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In vitro antioxidant properties and GC-MS analysis of solvent extracts of *Persea americana* Leaf

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ABSTRACT

Persea americana has been widely utilized in the traditional parlance for the management of diseases. The present study investigates the in vitro antioxidant mechanism(s) as well as the active principles responsible for the acclaimed therapeutic potentials of the plant. Powdered leaves of Persea americana leaves was extracted in ethanol, methanol and water and assessed for their total phenolic and flavonoids contents following established protocols. Free radical scavenging activity against 2,2-diphenyl-1- picryl hydrazyl (DPPH) and nitric oxide (NO) radicals as well as the ferric reducing antioxidant potential (FRAP) were also determined. Moreover, structure and relative abundance of phytochemical constituents of the methanolic extract of Persea americana leaves was determined on gas-chromatography-mass spectrometer (GC-MS). Results indicate that methanolic extract exhibited significantly higher DPPH and NO radical scavenging and ferric reducing power than aqueous and ethanolic extract. However, aqueous extract of the plant has the highest total phenolic and flavonoids content of the three extracts investigated. GC-MS chromatogram of the methanolic extract of Persea americana leaves reveals the presence of eighteen phytochemicals. Phytochemicals content of P. americana leaves depends on the extraction solvent, while its antioxidant activity is not only a function of amount of phenols and flavonoids but of the individual antioxidant capacity of the phytochemicals.

1. Introduction

In recent times, analysis of heterogenous mixtures such as cocktail of phytochemicals found in crude extract of plants has been achieved using gas chromatography coupled with mass-spectrometer (Kadhim *et al*., 2016; Mohammed *et al*., 2016; Pierangeli *et al*., 2009). This analytical technique involves the introduction of samples into gas chromatography column with the aid of heated injector. Components of the samples are then separated on a column based on polarity and molecular mass. The separated components then sequentially enter the mass spectrometer through a heated chamber generating the chromatogram and the mass spectra of the analytes. Gas chromatography as an analytical tool can provide useful qualitative and quantitative information on materials of interest even at trace level. Hence, its application in pollution studies, forensic analysis and purification of compounds (Vyas, 1999; Kaushik et al., 2002; Chaman and Verma, 2006; De-Fatima et al., 2006; Milne, 1993; Andrew, 2007). Research efforts have been tailored towards unravelling the phytochemical constituents of plants in recent times. This is beneficial in the discovery of new therapeutic agents that can be exploited as well as providing scientific validation on the folkloric use of these plants for medicinal purposes. Moreover, information related to structure and mechanisms of the active principles will also be provided. Plants have assumed a central position in the provision of bioactive compounds that can enhance human health. Phytochemicals as therapeutics are effective, efficient, relatively non-toxic and biofriendly (International Organization for Standardization, 2002; ISO/IEC, 2005; Bliesner, 2006; Amirav *et al*., 2008). Hence,

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there is an evolving branch of science involved in scientific validation of herbal products used as drugs with particular emphasis on their standardization. This is pertinent since several phytochemicals, which co-exists in plants parts, have complementary and overlapping pharmacologic mechanisms. This explains the surge in research outputs involving GC-MS analysis of medicinal plants for the identification of non-polar components and volatile essential oil, fatty acids, lipids and alkaloids (Hameed *et al*., 2016; Hussein *et al*., 2016; Sosa *et al*., 2016; Altameme *et al*., 2015).

Persea americana Mill. (Lauraceae) thrives in the sub-tropical region and has many medicinal benefits that have been used in folkloric practice as traditional medicament (Oliveira et al., 2013). Topical application of its oils has been used for wound healing and in the treatment of skin infections (Sosa et al., 2016; Altameme et al., 2015). Leaf extract of *P. americana* has analgesic, anti-inflammatory, anticonvulsant, antiviral, antihyperglycemic, antihyperlipidemic and antiulcerogenic properties [Oliveira *et al*., 2013; Narel *et al*., 2020; Lee *et al*., 2012).

Oxidative stress has been identified as the major culprit in the pathogenesis of several degenerative diseases, including cancer, diabetes, atherosclerosis among others (Wu et al., 2019). It occurs as a result of distortion (in favor of free radicals) in the balance between free radicals and antioxidants level in the physiological system (Monika and Geetha, 2016; Tabeshpour *et al*., 2017; Edeas *et al*., 2010; Majima *et al*., 2011). Whenever the antioxidant system capacity decreases, there is always a concomitant rise in the level of free radicals which can assume a deleterious dimension if not checked. Consequently, critical macromolecules such as proteins, DNA, carbohydrates and lipid are oxidized triggering serious health problems (Mao et al., 2011; Cesaratto *et al*., 2012; Wang *et al*., 2013a; Wang *et al*., 2013b; Sinha *et al*., 2013; Lu, 2008). Whenever, the endogenous antioxidant capacity is exhausted, there is a need for sourcing from exogenous antioxidant sources one of which is medicinal plants. This is due to the presence of antioxidant phytochemicals which are able to mitigate the deleterious effect of free radical by scavenging them. Considering the continued use of *P. americana* as therapeutic agent in the management of diseases, there is a dire need to unravel the phytochemicals present in its leaf extract and their possible mechanism of action *in vitro*. Hence, this study.

Materials and methods

Preparation of plant materials

Fresh leaves of *P. americana* were harvested from a farm in Ado Ekiti. It was identified botanically and authenticated at The Department of Plant Science, Ekiti State University, Ado Ekiti, Nigeria. Voucher specimen with herbarium number UHAE2020071 was deposited at the University herbarium. The leaves were air dried and pulverized using warring blender. The powdered samples were then extracted with methanol (80%) for 72 hours. The extraction solvent was allowed to evaporate completely, which was confirmed by constant weight of the extract. Ethical approval was obtained from the Office of Research and Development (ORD), Ekiti State University, Ado Ekiti.

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH.) radical was purchased from Fluka Chemicals, sodium nitroprusside (SNP), sodium nitrite, sulfanilamide, phosphate buffer saline (PBS), phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), ferric chloride ($FeCl^{3+}$), naphthylethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA), sodium carbonate (Na_2CO_3), aluminium trichloride, perchloric acid ($HClO_4$), ferrous sulphate, Folin-ciocalteu reagent (FCR) and trichloroacetic acid (TCA) were all purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of high analytical grade, obtained from standard commercial suppliers.

Determination of total phenolic content

The method of Singleton *et al*. [32] was followed in the determination of total phenolic contents of the leaf extracts of *P. Americana*. Appropriate volume of extract containing 200, 400 and 800 mg/ml of the extract was mixed with equal volume of distilled water. Thereafter, 2.5 ml of Folin - Ciocalteau's reagent (FCR) and 2 ml of 7.5% sodium carbonate were added to the reaction mixture. The resulting mixture was incubated at 45°C for 40 min. The mixture was allowed to cool and its absorbance read at 765 nm. Amount of phenol in the extracts was expressed as gallic acid equivalent.

Determination of total flavonoid content

Total flavonoids content of the extracts was determined as described by Meda *et al*. (2005) with slight modifications. One hundred microliters (100 µl) each containing 200, 400 and 800 mg/ml of the extracts was mixed separately with 100 µl of 20% aluminium trichloride (w/v). The mixture was acidified with 100 µl of acetic acid and made up to 5 ml with distilled water and left to stand for 40 min at 25 °C. Absorbance at the resulting mixture was then read at 415 nm. Blank samples contained all assay components except the extract while standard was prepared using quercetin to replace extract in the assay mixture. Amount of flavonoids in the plant extract was expressed as quercetin equivalents (QE).

In vitro nitric oxide radical (NO·) scavenging assay

Amount of NO· produced was determined following the method of Marcocci *et al*. (1994). The reaction mixture containing 5 mM SNP in phosphate buffered saline (pH 7.3), with or without the plant extracts at 200, 400 and 800 mg/ml was incubated for 180 min at 25°C. The NO· radical released reacts with oxygen to generate nitrite ion (NO.) which was measured at 30 min intervals by mixing 1.0 ml of incubation mixture with equivalent volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). Absorbance of the chromophore formed as a result of diazotization of nitrite ions with sulfanilamide and subsequent coupling with napthyl ethylenediamine dihydrochloride was read at 546 nm. The amount of nitrite produced in the presence or

absence of the plant extracts was estimated from a standard curve of sodium nitrite.

Free radical scavenging ability

DPPH radical scavenging activity of the extracts was determined following the method of Awah *et al*. (2010). Fifty microliters (50 µl) of each extract was mixed separately with 1.0 ml of 0.4 mM DPPH and made to 5ml with methanol. The reaction mixture was vortexed for 1 min and incubated in the dark for 20 min at 25 °C. Absorbance of the resulting solution was then read at 517 nm against blank containing DPPH and methanol. Percentage DPPH radical scavenged was determined with reference to the blank.

Reducing property

The ferric reducing potential of the extracts was determined according to the method of Pulido *et al.* (2000). Varying volumes of extracts containing 200, 400 and 800 mg/ml was mixed with 250 µl of 0.2 M sodium phosphate buffer pH 6.6 and 250 µl of 1% potassium ferrocyanide (w/v). The reaction mixture was incubated at 50 °C for 20 min. Two hundred and fifty (250 µl) of 10% trichloroacetic acid (TCA) was added to the mixture and centrifuged at 650 rpm for 10 min. One milliliter (1 ml) of the supernatant was mixed with 1 ml of the supernatant was mixed with equal volume of water and 100 µl of 0.1% (w/v) ferric chloride. Absorbance of the resulting mixture was measured at 700 nm against the blank containing all assay components except the extracts.

GC-MS Analysis

Chromatographic separation was achieved with the Vocol column (J & W, Agilent Technologies, CA, USA), which has dimensions of 1.8 µm × 60 m × 0.32 mm film thickness. Nitrogen was used as the carrier gas at a constant flow rate of 0.8 mL min^{-1}. The column temperature was initially held at 35°C for 3 min, then, the temperature was increased to 40°C with a heating rate of 3°C min^{-1}, and the temperature was held for 1 min. The second ramp was programmed from 40°C to 210°C with a heating rate of 5°C min^{-1}. A post-run of 16 min at 210°C was established. The mass spectrometer was used in electron ionization mode; all spectra were acquired using a mass range of m/z 30 – 800. The transfer line temperature was set at 210°C, the ion source temperature at 200°C, detector voltage 0.8 kV. The identification of VOCs was achieved by using the National Institute of Standards and Technology (NIST) reference library diluted samples (1: 10 chloroform, v/v) of 0.2 µl of the mixtures were always injected automatically in the splitless mode. Mass spectra were obtained by electron ionization at 70 eV, using a spectral range of m/z 30–1000. Most of the compounds were identified using the analytical method: mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library; Wiley 9 and NIST 08). and the comparison of the retention times (tR) and mass spectra of authentic standards. In addition, an in-house dedicated mass spectral library was built by using the mass spectra of authentic compounds to confirm the identities of detected VOCs. The following standards were used: acetoin, ethyl acetate, cis-grandisol [(1R,2S)-cis-2-isopropenyl-1-methylcyclobutaneethanol], isoamyl alcohol, isobutanol, 2-methyl-1-butanol, phenethyl acetate, and phenethyl alcohol. Each individual compound was quantified using standard curves calculated from three serial dilutions of analytical standards. The internal standard (tridecane) was used to improve the precision of quantitative analysis with the calibration curve by plotting the signal from analyte with the signal from the internal standard as a function of the analyte concentration of the standards. All the measurements were replicated three times for each assay and the results are presented as mean.

All the samples and replicates were continuously injected as one batch in random order to discriminate technical from biological variations. Additionally, the prepared pooled samples were used as quality controls (QCs), which were injected at regular intervals throughout the analytical run to provide a set of data from which the repeatability can be assessed.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of an experiment performed in triplicate and were analyzed by appropriate analysis of variance (ANOVA)followed by Duncan's multiple range test. Differences were considered significant at p = 0.05.

Results

Total phenolics and flavonoids content was higher in the aqueous extract than both methanolic and ethanolic extract of the leaf of *P. americana.* However, DPPH radical scavenging activity of the extract was in the order: methanolic extract > ethanolic extract >aqueous extract. A similar trend was also noticed when the extracts were applied as NO radical scavengers. Their effects were dose-dependent. *P. americana* leaf extract showed a dose dependent ferric reducing antioxidant potential (FRAP) with methanolic extract producing the highest reducing potential followed by aqueous and ethanolic extract of the plant's leaf. GC-MS analysis showed the presence of salicylic acid, bicyclo [2.2.2] octane-1-carboxylic acid, 4-methyl-, orcinol-1,3-cyclopentanediol, cis- hexadecane, 9-hexadecenoic acid, methyl ester, (Z), hexadecanoic acid, methyl ester, dodecanoic acid, octadecanoic acid, oleic acid among others.

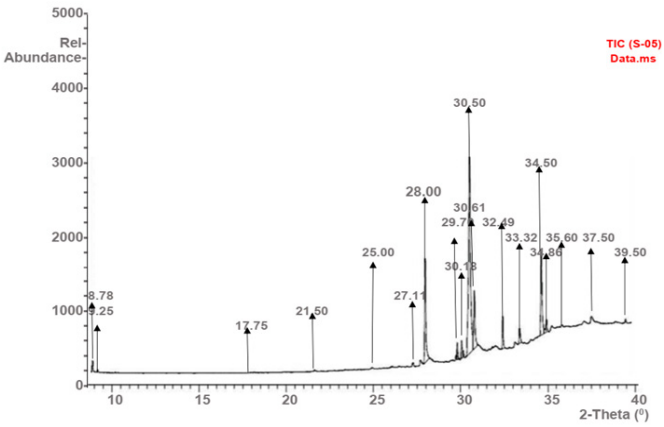
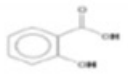
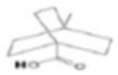
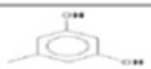
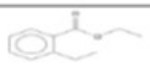
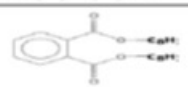
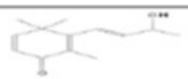
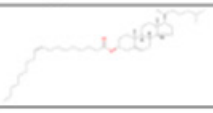


Figure 1.0: GC-MS Chromatogram of methanolic extract of *P. americana* leaf extract

Table 1.0: Bioactive constituents of methanolic extract of P. americana leaf

Peak #	RT	Compound Detected	Mol. Formula	MW	Peak Area %	Comp %wt	m/z	Structures
1	8.78	Salicylic acid	$C_7H_6O_3$	138	2.63	2.77	92, 120, 138	
2	9.25	Bicyclo[2.2.2]octane-1-carboxylic acid, 4-methyl-	$C_{10}H_{16}O_2$	168	1.38	1.42	81, 123, 168	
3	17.75	Orcinol	$C_7H_8O_2$	124	1.41	1.04	67, 95, 124	
4	21.50	1,3-Cyclopentanediol, cis-	$C_5H_{10}O_2$	102	1.43	0.54	55, 84, 102	
5	25.00	Hexadecane	$C_{16}H_{34}$	226	1.46	0.17	43, 71, 226	
6	27.11	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	268	1.64	3.96	55, 83, 268	
7	28.00	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	11.75	9.07	74, 87, 270	
8	29.70	9-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	2.67	3.28	55, 69, 296	
9	30.18	Benzoic acid, 2-ethyl, ethyl ester	$C_{11}H_{14}O_2$	178	2.73	2.24	43, 71, 178	
10	30.50	9,12-Octadecadienoic acid (Z,Z)-, methyl esteric acid	$C_{19}H_{34}O_2$	294	20.75	12.66	67, 81, 294	
11	30.61	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	7.90	8.60	55, 69, 296	
12	32.49	Dodecanoic acid	$C_{12}H_{24}O_2$	200	5.23	6.13	60, 73, 200	
13	33.32	Octadecanoic acid	$C_{18}H_{36}O_2$	284	3.99	4.38	43, 73, 284	
14	34.50	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	17.66	18.26	97, 115, 390	
15	34.86	Cyclopentasiloxane, decamethyl-	$C_{10}H_{30}O_5Si_5$	370	4.83	5.92	63, 267, 270	
16	35.60	Oleic acid	$C_{18}H_{34}O_2$	282	4.09	4.84	55, 69, 282	
17	37.50	2,3-Dehydro-4-oxo-β-ionol	$C_{18}H_{36}O$	206	4.20	5.25	43, 163, 206	
18	39.50	Cholest-5-en-3-ol (3β)-, 9-octadecenoate, (Z)-	$C_{45}H_{78}O_2$	651	4.18	5.12	81, 147, 651	

Discussion

Oxidative stress has become a major subject of intensive investigation. Perhaps, it is a deliberate effort at achieving the sustainable development goal (SDG) on health and well-being. Attaining this goal will require identification and exploitation of potential phytochemical agents that can mitigate the menace of free radicals. Reports have suggested that free radicals are the major culprits in the onset, progression and complication of all known diseases (Ogunmoyole *et al*., 2021). In the present study, all extracts of *P. americana* leaf used demonstrated potent and dose-dependent free radical scavenging activity against DPPH *in vitro*. (Figure 4). This observation can be linked to the presence of phenols and flavonoids in the extracts (Figures 2 and 3). These phytochemicals have been reported as potent radical scavengers *in vitro* (Kumar *et al*., 2010). DPPH radical scavenging have been suggested to involve the ability of antioxidants to donate protons (via reduction) to unstable DPPH radicals thereby attaining conformational stability. The reductive ability is physically noticeable by a change in the purple color of DPPH radicals to golden yellow depending on the strength of the antioxidant (Ogunmoyole *et al*., 2009). Since all extracts of *P. americana* scavenged DPPH radicals, it suggests that flavonoids and phenols in the extracts donated their protons to unstable DPPH radical stabilizing the molecule via reduction. Worthy of note however, is the fact that the total phenolic and

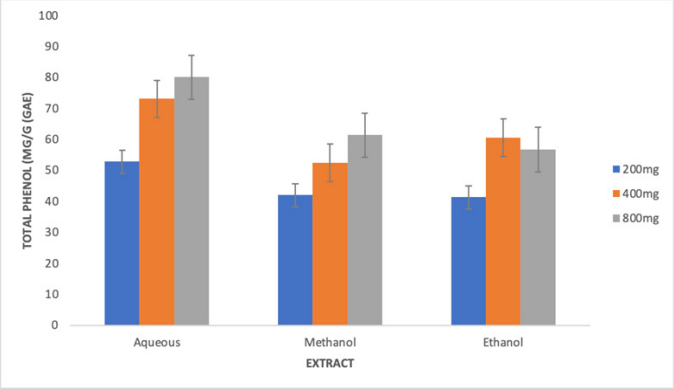


Figure 2: Total phenolic content of *P. americana* leaf extracts. Data represent mean±SD of an experiment performed in triplicate.

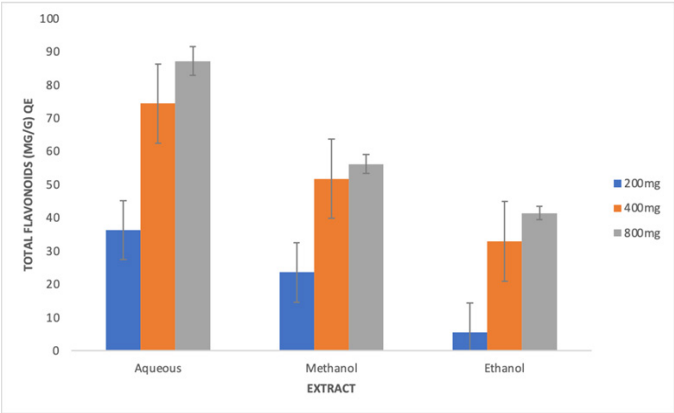


Figure 3: Total flavonoids content of *P. americana* leaf extracts. Data represent mean±SD of an experiment performed in triplicate.

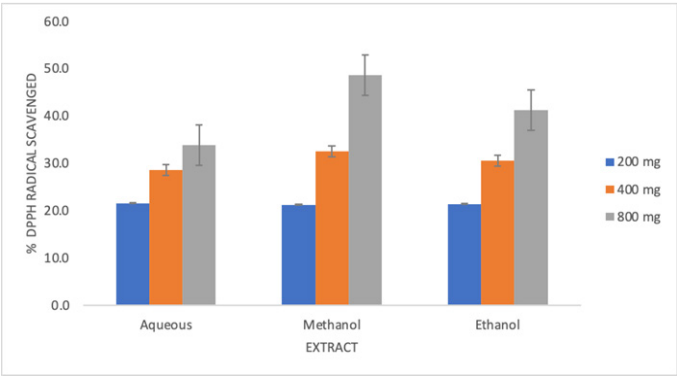


Figure 4: DPPH radical scavenging ability of *P. americana* leaf extracts. Data represent mean±SD of an experiment performed in triplicate.

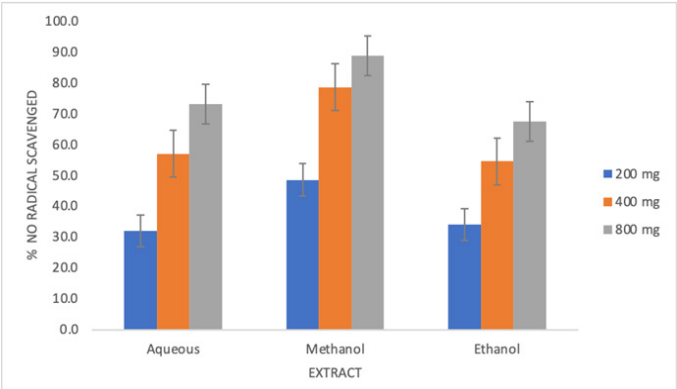


Figure 5: NO radical scavenging ability of *P. americana* leaf extracts. Data represent mean±SD of an experiment performed in triplicate.

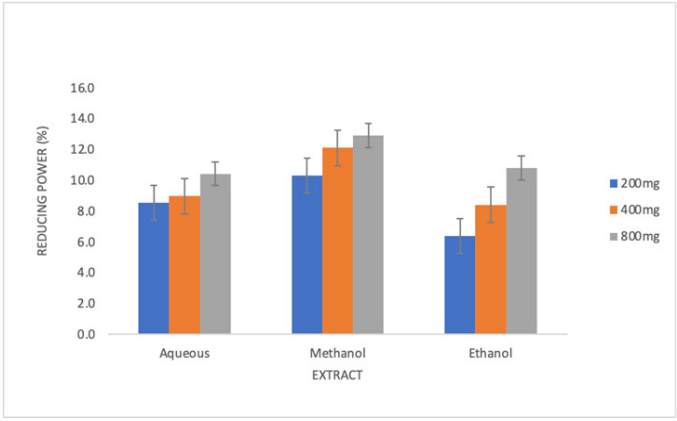


Figure 6: Ferric reducing antioxidant potential of *P. americana* leaf extracts. Data represent mean ± SD of an experiment performed in triplicate.

flavonoid content was highest in the aqueous extract, while methanolic extract showed the highest DPPH scavenging ability at the same dose. Perhaps, this suggests that the type of flavonoids and phenols extracted in methanol were stronger reductant, although relatively less in quantity, than aqueous and ethanol extract. Hence, the strength of flavonoid and phenolics compounds in the crude extract of plants is not necessarily a function of their quantity but their individual antioxidative capacity.

Reactive nitrogen species (RNS) generated from the reaction of nitric oxide with reactive oxygen species (ROS) has been used to determine the antioxidant capacity of agents (Tsai *et al*., 2006) Nitrite radical is highly reactive on interaction with critical macromolecules especially proteins (Amaeze *et al*., 2011). At physiological concentration, NO is critical to neurotransmission and vasodilation (Bhaskar and Balakrishnan, 2009; Andrew, 2007). However, whenever its concentration rises above tolerable threshold, nitric oxide can trigger inflammation, multiple sclerosis, ulcerative colitis and arthritis. Toxicity of NO is potentiated by its interaction with superoxide radicals forming the highly deleterious peroxynitrite radical (ONOO−) (Amaeze et al., 2011). Quenching of NO radical *in vitro* is a routine method for measuring the antioxidant activity of agents (Lakhanpal and Rai, 2009). Mechanistically, it involves spontaneous generation of nitric oxide from aqueous solution of sodium nitroprusside at physiological pH. The NO thus produced then reacts with oxygen to generate nitrite ions, usually measured by Griess reagent. Antioxidants, scavenge nitric oxide radicals by competing with oxygen, thereby inhibiting the production of nitrite radicals (Patel et al., 2010). In the present study, all extracts of *P. americana* leaf demonstrated potent, dose- dependent NO scavenging activity (Figure 5). This can be attributed to the presence of phenols and flavonoids in the extracts. Similar to DPPH radical scavenging, methanolic extract showed the highest NO radical scavenging activity among the three extracts employed. This is perhaps due to the strength of the extracted phenols and flavonoids compared to other extracts. Nitric oxide has been shown to be directly scavenged by flavonoids (Ebrahimzadeh *et al*., 2010).

The capacity of agents to reduce transition metals such as iron has been suggested as an antioxidant mechanism. This involves the transfer of proton from antioxidants to free radicals. All extracts of *P. americana* leaves demonstrated

marked ferric reducing power in a dose dependent manner (Figure 6). This observation is due to the presence of phytochemicals including phenols and flavonoids present in the extracts.

GC-MS analysis of the methanolic extract of *P. americana* leaf revealed the presence of eighteen bioactive compounds (Table 1; Figure 1). The presence of salicylic acid, a phenolic compound with reported antioxidative and anti-inflammatory potentials partly explains the observed *in vitro* antioxidant properties of *P. amercana* leaf extracts. Octadecanoic acid (2.67%) (Table 1) is the primary metabolite responsible for the formation of glycerol esters in plants. It has been reported to exhibit antitumor activity in mouse models with selective cytotoxicity for MOLT-4 leukemia cancer cell line. 9,12-Octadecadienoic acid has hypocholesterolemic, 5-alpha reductase inhibitor, antihistaminic, insectifuge, anti-eczemic, and anti-acne properties (Table 1). 1,2-benzenedicarboxylic acid, diisooctyl ester (Table 1) is a plasticizer compound with antimicrobial and antifouling properties [Paranthaman *et al.,* 2012; Venkata-Raman *et al*., 2012; Aneesh *et al*., 2013). Moreover, hexadecanoic acid methyl ester (11.76%) (Figure 1) possess antioxidant, nematicidal, pesticidal, anti-inflammatory, and antiandrogenic activities (Kim *et al*., 2014) In the present study, methanolic extract of *P. americana* leaf is rich in these phytochemicals, which may be responsible for the antioxidant properties observed. Other metabolites such as esters, alkanes, aldehydes, alkenes and ketones that were found in the methanolic extract of *P. americana* leaf exhibit antiulcer, anti-inflammatory, anti-arthritic, antidiabetic, antihyperlipidemic and anticancer properties (Kumar et al., 2010). Unarguably, these phytochemicals are responsible for the DPPH and NO radicals scavenging as well as ferric reducing antioxidant potential of *P. americana* leaf. Perhaps, this might explain the rationale behind the wide usage of the plant in management of multiple diseases in folkloric medicine.

Considering the avalanche of antioxidant phytochemicals present in the methanolic extract *of P. americana* leaves, it is safe to conclude that the plant is a potential therapeutic agent that can be exploited in the treatment of free radical induced pathological conditions.

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