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In-vitro antioxidant activity of alcoholic leaf extract and sub-fractions of *Alangium lamarckii* Thwaites

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ABSTRACT

Oxidative stress due to generation of reactive oxygen species and free radicals play a role in pathogenesis of various diseases, the aim of present study was to determine the antioxidant activity and total phenolic contents of alcoholic extract and its various sub-fractions of A. lamarckii (AL). Antioxidant activity of extract and sub-fraction were measured using a series of well-established assays including the 2,2-diphenyl-1-picrylhydrazyl (DPPH), the reducing power, the nitric oxide (NO.) and the hydrogen peroxide (H_2O_2) scavenging and hydroxyl radical assays. From all the fractions tested, ethyl acetate fraction had shown to possess the maximum scavengers of DPPH radical, nitric oxide, hydrogen peroxide and hydroxyl radical. The alcoholic extract exhibited a higher reducing power as compared to its overall sub-fraction. The total antioxidant capacity of the extract and sub-fractions were also comparatively evaluated and the antioxidant capacities were expressed as the number of equivalent of ascorbic acid. The strong antioxidant activities of AL may be attributed to its high level of polyphenolic compounds which was estimated spectrophotometrically using Folin-Ciocalteau assay method. The antioxidant property of the extract and sub-fractions of AL, as observed in the present study might be useful for the development of newer and more potent antioxidants.

Keywords: *Alangium lamarckii*; Antioxidant; DPPH; Reducing power; Total phenolics.

INTRODUCTION

Oxidative stress inside the living organism due to generation of active oxygen species and free radicals have shown to be actively involved in the pathogenesis of age-related diseases [1,2] such as cancer and coronary heart disease and neurodegenerative disorders such as Alzheimer's disease [3]. Compounds that can scavenge free radicals have great potential in ameliorating these

disease processes (4). Natural antioxidants occur in all parts of plants. Plants may contain many different antioxidant components such as phenolic compounds, nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [5-10]. There is currently immense interest in natural antioxidants and their role in human health and nutrition [11].

Alangium lamarckii Thwaites (Syn. *A. salviifolium*) belongs to family Alangiaceae is found commonly in Tropical forest of South India and occasionally grown in garden. The root, root bark, seeds and leaves of the plant is used in Indian system of medicine. The root and root bark of the plant are used as antihelmenthic and purgative, whereas fruits are used as cooling, nutritive and tonic. Leaves of this plant are useful for curing diabetes [12,13]. Decoction of bark has been used as an emetic in India (14). Methanol extract of *Alangium salviifolium* flowers have shown to antibacterial activity against both gram-positive and gram-negative bacteria [15]. Methanolic extract of root of *A. salviifolium* have shown analgesis and anti-inflammatory activities in albino mice [16]. The lyophilized powder extract of pulverized wood of *A. salviifolium* showed good antifungal activity against *Candida albicans* [17]. Alangium A and B from root bark and akoline, lamarkine, alangine, akharkantine from bark, have been reported [18]. Present study is intended to evaluate the *In-vitro* antioxidant activity of crude alcoholic extract and their sub-fractions.

EXPERIMENTAL SECTION

2.1 Materials & Chemicals

Diphenylpicrylhydrazyl (DPPH) free radical was obtained from Sigma Aldrich. Other chemicals, sodium carbonate, sodium phosphate, potassium ferricyanide, ammonium molybdate, rutin, ascorbic acid, gallic acid, 2-thiobarbituric acid (TBA), butylated hydroxy anisole (BHA), Follin Ciocalteu, 2-deoxyribose, ethylenediaminetetraacetic acid (EDTA) and H_2O_2 (30%, v/v) were obtained from Merck Ltd., (Mumbai, India) and from Qualigens Fine Chemical Pvt. Ltd., (Mumbai, India). All other chemicals and solvents used were of analytical grade. The absorbance measurements were recorded using the Ultraviolet-visible spectrophotometer (Shimadzu, Pharmaspec-1700).

2.2 Plant material and preparation of extract

The leaves of *Alangium lamarckii* Thwaites were collected from Panakudi, Tirunelveli District in the month of July 2009 and identified by Prof. V. Chelladurai, Research Botanist, Palayamkottai, Tamilnadu, India. For future reference the voucher specimen (Specimen No-COG/AL/09) and the prepared herbarium was deposited at the Department of Pharmaceutics, Banaras Hindu University, Varanasi (U.P), India.

The leaves were dried under shade, crushed to coarse powder and extracted with alcohol (95%) as an extraction menustrum. The yield of alcoholic extracts was 11.2% w/w. The crude alcoholic extract was subjected to sub-fractionation using various water immiscible solvent (petroleum ether, chloroform and ethyl acetate).

2.3 *Preliminary phytochemical screening*

The alcoholic extract obtained by cold maceration was then subjected to preliminary phytochemical screening to detect for the presence of various phytoconstituents using various qualitative reagents [19].

2.4 *In-vitro* Antioxidant Activity

2.4.1 Antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity was evaluated by the phosphomolybdenum method [20]. 0.3 ml of extract and sub-fraction in methanol (1mg/ml), ascorbic acid used as standard (25-300 µg/ml) and blank (methanol) were combined with 3 ml of reagent mixture (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) separately. All tubes were capped and incubated in a boiling water bath at 95°C for 90 minutes. After cooling to room temperature, the absorbance of each sample was measured at 695 nm against the blank. The antioxidant activity was expressed as the number of equivalent of ascorbic acid.

2.4.2 Assay of Reducing Power

Assay of Reducing Power was carried out by potassium ferricyanide method [21]. 1 ml of alcoholic extract and its sub-fractions (final concentration 25- 200 µg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [$K_3Fe(CN_6)$] (10g/L). The mixture was then incubated at 50°C for 20 minutes. To this mixture 2.5 ml of trichloroacetic acid (100g/L) was added, which was then centrifuged at 3000 rpm for 10 minutes. Finally, 2.5 ml of the supernatant solution was collected and mixed with 2.5 ml of distilled water and 0.5 ml $Fecl_3$ (1g/L) and absorbance was measured at 700 nm. Ascorbic acid was used as standard and phosphate buffer as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

2.4.3 Free radical scavenging activity

The free radical scavenging activity of *A. lamarckii* extract and its sub-fraction was measured by incorporating the DPPH (1, 1-diphenyl-2-picryl-hydrazil) assay method [22]. Briefly, 100 µM/ml solution of DPPH in methanol was prepared and 5 ml of this solution was added to 1ml each of alcoholic extract and their various sub-fractions in different concentrations (25-200 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. The free radical scavenging activity was calculated from $(1-A_1/A_0) \times 100$ [equation 1], where A_0 is the absorbance of the blank and A_1 is the absorbance of the test sample. The percentage inhibition was plotted against respective concentrations used and IC_{50} was calculated using ascorbic acid as control.

2.4.4 Nitric oxide scavenging assay

Nitric oxide scavenging assay was carried by using sodium nitroprusside [23]. This can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract/sub-fraction at various concentrations and the mixture was incubated at 25°C for 150 minutes. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and further incubated at room temperature for 5 minutes. Finally, 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and maintained at room temperature for 30 minutes. The absorbance was measured at 546 nm and percentage inhibition was calculated according to the following equation (1). Here rutin was used as standard.

2.4.5 Scavenging of hydrogen peroxide

Scavenging activity of alcoholic extract and its sub-fractions were evaluated by hydrogen peroxide [24]. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). 1 ml of various concentrations of the extract, sub-fractions and standards in methanol was added to 2 ml of hydrogen peroxide solution in PBS. Then finally the absorbance was measured at 230 nm after 10 minutes. All readings were performed in triplicates and the percentage inhibition were calculated using equation (1).

2.4.6 Scavenging of hydroxyl radical by deoxyribose method

Hydroxyl radical scavenging activity of the alcoholic extract and its sub-fractions were measured by degradation of Deoxyribose [25]. 1ml of the final reaction solution consisted of aliquots (500 µl) of various concentrations of the extract/sub-fractions, 1 mM $FeCl_3$, 1mM EDTA, 20 mM H_2O_2, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 hour at 37°C, and further heated in a boiling water-bath for 15 minutes after addition of 1 ml of 2.8% (w/v) trichloroacetic acid and 1 ml of 1% (w/w) 2-thiobarbituric acid. The colour development was measured at 532 nm against a blank containing phosphate buffer. All readings were performed in triplicates and the percentage inhibition were calculated using equation (1).

2.5 ***Determination of total phenol by the Folin-Ciocalteu assay***

Total phenolic (TP) content of crude alcoholic extract and their sub-fractions was determined by The Folin-Ciocalteu method [26]. The folin-Ciocalteu (FC) assay was carried out by pipetting 1 ml of extract into test tube and to it 8 ml water was added. To this 0.5 ml of FC reagent was added. The mixture was vortexes for 30 seconds and 1.5 ml of filtered 20% sodium carbonate solution was added after 15 minutes. The absorbance of the coloured reaction product was measured at 765 nm after 2 hours at ambient temperature. A calibration curve was created using different concentrations of standard gallic acid solutions. The level of TP in the extract was calculated from the standard calibration curve. Results were expressed on the basis of mg of Gallic Acid Equivalent per gram (mg GAE/g) of dried ethanolic extract.

Table 1: Total phenolic yield and scavenging activity of alcoholic extract of *Alangium lamarckii* leaf and their sub-fractions

Extract/sub-fraction	Total phenolic content (mg gallic acid equivalent /g of extract)*	IC_{50} values ± SEM*(µg/ml)			
		DPPH	Hydrogen peroxide	Nitric oxide	Deoxyribose
Alcoholic extract	150.62 ± 0.67	159.32 ± 1.81	202.43 ± 3.04	245.40 ± 1.33	216.57 ± 1.04
Petroleum ether	52.79 ± 2.39	344.98 ± 2.09	588.149 ± 13.64	591.37 ± 3.70	506.72 ± 4.55
Chloroform	107.71 ± 1.31	331.44 ± 5.64	501.67 ± 8.19	485.51 ± 4.60	436.60 ± 6.34
Ethyl acetate	294.66 ± 1.06	160.66 ± 2.60	230.27 ± 2.49	313.74 ± 0.10	257.70 ± 0.57
Aqueous	166.27 ± 0.60	250.96 ± 4.55	286.94 ± 0.26	410.28 ± 0.91	380.77 ± 3.26
Standards					
Ascorbic acid		77.91 ± 1.93	-	-	-
Rutin		-	87.07 ± 0.51	98.06 ± 6.09	-
BHA		-	-	-	123.55 ± 1.61

**Average of three independent determinations, values were mean ±SEM*

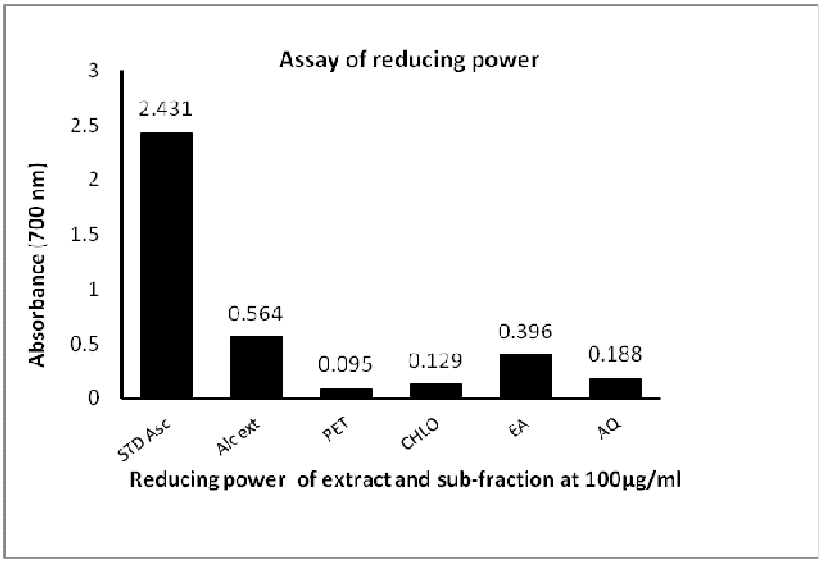

Fig 1: Reducing power of alcoholic extract and sub-fractions of *Alangium lamarckii* leaf

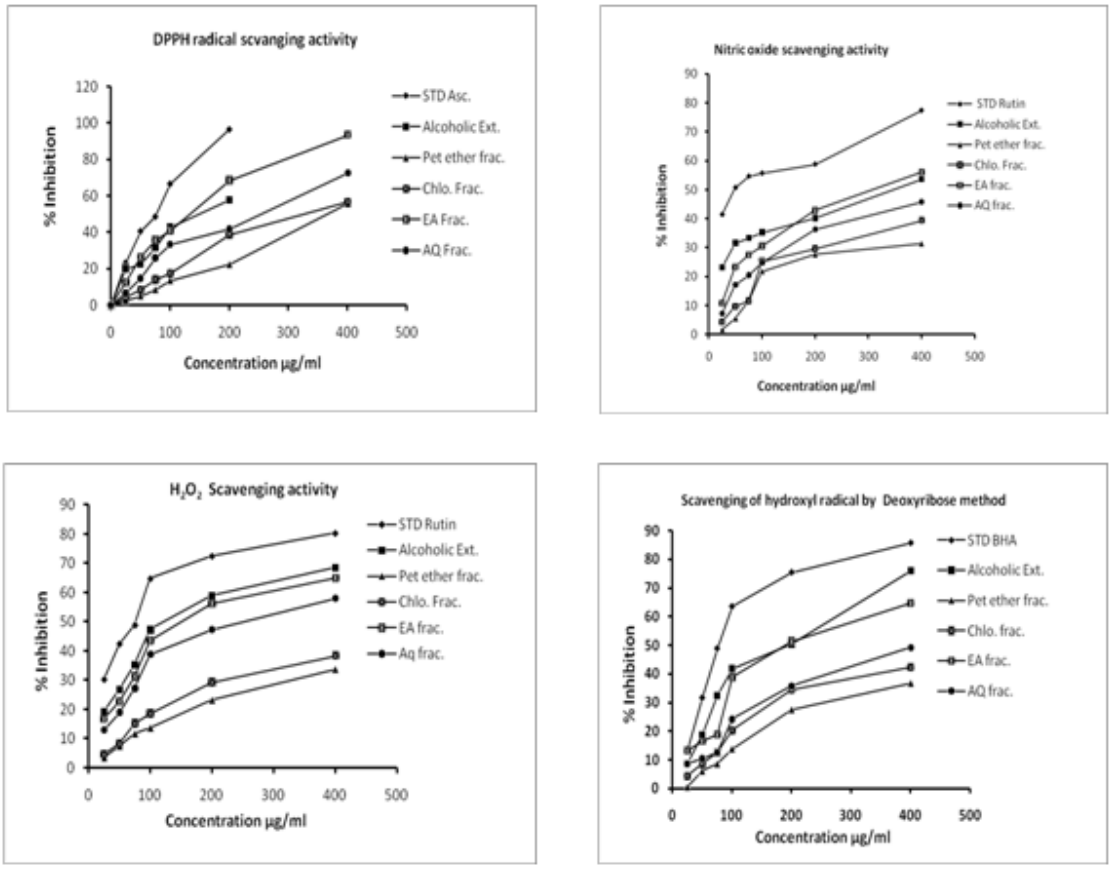

Fig 2: Anti-oxidant Scavenging activity of various standard, extract and different sub-fractions of *Alangium lamarckii* leaf on DPPH, Nitric oxide, H_2O_2 and hydroxyl radical

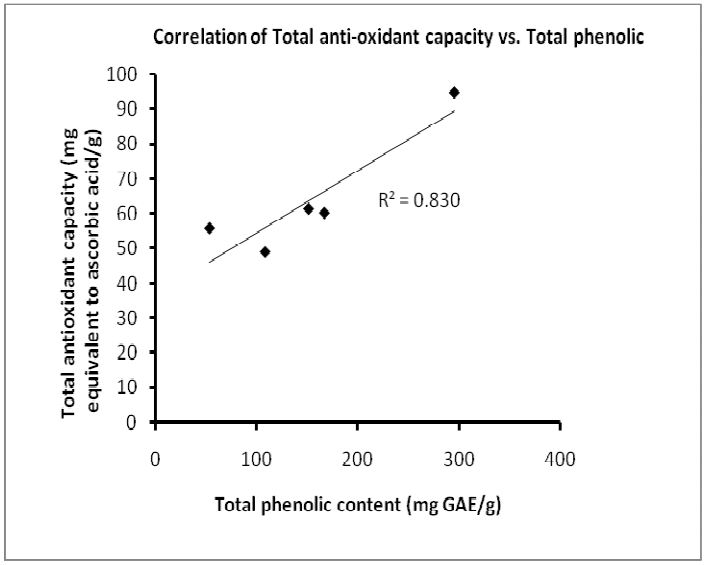

Fig 3: Correlation between the total antioxidant capacity and total phenolic content of *A. Lamarckii* leaf extract and their sub-fractions

RESULTS AND DISCUSSION

3.1 Preliminary phytochemical screening

Preliminary phytochemical screening of alcoholic extract of *Alangium lamarckii* leaf showed the presence of alkaloids, amino acids, phenolic compounds, tannins, sterols, terpenoids, carbohydrates and absence of flavanoids, saponins and glycosides.

3.2 Anti-oxidant assay

3.2.1 Determination of Total antioxidant capacity

The assay of total antioxidant capacity by Phosphomolbydenum method is based on reduction mechanism of Mo (VI) to Mo (V) by the antioxidant agents and the subsequent formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm [27]. The total antioxidant capacity in extract and sub-fractions was determined using the linear regression equation of the calibration curve ($y = 0.005x+0.042$, $r^2= 0.996$) and was expressed as the number of equivalent of ascorbic acid (µg/ml plant extract and their fractions). Ethyl acetate fraction of *A. lamarckii* has shown to have the highest antioxidant capacity (94.86±0.58 µg/ml ascorbic acid equivalent) than the ethanolic extract (61.4±0.69 µg/ml) and other sub-fractions (Fig 4).

3.2.2 Reducing power

The reducing capacity of a compound may serve as an indicator of its potential antioxidant activity [28]. The reducing ability of a compound generally depends on the presence of reductants which possess antioxidative potential by breaking the free radical chain, by donating a hydrogen atom. Ethanolic extract *A. lamarckii* and its sub-fractions exhibited a good reducing power. High absorbance indicates high reducing power. The reducing power of the petroleum ether fraction of *A. lamarkii* leaf was found to be the least as compared to that of standard ascorbic acid and the highest was seen with that of ethanolic extract (Fig. 1).

3.2.3 Free radical scavenging activity

The free radical scavenging activity of ethanolic extract *A. lamarckii* and its sub fraction was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and thereby bleach the DPPH absorption. DPPH is a purple colour dye having absorption maxima of 517 nm and upon reaction with a hydrogen donor the purple colour fades or disappears due to conversion of it to 2, 2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance. Alcoholic extract and its sub-fraction exhibited considerable free radical scavenging activity as indicated by their IC_{50} values and this can be shown in Table 1 and Fig. 2. IC_{50} indicate the potency of scavenging activity. Standard ascorbic acid was found to have an IC_{50} of 77.91±1.93 µg/ml. In comparison to ascorbic acid ethyl acetate fraction of alcoholic extract of *A. lamarckii* was shown to have a good IC_{50} of 160.66±2.60 µg/ml as compared to other sub-fractions. But it is rather comparable to its alcoholic extract with an IC_{50} value of 159.59±1.81. Petroleum ether fraction is seen to have the least free radical scavenging activity followed by chloroform and aqueous fractions.

3.2.4 Nitric oxide scavenging assay

Nitric oxide is a very unstable species and reacting with oxygen molecule produce stable nitrate and nitrite which can be estimated by using Griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546 nm. Alcoholic extract of *A. lamarckii* have potent nitric oxide scavenging activity (IC_{50} value 245.40±1.33 µg/ml) and petroleum ether fraction has showed the least nitric oxide scavenging activity (IC_{50} value 591.37±3.70 µg/ml) (Table 1 and Fig. 2). IC_{50} value of ethyl acetate fraction was 313.74±0.10 µg/ml, which is comparable to alcoholic extract. Nitric oxide (NO) has shown to play a crucial role in various physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical, which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilatation, antimicrobial and antitumor activities [29]. However, excess production of NO is associated with several diseases [30].

3.2.5 Scavenging of hydrogen peroxide

Scavenging of hydrogen peroxide of crude alcoholic extract, their sub-fractions and standard rutin are presented in figure (fig. 2). Hydrogen peroxide, although not a radical species play a role to contribute oxidative stress. The generation of even low levels of H_2O_2 in biological systems may be important. Naturally-occurring iron complexes inside the cell believed to react with H_2O_2 in vivo to generate highly reactive hydroxyl radicals and this may be the origin of many of its toxic effects [31]. Alcoholic extract and its ethyl acetate fraction showed good activity in depleting H_2O_2, with an IC_{50} value of 202.43±3.04 and 230.27±2.49 µg/ml respectively (Table 1).

3.2.6 Scavenging of hydroxyl radical by Deoxyribose method

The effect of *A. lamarckii* alcoholic extracts and their sub-fractions on inhibition of hydroxyl radical production was assessed by the iron (II)–dependent deoxyribose damage assay. The Fenton reaction generates hydroxyl radicals (OH^-) that degrade deoxyribose using Fe^{2+} salts as an important catalytic component [32]. Oxygen radicals may attack the sugar, which leads to sugar fragmentation. Addition of transition metal ions such as iron at low concentrations to deoxyribose causes degradation of the sugar into malondialdehyde and other related compounds

which form a chromogen with thiobarbituric acid (TBA). Table 1 represents the IC_{50} value of extract and its sub-fractions. Alcoholic extract showed potent hydroxyl radical scavenging effect by deoxyribose method, followed by ethyl acetate fraction. While the petroleum ether fraction showed least potent hydroxyl radical scavenging effect (Fig. 2).

3.3 Estimation of total phenolic content of crude alcoholic and their sub-fractions

Preliminary phytochemical investigation has shown the presence of phenolic compounds in alcoholic extract of *A. lamarckii*. Literature reveals that antioxidant activity of plant extract is mainly due to presence of phenolic compounds, which may exerts antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators [33]. In our present investigation, it was found that ethyl acetate fraction has the highest phenolic content followed by aqueous fraction and petroleum ether fraction has showed least phenolic content. The content of the total phenolics in extracts and sub-fractions was determined using the linear regression equation of the calibration curve ($y=0.008x+0.092$, $r^2= 0.995$) and is expressed as gallic acid equivalent (Table 1).

3.4 Relationship between the total antioxidant capacity and the total phenolic content

The extent of antioxidant capacity of extract and sub-fraction of *A. lamarckii* was correlated with their total phenolics contents. Many authors studied the correlation between the total content of phenolics and the antioxidant capacity. Several studied established a linear correlation between the total content of phenolics and the antioxidant capacity [34] whereas some studies reported that there is no correlation [35]. The correlation coefficient between the total antioxidant capacity monitored by phosphomolybdenum method and the total phenolic contents of the alcoholic extract and their sub-fractions were determined (Fig. 3). A linear correlation appeared between the total antioxidant capacity and the total phenolic contents of the extract and fractions with good correlation coefficient ($r^2=0.830$). The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the *A. lamarckii* extracts and its sub-fractions.

CONCLUSION

Overall, crude alcoholic extract and ethyl acetate fraction have showed impressive antioxidant activity and free radical scavenging activity. The remarkable antioxidant activity could be due to presence of high content of phenolics. However, further investigation of individual phenolic compounds, their in vivo antioxidant activity and the different antioxidant mechanisms is warranted.

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