IN VITRO EVALUATION OF ANTIOXIDANT POTENTIAL OF *ARTABOTRYS HEXAPETALUS*

Bathula Naveen Kumar[1],A.Srujana[2],Dasari Swathi[3],
Erpa Raasi[4],Mekala Dharmaraju[4],Lethakula venkatrao[4],Domala Pavan[4],Kola Shiva[4].
1.Department of Pharmacology,Mahammadiya Institute of Pharmaceutical Sciences.Khammam.
2.Department of Pharmacology,Bojjam Narsimhulu Pharmacy College for Women's
3.Doctor of Pharmacy,(Pharm.D)
4.Bachelor of Pharmacy, Mahammadiya Institute of Pharmaceutical Sciences.Khammam.

ABSTRACT
The aim of the current study was to assess the antioxidant potential, total phenolic and flavonoid contents of crude extracts of Artabotrys hexapetalus. The leaves and stem of Artabotrys hexapetalus 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 stem 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 hexapetalus*

INTRODUCTION

Free radicals are atomic or molecular species that can exist independently with one or more unpaired electrons in their outermost shell (Craft *et al.* 2012). They are generated as by-products during normal cellular metabolism (Barrera 2012). Due to their highly reactive and unstable properties in nature (Kamboj *et al.* 2014), they are capable of inducing oxidative damage to all the major classes of biomolecules including carbohydrates, lipids, proteins, and nucleic acids (Khasawneh *et al.* 2011). 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 senescence (Droge 2002).

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 completely (Lima-Saraiva *et al.* 2012). 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 toxicity (Kiran *et al.* 2012). Therefore, the exploration of alternative antioxidants from natural sources is highly desirable.

METHODOLOGY

Preparation of plant material

After removal of extraneous matter, the freshly collected leaves and stem were air-dried in the shade at room temperature for at least 2 weeks. The dried leaves and stem were then finely pulverized by grinding prior to extraction. The pulverized leaves (1.30 kg) and stem (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 according to the methods of Zongo *et al.* (2010) and Lee and Vairappan (2011). 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 (Na_2CO_3) (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) (Iqbal *et al.* 2012), 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 according to the methods of Tavares *et al.* (2010) and Yang *et al.* (2012). 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 µg/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 ($AlCl_3 \cdot 6H_2O$) (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 µg/mL to 25 µg/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 SkanIt Software 2.4.3 RE. The total flavonoid content was expressed as catechin equivalents (CE) in milligram per gram of extract (mg CE/g extract) (Vyas 2010), 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 according to the methods of Bunea *et al.* (2011) and Sampath and Vasanthi (2013). 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 ($K_2S_2O_8$) (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 µg/mL to 100 µg/mL).

The plate was shaken gently and incubated for 6 min at room temperature in the dark. Trolox (final concentrations ranging from 3.125 µg/mL to 100 µg/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 control (Yang *et al.* 2011).

Ferric reducing antioxidant power (FRAP) assay

The antioxidant potentials of crude extracts were assessed using FRAP assay according to the methods of Gan *et al.* (2010) and Song *et al.* (2010). 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 (Systerm, Malaysia)], 10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (Sigma-Aldrich, Switzerland) in 40 mM of hydrochloric acid (HCl) (Systerm, Malaysia), and 20 mM of ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) (Systerm, 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 ($FeSO_4 \cdot 7H_2O$) (final concentrations ranging from 50 µM to 250 µM) (Systerm, 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 $FeSO_4 \cdot 7H_2O$ 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 T_3 is the FRAP value, µmol/g of extract, in Fe(II); C_3 is the concentration of $FeSO_4 \cdot 7H_2O$ 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 antioxidants (Ghasemzadeh *et al.* 2012). 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 extracts (Ksiksi and Hamza 2012). 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 hexapetalus*

Folin-Ciocalteu assay is extensively used for the quantification of total phenolic content (Prasain *et al.* 2008). This method is based on the reduction of phosphomolybdic and phosphotungstic acid complexes to blue chromogens in the presence of phenolic compounds under alkaline conditions (Mehran *et al.* 2014).

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 7.1; Appendix E1). According to Rufino *et al.* (2010), 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 extract of Stem 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 7.2; Appendix E2). This may be due to the greater solubility of phenolic compounds in ethanol.

Total flavonoid contents of crude extracts of *Artabotrys hexapetalus*

Aluminium chloride assay is widely employed for the estimation of total flavonoid content (Corpuz *et al.* 2013). 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 flavonoids (Rajanandh and Kavitha 2010). These complexes subsequently produce pink chromogens upon reacting with sodium nitrite under alkaline conditions (Kaur 2010).

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 7.3; Appendix E1). 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 7.4; Appendix E2).

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

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

ABTS assay is commonly employed for the determination of antioxidant capacity of plant extracts (Contreras-Calderon *et al.* 2011). 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 antioxidants

(Ahmed 2012).

Figure 7.5 depicts the ABTS cation radical scavenging activities of crude extracts (Appendix E3). At 100 µg/mL concentration, ethanol extract of Stem and leaves exhibited the maximum scavenging effects of 99.86±0.06% and 99.76 ±0.26% against ABTS cation radical respectively, which were similar to that of the standard, trolox (99.72±0.15%), a water-soluble vitamin E analogue.

The cut-off point for antioxidant potentials of crude extracts was suggested to be 50 µg/mL (Omisore *et al.* 2005). According to Kuete and Efferth (2010) and Chew *et al.* (2011), 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 µg/mL), moderate antioxidant capacity (IC_{50} value ranging from 50 µg/mL to 100 µg/mL) and low antioxidant capacity (IC_{50} value more than 100 µg/mL). Under this categorisation, ethanol extract of Stem and leaves demonstrated high antioxidant capacity with respective IC_{50} values of 16.50 µg/mL and 30.77 µg/mL whereas trolox gave an IC_{50} value of 6.88 µg/mL. This implies that the corresponding extracts may function as effective scavengers of ABTS cation radical.

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

DPPH assay is widely used for the evaluation of radical scavenging activity of plant extracts (Ndhlala *et al.* 2010). 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 antioxidants (Murali *et al.* 2011).

The scavenging effects of crude extracts on DPPH radical are illustrated in Figure 7.6 (Appendix E4). 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 Stem (95.47±2.37%; IC_{50} value of 16.54 µg/mL), which had comparable scavenging effect to that of the positive control, ascorbic acid (95.34±0.64%; IC_{50} value of 7.59 µg/mL), a water-soluble form of vitamin C.

According to the scale proposed by Scherer and Godoy (2009), 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 Stem 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.

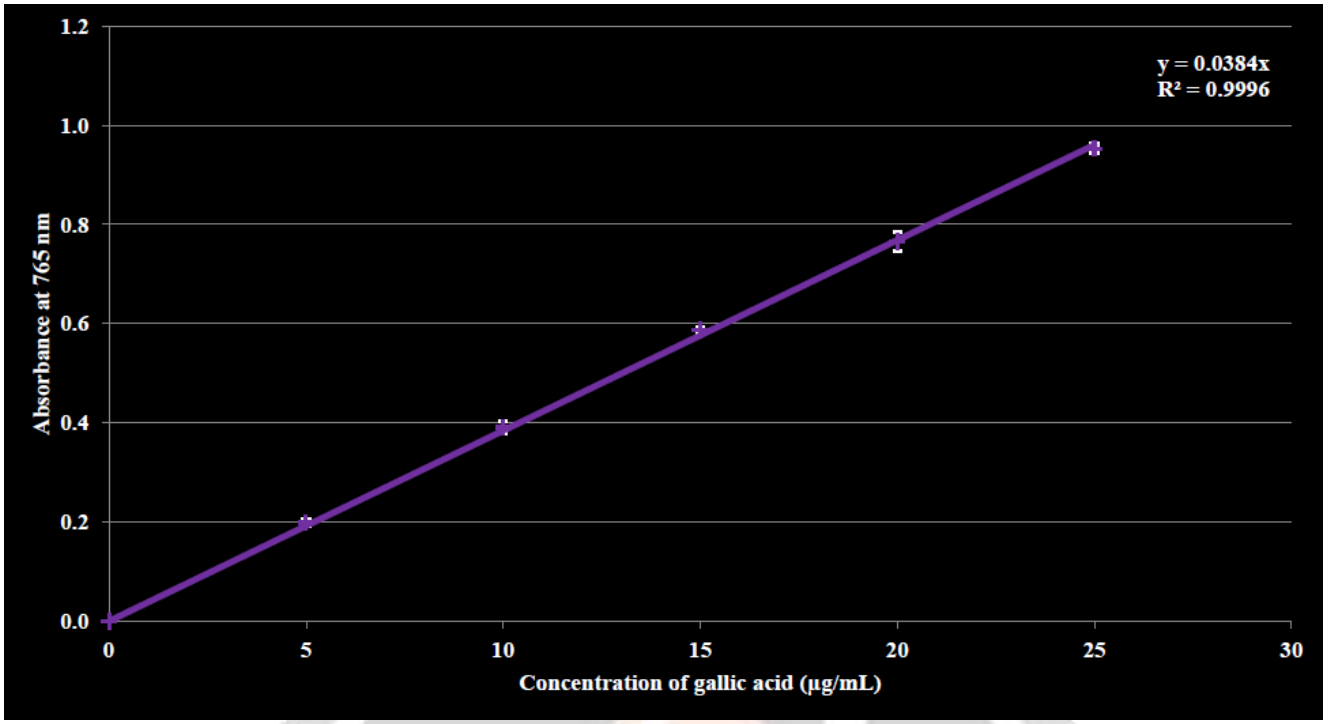

FIGURE 7.1 Standard curve of gallic acid for the determination of total phenolic contents of crude extracts of Artabotrys hexapetalus. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

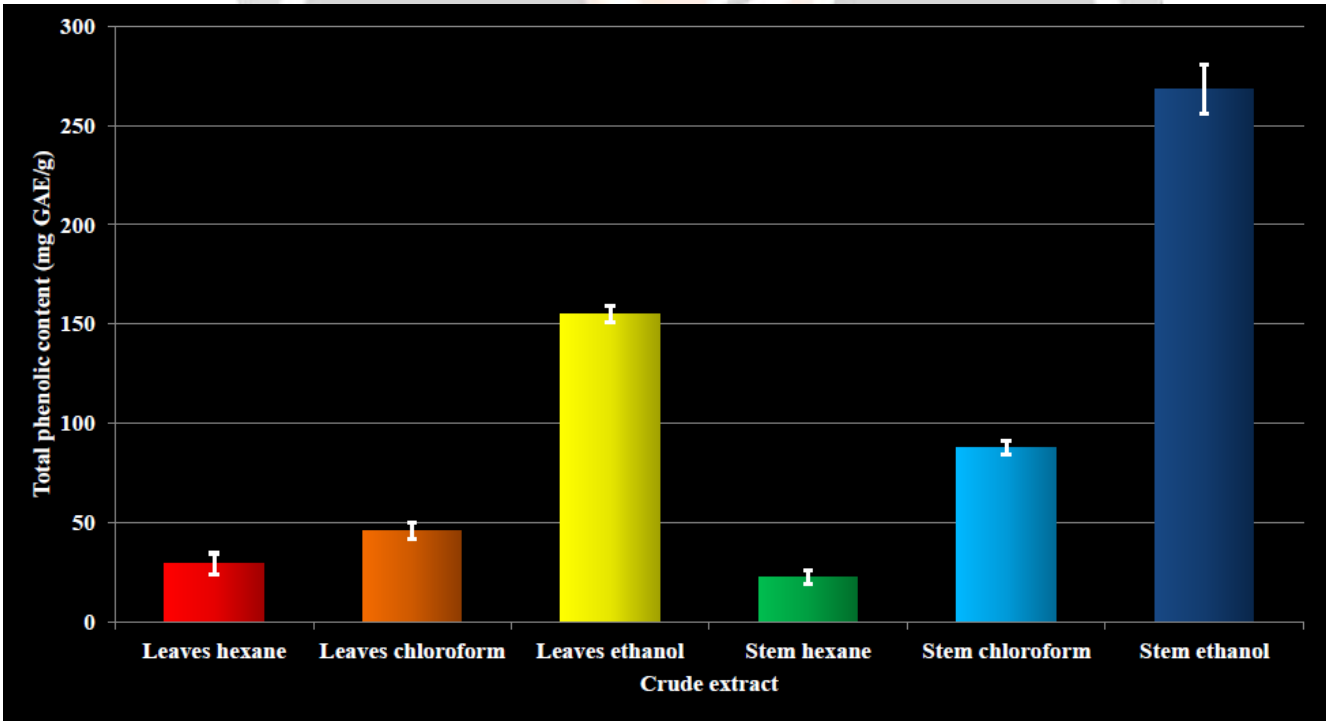

FIGURE 7.2 Total phenolic contents of crude extracts of Artabotrys hexapetalus. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

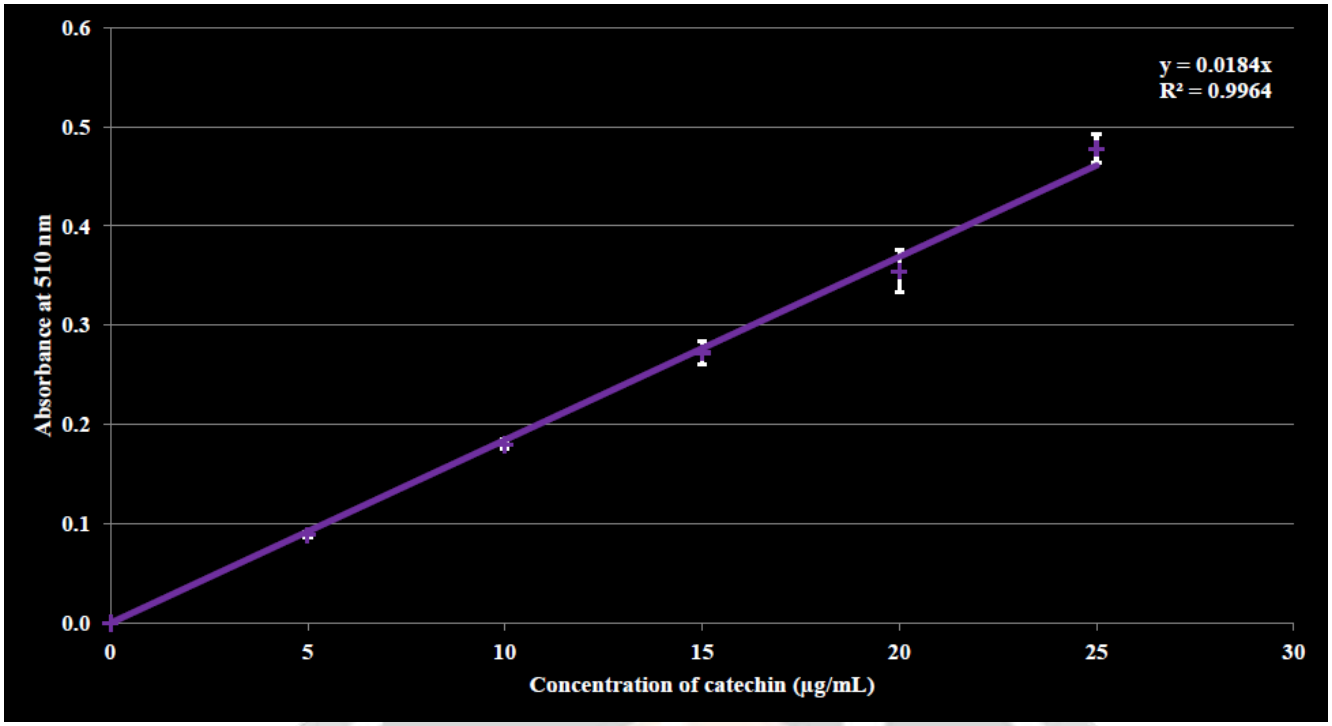

FIGURE 7.3 Standard curve of catechin for the determination of total flavonoid contents of crude extracts of Artabotrys hexapetalus. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

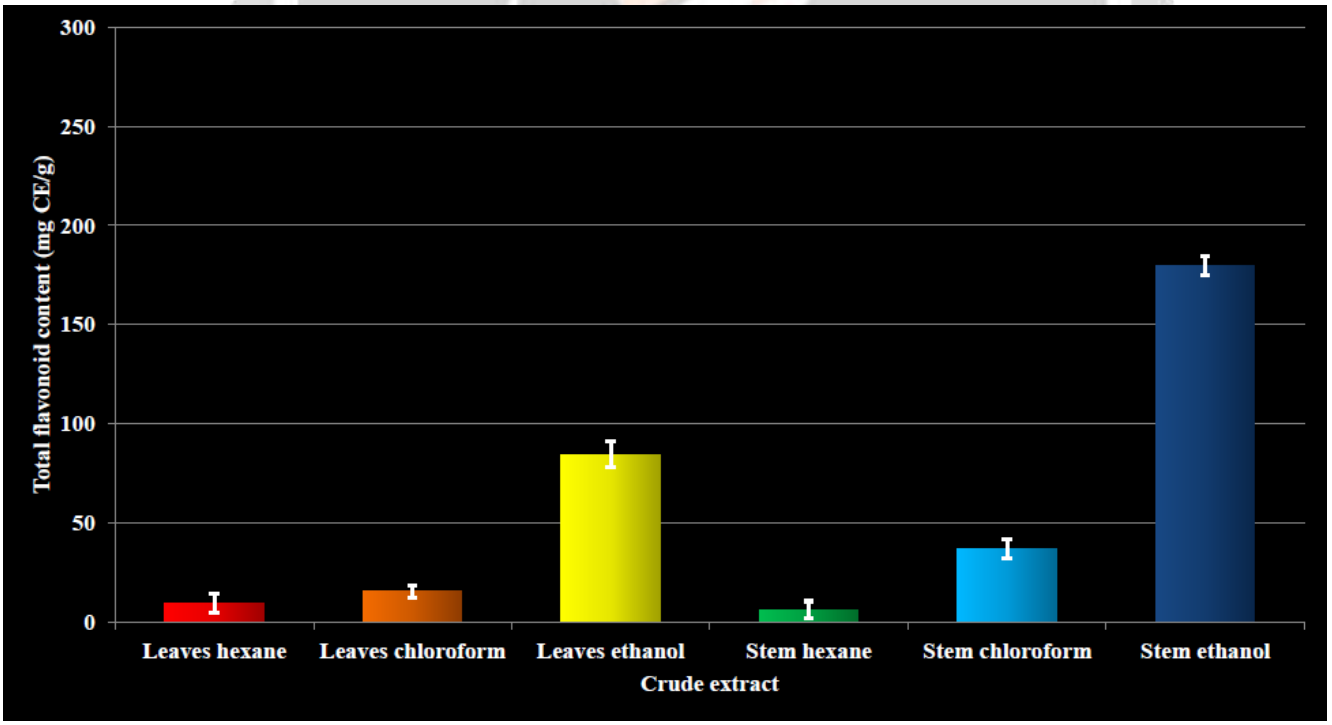

FIGURE 7.4 Total flavonoid contents of crude extracts of Artabotrys hexapetalus. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

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

FRAP assay is extensively applied for the measurement of reducing power of plant extracts (Magalhaes 2007). This method is based on the reduction of colourless ferric complex (Fe^{3+}-tripyridyltriazine) to blue-coloured ferrous complex (Fe^{2+}-tripyridyltriazine) in the presence of electron-donating antioxidants under acidic conditions (Irshad *et al.* 2012).

The FRAP values of crude extracts were obtained from the regression equation of the calibration curve of $FeSO_4 \cdot 7H_2O$ ($y = 0.0111x$, $R^2 = 0.9963$) (Figure 7.7; Appendix E5). Their FRAP values expressed as $FeSO_4 \cdot 7H_2O$ equivalents, Fe(II) were varied from 67.64±23.40 µmol/g to 1884.35±83.78 µmol/g (Figure 7.8; Appendix E6).

According to Wong *et al.* (2006) and Oonsivilai *et al.* (2008), crude extracts could be classified into four categories based on their antioxidant power: low [less than 10 µmol Fe(II)/g], medium [ranging from 10 µmol Fe(II)/g to 100 µmol Fe(II)/g], high [ranging from 100 µmol Fe(II)/g to 500 µmol Fe(II)/g], and extremely high [more than 500 µmol Fe(II)/g]. Based on this categorisation, ethanol extract of Stem and leaves exhibited exceptionally high antioxidant power with respective FRAP values of 1884.35±83.78 µmol Fe(II)/g and 979.57±57.17 µmol Fe(II)/g. In contrast, medium antioxidant power was detected in both hexane extracts of leaves [92.26±5.99 µmol Fe(II)/g] and Stem [67.64±23.40 µmol Fe(II)/g]. This may be attributed to the better electron-donating capabilities of ethanol extracts as compared to hexane extracts.performed in triplicate, n = 9.

With respect to the phytochemical analysis of crude extracts (Tan *et al.* 2013), the presence of cardiac glycosides, flavonoids, phenolic compounds, saponins and terpenoids in ethanol extract of Stem may explain its 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 extract. Considering that no literature review has been assembled to comprehensively address the antioxidant properties of *Artabotrys* species, this study could be the first report providing new insights into the antioxidant activity in the genus *Artabotrys*.

CONCLUSION

Evaluation of the *in vitro* antioxidant potential of *Artabotrys hexapetalus* revealed that ethanol extract of Stem may be a significant source of novel antioxidant compounds in consideration of its promising scavenging activity 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 hexapetalus*.

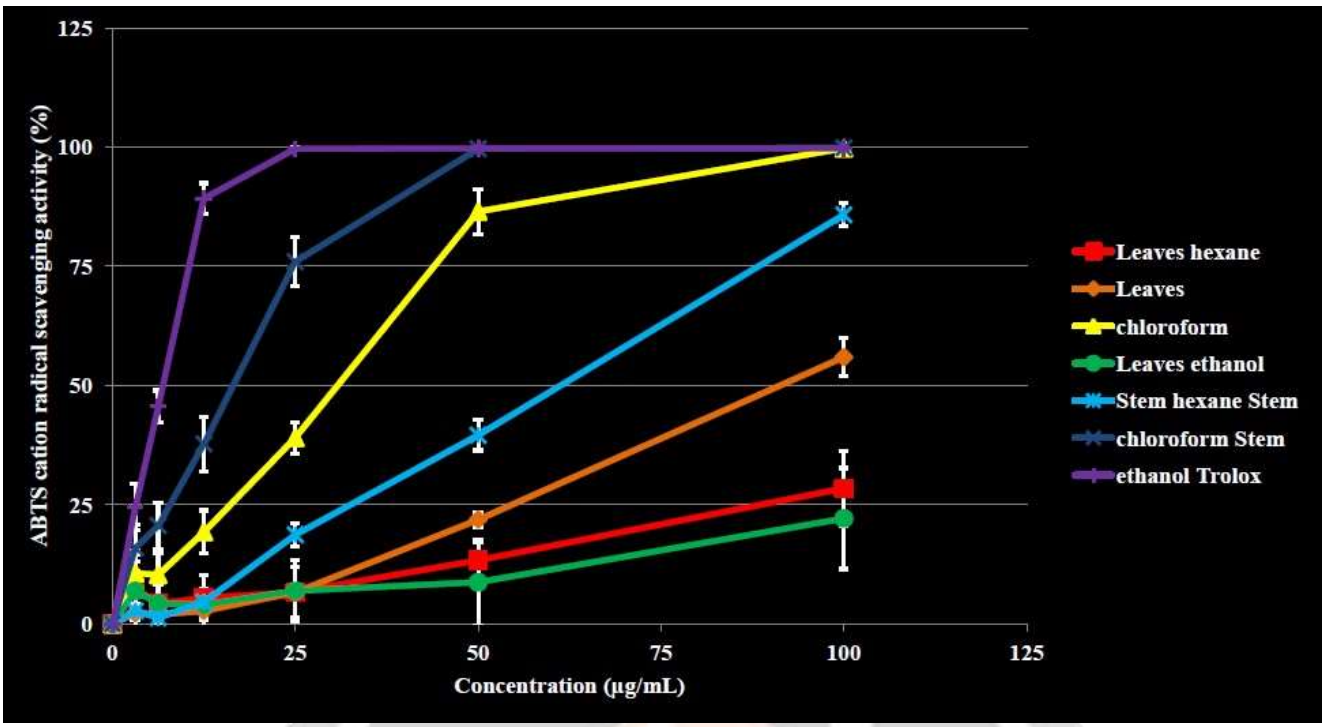

FIGURE 7.5 Antioxidant potentials of crude extracts of *Artabotrys hexapetalus* using ABTS cation radical scavenging assay. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

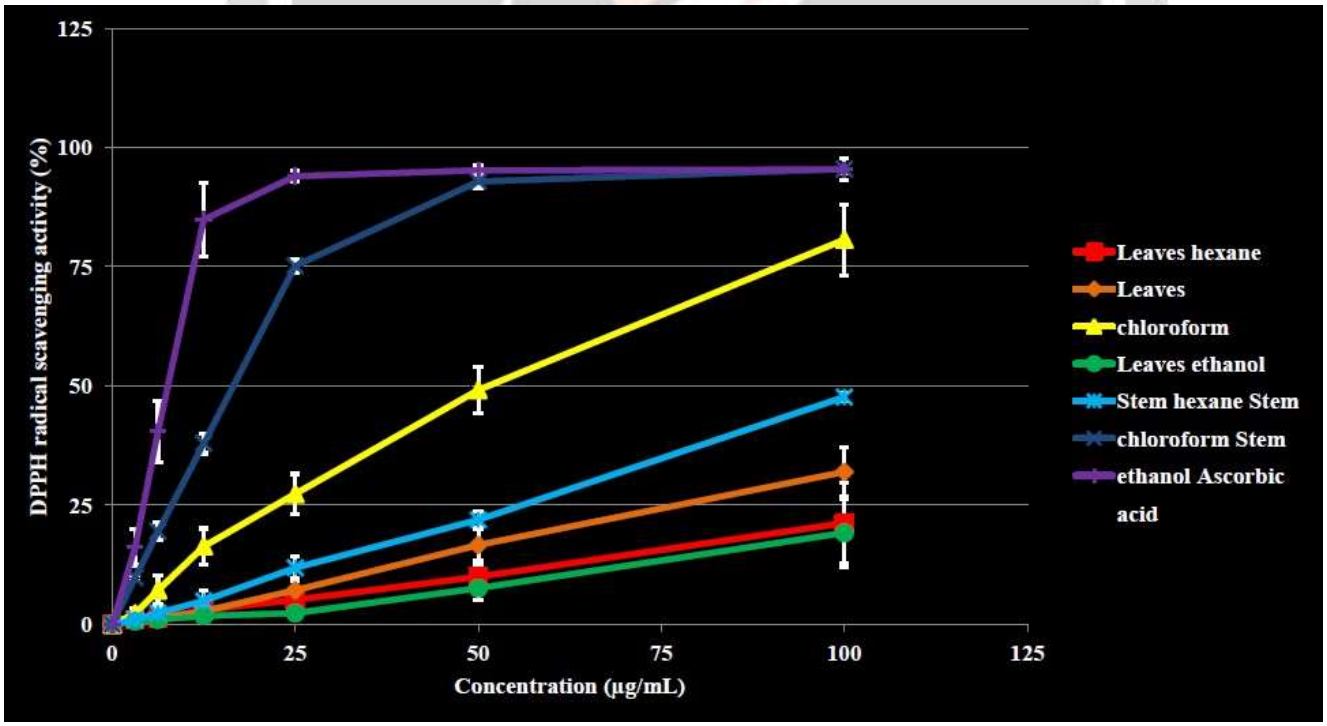

FIGURE 7.6 Antioxidant potentials of crude extracts of *Artabotrys hexapetalus* using DPPH radical scavenging assay. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

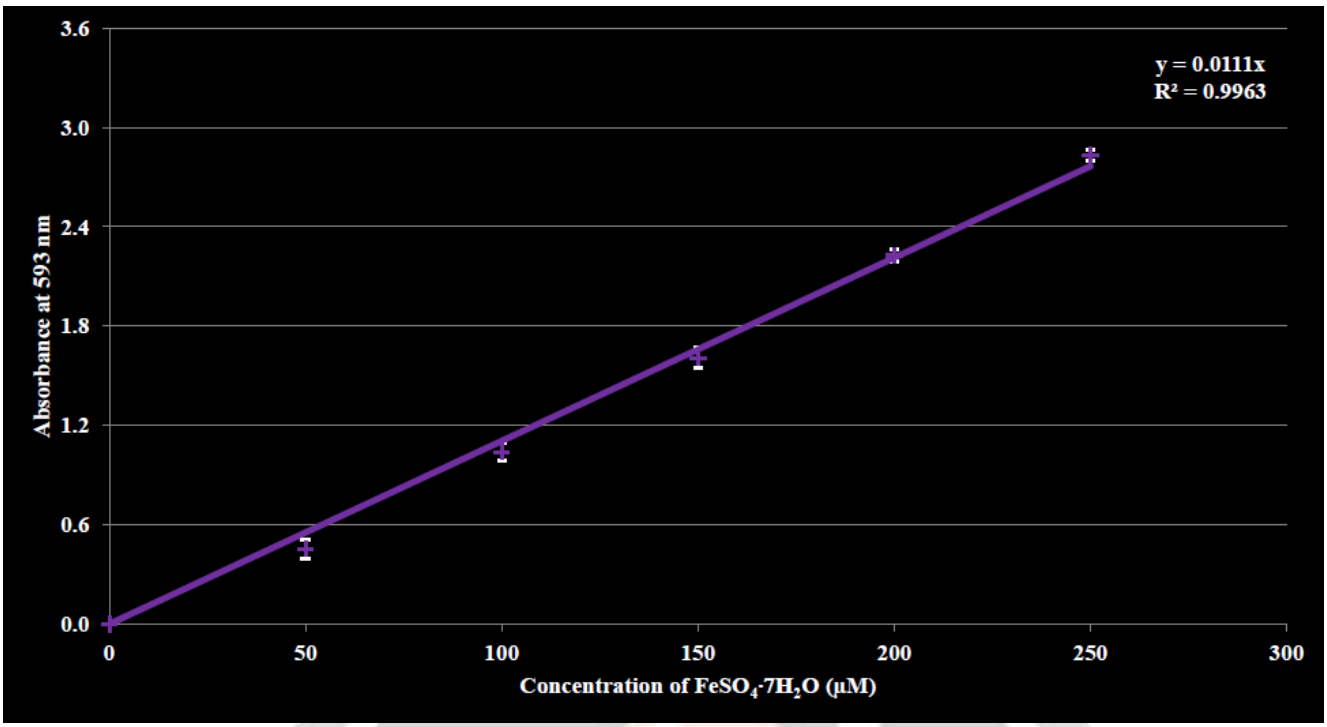

FIGURE 7.7 Standard curve of FeSO4·7H2O for the antioxidant potentials of crude extracts of Artabotrys hexapetalus using FRAP assay. Results are expressed as mean ± standard deviation (error bar) of three independent experiments performed in triplicate, n = 9.

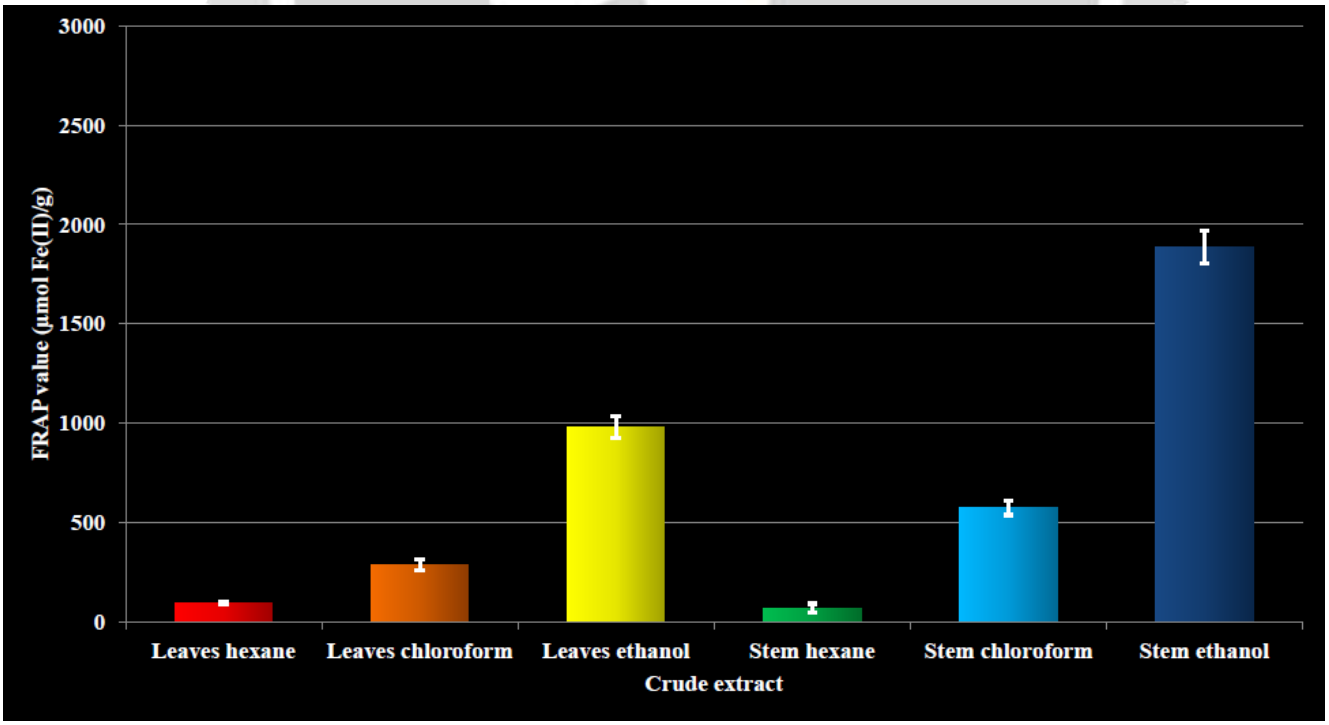

FIGURE 7.8 Antioxidant potentials of crude extracts of Artabotrys hexapetalus using FRAP assay. Results are expressed as mean ± standard deviation (error bar) of three independent experiments

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