Original Research Article
Phytochemical and antimicrobial studies of *Maprounea membranacea* Pax & K. Hoffm (Euphorbiaceae)

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* was extracted 02 times by percolation with MeOH for 48 h. A part of this residue (350 g) was suspended in distilled water and partitioned successively between hexane and 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 extracts 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, 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 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 structure of compounds were determined by nuclear magnetic resonance spectroscopy (NMR). 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). 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 bacterial strains were cultured on Muller-Hinton Agar and incubated at 37°C for 22h. The fungal strains were cultured in Potato Dextrose Agar 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 (MHA, Merck) 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 total of 0.1 ml of bacterial suspension was spread uniformly on Muller-Hinton Agar (MHA, Merck). Crude extract and its fractions were previously dissolved in DMSO and paper disks (Ø 5 mm) were impregnated with 12.5 µg 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 was used as standard.

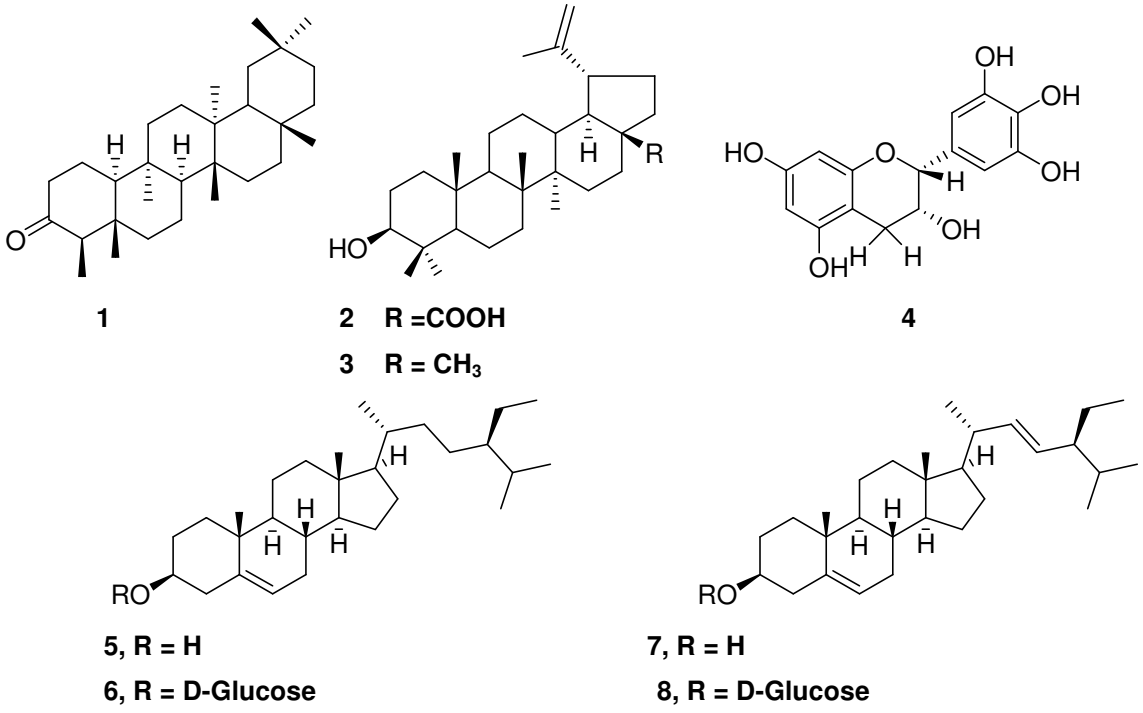
2.4.3 Antifungal activity test

Spores of all fungal 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 haemocy-tometer. Germination experiment was performed using liquid micro-dilution method in PD broth 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 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.

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].



1 **2 R =COOH** **4**

3 R = CH_3

5, R = H **7, R = H**

6, R = D-Glucose **8, R = D-Glucose**

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

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 (*Aspergillus niger*, *Aspergillus sp. Aspergillus flavus*, *Penicillium sp*). The results of antibacterial activities (Table 1) showed that samples displayed inhibition diameters

less than 7 mm against *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa.* According to the scale proposed by Mutai et al. (2009) [24], inhibition diameters of 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 our extracts would not be considered as active on the strains of bacteria tested. This last result is in accordance with those reported in the literature [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 bacterial 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+ bacteria has an outer peptidoglycan layer which is not an effective permeability barrier. Gram- bacterial 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 absence of activity on the germs would also suggest that the bacterial germs are not sensitive to the chemical compounds present in the extracts.

Table 1: Antibacterial activity (zone of inhibition, average mm) of crude extract and fractions of *Maprounea membranacea*

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.

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*, *Penicillium sp* to 100, 100, 97.33 and 94%, respectively. Nystatin, used as a standard chemical did not exhibit any spore germination inhibition at the same concentration of extracts. For the same extract, a difference of activity is observed as a function of the fungal species. This may be related to the strain difference used. Conidia of different species have different levels of sensitivity and optimal germination conditions [28].

The results of present investigation clearly indicate that the antibacterial and antifungal activity vary with the extract of the plant. Those results can be related to the reported activities of the isolated compounds against the strains tested.

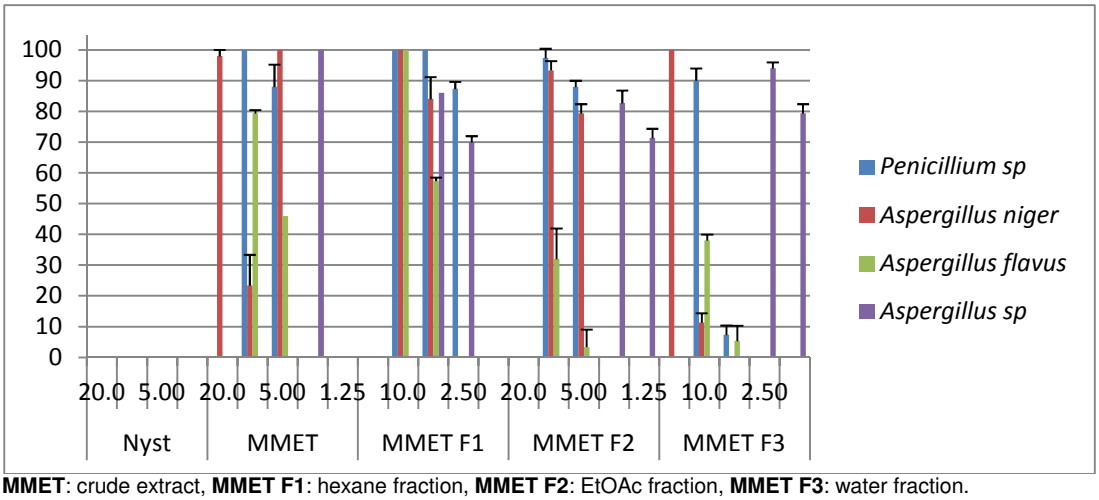
It was found that friedelin was highly active against most of the human pathogenic fungal strains such as *Aspergillus niger* and showed moderated activity against the gram+ strain *Staphylococcus aureus* [29]. Betulinic acid has also been reported to present some antibacterial activity against *Staphylococcus 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*.

Table 2 : CMI (µg/µl) of crude extract and fractions

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.



MMET: crude extract, **MMET F1**: hexane fraction, **MMET F2**: EtOAc fraction, **MMET F3**: water fraction.
Concentration in µg/µl

Fig. 2 : Percent spore germination inhibition

4. CONCLUSION

Phytochemical study of stem bark of *Maprounea membranacea* give eight know compounds in accordance with the chemotaxonomy in this plant family. Crude extract and franctions exhibiting significant *in vitro* fungicidal activity against four strains of fungi. The biological results of our present investigation suggest that this plant has the antifungal properties and can be used for infection treatment. Since *M. membranacea* is a medicinal plant, for an optimization of its use, it may appropriate to undergo more biological assays

REFERENCES

1. Annan K, Adu F, Gbedema SY. Friedelin: A bacterial resistance modulator from *Paullina pinnata* L. J of science and technology. 2009;29(1):152-159.

2. Singh DN, Verma N, Raghuwanshi S, Shukla PK, Kulshreshtha DK. Antifungal anthraquinones from *Saprosma fragrans*. Bioorgan Med Chem Letters. 2006;16(17):4512-4515. http://doi.org/10.1016/j.bmcl.2006.06.027

3. Hans–Joachim E. Taxonomic notes on neotropical *Maprounea* Aublet (Euphorbiaceae). Novon. 1999;9(1):32-35.

4. Mendonça de Senna L. Maprounea Aubl. (Euphorbiaceae). Taxonomic and anatomical considerations of the South American species. Rodrigues, Rio de Janeiro 1984; 36 (61): 51-78. English

5. N'gouemo P, Nguemby-Bina C, Baldy-Moulinier M. Some neuropharmacological effects of an ethanolic extract of *Maprounea africana* in rodents. J Ethnopharmacology. 1994;43(3):161-166. http://doi.org/10.1016/0378-8741(94)90037-X

6. Beutler JA, Kashman Y, Tischler M, Cardellina II JH, Gray GN, Currens MJ et al. A reinvestigation of *Maprounea* triterpenes. J Nat Products. 1995;58(7):1039-1046. doi**:** 10.1021/np50121a008

7. Tessier AM, Bouquet A, Paris RR. On some African poisonous euphorbiaceae. Medicinal Plants and Phytotherapy. 1975; 9 (3): 238-249. French.

8. Carney JR, Krenisky JM, Williamson RT, Luo J, Carlson TJ, Hsu VL et al. Maprouneacin, a new daphnane diterpenoid with potent antihyperglycemic activity from *Maprounea africana*. J Nat Products. 1999;62(2):345-347. doi**:** 10.1021/np980356c

9. Pengsuparp T, Cai L, Fong HHS, Kinghorn AD, Pezzuto JM, Wani MC et al. Pentacyclic triterpenes derived from *Maprounea africana* are potent inhibitors of HIV-1 reverse transcriptase. J Nat Products. 1994;57(3):415-418. doi**:** 10.1021/np50105a017

10. Marques MCS, Hamerski L, Garcez FR, Tieppo C, Vasconcelos M, Torres-Santos EC et al. In vitro biological screening and evaluation of free radical scavenging activities of medicinal plants from the Brazilian Cerrado. J Med Plant Research. 2013;7(15):957-962. doi: 10.5897/JMPR12-882

11. Wani MC, Schaumberg JP, Taylor HL, Thompson JB, Wall ME. Plant antitumor agents, 19. Novel triterpenes from *Maprounea africana*. J. Nat. Products. 1983;46(4):537-543. doi**:** 10.1021/np50028a019

12. David JP, Meira M, David JM, Guedes ML da S. Triterpenos e ferulatos de alquila de *Maprounea guianensis*. Quím Nova. 2004;27(1):62-65. Portuguese. http://dx.doi.org/10.1590/S0100-40422004000100013

13. Longue EJP, Nouga BA, Fomani M, Toze FAA, Kamdem WAF, Sewald N et al. Cytotoxic 24-nor-ursane-type triterpenoids from the twigs of *Mostuea hirsuta.* Z. Naturforschung. 2015;70(11) B:837-842. http://doi.org/10.1515/znb-2015-0083

14. Ragasa CY, Ebajo Jr V, De Los Reyes MM, Mandia EH, Brkljača R, Urban S. Triterpenes from *Calophyllum inophyllum* Linn. Int J Pharmacognosy and Phytochem Research. 2015;7(4):718-722.

15. Cichewicz RH, Kouzi AS. Chemistry, biological activity and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. Med Res Reviews 2004;24(1):90-114. doi: 10.1002/med.10053

16. Roy R, Jash SK, Roy S, Acharya R, Gorai D. Two Bioactive Constituents from *Combretum decandrum*. Intern J Nat Prod Research. 2016; 6(1):18-20

17. Guo X-Y, Wang N-L, Bo L, Li Y-H, Xu Q, Yao X.-S. Chemical constituents from *Pithecellobium clypearia* and their effects on T lymphocytes proliferation. J Chinese Pharm Sciences. 2007;16(3):208-213.

18. Zhang X, Geoffroy P, Miesch M, Julien-David D, Raul F, Aoude-Werner D et al. Gram-scale chromatographic purification of β-sitosterol, synthesis and characterization of β-sitosteroloxides. Steroids. 2005; 70(13): 886 - 895. http://doi.org/10.1016/j.steroids.2005.06.003

19. Supaluk P, Saowapa S, Apilak W, Ratana L, Somsak R, Virapong P. Bioactive Metabolites from *Spilanthes acmella* Murr. Molecules. 2009;14(2):850-867. doi:10.3390/molecules14020850

20. Venkata SPC, Indra P. Isolation of Stigmasterol and β-Sitosterol from the dichloromethane extract of *Rubus suavissimus*. Int Curr Pharm Journal 2012;1(9):239-242.

21. De Marino S, Gala F, Zollo F, Vitalini S, Fico G, Visioli F et al. Identification of minor secondary metabolites from the latex of *Croton lechleri* (Muell-Arg) and evaluation of their antioxidant activity. Molecules. 2008; 13(6): 1219 - 1229. doi:10.3390/molecules13061219

22. Leong KI, Alviarez PF, Compagnone RS, Suárez AI. Isolation and structural elucidation of chemical constituents of *Amanoa almerindae*. Pharm Biology. 2009;47(6):496-499. http://dx.doi.org/10.1080/13880200902838717

23. Bouquet A. Plantes médicinales du Congo-Brazzaville. Travaux et documents de l'ORSTOM 1972;13(1):1-127. French.

24. Mutai C, Bii C, Rukunga G, Ondicho J, Mwitari P, Abatis D et al. Antimicrobial activity of pentacyclic triterpenes isolated from *Acacia mellifera*. Afr J Tradit Complement Altern medicines. 2009;6(1):42-48.

25. Nikaidol H, Vaara M. Molecular basis of bacterial outer membrane permeability. Microbiological reviews. 1985;49(1):1-32.

26. Abeysinghe PD. Antibacterial activity of some Medicinal Mangroves against antibiotic resistant pathogenic bacteria. Indian J Pharm Sciences. 2010;72(2):167-172. doi**:** 10.4103/0250-474X.65019 PMID: 20838519

27. Gurinder JK, Daljit SA. Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspemum ammi*. BMC Complement Altern Med. 2009;9:30. doi: 10.1186/1472-6882-9-30

28. Osherov N, Gregory SM. The molecular mechanisms of conidial germination. FEMS Microbiology Letters. 2001;199:153-160.

29. Ali MS, Mahmud S, Perveen S, Rizwani GH, Ahmad VU. Screening for the antimicrobial properties of the leaves of *Calophyllum inophyllum* Linn. (Guttiferae). J Chem Soc Parkistan.1999;21(2):174-178.

30. Ahmed BKM, Krishna V, Gowdru HB, Rajanaika H, Kumaraswamy HM, Rajshekarappa S et al. isolation of bactericidal constituents from the steam bark extract of *Grewia tiliaefolia* Vahl. Res J Med Plant. 2007;1:72-82.

31. Singh B, Singh S. antimicrobial activity of terpenoids from *trichodesma amplexicaule* Roth. Phyt research. 2003;17:814-816.