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Antioxidant Properties of the Herb *Phyllanthus aramus* Leaves Extract

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Abstract: The herb *Phyllanthus aramus*, was used for long time as a medicinal plant in all tropical and sub-tropical areas of the world. The aim of this study was conducted to investigate and calculate the antioxidant potency of the leaves extract of this herb and to evaluate the bioactive molecules which could be used in future as a natural supplement or drug by using two different solvents, water and *n*-butanol. Leaves conents were extracted and total of phenolic contents, total flavonoids, radical scavenging potency, β-carotenoid contents and cell viability were assessed. The results showed high content of polyphenols in both extract. Also, the results showed that the highest total extractable components were revealed in water fraction. The results showed that IC_{50} in the extract of both solvents was low indicating a great antioxidant value.

Key words: *Phyllanthus aramus* · Cell viability · Polyphenols · Flavonoids · β-carotenoid · Antioxidants

INTRODUCTION

Medicinal herbs are significant source of pharmaceutical drugs. Latest trends have shown increasing demand of phytodrugs and medicinal herbs have proven that they have a potent antioxidant character [1].

In the past, herbs often represented the original sources of most drugs, but nowadays, alternative medicines are used widely in all over the world. With increasing recognition of herbal medicine as an alternative form of health care, screening of medicinal plants for biologically active extracts and/or compounds has become an important source of drugs [2]. *Phyllanthus aramus* is a medicinal herb, native in Southeast Asia. It was reported that *Phyllanthus* genus includes over 600 species distributed throughout tropical and subtropical areas [3].

Also, it was reported that *Phyllanthus* contains several chemically active constituents such as polyphenols, terpenoids, oleanolic acid and sterols [4]. The polyphenols are the most dominant constituents in the leaf, which has been reported to be effective in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems [5]. Phenolic compounds such as lipophilic flavones, caffeic acid derivatives (rosmarinic acid and 2, 3-dicaffeoyl tartaric acid), eupatorine, cichoric acid, sinensetin and methoxy flavones were found in herbs [6]. Three highly oxygenated 2, 3-secoisopimarane-type diterpenes, have been isolated from the leaves of some herbs [7]. The therapeutic benefit of medicinal plants is usually contributed to their antioxidant properties [8]. Phenolic compounds possess diverse biological activities such as anti-inflammatory, anticarcinogenic and antiatherosclerotic activities. These activities of phenolic compounds might be related to their antioxidant activities [9]. Other studies showed that there were significant correlations between phenolic compounds and antioxidant properties of medicinal plants. Therefore, the current study was conducted to investigate the antioxidant and antiapoptotic potency of the extract/fractions of the leaves of *Phyllanthus aramus* from Malaysia.

MATERIALS ANDMETHODS

Chemicals: All chemical used in this study were of analytical grade and purchased from Sigma Co.USA.

Plant Materials: Leaves of healthy *Phyllanthus aramus* (Euphorbiaceae) herbs were collected from Ipoh area, Malaysia. Authentication was done in University of Malaya.

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The freshly isolated leaves were cleaned by tap water for 3 times, then by phosphate buffer pH.7.4. Leaves were dried in oven at 40–50°C for 3 days. The dried leaves were weighed and then grounded to powder form and kept in an airtight brown bottle at room temperature.

Extraction of the Crude of Leaves: Leave extraction was done according to the method recommended before [10].

One hundred grams of the dried ground leaves of *Phyllanthus aramus* were extracted successively with 60% methanol (3×300mL) to obtain crude methanolic extract at room temperature for two days each, then combined and concentrated by removing solvent using rotary evaporator.The obtained crude (62.5 g) was fractionated using *n*-butanol and water individually, where the residue from each fractionation step was used to obtain the subsequent fraction. Each extraction process involved homogenisation using Ultra homogenizer and its fractions in the respective solvent was centrifuge at 13, 000 rpm for15 min followed by sonication at constant temperature of 25°C for 1 h. The crude and its fractions, *n*-butanol and water fractions were filtered through filter paper Whatman No. 1, then solvents were removed by using rotary evaporator as recommended before [11]. The yield of each extract and its fractions was measured before kept in refrigerator at -20°C for further analysis.

Total Phenolic Contents: Phenolic contents in the extracts were determined using Folin-Ciocalteu method [12], with minor modification. Briefly, stock solutions of extract and fraction of leaves were prepared in a concentration of 10 mg/mL in methanol. Five microliters of each solution were transferred to 96-well microplate. To this, 80 µL of Folin-Ciocalteu reagent (1: 10) were added and mixed thoroughly. After 5 min, 160 µL of sodium bicarbonate solution($NaHCO_3$ 7.5%) was added and the mixture was allowed to stand for 30min with intermittent shaking. Absorbance was measured at 765 nm using microplate reader. The total phenolic content was expressed as gallic acid equivalent (GAE) in mg/g extract, obtained from the standard curve of gallic acid. The gallic acid standard curve was established by plotting concentration (mg/mL) versus absorbance (nm).

($y = 0.001x + 0.045$; $R2 = 0.9975$),

where y is absorbance and x is concentration in GAE ($n = 3$).

Total Flavonoid Contents: otal flavonoid contents were determined by the $AlCl_3$ method using rutin as a standard. The test samples were dissolved in DMSO (10mg/mL). The sample solution (1mL) was mixed with one millilitre of $AlCl_3$ (2.0%). After 10 min of incubation at ambient temperature, the supernatant was transferred to 96-well plate. The absorbance of the supernatant was measured immediately at 435nm using microplate reader. Total flavonoid content was expressed as rutin equivalents (RE) in milligrams per gram sample. The curve was established by plotting concentration (µg/mL) versus absorbance (nm).

($y = 5.6752x - 0.0312$;$R2 = 0.99$).

where y = absorbance and x = concentration($n = 3$).

Radical Scavenging Determination (DPPH Method): Activity of extract and fractions of leaves against DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate), was determined spectrophotometrically.

When DPPH (violet colour) reacts with an antioxidantcompound, which can donate hydrogen, it is reduced andproduces a yellow colour. The changes in colour were measured at 517nm wavelength according to method reported before [13]. Radical scavenging activity of extract/fractions of *Phyllanthus aramus* leaves were measured as in the method designed before [14], as described below.

Stock solutions were dissolved in methanol (10 mg/mL). The working solution was prepared using methanol in aconcentration of 200 µg/mL. The solution of DPPH in methanol (2.5mg/mL) was freshly prepared. Five µL of this solution were mixed with 100 µL of serial dilutions of samples (15.625–2000 µg/mL) in 96-well plate. The samples were kept in the dark for 30 min at ambient temperature and then the change in absorption was measured according to the instruction of manifacturer. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured daily. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \, Inhibition = \left[\frac{A_B - A_A}{A_B} \right] X100$$

where A_B is the absorption of blank sample; A_A is the absorption of tested samples. The inhibitory concentration 50% was determined as well as the kinetics of DPPH scavenging reaction. Ascorbic acid was also tested against DPPH as positive control.

Beta-Carotene Bleaching Assay: The antioxidant activity of the different extracts/fractions of *Phyllanthus aramus* leaves was evaluated using the β-carotene-linoleic acid assay according to the method reported before [15]. An aliquot of 5.0mg from β-carotene was dissolved in 50 mL chloroform. Linoleic acid 200 µL and 600 mg Tween-20 were mixed with 1mL of the chloroform solution. The chloroform was evaporated under vacuum at 45°C for 20 min, then 50mL was used to culture MDA-M231 cells. Cultures were supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). Stock cultures of exponentially growing cells were trypsinized and plated (2 × 105cells/well) into 96-well plate and incubated at 37°C for 48h prior to use then washed twice with PRMI-1640 medium and recultured there in.

Oxygenated water was added and the mixture was vigorously shaken to obtain β-carotene-linoleic acid emulsion. Two hundred µL of this emulsion were distributed in a 96-well microplate. Methanolic solutions (10 µL, 2mg/mL) of the sample extracts and positive control BHA (butylated hydroxyanisole) were added into the plate. An equal amount of methanol was used as negative control. Absorbance was read at 470nm after incubation for every 20 min until 120 min at 50°C using a microplate reader. Tests were performed in triplicate.

Assessment of Cell Viability: Cell viability could be quantified using MTT, which yields a purple formazan product in living cells, but not in dead cells or their lytic debris. MTT was dissolved in aseptic PBS to a concentration of 5mg/mL as a stock solution. MTT was added at the end of incubation to a final concentration of 0.5 mg/mLand mixed with 100 µL of serial dilutions of samples (15.625–2000 µg/mL) in 96-well plate. The samples were kept in the dark for 30min at room temperature and then the change in absorption was measured. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured daily.

RESULTS AND DISCUSSION

The current study provide, for the first time, a standardized method to obtain rich antioxidant fractions from *Phyllanthus aramus* leaves.

In this study, amount of extractable compounds was calculated as milligram extract in each gram of powdered leaves. The results showed that the highest total extractable components were revealed by water fraction (166.18 mg/g), followed by *n*-butanol (108.22 mg/g). The total extractable contents were obviously varying between the fractions of *Phyllanthus aramus* leaves and increased gradually with the increment of the solvents' polarity.

Since plant phenolics represent one of the major groups of compounds behaves as primary antioxidants or free radical scavengers [17], it was reasonable to determine their total amount in the extract/fractions of *Phyllanthus aramus* using Folin-Ciocalteau method. The total phenolic content was expressed as gallic acid equivalent in mg/g. Results showed that *n*-butanol fraction`showed the higher TPC followed by WF.

Flavonoids are the most common and widely distributed group of plant phenolic compounds, which usually are very effective antioxidants [19-24]. In this study, total flavonoids content (TFC) of *Phyllanthus aramus* leaves was determined. The TFC was expressed as rutin equivalent in mg/g [25]. The results showed that the flavonoids content was in the order of their BF >WF with the values of 1.9 and 13.20 ± 4.2mg RE/g extract, respectively. This indicates that the lipophilic flavonoids are major phenolic compounds present in *Phyllanthus aramus* leaves.

It can be observed that the content of phenolics in the extracts/fractions of *Phyllanthus aramus* leaves correlates with their antiradical activity (e.g., correlation coefficient between data of DPPH assay and total phenolic compounds was 0.966, $P < .005$), confirming that phenolic compounds are likely contribute to the radical scavenging activity of these plant extract/fractions. Detailed examination of phenolic composition in plant extract/fractions of *Phyllanthus aramus* leaves is required for the comprehensive assessment of individual compounds exhibiting antioxidant activity. A previous study had found that phenolic compounds contributed significantly to the antioxidant capacity of the medicinal herbs as there was a direct relationship between antioxidant activity and total phenolic content [26].

DPPH Scavenging Activity Test: The DPPH assay has been widely used to test the free radical scavenging ability of plant materials and pure compounds [27]. The DPPH is a stable free radical (purple coloured), which is reduced to α, α-diphenyl-β-picrylhydrazine (yellow coloured) when reacting with an antioxidant agent. Antioxidants interrupt free radical chain oxidation by donating hydrogen from hydroxyl groups to form a stable end-product, which does not commence or proliferate further oxidation of lipids in human body [28].

The data obtained from this assay is commonly reported as IC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period.

The results show that the IC_{50} in the extract of both solvent was low which indicates high antioxidant activity. This is due to the high total phenolic contents present in the leaves of *Phyllanthus aramus* that act as free radical scavengers. It also observed that there is a positive linear relationship (P <.05) between DPHH inhibition and the polyphenolic content ofextract/fractions of *Phyllanthus aramus* leaves, whereby polar extract/fractions have shown the higher DPPH scavenging activities.

Results of the current study are in an agreement with those of Akowuah *et al*. [9] who indicated that the extracts of *Phyllanthus aramus* leaves are free radical inhibitors and primary antioxidants that react with free radicals and the polar extracts have the highest free radical scavenging activity. However, the current study investigated for the first time the antioxidant activities of *Phyllanthus aramus* leaves fractions.

β-Carotene Bleaching (BCB) Assay: In the BCB assay, the oxidation of linoleic acid produces peroxyl free radicals due to the abstraction of hydrogen atom from diallylicmethylene groups of linoleic acid [34-36]. The free radical then will oxidize the highly unsaturated β-carotene. The presence of antioxidants in the sample will reduce the oxidation of β-carotene by hydroperoxides which is formed in this system and decomposed by the antioxidants from the extracts [37]. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts against the oxidation by hydroperoxides. The degradation rate of β-carotene clearly depends on the antioxidant activityof *Phyllanthus aramus* leaves extract. There was a correlation between the degradation rate and the bleaching of β-carotene, where the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity. The high antioxidant activities of *n*-butanol fraction tested using β-carotene model may be correlated with their high phenolic content. These findings are in good agreement with those results reported before by Khopde [29] who found that extraction with polar solvents gave the highest amounts of phenolic compounds.

Cell Viability: To examine the effect of the extract of *Phyllanthus aramus* leaves on oxidant-induced apoptosis, the method designe by Han (14) was followed. Incubation of cells with H_2O_2 leads to the decrease of formazan production compared with the untreated one, which can be seen from the decrease of optical density at 570 nm and it means that cell viability in H_2O_2-treated wells was much lesser than the untreated one. However, when cell is incubated with extract/fractions of *Phyllanthus aramus* leaves H_2O_2 exposure, cell viability was higher than that of control. Results showed significant (P <.05) protection against hydrogen peroxide and inducing apoptosis [38].

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