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Phytochemical Composition and Antioxidant Property of *Mandillo*, *Crassocephalum macropappum* (Sch.Bip. ex. A.Rich.) S.Moore

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ABSTRACT: The object of this study was to analyze the major bioactive components and to evaluate biological activity of *Mandillo* [*Crassocephalum macropappum* (Sch.Bip. ex. A.Rich.) S. Moore], an Ethiopian endemic herbaceous plant. The stem, leaf, and aerial parts of this plant were separately extracted using different solvents before which various biological assays were performed. The ethanolic extract of aerial part showed the highest total phenolic and flavonoid contents (101.48 mg gallic acid equivalents/g and 293.25 mg quercetin equivalent/g, respectively). Interestingly, a phytochemical screening assay revealed the presence of saponins, tannins, anthraquinones, steroids, terpenoids, and flavonoids in the aerial part. The aerial part was also shown to have a strong 2,2-diphenyl-1-picryl hydrazyl scavenging potential ($IC_{50} \leq 100$ μg/mL) and a promising protective activity against oxidative DNA damage. Thus, the results of the present study reveal *Mandillo* contains highly bioactive components, and these properties may be as an antioxidant and to prevent oxidative DNA damage.

Keywords: antioxidant activity, *Mandillo*, phytochemical composition, DNA damage

INTRODUCTION

Human health may be impacted by chemical substances presents in plants, which produce physiological actions on the human body (Ooi et al., 2012). The most important of these plant bioactive constituents are alkaloids, tannins, flavonoids, phenolic compounds, saponins, anthraquinones, glycosides, steroids, and cardiac glycoside, in addition to other bioactive compounds, have a large importance in human drugs and food (Oloyede and Ogunlade, 2013; Wu et al., 2009).

According to Proestos et al. (2013), the use of certain plant products in food processing is recommended to minimize the undesirable effects of synthetic food preservatives in human health. Aromatic plants are well known as natural food preservatives due to their antioxidant and antimicrobial properties (Wu et al., 2009), which arises from their high phenolic contents and prevent food degradation (Alsabri et al., 2012; Proestos et al., 2013). A strong positive correlation between antioxidant activity and the contents of phenolic acids was also studied by Vladimir-Knežević et al. (2011); this study showed that antioxidants can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals. Antioxidants may also play an important role in human health, through adding flavor to food, without the addition of extra ingredients such as excess fat or salt.

In Ethiopia, different plant parts are used as food preservatives, or flavoring agents to improve organoleptic properties. The stem of *Mandillo* [*Crassocephalum macropappum* (Sch.Bip. ex. A.Rich.) S. Moore] is a herb added to *Enset* fermentation processes, believed to shorten the fermentation period, to improve sensory qualities, and to increase the shelf-life of *Kocho*, which has been in use by Shekacho society for a long time. Despite its traditional use as a food preservative, there are no prior data on phytochemical profile and antioxidant activity of *Mandillo*. This study therefore aimed to evaluate the phytochemical content and antioxidant of activities of *Mandillo* extracts.

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MATERIALS AND METHODS

Plant material and extraction

Fresh *Mandillo* was collected on November 2015 at Masha, Sheka, Southern Nation Nationalities and Peoples Regional State of Ethiopia. The plant samples were grouped into stem, leaf, and aerial parts. The three parts were dried at room temperature under shade for three days. The dried samples were powdered in a blender. Moisture contents of fresh and dried samples were analyzed in a drying oven (DHG-9055A, Zenith Lab Inc., CA, USA) at 105°C until a constant weight was obtained. The stock samples were kept in air tight bottles at 4°C for future extraction (Sibanda and Okoh, 2008).

The plant materials were extracted following the procedure described by Sultana et al. (2009) and Eom et al. (2008). The powder of the parts of *Mandillo* were extracted three times by incubating 100 g sample in 1,000 mL of 70% ethanol on an orbital shaker at 130 rpm at 40°C for 24 h. The extracts were filtered using Whatman No. 1 filter paper (Sigma-Aldrich Co., St. Louis, MO, USA), and concentrated by a rotary evaporator (RE300; Wolf Laboratories Ltd., York, UK) at 40°C. The crude extracts were kept at 4°C for further study.

Qualitative phytochemical analysis

Qualitative phytochemical analysis was carried out using the methods described by Tiwari et al. (2011), Akinyemi et al. (2005) and Sofowora (1993). Extracts were dissolved in diluted hydrochloric acid and then filtered. The presence of alkaloids was examined by Wagner's test. Filtrates were treated with iodine in potassium iodide (Wagner's reagent), following which a brown to reddish precipitate was not observed. Extracts were treated with a few drops of lead acetate solution, following which yellow precipitate confirmed the presence of flavonoids. Extracts (10 mg) were dissolved in 1 mL of chloroform and 1 mL of acetic anhydride, followed by the addition of 2 mL of concentrated H_2SO_4; formation of a reddish violet color indicated the presence of terpenoids. Extracts (0.5 g) were shaken with 2 mL of water, following which the formation of foam persisting for 10 min indicated the presence of saponins. Gelatin solution (1%) containing sodium chloride was added to the extracts, following which the formation of white precipitate indicated the presence of tannins. Extracts (5 mL) were hydrolyzed with concentrated H_2SO_4 and extracted with benzene. The extract was treated with 1 mL of diluted ammonia to give a rose to pink coloration, suggestive of the positive response for anthraquinones. Extracts (1 mL) was dissolved in 10 mL of chloroform and an equal volume of concentrated sulphuric acid (added via sides of the test tube), following which the upper layer turned red and the sulphuric acid layer turned a yellow with green fluorescence. This indicated the presence of steroids.

Determination of total phenolic contents

Total phenolic contents were determined according to the Folin-Ciocalteu's procedure as described by Alimpić et al. (2014). The stock *Mandillo* extract solutions were diluted with 0.1 mg/mL in methanol, and 100 μL was added to test tubes in triplicates. Folin-Ciocalteu's reagent (Sigma-Aldrich Co.) (1 mL; diluted 1:10) was dissolved in deionized water and added to each test tube. Sodium carbonate (1 mL; 7.5%) reagent was then added to the mixture. The tubes were vortexed and incubated at 25°C for 90 min. Absorbance was measured at 765 nm by an UV/VIS Lambda 950 (Perkin Elmer, Liantrisant, UK). The total phenolic content was determined using a standard curve of gallic acid (SD Fine-Chem Ltd., Mumbai, India), with concentrations ranging from 0.001~1.00 mg/mL dissolved in methanol. The total phenolic content was calculated using the following equation: $y=11.25x+0.068$; $R^2=0.998$. The values were expressed as gallic acid equivalents (GAE) in milligrams per gram of dry material (mg GAE/g).

Determination of total flavonoid contents

Total flavonoid contents were determined using the method described by Sultana et al. (2009). Plant extract stock solutions (1 mg/mL) were prepared, and 2 mL of each solution was added to 2 mL of 2% aluminum chloride. Methanol (2 mL) was used as a blank. The absorbance of each sample was measured at λ_{max} 415 nm following incubation for 30 min at room temperature, comparative to the blank sample. The total flavonoid content was determined following the equation: $y=23.95x+0.111$; $R^2=0.991$, through using a quercetin standard curve (Sigma-Aldrich Co.) ranging from concentrations of 0.001~40 μg/mL dissolved in 2 mL of 2% aluminum chloride, in triplicate. The result was expressed as milligram of quercetin equivalent (QE) per gram of dry samples (mg QE/g).

Evaluation of antioxidant scavenging capacity by 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay

Antioxidant scavenging capacity was analyzed according to the procedure described by Oloyede and Ogunlade (2013), with slight modification. Extracts were mixed with methanol to prepare the stock solution at a concentration of 1 mg/mL. Aliquots of 5, 10, 20, 35, 50, and 60 μL added to test tubes in triplicate. Serial dilution of the solution was carried out by adding 995, 990, 980, 965, 950, and 940 μL of methanol, respectively, to leave a final volume of 1 mL. The final concentrations were calculated as 0.005, 0.01, 0.02, 0.035, 0.05, and 0.06 mg/mL, respectively. Two mL of freshly prepared DPPH solution (0.06% w/v) in methanol was added to each test tube.

The reaction mixtures and the control (2 mL; DPPH in methanol) were vortexed and incubated at room temperature in the dark for 30 min. The absorbance was measured at λ_{max} 517 nm using a spectrophotometer. Ascorbic acid was used as the reference compound. The control was prepared by adding 1,000 μL of methanol to 2 mL of DPPH solution. The DPPH radical scavenging capacity of the plant extracts was calculated as follow:

$$\text{DPPH radical scavenging capacity (\%)} = \frac{\text{Absorbance of control} - \text{absorbance of test}}{\text{Absorbance of control}} \times 100$$

Evaluation of DNA-protective activity

The DNA protective activity of *Mandillo* extracts was evaluated relative to DNA-damaging chemicals according to the method of Kim et al. (2012) as cited by Rafiquzzaman et al. (2013). Hydroxyl radicals were generated using a mixture of 30 μL of ascorbic acid (10 mM final concentration) and 1 μL of copper sulfate (II) (1 mM final concentration). Bacteriophage λ DNA (40 μL, 0.1 μg/mL) was exposed to the solution in both the absence and presence of *Mandillo* extract (100 μL, 1 mg/mL). The mixture was incubated at 37°C for 1 h after which the samples were loaded onto 1% agarose gels and the fragments were separated by agarose gel electrophoresis (Rafiquzzaman et al., 2013). The result was compared to the control group.

Statistical analyses

All experiments were carried out in triplicate and the results were expressed as mean±standard deviation (SD). Statistical analyses were carried out with SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) by using one-way ANOVA followed by Duncan's multiple range test ($P<0.05$).

RESULTS AND DISCUSSION

Phytochemical secondary metabolites in *Mandillo* extracts

We conducted a preliminary qualitative phytochemical analysis to identify the secondary metabolites present in *Mandillo* extracts. Phytochemical analysis revealed that ethanolic extracts of *Mandillo* leaf, stem, and aerial parts contained numerous bioactive compounds, including anthraquinones, flavonoids, saponins, steroids, tannins, and terpenoids (Table 1). These secondary metabolites possess many biological properties, such as antioxidant, antitumor and anti-inflammatory activities, suggesting that *Mandillo* may have many medicinal applications (Senguttuvan et al., 2014). However, alkaloids (an abundant secondary plant metabolite) were not detected in *Mandillo* extracts (Chen et al., 2014; Compean and Ynalvez, 2014).

Table 1. Phytochemical components determined in the ethanol extract of *Mandillo* part

Chemical groups	Test results		
	Stem	Leaf	Aerial root
Alkaloids	−	−	−
Anthraquinones	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Steroids	+	+	+
Tannins	+	+	+
Terpenoids	+	+	+

−, not identified; +, qualitatively identified.

Quantification of total phenolic and flavonoid compounds in *Mandillo* extracts

The total phenolic and flavonoid contents of *Mandillo* ethanolic extracts were determined (Table 2). Aerial extract of *Mandillo* contained the highest phenolic contents (101.48±9.11 mg GAE/g) and flavonoid content (293.25 ±3.48 mg QE/g) of the three parts, followed by leaf extracts and stem extracts. The phenolic and flavonoid contents of the stem extracts were relatively low compared to those of the aerial and the leaf extracts. The phenolic and flavonoid contents of aerial extract were 2.9-fold and 6.2-fold higher than those of stem extracts, respectively. In contrary to the current report, most plants exhibit higher phenolic and flavonoid contents in their leaves and stems than their roots, indicating that the *Mandillo* plant may be an unique organism with un-known properties (Chai and Wong, 2012; Silva-Beltrán et al., 2015).

Antioxidant scavenging potential of DPPH radical activity

The free radical-scavenging potential of *Mandillo* extracts showed remarkable DPPH radical scavenging abilities compared to the control ascorbic acid (Table 3). Generally, the radical scavenging activity of *Mandillo* extracts were shown to be dose-dependent. Leaf and stem extracts showed moderately higher DPPH scavenging activities than those of aerial extracts at a concentration of 0.05～0.10 mg/mL, whereas no significant differences were observed at concentrations above 0.50 mg/mL.

Extracts containing higher phenolic and flavonoid contents exhibit higher antioxidant effects since polypheno-

Table 2. Total phenolic and flavonoid contents of *Mandillo* ethanolic extracts

Sample type	Total phenol content (mg GAE/mg)	Total flavonoid content (mg QE/g)
Stem	35.47±3.20[a]	47.60±4.42[a]
Leaf	95.26±2.49[b]	271.54±7.86[b]
Aerial	101.48±9.11[b]	293.25±3.48[c]

Means with different letters (a-c) within each column indicate significant differences by Duncan's multiple range test ($P<0.05$).

Table 3. DPPH scavenging activity of *Mandillo* ethanolic extracts

Conc. (mg/mL)	DPPH scavenging potential (%)			
	Ascorbic acid	Stem	Leaf	Aerial root
0.05	96.11±0.10^{aD}	65.81±0.02^{aC}	63.13±0.07^{aB}	52.09±0.03^{aA}
0.10	97.48±0.30^{bD}	72.67±0.03^{bB}	78.22±0.20^{bC}	62.15±0.04^{bA}
0.20	97.54±0.07^{bD}	78.93±0.11^{cA}	93.09±0.02^{fC}	92.25±0.03^{cB}
0.35	97.59±0.01^{bD}	89.20±0.05^{dA}	92.30±0.04^{eB}	93.85±0.05^{fC}
0.50	97.70±0.20^{bC}	93.62±0.03^{eB}	91.61±0.02^{dA}	93.66±0.03^{eB}
0.60	97.83±0.08^{bC}	93.52±0.01^{eB}	91.34±0.04^{cA}	93.42±0.02^{dB}

Means with different small letters (a-f) within each column and capital letters (A-D) within each row indicate significant differences by Duncan's multiple range test (P<0.05).

lic compounds possess antioxidant activity (Eom et al, 2011; Jiménez-Escrig et al., 2001). In contrary to these reports, *Mandillo* extracts in this study exhibited different patterns. In low concentrations (0.05 to 0.1 mg/mL), stem extracts showed higher antioxidant activities than those of aerial extracts, despite the stem extract contained the lowest phenolic and flavonoid contents. These results suggest that the *Mandillo* extracts may possess a novel compound acting as a major antioxidant substance. There are many positive correlations between phenolic contents and antioxidant capacity (DPPH method), whereas a few studies also report that this is not the case. Reihani and Azhar (2012) previously reported that there is no significant correlation between antioxidant activity and phenolic compounds, which may be due to steric hindrance or the presence of other reducing agents.

DPPH radical scavenging capacity is widely used to evaluate antioxidant activity of natural extracts (Chen et al., 2014; Oloyede and Ogunlade, 2013). In this study, *Mandillo* extracts exhibited significant scavenging potential upon DPPH radicals. All *Mandillo* extracts showed over 90% of DPPH radical scavenging effects at concentrations of 0.5~0.6 mg/mL when comparable to those of the natural standard antioxidant, ascorbic acid. Thus, these extracts possess strong antioxidative activities through inhibiting or preventing the oxidation of free radicals as a result through radical scavenging (Vladimir-Knežević et al., 2011).

DNA-protective activity

The antioxidant activities of green plants and herbs have been well studied by a number of investigators (Alsabri et al., 2012; Proestos et al., 2013; Wu et al., 2009). As indicated in Table 3, ethanolic extracts of *Mandillo* show incredible DPPH scavenging capacities. Hence, in this study, we evaluated the DNA-protective effects of *Mandillo* extracts against oxidation induced by oxidative stress. Among of *Mandillo* extracts, the aerial extract was chosen for the further study, through considering the phenolic and flavonoid contents and antioxidant activity of each extract. As expected, λ DNA were damaged by oxidative stress generated by Cu (II)-ascorbic acid (Fig. 1, lane 2). However, λ DNA treated with *Mandillo* extracts (Fig. 1, lane 3) showed clear bands, similar to the λ DNA control (Fig. 1, lane 1) despite exposure of the λ DNA to oxidative stress. According to Sultana et al. (2009), dietary supplementation of flavonoid compounds reduces oxidative damage to cell membrane lipids, proteins, and nucleic acids. This protective mechanism is due to the strong quenching property of free radicals, which is in line with the DNA protective effect recorded in the present study.

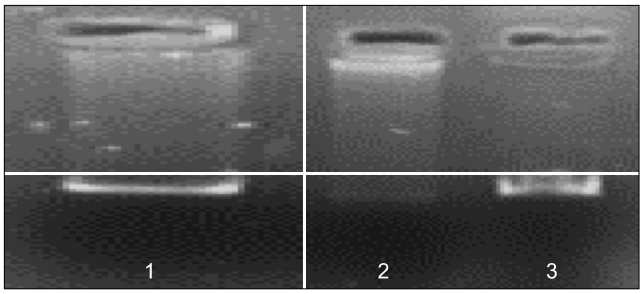

Fig. 1. Agarose gel electrophoresis showing separation of damaged DNA and the protective effect of *Mandillo* (aerial part) ethanolic extracts. The lanes are as follows: lane 1, λ DNA (control); lane 2, λ DNA treated with Cu (II)-ascorbic acid; lane 3, λ DNA treated with Cu (II)-ascorbic in the presence of the *Mandillo* aerial extract.

In this study, we analyzed the major photochemical compounds of *Mandillo* extracts, which is used as a natural food preservative in Ethiopia. We also evaluated the antioxidant capacity of *Mandillo*, including its DPPH radical scavenging activity and ability to protect DNA from damage caused by oxidative stress. Our results strongly suggest that this African herb possesses strong antioxidant activity, which may be useful as an antioxidant food or food ingredient.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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