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Research Article

COMPARATIVE PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTIOXIDANT ASSAY OF AQUEOUS AND ETHANOLIC EXTRACT OF *PHYLLANTHUS AMARUS* LEAVES

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Abstract

This study was carried out to evaluate the phytochemical constituents and antioxidant potentials of the aqueous and ethanolic leaf extracts of *Phyllanthus amarus*. Phytochemical constituents were determined using standard procedures. The antioxidant potentials of both extracts were evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Scavenging assay, reducing power activity by the method described by Oyaizu (Fe^3 - Fe^{2+} and measuring of Perl's Prussian blue complex) and total antioxidant capacity by phosphomolybdenum method (Prieto). Total phenol, flavonoid, saponin and tannin were evaluated by Folin-Ciocalteu, Aluminum chloride (Olajire), Vanillin-Sulphuric acid and Broad Hurst method respectively. In the DPPH scavenging assay, the IC_{50} values of ethanolic and aqueous extract were 5.36, and 0.21 mg/ml and the standard reference IC_{50} value for Gallic acid was 5.26mg/ml. Total antioxidant capacity of the aqueous and ethanolic extracts was 0.71mg/ml equivalent of ascorbic acid and 0.8mg/ml equivalent of ascorbic acid at higher concentration (4mg/ml). The ethanolic and aqueous extract exhibited a similar pattern of reducing power activity, slightly different from that of Ascorbic acid. However, in all three cases, the reduction was linear. Total phenolic content of ethanolic and aqueous extract yielded 0.40±0.01mg GAE/g extract and 0.39±0.09mg GAE/g extract. Total flavonoids content of ethanolic and aqueous extract gave 2.6±0.15µg CEq/g extract and 1.53±0.03µg CEq/g extract. Total Saponins content of the ethanolic and aqueous extract was found to be 1.19±0.02mg and 0.92±0.03mg SEq/g extract. The total tannins content in ethanolic and aqueous extract was found to be 0.13±0.01 and 0.02±0.01mg CEq/g extract. The result obtained showed that aqueous and ethanolic extracts of *Phyllanthus amarus* leaves demonstrated a substantial amount of biochemically valuable phytochemicals and antioxidant potential.

Keywords: *Phyllanthus amarus*, Phytochemical Screening, *in vitro* antioxidant potential, DPPH scavenging assay, Reducing power activity, Total antioxidant capacity, IC_{50}.

INTRODUCTION

The concept *medicinal plant* describes an assortment of herbs employed in the practice of traditional medicine due to their curative potency. These herbs are indeed great reservoirs of compounds that can be used to improve drug design; hence, they are recommended for their therapeutic value (Rasool, 2012). Guaranteeing the safety, standard and efficacy of medicinal plants and herbal medicines just recently became a primary concern in advanced and emerging nations (Jamshidi-Kia *et al*., 2018). The genus *Phyllanthus* consist of nearly 800 species distributed throughout the equatorial regions of the world, with *Phyllanthus amarus* being among the most notable species due to its widely reported pharmacological activities (Joseph and Raj, 2011; Zubair *et al*., 2017). Most of the plants belonging to the genus *Phyllanthus*, yield various natural products with useful medicinal properties. These secondary metabolites viz; alkaloids, flavonoids, lignin, phenols, tannins and terpenes have been isolated from these plants (Zubair *et al*., 2017). *Phyllanthus amarus*, also known as "stone breaker", "carry me seed", is an upright annual herb which usually grows not more than 60cm tall with small leaves and yellow flowers. It is commonly found in forest areas, dry land, grasslands, leached or impoverished soil in several countries including, but not limited to China, India, Nigeria, Cuba and Philipines (Mamza *et al*., 2012). Over the years, there has been convincing evidence of the therapeutic use of *P. amarus* to treat several diseases such as hypertension, jaundice, diabetes, kidney problems etc (Joseph and Raj, 2011; Zubair *et al*., 2017).

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Other studies revealed preclinical pharmacological activity and therapeutic potential of phytochemicals isolated from *Phyllanthus amarus,* including antioxidant, hypouricemic, hypolipidemic, and hepatoprotective activities (Puspita and Alhebshi, 2019).

Table 1 Scientific Classification of Phyllanthus amarus (Taxonomy)

Kingdom	Plantae
Sub-kingdom	Tracheabionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	*Euphorbiaceae*
Genus	*Phyllanthus*
Species	*Amarus*

Radicals are molecular species with one or more unpaired electrons which makes them unstable and highly reactive. They are capable of accepting electrons from macromolecules, therefore wreaking havoc in the process (Lobo *et al*., 2010). Oxygen-derived radicals referred to as reactive oxygen species (ROS) are of most concern to biological systems (Valko *et al*., 2004). ROS are produced during normal and pathological cell metabolism. Living organisms are armed with antioxidant defence system, which aids to counterbalance the harmful effects of free radicals (de Beer *et al*., 2002). However, when reactive oxygen species are generated in excess, they are likely to subdue this defence thereby triggering pathophysiological processes such as diabetes, inflammation, cancer, liver damage and cardiovascular diseases (Liao and Yin, 2000). The quest

for finding new and safe naturally occurring antioxidants for application in biopharmaceutical and nutraceutical industry is compelling. Phytochemical screening is one of the methods used to explore antioxidant compounds in plants (Do *et al*., 2014). This research work compared the phytochemical constituents and *in vitro* antioxidant activity of aqueous and ethanolic extract of *Phyllanthus amarus* leaves.

Aim of study

This study aimed at the screening for phytochemical constituents, determination of total phenolic, total Saponin, total tannin and flavonoid contents, as well as the evaluation of *in vitro* antioxidant activity of aqueous and ethanolic extract of *Phyllanthus amarus leaves.*

MATERIAL AND METHODS

Plant collection

The leaves of *Phyllanthus amarus* was collected from the botanical garden of University of Benin, Nigeria and was identified by an expert in the Department of Plant Biology and Biotechnology, University of Benin, Benin City.

Plant Sample Preparation

The leaves of this plant were air-dried in the laboratory at the Department of Biochemistry, University of Benin, Benin City. The leaves were later pulverized to powdery form in Pharmacognosy laboratory at the Faculty of Pharmacy, University of Benin. 250g of the powdered leaves of *Phyllanthus amarus* was saturated in 1.5liters of absolute ethanol for 24hours with frequent stirring of the mixture. After which the mixture was filtered with fine cheesecloth and the residue was discarded. The filtrate was used to soak another 250g of powdered leaves of the same plant above, allowed to stand for another 24hours with continuous stirring; after that, the mixture was again filtered. The residue was discarded, and the filtrate concentrated with the aid of a concentrator. The concentrates were then weighed and used as experimental sample. The same procedure was carried out using distilled water for the aqueous extract. The above isolation of crude extract was done at the Department of Biological Sciences, Birla Institution of Technology and Science, BITS-Pilani, Hyderabad, India.

Phytochemical screening

Phytochemical screening to identify the presence of bioactive agents was performed by standard procedures (Sofowora, 1993; Evans, 2009). After the addition of the appropriate reagents to the solution, the test samples were observed for colour change or precipitate formation.

Total phenolic contents (TPC)

The total phenolic contents were spectrophotometrically determined using the Folin-Ciocalteu reagent. This reagent is a colourimetric redox method for quantifying phenolic compounds based on the method of Slinkard and Singleton (Slinkard and Singleton, 1977) and the earlier work of Singleton and Rossi (Singleton and Rossi, 1965). The extracts were reacted with Folin-Ciocalteu reagent and then neutralized with sodium carbonate solution (7%). After 90 minutes, the absorbance of the solution was measured at 765nm. The concentrations of phenolic compounds were calculated using the equation obtained from the standard of Gallic acid curve. All tests were carried out in triplicate, and the results are given in Gallic acid equivalents (GAE).

Total flavonoid contents (TFC)

Total flavonoid content was estimated by Aluminum chloride method (Olajire and Azeez, 2011) using catechin as a standard. 1ml of the test sample and 4 ml of water was added to a volumetric flask (10 ml volume). 0.3ml of 5% Sodium nitrite, 0.3ml of 10% Aluminum chloride was added after 5 minutes. The reaction solution was left to stand for 6 minutes at room temperature. 1ml of 1M Sodium hydroxide was then added to the mixture. Following that, the final volume was made up to 10ml with distilled water. Absorbance of the sample was measured against the blank at 510nm using a spectrophotometer. The total flavonoid concentration was calculated using the equation that was obtained from the standard catechin curve. All the experiment was repeated three times and values were expressed as mean ± standard error of the mean, in terms of Catechin equivalent (CEq).

Total Tannin Content (TTC)

The tannin contents were determined by the method of Broadhurst and Jones (1978) with slight modification, using catechin as a reference compound. A 400µL volume of the extract was added to a 3ml solution of vanillin (4%in methanol) and 1.5ml of concentrated hydrochloric acid. After incubating for 15min, the absorbance was read at 500nm. The total tannin was expressed as mg Catechin equivalent (CEq)/g of extract. Catechin standard curve was prepared by using various concentrations (0.2-0.7mg/ml). The extract was prepared in triplicate for precision sake. Total tannin content was estimated from the equation generated from the standard curve.

Total Saponin contents (TSC)

Using the Vanillin –Sulphuric Acid Method (Tan *et al.*, 2014 and Nguyen *et al.*, 2017). 0.25ml of the sample was added to 0.25ml of 8 %(w/v) vanillin in ethanol and 2.5ml of 72 %(V/V) sulphuric acid and allowed to stand for 15minutes at 60°C in shaking water bath with standard and blank made up with the solvent used for extracting the plant samples. After cooling in water at ambient temperature for 5 minutes, absorbance was read at 560nm with a spectrophotometer. The concentration of Saponin was calculated using the equation from the calibrated curve. Data obtained for each extract of the quantitative test was compared, and P values less than 0.05 ($P<0.05$) were considered to be significantly different.

Antioxidant studies

In the present study, three widely applied antioxidant assay methods such as DPPH radical scavenging activity, reducing power assay and phosphomolybdenum method were used to determine the antioxidant potential of aqueous and ethanolic extracts of *Phyllanthus amarus* leaves.

Determination of free radical scavenging activity by DPPH method

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is one of the most widely employed methods because, in general terms, it is

easy, efficient and cost-friendly. The method was initially developed by Blois (Blois, 1958), with the modifications introduced by Brand-Williams, Cuvelier, and Berset (Brand-Williams, 1995), it is widely used as a reference point (Bondet *et al*., 1997; Chen *et al*., 2013). The results are typically expressed as Efficient Concentration (EC_{50}) also called the IC_{50} value, which is the amount of sample necessary to reduce the initial DPPH concentration by 50%. The parameter IC_{50} was introduced by Brand-Williams, 1995; Molyneux, 2004; Kedare and Singh, 2011. It is beneficial for comparing results because it is independent of the sample concentration. Briefly, 100µl of varying concentrations of the extract in ethanol was added to 10ml of a methanol solution of DPPH (0.57mM). The resulting solution was shaken vigorously and then incubated in the dark at room temperature for 30 min. Using a spectrophotometer, the absorbance of the mixture was read at 517nm. A mixture of 100µl methanol and 10ml methanol solution of DPPH was used as the blank. The DPPH radical scavenging potential was expressed as percentage inhibition using the following equation:

Gallic acid was used as a reference. The concentration of the extract providing 50% inhibition (IC_{50}) was determined from the graph of % inhibition plotted against extract concentration (Viturro *et al*., 1999).

Total antioxidant capacity (TAC)

The total antioxidant capacity of ethanol and aqueous extracts were evaluated by phosphomolybdenum (phosphate/Mo) method based on the work done by Prieto (Prieto *et al*., 1999). The assay is based on the reduction of Mo (VI) to Mo (V) by the extract, followed by the formation of green phosphomolybdate (V) complex. Briefly, 0.1ml extract was mixed with 1 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The test-tubes containing the reaction mixture were incubated at 95ºC for 90 min. Then the absorbance of the mixture was measured at 695nm using a spectrophotometer against blank after cooling at room temperature. Ethanol (0.1ml) in the place of extract was used as the blank. The standard curve was prepared by using ascorbic acid of various concentrations (0.2-0.6mg/ml) in ethanol. The result obtained is expressed as the number of equivalents with reference to ascorbic acid.

Reducing power Activity (RPA)

The reducing power of both extracts was determined according to the method previously described by Oyaizu (Oyaizu, 1986). According to this method, the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) form of iron is determined by measuring the absorbance of the final Perl's Prussian blue complex. Briefly, different concentrations of extracts (0.1-1g/ml) were mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 1 %). The resulting mixture was maintained at 50°C for 20 min. An aliquot (2.5ml) of trifluoroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000rpm for 10 min. The supernatant (2.5ml) was mixed with distilled water (2.5ml), and $FeCl_3$ (0.5ml, 0.1%) and the absorbance was measured at 700nm. Ascorbic acid was used as reference.

RESULTS

The qualitative phytochemical screening is presented in Table 2 below. The result revealed the presence of phenols, flavonoids, tannin, saponin and quinones in both extracts. However, Terpenoids and steroids were detected only in the ethanol extract, while glycosides were found present in the aqueous extract alone.

Table 2. Phytochemical Screening

Phytochemicals	Aqueous extract	Ethanol extract
Tannin	+	+
Saponin	+	+
Flavonoids	+	+
Glycosides	+	-
Quinones	+	+
Phenols	+	+
Terpenoids	-	+
Steroids	-	+

Key; + for present and – for absent

Total Phenolic Content

The total phenolic content in aqueous and ethanolic extract of *P. amarus* was determined from the Gallic acid calibration using the equation of the curve: , and the results obtained are shown in Table 3, expressed as mean±SEM. The content of total phenols in aqueous and ethanolic extracts is expressed as Gallic acid equivalents (GAE) per gram of dry extract. Aqueous extract yielded 0.39±0.03mg GAE/g extract, and the ethanolic extract gave 0.40±0.01mg GAE/g extract. From the result, both extracts had a nearly similar yield of total phenolic content at a *P*-value of 0.263.

Total flavonoid Content

The total flavonoids contents in both aqueous and ethanolic extracts were estimated from the Catechin calibration curve using the equation of the curve. In both extracts, total flavonoids yield was the lowest as seen in table 3; however, the total flavonoids yield of the ethanol extract was significantly higher ($P = .002$) than that of aqueous.

Total Saponin Content

Total Saponin contents were expressed as mg Saponin equivalent/ g of dry extract with reference to the standard curve. The results from the standard curve showed that both extracts had different yields of saponin ($P = .001$), as shown in Table 3.

Total Tannin Content

Total Tannin content is presented as mg catechin equivalent/g of dry extract with reference to catechin curve. It was observed that the tannins content of the ethanolic extract was significantly higher ($P = .001$) than that of the aqueous extract. This is shown in table 3.

Table 3 Showing Total Phenolic, flavonoid, Saponin and Tannin Contents in P.amarus leaves

Extracts	TPC (mg GAE/g extract)	TFC (µg CEq/g extract)	TSC (mg SEq/g extract)	TTC (mg CEq/g extract)
Aqueous	0.39 ± 0.01^a	1.53 ± 0.03^a	0.92 ± 0.03^a	0.02 ± 0.001^a
Ethanol	0.40 ± 0.002^a	2.60 ± 0.15^b	1.19 ± 0.02^b	0.13 ± 0.01^b

The results above are presented as Mean±SEM, n=3. Values with different superscript are significantly different.

Key: TPC-Total Phenolic content, TFC-Total flavonoid content, TSC-Total Saponin content and TTC- Total Tannin content.

DPPH's radical scavenging activity

The DPPH's radical scavenging activities of the plant extracts are presented in figure 1. The ethanol extract of *Phyllanthus amarus* had the better ability to inhibit DPPH radical at all concentrations than aqueous extract. Comparing the IC_{50} of the extracts with the Gallic acid reference, ethanol had similar value with Gallic acid, while aqueous extract had value different from both ethanol extract and Gallic acid. This ability, however, increased with increasing concentrations. The IC_{50} value of ethanol extract was found to be 5.36mg/ml while that of Aqueous was 0.21mg/ml. The reference IC_{50} was 5.26mg/ml. The antiradical power (reciprocal of IC_{50}) of both Gallic acid and ethanol extract were notably the same (0.19 and 0.189 $(mg/ml)^{-1}$ while that of aqueous was 4.76$(mg/ml)^{-1}$.

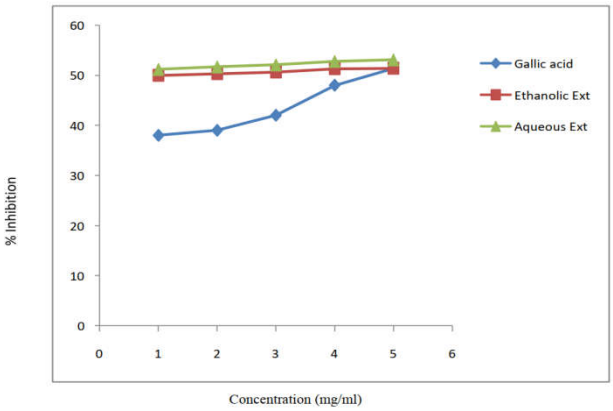

Fig 1: DPPH's radical scavenging activities of extracts of *Phyllanthusamarus* leaves

Table 4. IC50 of extracts of *Phyllanthus amarus*

Extracts	IC_{50} Values	ARP Values
Ethanol	5.36mg/ml	0.189$(mg/ml)^{-1}$
Aqueous	0.21mg/ml	4.76$(mg/ml)^{-1}$
Gallic Acid	5.26mg/ml	0.19$(mg/ml)^{-1}$

Key: APR- Antiradical Power

Reducing Power Activity

The reducing power activity of *Phyllanthus amarus* is shown in figure 2. Both extracts had a similar fashion of reducing ferric iron to ferrous iron (Fe^{3+}-Fe^{2+}). However, the ethanolic extract had a better reduction when compared with aqueous extract. Both extracts eschewed the pattern depicted by Ascorbic acid. The ethanolic extract had a reduced value at increased concentration compared to that of Ascorbic acid at the same concentration.

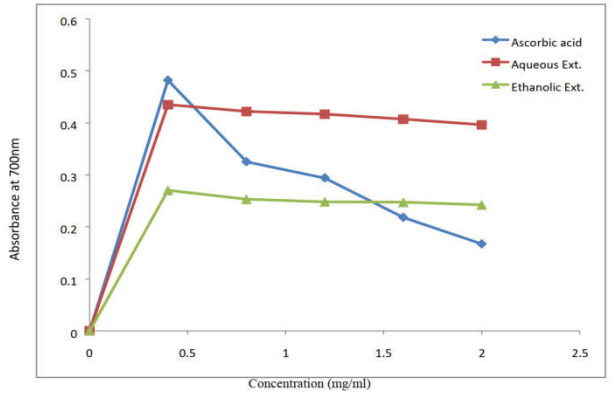

Fig.2. Reducing Power Activity of Phyllanthus amarus extracts

DPPH's scavenging power of ethanolic extract and Gallic acid at low concentrations

At low concentrations (0.2-0.5mg/ml), ethanolic extract and Gallic acid exhibited similar scavenging power (IC_{50}=0.92 for ethanol and 1.08 for Gallic acid) and ARP values for ethanol, and Gallic acid was found to be 1.09$(mg/ml)^{-1}$ and 0.93 $(mg/ml)^{-1}$ respectively. The curve is depicted in fig.3

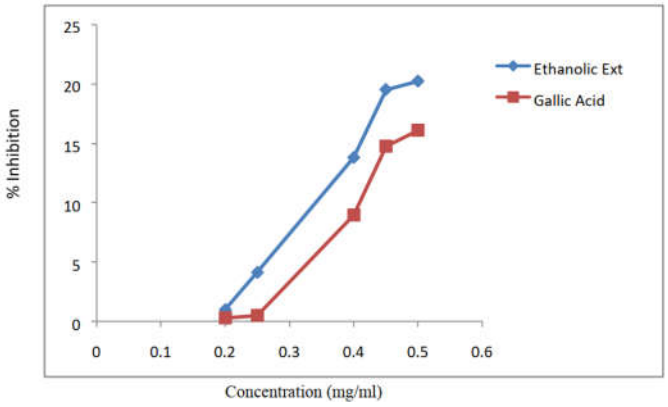

Fig. 3. The relationship between ethanolic extract and Gallic acid in DPPH scavenging activity at low concentrations

Total Antioxidant Capacity (TAC)

In the phosphomolybdenum assay, ethanolic and aqueous extracts exhibited similar degrees of activity, as shown in Figure 4. Both extracts showed increasing antioxidant activity with increasing concentration. It was observed, however, that the ethanolic extract had total antioxidant capacity equivalent to 0.8 mg/g ascorbic acid at higher concentration (4 mg/ml). In contrast, the aqueous extract had 0.71mg/g ascorbic acid equivalent at the same concentration.

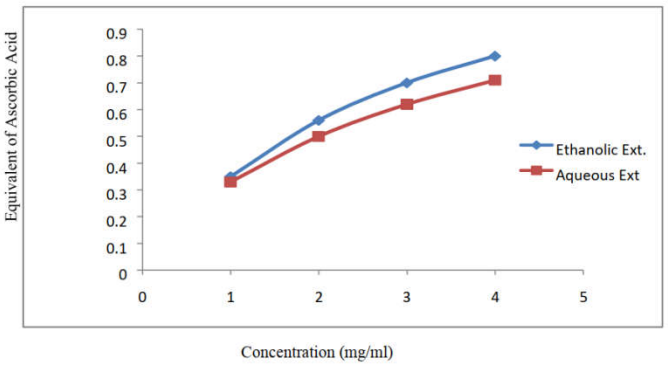

Fig. 4. Total antioxidant capacity of Ethanolic and Aqueous extracts of Phyllanthus amarus

DISCUSSION

Phytochemicals are naturally occurring, non-nutritive, biochemically active compounds with therapeutic usage. Their presence contributes to the medicinal properties of herbs (Jothi *et al*., 2017). Phenolic compounds such as phenolic acids, flavonoids, tannins, etc., are commonly known for their reduction potential. They also play a crucial role in mopping up and offsetting free radicals by quenching singlet and triplet oxygen, breaking down peroxides and chelation of metals (Ojezele *et al*., 2016). Hence, they are deemed as good antioxidants (Maswada, 2013). Saponins and steroids are noted for their cholesterol-lowering and anti-inflammatory activities (Ojezele *et al*., 2016). The quantitative evaluation showed that

ethanol is preferred for extracting active principles be due to its amphipathic nature which allows for the dissolution of both polar and non-polar constituents in plants (Ojezele *et al*., 2013). This was also reflected in its antioxidant activity when compared to the aqueous extract. Ethanol was also found more comfortable to permeate the cell membrane and extract the subcellular ingredients from the plant materials (Tiwari *et al*., 2011). A more detailed explanation for the lower yield in total flavonoid and tannin content of aqueous extract can be ascribed to the activity of the enzyme polyphenol oxidase, which degrades polyphenols (tannins and flavonoids) in the presence of water. However, the enzyme is inhibited in ethanol (Tiwari *et al*., 2011).

Oxidative stress take part in the generation of potentially harmful radicals which is a key factor in the development and progression of some chronic and degenerative diseases including cardiometabolic disorders, neurodegenerative diseases and cancer (Chen *et al*., 2013). Natural antioxidants, mostly found in food and medicinal plants such as fruits, vegetables, beverages, herbs and spices, are a fundamental part of human diet especially in preventing oxidative stress (Singh *et al*., 2016). As a fast and easy measure of antioxidant activity *in vitro*, the DPPH radical scavenging assay is based on reduction of the unstable diamagnetic radical DPPH to the stable diphenylpicrylhydrazine by protonation in the presence of a hydrogen donor (Adeogun *et al*., 2019). This method has been widely applied in evaluating the free radical scavenging potential of various extracts. Our study on the radical scavenging activity of *P. amarus* using DPPH test and reducing power activity revealed that both extracts possess strong antioxidants which may be attributed to its phytochemical constituents.

However, the ethanol extract (IC_{50} = 5.36mg/ml) was found to be more potent and relatively comparable with that of the Gallic acid reference (IC_{50} = 5.26mg/ml), as opposed to the aqueous extract (IC_{50} = 0.21mg/ml). At low concentrations, the ethanol extract was yet found to exhibit a similar design as the Gallic acid reference with closely marked values. The presence of reducing agents in *P. amarus* brought about the reduction of Iron (III) to Iron (II) via electron donation, as observed in the gradual colour change from green to blue (Oyaizu, 1986). Our values showed that even though the ethanol extract gave a slightly better reduction when compared with aqueous extract, yet both extracts displayed similar patterns which were different from that of the ascorbic acid reference. Total antioxidant capacity, as estimated by the phosphomolybdenum method under acidic condition, usually evaluates both water and lipid-soluble antioxidants, and express them in terms of ascorbic acid or α-tocopherol equivalent (Prieto *et al*., 1999). Our findings revealed that both extracts of *P. amarus* gave a progressive pattern in total antioxidant capacity with an increase in concentration; however, the ethanol extract was slightly higher compared to the aqueous extract. To a larger extent, this could be due to the difference in total flavonoid contents of the extracts. In reality, it may also be accounted for by other antioxidant compounds such as carotenoids, vitamins and others (Maswada, 2013). Tannins and saponins have been found to possess good antioxidant activity (Pande *et al*., 2014). The results obtained in the study aligns with other previous works, suggesting that *P. amarus* possess powerful antioxidant activity capable of preventing the deleterious consequences of oxidative stress (Londhe *et al*., 2008; Al-Jasabi *et al*., 2016; Singh *et al*., 2016).

CONCLUSION

On the basis of the result obtained in this study, the following conclusions can be reached. First, both the aqueous and ethanol extracts of *Phyllanthus amarus* contain bioactive compounds with possible therapeutic value. Furthermore, the ethanol extract has a significantly higher concentration of phytoconstituents as seen in the total flavonoid, saponin and tannin contents, and was also more effective in scavenging and reducing free radicals *in vitro* compared to the aqueous extract. Finally, these analyses carried out implies that *Phyllanthus amarus* is a good source of plant-based antioxidants capable of suppressing the activity of oxidative stress factors as well as its associated disease conditions. This antioxidant activity is accounted for by the rich presence of essential phytochemicals in the plant and contributes vastly to its medicinal uses.

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