Journal of Science and Practice of Pharmacy
December 2020; 7 (1): 400-405
Available at http://www.jsppharm.org **https://doi.org/10.47227/jsppharm.v7i1.7**
ISSN: 2449-0458 (print); 2449-0466 (electronic)

Original Research Article

Antibacterial screening and isolation of compounds from *Detarium microcarpum* stem bark against methicillin resistant *Staphylococcus aureus*

Kayode Muritala Salawu[12*], Yan Wang[2], Rukesh Maharjan[2], Edith Oriabure Ajaiyeoba[3]

[1]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, University of Ilorin, Ilorin, Nigeria.,[2]*H.E.J* Research Institute of Chemistry, International Center for Chemical and Biological Sciences (*ICCBS*), Karachi, Pakistan., [3]Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria.

* For correspondence: *Email: Salawu.mk@unilorin.edu.ng, Tel: +2348067818912*

Abstract

Purpose: Infectious diseases are leading cause of death globally and a primary cause of public health concern. The most worrisome aspect of microbial infection is the emergence of resistant strains of micro-organisms to commonly available antimicrobial agents. The emergences of resistant strains have added more pressure on the need to identify new agents that may be effective against resistant microbial strains. The focus of this study was to screen the crude extract and fractions of *D. microcarpum* stem bark for their antibacterial effects and isolate compounds with antibacterial activities.
Methods: The stem bark of *Detarium microcarpum* was collected, authenticated, air dried, extracted into 70% methanol. The extract was fractionated successively into n-hexane, dichloromethane and ethyl acetate. Concentrated extract and fractions were subjected to antibacterial assay using Microplate Alamar Blue Assay. Two compounds were isolated using column chromatographic techniques. The compounds were characterised using spectroscopic techniques and later subjected to antibacterial studies to determine their minimum inhibitory concentration (MIC).
Results: The crude extract displayed weak antibacterial activity across the panel of test microorganisms while the fractions displayed antibacterial activity in order of decreasing polarity. Compounds isolated from dichloromethane and ethyl acetate fractions were identified as methyl gallate (compound 1) and catechin gallate (compound 2), respectively. Interestingly compounds 1 and 2 displayed anti-MRSA activity with compound 2 (MIC of 200 µg/mL) displayed anti-MRSA activity.
Conclusion: Antibacterial and chromatographic analysis of *D. microcarpum* stem bark led to identification of compounds active against methicillin resistant *Staphylococcus aureus*.

Keywords: *Detarium microcarpum,* Antibacterial activity Methyl gallate, Catechin gallate

Indexing: Index Copernicus, African Index Medicus

Introduction

Infectious diseases caused by bacteria are major source of public health concern and the Centre for Disease Control and Prevention (CDC) reported that at least 2 million people are diagnosed annually with serious bacterial infections and about 20,000 deaths are recorded annually primarily because of therapeutic failure [1]. Wide spread and indiscriminate use of antibiotics has led to the development of many antibiotic resistance microbial strains which include methicillin resistant *Staphylococcus aureus* (MRSA). Hence, many infectious diseases are becoming more difficult to manage with available conventional antibiotics [2]. Hence the need to search for new antimicrobial agents from various sources, particularly plants that have been used

traditionally in the treatment of several infectious diseases.

Medicinal plants have been in use as an anti-infective from time immemorial [3] and are now considered as an important source for pharmaceutical raw materials and medicinal principles [4]. Globally several research efforts are being focused on identifying new anti-infective principles from natural source for the treatment of old scourges and emerging diseases caused by bacteria, fungi, viruses and plasmodium.

Several antimicrobial compounds including hypericin, berberine, hasubanalactam, pyranocoumarins have been recently isolated from medicinal plants [5]. The microplate Alamar Blue assay (MABA) is a sensitive, rapid, inexpensive method that is capable of evaluating cellular viability and offers the potential for screening large numbers of antimicrobial compounds. MABA has offered several advantages of being simple, reproducible, cost-effective and nontoxic. It is amenable to high throughput screening and MIC_{50} determination. The primary advantage of MABA is that growth can be evaluated fluorometrically or spectrophotometrically or visually [6].

Detarium microcarpum belonging to Caesalpiniaceae family is indigenous to Africa. The seeds, leaves and barks are often used as food and in the treatment of numerous infectious diseases including diarrhoea, dysenteries, heamorrhoids, leprosy and syphilis [7]. The focus of this study was to screen the crude extract and fractions of *D. microcarpum* stem bark for their antibacterial effects using microplate Alamar Blue assay and isolate antimicrobial compounds from the selected fractions.

Methods

Materials

Plant samples, hexane, dichloromethane, ethyl acetate, distilled methanol, butanol, distilled water, glass macerating jar, cotton wool, funnel, rotary evaporator, regulated water bath, evaporating dish, spatula.

Plant selection, collection and authentication

The stem bark of *Detarium microcarpum* was collected from the main campus of University of Ilorin, Kwara State, Nigeria. The plants were identified and authenticated at the Herbarium Unit of Forest Herbarium Ibadan (FHI) in the Department of Forestry Conservation and Preservation of the Forestry Research Institute of Nigeria (FRIN). Voucher number (FHI) of 111953 was issued.

Extraction and fractionation

Ten (10) kg of *D. microcarpum* was extracted into 70% methanol. The crude extract was concentrated *in vacuo* and stored until needed for further use. One thousand (1000) g of extract was fractioned exhaustively and successively into n-hexane, dichloromethane and ethyl acetate. The fractions obtained were concentrated *in vacuo*. The crude extract and fractions were subjected to antibacterial evaluation as shown below.

Isolation of antibacterial compounds

Thirty five grams (35 g) of dichloromethane fraction of *D. microcarpum* was chromatographed using a glass column (6 x 60 cm) with silica gel (60-120 μm) as the adsorbent (1050 g) using gradient elution using two solvents combination system of n-hexane (Hex), dichloromethane (DCM) and methanol (MeOH) in order of increasing polarity as mobile phase (5:0//Hex:DCM, 4:1//Hex:DCM, 3:2//Hex:DCM, 2.5:2.5//Hex:DCM, 2:3//Hex:DCM, 1:4//Hex:DCM, 0:5//Hex:DCM, 4:1//DCM:MeOH, 3:2//DCM:MeOH, 2.5:2.5//DCM:MeOH and 2:3//DCM:MeOH).

Column fractions obtained were pooled into seven sub-fractions (1-7) using their TLC profile. Sub-fraction 4 (273 mg) was purified by dissolving it in methanol and purified by passing it through a column of sephadex LH-20 using 100% methanol as the mobile phase, 265 mg of pure compound was obtained and labelled as Compound **1**.

Thereafter, forty grams (40 g) of ethyl acetate fraction of *D. microcarpum* was chromatographed using glass column (7 x 60 cm) with silica gel (60-120 μm) as the stationary phase and eluted using two solvents combination system of n-hexane, ethyl acetate and methanol in order of increasing polarity as mobile phase.

The fraction was further fractionated into seven sub-fractions (1-7). Sub-fraction 3 (10 g) was further chromatographed using two solvents combination system of n-hexane, ethyl acetate and methanol in order of increasing polarity as mobile phase using silica gel (mesh size 60-120

µm) as the stationary phase and a column (3 x 50 cm) as the stationary support. Fractions obtained were pooled to five fractions (A-D). Sub-fraction A (160 mg) was purified by dissolving in methanol and passed though long sephadex LH-20 column (1 x 50 cm) to obtain compound **2**(31 mg). Compounds isolated were characterized using combination of mass and NMR spectroscopic techniques. Spectroscopic data obtained were compared to those available in literature. Anti-MRSA activities of the isolated compounds were evaluated as shown below:

Preliminary susceptibility test

The antibacterial activity of the extract and fractions was done using antibacterial Microplate Alamar Blue assay [8].
Typed culture Gram negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145*, Salmonella typhi* ATCC 14028) and Gram positive (*Bacillus subtilis* ATCC 23857, methicillin resistant *Staphylococcus aureus* NCTC 13277) were used in the study.

Susceptibility test for isolated compounds

Test organisms were grown in Mueller Hinton medium and used to prepare inoculums. The inoculums were adjusted to 0.5 McFarland turbidity index. Stock solution of 60 mg/mL and 20 mg/mL of crude extract and pure compound was prepared in 100% DMSO, respectively.

Crude extracts were tested at 3000µg/mL against all five strains of bacteria, while the pure compounds were tested at different concentration (500, 250, 125 and 62.5 µg/mL) against methicillin resistant *S. aureus* (NCTC 13277) and *E. coli* (ATCC 25922).

A 100 µL of Mueller Hinton Broth (MHB) containing $1x10^6$ CFU/mL bacterial inoculum was added, such that total volume of solution become 200 µL and incubated briefly for 24 h at 37 ºC without shaking.

After 24 h the parafilm was removed, and the turbidity of the wells were checked to identify clear wells. After visual inspection, 5 µL from each well were plated in well and incubated overnight. After that, 20 µL of Alamar Blue dye was added in each well and further incubated for 2 h at 37 ºC in shaking incubator at 80 rpm. Thereafter, the absorbance was recorded at 570 and 600 nm by ELISA reader (Thermo Scientific, USA).

The values obtained were processed using the formula below to determine the % growth inhibition due to extract and compounds. Those wells with the lowest concentration having clear wells on visual inspection and wells having blue dye colour with more than 60% inhibition was considered as the minimum inhibitory concentration (MIC).

Standard antibacterial drug (Ofloxacin) at 25 was used as positive control while 2.5% DMSO was used as the negative control. The experiment was done in three wells/plates in three different days. Percent reduction of Alamar blue was calculated using the formula below:

$$\text{Percentage Inhibition} = \left[\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{red})\lambda_1 A'\lambda_2 - (\varepsilon_{red})\lambda_2 A'\lambda_1} X100\right]$$

Where:
ε_{OX} = molar extinction coefficient of Alamar blue oxidized form (blue)
ε_{red} = molar extinction coefficient of Alamar blue oxidized form (pink)
A = Absorbance of test wells,
A' = Absorbance of negative control well
λ_1 = 570 nm
λ_2= 600 nm

Results

Antibacterial effects of crude extract and fractions of D. microcarpum

Different fractions of *D. microcarpum* were tested against 5 different strains of bacteria using Microplate Alamar Blue assay and % inhibition is reported in Table-1. None of the extracst showed potent antibacterial activity against tested bacteria. Only DCM fraction showed 51% against *B. subtilis*, where the other fractions are inactive.

Tables 1: Antibacterial Activity of *D. microcarpum* stem bark crude extract and fractions

Samaple	Percent (%) Inhibition at 3000 µg/mL				
	E.coli	***B. subtilis***	***S. aureus***	***P. aeruginosa***	***S. typhi***

Extract	-	-	-	-	10
Hx	11	-	13	16	11
DCM	-	51	2	19	-
EtOAc	-	13	-	10	-
AQ	-	-	-	11	-
Standard Drug*	83	92	90	91	89

Key: HX= n-hexane fraction, DCM= Dichloromethane fraction, EtOAc= Ethyl acetate fraction AQ= Aqueous phase, - = no inhibition, ***= at 25µg/mL**

Isolation and structural elucidation of bioactive compounds

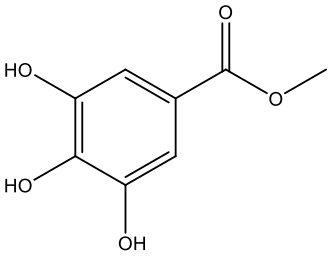
Preparative chromatographic analysis of dichloromethane and ethyl acetate fractions led to isolation of one compounds each from the dichloromethane and ethyl acetate fractions. The compounds were identified as methyl gallate and catechin gallate respectively as shown in Figure 1.

Compound **1** was isolated as a white crystalline solid and identified as methyl gallate with the following spectroscopic data; ^{1}H NMR (in CD3OD): 7.03 (2H, s) and 3.81 (3H, s),

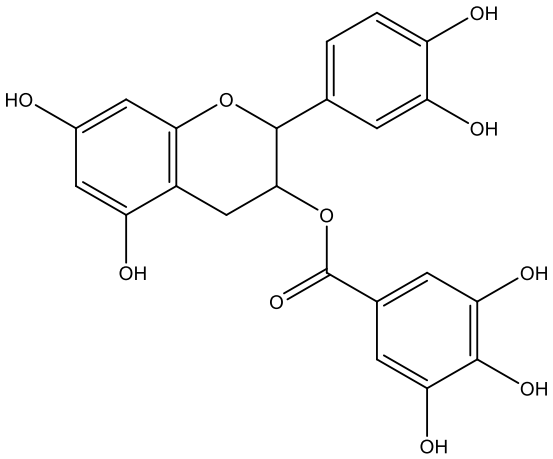
^{13}C NMR (in CD_3OD): 121.45 (C-1), 110.04 (C-2), 146.54 (C-3), 140.30 (C-4), 145.51 (C-5), 110.04 (C-6), 169.02 (C-7), 52.24 (C-8). MS *m/z*: 184 (M^+).

Compound **2** was isolated as a colourless crystalline solid and identified as catechin gallate with the following spectroscopic data;^{1}H NMR (in C_5D_5N): 5.44 (1H, bs), 6.10 (1H, bs), 3.64 (1H, dd, $J_{4a,b}$ = 4.8; $J_{4a,3}$ = 17.2), 3.46 (1H, dd, $J_{4b,a}$ = 4.8; $J_{4b,3}$ = 17.2), 6.65 (1H, d $J_{6,8}$ = 2), 6.66 (1H, d $J_{8,6}$ = 2), 7.13 (1H, d, $J_{3',2'}$ = 8), 7.67 (1H, d, $J_{6',2'}$ = 2), 7.84 (1H, s), 7.84 (2H, s).

^{13}C NMR NMR (in C_5D_5N): 78.29 (C-2), 69.27 (C-3), 26.94 (C-4), 157.34 (C-5), 96.93 (C-6), 158.50 (C-7), 95.75 (C-8), 158.68 (C-9), 99.04 (C-10), 130.65 (C-1'), 118.98 (C-2'), 146.98 (C-3'), 147.44 (C-4'), 116.18 (C-4'), 115.63 (C-5'), 121.14 (C-1"), 110.27 (C-2"), 1.47.44 (C-3"), 140.95 (C-4"), 147.44 (C-5"), 110.27 (C-6"), 166.69 (C-7"). Ms M/Z: 443.1 (M^++1).



Compound 1



Compound 2

Figure 1: Structures of Isolated compounds

The compounds were tested against multidrug resistant Gram-positive *S. aureus* (NCTC 13277) and *E. coli* (ATCC 25922) using Alamar Blue assay (Table 2). Interestingly the compounds**1** and **2** displayed anti-MRSA activity. However, Compound **2**(MIC of 200 µg/mL) displayed an anti-MRSA effect that is over two times better when compared to compound **1** (MIC of 500 µg/mL). These two compounds having anti-MRSA activity were active at higher concentration, therefore, it was anticipated that these compounds might be present in smaller quantity in crude extract due to which extract was found to be inactive.

Table 2: Antibacterial activity of compounds isolated from *D. microcarpum* stem bark

Test Samples	*Staphylococcus aureus* (NCTC 13277)		*Escherichia coli* (ATCC 25922)	
	% inhibition at 500 µg/mL	MIC (µg/mL)	% inhibition at 500 µg/mL	MIC (µg/mL)
Compound 1	82.20 ± 0.99	500	No Inhibition	NT
Compound 2	86.03 ± 0.84	200	No Inhibition	NT
Ofloxacin	85.13 ± 0.26	25	85.45 ± 0.18	50

Key: MIC= Minimum Inhibitory Concentration, NT: Not Tested

Discussion

Bacterial infection are major cause of death globally and several plant used traditionally are now been scientifically evaluated for antimicrobial activities with the aim of identifying the antibacterial principles present in the plants. Several parts of *D. microcarpum* have been used locally for the treatment of several infectious diseases particularly of bacterial origins such as venereal diseases, urogenital infections, diarrhoea, dysentery, leprosy, sore throat and wounds infections. Generally, the plant extract is used to prevent and cure infections of several origin and several literature have reported antimicrobial activities of extracts of *D. microcarpum* [9] however, there are no sufficient data on the bioactive compounds responsible for the antibacterial activity of the plant. The fractions of the crude extract displayed weak antibacterial activity but the compound **1**and **2** isolated from dichloromethane and ethyl acetate fractions, respectively displayed anti-MRSA activity.

An earlier report of antimicrobial activity of the *D. microcarpum* stem bark led to isolation of some constituents such as lupeol, lup-20(20)-en-2α,3ß-diol, ß-sitosterol, stigmasterol and campesterol [10]. However, this is the first report of isolation of methyl gallate from *D. microcarpum.*Though catechingallate (86.03 ± 0.84 % inhibition) and methyl gallate (82.20 ± 0.99 % inhibition) displayed similar anti-MRSA activity at 500 µg/mL and it was observed that catechin gallate (MIC of 200 µg/mL) displayed better anti-MRSA activity as compared to methyl gallate (MIC of 500 µg/mL). Methyl gallate and catechin gallate have been previously report to display anti-MRSA activity [11,12].

Conclusion

The extract of *D. microcarpum* displayed weak antibacterial activities against *E. coli, P. aeruginosa, S. typhi, B. subtilis and S. aureus*. However, the extract contains catechin gallate with anti-MRSA properties. This is the first report of the isolation of methyl gallate from *D. microcarpum*.

Acknowledgements

KMS was a recipient of The World Academy of Sciences (ICCBS) split-site PhD Scholarship at the International chemical and biological Sciences, Pakistan and the TEFUND SDA from the University of Ilorin during his PhD study at The University of Ibadan, Ibadan, Nigeria.

Conflict of Interest

The authors have declared that there is no conflict of interest.

Contribution of Authors

We declare that this study was done by the authors stated in this article and all liabilities pertaining to claims relating to the content of this work will be borne by us. KMS and EOA conceived and designed the work. KMS collected plant, extracted plant material and isolated the compounds, elucidated the molecular structures of the compounds and wrote manuscript. YW validate the structures of the compounds isolated. RM performed the Antimicrobial studies. MIC is a research collaborator.

References

1. Li, J., Echevarria, K.L. and Traugott, K.A. β-Lactam Therapy for Methicillin-Susceptible Staphylococcus aureus Bacteremia: A Comparative Review of Cefazolin versus Antistaphylococcal Penicillins. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy 2017;37(3):346-60.
2. Chabán, M.F., Karagianni, C., Joray, M.B., Toumpa, D., Sola, C., Crespo, M.I., Palacios, S.M, Athanassopoulos, C.M. and Carpinella, M.C. Antibacterial effects of extracts obtained from plants of Argentina: Bioguided isolation of compounds from the anti-infectious medicinal plant Lepechinia meyenii.

J Ethnopharmacol 2019;239:111930. DOI: 10.1016/j.jep.2019.111930.

3. Hamza, H.J. Antimicrobial activity of some plant extracts on microbial pathogens isolated from Hilla city hospitals, Med J Babylon 2015;12(2):398-407.
4. Singh, S., Saroj, K., Tripathi, V., Singh, A. and Singh, R. An antimicrobial principle from Sphaeranthus indicus L.(family compositae). Int J Crude Drug Res 1988;26(4):235-9.
5. Bereksi, M.S., Hassaïne, H., Bekhechi, C. and Abdelouahid, D.E. Evaluation of antibacterial activity of some medicinal plants extracts commonly used in Algerian traditional medicine against some pathogenic bacteria. Pharmacognosy Journal 2018;10(3).
6. Tyc, O., Tomás-Menor, L., Garbeva, P., Barrajón-Catalán, E. and Micol, V. Validation of the AlamarBlue® assay as a fast screening method to determine the antimicrobial activity of botanical extracts. PLoS One. 2016 Dec 29;11(12).
7. Gbadamosi, I., Moody, J. and Oyekini, A. Nutritional composition of ten ethnobotanicals used for the treatment of anaemia in Southwest Nigeria. European Journal of Medicinal Plants. 2012;2(2):140.
8. Pettit, R.K., Weber, C.A., Kean, M.J., Hoffmann, H., Pettit, G.R., Tan, R., Franks, K.S. and Horton, M.L. Microplate Alamar blue assay for Staphylococcus epidermidis biofilm susceptibility testing. Antimicrob. Agents Chemother 2005;49(7), 2612-2617.
9. Friday, C., Akwada, U. and Igwe, O.U. Phytochemical screening and antimicrobial studies of Afzelia africana and Detarium microcarpum seeds. Chem Int 2018;4(3):170-176.
10. Doughari, J. and Nuya, S. Vitro antifungal activity of deterium microcarpum. Pak J Med Sci Q 2008;24(1):91.
11. Abreu, P., Rosa, V., Araujo, E., Canda, A., Kayser, O., Bindseii, K.V., Siems, K. and Seeman, A. Phytochemical analysis and antimicrobial evaluation of Detarium microcarpum bark. Phar Pharmacol Lett. 1998;8:107-11.
12. Ahmed, M.D., Taher, M., Maimusa, A.H., Rezali, M.F. and Mahmud, M.I.A.D.M. Antimicrobial activity of methyl gallate isolated from the leaves of Glochidion superbum against hospital isolates of methicillin resistant Staphylococcus aureus. Nat Prod Sci 2017;23(1):5-8.