Oxidants and Antioxidants in Medical Science
www.oamsjournal.com
DOI: 10.5455/oams.290616.or.099

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ORIGINAL ARTICLE

In vitro and *in vivo* antioxidant properties of *Phyllanthus muellerianus* and its major constituent, geraniin

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ABSTRACT

Objective: *Phyllanthus muellerianus* (Kuntze) Exell. of the family Euphorbiaceae, is used as a wound healing agent in Ghana and other West African countries. Oxidative stress has been identified to delay wound healing process and subsequently leads to the development of chronic wounds. Hence, the aim of the present study was to investigate the *in vivo* and *in vitro* antioxidant properties of aqueous extract of the aerial parts of *P.muellerianus* (PLE) and its major constituent, geraniin.

Methods: Wound tissues excised from rats treated with PLE (0.25, 0.5 and 1% w/w) and geraniin (0.1, 0.2 and 0.4 % w/w) incorporated in aqueous creams were assayed for the levels of superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), ascorbate peroxidase (APx) and malondialdehyde (MDA). 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric reducing antioxidant power, total phenolic content and total antioxidant capacity assays were used to measure *in vitro* antioxidant activity.

Results: SOD, CAT and APx activity increased significantly in both PLE and geraniin-treated wound tissues compared to the untreated tissues. However, MPO activity decreased significantly in all three PLE and 0.2 and 0.4% w/w geraniin-treated wound tissues compared to the untreated tissues. MDA levels decreased significantly in all of the PLE and geraniin-treated wound tissues compared to the untreated tissues. Ferric reducing antioxidant power (FRAP) was observed to be significantly lower for PLE than geraniin.

Conclusion: PLE and geraniin exhibit both *in vivo* and *in vitro* antioxidant properties.

Received: May 12, 2016
Accepted: June 29, 2016
Published: October 7, 2016

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Key Words: Antioxidant, oxidant, wound healing, *Phyllanthus muellerianus*, geraniin

INTRODUCTION

Oxidative stress which is largely characterized by reactive oxygen and nitrogen species is implicated in the development of a number of chronic and degenerative diseases such as atherosclerosis, cancer, cirrhosis, diabetes, wound healing and aging. Although reactive oxygen species (ROS) are believe to play a role in cell signaling and defense against microbes, they can also damage biological molecules such as proteins, lipids and DNA when produced excessively and subsequently lead to disease state [1-4].

Antioxidants or inhibitors of oxidation are compounds which retard or prevent the oxidation and in general prolong the life of the oxidizable matter. In other words, antioxidants inhibit or prevent the progression of diseases that have their etiology in oxidative stress. Though the body has its own mechanism of defense against ROS; during oxidative stress the defense mechanism can be overwhelmed and lead to the development of some chronic and/or degenerative disease. Reports have shown that it is possible to reduce the risk of chronic diseases and prevent progression of diseases linked to ROS by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants [5-7]. This has necessitated the search for potent and non-toxic antioxidants agents to prevent the progression of oxidant implicated chronic diseases [8].

Reactive oxygen species, such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals (OH•), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), play a major role in the development of oxidative stress. High oxidative stress at wound sites promotes collagen breakdown and hence destruction of the extracellular matrix (ECM). When the ECM is destroyed, processes such as angiogenesis and re-epithelialization which are crucial for wounds to heal are reduced and leads to the development of chronic wounds [9, 10]. However, the human system has inherent antioxidant mechanisms which stabilize or deactivate free radicals, often before they attack and damage targets in biological cells or tissues [11]. But in most wounds, there is an imbalance between ROS and the intrinsic antioxidant capacity of the body which leads to cell (fibroblast and keratinocytes) and tissue damage through covalent bonding and lipid peroxidation [12]. Apart from the ability of these antioxidants to inhibit tissue damage and lipid peroxidation at wound site, antioxidants also stimulate the synthesis of collagen [13]. In addition, antioxidants have been shown to promote wound healing by stimulating the expression of many genes that code for antioxidant enzymes such as ascorbate peroxidase (APx), catalase (CAT) and superoxide dismutase (SOD) in wound bed [14, 15].

Phyllanthus muellerianus (Kuntze) Exell., belonging to the family Euphorbiaceae, is a widely distributed plant in West Africa and is used traditionally in treating wounds in Ghana and other parts of West Africa [16]. It is also used to manage menstrual disturbances, pain, dysentery, gonorrhea and stomach sores. Agyare *et al* [17] reported that the aqueous leaf extract of *P.muellerianus*

and its major isolate, geraniin stimulate cellular activity, differentiation and collagen synthesis of human skin keratinocytes and dermal fibroblasts. Also, aqueous aerial part extract of *P.muellerianus* and geraniin have been reported to exhibit wound healing activity via their stimulatory effect on rate of wound closure, increased hydroxyproline production, collagen deposition and TGF-β_1 levels in excision wound model as well as improved tensile strength in incision wound model [18]. The anti-infective and anti-inflammatory properties of *P.muellerianus* and geraniin have also been reported [19, 20]. Hence, the present study aimed to investigate the *in vivo* and *in vitro* antioxidant activities of *P.muellerianus* and its major metabolite, geraniin.

MATERIALS AND METHODS

Plant collection

The fresh matured aerial parts of *Phyllanthus muellerianus* were collected from the Kuntanase community (longitude 1.0°28'18"W, latitude 6.0°32'23"N), Ashanti Region, Ghana in February 2010. The plant was authenticated and a voucher specimen (A 001) deposited at Ghana Herbarium, University of Ghana, Accra, Ghana.

Preparation of aqueous extract

The plant material was washed under running water, air-dried at room temperature and ground to powder (1 kg) using a lab mill machine (Christy and Norris; Chelmsford, UK). Powdered plant material was extracted in 10 l of distilled water by heating at 90°C for 15 min under atmospheric pressure. The extract was filtered using a Buchner funnel and Whatman no. 10 filter paper and concentrated under reduced pressure at 45°C by a vacuum rotary evaporator (R-210, BUCHI; Flawil, Switzerland) and further lyophilized using a freeze drying system to yield the powdered plant extract (14.1 %w/w) which will subsequently be referred as PLE.

Source of geraniin

The geraniin (96% w/w HPLC grade) used in this study was kindly provided by Prof. Andreas Hensel, Institute of Pharmaceutical Biology and Phytochemistry, University of Muenster, Muenster, Germany. Geraniin was isolated from the aqueous extract of the aerial parts of *P.muellerianus* as described earlier[16].

Chemicals

Ammonium molybdate (99.9%), epinephrine (97%), Folin-Ciocalteau (2 M), hydrogen peroxide (30%), thiobarbituric acid (98%), trichloroacetic acid (99%), α-tocopherol (95.5%), 1,1-diphenyl-2-picryl-hydrazyl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tannic acid (98%), potassium ferricyanide (99%), ferric chloride were acquired from Merck (Poole, UK). Hydrogen peroxide (30%) and ascorbic acid (99%) were obtained from Bells (Birmingham, UK) and Fisher Scientific (Glasgow, UK), respectively. Ketamine hydrochloride was purchased from Pfizer (New York, NY, USA) and silver sulphadiazine (1% w/w) aqueous cream from Ayrton Drugs (Accra, Ghana).

Experimental animals

Male Sprague-Dawley rats (200 to 250 g) were acquired from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana. The rats were kept in groups of five in clean polypropylene cages in the experimental animal house of the Department of Pharmacology, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana under temperature of 25ºC, normal daylight and relative humidity of 55-60%. Rats were fed on commercial pellets (Ghana Agro-Food Company, GAFCO) *ad libitum*, with clean water accessible to the caged rats in clean bottles. Animals were allowed to acclimatize for a week before experiment was performed. All experimental protocols were approved by College of Health Sciences Animal Ethical Committee (Pharm/EtcC/X8122013) and performed according to internationally accepted principles for laboratory animal use and care (EEC Directive of 1986: 86/609 EEC). All animals were euthanized at the end of the experiment.

Determination of *in vivo* antioxidant properties of PLE and geraniin

The excision wound model as described by Morton and Malone [21] and Agyare *et al* [22] was employed. The dorsal fur of the rats was trimmed and depilated using clean razor blades. The shaved area was then treated with 70% v/v ethanol. The rats were anesthetized with an intramuscular injection of ketamine hydrochloride at a dose of 50 mg/kg body weight. With the aid of a pair of sterile surgical scissors and toothed forceps, full thickness wounds of approx. 20 mm in diameter were excised at the back of each rat to a depth of loose subcutaneous tissue. Hemostasis was achieved by even compression with sterile gauze and wounds were left untreated for a period of 24 h. Rats were randomized into 9 groups consisting of 5 rats per group and topically treated with aqueous cream base (vehicle), silver sulphadiazine (1% w/w) aqueous cream, PLE (0. 25, 0.50 and 1.0% w/w) aqueous creams, geraniin (0. 1, 0.2 and 0.4% w/w) aqueous creams and untreated group (control). All the wounds received daily standard wound cleansing with 0.9% w/v saline solution prior to the topical treatment with the creams (0.1 g of cream per daily treatment). Any rat showing a wound hematoma or wound infection was immediately euthanized with an overdose of ketamine hydrochloride (200 mg/kg) to avoid any discomfort. Also, data from these animals were not used in the final analysis. Rats were euthanized on day 9, post topical treatment. Harvested wound tissues from the various groups were stored immediately at –80°C till laboratory analysis.

Preparation of tissues for enzyme assay

Tissue samples were trypsinized and homogenized using a Potter-Elvehjem homogenizer in ice-cold 0.01 M Tris–HCl buffer (pH 7.4) to give a 10% homogenate. Protein concentration of homogenate

(supernatant) was determined using Bradford protein assay [23]. Homogenate (supernatant) was used for superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), ascorbate peroxidase (APx) and malondialdehyde (MDA) assays.

Superoxide dismutase

SOD activity was measured by the method described by Misra and Fridovich [24] based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. To 0.5 ml of tissue homogenate, 0.75 ml of ethanol and 0.15 ml of chloroform (placed in ice) was added and centrifuged at 10,000g for 20 min. To 0.5 ml of homogenate, 0.5 ml of 0.6 mM EDTA solution and 1 ml of carbonate bicarbonate buffer (0.1 M, pH 10.2) were added. The reaction was initiated by the addition of 0.05 ml of 1.3 mM adrenaline and the increase in absorbance at 480 nm due to the adrenochrome formed was measured with a spectrophotometer. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 25°C. The percentage inhibition was calculated according to following formula:

$$\% \text{ inhibition} = \frac{(\text{Absorbance}_{test} - \text{Absorbance}_{reference}) \times 100}{\text{Absorbance}_{test}}$$

$$\text{Units of activity per mg protein} = \frac{\% \text{ inhibition}}{50 \times \text{weight of protein}}$$

Catalase

Activity of catalase enzyme was measured using the method as described by Aebi [25]. It was determined by measuring decrease in hydrogen peroxide (20 s interval) concentration at 240 nm for 60 s. Briefly, to 100 µl of homogenate, 130 µl of 50 mM potassium buffer (pH 7) and 65 µl of 10 mM H_2O_2 was added. The blank was a mixture of 65 µl of the potassium phosphate and 130 µl of homogenate. The concentration of H_2O_2 was calculated from the absorbance using the formula given below, where 39.4 $mol^{-1}cm^{-1}$ is the molar extinction coefficient for H_2O_2:

$$[H_2O_2 \text{ mM}] = \frac{\text{Absorbance}_{240\text{ nm}} \times 1000}{39.4 \text{ mol}^{-1}\text{cm}^{-1}}$$

CAT activity was expressed as U/mg protein.

Myeloperoxidase

The MPO enzyme assay was determined by a modified method described by Bradley *et al* [26]. To 20 µl of 0.1 M phosphate buffer (pH 6), 20 µl of 0.01 M H_2O_2, 40 µl of 0.02 M *o*-dianisidine (freshly prepared) and 110 µl of deionized water was added. Then, 10 µl of the homogenate was added to make a final volume of 200 µl. The homogenate was added last and the change in absorbance at 460 nm was followed for 10 min (taken every 1 min). One unit of MPO was defined as that giving an increase in absorbance of 0.001 min^{-1} and specific activity was given as U/mg protein.

Ascorbate peroxidase

Ascorbate peroxidase enzyme activity was measured according to the method described by Nakano and Asada [27]. To 600 µl of 50 mM potassium buffer (pH 7), 100 µl of 0.1 mM EDTA, 100 µl of 0.5 mM ascorbic acid and 100 µl of homogenate was added. Then 100 µl of 1.25 mM H_2O_2 was added to the reaction mixture. The blank had all components other than the homogenate. The reaction was initiated by adding H_2O_2. The decrease in absorbance at 290 nm (extinction coefficient: 2.8 $mM^{-1}cm^{-1}$) was measured every 10 s for 1 min.

Determination of lipid peroxidation

Lipid peroxidation level in excised wound tissue samples expressed in amount of MDA produced was determined according to the method described by Heath and Parker [28]. For measurement of MDA content, 3 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added to 1 ml of the homogenate. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. The tube was centrifuged at 10,000g for 10 min. The absorbance of the supernatant was read at 532 and 600 nm; the absorbance at 600 nm (non-specific absorption) was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated using MDA's extinction coefficient of 155 $mM^{-1}cm^{-1}$.

In vitro antioxidant activity

DPPH free radical scavenging assay

The free radical scavenging effects of the PLE, geraniin and α-tocopherol (positive control) on 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was determined using the method described by Susanti *et al* [29]. In brief, 10 ml of PLE was weighed and dissolved in 10 ml of sterile distilled water to produce a stock solution of 1 mg/ml. Then, 2 ml of the stock was transferred into a clean test tube and diluted with equal volume of sterile distilled water. The resulting solution was serially diluted by twofold dilution to concentrations ranging from 0.0195 to 10 µg/ml; 1 ml of each concentration was added to 3 ml of freshly prepared DPPH solution (50 µM) in methanol. After 30 min of incubation in the dark at 25°C, the absorbance was measured at 517 nm using a UV spectrophotometer against the blank. The blank solution containing the reagent solution (50 µM DPPH) and solvent used for dissolving sample was prepared and treated under same conditions as the sample. The experiment was performed in triplicates. The procedure was repeated for geraniin and α-tocopherol (reference compound). The capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{(\text{Absorbance}_{DPPH} - \text{Absorbance}_{sample}) \times 100}{\text{Aborbances}_{DPPH}}$$

Ferric reducing antioxidant power

Antioxidant activity of PLE and geraniin by ferric (Fe^{3+}) reducing power assay (FRAP) was determined according to the method described by Benzie and Strain [30]. Different concentrations of PLE ranging from 1 to 100 µg/ml were prepared. To each concentration of PLE, 2.5 ml each of phosphate buffer (200 mM, pH 6.6) and 1% potassium ferricyanide were added. The mixture was placed in a water bath for 20 min at 50°C, cooled rapidly, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 3,000g for 10 min. To 2.5 ml of the supernatant, 2.5 ml of sterile distilled water and 0.5 ml of 0.1% ferric chloride ($FeCl_3$) was added. The intensity of iron (II)-ferricyanide complex was determined by measuring the absorbance of the Perl's Prussian blue color formation at 700 nm after allowing the mixture to react for 10 min. A blank solution containing reagent solutions and solvent used for dissolving sample was prepared and processed under same conditions as rest of the sample. The higher absorbance of the reaction mixture indicates increased reducing power. The procedure was repeated with α-tocopherol (reference compound) and geraniin with concentrations ranging from 1 to 100 µg/ml. The procedure was carried out in triplicates to ensure accuracy.

Total phenol content

Total phenolic content in PLE was determined with Folin-Ciocalteu reagent according to the method described by Slinkard and Singleton [31]. Concentration of PLE ranging from 1 to 100 µg/ml was prepared and 1 ml of each concentration of PLE was mixed with 1 ml of Folin-Ciocalteu reagent. The mixture was shaken vigorously and allowed to stand at 25°C for 5 min before the addition of 2 ml of 7.5% Na_2CO_3 and allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm against the reagent blank. The same procedure was repeated for tannic acid (reference compound) using concentrations ranging from 1 to 100 µg/ml. A standard curve was prepared by plotting the absorbance of tannic acid against concentration. The linearity obtained was in the range of 1 to 100 µg/ml. Using the standard curve, the total phenolic content was calculated and expressed as tannic acid equivalent in mg/g of PLE. The experiment was carried out in triplicate. The total phenolic content of PLE was calculated using the formula below:

$$TAE = \frac{(Absorbance_{sample} - C)}{m}$$

(TAE, tannic acid equivalent; m, slope of calibration/standard curve; C, intercept on the y-axis)

Total antioxidant capacity

The total antioxidant capacity of the PLE and geraniin was evaluated using the phosphomolybdenum method described by Prieto *et al* [32]. For this, 500 µl each of PLE solutions ranging from 1 to 100 µg/ml were combined with 5 ml of reagent solution (0.6 M H_2SO_4, 28 mM Na_2PO_4 and 4 mM $(NH_4)_2MoO_4$). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A blank solution containing reagent solution and solvent used for dissolving sample was prepared and it was incubated under same conditions as PLE. The same procedure was repeated for α-tocopherol (reference compound) using concentrations ranging from 1 to 100 µg/ml. A standard curve was prepared by plotting absorbance of α-tocopherol against concentration. The linearity obtained was in the range of 1 to 100 µg/ml. Using the standard curve, the total antioxidant capacity was calculated and expressed as α-tocopherol equivalent in mg/g of extract. The procedure was carried out in triplicate. Also, the procedure was repeated with geraniin at concentrations ranging from 1 to 100 µg/ml. The total antioxidant capacity of PLE and geraniin was determined using following formula:

$$\alpha\text{-TE} = \frac{(Absorbance_{sample} - C)}{m}$$

(α-TE, α-tocopherol equivalent; m, slope of calibration/standard curve; C, intercept on the y-axis)

RESULTS

In vivo antioxidant activity

Superoxide dismutase activity in excised wound tissues

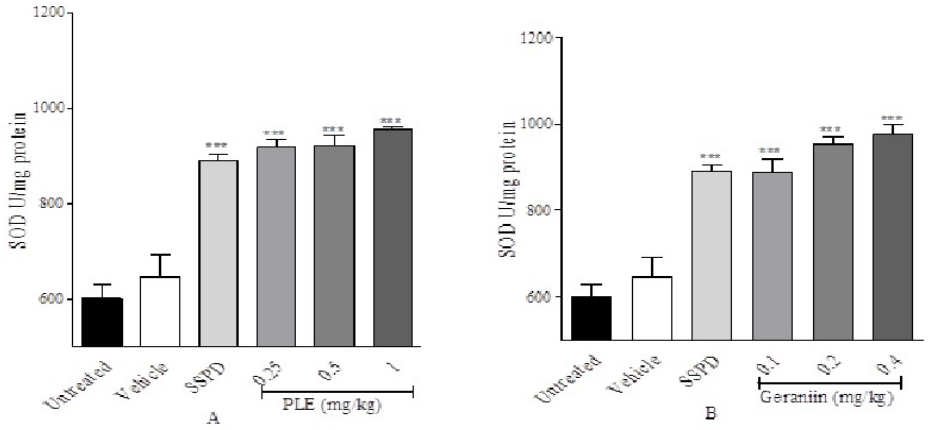

Figure 1. Influence of PLE and geraniin on superoxide dismutase (SOD) activity in excised wound tissues. **(A)** PLE-treated wound tissues; **(B)** geraniin-treated wound tissues. PLE, aqueous extract of the aerial parts of *P.muellerianus*; SSPD, 1% silver sulphadiazine-treated wound tissues.

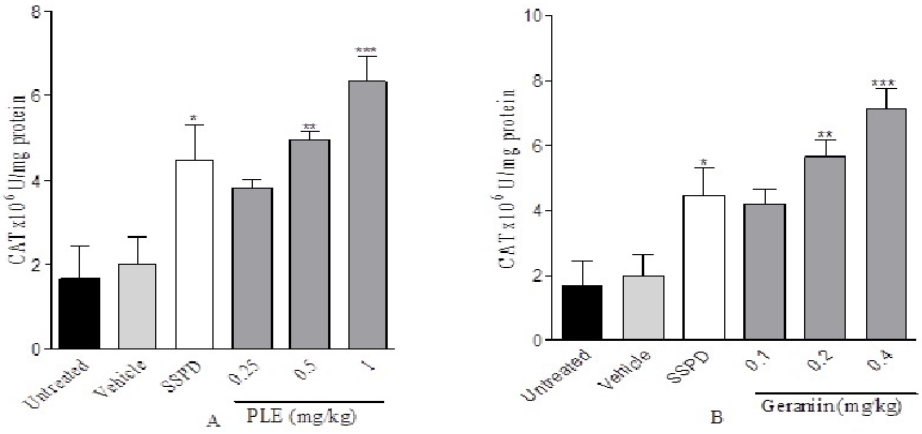

Figure 2. Influence of PLE and geraniin on catalase (CAT) activity in excised wound tissues. **(A)** PLE-treated wound tissues; **(B)** geraniin-treated wound tissues. PLE, aqueous extract of the aerial parts of *P.muellerianus*; SSPD, 1% silver sulphadiazine-treated wound tissues.

PLE-treated wound tissues at concentrations of 0.25, 0.5 and 1% w/w showed significant (P< 0.001) increase in the activity of SOD compared to the untreated wound tissues (Figure 1A). In geraniin-treated wound tissues, there was a significant (P < 0.001) increase in SOD activity compared to the untreated wound tissues (Figure 1B).

Catalase activity in excised wound tissues

Wound tissues treated with 0.5 and 1% w/w PLE significantly (P < 0.01 and 0.001, respectively) increased CAT activity compared to the untreated wound tissues; there was no significant (P > 0.05) change in CAT activity of 0.25% w/w PLE-treated wound tissues (Figure 2A). CAT activity significantly increased in wound tissues treated with 0.2 (P < 0.01) and 0.4% w/w (P < 0.001) geraniin compared to the untreated wound tissues; there was no significant (P > 0.05) difference in CAT activity of 0.1% w/w geraniin-treated wound tissues (Figure 2B).

Myeloperoxidase activity in excised wound tissues

Compared to untreated wound tissues, there was a significant reduction in MPO activity in 0.25 (P < 0.01), 0.5 (P < 0.01) and 1% w/w (P < 0.001) PLE-treated wound tissues (Figure 3A). Also, 0.2 (P < 0.01) and 0.4% w/w (P < 0.001) geraniin-treated wound tissues showed significant reduction in MPO activity compared to the untreated wound tissues. There was no significant (P > 0.05) difference between 0.1% w/w geraniin-treated wound tissues compared to the untreated wound tissues (Figure 3B).

Ascorbate peroxidase activity in excised wound tissues

PLE- and geraniin-treated wound tissues showed marked increase in APx enzyme activity compared to the untreated wound tissues. In wound tissues treated with 0.25, 0.5 and 1% w/w PLE, there was significant (P < 0.001) increase in APx activity compared to the untreated wound tissues (Figure 4A). Also, 0.1, 0.2 and 0.4 % w/w geraniin-treated wound tissues significantly (P < 0.001) increased the activity of APx activity compared to the untreated wound tissues (Figure 4B).

Lipid peroxidation level in excised wound tissues

Wound tissues treated with 0.25, 0.5 and 1% w/w PLE showed significant (P < 0.05, 0.05 and 0.01, respectively) reduction in MDA content when compared to the untreated wound tissues (Figure 5). Furthermore, wound

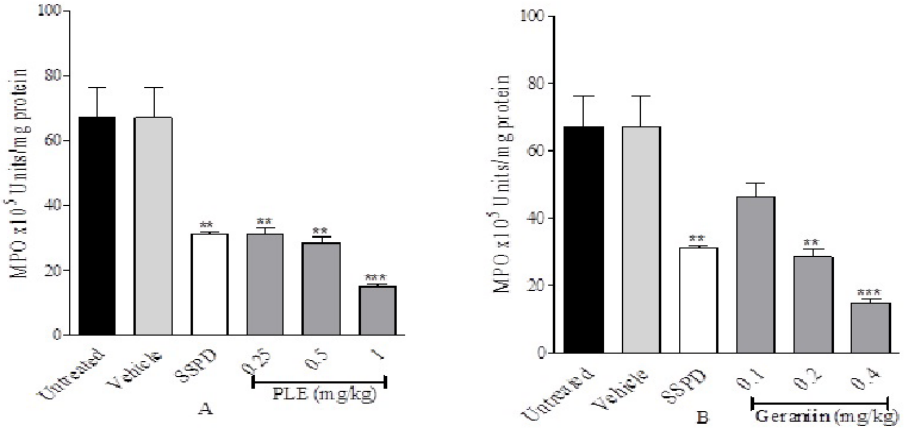

Figure 3. Influence of PLE and geraniin on myeloperoxidase (MPO) activity in excised wound tissues. **(A)** PLE-treated wound tissues; **(B)** geraniin-treated wound tissues. PLE, aqueous extract of the aerial parts of *P.muellerianus*; SSPD, 1% silver sulphadiazine-treated wound tissues.

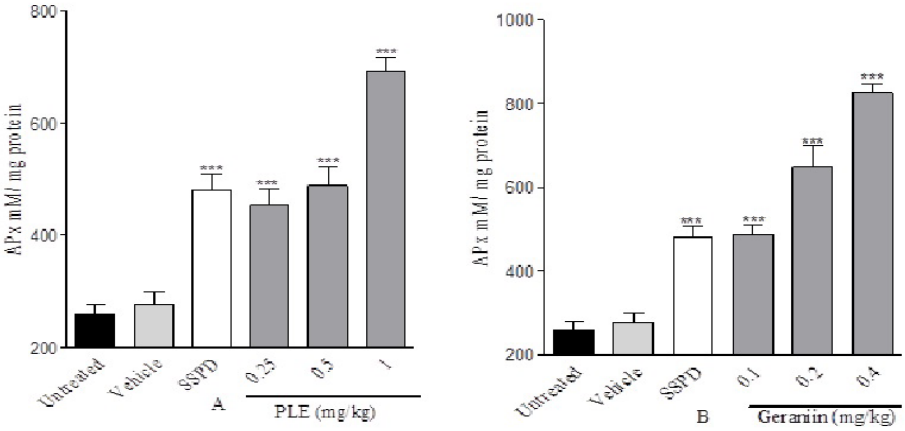

Figure 4. Influence of PLE and geraniin on ascorbate peroxidase (APx) activity in excised wound tissues. (**A)** PLE-treated wound tissues; **(B)** geraniin-treated wound tissues. PLE, aqueous extract of the aerial parts of *P.muellerianus*; SSPD, 1% silver sulphadiazine-treated wound tissues.

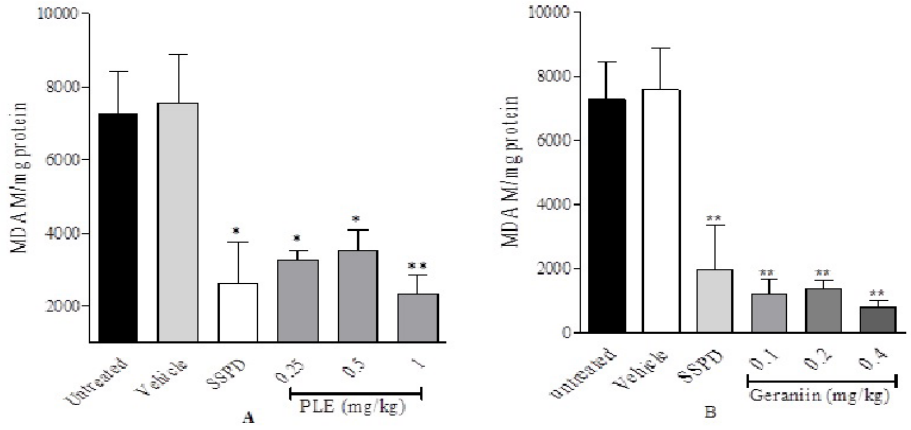

Figure 5. Influence of PLE and geraniin on lipid peroxidation level in excized wounds. (**A)** PLE-treated wound tissues; **(B)** geraniin-treated wound tissues. PLE, aqueous extract of the aerial parts of *P.muellerianus*; SSPD, 1% silver sulphadiazine-treated wound tissues.

tissues treated with 0.1, 0.2 and 0.4 % w/w geraniin significantly ($P < 0.01$, 0.001 and 0.001, respectively) reduced MDA content compared to the untreated wounds (Figure 5).

In vitro antioxidant activity

DPPH free radical scavenging activity

Both PLE and geraniin exerted apparent antioxidant activity (free radical scavenging activity) (Figure 6). The IC_{50} of PLE and geraniin were 0.12 and 1.85 μg/ml, respectively. The highest antioxidant activity was observed in the reference compound (α-tocopherol) with an IC_{50} of 0.03 μg/ml (Table 1)

Ferric reducing power antioxidant activity

The reducing power of geraniin and PLE were compared to α-tocopherol at the same concentration. The reducing power ability of PLE and geraniin were found to be significantly ($P < 0.001$) lower at concentrations of 1, 3, 10, 30, 100 μg/ml compared to α-tocopherol at the same concentrations (Figure 7).

Total phenolic content of PLE

The total phenolic content of PLE was compared to that of tannic at the same concentration range. The absorbance of PLE at concentrations of 1, 3, 10, 30 and 100 μg/ml were significantly ($P < 0.001$) lower than that of tannic acid at the same concentrations (Figure 8). The total phenolic content of PLE which is expressed as TAE was calculated to be 39.97 ± 0.07 mg/g (Table 2).

Total antioxidant capacity

The absorbance of PLE when compared to α-tocopherol, was observed to be significantly low at concentrations of 3 ($P < 0.05$), 10 ($P < 0.001$), 30 ($P < 0.001$) and 100 μg/ml ($P < 0.001$) (Figure 9). Also, the absorbance of geraniin at concentrations of 30 and 100 μg/ml was significantly ($P < 0.001$) lower compared to α-tocopherol (Figure 9). The total antioxidant capacity of PLE and geraniin which is expressed as α-TE was calculated to be 179.8 ± 0.01 and 740.8 ± 0.1 mg/g (Table 3).

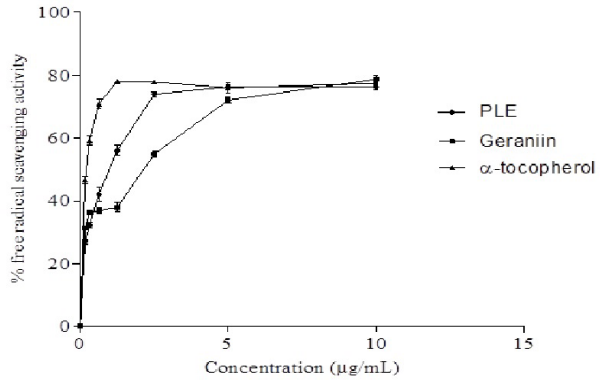

Figure 6. DPPH free radical scavenging activity of PLE, geraniin and α-tocopherol (reference compound).

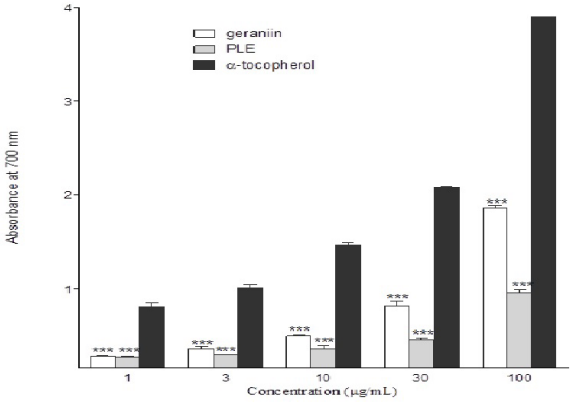

Figure 7. Reducing power of PLE and geraniin. The data was analyzed using one-way ANOVA followed by Dunnett's post hoc test (***P < 0.001). Values are mean ± SEM, n = 3; PLE, aqueous extract of the aerial parts of *P.muellerianus*.

Table 1. IC_{50} of *Phyllanthus muellerianus* extract, geraniin and α-tocopherol

Extract/compound	IC_{50} (µg/ml) ± SEM	IC_{50} (µM) ± SEM
PLE	0.12 ± 0.1	-
Geraniin	1.85 ± 0.13	1.94 ± 0.13
α-tocopherol (vitamin E)	0.03 ± 0.04	0.08 ± 0.05

PLE, aqueous extract of the aerial parts of *P.muellerianus*; IC_{50}, concentration that causes 50% inhibition of DPPH free radical; SEM, standard error of the mean.

Table 2. Total phenolic content of *Phyllanthus muellerianus* extract

Extract	Tannic acid equivalent
PLE	39.97 ± 0.07 mg/g

PLE, aqueous extract of the aerial parts of *P.muellerianus*

Table 3. Total antioxidant capacity of *Phyllanthus muellerianus* extract and geraniin

Extract/compound	α-tocopherol equivalent
PLE	179.8 ±0.01 mg/g
Geraniin	740.8 ±0.03 mg/g

PLE: aqueous extract of aerial parts of *P. muellerianus.*

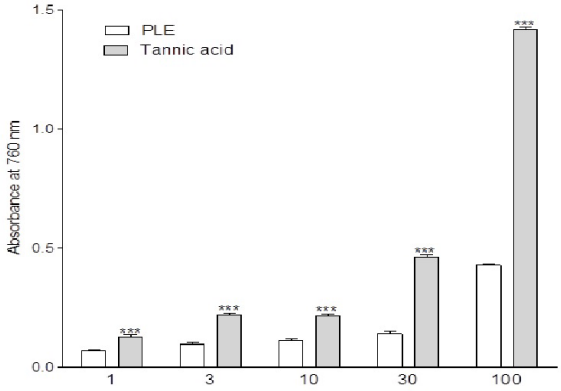

Figure 8. Total phenolic content of PLE. The data was analyzed using one-way ANOVA followed by Dunnett's post hoc test (***P < 0.001). Values are mean ± SEM, n = 3; PLE, aqueous extract of the aerial parts of *P.muellerianus*.

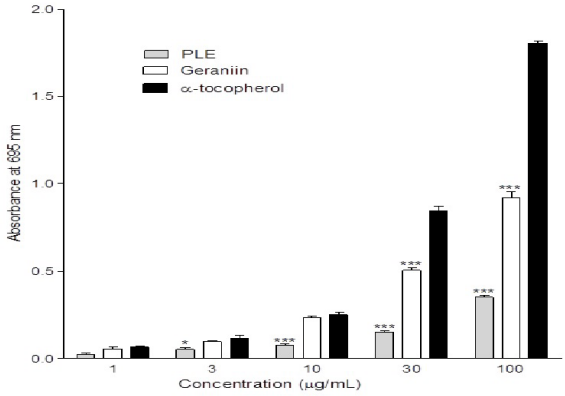

Figure 9. Total antioxidant capacity of PLE and geraniin. PLE: aqueous extract of the aerial parts of P. muellerianus. The data was analysed using One-way ANOVA followed by Dunnett's post hoc test (P < ***0.001 and *0.05). Values are mean ± SEM, n = 3; PLE, aqueous extract of the aerial parts of *P.muellerianus*.

DISCUSSION

Phyllanthus muellerianus extract and geraniin stimulated the production of SOD (Figure 1), CAT (Figure 2) and APx (Figure 4) in the present study. SOD, CAT and APx are the first line of defense against ROS [33, 34]. In the wound bed, SOD catalyses the dismutation of superoxide radicals to hydrogen peroxide and dioxygen [35, 36]. The toxic hydrogen peroxide produced is then neutralized by either catalase or the peroxidases (glutathione peroxidase and APx) to prevent cell damage, lipid peroxidation and myeloperoxidation [35, 37, 38]. High ROS levels damage the extracellular matrix and therefore are implicated in delayed wound healing. High levels of SOD, CAT, and APx at wound site may indicate high antioxidant activity and improved the wound healing process. This may suggest that PLE and geraniin enhanced the wound healing process by stimulating the production and/or activity of SOD, CAT and APx in wound bed. Also, PLE and geraniin reduced MDA level which is a measure of the degree of lipid peroxidation (Figure 5). The ability of PLE and geraniin to reduce lipid peroxidation may suggest that PLE and geraniin can reduce the oxidation of fatty acids such as omega-3 fatty acids which is essential

in the wound healing process [39]. In addition, PLE and geraniin also reduced MPO activity (Figure 4). MPO stimulates the production of neutrophils which lead to prolonged inflammatory response and cell damage at wound site [40, 41]. Hence low MPO activity in the presence of PLE and geraniin treatment suggests that the two agents can prevent cell damage in wound bed and improve wound healing [42].

The DPPH test provides information on the reactivity of the test compounds with a stable free radical, though DPPH radical is absent in biological systems. However, the degree of reduction in absorbance is an indication of the radical scavenging (antioxidant) power of an agent [43, 44]. It can be observed that the free radical scavenging activity of PLE and geraniin increased with increasing concentration (Figure 6). A study by Motalleb *et al* [45] showed that the scavenging effect on the DPPH radical increases sharply with the increasing concentration of test samples. The presence of phenolic compounds like flavonoids and tannins in PLE are likely to be responsible for the free radical scavenging effects observed because they act as primary antioxidants [46, 47]. Polyphenolic compounds are known to have antioxidant activity and therefore the activity of geraniin, a polyphenol and PLE which contains high amount of geraniin and other polyphenols may be due to these compounds [48-50]. The polyphenols exert their antioxidant activity through their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [51]. PLE and geraniin may neutralize or inactivate free radicals that may be present in wounds during oxidative burst or stress.

According to Hodzic *et al* [52], FRAP presents a simpler and quicker method for determining antioxidant activity. Besides that, the reaction is reproducible and linearly related to molar concentration of the antioxidants. The disadvantage of the method is that it does not react fast with some antioxidants such gluthathione [53]. Schafer and Buettner [54] reported that FRAP assay still can be used for assessment of antioxidant activity of plant materials as humans only absorb limited amount of gluthathione. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agents. The reducing power might be due to hydrogen donating ability, and is generally associated with the presence of reductones [53]. It was observed that the reducing power ability increases with increasing concentrations of PLE and geraniin (Figure 7). The results also revealed that at higher concentrations geraniin showed higher reducing power ability than PLE. Oszmianski *et al* [55] reported that ellagitannins from leaves of wild raspberries showed high FRAP value and also Zhang and Lin [56] have shown that ellagitannins in fruits of *Syzygium cumini* have high FRAP value and our findings are consistent with these reports. High FRAP activity of PLE and geraniin may be due to the high tannin content since the antioxidant activity of tannins is mediated through reducing power and scavenging activity [57, 58]. The reducing power ability of PLE and geraniin may suggest that the ROS such as H_2O_2, $O_2^{\bullet -}$ and $OH^{\bullet}$ may be neutralized by PLE and geraniin via hydrogen atom transfer.

It must be noted that the reagent used for total phenolic content in the study (see Table 2) does not react exclusively with phenolics, but also with other reducing agents; for example, ascorbic acid [59, 60]. Hence, results of this test therefore reflect the total reducing capacity of PLE (Table 3). Recent reports have shown that many flavonoids and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants [61]. Hence the observed higher total antioxidant capacity of geraniin, a known polyphenolic compound compared to PLE.

Hence, it can be concluded from the study that the aqueous aerial parts extract of *Phyllanthus muellerianus* possesses both *in vitro* and *in vivo* antioxidant activity which may play a vital role in its wound healing activity. The study also gives credence to the traditional use of *P.muellerianus* as a wound healing agent. The observed antioxidant activity of PLE may largely be attributed to its major isolate, geraniin.

ACKNOWLEDGEMENTS

The authors express their profound gratitude to Mr. Thomas Ansah of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana for his technical assistance and Mr. Eric Gyebi for collecting the plant material. Again, the authors acknowledge the technical support of Mr. Francis Amankwah of the Department of Pharmaceutics (Microbiology Section), KNUST, Kumasi, Ghana.

REFERENCES

1. Kadenbach B, Ramzan R, Vogt S. Degenerative diseases, oxidative stress and cytochrome c oxidase function. Trends Mol Med 2009; 15:139-47.
2. Fang YZ, Yang S, Wu G. Free radicals, antioxidants, and nutrition. J Nutr 2002; 18:872-9.
3. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 2009; 7:65-74.
4. Fridovich I. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? Ann NY Acad Sci 1999; 893:13-8.
5. Gokhale SB, Kokate CK, Purohit AP. A Text Book of Pharmacognosy. Nirali Prakshan, Pune, India, pp 345-8, 1993.
6. Narayanaswamy N, Balakrishnan KP. Evaluation of some medicinal plants for their antioxidant properties. Int J Pharm Tech Res 2011; 3:381-5.
7. Sen S, Chakraborty R. The role of antioxidants in human health. In: Silvana A, Hepel M (eds) Oxidative Stress: Diagnostics, Prevention, and Therapy, ACS symposium series, Washington DC: American Chemical Society, pp 1-37, 2011.
8. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: impact on human health. Pharmacog Rev 2010; 4:118-26.
9. Siwik DA, Pagano PJ, Colucci WS. Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. Am J Physiol Cell Physiol 2001; 280:C53-60.
10. Rodriguez PG, Felix FN, Woodley DT, Shim EK. The role of oxygen in wound healing: a review of the literature. Dermatol Surg 2008; 34:1159-69.
11. Nunes XP, Silva FS, Almeida JR, Barbosa Filho JM, de Lima JT, de Araujo Ribeiro LA, Junior LJ. Biological oxidations and antioxidant activity of natural products. In: Rao V (ed) Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health, INTECH Open Science 2012; doi:10.5772/26956.
12. Gulcin I. Antioxidant activity of food constituents: an overview. Arch Toxicol 2012; 86:345-91.
13. Kavitha O, Thampan RV. Factors influencing collagen biosynthesis. J Cell Biochem 2008; 104:1150-60.
14. Shukla A, Rasik AM, Patnaik GK. Depletion of reduced glutathione, ascorbic acid, vitamin E and antioxidant defence enzymes in a healing cutaneous wound. Free Radical Res 1997; 26:93-101.
15. Keller U, Kumin A, Braun S, Werner S. Reactive oxygen species and their detoxification in healing skin wounds. J Invest Dermatol Symp Proc 2006; 11:106-11.

16. Agyare C, Asase A, Lechtenberg M, Niehues M, Deters A, Hensel A. An ethnopharmacological survey and *in vitro* confirmation of ethnopharmacological use of medicinal plants used for wound healing in Bosomtwi-Atwima-Kwanwoma area, Ghana. J Ethnopharmacol 2009; 125:393-403.
17. Agyare C, Lechtenberg M, Deters A, Petereit F, Hensel A. Ellagitannins from *Phyllanthus muellerianus* (Kuntze) Exell.: Geraniin and furosin stimulate cellular activity, differentiation and collagen synthesis of human skin keratinocytes and dermal fibroblasts. Phytomed 2011; 18:617-24.
18. Boakye YD, Agyare C, Ayande PG, Asiamah EA, Titiloye NA. Wound healing activity of geraniin and aqueous leaf extract of *Phyllanthus muellerianus* (Kuntze.) Exell (Euphorbiaceae). Planta Med 2014; 80:WS20.
19. Boakye YD, Agyare C, Hensel A. Anti-infective properties and time-kill kinetics of *Phyllanthus muellerianus* and its major constituent, geraniin. Med Chem (Los Angeles) 2016; 6:95-104.
20. Boakye YD, Agyare C, Abotsi WK, Ayande PG, Ossei PP. Anti-inflammatory activity of aqueous leaf extract of *Phyllanthus muellerianus* (Kuntze) Exell. and its major constituent, geraniin. J Ethnopharmacol 2016; 187:17-27.
21. Morton JJ, Malone MH. Evaluation of vulneray activity by an open wound procedure in rats. Arch Int Pharmacodyn Ther 1972; 196:117-26.
22. Agyare C, Dwobeng AS, Agyepong N, Boakye YD, Mensah KB, Ayande PG, Adarkwa-Yiadom M. Antimicrobial, Antioxidant, and Wound Healing Properties of *Kigelia africana* (Lam.) Beneth. and *Strophanthus hispidus* DC. Adv Pharmacol Sci 2013; 2013:692613.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54
24. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; 247:3170-5.
25. Aebi H. Catalase *in vitro*. Methods Enzymol 1984; 105:121-6.
26. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 1982; 78:206-9.
27. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 1981; 22:867-80.
28. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968; 125:189-98.
29. Susanti D, Sirat HM, Ahmad F, Ali RM, Aimi N, Kitajima M. Antioxidant and cytotoxic flavonoids from the flowers of *Melastoma malabathricum* L. Food Chem 2007; 103:710-6.
30. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 1999; 299:15-27.
31. Slinkard K, Singleton VL. Total phenol analysis: automation and comparison with manual methods. Am J Enol Vitic 1977; 28:49-55.
32. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem 1999; 269:337-41.
33. Fridovich I. The biology of oxygen radicals. Science 1978; 201:875-80.
34. Ponrasu T, Subamekala MK, Ganeshkumar M, Suguna L. Role of *Annona squamosal* on antioxidants during wound healing in streptozotocin nicotinamide induced diabetic rats. J Pharmacol Phytochem 2013; 2:77-84.
35. Mavelli I, Rigo A, Federico R, Ciriolo MR, Rotilio G. Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. Biochem. J 1982; 204:535-40.
36. Kusvuran S, Ellialtioglu S, Yasar F, Abak K. Antioxidative enzyme activities in the leaves and callus tissues of salt-tolerant and salt-susceptible melon varieties under salinity. Afr J Biotech 2014; 11:635-41.
37. Scandalios JG. The antioxidant enzyme genes Cat and Sod of maize: regulation, functional significance, and molecular biology. Isozymes Curr Top Biol Med Res 1987; 14:19-44.
38. Mikhal'chik EV, Titkova SM, Anurov MV, Ettinger AP, Korkina LG. Protective effect of complex antioxidant preparation containing vitamins and amino acids in rats with burn trauma complicated by endotoxemia. Bull Exp Biol Med 2006; 141:688-90.
39. McDaniel JC, Belury M, Ahijevych K, Blakely W. Omega-3 fatty acids effect on wound healing. Wound Repair Regen 2008; 16:337-45.
40. Suchithra TV, Zuhara KF. Myeloperoxidase activity in infection complicated and uninfected diabetic patients. Indian J Biochem Biophys 2008; 45:179-83.
41. Kothari N, Keshari RS, Bogra J, Kohli M, Abbas H, Malik A, Dikshit M, Barthwal MK. Increased myeloperoxidase enzyme activity in plasma is an indicator of inflammation and onset of sepsis. J Crit Care 2010; 26:435.e1-7.
42. Wang P, Peng X, Wei ZF, Wei FY, Wang W, Ma WD, Zu YG. Geraniin exerts cytoprotective effect against cellular oxidative stress by upregulation of Nrf2-mediated antioxidant enzyme expression via PI3K/AKT and ERK1/2 pathway. Biochim Biophys Acta 2015; 1850:1751-61.
43. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005; 53:1841-56.
44. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005; 53:4290-302.
45. Motalleb G, Hanachi P, Kua SH, Fauziah O, Asmah R. Evaluation of phenolic content and total antioxidant activity in *Berberis vulgaris* fruit extract. J Biol Sci 2005; 5:648-53.
46. Jain A, Soni M, Deb L, Jain A, Rout SP, Gupta VB, Krishna KL. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. leaves. J Ethnopharmacol 2008; 115:61-6.
47. Belguidoum M, Dendougui H, Kendour Z. *In vitro* antioxidant properties and phenolic contents of *Zygophyllum album* L. from Algeria. J Chem Pharm Res 2015; 7:510-4.
48. Okudu T, Yoshida T, Hatano T. Food phytochemicals for cancer prevention II. In Ho CT, Osawa T, Huang MT, Rosen TR (eds), Chemistry and Anti-Oxidative Effects of Phenolic Compounds from Licorice, Tea and Compositae and Labiateae Herbs, Washington DC, USA, pp 132-143, 1994.
49. Tepe B, Sokmen M, Akpulat HA, Sokmen A. Screening of the antioxidant potentials of six Salvia species from Turkey. Food Chem 2006; 95:200-4.
50. Kahkonen M, Kylli P, Ollilainen V, Salminen JP, Heinonen M. Antioxidant activity of isolated ellagitannins from red raspberries and cloudberries. J Agric Food Chem 2012; 60:1167-74.
51. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem 2001; 49:5165-70.
52. Hodzic Z, Pasalic H, Memisevic A, Srabovic M, Saletovic M, Poljakovic M. The influence of total phenols content on antioxidant capacity in the whole grain extracts. Eur J Sci Res 2009; 28:471-7.
53. Guo C, Yang J, Wei J, Li Y, Xu J, Jiang Y. Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. Nutr Res 2003; 23:1719-26.
54. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Biol Med 2001; 30:1191-212.
55. Oszmianski J, Wojdylo A, Nowicka P, Teleszko M, Cebulak T, Wolanin M. Determination of phenolic compounds and antioxidant activity in leaves from wild *Rubus* Species. Molecules 2015; 20:4951-66.
56. Zhang LL, Lin YM. Antioxidant tannins from *Syzygium cumini* fruit. Afri J Biotechnol 2009; 8:2301-9
57. Shon MY, Kim TH, Sung NJ. Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus of Hymenochaetaceae*) extracts. Food Chem 2003; 82:593-7.
58. Minussi RC, Rossi M, Bologna L, Cordi L, Rotilio D, Pastore GM, Duran N. Phenolic compounds and total antioxidant potential of commercial wines. Food Chem 2003; 82:409-16.
59. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem 2005; 91:571-7.
60. Yang JH, Tseng YH, Lee YL, Mau JL. Antioxidant properties of methanolic extracts from monascal rice. Food Sci Tech 2006; 39:740-7.
61. Khan RA, Khan MR, Sahreen S, Ahmed M. Assessment of flavonoids contents and *in vitro* antioxidant activity of *Launaea procumbens*. Chem Cent J 2012; 6:43.

Boakye YD, Agyare C, Dapaah SO. *In vitro* and *in vivo* antioxidant properties of *Phyllanthus muellerianus* and its major constituent, geraniin. Oxid Antioxid Med Sci 2016; 5(2): ?-?. DOI: 10.5455/oams.290616.or.099

Conflict of Interest: None declared