Original Research Article

Persea americana Leaf Ethyl Acetate Extract Phytochemical, *In-Vitro* Antioxidant and *In-Vivo* Potentials to Mitigate Oxidative Stress in Alloxan-Induced Hyperglycaemic Rats

ABSTRACT

The purpose of this study was to investigate the *in-vivo* and *in-vitro* potentials of ethyl acetate extract of *P. americana* leaf in alloxan-induced diabetic rats. Quantitative phytochemicals analyzed includes; flavonoids, saponins, tannins, alkaloids and phenolics. Measurement of antioxidant activity using 1,1-Diphenyl-2-picrylhydrazyl, total antioxidant capacity, hydroxyl radical, hydrogen peroxide, superoxide radical and ferric reducing activity of the extract was carried out. Hyperglycemia was induced by intraperitoneal injection of alloxan monohydrate to albino rats. *In-vivo* anti-oxidant potentials of the extract were evaluated by measuring liver homogenate activity of superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and malondyaldehyde in alloxan-induced diabetic rats administered with the extract. A total of 30 Albino rats were used for this experiment and they were divided into six groups of 5 rats each. Group A; normal control, Group B; diabetic control, Groups C-E; experimental groups administered with different doses (100, 200 and 400mg/kg body weight respectively); of the extract and Group F; glucophage (84mg/kg body weight, standard drug) for 4 weeks. This study was conducted in the Department of Biochemistry, Bayero University, Kano, in August, 2018. Data was analyzed using one-way ANOVA with P=.05 value considered as significant. Results of the quantitative phytochemical investigation shows that the extract is rich in phenolics (184.1±0.6), flavonoids (115.8±2.1), alkaloids (41.5±1.8), with least concentration of tannis (21.2±0.8) and saponins (15.2±2.3). The extract exhibited high radical scavenging activity against synthetic free radicals (DPPH), reactive oxygen species (peroxide, superoxide and hydroxyl acid) and high ability to reduce Fe^{3+} to Fe^{2+} (FRAP). The activities of antioxidant enzymes of the treated rats were increased significantly (P=.05) while the level malondyaldehyde was significantly decreased (P=.05) in the treated groups. Ethyl acetate leaf extract of *Persea americana* contains phytochemical substances which improved antioxidant status and can be use as herbal therapy for the management of oxidative stress induced by diabetes mellitus and associated complications.

Key words: Antioxidants, Phytochemicals, *Persea americana,* Oxidative stress, Diabetes mellitus

1. INTRODUCTION

Diabetes mellitus ranked among the leading causes of death in developed countries and is one the most prevalent metabolic disorder in the world [1]. Although several etiologies have been implicated, defects in insulin secretion, insulin action, or both are often the primary characteristic of the disease [2]. Long-term complications of diabetes include coronary heart diseases, retinopathy, nephropathy, and foot ulceration [3]. Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxyuracil), one of the commonly used drugs for the induction of experimental diabetes in rats, is a diabetogenic agent that selectively destroys pancreatic β-cells [4]. Oxidative stress is defined as a state of overload due to imbalanced formation and elimination of highly reactive molecules including reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) [5, 6]. OS play a major role in reduced secretion by pancreatic β-cells, systemic inflammation,

endothelial damage and impaired glucose consumption in peripheral tissues [7]. OS is associated with numerous deleterious consequences in which radicals speed up cellular destruction in many diseases and can attack proteins, lipids, DNA and cell death [8]. Medicinal plant is an important part of traditional health care system and a veritable health care source for the vast majority of the world population. It was estimated that 70-80% of people worldwide use herb for management of mild to moderate illnesses [9-13]. The Avocado (*Persea americana* Mill.), unflatteringly known in the past as alligator pear, midshipman's butter and vegetable butter. It has traditionally been used due to its antibacterial, antifungal, hypotensive, anti-inflammatory, and immune-enhancing effects [14, 15]. Furthermore, Avocado juice made from ripe fruit was very popular due to its numerous health benefits. Phenolic substances including lignoids, proanthocyanidins, and flavonoids with highly potent antioxidant properties have been reported as the predominating phytochemicals in avocado [16 - 20]. This present study seeks to validate the traditional use of *Persea americana* leaf extract in the management of DM and several oxidative stress induced diseases.

2. MATERIALS and METHODS

2.1 Sample Collection, Identification and Preparation

The leaf of *Persea americana* was collected from Jos, Plateau state, Nigeria. It was authenticated by a Botanist at Plant Science Department, Bayero University, Kano with accession number BUKHAN 0326. The sample was chopped into small pieces and then shades dried and ground into powdered form. One hundred grams of the dried sample was exhaustedly extracted by soxhlet extraction using ethyl acetate as solvent.

2.2 Quantitative Analysis, *In-vitro* and *In vivo* Analysis

2.2.1 Determination of total phenolic content (TPC)

The quantification of TPC with different solvents of EPAL was carried out using the prescribed procedure reported by Wolfe K et al., using Folin Ciocalteu reagent [21]. Gallic acid was used as standard. TPC was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.

2.2.2 Determination of total flavonoid content (TFC)

The TFC with different solvents' extracts were determined using the method employed. TFC was calculated as quercetin (mg/g) equivalent using the equation obtained from a calibration curve of quercetin [22].

2.2.3 Determination of Alkaloids

A total of 200 mL of 20% acetic acid was added to 5 g of leaf and root powders taken in a separate 250 mL beaker and covered to stand for 4 h. This mixture containing solution was filtered and the volume was reduced to one quarter using water bath. To this sample, concentrated ammonium hydroxide was added drop-wise until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed [23]. The percentage of total alkaloid content was calculated as:

Percentage of total alkaloids (%) = Weight of residue×100/Weight of sample taken

2.2.4 Determination of Tannins

Tannin content was assessed using the vanillin assay [24], and 50 μL of each extract was added to 1.5 mL of vanillin (4 %) and 750 μL of HCl. After 20 min at room temperature, the absorbance was calculated at 500nm. Results were expressed as milligram catechin equivalents per gram of extract. The three extracts were analyzed in triplicate.

2.2.5 Determination of Saponins

Estimation of saponins content was determined by the method described by Makkar *et al.* based on vanillin-sulphuric acid colorimetric reaction with some modifications [25]. About 50 µL of plant extract was added with 250 µL of distilled water. To this, about 250 µL of vanillin reagent (800 mg of vanillin in 10 mL of 99.5% ethanol) was added. Then 2.5 mL of 72% sulphuric acid was added and it was mixed well. This solution was kept in a water bath at 60 °C for 10 min. After 10 min, it was cooled in ice cold water and the absorbance was read at 544 nm. The values were expressed as diosgenin equivalents (mg DE/g extract) derived from a standard curve.

2.2.6 *In vitro* Antioxidant Assays

Five different concentrations were used (1.2, 1, 0.8, 0.6, 0.4 and 0.2µg/ml) for the assay. All experiments were conducted in triplicates and all the negative control (blank) was prepared using the same procedure replacing the ethyl acetate extract of *P. americana* leaf (EPAL) with distilled water. The free radical scavenging activity of the EPAL were evaluated with various solvents based on its scavenging activities on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by [26]. Determination hydroxyl radical scavenging potential of EPAL with various solvents to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the modified method [27]. Determinations of superoxide anion radical scavenging potential of EPAL with various solvents were achieved according to the method [28]. The chelating of Fe^{2+} by EPAL with various solvents was estimated as described by [29]. Ferric ions reducing power of the with various solvents' extracts and standards were determined according to the method adopted by [30]. Total antioxidant capacity (TAC), was measured by a spectrophotometric method using a phosphomolybdenum method, which was based on the reduction of Mo^{6+} to Mo^{5+} by the sample analytes and the subsequent formation of green phosphate/Mo^{5+} compound with a maximum absorption at 695 nm. Sharp absorbance values proved the possession of significant antioxidant activity [31]. TAC was identified using the standard curve of vitamin C (equation: y = 2.046x + 0.043, r2= 0.991). To these above said antioxidant assays, the percentage inhibitory / scavenging activity of the EPAL / standard was calculated using following equation-

$$\text{EPAL} = \frac{A0 \quad \times \quad A1}{A0} * 100$$

Where A0 is the absorbance of the control, and A1 is the absorbance of the EPAL / standard. The half maximal inhibitory concentration (IC_{50}) value were calculated from the linear regression equation using following equation- y = m x + c, Where y is the percentage activity and equals 50, m is the slope, c is the intercept and x is the IC_{50} value.

2.2.7 *In vivo* Antioxidant Activity (oxidative stress markers)

Preparation of liver homogenate

Liver homogenate was prepared according to the method described [32]. Dissected livers were excised, washed with ice-cold 0.9% NaCl (w/v) to remove the blood, cut into small pieces by fine scissors, and then homogenized (10%w/v) separately in ice-cold 1.15% kcl-0.01M sodium phosphate buffer, pH 7.4 with a homogenizer. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. Supernatant of the liver homogenate was collected into sterilized tubes and stored at -20°C until analysis.

Superoxide dismutase

Liver homogenate of (0.1 ml) was diluted in 0.9 ml of distilled water to make 1:10 dilution. An aliquot (0.20 ml) of the diluted homogenate was added to 2.5 ml of 0.05 M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3 mm Adrenaline. The reference mixture contained 2.5 ml of 0.05 M carbonate buffer, 0.3ml of 0.3 mm Adrenaline and 0.20 ml of distilled water. Absorbance was measured at 30sec intervals up to 150 sec at 480nm in a spectrophotometer [33].

Calculation: Increase in absorbance per minute = (A5 – A1) 2.5

$$\% \text{ Inhibition} = \frac{100 - \text{Increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 100$$

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adrenochrome in 1 minute.

$$\text{SOD Activity} = \frac{\% \text{ inhibition}}{50} \times \text{weight of tissue}$$

Catalase

Into two test tubes T0 and T1, 2ml of homogenate supernatant, 2ml of phosphate buffer and 0.4ml of H_2O_2 was added and mixed. Immediately after, 1 ml of potassium dichromate/glacial acetic acid was added to T0. After 10 minutes, 1 ml of potassium dichromate /glacial acetic acid was also added to T1.

The contents were mixed and incubated at 800°C for 10 minutes. The absorbance was measured at 570nm against blank in a spectrophometer [34].

Calculation: $\frac{CT}{CS} = \frac{AT}{AS}$

CT = Concentration of Test, CS = Concentration of Standard = 0.2M, AT = Absorbance of Test (T0 - T1), AB = Absorbance of Blank (T0)

Catalase Activity = $\frac{CT \times \text{total vol. Of homogenate}}{10 \text{ minutes}}$ × weight of tissue × vol. Of homogenate used

Glutathione Reductase

To 150 µl of tissue homogenate, 1.5ml of 10 % TCA was added and centrifuged at 1500g for 5min. To a fresh test tube, 1 ml of the supernatant was added then 0.5 ml of Ellman's reagent was added and mixed then and 3 ml of phosphate buffer was added. The contents were mixed and the absorbance was read at 412 nm against the blank in a spectrophotometer [35].

Calculation: CT = concentration of the test, CS =Standard concentration, AT = Absorbance of test, AS = Absorbance of standard

Gluthathione = CT × total volume of homogenate / Volume of homogenate used × weight of tissue

Glutathione Peroxidase

To non-enzymatic wells - add 120 µl of assay buffer and 50 µl of co-substrate mixture to three wells. To positive control wells (bovine erythrocyte GPx) - add 100 µl of assay buffer, 50 µl of co-substrate mixture, and 20 µl of diluted GPx (control) to three wells. To sample wells - add 100 µl of assay buffer, 50 µl of co-substrate mixture, and 20 µl of sample to three wells. To obtain reproducible results, the amount of GPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min. Initiate the reactions by adding 20 µl of cumene hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the cumene hydroperoxide as quickly as possible. Carefully shake the plate for a few seconds to mix. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points [36].

Malondialdehyde (MDA)

Aliquots of homogenate (1 ml) were incubated at 37°C for 3 hr in a metabolic shaker. Then 1 ml of 10% aqueous trichloroacetic acid (TCA) was added and mixed. The mixture was then centrifuged at 800 g for 10 min. 1 ml of the supernatant was removed and mixed with 1 ml of 0.67% thiobarbituric acid in water and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 ml distilled water. The absorbance of the solution was then read at 535 nm in a spectrophotometer. The content of malondialdehyde (nmol/g wet tissue) was then calculated, by reference to a standard curve of malondialdehyde solution [37, 38].

2.3 Experimental Animals

Albino wistar rats (70-100g body weight) were purchased from Physiology department, Bayero University, Kano (B.U.K). They were kept in animal cages at the animal room of same Physiology department at a temperature of 25 degrees and humidity of 57%. They were acclimatized for 3 days and had free access to feed and water *ad libitum* prior to the studies. Ethical conditions governing the conducts of experiments with life animals as stipulated were strictly observed. Also, the experimental protocol was approved by the College of Health Science ethical committee.

2.3.1 Experimental Design

A total of 30 Albino rats were used for this experiment and they were divided into six groups of 5 rats each.

Group 1- normal rats
Group 2- diabetic control rats
Group 3- diabetic rats treated with EPAL (100 mg/kg).
Group 4- diabetic rats treated with EPAL (200 mg/kg).
Group 5- diabetic rats treated with EPAL 400mg/kg).
Group 6- diabetic rats treated with glucophage (84mg/kg) [39].

The studies lasted for a period of four weeks. Liver tissue was harvested for oxidative stress markers determination [superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH), glutathione peroxidase (GPx) and malondialdehyde (MDA)].

2.4 Statistical Analysis

Statistical package for social sciences (SPSS) version 17 software was used for all calculations and statistical analysis. Analyses were performed using student t-test at 95% confidence level with P =.05 being significant. Results were presented as mean ± standard deviation.

3. RESULTS

3.1 Quantitative Phytochemical Analysis

The result of quantitative analysis of ethyl acetate extract of *P. americana* leaf revealed the presence of significant (P=.05) high concentration of phenolics (184.1±0.6) which was then followed by flavonoids (115.8±2.1), with saponins (15.2±2.3) having the least concentration (Table 1).

3.2 *In vitro* antioxidant activity

Antioxidant capacity of EPAL was examined using six different assays. EPAL displayed significant (P=.05) radical scavenging potential against DPPH, Superoxide anion, total antioxidant capacity, hydroxyl radical and hydrogen peroxide. The activity was found to increase with increase in concentration of EPAL (Table 2). Figure 1 shows the dose response curves for the reducing power (FRAP) of EPAL. The result of this assay shows that the extract possesses high radical scavenging activity when compared with vitamin C.

3.3 *In-vivo* antioxidant activity

Superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase activities were assayed in alloxan-induced diabetic rats administered with different doses of ethyl acetate extract of *P. americana* leaf. The levels of these oxidative stress markers were found to significantly increase (P=.05)

when compared with diabetic control group. However, the level of malondyaldehyde was found to increase significantly in diabetic control group (P=.05) when compared with the treated groups (Table 3).

Table 1: Quantitative Phytochemical Analysis of Ethyl acetate Leaf extract of *P. americana*

Phytochemicals	**Leaf**
Alkaloids (%)	41.5 ± 1.8^{b}
Flavonoids (mg RE/g)	115.8 ± 2.1^{a}
Tannis (%)	21.2 ± 0.8^{c}
Saponins (g)	15.2 ± 2.3^{e}
Phenolics(mg GAE/g)	184.1 ± 0.6^{d}

Results are presented as Mean ± SD (n=5). Values bearing different superscripts are significantly different (P=.05)

Table 2: *In vitro* Antioxidant Activity of Ethyl acetate Extract of *P. americana* Leaf

Antioxidant Assay	**IC_{50}**
DPPH	0.279
TAC	0.307
Hydroxyl radical	0.614
Hydrogen peroxide	0.199
Superoxide anion	0.378

Key: DPPH= 1, 1-diphenyl-2-picrylhydrazyl, TAC= Total antioxidant capacity

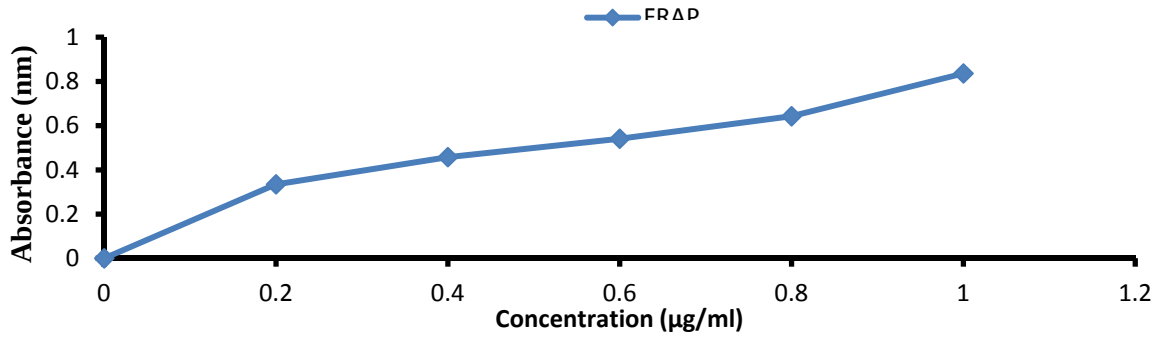

Figure 1: Ferric Reducing Antioxidant Power (FRAP) Activity of Ethyl acetate Fraction of *P. americana* Leaf

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221 **Table 3: Levels of Oxidative Stress Markers of Alloxan-induced Diabetic Rats Administered with Ethyl acetate Extract of *P. americana***
222 **leaf and Glucophage for 4 weeks**

GROUPS	SOD (U/g)	CAT (µmol/g)	GSH (µg/mg)	GPX(µmol/min/g)	MDA (µmolMDA/g)
NC	44.20 ± .58a	21.00 ± .71a	26.80 ± .56a	18.80 ± 2.91a	9.70 ± .62a
DC	23.80 ± .97b	11.66 ± .21b	11.40 ± .53b	11.20 ± .73b	35.70 ± 1.14b
EPAL(100mg/kg)	33.60 ± .75c	16.04 ± .43c	16.96 ± .60c	10.00 ± 1.14b	11.56 ± .56c
EPAL(200mg/kg)	33.00 ± 1.3c	17.60 ± .68c	18.62 ± .96c	16.60 ± 2.66a	13.80 ± .40c
EPAL(400mg/kg)	34.60 ± .93c	15.30 ± .62c	19.00 ± 2.3c	18.80 ± 2.15a	13.50 ± .55c
GP (84mg/kg)	36.20 ± .86c	18.98 ± 1.74a	20.40 ± 1.12c	17.60 ± 2.98a	13.40 ± .75c

223 Results are presented as Mean ± SD, n=5. Values with the different superscripts in the same column are significantly different (P=.05) with respect
224 to normal control, NC= Normal control, DC= Diabetic control, EPAL=Ethyl acetate extract of *P. americana* leaf, GP= Glucophage (standard drug)

225

DISCUSSION

The activities of plant extracts in effecting any therapeutic or biological changes in diseased animals or living tissues are direct functions of the chemical constituents inherently present in them after extraction [40]. The use of plants in treating diseases is as old as civilization [41] and herbal medicine is still a major part of habitual treatment of different diseases [42]. Diverse uses of plants in treatment of wide variety of diseases can be attributed to the presence of the phytochemicals or biologically active compounds which could serve as a potential a source of drugs in herbal medicine. The quantitative phytochemical assays in this present study indicated the concentration of the different secondary metabolite. Phenolics and flavonoids are known to possess varying antioxidant activities [43, 44]. Antioxidant activity of a medicinal plant cannot be concluded based on a single antioxidant test model [45]. Similar findings have been documented for the antioxidant and anti-inflammatory properties of Avocado [46]. It is noteworthy that the tested extract demonstrated the ability to neutralize the ROS at different degree which may due to the presence of polyphenols which has capability to directly scavenge superoxide and other ROS like hydroxyl and peroxyl radicals [47 - 48]. Flavonoids are water soluble anti-oxidants and free radical scavengers which prevent oxidative cell damage by donating a hydrogen atom, breaking the free radical chain, or electron to stabilize the radical species [49]. Phytochemicals are currently receiving attention as a potential protector against variety of human disease, major flavonoids has been shown to have neutralizing effect on free radical and ROS like hydroxyl radical, superoxide radical, hydrogen peroxides [50], Saponins, triterpenes and phytosterol have been demonstrated to scavenge superoxide anion [51].

The DPPH test reveals that the leaf has free radical scavenging ability. It is a free radical compound widely used to test the free radical scavenging ability of flavonoids. This research is in line with the work [16, 52], who suggested that avocado leaves have strong antioxidant activity, which may help in preventing or slowing down the progression of various diseases associated with oxidative stress. Hydrogen peroxide is a non-radical molecule generated *in-vivo* by several enzymes or by dismutation of two molecules of superoxide anions, a reaction catalysed by superoxide dismutase. Phenolics identified from EPAL in this study are antioxidant in nature, their scavenging activity may be as a result of their

253 ability to donates electrons to H_2O_2 neutralizing it to water. Hydrogen peroxide reacts with Fe^{2+} ion by
254 Fenton reaction to form a highly reactive hydroxyl radical ($OH^{\cdot}$) which in turn reacts with organic
255 molecules [53]. Ursolic acid, a phenolic compound was found to exhibit hydroxyl radical scavenging
256 activity, perhaps through its hydrogen donating ability as well as scavenged superoxide anion [54, 55].
257 Ethyl acetate extract of *P. americana* leaf was found to be rich in phyto-reductants such as flavonoids and
258 phenolics, which could be the main contributor to their antioxidative properties as many studies affirmed
259 that flavonoids and phenols offered the highest ability of scavenging activity in medicinal plants.

260 Oxidative stress is currently suggested as the mechanism underlying diabetes and its complications [56.
261 57]. From the present study, it has been observed that in alloxan-induced hyperglycemic rats, there were
262 significant decrease in SOD, CAT, GPx and GSH activities and enhanced lipid peroxidation (MDA) in the
263 liver. The decreased SOD activity may be due to high level of free radicals with decreased antioxidant
264 defense mechanisms [58, 59]. The significantly increased SOD level in diabetic rats following the
265 administration of *P. americana* extract suggests a positive modulatory role for the extract in the
266 amelioration of the induced oxidative stress. The observed significant elevation of GSH content of the
267 liver of ethyl acetate extract of *P. americana* (EPAL) treated rats indicate that, the extract might have
268 either increased the biosynthesis of GSH or lowered the utilization of GSH due to decreased oxidative
269 stress, or both. Induction of the hepatic GSH antioxidant system by chemopreventive agents was
270 reported in several studies [60]. Glutathione peroxidase (GPx) is a relatively stable enzyme, but it may be
271 inactivated under conditions of severe oxidative stress [61]. Decreased activity of GPx in diabetic control
272 could be directly explained by the low content of GSH found in these rats since GSH is a substrate for
273 GPx. Glutathione, the most important antioxidant metabolite, plays an important role in maintaining good
274 levels of GPx activity. This would cause an increased accumulation of superoxide radicals, which could
275 further stimulate lipid peroxidation. Decline in the activity of SOD in diabetic tissue and blood has been
276 reported in many studies [62]. A study [63] suggested that hyperglycaemia increased hydrogen peroxide
277 production and down-regulated CAT gene expression. A study carried out by [64] oral administration of *P.*
278 *americana* fruit extract to hyperglycaemic rats tends to bring liver peroxides to near control levels, which
279 could be as a result of improved antioxidant status. Catalase protects pancreatic cells from damage by
280 hydrogen peroxide [65, 66].

The increased MDA level in diabetic control rats may be due to the generation of free radical species potentiated by exposure to alloxan. Further, EPAL exerts a protective effect by scavenging MDA and elevating the activities of antioxidant enzymes in treated rats. The decreased activity of SOD in liver of diabetic control rats may be due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes. Medicinal plants with high flavonoid content have been reported to decrease MDA level in plasma, and thus provide protection against many chronic diseases by virtue of their free radical scavenging properties [67].The results on radical scavenging activity of EPAL in this study correspond with that of the oxidative stress markers. Several phytochemicals were reported to act against the deleterious effects of oxidative stress such as triterpenes in aloe vegetables [68], total saponins from *Panax ginseng* [63], polyphenols [55] and flavonoids from *Sideritis raeseri* [69]. This study suggests that *P. americana* leaf is potentially used for averting some diseases associated with oxidative stress

CONCLUSION

Based on this research, the leaf of *P. americana* has great and promising potential as pharmaceutical agent, particularly to be developed as anti-oxidative agent. This natural approach is thought to be safer and more effective compared to its synthetic agents. Therefore the fruit extract of *P. americana* may play an important role in the development of nutraceuticals and also in the management of oxidative stress induced diabetic mellitus.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

ETHICAL APPROVAL

All authors hereby declare that; principle of laboratory animals care (NHI publication number 829 revised 1985) were followed, as well as all experiment have been examined and approved by the appropriate ethic committee.

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