Research in Pharmaceutical Sciences, May 2016; 11(3): 219-226
Received: June 2015
Accepted: Aug 2015

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Wound-healing and antimicrobial properties of dichloromethane fraction of *Dialium guineense* (Wild) fruit coat

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Abstract

This research established the scientific bases for the folkloric use of the neglected *Dialium guineense* fruit coat in wound and microbial infection management in Nigeria. The phytochemical analysis of the crude extract, fractions and sub-fractions was performed by standard methods. Agar well diffusion protocol was adopted for the antimicrobial assay while the wound healing properties was determined by full thickness skin excision wound model. Phytochemical analysis showed high relative proportion of alkaloids (6.05 ± 0.98 %), saponins (3.91 ± 0.02 %) and tannins (1.86 ± 0.05 %). The only active fraction (DF) and sub-fraction (DF-5) were effective against Gram-positive (inhibition zone diameters, IZDs, 8-10 mm and 11-15 mm) and Gram-negative (IZDs, 15-19 mm and 16-21 mm) bacteria and fungi (6-8 mm) compared with 20-24 mm and 18-19 mm of the standard (ciprofloxacin) respectively. Fifty mg/kg of the DF-5 showed nearly equal percentage wound healing post-surgery days to Cicatrin®. The 50 mg/kg dose of DF and DF-5 showed more than 50 % wound healing at 10th day post-surgery, 50 mg/kg crude extract showed 54 % on day 14 while distilled water showed 56 % wound healing on day 17 with no sign of infection in all animal groups. All the treatments were significantly (P<0.01*)* different from control (distilled water) in wound healing by the 10th and 17th post-surgery days. The studies revealed that the fruit coat, which hitherto was treated as wastes could be explored for antimicrobial and wound healing properties against the backdrop of continually emerging antibiotic resistant strains of microorganisms.

Keywords: Antimicrobial; Wound-healing; *Dialium guineense*; Cicatrin®; Phytochemical

INTRODUCTION

Man's dependence on plants for food ingredients, dietary supplements or nutraceuticals, pharmaceutical and cosmetic products has remained a reference points in modern dietetics, chemotherapy, drug development and cosmetology. This has led scientists to continually search for plant materials that are useful to man. Surprisingly, some unexpected plants or plant materials that can prove very potent in certain ailments, have been overlooked, neglected or abandoned for other preferred parts. *Dialium guineense* (sub-family Caesalpinaceae) fruit coat is the black brittle outermost layer covering a more or less circular, flattened or sometimes glabrous pulp of about 2 cm diameter (1). In Nigeria, the pulp is consumed raw or soaked in water and drank as beverage while discarding the coat (2). Various activities of the leaves, fruit pulp, root, stem bark and seeds (3-15) of *D. guineense* have been reported. Proximate and elemental compositions have also been demonstrated (16-20). Peels or coats of various fruits, hitherto discarded after consumption of the pulp, have been reported to be beneficial (21-26), thus encouraging researcher to search for pharmacologically active species from 'waste' resources of plants. In some parts of Eastern Nigeria, the fruit coat of *D. guineense* is ground into a paste with water and placed on a wound for healing. Many traditional practitioners are also

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of the opinion that the paste can be licked to ameliorate gastrointestinal disturbances. The veracity or otherwise of these claims have not been reported to the best of our knowledge. Depending on pH, peristalsis, redox potential, bacterial adhesion, bacterial cooperation, mucin secretion, nutrient availability, diet, and bacterial antagonism, there are prevalence of allochthonous microorganism in the gastrointestinal tract, which are pathogenic in nature (27). These organisms, especially resistant ones, are known to compromise human immune system, if left unattended to by either multi-drug chemotherapy or use of plant-derived products like *D. guineense* (28). A wound is sharp injury that damages the dermis of the skin and may be infected depending on host resistance. Wound infection is characterized by increased local temperature, pain, exudate, swelling and erythema (29). Systematically initiated wound healing processes characterized by hemostasis, inflammation, proliferation and maturation; and microbiostatic or microbiocidal activity of external agent provides excellent remedy for wound healing. To this end, this paper tried to evaluate the triple application of the extract and fractions of *D. guineense* fruit coat in initiating systematic wound healing processes, suppressing the aerobic microorganisms that can infect wounds and manage microbial infections.

MATERIALS AND METHODS

Collection of plant materials

The plant materials were collected from Enugu Ezike, South Eastern Nigeria during the 2011 summer season, identified and authenticated by Mr. Alfred Ozioko of the International Centre for Ethno-medicine and Drug Development (INTERCEDD), Nsukka. A voucher specimen (No. Intercedd/1505) of the plant and its fruit coat have been deposited at the center.

Extraction of the fruit coat

The fruits and seed contents were manually separated from the whole plant materials, dried under the shade at room temperature and pulverized to fine particle sizes. One thousand g of the powdered sample was macerated in 4 × 500 ml of methanol (95 % v/v) for 24 h and the combined extracts filtered *in-vacuo*. The filtrate was concentrated to dryness in a rotary thin-film evaporator (BUCH R-210215, Germany) and the crude extract stored in airtight container. The total extract yield was 11.5 % (w/w) of the total dry weight of sample.

Fractionation of crude methanol extract

The dried methanol extract (100 g) was triturated with 200 g of silica gel, transferred into a 500 ml flask and partitioned successively in n-hexane, dichloromethane, ethyl acetate, methanol and water respectively. Each liquid fraction was concentrated in a rotary thin-film evaporator or lyophilized in a freeze dryer for the aqueous fraction. Preliminary antimicrobial and wound healing activities tests performed with the fractions showed promising activities in the dichloromethane fraction only. This fraction was subjected to silica gel column for more separation. Dichloromethane soluble fraction, DF (8.5 g), was chromatographed on a 60 × 4.5 cm column packed with 240 g silica gel (size 100-400) and eluted sequentially by a linear gradient mobile phase of n-hexane: ethyl acetate starting with 100 % n-hexane to 100 % ethyl acetate. The eluates were collected and pooled into eight sub-fractions (DF-1 to DF-8) based on their thin-layer chromatography spots. All the extracts, fractions and sub-fractions were subjected to *in-vitro* antimicrobial and wound healing assays for further isolation processes. Only the DF and sub-fraction (DF-5) were found to possess significant antimicrobial and wound healing activities.

Animals

A total of twenty-five healthy albino rats of either sex (180-200 g) procured from the Animal Husbandry unit of the Department Veterinary Medicine, University of Nigeria, Nsukka were used for wound healing activity experiment. They were divided into five groups of five rats each, maintained as per recommendations in the Guide for the Care and Use of Laboratory Animals (DHHS, NIH Publication No. 85–23, 1985) for 14 days prior

to the experiment with full access to clean water and food.

Phytochemical analysis of crude extracts

Qualitative and quantitative phytochemical properties of the crude extract of *D. guineense* fruit coat were determined by previously validated protocol (30). The phytochemical components assessed were tannins, saponins, alkaloids, steroids, phenols and flavonoids. Only the qualitative analysis was performed on all the fractions and sub-fractions.

Antimicrobial activities

The antimicrobial property of the methanol extract, fractions and sub-fractions was evaluated by agar well diffusion method (28). The test microorganism used were selected at least one from gastrointestinal, urinary and respiratory (upper and lower) tracts or strains from multi-drug resistant isolate from the in-patient wards of a hospital in Nsukka urban comprising of one fungus and three Gram positive and negative bacteria each. They included; *Staphylococcus aureus, Streptococcus pneumonia, Escherichia coli, Enterococcus faecalis, Salmonella typhi, Pseudomonas aeruginosa* and *Candida albicans.* Twenty ml Saboroud dextrose agar (Sigma-Aldrich, Germany) and molten Mueller-Hinton agar (Thermo Fisher Scientific, UK) was prepared for each of the fungi and bacteria respectively, sterilized and allowed to cool and then transferred to sterile Petri dishes (Tomar Scientific, India) to solidify. One hundred µl of the broth cultures of the organism (standardized to 0.5 McFarland's turbidity standard, which is equivalent to 1.5×10^8 cfu/ml with normal saline) was spread on the solidified dishes and six wells (6 mm diameter, 2 mm deep) bored in each of the inoculated plates. One hundred µl of 20 mg/ml extract, fractions and sub-fractions in dimethyl sulfoxide (DMSO), DMSO alone and standard (20 µg/ml) drug (ketoconazole for fungi and ciprofloxacin for bacteria) was added into each of the wells. The plates were left for 20 min for pre-diffusion of the extract, fractions, sub-fractions, DMSO or drug before they were incubated at 37 °C for 24 h for bacteria and 25 °C for 48 h for fungi; after which the test strains sensitive to the extracts have their inhibition zone diameters (IZD) measured.

Evaluation of wound healing properties of the extract

The rats were anaesthetized by chloroform exposure, the hair on the skin of the back shaved, the shaved area disinfected with ethanol and wound sites prepared as previously described (31). The wound area was measured by placing a transparent tracing paper on 1 sq mm graph sheet and counting the number of squares covered by the graph sheet. Groups I-III animals were treated 50 mg/kg each of the methanol extract, DF and DF-5. Groups IV and V received Cicatrin® (combination of neomycin and bacitracin) solution and distilled water respectively. All treatments were done topically at 100 µl once daily for 17 days and each application was done after cleaning the wound with dilute solution of disinfectant. The wound area of all the animals were measured under chloroform anesthesia on day 1, 4, 7, 10, 14 and 17 post wound infliction and the percentage of wound healing for each day calculated thus:

% Wound healing = Wound area on surgery – Wound area on day n / (Wound area on surgery day) × 100 (1)

where, n represents days 1, 4, 7, 10, 14 and 17 post treatments.

Statistical analysis

The results of the percentage wound healing post-surgery days were given as mean ± standard deviation (SD) of the replicates. One way analysis of variance (ANOVA) was done to test for the significant difference between the means of samples and control at $P<0.01$ by post-hoc using 2-sided Dunnett's test of the GraphPad Prism software. A P-value <0.01 was considered to be significant.

RESULTS

The phytochemical composition of *D. guineense* fruit coat methanol extract showed the presence of alkaloids and saponins in high concentration and flavonoids, phenols and steroids in trace quantities (Table 1).

Table 1. Phytochemical constituents of *Dialium guineense* fruit coat.

Phytochemicals	Tannins	Saponins	Flavonoids	Alkaloids	Steroids	Phenols
MeOH extract	++	+++	+	+++	+	+
Quantitative (%)	1.86 ± 0.05	3.91 ±	0.67 ± 0.02	6.05 ± 0.98	0.21 ± 0.06	0.32 ± 0.08
n-Hex fraction	-	-	-	+	-	+
DCM fraction	-	++	+	++	+	+
EtOAc fraction	+	+	+	+++	+	+
MeOH fraction	+	+	-	+++	+	+
H_2O fraction	++	++	-	-	-	-
DF-1	-	-	-	+	-	-
DF-2	-	-	-	-	-	-
DF-3	+	-	+	-	-	+
DF-4	-	-	-	-	-	-
DF-5	-	++	-	++	+	-
DF-6	-	-	-	+	-	-
DF-7	-	-	-	-	-	-
DF-8	-	-	-	-	-	-

MeOH; methanol, n-Hex; n-hexane, DCM; dichloromethane, EtOAc; ethyl acetate, H_2O; water, DF; sub-fractions of dichloromethane soluble. Quantitative abundances expressed as mean ± SEM for 3 determinations for methanol extract only, qualitative relative abundances expressed as +low, ++medium and +++high.

Table 2. Antimicrobial activity of methanol extract and fractions of *Dialium guineense* fruit coat.

Microorganisms	*Inhibition zone Diameters, IZDs (mm)				
	Crude extracts	DF-5	DF	Cipro/keto	DMSO
Staphylococcus aureus	8.0 ± 0.6	13.0 ± 0.2	10.0 ± 0.4	22.0 ± 1.5	Nil
Enterococcus faecalis	10.0 ± 0.3	11.0 ± 0.0	9.0 ± 0.1	20.0 ± 0.0	Nil
Streptococcus pneumonia	6.0 ± 0.1	15.0 ± 0.6	8.0 ± 0.0	24.0 ± 0.1	Nil
Escherichia coli	14.0 ± 0.3	16.0 ± 0.7	15.0 ± 0.5	18.0 ± 0.0	Nil
Salmonella typhi	15.0 ± 0.4	21.0 ± 0.1	19.0 ± 0.9	19.0 ± 0.0	Nil
Pseudomonas aeruginosa	17.0 ± 0.5	18.0 ± 0.3	19.0 ± 0.1	18.0 ± 0.6	Nil
Candida albicans	3.0 ± 0.0	8.0 ± 0.0	6.0 ± 0.5	21.0 ± 0.5	Nil

Cipro; ciprofloxacin, keto; ketoconazole, IZDs; inhibition zone diameters. *values expressed as mean of 3 replicates to the nearest mm (mean ± SEM), Nil;no inhibition.

Quantitative phytochemical composition of alkaloids and saponins in the crude methanol extract were 6.05 and 3.91 % respectively while tannins, flavonoids, steroids and phenols were 0.21-1.86 %. The fractions and sub-fractions showed various degrees of phytochemicals.

The antimicrobial activities were assessed based on the diameter of inhibition zone (IZD) recorded. The results showed that the crude methanol extract significantly inhibited the growth of the entire Gram-negative (IZD, 14-17 mm) more than Gram-positive (IZD, 6-10 mm) bacteria, with least inhibition of fungal growth (Table 2). Similar trends of inhibition were also observed in the fractions and sub-fractions of the crude extract. The activities in all the tested strains were, however, lower compared with the standard agents (18-24 mm) with the exception of DF and its DF-5 against *S. typhi* and *P. aeruginosa*, which showed higher IZDs against *S. typhi* (19 and 21 mm) and *P. aeruginosa* (19 and 18 mm) respectively.

The changes in wound area of animals treated with the methanol extract, fractions, sub-fractions and controls are shown in Fig. 1. The wound healing properties showed a gradual decrease in wound area on days post-surgery with highest reduction observed in positive control (Cicatrin®) and the group treated with 50 mg/kg DF-5 sub-fraction. However, the wound area reduction showed an improved effect with each level of purification of the extract with lesser reduction in Group I animals. Group II animals showed the highest reduction between days 7 and 10 and days 14 and 17. By the 17^{th} day, however, the wound

areas had disappeared completely in animals treated with 50 mg/kg DF-5 sub-fraction and Cicatrin®. The choice of the 50 mg/kg treatment dose was selected from one-point-trial study with 10, 20 50, 80, 100 and 200 mg/kg doses of the crude methanol extract, which showed a dose-dependent wound healing activity with the minimum wound closure effect observed in the 50 mg/kg treated animal. For valid comparison, however, other treatments which were anticipated to possess higher wound closure effects were tested at the same dose of 50 mg/kg.

The percentage wound healing was as shown in Table 3. After day one of the treatments, none of the groups showed any sign of wound healing. The healing process became visible on the 4th day post treatment with the Cicatrin® recording 25 % wound area closure, DF-5 showed 12 %, dichloromethane soluble fraction showed 4.7 % while the crude methanol extract produced 4.5 % wound closure. Moreover, the dichloromethane soluble fraction and its active sub-fraction showed more than three times wound area closure between the 4th and 7th day post-surgery compared to about two times closure seen with Cicatrin®, distilled water and crude methanol extract. All the treatments recorded more than 50 % healing by the 14th day except water. 50 mg/kg DF-5 sub-fraction and positive standard (Cicatrin®) showed 100 % healing by the 17th day post-surgery and by the 10th day, 50 mg/kg DF fraction and DF-5 sub-fraction and Cicatrin® showed more than 50 % wound closure. However, all the treatments recorded a gradual rise in wound healing percentages post-surgery days. On the 4th and 7th post-surgery days, DF-5 and Cicatrin® significantly resulted in different percentage wound healing at P<0.01.

The crude methanol extract did not significantly improve the wound healing on the 17th day post-surgery.

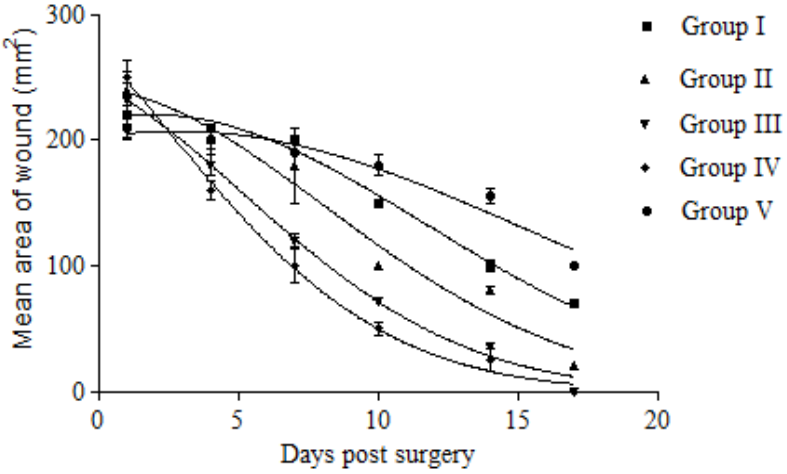

Fig. 1. Percentage wound healing activity of *Dialium guineense* fruit coat Post-surgery days.

Table 3. Percentage wound healing properties of *Dialium guineense* fruit coat.

Groups	Treatment/dose (mg/kg)	Percentage wound healing post-surgery days (day)					
		1 d	4 d	7 d	10 d	14 d	17 d
I	Methanol extract/50	None	4.6 ± 0.3	9.1 ± 0.8	*31.8 ± 0.3	*54.6 ± 1.2	68.2 ± 2.1
II	DCM fraction/50	None	4.8 ± 0.5	14.3 ± 0.4	*52.4 ± 0.1	*61.9 ± 1.8	*90.5 ± 1.7
III	DF-5 sub-fraction/50	None	*12.2 ± 0.2	*41.5 ±1.0	*64.9 ± 1.9	*82.4 ± 0.3	*100.0 ± 0.0
IV	Cicatrin®/50	None	*25.0 ± 1.1	*50.0 ± 0.4	*75.0 ± 0.6	*87.5 ± 0.5	*100.0 ± 0.0
V	Distilled water (100 µl)	None	4.4 ± 0.9	11.3 ± 0.3	21.7 ± 1.5	32.2 ± 0.1	56.5 ± 0.8

DCM; dichloromethane, DF;- dichloromethane sub-fraction, Results expressed as mean ± SD (n=5), *P<0.01 as compared with control (one way ANOVA) followed by Dunnett's test. Analysis was done by direct comparison of each treatment and the corresponding control (distilled water) for every post-surgery day.

DISCUSSION

The findings in this study suggested that the phytochemical constituents of *D. guineense* could be responsible for both the antimicrobial and wound healing properties of the fruit coat. Alkaloids, saponins and tannins were found to be relatively in higher abundance compared to other phytochemicals. Previous researchers had reported antimicrobial activities of saponins (32-34), alkaloids (35,36) and tannins (37-40); and wound healing properties of these phytochemicals (41,42) as well in different plants within the sub-family Caesalpinaceae. Similarly, only the antimicrobial activities (7,11-14,28) of different morphological parts of *D. guineense* had been reported, but not in the fruit coat; however, claims of wound healing properties of any part of *D. guineense* had remained folkloric till date. The antimicrobial activities also suggested that extract was more potent against Gram-negative than Gram-positive microorganisms; and the activities were found against the respiratory (upper and lower), gastrointestinal and urinary tracts infecting microorganisms. Our present findings (fruit coat) proved to have higher antimicrobial activities compared with the previously reported ethanol extract (100 mg/ml) of the *D. guineense* stem bark (7) which showed IZDs of 14-18 mm (*S. aureus*), 12 mm (*S. pneumonia*), 13 mm (*E. coli*), 18 mm (*S. typhi*), 11-14 mm (*P. aeruginosa*), and 16 mm (*C. albicans*). However, our study showed lower antimicrobial activities compared with the results of methanol leaf extract (0.25 mg/ml) with IZDs of 22.5 ± 1.6 (*S. aureus*), 19.2 ± 1.8 (*E. coli*), 18.2 ± 1.9 (*S. typhi*), 18.9 ± 1.5 (*P. aeruginosa*), and 22.4 ± 0.7 (*C. albicans*) (11). Wound healing is a complex process involving cascades and coordinated interactions between the immunological and biological components of the animals and the phytochemical constituents of *D. guineense* fruit coats. Several mediatory effects have been postulated for these processes and these phytochemical identified could be responsible for either the stimulatory or inhibitory effects of associated mediators for effective wound healing (43). The molecular and biochemical mechanisms behind the cascades of wound healing could, however, not be determined at this stage of the study. However, different theories have been postulated; the up-regulation of endothelial cells by the secreted soluble factors (fibroblast, transforming, epithelial, and vascular growth factors) from wound sites causing increased vascular permeability, angiogenesis and then healing (43) that characterizes the early wound healing processes which are followed by the remodeling phase, which involves the development of new epithelium and scar tissue formation. These theories take cognizance of the fact that the model adopted in this study follows acute wound healing (within 17 days) processes, even though the process of wound healing is continuous. The wound healing observed in negative control group (water-treated) could be attributed to the innate immunity of the rats used in the study. The raging controversy surrounding the use of dilute disinfectant solution in cleaning wounds prior to treatments in full thickness skin excision wound model has been addressed by several authors (44-46). This means that all the wound healing activities observed in this study could be attributed to the *D. guineense* extract, fraction and sub-fraction or the controls (Cicatrin® and distilled water) and not from the dilute solution of ethanol used as disinfectant. One of the outstanding findings here suggests that the same compound or closely chemically related compounds could be responsible for both the antimicrobial and wound-healing properties of *D. guineense* fruit coat even though the mechanisms of both activities could be different.

CONCLUSION

In view of the emerging microbial resistance to antibiotics, the fruit coat of *D. guineense* could be harnessed to provide lead compounds with antimicrobial and wound-healing properties. Further isolation and characterization of the phytochemical compounds responsible for the activities are ongoing.

ACKNOWLEDGEMENTS

Authors are grateful to IPBP-WWU, Münster for the use of her GraphPad Prism Data Analysis software.

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