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Phytochemical and Antimicrobial Studies of *Maprounea membranacea* Pax & K. Hoffm (Euphorbiaceae)

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Authors' contributions

This work was carried out in collaboration between all authors. Author JPLE designed the study, performed the extraction, fractionation and isolation of compounds, wrote the protocol and the first draft of the manuscript. Author JLS worked on literature search, reviewed the study and wrote the final draft of manuscript. Author MNAA performed the biological studies. Author MKL carried out the NMR experiments. Authors AFKW, LMM and JDW managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The aim of the present study was to carry out the isolation, purification and structural identification of secondary metabolites from methanolic extract of stem bark of *Maprounea membranacea* and further to evaluate the antimicrobial activity of its fractions. According to the literature and due to their use in traditional medicine, plants of the genus *Maprounea* may be good candidate for new antimicrobial drugs. In this study, the air-dried and powdered stem bark (6.3 kg) of *M. membranacea*

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was extracted 02 times by percolation with methanol (MeOH) for 48 h. A part of this residue (350 g) was suspended in distilled water and partitioned successively between hexane and ethyl acetate (EtOAc) to obtain hexane, ethyl acetate and water soluble fractions. The two first fractions were then subjected to repeated column chromatographic separation. The structures of the isolates were established by means of spectroscopic methods. The crude extract and its fractions were also screened *in vitro* for their activity against bacteria and fungi. Eight known compounds including friedelin, betulinic acid, lupeol, epigallocatechin, β-sitosterol, β-sitosterol glucoside, stigmasterol and stigmasterol glucoside were isolated from hexane and ethyl acetate fractions of methanolic extract of stem bark. The structures of these compounds were determined by comprehensive spectroscopic analyses (1D, 2D NMR) and by comparison of their data with those reported in the literature. The agar diffusion test resulted in low to missing antibacterial activities. In addition, all fractions and crude extract displayed significant antifungal activities with MIC value ranging from 1.25-40.0 µg/µl.

Keywords: Euphorbiaceae; Maprounea membranacea; triterpenoids; flavonoids; antimicrobial activity.

1. INTRODUCTION

The effectiveness of the arsenal of antibiotics now in use is decreasing in the face of emerging bacterial resistance and has been the subject of various researches. Also, the crude mortality rate from opportunistic fungal infections still exceeds 50% in most human studies and has been reported to be as high as 95% in bone marrow transplant recipients infected with *Aspergillus sp.* [1,2].

Plants can represent an important source to provide alternatives to some orverused antimicrobial compounds currently available.

Maprounea Aublet is a small genus of shrubs and trees occurring in the Neotropics and in Africa [3]. It consists of four species, two in Africa and two in Northern South America [4]. *Maprounea membranacea* Pax & K. Hoffm, is a woody species native to central and eastern Africa. Members of the genus *Maprounea* have a long history of therapeutic use in traditional medicine in many African countries and have been reported to have beneficial effects on several diseases, including epilepsy and syphilis [5,6]. The stem bark of *Maprounea membranacea* has been investigated previously and yielded pentacyclic triterpenoids as 2*α*-hydroxyaleuritolic acid 2-*p*-hydroxybenzoate, 2*α*-hydroxyaleuritolic acid 2,3-*bis*-*p*-hydroxybenzoate, aleuritolic acid 3-*p*-hydroxycinnamate and 3*α*-hydroxyaleuritolic acid 2*β*-*p*-hydroxybenzoate [6]. Tetracyclic terpenes of cucurbitacines series including cucurbitacine and 23, 24-dihydrocucurbitacine A have been also isolated from the stem of the same plant [7]. Extracts and several of the compounds isolated from the genus *Maprounea* were reported to have potent antihyperglycemic activity [8], neuropharmacological effects [5], potent inhibitory activity against HIV-1 reverse transcriptase [9], antimicrobial activity [10] and also cytotoxic effects [11,12].

In the present study, the isolation and characterization of eight known compounds are reported for the first time in the *Maprounea* genus together with antifungal and antibacterial activities of the crude extract and its fractions.

2. MATERIALS AND METHODS

2.1 Plant Material

The stem bark of *Maprounea membranacea* was collected from Japoma, Littoral region of Cameroon in November 2015 and identified by Mr NANA Victor from the National Herbarium, Yaoundé, Cameroon, where a voucher specimen was deposited under ref. 9061 SRF/CAM. The stem bark were dried at room temperature and crushed using an electric blender, from which a brown powder was then obtained.

2.2 Extraction and Isolation of Compounds

The air dried powder of the stem bark of *Maprounea membranacea* Pax (6.3 kg) was extracted 02 times by percolation with MeOH for 48 h. The resulting solutions were filtered, combined, and concentrated under low pressure to give 795 g of a syrupy residue. A part of this residue (350 g) was suspended in distilled water and partitioned successively between hexane: MeOH/H_2O (9:1) and EtOAc: H_2O (6:4) to obtain hexane (F1, 12.45 g), ethyl acetate (F2, 14.20 g) and water (F3, 318.0 g) soluble fractions.

Fraction F1 was further subjected to column chromatography (6.7 cm i.d × 42.5 cm) over silica gel, using hexane-EtOAc (100:0 ⟶ 20:80) gradiently to afford stigmasterol-3-*O*-*β*-*D*-glucopyranoside (48.3 mg), a mixture of stigmasterol and *β*-sitosterol (10.1 mg) and four major fractions (F1.1 – 1.5). Fraction F1.1 was purified by isocratic with hexane-EtOAc (98:2) to give friedelin (18.4 mg).

The EtOAc fraction (14.20 g) was also subjected to a silica gel column chromatography (6.7 cm i.d × 42.5 cm), eluted with hexane-EtOAc (100:0 ⟶ 20:80) through EtOAc-MeOH (1:0 ⟶ 9:1) to yield betulinic acid (100 mg), *β*-sitosterol-3-*O*-*β*-*D*-glucopyranoside (30.5 mg) and six main fractions (F2.1 – 2.6). Further purification of fraction F2.2 by silica gel column in isocratic elution conditions with hexane-EtOAc (93:7) yielded lupeol (8.8 mg) while purification of fraction F2.5 in same conditions using hexane-EtOAc (4:6) for elution yielded epigallocatechin (8.2 mg).

2.3 Structural Identification

The structures of compounds were determined by Nuclear Magnetic Resonance (NMR) spectroscopy. The ^{1}H- and ^{13}C-NMR spectra were recorded at 400 MHz and 100 MHz respectively on Bruker DRX 500 NMR spectrometers. Column chromatography was carried out on silica gel (70-230 mesh, Merck). Thin-layer chromatography (TLC) was performed on Merck precoated silica gel 60 F_{254} aluminium foil, and spots were visualized using anisaldehyde spray reagent. The presence of phenolic compounds and triterpenoids were detected using $FeCl_3$ reagent and Lieberman-Buchard respectively.

2.4 Antimicrobial Assays

2.4.1 Microorganisms and growth conditions

Three bacteria and four fungal species obtained from the Laboratory of biochemistry of "University of Douala" were used for experiment: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Aspergillus niger*, *Aspergillus sp, Aspergillus flavus*, *Penicillium sp.* The bacteria strains were cultured on Muller-Hinton Agar (MHA) medium and incubated at 37°C for 22 h. The fungal strains were cultured in Potato Dextrose Agar (PDA) plates and incubated at 27°C for 7 days.

2.4.2 Antibacterial susceptibility assay

The antibacterial activity of the methanol extract of *M. membranacea* was investigated by the Agar-disk diffusion method as previously described [13].

Muller-Hinton Agar medium was used to grow the test isolates for 22 h at 37°C. A 0.5 McFarland standard suspension was prepared in distilled water. A volume of 0.1 ml of bacterial suspension was spread uniformly on MHA medium. Crude extract and its fractions were previously dissolved in dimethyl sulfoxide (DMSO) (at 80 µg/µl, 40 µg/µl and 20 µg/µl concentration) and paper disks (Ø 5 mm) were impregnated with 12.5 µl of different concentrations (1 mg/disc, 0.5 mg/disc and 0.25 mg/disc) each, dried for 3 h under sterile conditions and placing onto the surface of the inoculated media. The plates were then incubated at 37°C for 48 h. The zones of inhibition were measured as indicators of sensibility of bacterial test. All the tests were done in triplicate.

Discs impregnated with DMSO were used as control. Ciprofloxacin disc (5 µg/disc) was used as standard.

2.4.3 Antifungal activity test

Spores of all fungal species were harvested to 7-days-old cultures with a wire loop, using two consecutive rinsing with sterile distilled water. The spore suspensions were filtered through layers of sterile muslin to remove hyphae and adjusted to 10^6 spores/ml using a haemocytometer. Germination experiment was performed using liquid micro-dilution method in Potato Dextrose Broth (PDB) medium supplemented with extract at final concentration varying 40.0 to 1.25 µg/µl. Microcupules were inoculated with 100 µl of spore suspension and incubated at 27 ± 2°C during 24 h. Nystatin previously solubilised in DMSO was serially diluted two folds and added to wells to give a range of concentration from 20.0 to 1.25 µg/µl used as reference antifungal. The turbidity was taken as an indication of growth, and the Minimum Inhibitory Concentration (MIC) was confirmed by microscopic observations. Other hand, germination was recorded as percentage of total conidia germinated under light microscope using a haemocytometer. Inhibition germination (IG) was calculated following the formula %IG= (Nt –Na) x 100/Nt. Nt= number of spores germinated in the microcupules of

negative control (DMSO), Na= number of spores germinated in the assay.

2.5 Statistical Analysis

All data were verified for consistency, coded, and keyed in an Excel sheet. Thereafter, statistical analyses were performed with Statview software version 5.0 (SAS Institute Inc., USA). Data were summarized in table as percentages or mean ± standard deviation (SD) for qualitative and quantitative variables respectively, where appropriated. Mann-Whitney test and Kruskal-Wallis test were used to compare mean. Significant levels were measured at 95% CI with significant differences recorded at *p-value*< 0.05.

In the comparative analysis of inhibition germination percentage, the concentration values of samples which have induced a total destruction of conidia were not considered.

3. RESULTS AND DISCUSSION

The methanolic crude extract of *Maprounea membranacea* stem bark was partitioned with hexane and ethyl acetate. Purification over silica gel of these fractions resulted into the isolation of eight known compounds 1-8 (Fig. 1). The compounds obtained in this study, friedelin (**1**) [14], betulinic acid (**2**) [15], lupeol (**3**) [16], epigallocatechin (**4**) [17], β-sitosterol (**5**) [18], β-sitosterol glucoside (**6**) [19], stigmasterol (**7**) [20] and stigmasterol glucoside (**8**) [19] were identified by comparison of their physical and spectroscopic data with literature reports.

The presence of these compounds in *M. membranacea* stem bark is in chemotaxonomic accordance with previous reports from other species of the family Euphorbiaceae [21,22]. But, the occurrence of a flavonoid in this plant for the first time promise new perspectives of phytochemical study in this genus because only one chemical study report the presence of flavonoids in *Maprounea* genus [23].

Following isolation, crude extract and its fractions were evaluated for their antibacterial and antifungal properties *in vitro* against three bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*) and four fungal species (*Aspergillus niger*, *Aspergillus sp. Aspergillus flavus*, *Penicillium sp*). The results of antibacterial activities showed that at the concentration of 20 µg/µl and 40 µg/µl, samples did not display any zone of inhibition regarding the three bacteria strains tested. However, at 80 µg/µl samples displayed low zone of inhibition though they were less than 7 mm (Table 1). According to the scale proposed by Mutai et al [24], zone of inhibition less than 7 mm, between 8-11 mm and those greater than 12 mm, correspond to non active, active and very active extracts, respectively. On the basis of these scales, the plants extracts are considered inactive against the strains of bacteria tested. This result is in accordance with previous studies [9] which revealed that crude extracts of the leaves and bark of *Maprounea guianensis* was reported to be significantly active against *Candida albicans*, *Candida krusei* and *Cryptococcus neoformans* but inactive against *Pseudomonas aeruginosa.* In addition, the sensitivity test did not show any variation based on the structure of the bacteria cell wall. It has been proved that the outer membrane is very important in the physiology of gram-negative bacteria in making them resistant to host defense factors which are very toxic to gram-positive bacteria [25]. Indeed, the envelope of Gram-positive bacteria has an outer peptidoglycan layer which is not an effective permeability barrier. Gram-negative bacteria having an outer phospholipidic membrane carrying the structural lipopolysaccharide components make the cell wall impermeable to lipophilic solutes while porins constitute a selective barrier to hydrophilic solutes with an exclusion limit of 600Da [26,27]. The lack of activity of the extracts against tested bacteria suggests that the tested bacteria are not sensitive to the chemical compounds present in the extracts or resisted the concentrations of extracts used in this study.

However, all samples displayed antifungal activities against the four strains tested (Table 2 and Fig. 2). A total destruction of spores was observed at variable extract concentrations and according to fungi strains as presented in Table 2. The crude extract exhibited the highest activity against *Aspergillus sp* with a MIC of 1.25 µg/µl. The crude extract, hexane, EtOAc and water fractions also showed potent antifungal activity in reducing the spore germination of *Aspergillus niger*, *Aspergillus sp, Aspergillus flavus* and *Penicillium sp* to 100, 100, 97.33 and 94%, respectively. The hexane fraction was the most active on *Aspergillus flavus* and *Aspergillus niger* at the concentrations of 10 µg/µl and 5 µg/µl. At the concentration of 5 µg/µl, no significant difference has been found between all the samples tested on *Penicillium sp* (H = 5.728; p-value = 0.0571). Nevertheless, crude extract was significantly more active on this strain at the concentration of 10 µg/µl (H = 11.975; p-value =

0.0075). Nystatin, used as a standard chemical did not exhibit any spore germination inhibition at the same concentration of extract/fractions. These observations may be related to the fact that conidia of different species have different levels of sensitivity and optimal germination conditions [28].

The biological results of present investigation can be related to the reported activities of the isolated compounds against the strains tested.

Indeed, it was found that friedelin was highly active against most of the human pathogenic fungal strains such as *Aspergillus niger* and showed moderated activity against the Gram-positive strain *Staphylococcus aureus* [29]. Betulinic acid has also been reported to present some antibacterial activity against *Staphylococcus aureus* and to be inactive against *Enterococcus faecalis*. Lupeol displayed moderate zone of inhibition in Aspergillus *niger, Aspergillus flavus* and significant zones of inhibition in *Pseudomonas aeruginosa* at a concentration of 0.3 µg/µl [30,31].

The results presented herein add new biological activity data of the extracts and fractions obtained from members of the genus *Maprounea*.

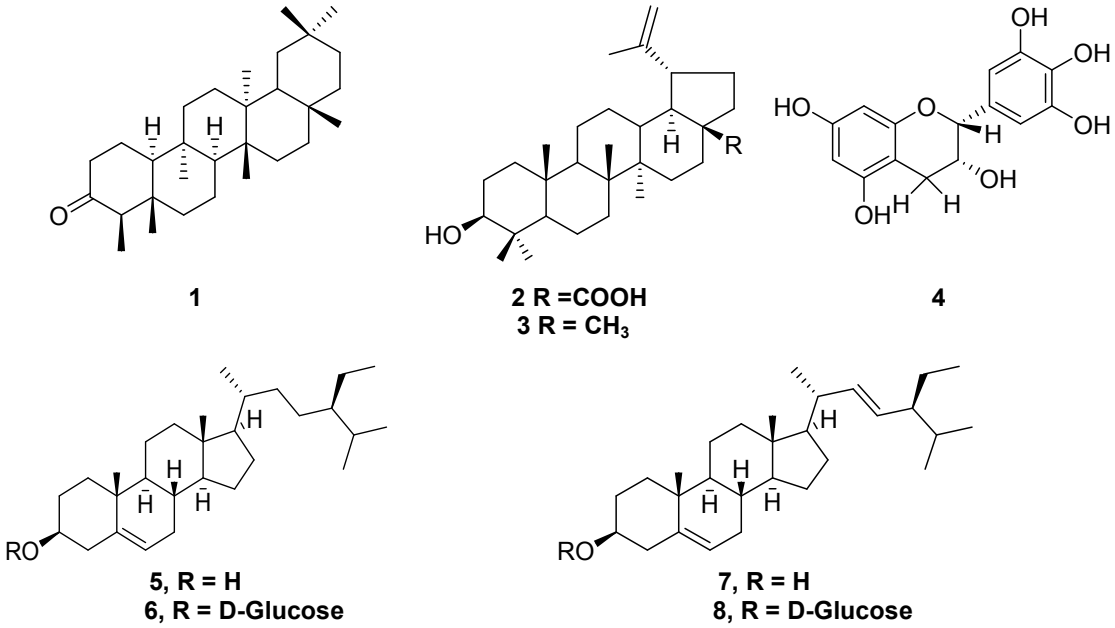

Fig. 1. Structures of compounds isolated from *Maprounea membranacea*

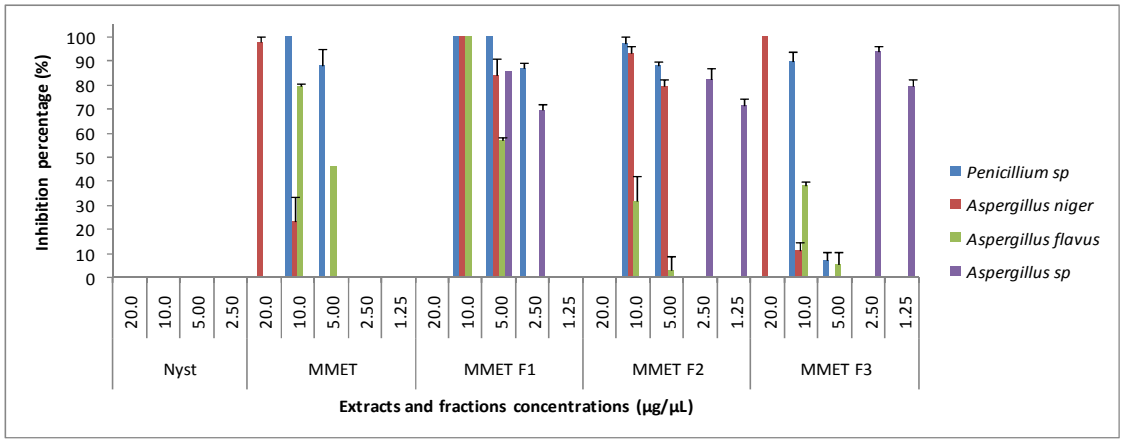

Fig. 2. Plant extract and fractions efficiency on spore germination inhibition

MMET: Crude extract, MMET F1: Hexane fraction, MMET F2: EtOAc fraction, MMET F3: Water fraction

Table 1. Antibacterial activity (zone of inhibition, average mm) of crude extract and fractions of *Maprounea membranacea* (sample concentration: 80 µg/µl)

Sample	Bacteria gram-positive	Bacteria gram-negative	
	S. aureus	***E. faecalis***	***P. aeruginosa***
MMET	3.67 ± 0.23	3.67 ± 0.47	0
MMET F1	2.33 ± 1.88	2.17 ± 0.23	0
MMET F2	2.00 ± 0.82	2.50 ± 0.00	0
MMET F3	2.33 ± 0.47	2.00 ± 0.00	0
Ciprofloxacin	23.0 ± 0.0	11.0±0.0	34.0±0.0

MMET: Crude extract, MMET F1: Hexane fraction, MMET F2: EtOAc fraction, MMET F3: Water fraction

Table 2. MIC (µg/µl) of total destruction of spores

Fungi	MMET	MMET F1	MMET F2	MMET F3	Nystatin
Aspergillus niger	40.0	20.0	20.0	40.0	> 20
Aspergillus sp	1.25	10.0	5.0	5.0	> 20
Aspergillus flavus	20.0	20.0	20.0	20.0	10.0
Penicillium sp	20.0	20.0	20.0	20.0	20.0

MMET: Crude extract, MMET F1: Hexane fraction, MMET F2: EtOAc fraction, MMET F3: Water fraction

4. CONCLUSION

Phytochemical study of stem bark of *Maprounea membranacea* gave eight known compounds in accordance with the chemotaxonomy in this plant family. Crude extract and fractions exhibited *in vitro* fungicidal activity against four strains of fungi. Considering the MIC values, the crude extract, hexane and ethyl acetate fractions may represent a therapeutic alternative in infections involving *Aspergillus sp* and *Aspergillus niger.* Since *M. membranacea* is a medicinal plant, for an optimization of its use, it may be appropriate to undergo more biological assays.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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