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***In vitro* antioxidant potential, total phenolic and flavonoid contents of *Artabotrys crassifolius* Hook.f. & Thomson**

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ABSTRACT

The aim of the current study was to assess the antioxidant potential, total phenolic and flavonoid contents of crude extracts of *Artabotrys crassifolius*. The leaves and barks of *Artabotrys crassifolius* were extracted sequentially with hexane, chloroform and ethanol. The corresponding crude extracts obtained were then subjected to TPC, TFC, ABTS, DPPH and FRAP assays. Among the extracts tested, ethanol extracts of barks and leaves showed promising radical scavenging activity which necessitates further isolation and characterisation of the bioactive compounds present in the respective extracts.

Keywords: Antioxidant, Phenolic, Flavonoid, *Artabotrys crassifolius*

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INTRODUCTION

Free radicals are atomic or molecular species that can exist independently with one or more unpaired electrons in their outermost shell¹. They are generated as by-products during normal cellular metabolism². Due to their highly reactive and unstable properties in nature³, they are capable of inducing oxidative damage to all the major classes of biomolecules including carbohydrates, lipids, proteins, and nucleic acids⁴. These damages are further implicated in the pathogenesis of atherosclerosis, cancer, diabetes mellitus, ischemia and reperfusion injury, neurodegenerative diseases, obstructive sleep apnea, rheumatoid arthritis as well as senescence⁵.

Although the human body possesses the comprehensive network of antioxidant defence and repair systems, these endogenous protective mechanisms are inadequate to counteract the damaging effects of free radicals completely⁶. More importantly, the application of currently available synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), gallic acid esters and tert-butylhydroquinone (TBHQ) is often restricted because of their low solubility, moderate antioxidant activity and possible toxicity⁷. Therefore, the exploration of alternative antioxidants from natural sources is highly desirable.

Being as one of the most evolved and complex ecosystems in the world, the tropical rainforest of Malaysia serves a vast untapped biodiversity of natural resources⁸. This unique natural heritage has brought renewed interest in the screening of indigenous medicinal plants for bioactive compounds. *Artabotrys* R.Br. is one of the largest genera of the custard-apple family, Annonaceae. The genus *Artabotrys* encompasses more than 100 species of woody climbers and scandent shrubs distributed mainly in tropical and subtropical regions of the world, especially tropical Africa and Eastern Asia. Furthermore, many species of this genus have been traditionally used for the treatment of different human ailments, particularly malaria, scrofula and cholera⁹. In continuation of our previous work on the pharmacological properties of medicinal plants¹⁰, the present study was conducted to assess the antioxidant potential, total phenolic and flavonoid contents of crude extracts of *Artabotrys crassifolius*.

MATERIALS AND METHODS

Collection and identification of plant material

The leaves and barks of *Artabotrys crassifolius*, with the local name of *akar mempising*, were collected from Kuala Kangsar, Perak, Malaysia (4°46'N, 100°56'E) in March 2011. The plant was identified and authenticated by Mr. Kamarudin Saleh, Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM). Voucher specimens were prepared and deposited in the

Kepong Herbarium (KEP) of FRIM (PID 080311–05), and the School of Pharmacy, Faculty of Science, The University of Nottingham Malaysia Campus (UNMC 65) for future reference.

Preparation of plant material

After removal of extraneous matter, the freshly collected leaves and barks were air-dried in the shade at room temperature for at least 2 weeks. The dried leaves and barks were then finely pulverized by grinding prior to extraction. The pulverized leaves (1.30 kg) and barks (4.79 kg) were extracted sequentially with solvents of increasing polarity starting from hexane, chloroform and 95% (v/v) of ethanol. Each extraction was performed in triplicate at a solid-to-solvent ratio of 1:5 (w/v) in a 40°C water bath for three days. The respective extract was subsequently filtered through Whatman filter papers No. 1 and the collected filtrate was concentrated to dryness under reduced pressure at 40°C using rotary evaporator. Eventually, the dried extract obtained was weighed and stored at –20°C until further use. For stock solutions, each crude extract was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL and stored at 4°C.

Determination of total phenolic content (TPC)

The total phenolic contents of crude extracts were assessed using Folin-Ciocalteu (FC) assay^{11,12}. In a 96-well microtiter plate (Jet Biofil, China), 100 µL of 10% (v/v) of FC reagent (R & M Chemicals, UK) was added to 5 µL of each crude extract (final concentration of 50 µg/mL). After 5 min incubation at room temperature, 80 µL of 7.5% (w/v) of sodium carbonate (Na₂CO₃) (R & M Chemicals, UK) was added to each well containing the previous mixture. The plate was shaken gently and incubated for 30 min at room temperature in the dark. Gallic acid (final concentrations ranging from 5 µg/mL to 25 µg/mL) (R & M Chemicals, UK) was used to establish the standard curve. Eventually, the absorbance was measured at 765 nm against the corresponding blank solution using Varioskan Flash microplate reader with SkanIt Software 2.4.3 RE (Thermo Scientific, Malaysia). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram of extract (mg GAE/g extract)¹³, which was calculated by the following equation:

$$T_1 = C_1 \times \frac{V}{M} ,$$

where T_1 is the total phenolic content, mg/g of extract, in GAE; C_1 is the concentration of gallic acid established from the calibration curve, mg/mL; V is the volume of crude extract, mL; M is the weight of crude extract, g.

Determination of total flavonoid content (TFC)

The total flavonoid contents of crude extracts were assessed using aluminium chloride colourimetric assay^{14,15}. In a 96-well microtiter plate, 15 μL of 5% (w/v) of sodium nitrite (NaNO_2) (Merck, Germany) was added to 5 μL of each crude extract (final concentration of 50 $\mu\text{g}/\text{mL}$). The plate was allowed to stand for 6 min at room temperature and subsequently 30 μL of 10% (w/v) of aluminium chloride hexahydrate ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$) (R & M Chemicals, UK) was added to the mixture. After a further 5 min incubation at room temperature, 100 μL of 1 M of sodium hydroxide (NaOH) (Merck, Germany) was added to the mixture and immediately diluted by the addition of 55 μL of distilled water. (+)-Catechin hydrate (final concentrations ranging from 5 $\mu\text{g}/\text{mL}$ to 25 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich, Indonesia) was used to establish the standard curve. Eventually, the absorbance was measured at 510 nm against the corresponding blank solution using Varioskan Flash microplate reader with Skan. It Software 2.4.3 RE. The total flavonoid content was expressed as catechin equivalents (CE) in milligram per gram of extract ($\text{mg CE}/\text{g extract}$)¹⁶, which was calculated by the following equation:

$$T_2 = C_2 \times \frac{V}{M} ,$$

where T_2 is the total flavonoid content, mg/g of extract, in CE; C_2 is the concentration of catechin established from the calibration curve, mg/mL ; V is the volume of crude extract, mL ; M is the weight of crude extract, g .

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) cation radical scavenging assay

The antioxidant potentials of crude extracts were assessed using ABTS cation radical scavenging assay^{17,18}. The ABTS cation radical was produced by reacting 7 mM of ABTS diammonium salt solution (Sigma-Aldrich, Canada) with 2.45 mM of potassium peroxodisulphate solution ($\text{K}_2\text{S}_2\text{O}_8$) (Fluka, Germany) in equal volume. The mixture was allowed to stand in the dark at room temperature for 12 h to 16 h. Prior to assay, the ABTS working solution was prepared by diluting the stock solution with methanol (Friendemann Schmidt, Australia) to an absorbance of 0.70 ± 0.02 at 734 nm. In a 96-well microtiter plate, 195 μL of the diluted ABTS solution was added to 5 μL of each crude extract (final concentrations ranging from 3.125 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$). The plate was shaken gently and incubated for 6 min at room temperature in the dark. Trolox (final concentrations ranging from 3.125 $\mu\text{g}/\text{mL}$ to 100 $\mu\text{g}/\text{mL}$) (Acros Organics, Belgium) was used as positive control. Eventually, the absorbance was measured at 734 nm against the corresponding blank solution using Varioskan Flash microplate reader with SkanIt Software 2.4.3 RE.

The percentage of ABTS cation radical scavenging activity was calculated by the following equation:

$$\text{ABTS cation radical scavenging activity (\%)} = \frac{[A_0 - (A_1 - A_2)]}{A_0} \times 100\%$$

where A_0 is the absorbance of negative control; A_1 is the absorbance of reaction mixture; A_2 is the absorbance of crude extract or positive control. The IC_{50} value was determined from the plotted graph of scavenging activity against the concentration of crude extracts or positive control¹⁹.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant potentials of crude extracts were assessed using DPPH radical scavenging assay^{20,21}. In a 96-well microtiter plate, 195 μL of 0.1 mM of DPPH (Sigma-Aldrich, USA) was added to 5 μL of each crude extract (final concentrations ranging from 3.125 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$). The plate was shaken gently and incubated for 30 min at room temperature in the dark. L(+)-ascorbic acid (final concentrations ranging from 3.125 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$) (System, Malaysia) was used as positive control. Eventually, the absorbance was measured at 517 nm against the corresponding blank solution using Varioskan Flash microplate reader with Skan. It Software 2.4.3 RE. The percentage of DPPH radical scavenging activity was calculated by the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[A_0 - (A_1 - A_2)]}{A_0} \times 100\%$$

where A_0 is the absorbance of negative control; A_1 is the absorbance of reaction mixture; A_2 is the absorbance of crude extractor positive control. The IC_{50} value was determined from the plotted graph of scavenging activity against the concentration of crude extracts or positive control²². The antioxidant activity index (AAI)²³ was calculated as follows:

$$\text{AAI} = \frac{\text{Final concentration of DPPH } (\mu\text{g/mL})}{\text{IC}_{50} \text{ value of crude extract } (\mu\text{g/mL})}$$

Ferric reducing antioxidant power (FRAP) assay

The antioxidant potentials of crude extracts were assessed using FRAP assay^{24,25}. The FRAP reagent was freshly prepared by mixing 300 mM of acetate buffer (pH 3.6) [mixture of sodium acetate trihydrate (Merck, Germany) and glacial acetic acid (System, Malaysia)], 10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, Switzerland) in 40 mM of hydrochloric acid (HCl) (System, Malaysia), and 20 mM of ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)

(System, Malaysia) in a volume ratio of 10:1:1 respectively. Prior to assay, the FRAP reagent was warmed to 37°C in a water bath (Julabo, Germany). In a 96-well microtiter plate, 195 µL of the FRAP reagent was added to 5 µL of each crude extract (final concentrations ranging from 3.125 µg/mL to 100 µg/mL). The plate was shaken gently and incubated at 37°C for 4 min. Ferrous sulphate heptahydrate (FeSO₄·7H₂O) (final concentrations ranging from 50 µM to 250 µM) (System, Malaysia) was used to establish the standard curve. Eventually, the absorbance was measured at 593 nm against the corresponding blank solution using Varioskan Flash microplate reader with SkanIt Software 2.4.3 RE. The FRAP value was expressed as FeSO₄·7H₂O equivalents, Fe(II) in micromole per gram of extract [µmol Fe(II)/g extract], which was calculated by the following equation:

$$T_3 = C_3 \times \frac{V}{M} ,$$

where T₃ is the FRAP value, µmol/g of extract, in Fe(II); C₃ is the concentration of FeSO₄·7H₂O established from the calibration curve, µmol/L; V is the volume of crude extract, L; M is the weight of crude extract, g.

Statistical analysis

Statistical analysis of experimental data was performed using Microsoft Office Excel data analysis software. Data were presented by descriptive analysis as mean and standard deviation of three independent experiments performed in triplicate.

RESULTS AND DISCUSSION

Plants represent an invaluable source of raw materials for the development of natural antioxidants²⁶. Nevertheless, due to the diverse and complex nature of the phytochemical constituents, there is no single universal method that can accurately evaluate the antioxidant activities of plant extracts²⁷. In the current study, TPC, TFC, ABTS, DPPH and FRAP assays were performed to assess the antioxidant potentials of crude extracts.

Total phenolic contents of crude extracts of *Artabotrys crassifolius*

Folin-Ciocalteu assay is extensively used for the quantification of total phenolic content²⁸. This method is based on the reduction of phosphomolybdic and phosphotungstic acid complexes to blue chromogens in the presence of phenolic compounds under alkaline conditions²⁹.

The total phenolic contents of crude extracts were calculated from the regression equation of the calibration curve of gallic acid ($y = 0.0384x$, $R^2 = 0.9996$) (Figure 1). According to Rufino *et al.*³⁰, the content of total phenolics of crude extracts could be categorised into three classes: low

(less than 10 mg GAE/g), medium (ranging from 10 mg GAE/g to 50 mg GAE/g) and high (more than 50 mg GAE/g). Under this classification, ethanol extracts of barks and leaves demonstrated remarkably high total phenolic contents of 268.29 ± 12.36 mg GAE/g and 154.91 ± 4.26 mg GAE/g respectively (Figure 2). This may be due to the greater solubility of phenolic compounds in ethanol.

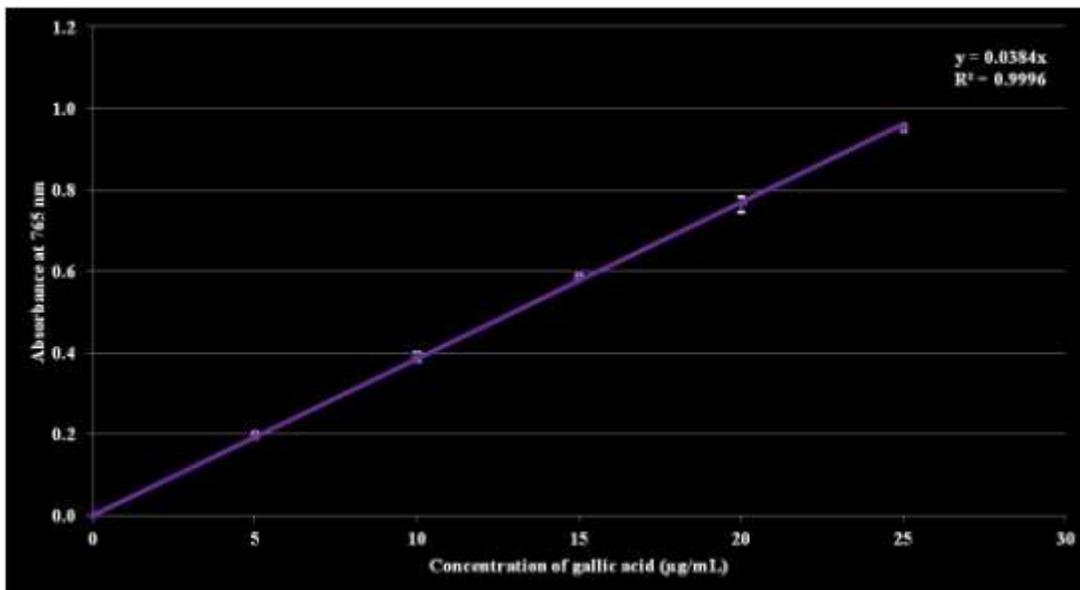


Figure 1: Standard curve of gallic acid for the determination of total phenolic contents of crude extracts of *Artabotrys crassifolius*.

Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

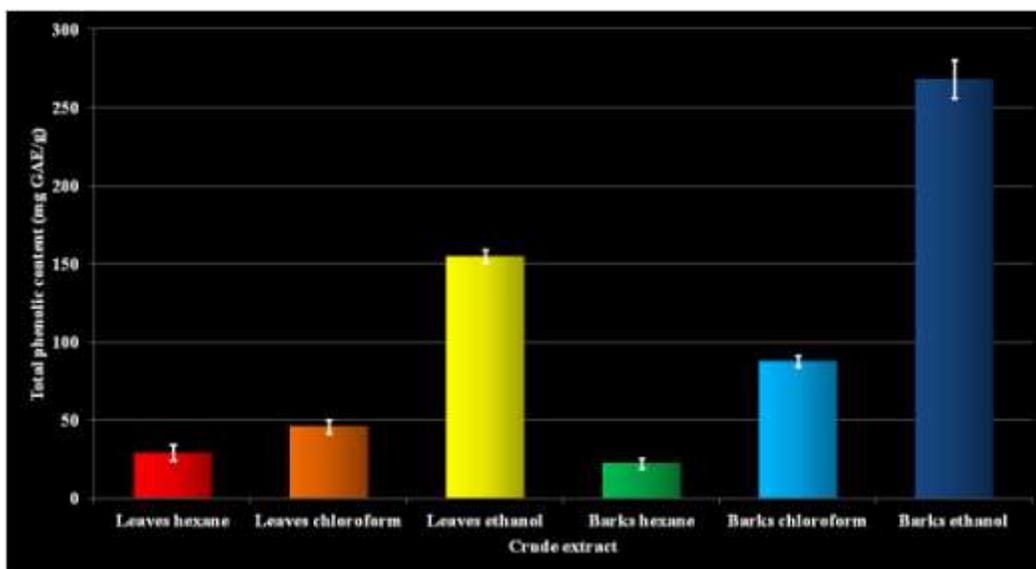


Figure 2: Total phenolic contents of crude extracts of *Artabotrys crassifolius*.

Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

Total flavonoid contents of crude extracts of *Artabotrys crassifolius*

Aluminium chloride assay is widely employed for the estimation of total flavonoid content³¹. This method is based on the development of acid-stable complexes between aluminium chloride and the C-4 keto group along with either the C-3 or C-5 hydroxyl group of flavones and flavonols. Additionally, aluminium chloride also forms acid-labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids³². These complexes subsequently produce pink chromogens upon reacting with sodium nitrite under alkaline conditions³³.

The total flavonoid contents of crude extracts were determined from the regression equation of the calibration curve of catechin ($y = 0.0184x$, $R^2 = 0.9964$) (Figure 3). Their content of total flavonoids expressed as catechin equivalents was recorded in the range from 6.29 ± 4.27 mg/g to 179.54 ± 4.98 mg/g (Figure 4).

Among the crude extracts examined, ethanol extract of barks displayed the highest total flavonoid content (179.54 ± 4.98 mg GAE/g), which was approximately 2.13-fold greater than that of ethanol extract of leaves (84.47 ± 6.61 mg GAE/g). On the contrary, the content of total flavonoids was found to be comparatively low in both hexane extracts of leaves (9.48 ± 4.53 mg GAE/g) and barks (6.29 ± 4.27 mg GAE/g). This indicates that ethanol has superior extraction capacity as well as selectivity for flavonoids in comparison to hexane.

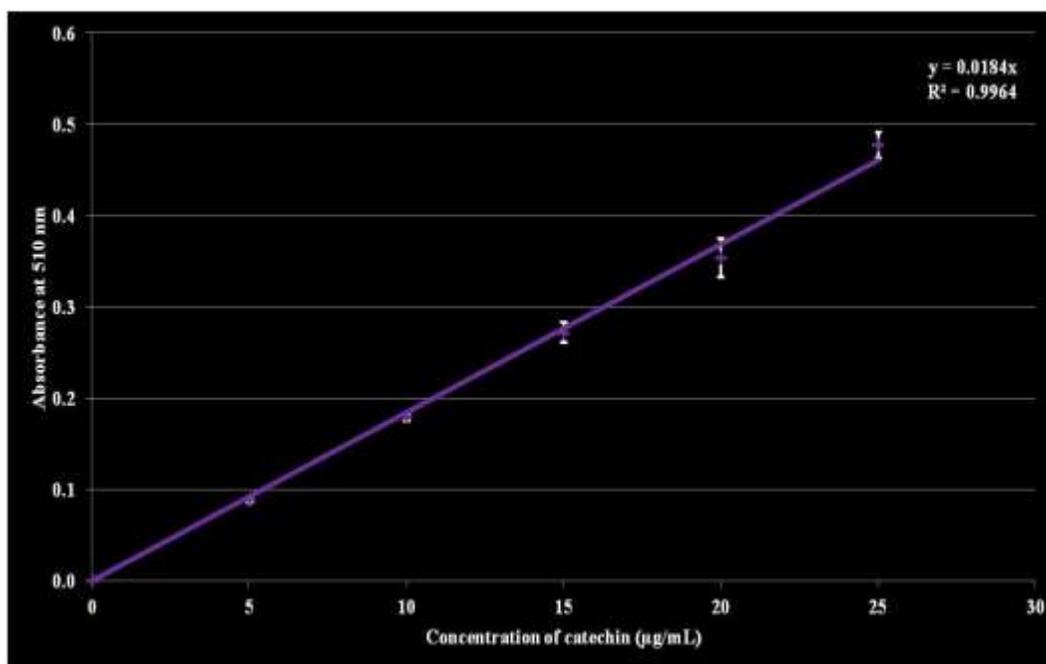


Figure 3: Standard curve of catechin for the determination of total flavonoid contents of crude extracts of *Artabotrys crassifolius*. Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

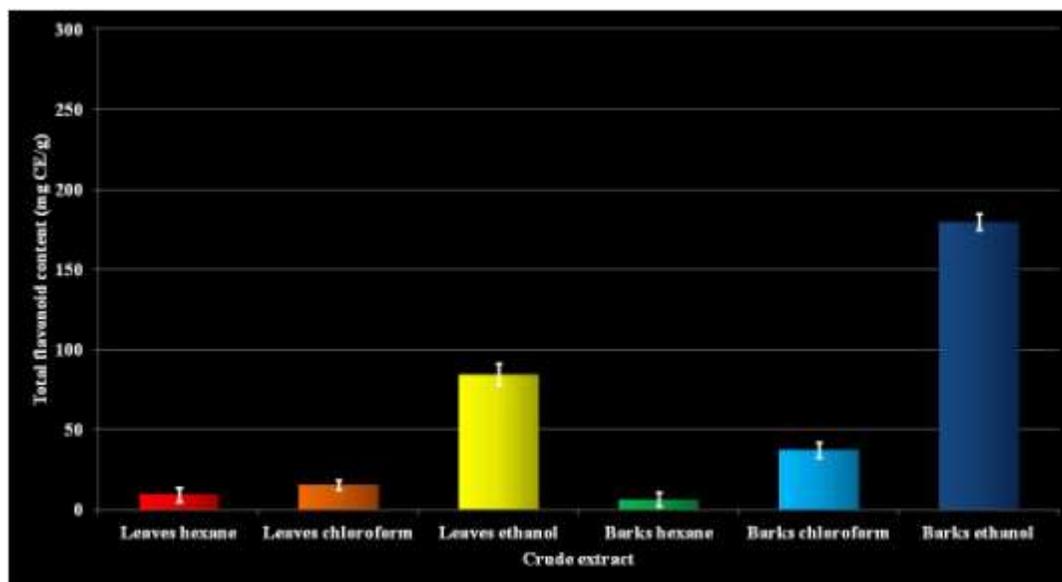


Figure 4: Total flavonoid contents of crude extracts of *Artabotrys crassifolius*. Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

Antioxidant potentials of crude extracts of *Artabotrys crassifolius* using ABTS cation radical scavenging assay

ABTS assay is commonly employed for the determination of antioxidant capacity of plant extracts³⁴. This method is based on the reduction of blue-green chromogens produced from the reaction between ABTS and potassium peroxodisulphate in the presence of electron-donating antioxidants³⁵.

Figure 5 depicts the ABTS cation radical scavenging activities of crude extracts. At 100 $\mu\text{g/mL}$ concentration, ethanol extracts of barks and leaves exhibited the maximum scavenging effects of $99.86 \pm 0.06\%$ and $99.76 \pm 0.26\%$ against ABTS cation radical respectively, which were similar to that of the standard, trolox ($99.72 \pm 0.15\%$), a water-soluble vitamin E analogue.

The cut-off point for antioxidant potentials of crude extracts was suggested to be 50 $\mu\text{g/mL}$ ³⁶. According to Kuete and Efferth³⁷ and Chew *et al.*³⁸, the radical scavenging ability of crude extracts could be classified based on their IC_{50} values as follows: high antioxidant capacity (IC_{50} value less than 50 $\mu\text{g/mL}$), moderate antioxidant capacity (IC_{50} value ranging from 50 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$) and low antioxidant capacity (IC_{50} value more than 100 $\mu\text{g/mL}$). Under this categorisation, ethanol extracts of barks and leaves demonstrated high antioxidant capacity with

respective IC₅₀ values of 16.50 µg/mL and 30.77 µg/mL where as trolox gave an IC₅₀ value of 6.88 µg/mL. This implies that the corresponding extracts may function as effective scavengers of ABTS cation radical.

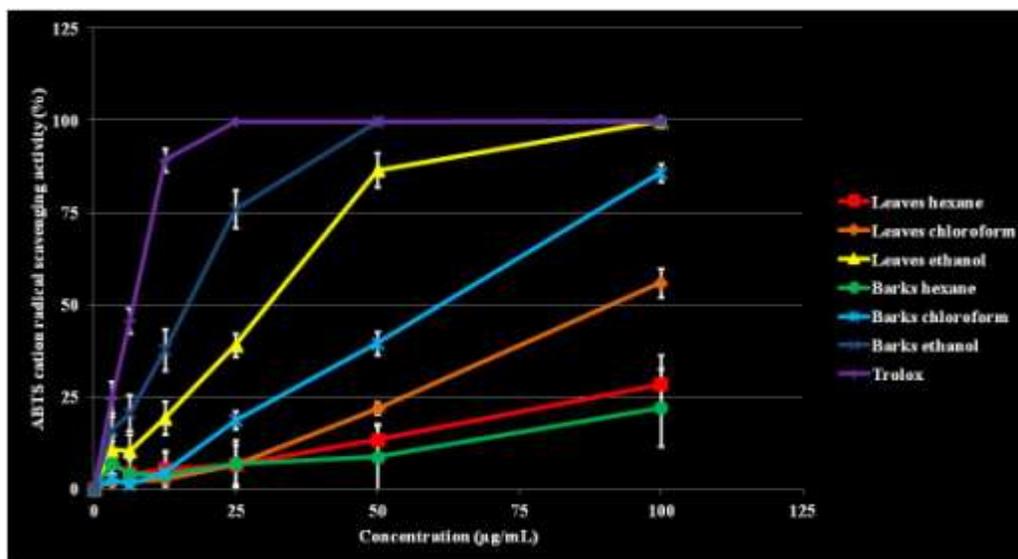


Figure 5: Antioxidant potentials of crude extracts of *Artabotrys crassifolius* using ABTS cation radical scavenging assay.

Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

Antioxidant potentials of crude extracts of *Artabotrys crassifolius* using DPPH radical scavenging assay

DPPH assay is widely used for the evaluation of radical scavenging activity of plant extracts³⁹. This method is based on the reduction of purple-coloured DPPH radical (2,2-diphenyl-1-picrylhydrazyl) to yellow-coloured non-radical form of DPPH (2,2-diphenyl-1-picrylhydrazine) in the presence of hydrogen-donating antioxidants⁴⁰.

The scavenging effects of crude extracts on DPPH radical are illustrated in Figure 6. All the crude extracts showed a concentration-dependent increase in scavenging DPPH radical. At a concentration of 100 µg/mL, the highest DPPH radical scavenging activity was observed in ethanol extract of barks (95.47 \pm 2.37%; IC₅₀ value of 16.54 µg/mL), which had comparable scavenging effect to that of the positive control, ascorbic acid (95.34 \pm 0.64%; IC₅₀ value of 7.59 µg/mL), a water-soluble form of vitamin C.

According to the scale proposed by Scherer and Godoy⁴¹, crude extracts could be considered to show poor antioxidant activity when AAI less than 0.5, moderate antioxidant activity when AAI ranging from 0.5 to 1.0, strong antioxidant activity when AAI ranging from 1.0 to 2.0, and very

strong antioxidant activity when AAI more than 2.0. Based on this classification, ethanol extract of barks displayed very strong antioxidant activity with an AAI value of 2.32, while the AAI value of ascorbic acid was found to be 5.07. This suggests that the respective extract may possess compounds with hydrogen-donating ability that can efficiently scavenge DPPH radical.

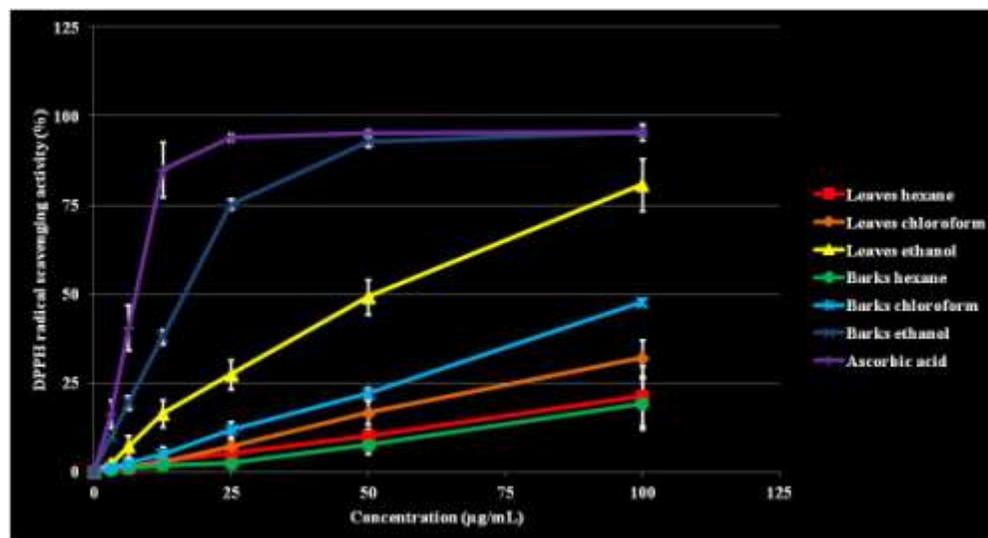


Figure 6: Antioxidant potentials of crude extracts of *Artabotrys crassifolius* using DPPH radical scavenging assay.

Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

Antioxidant potentials of crude extracts of *Artabotrys crassifolius* using FRAP assay

FRAP assay is extensively applied for the measurement of reducing power of plant extracts⁴². This method is based on the reduction of colourless ferric complex (Fe^{3+} -tripirydyltriazine) to blue-coloured ferrous complex (Fe^{2+} -tripirydyltriazine) in the presence of electron-donating antioxidants under acidic conditions⁴³.

The FRAP values of crude extracts were obtained from the regression equation of the calibration curve of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ($y = 0.0111x$, $R^2 = 0.9963$) (Figure 7). Their FRAP values expressed as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ equivalents, Fe(II) were varied from 67.64 ± 23.40 $\mu\text{mol/g}$ to 1884.35 ± 83.78 $\mu\text{mol/g}$ (Figure 8).

According to Wong *et al.*⁴⁴ and Oonsivilai *et al.*⁴⁵, crude extracts could be classified into four categories based on their antioxidant power: low [less than 10 $\mu\text{mol Fe(II)/g}$], medium [ranging from 10 $\mu\text{mol Fe(II)/g}$ to 100 $\mu\text{mol Fe(II)/g}$], high [ranging from 100 $\mu\text{mol Fe(II)/g}$ to 500 $\mu\text{mol Fe(II)/g}$], and extremely high [more than 500 $\mu\text{mol Fe(II)/g}$]. Based on this categorisation, ethanol extract of barks and leaves exhibited exceptionally high antioxidant power with

respective FRAP values of $1884.35 \pm 83.78 \mu\text{mol Fe(II)/g}$ and $979.57 \pm 57.17 \mu\text{mol Fe(II)/g}$. In contrast, medium antioxidant power was detected in both hexane extracts of leaves [$92.26 \pm 5.99 \mu\text{mol Fe(II)/g}$] and barks [$67.64 \pm 23.40 \mu\text{mol Fe(II)/g}$]. This may be attributed to the better electron-donating capabilities of ethanol extracts as compared to hexane extracts.

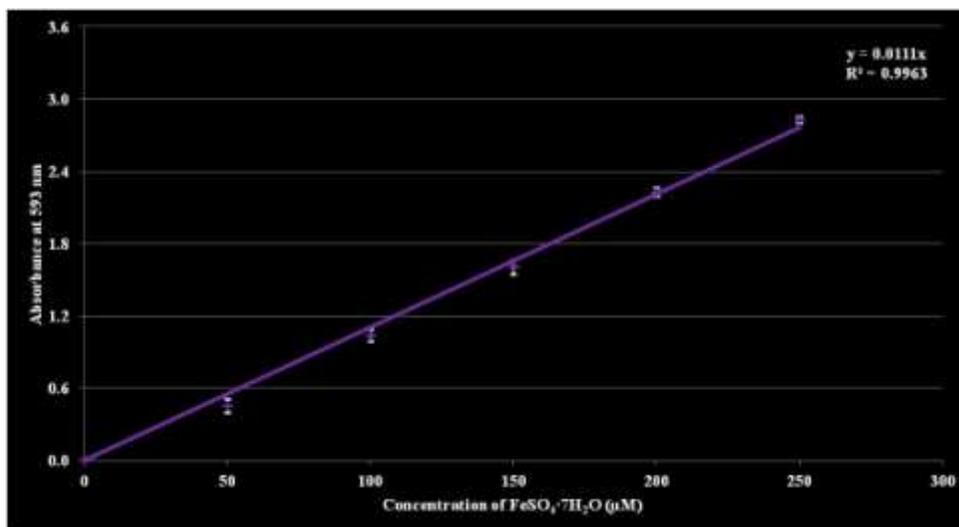


Figure 7: Standard curve of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ for the antioxidant potentials of crude extracts of *Artabotrys crassifolius* using FRAP assay. Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

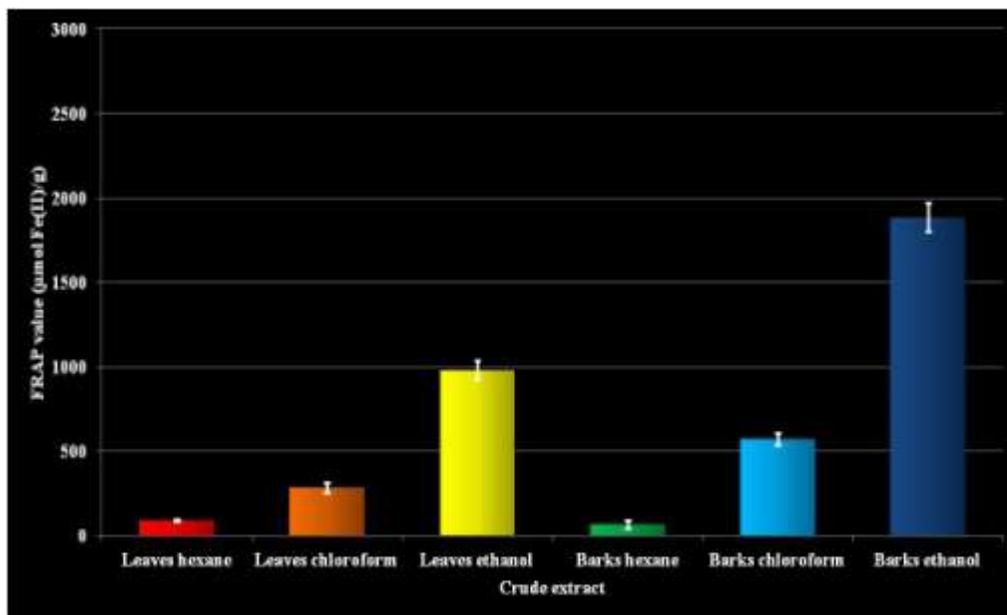


Figure 8: Antioxidant potentials of crude extracts of *Artabotrys crassifolius* using FRAP assay. Results are expressed as mean \pm standard deviation (error bar) of three independent experiments performed in triplicate, $n = 9$.

With respect to the phytochemical analysis of crude extracts⁸, the presence of cardiac glycosides,

flavonoids, phenolic compounds, saponins and terpenoids in ethanol extracts of barks and leaves may explain their superior activity in comparison to the other crude extracts tested. This necessitates further isolation and characterisation of the potentially active principles from the respective crude extracts.

CONCLUSION

Evaluation of the *in vitro* antioxidant potential of *Artabotrys crassifolius* revealed that ethanol extracts of barks and leaves may be a significant source of novel antioxidant compounds in consideration of their promising scavenging activities predominantly against ABTS cation radical. Consequently, further studies are needed to isolate and characterise the bioactive compounds responsible for the observed antioxidant properties of *Artabotrys crassifolius*.

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