

## STUDIES ON IN VITRO FREE RADICAL SCAVENGING ACTIVITY OF *BIXA ORELLANA* L. BARK EXTRACT

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### ABSTRACT

In vitro antioxidant activity of methanolic bark extract of *Bixa orellana* L. (Bixaceae) was screened by various antioxidant assay models such as 1,1-diphenyl-2-picryl hydroxyl quenching assay, reducing power capacity, superoxide scavenging assay, scavenging capacity towards hydroxyl ion radicals, nitric oxide radical, hydrogen peroxide inhibition activities, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid cation decolorization test and inhibition of lipid peroxidation. Total phenolics, total flavonoid, flavonol and ascorbic acid contents were also determined. The bark yielded 72 mg phenolic content, 83mg flavonoid content, 250mg flavonol content and 47.2 mg Vitamin C. The extract exhibited high antiradical activity against DPPH, ABTS<sup>+</sup>, NO, ·OH, O<sub>2</sub><sup>-</sup> LPO and H<sub>2</sub>O<sub>2</sub> with IC<sub>50</sub> value of 16 ± 0.70, 80 ± 0.70, 70 ± 1.45, 90 ± 0.41, 250 ± 1.52, 86.5 ± 1.89 and 230 ± 2.85µg/ml respectively. High correlation between polyphenolics and scavenging potential of different reactive oxygen species indicated that polyphenols are considered as main antioxidants.

**Keywords:** *Bixa orellana*, Antioxidant activity, DPPH, Reducing capacity, ABTS, Nitric oxide scavenging activity.

### INTRODUCTION

Free radicals including reactive oxygen species (ROS) which is generated from metabolism and or by the environmental factors interact directly to the biological systems and their unbelievable generation of free radicals have been implicated with variety of chronic diseases including cancer, diabetes, atherosclerosis, neurodegenerative disorders and arthritis<sup>1,2</sup>.

Antioxidants are compounds that can slow down or totally inhibit the oxidation of lipid or other molecules by inhibiting the propagation of oxidizing chain reactions<sup>3</sup>. The body's antioxidant defense system helps to protect the cells from excess reactive oxygen species production and is comprised of both endogenous (bilirubin, uric acid, superoxide dismutases, catalase, glutathione peroxidase, etc.) and exogenous (carotenoids, tocopherols, ascorbate, bioflavonoids, etc.) compounds<sup>4</sup>. Natural antioxidant mechanisms is inefficient, hence dietary intake of antioxidant compounds becomes important.

Epidemiological studies have indicated the relationship between the plant antioxidants and reduction of chronic diseases<sup>5, 6</sup>. These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids, and carotenoids<sup>7</sup>. The studies in recent years have shown that phenolic substances in plants scavenge active oxygen species and effectively prevent oxidative cell damage<sup>8</sup>. Use of herbal products could be a better option to meet the objective of finding a suitable treatment for reducing the free radicals generation.

*Bixa orellana* L., belonging to the family Bixaceae, is used for the treatment of liver disorders<sup>9</sup>. It is a small handsome evergreen tree, leaves are cordate, acuminate, flowers are white or pink in terminal panicle, fruits reddish brown, and seeds are trigonous covered with a red pulp. The roots bark and seeds of *B. orellana* are antiperiodic, antipyretic and astringent. They are useful in intermittent fevers and gonorrhoea. The pulp surrounding the seed is a mosquito repellent and is useful to treat dysentery. The decoction of the root is used for liver diseases. The whole plant is bitter, purgative, cures leprosy, biliousness, kidney disorders and vomiting. Seeds and leaves of the annatto tree were used by the Aztecs to prepare remedies for a variety of illnesses such as tonsillitis, asthma, pleurisy, rectal disorders, headache, jaundice, sunstroke, and burns<sup>10</sup>. To the best of our knowledge, no work has been reported on the antioxidant properties of this plant. The present study was undertaken to evaluate the effects of methanolic bark extract of *B. orellana* on free radicals generated in various assay models.

### MATERIALS AND METHOD

#### Plant Material

The bark of *B. orellana* was collected from Karpagam University campus, Coimbatore, Tamil Nadu State, India, identified by Dr. R. Gopalan, taxonomist, Karpagam University, Coimbatore and was authenticated in the Botanical Survey of India (BSI), Coimbatore. Voucher specimen was deposited in the herbarium of the Karpagam University (Voucher no: KU 625).

#### Preparation of *B. orellana* Bark Extract (BME)

The *B. orellana* bark was shade dried at room temperature and finely powdered. The bark powder (1000 g) was exhaustively extracted with 5 L of methanol [MeOH 1:5 (w/v)] using soxhlet apparatus. The extract was centrifuged (3000 x g) thrice and the clear supernatants were filtered over Whatman No. 1 filter paper. The extract was evaporated to dryness *in vacuo* by rotary flash evaporator (Buchi type, Switzerland) under reduced pressure at 45° C. Different concentrations were prepared from the resultant crude methanolic extract to assess in vitro antioxidant capacity.

#### Measurement of Total Phenolics (TP)

The content of total phenolics was determined by using Folin-Ciocalteu method<sup>11</sup>. Briefly, diluted extract (1mL) was added with diluted Folin-Ciocalteu reagent (1 N, 1 ml). After 3 min of reaction, Na<sub>2</sub>CO<sub>3</sub> (35%, 2 ml) was added and the mixture was incubated for 30 min at room temperature. The absorbance was read at 765 nm using UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The analyses were performed in triplicate. The TP was expressed as mg gallic acid equivalents from a gallic acid standard curve (mg GAE / 100 g fresh material, R<sup>2</sup> = 0.9968).

#### Measurement of Total Flavonoids (TF)

The content of total flavonoid in *B. orellana* bark extract was based on the method<sup>12</sup>. A volume of 0.5 ml of 2 % AlCl<sub>3</sub> ethanol solution was added to 0.5 ml of sample solution. After one hour at room temperature, the absorbance was measured at 420 nm with UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan). The TF was estimated from a quercetin standard curve and the results were expressed as mg quercetin equivalents (mg QE/100 g fresh material, R<sup>2</sup> = 0.992).

#### Measurement of Total Flavonols (TFI)

Total flavonols in the plant extract were estimated using the method of Kumaran and Karunakaran 2007<sup>13</sup>. To 2.0 ml of sample, 2 ml of AlCl<sub>3</sub> (2%) ethanol and 3 ml (50 g/l) sodium acetate solutions were

added. After 2.5 h at 20°C, the absorption was measured at 440 nm. Extract sample was evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve:  $y = 0.0255x$ ,  $R^2 = 0.9812$ , where  $x$  was the absorbance and  $y$  was the quercetin equivalent (mg/100g).

#### Measurement of Ascorbic Acid (AA)

The amount of ascorbic acid is determined using the method of Sadasivam and Manickam (1996)<sup>14</sup>. A volume of 5ml of the standard solution were taken (Vitamin C) into a 100 ml conical flask. Add 10 ml of 4 % oxalic acid and titrate against the dye. End point is the appearance of pink color which persists for a few minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. Extract the sample (0.5-5 g depending on the sample) in 4 % oxalic acid and make up to a known volume (100 ml) and centrifuge. A volume of 5 ml of the supernatant, and 100 ml of 4 % oxalic acid were taken and titrated against the dye. Amount of ascorbic acid was calculated and expressed as mg/100g sample

#### Antioxidant Activity Determination

The antioxidant activity of methanolic extract was evaluated using DPPH radical quenching assay, reducing capacity, scavenging capacity towards superoxide radical ( $O_2^{\cdot-}$ ), scavenging capacity towards hydroxyl ion ( $OH^{\cdot}$ ) radicals, scavenging capacity towards nitric oxide (NO), ABTS<sup>+</sup> cation decolorization test, hydrogen peroxide ( $H_2O_2$ ) scavenging activity and lipid peroxidation assay (TBARS).

#### DPPH Radical Quenching Activity

DPPH radical scavenging activity was adopted from those previously described with slight modifications<sup>15</sup>. Briefly, various concentrations (20- 100 µg/ml) of sample were mixed with 5 ml of 0.1mM methanol solution of DPPH and vortexed. The tubes were allowed to stand at room temperature for 20 min. The control was prepared as above without any extract, and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Mean values were obtained from triplicate experiments. Butylated hydroxy toluene (BHT) was taken as reference standard. The percentage quenching of DPPH was calculated as follows: Inhibition of DPPH (%) = Control 517nm - Sample 517nm /Control 517nm x 100, where, Sample 517nm was absorbance of the sample and Control 517nm was absorbance of control. The results were expressed as  $IC_{50}$ , which means the concentration at which DPPH radicals were quenched by 50%.

#### Reducing Capacity

The ferric reducing power of the bark extract was quantified according to the method of Oyaizu (1986)<sup>16</sup>. Various concentrations of the extracts (200-1000µg/ml) were prepared. To all the extracts in test tubes 2.5 ml of sodium phosphate buffer followed by 2.5 ml of 1% potassium ferricyanide [ $K_3Fe(CN)_6$ ] solution was added. The contents were vortexed well and then incubated at 50° C for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid (TCA) was added to all the tubes and centrifugation was carried out at 3000 g for 10 minutes. Afterwards, to 5ml of the supernatant, 5 ml of deionized water was added. To this about 1 ml of 1% ferric chloride was added to each test tube and incubated at 35°C for 10 minutes. The absorbance was read at 700 nm. Mean values were obtained from triplicate experiments. The reducing power of the extract was linearly proportional to the concentration of the sample. Increased absorbance of the reaction mixture indicated increased reducing power. Butylated hydroxytoluene (BHT) was taken as reference standard.

#### Scavenging Capacity towards Superoxide Radical ( $O_2^{\cdot-}$ )

Super oxide anion radicals ( $O_2^{\cdot-}$ ) generated in the phenazine methosulfate-reduced form of nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium chloride (NBT) by the extract with some changes<sup>17</sup>. The  $O_2^{\cdot-}$  were generated in 1.25 ml of Tris-HCl (16mM, pH 8.0), 0.25 ml of NBT (150 µM), 0.25 ml of NADH (468µM) and different concentrations (100-1000µg/ml) of BME and standard.

The reaction was initiated by addition of 0.25 ml of phenazine methosulfate (60µM) to the mixture. Following incubation at ambient temperature for 5 min the absorbance was read at 560 nm. Blank was used as the same way using methanol instead of test sample. BHT was used for comparison. The percentage scavenging of  $O_2^{\cdot-}$  was calculated as follows: Inhibition of  $O_2^{\cdot-}$  (%) = Control 560nm - Sample 560nm / Control 560nm x 100, where, Sample 560nm was absorbance of the sample and Control 560nm was absorbance of control.  $IC_{50}$  was calculated as 50% reduction in absorbance brought about by sample compared with blank.

#### Scavenging Capacity towards Hydroxyl Ion ( $OH^{\cdot}$ ) Radicals

The  $OH^{\cdot}$  scavenging activity of the BME was determined according to the method described previously<sup>18</sup>. Different concentrations (50-250µg / ml) of extract were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate 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-(difference in absorbance of sample/difference in absorbance of blank) × 100.

#### Scavenging Capacity towards Nitric Oxide (NO)

Sodium nitroprusside (SNP) in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction<sup>19</sup>. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. The reaction mixture (3 ml) containing SNP (10mM, 2 ml), phosphate buffer saline (0.5 ml) and the BME at different concentrations and standards (50-250µg mL) were incubated at 25° C for 150 min. After incubation, 0.5 ml of the incubated solution containing nitrite was pipetted and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of N-1-naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25° C. The absorbance of pink colored chromophore formed during diazotization was measured at 540 nm. BHT was used for comparison. The percentage scavenging of NO was calculated as follows: Inhibition of NO (%) = Control 540nm - Sample 540nm/Control 540nm x 100, where, Sample 540nm was absorbance of the sample and Control 540nm was the absorbance of control.

#### Hydrogen Peroxide ( $H_2O_2$ ) Scavenging Activity

The  $H_2O_2$  scavenging activity of the extract was determined by the method of Ruch et al. (1989)<sup>20</sup>. The extract at different concentration (100-500µg mL<sup>-1</sup>) was dissolved in 3.4 ml of 0.1 M phosphate buffer pH 7.4) and mixed with 600µl of 43mM solution of  $H_2O_2$ . The absorbance value of the reaction mixture was recorded at 230 nm and BHT was considered as the standard. The percentage scavenging of NO was calculated as follows: Inhibition of NO (%) = Control 230nm - Sample 230nm/Control 230nm x 100, where, Sample 230nm was absorbance of the sample and Control 230nm was the absorbance of control.

#### 2, 2'-Azinobis (3-ethylbenzothiazoline sulphonic acid) (ABTS<sup>+</sup>) Cation Decolorization Test

The ABTS<sup>+</sup> radical cation was produced by oxidizing ABTS<sup>+</sup> with potassium persulfate ( $K_2S_8O_8$ )<sup>21</sup>. The ABTS<sup>+</sup> solution (7mM) was oxidized with  $K_2S_8O_8$  (2.4mM) for 12 h at room temperature in the dark. The ABTS<sup>+</sup> solution was then diluted by mixing 990µl ABTS<sup>+</sup> solution with 60 ml methanol to obtain an absorbance of  $0.706 \pm 0.001$  at 734 nm. BME and BHT of various concentrations (100-

500µg mL<sup>-1</sup>) were allowed to react with 1.0 ml of the ABTS<sup>•+</sup> solution and the absorbance was measured at 734 nm after 7 min. BHT was used for comparison. Percentage inhibition was calculated as  $\text{ABTS}^{\bullet+}$  radical scavenging activity (%)  $\text{Control } 734\text{nm} - \text{Sample } 734\text{nm} / \text{Control } 734\text{nm} \times 100$ , where,  $\text{Sample } 734\text{nm}$  was absorbance of the sample and  $\text{Control } 734\text{nm}$  was absorbance of control.

#### Lipid Peroxidation Assay (LPO)

A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid rich medium<sup>22</sup>. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids thiobarbituric acid (TBA) yielding a pinkish red chromogen with an absorbance maximum at 532nm. Egg homogenate (0.5ml of 10% v/v) and 0.1ml of extract were added to a test tube and made up to 1ml with distilled water 0.005 ml of FeSO<sub>4</sub> (0.07M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of (w/v) TBA (0.8%) in 1.1% sodium dodecyl sulphate (SDS) and 0.5 ml TCA (20%) were added. The resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5.0 ml of butan-*n*-ol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was read at 532 nm. Inhibition of lipid peroxidation by the extract was calculated according to  $[(1-E/C) \times 100]$ , where C is the absorbance value of the fully oxidized control and E is (Abs<sub>532</sub>+TBA - Abs<sub>532</sub>-TBA).

#### Statistical Analysis of Data

The experimental data were expressed as mean values  $\pm$  SD of at least three independent measurements. Linear regression analysis was used to calculate the efficient concentration (IC<sub>50</sub>) values. One way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were carried out. The *P* values of less than 0.05 were adopted as statistically significant. Regression analysis was used to determine correlation between the antioxidant properties and polyphenolic contents.

### RESULT AND DISCUSSION

#### TP, TF, Tfl and AA Contents

The total phenolic, flavanoid, flavanol, ascorbic acid contents of the methanolic bark extract of *B. orellana* were determined and the results are presented in table 1. The antioxidant activity of phenolic compounds is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers<sup>23</sup>. Total phenolic content of bark extract was found to be 72mg/100g of Gallic acid equivalent (GAE). Polyphenols are present in a variety of plants utilized as important components of both human and animal diets<sup>24</sup>. The flavanol and flavanoid content of *B. orellana* was found to be 83mg /100g and 250 mg /100g of quercetin equivalent respectively, where as the AA content was found to have 47 mg /100g of Vitamin C equivalent (Table 1).

**Table 1: Polyphenols and ascorbic acid content of *B. orellana* bark extract**

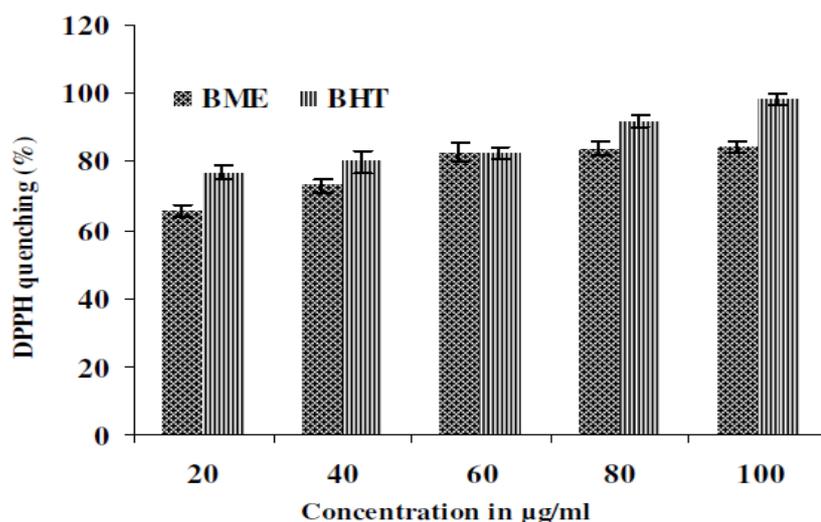
Parameters	(mg/100g)
Total phenols <sup>a</sup>	72.0 $\pm$ 0.029
Total flavanoid <sup>b</sup>	83 $\pm$ 0.014
Total flavanol <sup>b</sup>	250 $\pm$ 0.120
Ascorbic acid <sup>c</sup>	47.2 $\pm$ 0.84

Each value in the table was obtained by calculating the average of three experiments  $\pm$  standard deviation (*n* = 3). <sup>a</sup> mg Gallic acid/100g of sample. <sup>b</sup> mg Quercetin/100g of sample, <sup>c</sup> mg Vitamin C/100g of sample.

#### Antioxidant Activity

##### DPPH Quenching Capacity

DPPH<sup>•</sup> is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant<sup>25</sup>. Since the DPPH<sup>•</sup> assay can accommodate many samples in a short period and it is sensitive enough to detect active ingredient at low concentration, the DPPH scavenging activity has been widely used to evaluate the antiradical activity of various samples<sup>26,27</sup>. The DPPH<sup>•</sup> scavenging percentage rate of BME is illustrated in Fig 1. In the present study, the extract exhibited a concentration dependent antiradical activity by quenching DPPH radical. Our finding shows that the extract exhibit almost equal scavenging power when compared with the standard BHT.



**Fig.1: DPPH<sup>•</sup> quenching activity of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).**

#### Reducing Capacity

The reduction of the ferric ion (Fe<sup>3+</sup>) to ferrous ion (Fe<sup>2+</sup>) is measured by the intensity of the resultant blue-green solution which absorbs at 700 nm, and an increased absorbance is indicative of

higher reducing power. The dose dependent reducing capacity of BME was shown in Fig 2. It caused significant elevation of reducing power with OD value of 1.133 at 1000µg/ml which was significantly more pronounced than that of BHT 1.168 at 1000µg/ml. The higher

values of reducing power indicate that some constituents are

electron donors which would react with the free radicals.

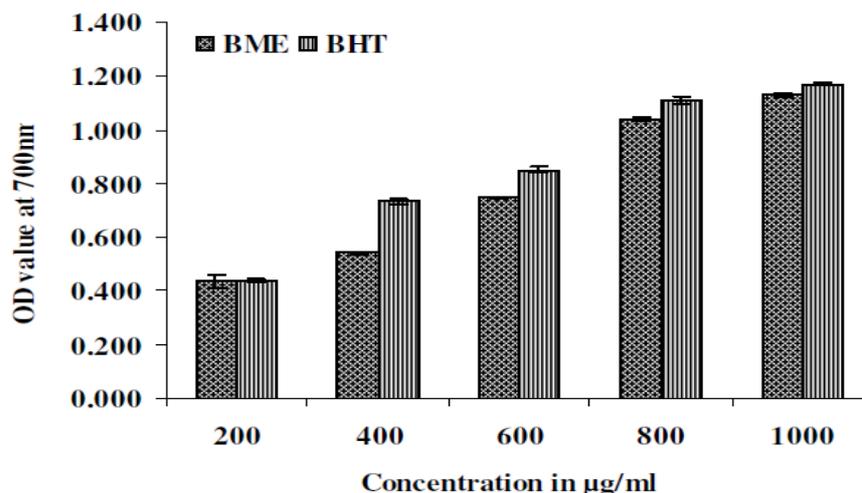


Fig. 2: Reducing capacity of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).

In our present study assay of reducing power was based on the reduction of  $Fe_3^+$  in the presence of reductants (antioxidant) in the tested extract sample. The increased reducing ability may be due to the formation of reductants which would react with free radicals to stabilize and terminate radical chain reaction and converting them to more stable products<sup>28</sup>.

#### Superoxide ( $O_2^-$ ) Scavenging Capacity

Superoxide radical is the main source for the formation of other ROS such as hydroxyl radical and  $H_2O_2$ <sup>29</sup>. Result of the  $O_2^-$  scavenging activity of BME measured by PMS-NADH-NBT superoxide generating system is illustrated in Fig 3. The scavenging effect of extract at a concentration of 500µg/ml is 86.4 %. In the PMS-NADH-NBT system  $O_2^-$  derived from dissolved oxygen by PMS-NADH coupling reaction

reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of  $O_2^-$  in the reaction mixture. The result was comparable with standard BHT which was found to have 86.7 % inhibition at 500µg/ml.

#### Hydroxyl radical ( $\cdot OH$ ) Scavenging Capacity

Hydroxyl radical ( $\cdot OH$ ), the most reactive free radical has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity<sup>30</sup>. When BME was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction (Fig 4). The BME had scavenged hydroxyl radical by 68.5% at 100µg and 82.1% at 250µg/ml respectively and percentage inhibition was proportional to the concentration of the extract.

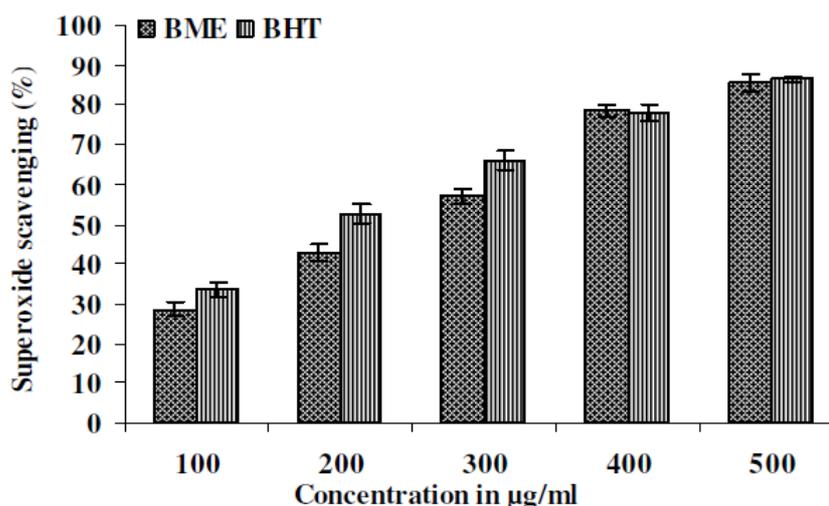


Fig. 3: Superoxide ( $O_2^-$ ) scavenging activities of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).

#### Nitric oxide radical (NO) Scavenging Capacity

Extent of nitric oxide radical scavenged was determined by the decrease in intensity of pink colored chromospheres at 540 nm.

From the results obtained, it was found that BME exerted marked inhibitory potential against nitric oxide generation (Fig 5). From the results obtained, it is inferred that the extract possessed antiradical activity. Nitric oxide scavenging potential of the BME is comparable

with that of BHT. The BME showed significantly high inhibitory potential 93.1% at the concentration of 250µg/ml against nitric oxide generation

The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by

directly competing with oxygen in the reaction with nitric oxide. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO<sup>-</sup>)<sup>31</sup>. Nitric oxide is a secondary messenger and plays an important role in the control of blood pressure. The present study proved that the extract studied has potent nitric oxide scavenging activity.

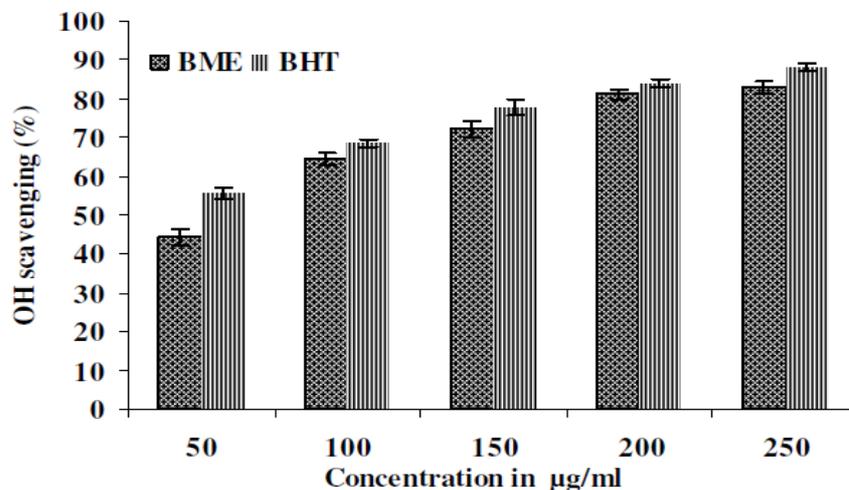


Fig. 4: Hydroxyl radical (OH<sup>·</sup>) scavenging activities of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).

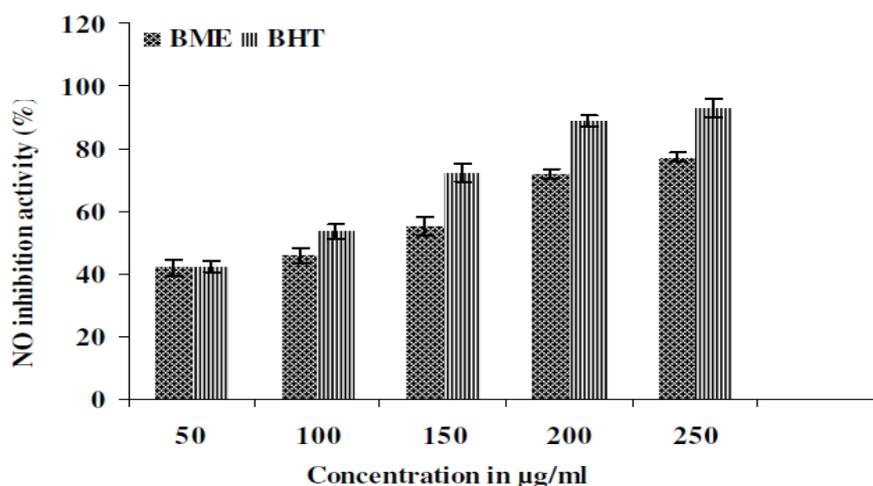


Fig. 5: Nitric oxide (NO) scavenging activities of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).

#### H<sub>2</sub>O<sub>2</sub> Inhibition Capacity

Elimination of H<sub>2</sub>O<sub>2</sub> is very important for antioxidant defense in cell or food systems since H<sub>2</sub>O<sub>2</sub> may give rise to hydroxyl radicals which are toxic to cell<sup>32</sup>. Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Fig 6 demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner. The BME was found to have 33.4 % at 100µg and 80.1% at 500µg/ml where as the standard BHT was amounted to have 54.5% at 100µg and 85.1% at 500µg/ml. From the results obtained, it is inferred that the extract possessed inhibition capacity.

#### ABTS<sup>·+</sup> Scavenging Capacity

The ABTS<sup>·+</sup> scavenging assay can be an index reflecting the antioxidant activities of test sample. The BME was an effective scavenger of the ABTS<sup>·+</sup> radical and was comparable to BHT (Fig 7). The extract expressed the maximum scavenging activity of 97.7% which was comparable to that of BHT (98%). The decolourization of ABTS<sup>·+</sup> radical also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to deactivate the radical species<sup>33</sup>. The findings are considered to be noteworthy when compared to the results of previous studies on medicinal plants<sup>34, 5</sup>.

#### Inhibition of LPO

The damage caused by LPO, is highly detrimental to the functioning of the cell<sup>35</sup>. The effect of BME on peroxidation of lipids in the presence of ferrous sulphate is shown in Fig 8.

It showed an inhibition of 80.2% at a concentration of 500µg/ml. The BME inhibited generation of lipid peroxides in a concentration

dependent manner. Therefore BME has a strong resistance on peroxidation.

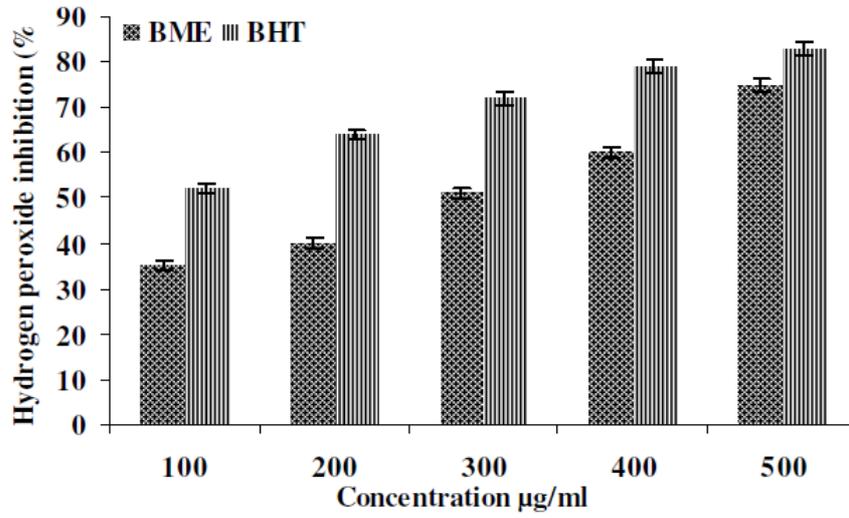


Fig. 6: Hydrogen peroxide radical (H<sub>2</sub>O<sub>2</sub>) scavenging activities of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT)

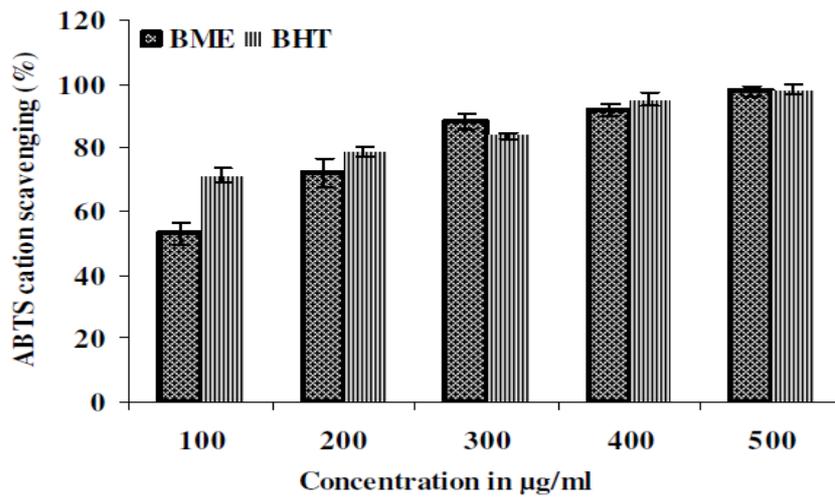


Fig. 7: ABTS<sup>+</sup> cation scavenging activity of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).

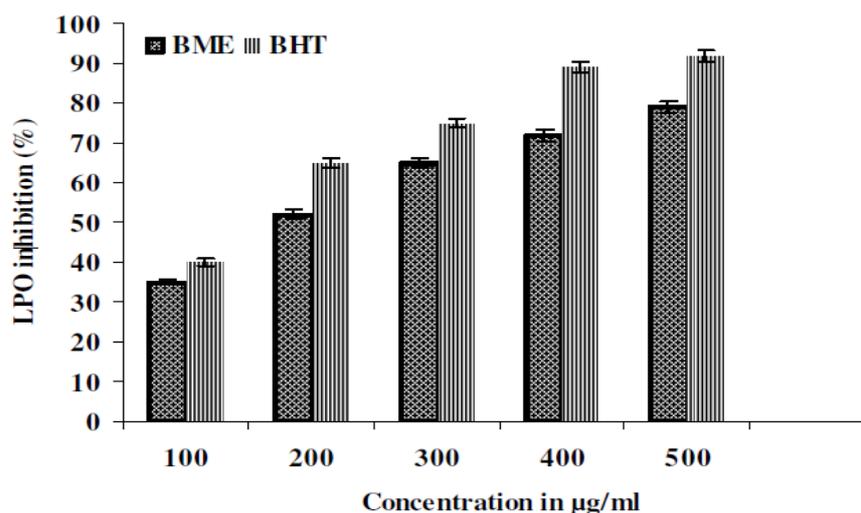


Fig. 8: Inhibition of lipid peroxidation (LPO) of *B. orellana* methanol extract (BME) and Butylated Hydroxy Toluene (BHT).

#### IC<sub>50</sub> Values and Multiple Correlations

Result expressed as IC<sub>50</sub> values (µg of extract / ml) were summarized in Table 2. The IC<sub>50</sub> values of BME in DPPH<sup>•</sup>, ABTS<sup>+</sup>, NO<sup>•</sup>, OH<sup>•</sup>, O<sub>2</sub><sup>-</sup> LPO and H<sub>2</sub>O<sub>2</sub> scavenging activities were found to be 16 ± 0.70, 80 ± 0.70, 70 ± 1.45, 90 ± 0.41, 250 ± 1.52, 86.5 ± 1.89 and 230 ± 2.85 µg/ml respectively. Among all the assays DPPH is found to have better inhibiting capacity with 16µg/ml. The IC<sub>50</sub> values of standard BHT were also depicted in Table 2. It was reported that

lower IC<sub>50</sub> values indicated that the extracts were effective in antioxidant properties<sup>36</sup>.

Correlation between the antioxidant capacities measured by different assays and the polyphenolic contents were presented in table 3. It was observed that the TP, TF and TFI showed a high positive correlation with antioxidant activities assays,

DPPH<sup>•</sup> (R<sup>2</sup>=0.848), ABTS (R<sup>2</sup>=0.991) and reducing capacity (R<sup>2</sup>=0.972).

Table 2: IC<sub>50</sub> Values of *B. orellana* methanolic bark extract (BME) and standard butylated hydroxyl toluene (BHT).

Assay models	IC <sub>50</sub> µg/ml	
	BME	BHT
DPPH <sup>•</sup> scavenging activity	16 ± 0.70 <sup>a</sup>	10.00 ± 0.65 <sup>a</sup>
ABTS <sup>+</sup> scavenging activity	80 ± 0.70 <sup>b</sup>	41.50 ± 0.08 <sup>b</sup>
NO scavenging activity	70 ± 1.45 <sup>c</sup>	33.50 ± 2.12 <sup>c</sup>
OH <sup>•</sup> scavenging activity	90 ± 0.41 <sup>e</sup>	41.50 ± 2.23 <sup>b</sup>
O <sub>2</sub> <sup>-</sup> scavenging activity	250 ± 1.52 <sup>g</sup>	56.25 ± 1.25 <sup>d</sup>
LPO inhibition	86.5 ± 1.89 <sup>d</sup>	43.87 ± 2.02 <sup>bc</sup>
H <sub>2</sub> O <sub>2</sub> scavenging activity	230 ± 2.85 <sup>f</sup>	52.00 ± 1.76 <sup>d</sup>

Each value in the table was obtained by calculating the average of three experiments ± standard deviation (n = 3). Values in a column with different superscripts indicate significantly different at P < 0.05.

It was also noticed that there was a strong correlation between TF and ABTS<sup>+</sup> test (R<sup>2</sup> =0.991). It is interesting to find that lipid peroxidation (LPO) and TF exhibited a perfect correlation (R<sup>2</sup> =1.0) (Table 3). Direct correlation among the antioxidant tests were demonstrated by linear regression method. The results showed that the correlation between ABTS and DPPH was found to have positive correlation of R<sup>2</sup>=0.832.

The correlation between reducing power and TBARS has a moderate correlation (R<sup>2</sup>= 0.852) respectively. Many supportive

reports emphasize the positive correlation between phenolic content and antioxidant efficacy<sup>37, 38</sup>. A positive correlation between antioxidant activity and polyphenol content was found, suggesting that the antioxidant capacity of the plant extracts is due to a great extent to their polyphenols<sup>39, 40</sup>. Strong positive correlations between antioxidant activity and flavanoid and phenol contents suggests that the antioxidant capacity of the bark extract of *B. orellana* is due to a great extent of phytochemical like flavonoids and other phenols.

Table 3: Correlation coefficient of total phenolic, flavanoids and flavanol with different antioxidant assays

Parameters	TP	TF	TFL
TP	1		
TF	1	1	
TFI	0.956	1	1
DPPH <sup>•</sup>	0.842	0.868	0.846
NO	0.957	0.958	0.957
OH <sup>•</sup>	0.902	0.902	0.923
ABTS <sup>+</sup>	0.991	0.991	0.903
RC	0.972	0.972	0.972
LPO	1	0.926	0.926
O <sub>2</sub> <sup>-</sup>	0.984	0.984	0.823

H <sup>2</sup> O <sup>2</sup>	0.859	0.873	0.916
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TP - Total phenolics; TF- Total flavanoid; TFI - Total flavanol; DPPH- Diphenyl picryl hydrazyl; ABTS- 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); NO - Nitric oxide; OH<sup>-</sup> - Hydroxyl radical scavenging; RC - Reducing capacity, O<sub>2</sub><sup>-</sup> - Super oxide anion scavenging activity, H<sub>2</sub>O<sub>2</sub> - Hydrogen peroxide, LPO - Lipid peroxidation.

## CONCLUSION

Herbal extracts could be used as a complementary agent in providing better clinical recoveries when given along with our regular food practices. Hence the potential medicinal use of *B. orellana* was supported by the radical scavenging capacity as explained above can also be used to attenuated oxidative stress via its antioxidant properties. However, further studies on isolating active principles responsible for the overall antioxidant activity of the extract through LC-MS will be carried out as the future study.

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