Antibacterial, Antioxidant and Anti-inflammatory Properties of Margaritaria discoidea, a Wound Healing Remedy from Ghana

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**ABSTRACT**

*Margaritaria discoidea* (Baill.) Müll Arg. (Euphorbiaceae), has folkloric use in the treatment of wounds and skin infections. We report on the antibacterial, antioxidant and anti-inflammatory effects of the leaves and stem bark of *Margaritaria discoidea*. A 70% ethanolic extract of the various plant parts were tested for antibacterial activity using the agar well diffusion and micro dilution assays. Free radical scavenging, total antioxidant and phenol content were estimated. Using the carrageenan-induced foot pad oedema in chicks, the anti-inflammatory activity of the extract was assessed. The bark extract gave the higher activity with a zone of inhibition of 16.33 ± 0.88 mm against *Bacillus subtilis*. The MIC's for the bark extracts ranged from 500 µg/mL to over 1000 µg/mL against the test organisms. However, the leaf extract had no activity against all organisms tested. The leaf and bark extracts demonstrated free radical scavenging activity yielding IC\(_{50}\) values of 0.0185 and 0.0181 mg/mL respectively. In the total antioxidant assay, ascorbic acid equivalents ranged from 0.49 mg/g in the bark to 0.56 mg/g in the leaf. Both extracts had high phenolic content correlating with their antioxidant activity. The extracts showed significant anti-inflammatory activity. The ED\(_{50}\)s of the leaf and bark extracts were 12.20 and 8.27 mg/kg body weight respectively. The results were comparable to those of diclofenac and dexamethasone, the reference drugs used in this study. On the basis of the antimicrobial, antioxidant and anti-inflammatory activities observed for both extracts, the ethnomedicinal use of the plant in the management of wounds and skin infections is supported.

**Key words:** Antimicrobial; Carrageenan-induced oedema; Free radical; *Margaritaria discoidea*; Wound healing.

**INTRODUCTION**

Over 80\% of the world’s population depends on traditional medicines for numerous skin disorders.\(^{[1]}\) Skin disorders, primarily wounds, are the third most common causes of people seeking medical care in developing countries, including Ghana. Medical costs and lost productivity from the workforce suggest that chronic wounds cost several billions of dollars annually. Contributing to these staggering costs is treatment regimen that is expensive and/or ineffective and associated with recurrence rate.\(^{[2]}\)

Wound healing is the body’s natural process of regenerating dermal and epidermal tissue. When an individual is wounded, a cascade of biochemical events is initiated, leading to the repair of the damaged tissues. These events overlap but can be categorized as: the inflammatory, proliferative and remodelling phases;\(^{[3]}\) each phase is characterized by the infiltration into the wound site of specific cells, all of which interact by chemical signals to optimize repair.

Open wounds are prone to infection, especially by bacteria, and also provide an entry point for systemic infections. To arrest this, neutrophils must be released into the wound site. The over-abundant neutrophil infiltration is responsible for the chronic inflammation characteristic of non-healing ulcers. These neutrophils release the enzymes collagenase and elastase which are responsible for the destruction of the connective tissue matrix and important growth factors respectively.\(^{[4]}\) Antimicrobial agents may prevent this occurring and may underlie their use in treating wounds.
Antioxidants counter the excess proteases and reactive oxygen species (ROS) often formed by neutrophil accumulation in the wound site and protect protease inhibitors from oxidative damage. Fibroblasts and other cells may be killed by excess ROS and antioxidant agents may reduce the possibility of these adverse events occurring.[5]

When an individual is wounded, it is accompanied within a short time by the classical symptoms of inflammation: redness, increased heat, swelling, pain and loss of function. The release of eicosanoids, prostaglandins and leukotrienes are responsible for these symptoms. Fundamentally, inflammation is a protective response, with the aim of ridding the individual of the injurious stimuli and initiates the healing process. However, in chronic inflammation, the orderly process of healing is lost and there is the formation of chronic non-healing ulcers. Healing proceeds only after the inflammation is controlled.[6] The synthesis of eicosanoids, prostaglandins and leukotrienes are processes that can be inhibited by anti-inflammatory agents.

Medicinal plants have played and continue to play an invaluable role as drugs or lead compounds in the management of diseases.[7] There has been a growing interest in medicinal plants traditionally used in the treatment of wounds, and infectious diseases.[2,5] This study involves the scientific investigation of the leaves and stem bark of Margaritaria discoidea (Baill.) Müll Arg. (Euphorbiaceae), which finds use in ethnomedicine in Ghana as a wound healing agent.[8]

The leaves of Phyllanthus discoideus (Baill.) Müll Arg also referred to as Margaritaria discoidea demonstrated antibacterial[9,10] activity. The alkaloids responsible for the antibacterial activity have been isolated and characterised.[11] The present study aimed to verify the anti-inflammatory, antioxidant and antibacterial effects of the leaves and bark of the plant.

**MATERIALS AND METHODS**

**Plant materials**

The stem bark of Margaritaria discoidea – voucher specimen number (KNUST/HM1/2010/S003) and the leaves of Margaritaria discoidea – voucher specimen number (KNUST/HM1/2010/L30) were collected from Kente, Amansie Central District in the Ashanti Region of Ghana in June 2009. The plants were initially identified locally by a resident herbalist and authenticated at the Department of Pharmacognosy, College of Health Sciences, Kwame Nkrumah University of Science and Technology herbarium where herbarium specimens have been deposited.

**Chemicals**

All chemicals used were of analytical grade and purchased from Sigma Aldrich Co Ltd. Irvine, UK. Organic solvents were also of analytical grade and purchased from BDH Laboratory Supplies (England). Precoated aluminium-backed silica gel F\textsubscript{254} TLC plates (0.25 mm thickness), product code OB 315394 were purchased from Merck KGaA, Germany. Carrageenan sodium salt was purchased from Sigma Chemicals, St. Louis, MO, USA. Diclofenac and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium respectively.

**Microorganisms used**

Eight bacterial species, including four Gram-positive bacteria (Enterococcus faecalis ATCC 29212, Bacillus thuringiensis ATCC 13838, Staphylococcus aureus ATCC 25923 and Bacillus subtilis NCTC 10073) and four Gram-negative bacteria (Salmonella typhi NCTC 6017, Escherichia coli NCTC 9002, Proteus vulgaris NCTC 4635 and Pseudomonas aeruginosa ATCC 27853) were used for the antibacterial tests.

**Animals**

Cockerels (Gallus gallus; strain shaver 579, Akropong Farms, Kumasi, Ghana) were obtained 1-day post-hatch and were housed in stainless steel cages (34 × 57 × 40 cm\textsuperscript{3}) at a population density of 12-13 chicks per cage. Feed (Chick Mash, GAFCO, Tema, Ghana) and water were available ad libitum through 1-quart gravity-fed feeders and water trough. Room temperature was maintained at 29°C, and overhead incandescent illumination was maintained on a 12-hour light-dark cycle. Daily maintenance of the cages was conducted during the first quarter of the light cycle. Chicks were tested at 7 days of age. Group sample sizes of 5 were used throughout the study.

**Extraction of plant materials**

The leaves and stem barks of Margaritaria discoidea were dried and coarsely powdered. 50 g (dry weight) of each powdered plant part were extracted with 70% ethanol (1.5 L) using soxhlet extraction.

**Antimicrobial assays**

**Agar well diffusion method**

The antimicrobial activities of the different extracts were determined using the agar well diffusion method as outlined by Vanden Berghe and Vlietnick.[12] Crude extracts were prepared at concentrations of 5 mg/mL using methanol. Wells of 9 mm diameter were made in 20 mL nutrient agar (Oxoid) seeded with 2 loopfuls of a suspension of test organisms under aseptic conditions. The wells were filled with 100 µL of the extracts, allowed to diffuse for 1 hour at room temperature and incubated at 37°C for 24 hours, after which they were examined for zones of inhibition. Amoxycillin was included as positive control. All experiments were carried out in triplicates.

**Micro dilution assay**

Minimal inhibitory concentration (MIC) values of the extracts were determined based on a micro-well dilution...
The inocula of microorganisms were prepared from 12-hour broth cultures and serial dilutions were made to achieve a suspension of approximately $10^5$ CFU/mL. The plant extracts were screened at concentrations of between 1000 µg/mL and 7.8 µg/mL.

The 96-well sterile plates were prepared by dispensing into each well 100 µL of double strength nutrient broth and 100 µL of test samples together with 20 µL of the inoculum. The microplates were incubated at 37°C for 24 hours. Growth of the microorganisms was determined by adding 20 µL of a 5% solution of tetrazolium salt (MTT) and incubating for further 30 minutes. Dark wells indicated the presence of microorganisms as the dehydrogenase enzymes in the live bacteria react to form a dark complex with the tetrazolium salt. Amoxycillin was included as positive control. All experiments were carried out in triplicates.

**Antioxidant assays**

**Rapid screening for antioxidants**

Extracts were monitored initially for antioxidant activity on TLC (solvent system: chloroform, methanol 9:1) using 20 mg/L of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol, and antioxidant compounds in the extracts gave clear zones against a purple background.

**Free radical scavenging activity of the ethanolic extracts**

Assay was performed by the DPPH method described by Blois (15). 20 mg/L solution of DPPH in methanol was prepared and 3 mL of this solution were added to 1 mL of the ethanolic test extracts at 3, 1.5, 0.75 and 0.375 mg/mL. After 30 min, the absorbance was measured at 517 nm. Inhibition of radical scavenging was calculated according to the following equation.

$$\text{DPPH scavenging activity} (\%) = \left[ 1 - \frac{A_0 - A_1}{A_0} \right] \times 100$$

With $A_0$ being the absorbance of the control and $A_1$ is the absorbance in the presence of the test sample.

**Total phenols determination**

Total phenols were determined by Folin-Ciocalteau’s reagent. Different doses were tested for both tannic acid and the plant extracts: tannic acid (0.03-1 mg/mL); extract (0.125-2.5 mg/mL). 1 mL of each plant extract or ascorbic acid (standard antioxidant compound) was mixed with 1 mL of reagent solution (0.6 M H$_2$SO$_4$, 28 mM Na$_2$HPO$_4$ and 4 mM ammonium molybdate) and 1 mL of aqueous Na$_2$CO$_3$ (2%). The mixtures were incubated at 95°C for 90 min, and then centrifuged at 3000 rpm for 10 min and absorbance of the supernatant determined at 695 nm. Distilled water (1 mL) was added to 3 mL Molybdate, processed in the same way as the test drugs and used as blank. The standard curve was prepared. Total antioxidant values are expressed in terms of ascorbic acid equivalent (mg/g of dry mass).

**Total Antioxidant Capacity**

Total antioxidant capacity of extract was determined as described by Prieto. Ascorbic acid served as positive control. Different doses were tested for both ascorbic acid and the plant extracts: ascorbic acid (0.03-1 mg/mL); extract (0.125-2.5 mg/mL). 1 mL of each plant extract or ascorbic acid (standard antioxidant compound) was mixed with 3 mL of reagent solution (0.6 M H$_2$SO$_4$, 28 mM Na$_2$HPO$_4$, and 4 mM ammonium molybdate) and 1 mL of aqueous Na$_2$CO$_3$ (2%). The mixtures were incubated at 95°C for 60 min, and then centrifuged at 3000 rpm for 10 min and absorbance of the supernatant determined at 695 nm. Distilled water (1 mL) was added to 3 mL Molybdate, processed in the same way as the test drugs and used as blank. The standard curve was prepared. Total antioxidant values are expressed in terms of ascorbic acid equivalent (mg/g of dry mass).

**Anti-inflammatory assay**

**Carrageenan-induced oedema**

Anti-inflammatory activity was determined by the method of Roach and Sufka, modified by Wood et al. Chicks were randomly divided into groups of 5 and had access to food and water ad libitum. Foot volumes were measured by water displacement plethysmography as described by Fereidoni et al. Oedema was induced by subplantar injection of carrageenan (10 µL of a 2%/w/v solution in saline) into the right footpad of the chicks. Three test groups received the extracts (30, 100 and 300 mg/kg, p.o.), the standard groups received diclofenac (5, 15 and 50 mg/kg, i.p.) and dexamethasone (1, 3 and 10 mg/kg, i.p) and the control animals received the vehicle only. All treatments were administered 30 min for i.p. route and 1 hour for oral route before carrageenan injection.

**Statistical analysis**

Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0 then averaged for each treatment group. The time-course curves for foot volume were subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni’s post hoc t test. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). The inhibition percentage of oedema was calculated for each animal group in comparison with its vehicle-treated group.

Differences in AUCs were analyzed by ANOVA followed by Newman-Keuls post hoc t test. ED$_{50}$ (dose responsible
for 50% of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

\[
Y = \frac{a + (b - a)}{1 + 10^{a\log_{10}D_{50} - X}}
\]

Where \(X\) is the logarithm of dose and \(Y\) is the response. \(Y\) starts at \(a\) (the bottom) and goes to \(b\) (the top) with a sigmoid shape.

GraphPad Prism for Windows version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis and ED\(_{50}\) determinations. \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Antimicrobial effects**

**Agar well diffusion**

The largest diameter of zone of inhibition, 16.33 mm was given by the 70% ethanolic extract of *Margaritaria discoidea* bark against *Bacillus subtilis NCTC 10073* (Table 1). The extract of *Margaritaria discoidea* leaves showed no activity against the test organisms used.

**Micro-dilution assay**

Minimum inhibitory concentrations were observed for the extracts that showed activity in the agar well diffusion assay. The MIC values ranged from 500 \(\mu\)g/mL to more than 1000 \(\mu\)g/mL (Table 2).

**Antioxidant effects**

**TLC-screening for antioxidant compounds**

The active compounds were detected as yellow spots on a violet background. Both extracts were subjected to further testing.

**Free radical scavenging activity**

The abilities of the test extracts which were detected in the TLC screening to donate hydrogen atoms or electrons were measured spectrophotometrically. Both extracts reduced DPPH to the yellow coloured product, diphenylpicrylhydrazine, and the absorbance at 517 nm declined. The leaves and bark of *Margaritaria discoidea* showed effect with IC\(_{50}\)s of 0.0185 and 0.0181 mg/mL respectively (Table 3).

**Total phenol contents**

The total phenols were 23.49 ± 0.01 and 19.61 ± 0.02 mg/g in the leaf and bark extracts respectively as shown in Figure 1.

**Total antioxidant capacity**

The total antioxidant capacity of the leaf was 0.559 ± 0.00 whereas the bark was 0.488 ± 0.00 as shown in Figure 2.

**Anti-inflammatory activity**

**Carrageenan-induced oedema**

Figure 3 shows the time course curve and AUC for the effect of diclofenac, dexamethasone, *Margaritaria discoidea* leaf (MDL) and *Margaritaria discoidea* bark (MDB) on for 50% of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

\[
Y = \frac{a + (b - a)}{1 + 10^{a\log_{10}D_{50} - X}}
\]

Where \(X\) is the logarithm of dose and \(Y\) is the response. \(Y\) starts at \(a\) (the bottom) and goes to \(b\) (the top) with a sigmoid shape.

GraphPad Prism for Windows version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis and ED\(_{50}\) determinations. \(P < 0.05\) was considered statistically significant.

**Phytochemical screening**

The presence of phenolics, alkaloids, phytosterols, terpenoids, flavonoids, reducing sugars and saponins were detected by simple qualitative phytochemical methods.\(^{[21]}\)

**Table 2: Mean minimum inhibitory concentration (MIC; \(\mu\)g/mL) of *M. discoidea* bark on various microorganisms. n=3.**

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (\mu)g/mL</th>
<th>MDB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis NCTC 10073</em></td>
<td>500</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus thuringiensis ATCC 13838</em></td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus ATCC 25923</em></td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis ATCC 29212</em></td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td><strong>Gram negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proteus vulgaris NCTC 4635</em></td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa ATCC 27853</em></td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli NCTC 9002</em></td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella typhi NCTC 6017</em></td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

All experiments were carried out in triplicates. 200 \(\mu\)g/mL of amoxicillin served as positive control. NT = Not tested because extract was not active by the agar well diffusion assay. MIC readings for all wells were the same. Extracts: MDB = *Margaritaria discoidea* bark.

**Table 3. IC\(_{50}\) values (mg/mL) for free radical scavenging activity by extracts.**

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC(_{50}) DPPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDB</td>
<td>0.0185</td>
</tr>
<tr>
<td>MDB</td>
<td>0.0181</td>
</tr>
</tbody>
</table>

Extracts: MDB = *Margaritaria discoidea* leaves, MDB = *Margaritaria discoidea* bark.

with maximal inhibitory effect of total oedema by 84.94% at 30 mg/kg. Diclofenac exhibited a significant effect \(F_{3,12} = 16.22, p = 0.0002\) and a 78.75% maximal inhibitory effect of total oedema at 50 mg/kg. Dexamethasone, a steroidal anti-inflammatory agent showed a significant

carrageenan-induced oedema in chicks. On the time-course curve, MDB treatment exhibited a significant effect \(F_{3,12} = 37.04, p < 0.0001\) with maximal inhibitory effect of total oedema by 92.64% at 300 mg/kg. MDL treatment exhibited a significant effect \(F_{3,12} = 29.29, p < 0.0001\) }

Based on the ED50 values (Table 4) obtained from the dose response curves (Figure 3), MDB was more potent of the plant extracts. The standard drugs, diclofenac and dexamethasone were approximately twice as effective as MDB as an anti-inflammatory agent.

**Phytochemical screening.** The results of the phytochemical screening on the powdered plant parts are as shown in Table 5.

### DISCUSSION

In a series of in vitro tests, the 70% ethanolic extracts exhibited antibacterial, antioxidant and anti-inflammatory effects. The ethanolic extracts of the stem bark of *Margaritaria discoidea* was active with MIC values ranging from 500 µg/mL to more than 1000 µg/mL against Gram-positive (*Enterococcus faecalis* ATCC 29212, *Bacillus thurigienis* ATCC 13838, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* NCTC 10073) and Gram-negative (*Escherichia coli*...
NCTC 9002 and Proteus vulgaris NCTC 4635). The largest diameter of zone of inhibition 16.33 mm observed for the agar well diffusion method, was against Bacillus subtilis NCTC 10073 (Table 1). The extract of the leaves of *Margaritaria discoidea* showed no activity against all organisms employed. However in a previous study, the alkaloid fraction obtained from the lyophilized aqueous extract of the leaves was found to show antibacterial activity.[10]

Infected wounds heal less rapidly and often result in the formation of unpleasant exudates and toxins, which would lead to the killing of regenerating cells in the healing process. [8] The most familiar pathogen to be isolated from infected wounds is *Staphylococcus aureus* which may be isolated from approximately 1/3 of all infected wounds.[23] Interestingly, the extract of the bark of *Margaritaria discoidea* showed activity against *Staphylococcus aureus* ATCC 25923. The respective MIC was >1000 µg/mL. The stem bark extract thus has inhibitory effects on *Staphylococcus aureus* and other pathogens, and may serve as anti-infectives and hence its usefulness.

Quite a number of plant secondary metabolites of different classes have been shown to possess antimicrobial effect.[23] Subsequently, the presence of one or more of these metabolites in the extracts may be responsible for the effects observed in the antimicrobial assays (Table 5).

The ethanolic extracts of the leaves and stem bark of *Margaritaria discoidea* had similar scavenging activities on DPPH with IC<sub>50</sub> of 0.018 mg/mL. In the total antioxidant assay, the extract of *Margaritaria discoidea* leaf was more active with ascorbic acid equivalent being 0.559 mg/g dry weight of extract.

Phenolic compounds are a class of antioxidant agents which act as free radical terminators.[24] Phenolic compounds, due to their redox properties, act as reducing agents, hydrogen donors or singlet oxygen quenchers.[25] In this study, an attempt was made to establish the co-relationship between total phenolic content and antioxidant activity. The correlation developed between total antioxidant activity (Y) and total phenolic content (X) of all the plant extracts had correlation coefficient, R of 1.00 and 0.99 for leaf and bark respectively. Phenolic content of the extract correlated highly with their total antioxidant capacities. The results obtained suggest the potential of the extracts as antioxidant agents.

The carrageenan-induced oedema, an animal model of acute inflammation, involves the synthesis and/or release of histamine, serotonin, kinins, prostaglandins and cyclooxygenase-2.[26] Inflammatory mediators released during acute inflammation are potent vasodilator substances, which increase the vascular permeability and subsequently cause the observed oedema.[27] The extracts reduced the oedema produced. The leaf extract of *Margaritaria discoidea* showed a maximal inhibitory effect of total oedema by 84.94% at 30 mg/kg. The maximal inhibitory effect of total oedema by 92.64% at 300 mg/kg was exhibited by the bark of *Margaritaria discoidea*. The results of our work compare with that of Adedapo et al.[28] So it may be suggested that their anti-inflammatory activity is backed by inhibiting the synthesis, release or action of the inflammatory mediators.

Flavonoids have been identified to inhibit the action of prostaglandins which are involved in the last phase of acute inflammation.[23,29] Glycosides have also showed *in vitro* inhibition of the chemical mediators of the inflammatory process.[31] It may be suggested that the observed anti-inflammatory activity may be due to the presence of flavonoids and glycosides present in the plant extracts (Table 5).

In conclusion, the different phases of the wound healing process overlap and ideally a plant-based remedy should affect at least two different processes before it can be said to have some scientific support for its traditional use.[9] These findings, suggest that the extracts of all the plant materials possess antibacterial, antioxidant and anti-inflammatory properties which are likely to contribute to their beneficial effect in wound healing. Further isolation of the various compounds responsible for these activities is in progress in our laboratories.

**ACKNOWLEDGEMENT**

We gratefully acknowledge the support rendered by Mr. Kofi Asare, a local herbalist and the technicians of the Departments of Pharmacognosy, Pharmaceutics and Pharmacology of KNUST. Special thanks to the University of Ghana Medical School for providing the typed strains of bacteria.

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