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Biochemical Studies on the Antioxidant Activities of *Phyllanthus amarus* Fruit Extract

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Abstract: The herb *Phyllanthus amarus* 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 fruit extract of this herb and to evaluate the bioactive molecules which could be used in future as a natural supplement or drug by using methanol-water as a solvent. Fruit contents were extracted and total of phenolic contents, total flavonoids; radical scavenging potency,cell viability were assessed. The results showed high content of polyphenols in the extract, the highest total extractable components were revealed. Also, it showed that IC_{50} in the extract was low indicating a great antioxidant value.

Key words: *Phyllanthus amarus* fruits · Antioxidants · Polyphenols

INTRODUCTION

Phyllanthus amarus is an indigenous medicinal herb, which has a folk reputation in tropical and sub-tropical areas of the [1]. Therefore, we are planning to assess its antioxidant potency, flavonoids strength and total polyphenol contents.

The literature survey reveals that this herb controls the adverse effect of many diseases in human being [2, 3]. It elicits a wide spectrum of pharmacological activities. In recent years, attention has been paid to the role of this herb in human health. Several epidemiological studies have indicated that a high intake of *Phyllanthus amarus* products is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis, diabetes, hepatotoxicity and cancer [1, 4]. It was found that this herb has a wide group of secondary metabolites that can range from simple molecules, such as phenolic acids to highly polymerized constituents such as tannins, with a large number of derivatives [5]. All these studies, have been reported that this herb have multiple biological effects such as antioxidant activity, which can terminate or retard the oxidation process by scavenging free radicals [6-8]. These antioxidants are considered as protective agents for reducing oxidative damage of human body from reactive oxygen species (ROS). Also, they act as anti-inflammatory agents, inhibition of platelet aggregation, inhibition of mast cell histamine release, antimicrobial activities. Moreover, antioxidant phenolics of this herb have been suggested to play a preventive role in the development of cancer and heart diseases [9].

The extract of the whole plant shows that it induces apoptosis in tumour cells by activation of caspases and modulation of other pathways involved in cell proliferation and migration. The extract may therefore play a complementary or synergistic role in chemoprevention of different diseases. Further studies of bioavailability and *in vivo* activities are needed.

The antioxidant properties of the extract of the fruit of this herb have not been studied till now. Therefore, the aim of the present investigation is to study the biochemical characters of the active pharmaceutical group of the fruit of this herb, as well asto investigate the antioxidant properties and ROS scavenging activities.

MATERIALS AND METHODS

Materials: In this study, all chemicals used were of analytical grade, all assays reagents and cell lines were purchased from local supplier. Folin–Ciocalteu reagent,2, 20-Azino-bis(3-ethylbenzothiazoline-6- sulphonic acid) (ABTS), 2,2-diphenyl-1-picryl-hydrazil(DPPH), gallic acid, catechin, trolox, 2,4,6-tripyridyls-triazine (TPTZ), were products of Sigma and purchased from local supplier.

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Ripe healthy fruits of *Phyllanthus amarus* were collected from different areas of Kedah and kept frozen after cleaning.

Methods:

1-Extraction of *Phyllanthus amarus* Fruits: Collected healthy ripe fruits were cleaned 3 times by tap water then by phosphate buffer (pH 7.4), dried by using reflected air current and then powdered.

120g of this powder were homogenized in phosphate buffer (pH 7.4) using Ultra homogenizer and then extracted with methanol water in ratio 4:1 under Soxhlet extractor for eight hours as reported before [6]. The extract was purified by using Buchner funnel. The extractive value was calculated on dry weight basis from the formula given below:

$$\% \text{ extractive value (yield \%)} = \frac{\text{weight of dry extract}}{\text{weight taken for extraction}} \text{ X } 100$$

The extract kept in brown container in -80°C.

DPPH Based Free Radical Scavenging Activity: The free radical scavenging activities were assayed using stable DPPH following standard method [10]. The reaction mixture contained 1.8 ml of 0.1mM DPPH and 0.2 ml of each serial dilution (0.5-2) of methanol extract. Simultaneously, a control was prepared withoutsample extracts. The reaction mixture was allowed to be incubated for 5 min at room temperature in the dark and scavenging activity of the fraction was quantified spectrophotometrically by decolourization at 515nm. Percentage of free radicals scavenging activity was expressed as percent inhibition from the given formula:

$$\text{absorbance of control-absorbance of sample} = \frac{\% \text{ inhibition of DPPH radical}}{\text{absorbance of control}} \text{ X } 100$$

Determination of Total Phenolic Contents (TPC) in the Extract: The TPC of the PA extract was quantified based on the previous study [11] with some modifications. To 0.5 mL of the extract was added to 2.5 mL of 10% (v/v) Folin–Ciocalteu reagent indistilled water. The mixture was kept for 6 min and then 2 mL of 7.5% (w/v) Na_2CO_3 solution was added.

The absorbance of the mixture was measured at 765 nm using a UV-VIS spectrophotometer after incubation in the dark at room temperature for 1 h.

Methanolwas used as a control. TPC was quantified as mg gallic acid equivalents (GAE)/g dried extract.

The reaction mixture will be prepared by mixing 0.5 ml methanolic-water solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank will be concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent was dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples will be thereafter incubated in a thermostat at 45°C for 45 min. The absorbance was determined spectrophotometrically at 765 nm. The same procedure was repeated for the standard solution of gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration ofphenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts will be expressed in terms of gallic acid equivalent (mg of GA/g of extract).

ABTS Radical Scavenging Capacity (ARSC): The ARSC of the PA extracts and fractions was evaluated using the formerly established method [12] with some modifications. Briefly, a stock solution was prepared by mixing 7.4 mMABTS and 2.6 mM $K_2S_2O_8$ solutions (1:1 ratio), kept in the dark at room temperature for 12 h andstored at -20°C. Prior to use, mixing 1 mL of stock solution with 60 mL of methanol was performed to obtain a working solution with absorbance of 1.1 - 0.02 at 734 nm. 0.15 mL of the extract wasmixed with 2.85 mL of the working solution and incubated in the dark at room temperature for2 h. The absorbance of mixture was measured at 734 nm. Methanol was used as acontrol. The results were calculated as mg trolox equivalents (TE)/gdried sample.

Ferric Reducing Antioxidant Power (FRAP): The FRAP of the PA extracts and fractions was estimated based on the former report [13] with some modifications. Three reagents were prepared including 300 mM acetate buffer solution at pH 3.6(reagent A); 10 mM TPTZ solution in 40 mMHCl (reagent B); and 20 mM FeCl3.6H2O solution(reagent C). Prior to use, reagents A, B and C were mixed at a ratio of 10:1:1 to make a fresh FRAP solution. 0.15 mL of the extract was mixed with 2.850 mL of the fresh FRAP solution and incubated inthe dark at room temperature for 30 min. The absorbance of mixture was read at 593 nm. Methanol was used as a control and positive control, respectively. The results were calculated asmg trolox equivalents (TE)/g dried sample.

Determination of Flavonoid Content in *Phyllanthus amarus* Fruit Extract: The content of flavonoids in the extract was determined according to the method [14]. The

sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$, solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in the herb extract was expressed in terms of rutin equivalent (mg of RU/g of extract).

MTT Assay: The *Phyllanthus amarus* fruit extract was tested for *in vitro* cytotoxicity using HCT-116 cells by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [15, 16]. HCT (70-80%) confluent cell lines were trypsinized followed by viability checking and centrifugation. In a 96 well plate, 5 x 10^4 cells/ well was seeded and incubated for 24 hrs at 37°C in a humidified 5% CO_2 incubator. Plant extracts of varying concentrations ranging from 0-320 ìg/ ml (two fold variations) in RPMI media without fatal bovine serum (FBS) and antibiotics was incubated for 24 hrs. After incubation with fruit extracts, the media was removed from the wells and added 100ml/well (50 ìg/well) of the MTT (5 mg/10 ml of MTT in 1x PBS, thesolution was filtered through a 0.2 ì m filter and stored at 2-8 °C for frequent use or frozen for extended periods) working solution was added and incubated for 3 to 4 hours. After incubation with MTT reagent, the media will be removed from the wells and added 100 ìl of DMSO to rapidly solubilize the formazan. The absorbance for each well was measured at 590 nm in a microtitre plate reader and the percentage inhibition will be calculated using the formula:

$$\text{Inhibition \%} = \frac{\text{Control}_{A590} - \text{Sample}_{A590}}{\text{Control}_{A590}} \text{X } 100$$

Statistical Analysis: All data are expressed as means ± SD (n=3). Data were analysedby an analysis of variance ($P < 0.05$) and the results were processed by SPSS software.

RESULTS AND DISCUSSION

The current study provides, for the first time, a standardized method to obtain rich antioxidant fractions from *Phyllanthus amarus* fruit. Also, it showed that the amount of extractable compounds was calculated as

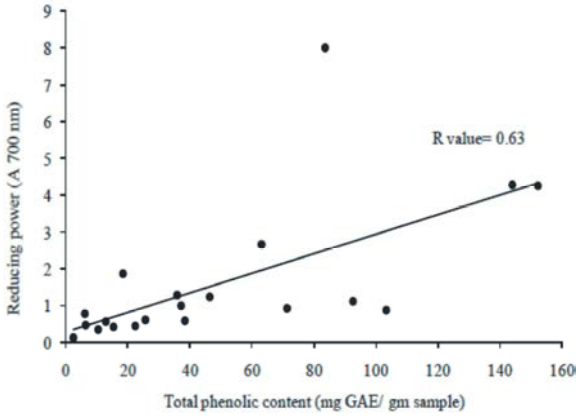

Fig. 1: Total phenolic content of fruit extract with its relative activity.

Table 1: Total phenolic contents of *P.aramus* fruit extract along with its RP activity

	TP content (mg GAE*/gm sample)	RP†(A ‡700nm)
P.aramus fruit extract	63.01±0.2	12.67±0. ±80

milligram extract in each gram of powdered fruits. The results showed that the highest total extractable components revealed were (194.31 mg/g) (Table 1, Fig 1).

Since plant phenolics represent one of the major groups of compounds behaves as primary antioxidants or free radical scavengers, it was reasonable to determine their total amount in the extract/fraction of *Phyllanthus amarus* fruit using Folin-Ciocalteau method [17].

The total phenolic content was expressed as gallic acidin mg/g. Results showed high content of TPC (Table 2).

Flavonoids are the most common and widely distributed group of plant phenolic compounds, which usually are very effective antioxidants [18-20]. In this study, total flavonoids content (TFC) of *Phyllanthus amarus* fruits was determined. The TFC was expressed as rutin equivalent in mg/g. The results showed that the flavonoids content value was 13.20±4.2mg RE/g extract. This indicates that the lipophilic flavonoids are major phenolic compounds present in *Phyllanthus amarus* fruit [21].

It can be observed that the content of phenolics in the extract/fractions of *Phyllanthus amarus* fruits are correlate 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 the fruit of this herb

Table 2: Statistical data of regression equations of calibration curve for gallic acid & linearity and specificity test for TP content from fruits of *Phyllanthus aramus*

Regression Analysis	Calibration Curve for Gallic acid	Linearity of (FC reaction method)	Specificity of (FC reaction method)
Regression equation	y = 0.1071x + 0.004	y=0.0529x +0.001	y=0.0529x+0.0203
Slope	0.1071	0.0529	0.0529
Intercept	0.004	0.001	0.0203
Regression coefficient (R2)	0.998	0.998	0.998

extract/ fractions and had antioxidant activities. These results also confirmed by both ABTS and FRAP assays.

The DPPH assay has been widely used to test the free radical scavenging ability of plant materials and pure compounds [22, 23]. The DPPH is a stable free radical (purple coloured), which is reduced to α, α-diphenyl- -picrylhydrazine (yellow coloured) when reacting with an antioxidant agent [24]. 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 [25].

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. It was (72.72% at the high dose 4000 ìg/ml).

These results suggest that the hydrochloride groups of the fruit of this herb display scavenging effect on DPPH radicals' generation that could help prevent or ameliorate oxidative damage.

The results show that the IC_{50} in the extract was low which indicates high antioxidant activity. This is due to the high total phenolic contents present in the fruits of *Phyllanthus amarus* that act as free radical scavengers [26].

It also observed that there is a positive linear relationship (P <.05) between DPPH inhibition and the polyphenolic content of extract/fractions of *Phyllanthus amarus* fruits, where by polar extract/fractions have shown the higher DPPH scavenging activities [27].

Results of the current study are in an agreement with previous published reports which indicated that the extracts of *Phyllanthus amarus* fruits are free radical inhibitors and primary antioxidants that react with free radicals and the polar extracts have the highest free radical scavenging activity [28-30]. However, the current study investigated for the first time the antioxidant activities of *Phyllanthus amarus* fruit fractions.

To examine the effect of the extract of *Phyllanthus amarus* fruit on oxidant-induced apoptosis, the method designed by Choi *et al*. [31] was followed incubation of cells with H_2O_2 leads to the decrement of formazan production compared with the untreate done 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 amarus* fruits H_2O_2 exposure, cell viability was higher than that of control. Results showed significant (P <.05) protection against hydrogen peroxide and inducing apoptosis.

Cell viability could be coefficient between data of DPPH assay and total quantified using MTT, which yields a purple formazan phenolic compounds was confirming that product in living cells, but not in dead cells or their lytic phenolic compounds are likely contribute to the radical debris [32].

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