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Antimicrobial of extract and compounds from the bark of *Drypetes afzelii* (Pax) Hutch

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Abstract

The objective of the present study is to evaluate *in vitro* the antimicrobial activities of the extract and compound of *Drypetes afzelii* used in Cameroonian medicine. The evaluation of the antibacterial and antifungal activities was determined on eighteen of microorganisms which 6 Gram-positives bacteria, 10 Gram-negatives bacteria and 2 fungi. This study focused on the determination of the Minimal inhibition and microbicidal concentrations. Nystatin and Gentamicin were used as reference antibiotics. The fractionation was realized by chromatographic methods (TLC, HPLC, MPLC) and the structures of the isolated compounds were established using spectroscopic analysis (one and two-dimensional nuclear magnetic resonance (NMR)). Five compounds were isolated from the bark of *Drypetes afzelii* whose namely the terpenoid (lupeol), lignan (sesamin) and flavonoids (Acacetin, Isosakuranetin, 5,7,4'-trihydroxyflavanone). The most sensitive bacteria were *Bacillus stearothermophilus* and *Klebsiella pneumoniae* with MIC values 20.1-20.2 µg/mL for *Bacillus stearothermophilus* and 20.2 µg/mL for *Klebsiella pneumoniae*. The MMC values of 25.2-40.3 µg/mL for *Citrobacter freundii* and 40.3 µg/mL for *Klebsiella pneumoniae*. The results indicated that this plant has great interest for the future production of the new drugs against infectious diseases.

Keywords: *Drypetes afzelii*, antimicrobial activity, extract, compounds.

1. Introduction

Infectious diseases account for approximately one half of all deaths in tropical countries and they are considered a major threat to human health because of the unavailability of vaccines or limited chemotherapy. They represent an important cause of morbidity and mortality among the general population, particularly in developing countries [1, 2]. The fungi form part of an individual's microbiota, especially around the mucosa areas such as the oral cavity, and play an important role both in health and in the development of oral diseases [3, 4]. Traditional medicine was once regarded as the sole source of treatment, making it a focus in the search for solution to increasing drug resistance among pathogenic microorganisms [5]. The recognition of bacterial resistance problem brings the need to analyze the antimicrobial properties of new substances [6]. Medicinal plants are of great importance to the health of individuals and communities in general. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body [7, 8]. It is well known that the use of medicinal plants contributes significantly to primary health care for about 80% of Africa [9]. Many plants are used in Cameroon in the form of crude extracts, decoction, infusion or percolation to treat infections. Many of these plants are without any scientific evidence of efficacy. In our research group, the investigation of the pharmacological properties of such plants includes the evaluation of the activity of the crude organic extracts as well as that of the isolated compounds. We have focused our interest on many plants families such Clusiaceae, Euphorbiaceae, Moraceae, Solanaceae, etc. Within the Euphorbiaceae family, plants of the genus *Drypetes* are mostly represented. The genus *Drypetes* (Putranjivaceae) contains about 220 species of dioecious trees and shrubs, mostly of the old world tropics [10]. The genus *Drypetes* belongs to the family Euphorbiaceae and constitutes about 160 species, worldwide with many species being found in Africa [11]. We have previously reported the interesting antimicrobial activity of *Alchornea cordifolia*, *Hyphaene thebaica* and *Garcinia brevipedicellata* [12-14]. In our continuing search of antimicrobial drugs, we undertook to evaluate the antibacterial and antifungal activities of

the crude extract of *Drypetes afzelii* (EXT) as well as that of compounds isolated from this extract, a plant used to traditionally treat infections.

Therefore, the present study sought to evaluate *in vitro* the antibacterial and antifungal activities of extract and compounds of *Drypetes afzelii*.

2. Materials and method

2.1. Plant material

The barks of *Drypetes afzelii* Hutch were collected in June 2014 at SA'A, Center region of Cameroon. The botanical identification of the plants was done by the National Herbarium in Yaounde, where the voucher specimens were conserved under the reference number 42256/HNC.

2.2. Isolation and General Procedures

The fractionation of 50 g of the extract was performed by open column adsorption chromatography of silica gel normal phase with a mixture of increasing polarity Ligroin/AcOEt to give 12 fractions. These fractions were submitted to antimicrobial tests. Bio guided Splitting by these activities were carried out in order to isolate the most active substances they contain. On TLC, 12 fractions were pooled to obtain new fractions 7: the fractions 1-6 (19 g), 8 (7.6 g) whose tests show no activity have not been exploited for the isolation. The purification of fraction 5 (3.4 g) on open column of gel de silica, eluted by Ligroin/AcOEt (5/1) system then by Ligroin/AcOEt (2/1) system, has allowed us to isolate the Lupeol 1(17 mg). The fraction 9 (7.8 g) was subjected to the liquid semi-preparative high performance liquid chromatography (HPLC Semi-preparative) to obtain Sesamin 2 (5 mg), Acacetin 3(18 mg) and Isosakuranetin 4 (127 mg) by a MeOH/H₂O (25/75) system. The fraction 12 (8.5 g) was subjected to medium pressure liquid chromatography (MPLC) to give 5, 7,4'-trihydroxyflavanone 5 (25 mg).

Aluminium sheet pre-coated with silica gel 60 GF254 Merck was used for thin layer chromatography and the isolated spots were visualized using both ultra-violet light (254 and 366 nm) and 50% H₂SO₄ spray reagent. The chemical structure of each of the isolated compound was determined on the basis of spectral data produced by one and two-dimensional nuclear magnetic resonance (NMR), recorded on Brüker DRX-400 instrument. This spectrometer was equipped with 5-mm, 1H- and 13C- NMR probes operating at 400 and 100 MHz, with tetramethylsilane as internal standard. Mass spectra were recorded on an API QSTAR pulsar mass spectrometer. The chemical structures of the compounds isolated from *Drypetes afzelii* are given on figure1.

2.3. Microbial strains

Eighteen of microorganisms namely *Bacillus cereus* LMP0404G, *Bacillus megaterium* LMP0204G, *Bacillus stearothermophilus* LMP0104G, *Bacillus subtilis* LMP0304G, *Staphylococcus aureus* LMP0206U, *Streptococcus faecalis* LMP0207U (Gram-positive bacteria), *Escherichia coli* LMP0101U, *Shigella dysenteriae* LMP0208U, *Proteus vulgaris* LMP0103U, *Proteus mirabilis* LMP0504G, *Shigella flexneri* LMP0313U, *Klebsiella pneumoniae* LMP0210U, *Pseudomonas aeruginosa* LMP0102U, *Salmonella typhi* LMP0209U, *Morganella morganii* LMP0904G, *Enterobacter aerogenes* LMP1004G,

Citrobacter freundii LMP0804G, *Enterobacter cloacae* LMP1104G (Gram negative bacteria), *Candida albicans* LMP0204U, *Candida glabrata* LMP0413U (fungi) were used in this study. 'Institut Appert de Paris' provided three *Bacillus* species, while the A.F.R.C Reading Laboratory of Great Britain provided *Bacillus cereus*. Other strains were clinical isolates from 'Centre Pasteur of Cameroon', Yaoundé. The microbial isolates were maintained on agar slant at 4 °C in the Laboratory of Applied Microbiology and Molecular Pharmacology (LMP) (Faculty of Science, University of Yaoundé I) where the antimicrobial tests were performed. The strains were sub-cultured on a fresh appropriate agar plate 24 hours prior to any antimicrobial test.

2.4. Culture media

Nutrient Agar (NA) (Oxoid) containing Bromocresol purple was used for the activation of *Bacillus* species while NA was used for other bacteria. Sabouraud Glucose Agar (Oxoid) was used for the activation of the fungi. The Mueller Hinton Agar (MHA) (Oxoid) was used in sensitivity assay. Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP) was used for MIC and MMC determinations.

2.5. Chemicals

Nystatin (Maneesh Pharmaceutic PVT. Ltd., Govandi, Mumbai, 400 043 India) and Gentamicin {Jinling Pharmaceutic (Group) corp., Zhejiang Tieng Feng Pharmaceutic Factory, No. 11 Chezhan Road, Huzhou city, Zhejiang, China) were used as reference antibiotics (RA) against fungi and bacteria respectively. The Dimethylsulfoxide (DMSO) (SIGMA) was used as solvent for the tested samples.

2.6. MIC and MMC determinations

The MICs of test samples and RA were determined as follows: the test sample was first of all dissolved in DMSO. The solution obtained was added to the NBGP to a final concentration of 250.6 µg/ml for the crude extracts and 175.4 µg/ml for the compounds and RA. This was serially diluted two fold to obtain concentration ranges of 50.1 to 250.6 µg/ml for the crude extracts and 24.4 to 175.4 µg/ml for the compounds and RA. One hundred microliters of each concentration was added in a well (96- wells microplate) containing 95 µl of NBGP and 5µl of the standard inoculum. The final concentration of DMSO in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO/NBGP affected neither the growth of the test organisms nor the change of color due to this growth). The negative control well consisted of 195 µl of NBGP and 5 µl of the standard inoculum [8]. The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37 °C for 24 hours. The assay was repeated trice. Microbial growth was determined by observing the change of color in the wells (red when there is no growth and yellow when there is growth). The lowest concentration showing no color change was considered as the MIC. For the determination of MMC, a portion of liquid (5 µl) from each well that showed no change in color was plated on MHA and incubated at 37 °C for 24 hours. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC.

3. Results

The structures of the isolated compounds were established using spectroscopic analysis, especially, NMR spectra in conjunction with 2D experiments, COSY, HMQC and HMBC, and direct comparison with published information and with authentic specimens obtained in our research group for some cases.

The compounds isolated from the bark of *Drypetes afzelii* were found to be terpenoid known as lupeol 1, a lignan known as sesamin 2 and flavonoids identified as Acacetin 3,

Isosakuranetin 4, and 5,7,4'-trihydroxyflavanone 5. In this study, the antibacterial and antifungal activities of the crude extract and the 5 compounds were evaluated and the results are reported in Table 1.

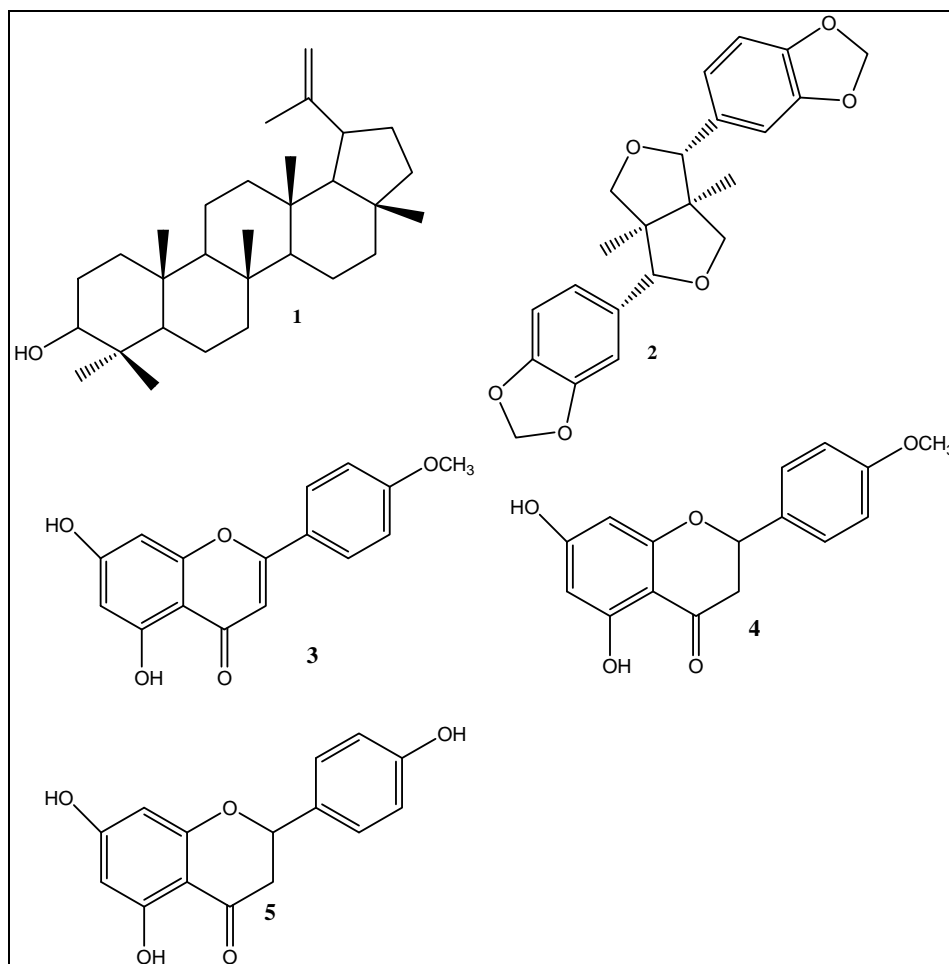
The MIC values ranging from 24.4-250.6 µg/ml (Table 1) were obtained with the crude extract from *Drypetes afzelii* on 16 of the 18 microbial species. The activities of compounds 1,2,3,4,5 were evaluated. Compounds 1 was active on 11 (61%), 2 on 9 (50%), 3 on 12 (66, 7%), 4 on 9 (50%) and 5 on 15 (83, 3%) of the 18 tested microorganisms respectively.

Table 1: Tableau 1: MICs values of compounds

Microorganisms	Extract and compounds												Reference antibiotics	
	EXT		1		2		3		4		5		^a RA	
	^b MIC	^c MMC	^b MIC	^c MMC	^b MIC	^c MMC	^b MIC	^c MMC	^b MIC	^c MMC	^b MIC	^c MMC	^b MIC	^c MMC
Gram-positives bacteria														
<i>Bacillus cereus</i>	25	125	25	150	25	200	-	-	25.2	100.2	25	50	2.44	2.2
<i>Bacillus megaterium</i>	25	175	-	-	25	100	20.2	100.1	50.2	100.1	-	-	2.44	2.2
<i>Bacillus stearothermophilus</i>	50	150	50	100	-	-	-	-	20.1	60.3	25.2	50.2	2.3	4.5
<i>Bacillus subtilis</i>	250.3	250.2	50.2	100.1	-	-	-	-	-	-	-	-	4.3	8.2
<i>Staphylococcus aureus</i>	25.2	150.1	25.6	75.2	25.2	75.3	25.3	75.3	25.1	75.2	25.3	125.2	6.5	18.6
<i>Staphylococcus faecalis</i>	25.1	125.2	50.3	100.1	-	-	25.1	50.2	50.1	150.3	25.2	125.2	4.3	16.1
Gram- negatives bacteria														
<i>Citrobacter freundii</i>	50.3	250.6	25,3	100,1	25.2	25.2	25.4	75.5	25.2	50.3	-	-	4.1	16.2
<i>Enterobacter aerogenes</i>	50.1	200.8	-	-	-	-	-	-	-	-	-	-	4.1	8.3
<i>Enterobacter cloacae</i>	25.5	250.2	25,3	50,2	-	-	-	-	-	-	50.1	100.1	3.1	9.3
<i>Escherichia coli</i>	24.4	290.8	75	75,2	-	-	-	-	-	-	25.1	150.1	2.4	6.4
<i>Klebsiella pneumoniae</i>	25.6	200.3	25	75	20.2	40.3	25.1	75.2	100.1	100.2	25.3	175.4	2.2	8.3
<i>Morganella morganii</i>	25.4	165.3	-	-	-	-	-	-	-	-	-	-	4.2	8.3
<i>Proteus vulgaris</i>	50.2	200.2	50,3	100.2	44.4	62.2	50.2	125.3	25.2	100.1	25.5	125.5	3.2	9.3
<i>Shigella dysenteriae</i>	25.5	175.5	25,3	100.2	-	-	50.3	50.5	-	-	25.2	75.2	2.1	8.3
<i>Shigella flexneri</i>	25.5	150.0	25	75.4	25.3	50.2	25.1	52.2	25.1	125.1	50.1	150.1	2.2	6.2
<i>Salmonella typhi</i>	25.5	175.3	55	55.5	-	-	-	-	-	-	50.2	125.1	2.1	8.2
Fungi														
<i>Candida albicans</i>	50.1	200.3	25.2	50.2	-	-	-	-	-	-	-	-	3.4	9.4
<i>Candida glabrata</i>	25.3	175.1	25.3	55.1	25.3	75.2	50.1	200.2	50.3	150.3	-	-	3.2	3.3

Legend: Not active (-); Minimal inhibition concentration (^bMIC); Minimal microbicidal concentration (^cMMC); ^aRA:

Reference antibiotics [Gentamycin (G) for bacteria, Nystatin (N) for yeasts];



Scheme 1: Compounds isolated from bark of *Drypetes afzelii*

Legend: lupeol 1; sesamin 2; Acacetin 3, Isosakuranetin 4, 5,7,4'-trihydroxyflavanone 5.

4. Discussion

The extract of the bark from *Drypetes afzelii* and one of its compounds were tested against eighteen of selected Gram positive and Gram negative bacteria as well as fungi (Table 1).

The most sensitive bacteria were *Bacillus stearothermophilus* and *Klebsiella pneumoniae* with MIC values 20.1 – 20.2 µg/mL for *Bacillus stearothermophilus* with compound 4 and 20.2 µg/mL for *Klebsiella pneumoniae* for compound 2 and with MMC values of 25.2– 40.3 µg/mL for *Citrobacter freundii* with compound 3 and 40.3 µg/mL for *Klebsiella pneumoniae* for compound 3. The most resistant bacterium was *Bacillus subtilis* with a MIC of 250.3 µg/mL *Escherichia coli* with an MMC of 290.8 µg/mL both with extract of the plant.

Gentamicin and Nystatin were used as reference antibiotics because they are the germicidal agent used in large scale in healthcare services to treat infections. In all the cases, they showed inhibition superior to extract and compounds in relation to the all microorganisms (Gram positives and negatives bacteria as fungi).

The present study showed, in spite of the high concentrations, in comparing to control, plant extract was active on all bacterial and fungal strains (100% active).

Staphylococcus faecalis, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Shigella flexneri* are sensible to the all compounds and the extract (100%). Concerning *Candida grabata*, *Citrobacter freundii*, *Staphylococcus faecalis*, *Candida grabata*, *Citrobacter freundii*, *Staphylococcus*

faecalis and *Bacillus cereus* each is having an 85.7% of sensibility.

A similar study was conducted with the methanol extract from the stem bark of *Drypetes tessmanniana*, another genus of *Drypetes*, and the results of the MMC values suggested that the microbicidal effect of most of the tested samples on the studied microorganisms could be expected [15]. Several workers have reported that many plants possess antimicrobial properties including the parts which include; flower, bark, stem, leaf, etc. It has been shown that when solvents like ethanol, hexane and methanol are used to extract plant, most of them are able to exhibit inhibitory effect on both gram positive and gram negative bacteria. Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent [16, 17].

The antifungal evaluation showed, the most sensitive candida were *Candida albicans* with MIC values 25.2 µg/mL for compound 1 and 25.3 µg/mL for *Candida grabata* with the extract, compounds 1 and 2, although MMC values of 50.2 µg/mL for *Candida albicans* with compound 2 and 55.1 µg/mL for *Candida grabata* with compound 3. In this present study, it is clearly observed that the antifungal activity was obtained with lupeol (terpenoid). The phytochemical analysis of the bark of *Drypetes afzelii* led to the isolation of five compounds such as triterpenoid (lupeol), a lignan (sesamin) and flavonoids (Acacetin, Isosakuranetin, and 5,7,4'-trihydroxyflavanone). The presence of the majority of these metabolites is previously reported in other

species of *Drypetes* [18]. Thus, the isolation of triterpenoids from *Drypetes* species indicates that these compounds could be chemotaxonomic markers for the *Drypetes* genus [8]. Our results demonstrated that lupeol (terpenoid) was the best effective and exhibited a broad spectrum inhibitory action to the experimented bacterial and fungal strains (100% of inhibition in MMC and 83.3% for MIC).

Previous studies have shown antibacterial properties of these secondary metabolites, including flavonoids, saponins, steroids and terpenes [19-23]. Flavonoids are largely responsible for antifungal activity [24, 25]. Many previous studies have shown antibacterial properties of secondary metabolites, including flavonoids and terpenoids. Euphorbiaceae family that showed condensate and hydrolysable tannins, flavonoids, among others, responsible for their biological activities. The presence of flavonoid in the plant suggests that it can be used as anti-spasmodic, antifungal and anti-bacterial drug plant. It is reported that flavonoids have the ability to inhibit spore germination and growth of plant pathogens and complex with extra cellular and soluble proteins and with bacterial cell walls [26, 27].

Indeed may be responsible for the antifungal activity [28]. These findings confirm the reason for the use of the plant in the treatment of diarrhea, spasmodic bronchitis and other microbial infections [29, 30]. Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipid and exerts its action by causing leakages from liposomes [31].

These constituents acting alone or in combination could be responsible for the observed effect of this plant.

5. Conclusion

The results of this present study confirm the antibacterial and antifungal potentials of *Drypetes afzelii*. The presence of various phytochemicals in the tested plant reveals that this plant may be a good source for production of new drugs. Its effects on both gram-positive and Gram-negative organisms as well as *Candida grabata* (yeast) indicates the broad spectrum activities.

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