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In-Vitro Assessment of Antioxidant and Antimicrobial Activities of Methanol Extracts of Six Wound Healing Medicinal Plants

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Abstract

In this study, quantitative values of antioxidant activity of crude methanolic extracts of five Wound healing medicinal *plants* (*Amaranthus spinosus*, *Anogeissus leiocarpus*, *Spondia monbin*, *Corchorus olitorius,* and *Mallotus oppositifolia*) were investigated. The investigation used DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical as a substrate and Ferric reducing antioxidant power (FRAP) assay to determine both scavenging ability and the reducing properties. Antioxidant was further analysed quantitatively for flavonoid content, total phenolic content in the crude methanolic extracts using spectrophotometric assay.

The result showed that all plants exhibited scavenging ability and strong reducing activity although the ability differed markedly among the various plant samples. The highest scavenging ability (% inhibition) was exhibited by *A. leiocarpus* (95.86 ± 0.1) followed by *C. olitorius* (94.19 ± 0.06) while the lowest was from *A. spinosus* (40.87±2.5). The reducing power was also highest in *A. leiocarpus* followed by *S. monbin*; while *A. spinosus* showed the least reducing power. In quantitative analysis, again *A. leiocarpus* was found to have the highest phenolic content (1294.81± 3.0 mg/g) with *A. spinosus* recording the least phenol and flavonoid content.

The crude methanol extracts were also screened for their antimicrobial activity against four common pathogenic microorganisms (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae and Citrobacter sp.*) associated with wound infection by well diffusion method. All the extracts were found to inhibit the growth of both gram (+) and gram (-) bacteria organisms tested.

Keywords: Radical scavenging effect, phenolic compounds, antioxidant activity

1. Introduction

The quest for plants with medicinal properties continues to receive attention for a complete range of biological activities. Currently, large and ever expanding global population prefers the use of natural products in treating and preventing medical problems including wound healing.

Wounds are the result of injuries to the skin that disrupt the soft tissue. Wounds affect physical and mental health of millions of patients and impose significant cost on patients.

Wound infection is one of the most commonly encountered and clinical impediments to wound healing.

In the past, commercial antibiotics were successful to fight these infections however; the future effectiveness of antimicrobial therapy is somewhat in doubt due to the emerging problem pose by the resistance of these microorganisms to the available antibiotics. Several plants and herb species used traditionally have potential antimicrobial and antiviral properties and this has raised the optimism of scientists about the future of phyto-antimicrobial agents (Gandhiraja et al. 2009).

Many plants and herbs contain a wide variety of phenolic compounds such as flavonoids thus, act potentially as antioxidants to scavenge free radicals and inhibit lipid peroxidation (Kumawat et al., 2012).

The enhanced wound healing potency of various herbal extracts may be attributed to antioxidants and the antimicrobial property of the phytoconstituents present. Hence the therapeutic benefit of medicinal plants is often attributed to their antioxidant property (Nayak et. al., 2006).

Antioxidants are noted to significantly prevent tissue damage that stimulates wound healing process. Botanicals with antioxidant or free radical-scavenging activity thus can play a significant role in healing of wounds (Kamath et. al., 2003).

There has been an increasing interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury. As a result, many vegetables, fruits and many other plant species are already exploited commercially either as antioxidant additives or a nutritional supplements (Schuler, 1990) or have been

investigated in the search for novel antioxidants (Chu, 2000; Koleva et al., 2002). In similar fashion, this study was designed to examine the antioxidant potential and antimicrobial abilities of five selected plants namely: *Amaranthus spinosus, Anogeissus leiocarpus, Spondia monbin, Corchorus olitorius* and *Mallotus oppositifolia*, used in the Kpando Municipality as folkloric medicine in the treatment of wound and wound infection.

Amaranthus spinosus Linn. *b*elongs to Amaranthaceae family. It is commonly known as Prickly amaranth or Pig weed and is an annual or perennial herb, native to tropical America and found throughout India as a weed in cultivated as well as fallow lands. It is a common weed of waste places, roadsides and pathsides and near rivers in Ghana (Dokosi, 1998). The plant has a long history of usage in traditional medicine against various ailments around the world. In Ayurvedic and other traditional medicinal practices the plant has been used against diseases like bilious complaints, cough, worms, jaundice, fever, inflammation, rheumatism, anaemia and vermifuge.

Mallotus oppositifolius (Geiseler) Müll. Arg., (Euphorbiaceae), is an endemic shrub from tropical Africa forests and savannas. The plant is widely used in folkloric medicine against infections, intestinal worms and malaria. Different parts of the plant have also been used to demonstrate anti-inflammatory, antioxidant, antidiarrheic, antibacterial, antifungal and antitrypanosomal properties (Kabran et al, 2012).

Spondias mombin is a tree to 30 m high; bark greyish-brown, thick, rough, often deeply grooved, with blunt, spinelike projections; trunk with branches 2-10 m above ground level to form a spreading crown up to 15 m in diameter and forming an open or densely closed canopy, depending on the vigour of the individual; seedlings with deep taproot, probably persisting in mature tree, which also possesses a shallower root system near the surface.

S. mombin is native to Central America and northern South America and can be found under semi-wild cultivation in most lowland areas of the American tropics. *S. mombin* occurs in a great variety of humid tropical climates, often in secondary vegetation derived from evergreen lowland forest or semi-deciduous forest. It has been introduced to most tropical locations and performs well under varied conditions. The tree is tolerant of most soil types and rainfall patterns.

Both bark and flowers are used in folk medicine to make cure-all teas for digestive tract ailments, lower back pain, rheumatism, angina, sore throat, malarial fever, congestion, diarrhoea, urethritis, metrorrhagia, and as contraceptive. Plant extracts exhibit antibacterial properties, and a decoction of the bark or root bark is considered antiseptic. The roots are regarded as febrifugal, and leaf decoctions used for colds, fevers and gonorrhea (Anon, 1986 and FAO, 1982).

Corchorus olitorius belongs to the family Tiliaceae. It has reddish stem. The leaves are alternating, dark green with long petiole. The flowers are bisexual with free petals. Seeds are numerous and seedlings grow with epigeal germination. The plant is found in most West African countries like Ghana, Cote d'ivoire, Nigeria, Benin, just to mention a few (Schippers, 2000). In West Africa, specifically Nigeria, the leaf of *C. olitorius* is used for preparation of a slimy sauce which is found suitable for easy consumption of starchy balls made from cassava, yam or millet.

In East Africa, for instance Kenya, the root scrapings are used to treat toothache and the root decoction is taken as a tonic (Khandakar, 2003).

In Southern Africa, example in Congo, the leafy twigs are used for treatment of cardiovascular diseases. An infusion from the leaves is also taken in Tanzania against constipation (BurKill, 2000).

Anogeissus leiocarpus (DC) Guill and Perr belongs to the Combretaceae family (Common name: Axle-wood tree). *A. leiocarpus* is used medically for the treatment of ascaricide, gonorrhoea, general body pain, blood clots, asthma, coughing and tuberculosis (Mann et al., 2008). The plant is also used in Nigeria as an antimicrobial agent against bacterial infections. The leaves of the plant are used externally as a decoction in the eastern part of Nigeria for the treatment of skin diseases and the itch of psoriasis. The powdered bark is applied to wounds, sores, boils, cysts and diabetic ulcers with good results. The powdered bark has also been mixed with 'green clay' and applied as an unusual face mask for serious blackheads (Mann et al., 2008). The infusion and decoctions are used as cough medicine and the pulped roots applied to wounds and ulcers, the powdered bark is also rubbed to reduced tooth ache on gums (Ibrahim et al., 1997). *A. leiocarpus* is traditionally acclaimed to be effective in treating infectious wounds in man and animals.

2. Materials and method

2.1 Plant materials

All the plant samples except *A. leiocarpus* were collected from Cape Coast in the month of May 2012. *A. leiocarpus* sample was collected from Kpando in June 2012. The plant samples were authenticated by Mr. Agyakwa the curator

at the herbarium, Department of Environmental Science, School of Biological Sciences, UCC where voucher specimens were kept. The plants were collected fresh and then shade dried and powdered.

2.2 Treatment of Plant samples

The powdered plant materials of the various samples; *A. spinosus*, *S. monbin*, *A. leiocarpus, C. olitorius, M. oppositifolius* were extracted successively with methanol in a Soxhlet's apparatus (two days for each plant) and the solvent removed under reduced pressure. The resultant semi solid crude extracts were dried further in a dessicator.

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2.3 *Determination of flavonoid contents*

The aluminum chloride colorimetric method was used to measure the flavonoid content of all plant extracts (Nguyen and Eun, 2011). Extract solution (0.25ml, 1mg/ml) of each plant extract was added to 1.25 ml of distilled water. Sodium nitrite solution (0.075ml, 5%) was then added to the mixture followed by incubation for 5 minutes after which 0.15ml of 10% aluminium chloride was added. The mixture was allowed to stand for 6 min at room temperature before 0.5ml of 1 M sodium hydroxide was finally added and the mixture diluted with 0.275 ml distilled water. The absorbance of the reaction mixture was measured at 510 nm with a UV/VIS spectrophotometer immediately. Quercetin was used as the standard for the calibration curve. Flavonoid contents were expressed as mg quercetin equivalent (QE)/g dry weight (D.W.).

2.4 *Determination of Phenolic content*

Total phenol content was estimated using Folin-Ciocalteu reagent based assay as previously described (McDonald et al., 2001) with little modification. To one ml of each extract (100µg/ml) in methanol, 5ml of Folin -Ciocalteu reagent (diluted ten-fold) and 4 ml (75 g/l) of Na_2CO_3 were added. The mixture was allowed to stand at 20^oC for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer. 1 ml aliquots of 20, 40, 60, 80, 100µg/ml methanolic gallic acid solutions were used as standard for calibration curve. All determinations were performed in triplicate. Total phenol value was obtained from the regression equation: y = 0.00048x + 0.0055 and expressed as mg/g gallic acid equivalent using the formula, C = cV/M; where C = total content of phenolic compounds in mg/g GAE, c = the concentration of gallic acid (mg/L) established from the calibration curve, V = volume of extract (0.5ml) and m = the weight of pure plant methanolic extract (0.052g).

2.5 *Ferric Reducing Antioxidant Power Assay (FRAP)*

The reducing antioxidant power of plant methanolic extracts was determined by the method of Oyaizu (1986). Different concentrations of plant extracts (250 – 1000 ppm) in 1 ml of distilled water were mixed with phosphate buffer (3.0 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^oC for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd). Increased absorbance of the reaction mixture indicates increase in reducing power. Ascorbic acid was used as standard.

2.6 Scavenging activity against 1,1-diphenyl-2-picryl hydrazyl radical (DPPH)

The crude extracts of the different plant were screened for DPPH radical Scavenging activity. DPPH radical scavenging activity was measured according to the method of Braca *et al.*, 2003 and Rajeswara et al., 2012 and Coutinho et al, 2008. Extract solutions were prepared by dissolving 0.05g of dry extract in 50ml of methanol. An aliquot of 2ml of 0.004% DPPH solution in methanol and 1ml of plant extract in methanol at various concentrations (200, 400 and 800ppm) were mixed and incubated at 25°C for 30 min. and absorbance of the test mixture was read at 517nm using a spectrophotometer (T 70 UV-VIS Spectrometer, PG Instruments Ltd) against a DPPH control containing only 1 ml of methanol in place of the extract. The DPPH solution in methanol was prepared daily before the absorbance measurements. DPPH is a purple coloured stable free radical. When reduced it becomes the yellow colored Diphenyl picryl hydrazine. All experiments were performed thrice and the results were averaged. Ascorbic acid was used as a standard (Ramnik *et al.*, 2008). Percent inhibition was calculated using the following expression

% Inhibition = $(A_{blank} - A_{sample} / A_{blank}) \times 100$

Where A_{blank} and A_{sample} stand for absorption of the blank sample and absorption of tested extract solution respectively.

2.7 Antibacterial Activity

2.7.1 Test Microorganisms

Microbial cultures of four different strains of both Gram positive and Gram negative bacteria were used for determination of antibacterial activity. Three standard bacterial strains viz. *Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC4352) and one clinical isolate Citrobacter sp.* From Korle-Bu Teaching Hospital Central Laboratory, Accra (Ghana).

2.7.2 Antimicrobial activity assay

Well diffusion method using Müeller-Hinton agar plates were used to demonstrate the antimicrobial properties of the crude extracts (Forbes et al, 1990). A suspension of the bacteria compared to 0.5 Macfarland standard was seeded on the Mueller-Hinton agar plates. Wells of 6mm in diameter and 2cm apart were punctured in the culture media using sterile cork borers. 80µl of the crude extracts was administered to fullness in each well and the plates were incubated overnight at 37° C. Growth was determined by measuring the diameter of the zone of inhibition. The solvents were used as the negative controls whiles 10µg ampicillin disc (Oxoid) was used as the positive control. The control zones of the solvents were deducted from the zones of inhibition created by the crude extracts. The experiments were carried out in triplicates and results were calculated as mean ± SD.

3. *Statistical analysis*

Microsoft Excel (2007) was used to enter and capture data. Graph and tables were extracted from this data. Data was then exported to SPSS for further analysis. The measured diameter of inhibition for each microorganism was analyzed using one-way analysis of variance (ANOVA). P value < 0.05 was considered as significant. SPSS 16.0 was employed for statistical analysis.

4. Result and Discussion

The plants parts were collected based on the information gathered on the folkloric use by the indigenous people in the Kpando municipality for the treatment of wounds (Table 1).

Table 1: Identity of plant samples used in the research

Plant	Family	Local Name	Plant part used
Amaranthus spinosus	Amaranthaceae	matonui	roots
Spondia monbin	Anacardiaceae	akuko	leaves
Corchorus olitorius	Tiliaceae	ademe	leaves
Anogeissus leiocarpus	Combretaceae	hehe	leaves
Mallotus oppositifolia	Euphorbiaceae	nyeti	leaves

4.1 Antioxidant activity

DPPH radical scavenging activity assay assesses the capacity of the extract to donate hydrogen or to scavenge free radicals. The results showed that all the plants exhibited scavenging ability and strong reducing ability although these abilities differed markedly among the plant species. The highest scavenging ability was found in *A. leiocarpus* (95.86 ± 0.1) followed by *C. olitorius* (94.19 ± 0.06) while the least value was from *A. spinosus* (40.87 ± 2.5) (Table 2). The reducing power was also highest in *A. leiocarpus* followed by *S. monbin*; while *A. spinosus* showed the least reducing power (fig. 1). Again, in quantitative analysis, *A. leiocarpus* was found to have exhibited the highest phenolic content while *A. spinosus* recorded the lowest phenol and flavonoid content. The flavonoid content of the extracts in terms of quercetin equivalent (the standard curve equation: $y = 0.0092x + 0.0249$, $r^2 = 0.985$) were

between 63.16 ± 10.7and 450.23 ± 24.5 mg g^{-1} for *A. spinosus* and *A. leiocarps* extracts respectively (Table 2). Table 2 also shows the contents of total phenols that were measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y = 0.00048x + 0.0055$, $r^2 = 0.9873$). The total phenol content arranged in increasing order is as follows: 48.01 ± 2.0, 477.5 ± 6.2, 540.67 ± 12.6, 698.12 ± 6.4 to 1294.81± 3.0 mg g^{-1} for *A. spinosus*, *C. olitorius*, *M. oppositifolius*, *S. monbin* and *A. leiocarpus* extracts respectively.

From the results obtained the phenolic content generally varied from one plant to the other in the same fashion as the reducing ability and the percentage inhibition of DPPH. The moderate positive correlation ($R^2 = 0.52$) between DPPH radical scavenging and total phenolic content of all the plant extracts supported this. This strengthens the fact that phenolic content of plants contribute directly to their antioxidant properties. The DPPH scavenging capacity of the plant extracts may therefore be partially related to the phenolic compounds present. However, there was very low positive correlation ($R^2 = 0.24$) between phenolic content and flavonoid content. Other phytoconstituents such as amino acids, alkaloids present in the plant could therefore contribute to the overall antioxidant activity. The values recorded for all plants except *A. spinosus* were not significantly different from the standard antioxidant (ascorbic acid). The plant samples thus can be said to be relatively good sources of antioxidants.

4.2 Antimicrobial activity

The antimicrobial activities of the selected plants in terms of the diameter of zone of inhibition are shown in Table 3. The methanolic extract of the plants showed considerable antibacterial activity against the test organisms.

The highest zone of inhibition was found in the methanolic extract of *Corchorus olitorius* against *E. coli*. The maximum zone of inhibition was against *Citrobacter species* for the methanolic extract of *A. leiocarpus*. It is evident from the zones of inhibition that all the plant species have antimicrobial properties and are therefore potentially good sources of antimicrobial substances with a broad spectrum of activities in preventing the growth of all the tested microorganisms. However, *A. leiocarpus* exhibited the least inhibitory activity against all the tested organisms.

The antibacterial activity of all the plants may be indicative of the presence of metabolic toxins or broad spectrum antimicrobial compounds that act against gram +ve and gram –ve bacteria.

5. Conclusion

In conclusion, all the plants analysed exhibited scavenging ability and strong reducing ability with *A. leiocarpus* exhibiting the highest scavenging ability and phenolic content. All the plants showed considerable antibacterial activity against the test organisms. The plant species are therefore potential good sources of antioxidants and antimicrobial substances with a broad spectrum of activities in preventing the growth of all the tested microorganisms.

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Table 2: DPPH free radical scavenging activity, Total phenolic content and flavonoid content of the extracts

Sample	Inhibition (I %)			Phenolic Content (mg of QE/g of extract).	Flavonoid content (mg of QE/g of extract).
	200	400	800		
A. spinosus	40.87 ± 2.5	41.71 ± 0.5	46.57 ± 1.9	48.01 ± 2.0	63.16 ± 11
S. monbin	86.77± 0.1	88.80 ± 1.7	90.12 ± 1.2	698.12 ± 6.4	328.28 ± 24
C. olitorius	88.85± 0.1	93.56± 0.1	94.19±0.06	477.5 ± 6.2	450.22 ± 25
A. leiocarpus	91.15 ± 1.7	91.39 ± 0.9	95.86 ± 0.1	1294.81± 3.0	330.72 ± 29
M. oppositifoius	89.11 ± 0.3	90.07 ± 0.5	91.36 ± 0.6	540.67 ± 13	75.70 ± 3.5
Ascorbic acid	92.75 ± 0.2	93.61 ± 9.4	96.78 ± 1.9	-	-

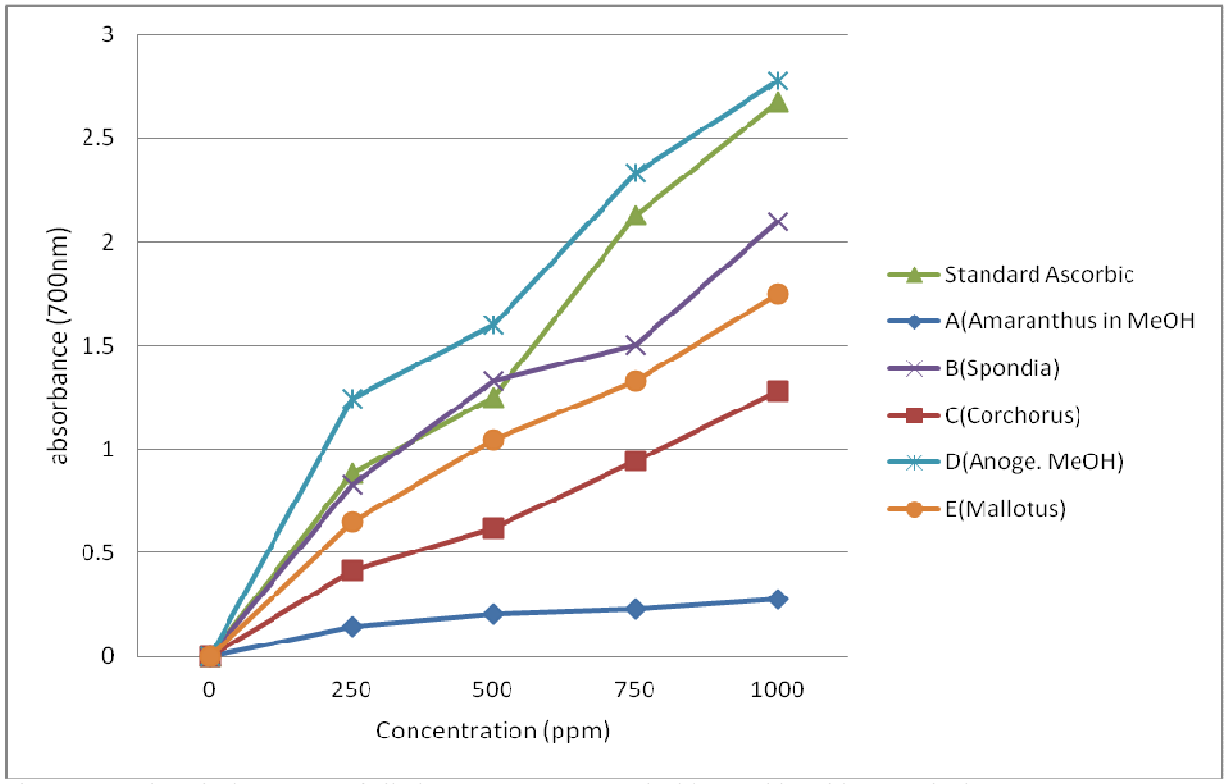

Figure 1: Ferric reducing power of all plant extracts compared with ascorbic acid as standard

Table 3: Antibacterial activities of the various plant extracts.

Organism	Zone of inhibition (mm) of methanolic extracts of plants (1.0 mg/ml)				
	A. spinosus	*S.monbin*	*C. olitorius*	*A. leiocarpus*	*M. oppositifolius*
Staphylococcus aureus.	11.12 ± 0.02^{a}	12.38 ± 0.02^{a}	10.9 ± 0.02^{e}	8.79 ± 0.2^{h}	10.31 ± 0.21^{a}
Klebsiella pneumonia	13.52 ± 0.2^{b}	11.62 ± 0.2^{c}	10.66 ± 0.2^{e}	8.79 ± 0.2^{h}	11.82 ± 0.2^{g}
Citrobacter sp.	10.73 ± 0.2^{a}	13.20 ± 0.2^{d}	11.86 ± 0.2^{f}	10.9 ± 0.2^{i}	11.08 ± 0.2^{j}
Escherichia coli	11.60 ± 0.2^{c}	12.26 ± 0.2^{a}	14.23 ± 0.2^{g}	9.03 ± 0.2^{h}	10.4 ± 0.2^{a}

Values are expressed as the mean ± standard deviation (n = 3). Means with different superscript letters within a column are significantly different ($p < 0.05$).
Inhibition zones 15 mm was declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities.