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Characterization of phytochemicals from the root extract of *Milletia leucantha* and their weak anti-microbial properties

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Abstract

Background: Cures for infectious diseases are slowly becoming elusive due to resistance mechanisms of disease-causing microbes. As a result, most conventional medicines are now being rendered ineffective, causing increased difficulty in the treatment of infections and an upsurge of fatalities. In the present study, the anti-bacterial and anti-fungal properties of extracts and compounds of *Milletia leucantha* were investigated.

Methods: The roots of *M. leucantha* were exhaustively extracted by 1:1 v/v CH_2Cl_2/MeOH. The extract was then subjected to column chromatography and from the fractions obtained some compounds were purified and others detected, this led to the identification of four compounds. The compounds were characterized based on spectroscopic techniques including 1D and 2D NMR and comparison of obtained data with that of previously reported compounds. Two extracts 1:1 v/v CH_2Cl_2/MeOH and 95:5 v/v MeOH/H_2O and compound **1** were screened for anti-microbial properties using disc diffusion assay.

Results: The four compounds were identified as, one flavonoid; afromosin (**1**), three terpenoids; lupeol (**2**), a mixture of stigmasterol (**3**) and β-sitosterol (**4**). The 95:5 v/v MeOH/H_2O extract, the 1:1 v/v CH_2Cl_2/MeOH extract and compound **1** showed low activity against the tested micro-organisms.

Conclusion: The results of this study suggest that the four compounds namely; afromosin (**1**), three terpenoids; lupeol (**2**), a mixture of stigmasterol (**3**) and β-sitosterol (**4**) were isolated from the 1:1 v/v CH_2Cl_2/MeOH root extract of *M. leucantha*. The root extracts and the compound tested, afromosin (**1**) showed minimal to no antimicrobial activity.

Keywords: Secondary metabolites; antimicrobial; *Milletia leucantha;* Fabaceae.

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Background

Microbial infections have become an increased cause for concern, due to a large number of fatalities [1]. Infections such as diarrhea, tuberculosis, and pneumonia are the common causes of morbidity in Kenya [2], where children and immune compromised persons being the most vulnerable [2]. The causative agents of these diseases have developed numerous resistance mechanisms [3], rendering medications ineffective. Newly developed drugs have not been spared [4] as they have also been rendered ineffective. Since plants have proved to be a vital resource in pharmaceutical research, the isolation of new antimicrobials is, therefore, necessary to replace the ineffective drugs.

Milletia leucantha belongs to the family Fabaceae with approximately 730 genera and 19000 species [5]. The *Milletia* genus has over 323 species distributed in tropical Africa, Asia and Australia, where about 260 species are believed to be medicinal [6]. In Kenya, this species is represented by six genus [7] including *M. leucantha*. *M. leucantha* is a scandent shrub, whose leaflets are oppositely pinnate with a terminal leaflet. The leaflets are also slightly pubescent underneath. It is commonly found in semi-deciduous forests, on rocky hills or secondary bushlands [7].

Plants from this genus are used for ethnomedicine to manage a number of diseases in traditional African and Asian set up including infertility, gonorrhea, malaria, dysentery, coughs and stomach pains [6]. Species from this genera also have a variety of applications in fishing [8], neutralizing snakebites [6], repelling houseflies and cabbage worms [9], while others have been used as timber [9], pesticides and vermicides [8]. *M. leucantha* commonly known as Sathorn in Thailand has been used traditionally as a preservative for chili sauce [10], while its stem is used to make furniture [11].

Compounds isolated from *M. leucantha* include; chalcones, flavones, flavonols [11], pterocarpans and other phenolic compounds [12]. A number of compounds from this species have been reported to exhibit various biological activity such as cytotoxicity against KB, MCF-7, NCI-H187 [11] and NCI-H460 cells [13], anti-viral, anti-inflammatory [13] and anti-cancer properties [12]. The Kenyan *M. leucantha* has not been phytochemically investigated so far. Information available on this species is only on variants of Thailand origin [12, 13]. The composition of metabolites in plants is significantly influenced by environmental factors since these compounds play a major role in the adaptation. Hence, in this study, the Kenyan species was phytochemically investigated. Based on the traditional application of this plant as a preservative [10], its root extracts and compounds isolated in sufficient yields were tested against different bacterial and fungal strains to establish their potency towards authenticating their traditional use.

Methods

Plant collection and extraction

The plant sample was collected from Nzaui forest (GPRS 1° 53' 59" S, 37° 31' 59" E) in Makueni County, Kenya. The plant material was identified by Mr. Patrick Mutiso a taxonomist from the University of Nairobi. The sample was then taken to the School of Biological Sciences (SBS), University of Nairobi (UoN) where the voucher specimen WWL2017/001 was deposited. The plant material was cut into small pieces and air-dried for a week under a shade. The dried sample was then ground into a powder and weighed (3.6 Kg) and thereafter extracted exhaustively with 1:1 v/v CH_2Cl_2/MeOH by marceration for three intervals, at room temperature, each time for 24 hours. The resultant extracts were filtered and concentrated *in vacuo* to give 147.25 g of the crude extract. Thereafter, 95:5 v/v MeOH/H_2O was used to repeat the process of extraction this time for two intervals of 24 hours each, to give 11 g of the extract.

Isolation of compounds

The 1:1 v/v CH_2Cl_2/MeOH extract was subjected to silica gel column chromatography and eluted with gradients of *n*-hexane/CH_2Cl_2 then by CH_2Cl_2/MeOH as the mobile phases. A total of 480 fractions of 400 mL each were obtained and combined into 15 fractions based on their analytical TLC profiles, coded LWW. Two sub-fractions (LWW 38L and LWW 62A) eluted at 10% *n*-hexane/CH_2Cl_2, yielded afromosin (**1**), lupeol (**2**) and a mixture of stigmasterol (**3**) and β-sitosterol (**4**). Recrystallization of fraction LWW 38L (0.96 g) yielded lupeol (**2**) (6 mg), while the mother liquor (0.3 g) was loaded onto a chromatotron and eluted with (10 – 20%) EtOAc in *n*-hexane in order of increasing polarity, resulting in a mixture of stigmasterol (**3**) and β-sitosterol (**4**) (5 mg). Purification of fraction LWW 62A (2.31 g) was achieved through column chromatography using silica gel as the stationary phase and eluted with *n*-hexane/EtOAc as the mobile phase. Where one of its fractions (0.29 g) was further purified by loading it into a Sephadex LH-20 column and eluted with (1:1) CH_2Cl_2/MeOH to yield afromosin (**1**) (5 mg).

Anti-microbial Assay

The *in vitro* disc diffusion method was carried out according to the Clinical and Laboratory Standards Institute, CLSI M44 method as described by literature [14]. The test organisms used for screening were *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* ATCC 23922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 1388. While four yeasts were used that is *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 90018, *Trichophyton mentagrophytes* and *Cryptococcus neoformans*. The test organisms were acquired from the American Type Culture Collection (Manassas, VA), except for methicillin-resistant *Staphylococcus aureus* (MRSA), *Trichophyton mentagrophytes* and *Cryptococcus neoformans* obtained from a clinical source. The positive controls used were chloramphenicol which was used for the bacteria, while nystatin was used for the fungi, whereas 5% DMSO was used as the negative control for the tests.

Briefly, 0.5 McFarland standard was prepared on Mueller hinton broth for each test organism, uniformly spread on Mueller hinton agar and left to dry at room temperature. The samples were dissolved in 2 mL DMSO to create concentrations of 95:5 v/v MeOH/H_2O extract (500 mg/mL), 1:1 CH_2Cl_2/MeOH extract (205 mg/mL), compound **1** (2.75 mg/mL). Thereafter, 20 µL of the samples were impregnated onto sterile 6 mm blank paper discs. The discs were aseptically placed on the surface of the inoculated plates and incubated at 35 ºC ambient air for 24 hours. The tests were done in triplicate, where the diameter of the zones of inhibition were expressed in mm. The activities of the test samples were classified according to literature [15]; where diameters ≤ 8 mm as non-sensitive, 9-14 mm as sensitive, 15-19 mm as very sensitive and finally ≥ 20 mm as extremely sensitive.

Results

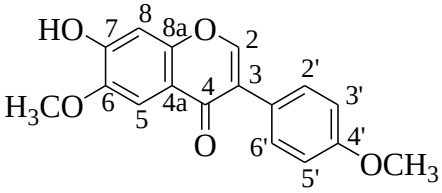
Phytochemistry

Phytochemical investigation of the root of *M. leucantha* led to the isolation of four known compounds. Their structures were established as afromosin (**1**) $C_{17}H_{14}O_5$, mp 228-230 ºC [16], lupeol (**2**) $C_{30}H_{50}O$ 216-218 ºC [17], stigmasterol (**3**) $C_{29}H_{48}O$ mp 144-146 ºC and β-sitosterol $C_{29}H_{50}O$ mp 147-149 ºC (**4**) [18]. They were characterized using 1D and 2D NMR spectroscopic techniques, where the spectral data obtained were compared to those of previously reported compounds. The spectral data obtained is as shown in the supplementary material, while the chemical structures of the compounds are represented in Figure 1.

Anti-microbial Assay results

The tested samples showed minimal inhibition zones as indicated in Table 1. The 95:5 v/v MeOH/H_2O extract showed better activity of 7 mm against *S. aureus* and 6 mm for *T. mentagrophytes* and *C. parapsilosis* as compared to CH_2Cl_2/MeOH extract that showed no activity with the said micro-organisms. The outcome of an extraction process depends on the type of solvents used, their temperature [19], as well as the nature and proportion of phytochemicals present [20]. In addition, the interactions of these substances with the solvent used [19], that is, if the substances are either lipophilic or hydrophilic also influences the yield of the extract and subsequent activity. Further, antimicrobial properties of plant extracts are often pathogen specific [21], this implies that extracts may show better activity in a strain of micro-organisms and little or no activity in another. This is the case with the 95:5 v/v MeOH/H_2O extract as mentioned above showing variable inhibitions zones with the test pathogens. The two extracts also exhibited inhibition zones of 6 mm with *E. Coli*, *C. albicans* and *C. neoformans* while no inhibition was recorded with MRSA, *P. aeruginosa* and *K. pneumoniae*.

Compound **1** showed an inhibition zone of 6 mm with the tested pathogens, with the exception of MRSA, *P. aeruginosa* and *K. pneumoniae*. Flavonoids are revered antimicrobials, their potency is determined by the structure and positioning of functional groups [22] such as hydroxylation and methoxylation [21]. Further the presence of the benzene moiety at position C-2 in a flavonoid structure is better suited for interactions with bacterial membranes, as opposed to the isofavanoid structure [21] making them better antimicrobials. Furthermore the presence of a hydroxyl group at position C-3 has been shown to improve the antibacterial activity of flavonoids [21]. A study by Park *et al* [23] showed that afromosin (**1**) was found to show no activity against *Helicobacter pylori,* the inactivity thought to be due to free rotation between the B and C rings [23]. Studies done on other *Milletia* species seem to suggest that prenylation [24] or pyrano rings [25] in an isoflavone structure enhance their antimicrobial activity. The minimal antimicrobial activity against MRSA, *P. aeruginosa* and *K. pneumoniae* could be due to the production of extended spectrum beta-lactamases (ESBL) enzymes by these microbes which make them to be highly resistant to most drugs [26].



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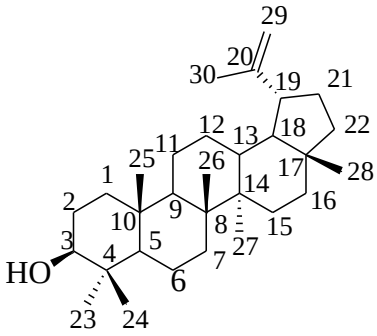

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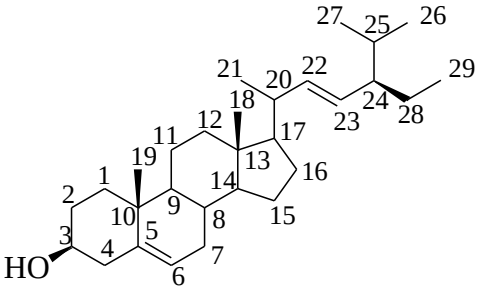

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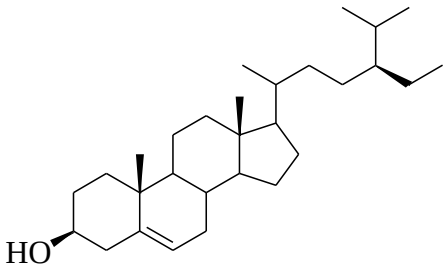

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Figure 1. Chemical structures of compounds 1-4 isolated from the roots of *Milletia leucantha*

Table 1. Bio-assay results for the extracts and compound obtained from the roots of *Milletia leucantha*

Test Samples	Zones of inhibitions in mm								
	Test pathogens								
	***S. aureus* ATCC 25923**	**MRSA (clinical source)**	***E. coli* ATCC 23922**	***P. aeruginosa* ATCC 27853**	***K. pneumoniae* ATCC 1388**	***C. albicans* ATCC 90028**	***C. neoformans* (clinical source)**	***T. mentagrophytes* (clinical source)**	***C. parapsilosis* ATCC 90018**
5 % H_2O/MeOH extract (500 mg/mL)	7	NI	6	NI	NI	6	6	6	6
MeOH/CH_2Cl_2 extract (205 mg/mL)	NI	NI	6	NI	NI	6	6	NI	NI
compound **1** (2.75 mg/mL)	6	NI	6	NI	NI	6	6	6	6
5 % DMSO	NI	NI	NI	NI	NI	NI	NI	NI	NI
Chloramphenicol	9	14	13	6	18	-	-	-	-
Nystatin	-	-	-	-	-	12	13	10	11

NI: No inhibition recorded

Conclusion

Four known compounds namely, afromosin (**1**), three terpenoids; lupeol (**2**), a mixture of stigmasterol (**3**) and β-sitosterol (**4**) were isolated from the 1:1 v/v CH_2Cl_2/MeOH root extract of *M. leucantha*. The two *M. leucantha* root extracts; (1:1 v/v) of CH_2Cl_2/MeOH and (95:5 v/v) MeOH/H_2O extracts, together with compound (**1**) showed minimal inhibition zones of ≤ 8 mm against the tested pathogens. They were therefore considered inactive according to [15]. Further phytochemical studies are required to isolate and characterize additional compounds especially using better analytical techniques.

Additional file

Supplementary file: NMR spectra of the isolsted compound 1308 kB [PDF]; available at: https://www.investchempharma.com/imcp43-supplementary-file-wangari-et-al/

Abbreviations

ATCC - American Type Culture Collection
CLSI - Clinical and Laboratory Standards Institute
CC - Column chromatography
1D & 2D NMR - 1 and 2-Dimensional Nuclear Magnetic Resonance spectroscopy
ETOAc - Ethyl Acetate
ESBL - Extended spectrum beta-lactamases
TLC - Thin layer column chromatography
MRSA - methicillin resistant *Staphylococcus aureus*
NI - No inhibition recorded

Authors' Contribution

LWW carried out the study and wrote the manuscript. LKO and EKO designed the experiments. LKO and EKO read and corrected the manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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