might be useful in the development of raw materials of medicine.

REFERENCES