Oxidants and Antioxidants in Medical Science

**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.

**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 believed 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 nontoxic antioxidants agents to prevent the progression of oxidant implicated chronic diseases [8].

Reactive oxygen species, such as superoxide anion (O$_2^-$), hydroxyl radicals (OH·), singlet oxygen ($^{1}O_{2}$) and hydrogen peroxide (H$_2$O$_2$), 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 stimulatory effect on rate of wound closure, increased 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 25°C, 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/Etc/C/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:

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

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 ml of homogenate, 130 ml of 50 mM potassium buffer (pH 7) and 65 ml of 10 mM H₂O₂ was added. The blank was a mixture of 65 ml of the potassium phosphate and 130 ml of homogenate. The concentration of H₂O₂ was calculated from the absorbance using the formula given below, where 59.4 mol/cm³ is the molar extinction coefficient for H₂O₂:

\[
[H₂O₂ \text{mM}] = \frac{\text{Absorbance}_{240\text{nm}} \times 1000}{59.4 \text{mol/cm}^3}
\]

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 ml of 0.1 M phosphate buffer (pH 6), 20 ml of 0.01 M H₂O₂, 40 ml of 0.02 M o-dianisidine (freshly prepared) and 110 ml of deionized water was added. Then, 10 ml of the homogenate was added to make a final volume of 200 ml. 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 H₂O₂ was added to the reaction mixture. The blank had all components other than the homogenate. The reaction was initiated by adding H₂O₂. The decrease in absorbance at 290 nm (extinction coefficient: 2.8 mM⁻¹cm⁻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⁻¹cm⁻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 5 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:

\[
\% \text{Inhibition} = \frac{(\text{Absorbance}_{\text{DPPH}} - \text{Absorbance}_{\text{sample}}) \times 100}{\text{Absorbance}_{\text{DPPH}}}
\]
**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$_2$CO$_3$ 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:

\[
TAE = \frac{(\text{Absorbance}_{\text{sample}} - C)}{m}
\]

(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$_2$SO$_4$, 28 mM Na$_2$PO$_4$ and 4 mM (NH$_4$)$_2$MoO$_4$). 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:

\[
\alpha-\text{TE} = \frac{(\text{Absorbance}_{\text{sample}} - C)}{m}
\]

(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.*
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 enzyme 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
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\textsubscript{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\textsubscript{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).
Table 1. IC₅₀ of *Phyllanthus muellerianus* extract, geraniin and α-tocopherol

<table>
<thead>
<tr>
<th>Extract/compound</th>
<th>IC₅₀ (µg/ml ± SEM)</th>
<th>IC₅₀ (µM ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLE</td>
<td>0.12 ± 0.1</td>
<td>-</td>
</tr>
<tr>
<td>Geraniin</td>
<td>1.85 ± 0.13</td>
<td>1.94 ± 0.13</td>
</tr>
<tr>
<td>α-tocopherol (vitamin E)</td>
<td>0.03 ± 0.04</td>
<td>0.08 ± 0.05</td>
</tr>
</tbody>
</table>

PLE: aqueous extract of the aerial parts of *P. muellerianus*. IC₅₀, concentration that causes 50% inhibition of DPPH free radical; SEM, standard error of the mean.

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

<table>
<thead>
<tr>
<th>Extract</th>
<th>Tannic acid equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLE</td>
<td>39.97 ± 0.07 mg/g</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Extract/compound</th>
<th>α-tocopherol equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLE</td>
<td>179.8 ± 0.01 mg/g</td>
</tr>
<tr>
<td>Geraniin</td>
<td>740.8 ± 0.03 mg/g</td>
</tr>
</tbody>
</table>

PLE: aqueous extract of 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 for maintaining skin integrity.
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 as glutathione [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 glutathione. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferri 

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 results. 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 \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \) and \( \text{OH}^- \) 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.

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Conflict of interest: None declared