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Antimicrobial activities of chemical constituents from the flowers of *Hypericum lanceolatum* Lam. (Hypericaceae)

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ABSTRACT

The emergence of multi drug resistant bacteria and fungi has become a crucial problem in the fight against infectious diseases worldwide. Up till now, the search for new potential antimicrobial agents is a challenge. Hence, the aim of this study was to investigate the antibacterial and antifungal properties of the chemical constituents from the flowers of *Hypericum lanceolatum* Lam (Hypericaceae). Bioguided fractionation led to the isolation of eight known compounds (1-8) from the dichloromethane (DCM)/methanol (MeOH) (1:1; v/v) extract of the flowers of *H. lanceolatum* using silica gel column chromatography, preparative thin layer chromatography (TLC) and Sephadex LH-20. The structures of these compounds were determined by interpretation of their nuclear magnetic resonance (NMR) spectroscopic data, in comparison of these data with those from the literature. The crude-extract derived fractions and compounds were evaluated for their antimicrobial properties. They exhibited both antibacterial and antifungal activities with minimal inhibitory concentration (MIC) values ranging between 16 and 2048 µg/ml. Compounds 1, 2, 4 and 5 were the most active substances (MIC = 16 – 64 µg/ml), while *Staphylococcus aureus* and *Pseudomonas aeruginosa* were sensitive to all the isolated compounds. The results of the present study support the use of *H. lanceolatum* in traditional medicine to fight microbial infections associated with the studied microorganisms.

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INTRODUCTION

The genus *Hypericum* belongs to the family Hypericaceae and comprises more than 450 species widely distributed in temperate regions of the world and tropical highlands (Mabberley, 1997; Crockett and Robson, 2011). Plants of this genus are used worldwide as traditional medicine against several diseases. In China, *Hypericum sampsonii* Hance is used for the treatment of disorders such as backache, burns, diarrhea, snake bite, and swellings (Xiao et al., 2007). *Hypericum laricifolium* Juss. is used in the traditional Ecuadorian medicine as diuretic and to induce menstruation (El-Seedi et al., 2003). In the folk medicine of Papua New Guinea, leaves of *Hypericum papuanum* Ridl. are applied to treat sores. *Hypericum lanceolatum* Lam. is a small tree or shrub occurring in the mountainous

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region of West Cameroon (Hutchinson and Dalziel, 1954). In Cameroonian traditional medicine, the leaves of *H. lanceolatum* Lam are extracted with palm wine and used for the treatment of skin infections, epilepsies and tumors, while the roots are boiled in water and used to treat venereal diseases, gastrointestinal disorders and infertility (Zofou et al., 2011; Wabo et al., 2012; Fobofou et al., 2014). The most common secondary metabolites found within the genus *Hypericum* include anthrones, flavonoids, anthraquinones, xanthones, coumarins, benzophenones, phloroglucinols, and less frequently chalcones, benzopyrans, steroids and terpenoids (Wu et al., 1998; Ang'edu et al., 1999; Wirz et al., 2000; Tanaka et al., 2009; Schmidt et al., 2012; Wabo et al., 2012; Fobofou et al., 2014). The structures and biological activities of secondary metabolites from *Hypericum* species have attracted wide attention in medicinal and synthetic chemistry fields since the isolation of hyperforin in 1975 (Gartner et al., 2005; Gey et al., 2007). They possess among others, antidepressant (Verotta, 2003), antibacterial (Shui et al., 2012; Li et al., 2015) and anti-inflammatory activities (Crockett et al., 2008). Hence, the aim of this study was to investigate the antibacterial and antifungal properties of secondary metabolites isolated from the flowers of *H. lanceolatum*.

MATERIALS AND METHODS

General experimental procedure

Various column chromatography were carried out using silica gel type Merck 60F_{254} (70-230 and 230-400 mesh, Darmstadt, Germany) or Sephadex LH-20 as stationary phase. Prefabricated silica 60F_{254} aluminium foil plates were used for analytical TLC. After visualization under ultraviolet lights, these plates were sprayed with 10% aqueous H_2SO_4 and then heated up to 80^oC. ^1H and ^{13}C NMR spectra of compounds 1-8 were recorded on an Agilent DD2 400 NMR spectrometer at 399.915 and 100.569 MHz, respectively. The ^1H NMR chemical shifts (δ) are reported relative to TMS, using residual solvent peaks as internal standards. ^{13}C NMR chemical shifts are referenced to internal solvent signals. The chemical shifts (δ) are expressed in parts per million (ppm) with TMS (Me_4Si, δ=0) as an internal reference (see Supplementary material).

Plant material

The flowers of *H. lanceolatum* were collected at Mount Bamboutos (coordinates: 5° 44′ 00″N 10° 04′ 00″E), West Region of Cameroon in December 2015 and identified by Mr. Nana Victor of the National Herbarium of Cameroon, Yaoundé (Cameroon), where a voucher specimen (No. 32356 HNC) is deposited.

Extraction and isolation

Flowers of *H. lanceolatum* (1 Kg) were extracted with 8 L of DCM/MeOH (1:1; v/v) three times during 72 h at room temperature to yield a crude extract (211.4 g) after solvent evaporation under reduced pressure. After a while, two phases (solid and liquid) were observed in the laboratory glassware containing the crude extract, and were then separated by decantation. The liquid phase (PL: 46.3 g) was subjected to silica gel column chromatography eluted with gradients of *n*-hexane/EtOAc and EtOAc/MeOH. Fifty-five fractions of 500 mL each were collected and combined on the basis of their TLC profiles into four main fractions coded AL–DL (AL: 1–10; BL: 11–17; CL: 18–38; DL: 39-55). These fractions were tested for their antibacterial and antifungal activities and the most active fractions were further subjected to purification in order to isolate the active principles. Fraction BL (12.4 g) was separated by column chromatography over silica gel using a gradient of DCM/MeOH to give three sub-fractions (BL_1–BL_3). The sub-fraction BL_2 was subjected to a Sephadex LH-20 column chromatography eluted with DCM/MeOH (1:1) to give 1 (150 mg) and 3 (19.1 mg). Fraction CL (10.1 g) was subjected to a Sephadex LH-20 column chromatography eluted with DCM/MeOH (1:1; v/v) followed by a column chromatography over silica gel eluted with DCM/MeOH (8:2; v/v) to give 6 (5 mg). Fraction DL (8.1 g) was subjected to a silica gel column chromatography eluted with gradients of DCM/MeOH to give 5 (12 mg) and 2 (110.2 mg). The solid phase (PS: 153.3 g) was subjected to a silica gel column chromatography eluted with gradients of *n*-hexane/EtOAc and EtOAc/MeOH respectively. Forty-five fractions of 400 mL each were collected and combined on the basis of their TLC profiles into four main fractions coded AS–DS (AS: 1–8; BS: 9–26; CS: 27–32; DS: 33-45). Each fraction was also tested for its antimicrobial activity and the most active fractions were further subjected to purification in order to isolate the active principles. Fraction BS (6.7 g) precipitated partially in EtOAc. Filtration followed by purification (of the white powder obtained) using a column chromatography over Sephadex LH-20 and eluted with DCM/MeOH (1:1; v/v) afforded compounds 7 (17.2 mg) and 8 (35.7 mg). Compound 4 (12.2 mg) was isolated from fraction CS after a series of silica gel column chromatography.

Antimicrobial assay

The antimicrobial activity was performed against four bacterial and three fungal species. The selected microorganisms were the Gram-positive (*S. aureus* ATCC25923) and Gram-negative [*P. aeruginosa* ATCC27853, *Escherichia coli* S2(1), *Shigella flexneri* SDINT] bacteria and yeast strains of *Candida albicans* ATCC10231, *Candida tropicalis* PK233 and *Cryptococcus neoformans* H99. These microorganisms were taken from our laboratory collection. The fungal and bacterial strains

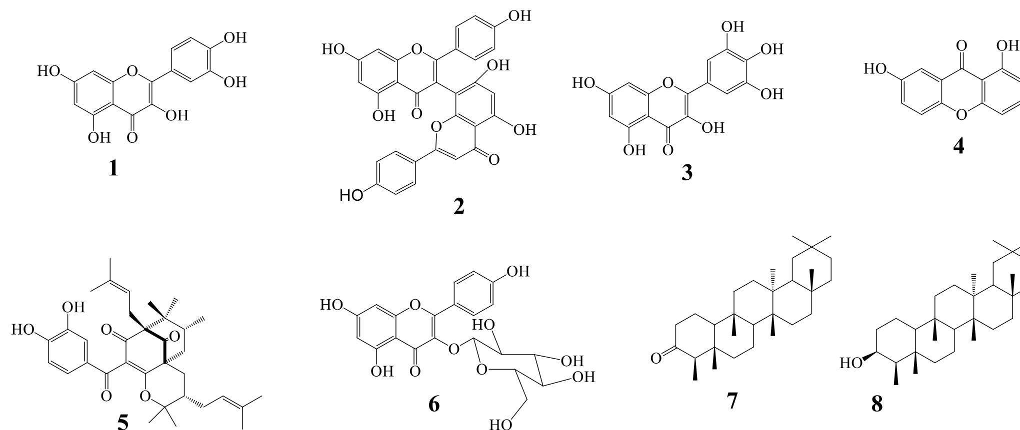

Figure 1. Chemical structures of isolated compounds from *H. lanceolatum.*
1, quercetin; **2**, I3,II8-biapigenin ; **3**, myricetin; **4**, euxanthone; **5**, isogarcinol; **6**, kaempferol-3-O-β-D-glucopyranoside; **7**, friedelanone; **8**, friedelan-3-β-ol.

were grown at 30 and 37°C, respectively and maintained on Sabouraud Dextrose Agar (SDA, Conda, Madrid, Spain) and nutrient agar (NA, Conda) slants, respectively. The antibacterial and antifungal activities were evaluated by determining the minimum inhibitory concentrations (MICs) and minimum microbicidal concentrations (MMCs) as previously described (Tamokou et al., 2009). Briefly, MICs of extracts and isolated compounds were determined by broth micro dilution method. Each test sample was dissolved in dimethylsulfoxide (DMSO) to give a stock solution. This was serially diluted two-fold in Mueller-Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for yeasts to obtain a concentration range of 2048 to 16 µg/mL (for extracts) and 256 to 0.125 µg/mL (for pure compounds). Then, 100 µL of each sample concentration was added to respective wells (96-well micro plate) containing 90 µL of SDB/MHB and 10 µL of inoculum to give final concentration ranges of 256 to 0.125 µg/mL. The final concentrations of microbial suspensions were 2.5×10^5 cells/mL for yeasts and 10^6 CFU/mL for bacteria. Dilutions of nystatin (Sigma-Aldrich, Steinheim, Germany) and ciprofloxacin (Sigma-Aldrich, Steinheim, Germany) were used as positive controls for yeasts and bacteria respectively. Microorganisms left untreated + 1% (v/v) DMSO + SDB/MHB were used as negative control. MICs were assessed visually and were taken as the lowest sample concentration at which there was no growth or virtually no growth. The lowest concentration that yielded no growth after the sub-culturing was considered as the MMCs. All the tests were performed in triplicate and repeated three times with similar results.

RESULTS

Chemical analysis

The DCM/MeOH (1:1; v/v) extract of the flowers of *H. lanceolatum* was subjected to silica gel and Sephadex LH-20 chromatography to afford eight known compounds including quercetin (Jorge et al., 1996), I3,II8-biapigenin (Berghöfe and Hölzl, 1987), myricetin (Jorge et al., 1996), euxanthone (Ee et al., 2005), isogarcinol (Kuete et al., 2013), kaempferol-3-*O*-*β*-D-glucopyranoside (Duc et al., 2018), friedelanone (Igoli and Gray 2008) and friedelan-3-*β*-ol (Queiroga et al., 2000) (Figure 1). Their structures were determined using NMR spectroscopic methods, and by comparison of the obtained data with those reported in the literature.

Antimicrobial activities

The antimicrobial activities of the crude extract, fractions and isolated compounds were assessed using broth micro dilution method against four bacteria and three yeasts and their results are summarized in Table 1. The inhibition effects of the crude extract, fractions and compounds were observed on all the tested microorganisms (100%). The MIC values of the crude extract against bacteria were higher than those of the PS and PL fractions. However, the MIC values of these two fractions against yeasts were higher than those of the crude extract. These results suggest that the fractionation enhanced the antifungal activity and diluted the antibacterial activity of the crude extract. The PS fraction displayed the largest antibacterial activity as compared to the PL fraction while their antifungal activities were found to be similar. The fractionation of the PS fraction enhanced its antibacterial and antifungal activities in CS subfraction and diluted those of other resulting subfractions (AS, BS and DS). Moreover, the fractionation of the PL fraction enhanced its antimicrobial activities in BL and CL subfractions and diluted those of other resulting subfractions (AL and DL). Among the crude extract and fractions, the CL fraction was the most active against bacterial and fungal species (MIC

Table 1. Minimum inhibitory (MIC) and minimum microbicidal concentrations (MMC) of extracts and isolated compounds from *H. lanceolatum* against bacterial and yeast strains.

Samples	Inhibition parameters	*S. aureus* ATCC25923	*P. aeruginosa* ATCC27853	*E. coli* S2(1)	*S. flexneri* SDINT	*C. albicans* ATCC10231	*C. tropicalis* PK233	*C. neoformans* H99
Crude extract	MIC	256	256	512	256	512	512	512
	MMC	256	256	512	256	1024	1024	512
	MMC/MIC	1	1	1	1	2	2	1
PS fraction	MIC	128	128	256	256	2048	1024	1024
	MMC	512	512	1024	1024	>2048	>2048	>2048
	MMC/MIC	4	4	4	4	/	/	/
AS fraction	MIC	256	256	512	512	1024	1024	512
	MMC	1024	512	2048	2048	>2048	2048	2048
	MMC/MIC	4	2	4	4	/	2	4
BS fraction	MIC	128	256	256	512	2048	1024	1024
	MMC	256	512	512	1024	>2048	2048	2048
	MMC/MIC	2	2	2	2	/	2	2
CS fraction	MIC	64	64	256	256	2048	512	256
	MMC	128	128	256	256	>2048	2048	1024
	MMC/MIC	2	2	1	1	/	4	4
DS fraction	MIC	128	128	256	512	2048	2048	1024
	MMC	256	512	512	1024	>2048	>2048	2048
	MMC/MIC	2	4	2	2	/	/	2
PL fraction	MIC	512	512	1024	1024	2048	1024	1024
	MMC	1024	1024	>2048	>2048	>2048	>2048	>2048
	MMC/MIC	2	2	/	/	/	/	/
AL fraction	MIC	512	1024	1024	1024	1024	1024	1024
	MMC	2048	1024	2048	2048	>2048	>2048	>2048
	MMC/MIC	4	1	2	2	/	/	/
BL fraction	MIC	256	256	512	512	512	256	256
	MMC	1024	1024	2048	2048	512	512	512
	MMC/MIC	4	4	4	4	1	2	2
CL fraction	MIC	64	128	256	128	256	128	64
	MMC	128	128	256	256	256	256	256
	MMC/MIC	2	1	1	2	1	2	4
DL fraction	MIC	1024	1024	2048	1024	1024	512	512
	MMC	2048	2048	>2048	>2048	2048	1024	1024
	MMC/MIC	2	2	/	/	2	2	2
1	MIC	32	32	32	32	64	64	64
	MMC	32	64	64	64	128	128	64
	MMC/MIC	1	2	2	2	2	2	1
2	MIC	16	16	16	32	32	32	16
	MMC	16	16	32	32	64	64	32
	MMC/MIC	1	1	2	1	2	2	2
3	MIC	32	32	64	64	128	128	64
	MMC	64	64	64	64	>256	>256	64
	MMC/MIC	2	2	1	1	nd	nd	1
4	MIC	32	32	32	32	64	64	32
	MMC	32	32	64	64	128	64	64
	MMC/MIC	1	1	2	2	2	1	2
5	MIC	32	32	32	32	32	32	16
	MMC	32	32	64	64	64	32	32
	MMC/MIC	1	1	2	2	2	1	2

Table 1. Contd.

6	MIC	32	32	64	128	128	64	32
	MMC	64	64	128	256	>256	128	64
	MMC/MIC	2	2	2	2	nd	2	2
7	MIC	64	64	128	128	128	128	64
	MMC	64	128	128	256	256	128	128
	MMC/MIC	1	2	1	2	2	1	2
8	MIC	64	64	64	64	128	128	64
	MMC	64	128	128	64	256	128	64
	MMC/MIC	1	2	2	1	2	1	1
Reference drugs*	MIC	1	4	8	16	2	0.5	1
	MMC	1	4	8	16	2	1	1
	MMC/MIC	1	1	1	1	1	2	1

*, Ciprofloxacin for bacteria; nystatin for fungi; nd: not determined; **MIC,** minimum inhibitory concentration; **MMC,** minimum microbicidal concentration. The lower the MIC and/or **MMC**, the higher the activity.

= 64 – 256 µg/mL). Moreover, regardless of the microorganisms tested, all the isolated compounds were more active than their respective fractions. Compound 2 displayed the largest antibacterial and antifungal activities followed in decreasing order by compounds 5, 4, 1, 6, 3, 8 and 7. However, the MIC values of compound 2 were lower than those of reference drugs ciprofloxacin and nystatin against all the tested microorganisms. Growth of *S. aureus* and *P. aeruginosa* was inhibited at lowest concentration of 16 µg/ml by compound 2, and 32 µg/ml by compounds 1, 3, 4, 5 and 6. The lowest MIC value (16 µg/ml), corresponding to the highest antimicrobial activity was recorded with compound 2 on *E. coli*, and with compounds 2 and 5 on *C. neoformans*. Frequently, the MMC values were either equal or two times greater than their corresponding MIC values. The lowest MIC value (16 µg/ml) was found with compound 2 against *S. aureus* and *P. aeruginosa*. *P. aeruginosa*, *S. aureus* and *C. neoformans* were the most sensitive microorganisms with median MIC value of 64 µg/ml while *C. albicans* exhibited the least susceptibility displaying median MIC of 256 µg/ml for all tested agents.

DISCUSSION

The result of the present study demonstrated that the antibacterial activity of the crude extract was higher than those of the PS and PL fractions while the antifungal activity of these two fractions was higher as compared to that of the crude extract. This result suggests that the fractionation enhanced the antifungal activity and diluted the antibacterial activity of the crude extract. The present study also showed that subfractions were in general more active than their preceding fractions. This may be due to the exclusion by fractionation of some constituents of the fractions which may tend to dilute the active principle and reduce their activities. On the other hand, fractionation may have increased the concentrations and the activity of antimicrobial principles in these subfractions. Our findings underlined differences in susceptibility of the various microorganisms with respect to the tested agents. As documented in several previous reports, this variability may be due to genetic differences between the strains (Tamokou et al., 2012). The antimicrobial activity of plant extracts is considered to be highly active if the MIC < 100 µg/mL; significantly active when 100 ≤ MIC ≤ 512 µg/mL; moderately active when 512 < MIC ≤ 2048 µg/mL; weakly active if MIC > 2048 µg/mL and not active when MIC > 10 mg/mL (Tamokou et al., 2017). Hence, the crude extract of *H. lanceolatum* were significantly active (100 ≤ MIC ≤512 µg/mL) whereas the fractions were highly, significantly and moderately active against all the tested microorganisms. The antibacterial and antifungal activities of crude extract/fractions support the use of *H. lanceolatum* in traditional medicine for the treatment of microbial infections. The MMC values obtained in this study were generally about 2-fold greater than the MICs on the corresponding microbial species, suggesting that doubling the concentration of the extracts and compounds that inhibit the growth of the microorganism may lead to microbial killing effect (Tamokou et al., 2009).

Antimicrobial cutoff points have been defined in the literature to enable the understanding of the potential of compounds as follows: highly active: MIC below 1 µg/mL (or 2.5 µM), significantly active: 1 ≤ MIC ≤ 10 µg/mL (or 2.5 ≤ MIC < 25 µM), moderately active: 10 < MIC ≤ 100 µg/mL (or 25 < MIC ≤ 250 µM), low activity: 100 < MIC ≤ 1000 µg/mL (or 250 < MIC ≤ 2500 µM) and not active: MIC > 1000 µg/mL (or > 2500 µM) (Tamokou et al., 2017). Based on this, most of the antimicrobial activities of the tested terpenes and phenolic compounds could be considered as significant, moderate and weak depending on the sensitive microorganisms. Compound 2 (I3,II8-

biapigenin) was found to be the most active agent isolated from *H. lanceolatum* in this study. It inhibited growth of *S. aureus, E. coli* and *P. aeruginosa* at lowest concentration of 16 µg/ml. This compound was isolated for the first time from *H. perforatum* (Berghöfe and Hölzl, 1987) and was reported as one of the active ingredient of *H. triquetrifolium* with antimicrobial activity against Gram negative and Gram positive bacteria (Pistelli et al., 2005). The antimicrobial activities of purified terpenes and phenolic compounds corroborate with those of the early reports against bacteria and fungi (Tamokou et al., 2009; Tatsimo et al., 2012; Tamokou et al., 2012; Djouossi et al., 2015). The antimicrobial inhibitory mechanisms of phenolic compounds found active in this study, may be due to iron deprivation or hydrogen bounding with vital proteins such as microbial enzymes (Scalbert, 1991). Lipophilic flavonoids may disrupt microbial membranes whereas terpenes may have the ability to disrupt microbial membrane and this may explain their antimicrobial properties (Cowan, 1999).

Conclusion

Bioguided fractionation of the DCM/MeOH extract of the flowers of *H. lanceolatum* yielded eight known compounds namely quercetin (1), I3,II8-biapigenin (2), myricetin (3), euxanthone (4), isogarcinol (5), kaempferol-3-*O*-*β*-D-glucopyranoside (6), friedelanone (7) and friedelan-3-*β*-ol (8). The crude extract, fractions and isolated compounds exhibited both antibacterial and antifungal activities with MIC values ranging between 16 and 2048 µg/ml. Compounds 1, 2, 4 and 5 were the most active substances (MIC = 16 – 64 µg/ml), while *S. aureus* and *P. aeruginosa* were sensitive to all the isolated compounds. The results of the present study support the use of *H. lanceolatum* in the traditional medicine to fight microbial infections associated with the studied microorganisms. However, additional experimentation on appropriate models is required to verify this hypothesis.

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