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Antimicrobial Constituents from Leaves of *Dolichandrone spathacea* and Their Relevance to Traditional Use

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Keywords
Dolichandrone spathacea, Bignoniaceae, iridoid glycosides, triterpenoid saponins, antibacterial activity

received 14.07.2017
revised 14.11.2017
accepted 11.12.2017

Bibliography
DOI https://doi.org/10.1055/s-0043-125339
Planta Med Int Open 2018; 5: e14–e23

ISSN 2509-9264

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ABSTRACT

Five new compounds, three iridoid glycosides (**1**-**3**) and two triterpenoid saponins (**4**, **5**), along with thirty-two known compounds were isolated from the methanolic extract of the leaves of *Dolichandrone spathacea*. This traditional medicinal plant is widely used in Asia and India as antiseptic, for bronchitis and thrush treatment, and the methanolic extract has been shown to possess antibacterial activity against methicillin-resistant *Staphylococcus aureus*. The new iridoids were esterified derivatives of 6-ajugol and 6-catalpol, and the new saponins were glucosides of two polyhydroxy triterpenes with ursan skeleton. Their structures were elucidated by spectroscopic methods, including 1D and 2D NMR experiments and HR-ESI-MS analysis, and from comparison with the literature. This study aimed at investigating extracts and isolated compounds for their antimicrobial activities against bacterial and yeast strains, in order to validate the uses of the plant in folk medicine. The 6-O-esterified iridoids had weaker antibacterial activity; verbascoside and p-methoxycinnamic acid, the major compounds of the methanol extract, possessed strong antibacterial activity, which could account for the traditional antiseptic and anti-infectious uses of the leaves of *D. spathacea*.

Introduction

Dolichandrone spathacea (L.f.) Seem. (Bignoniaceae) also known as mangrove trumpet tree, is a common tree growing wild in river banks and mangroves of the Asia-Pacific area [1]. The leaves are used as an antitumor, antiseptic, and to treat oral thrush (as mouthwash), nervous diseases and flatulence in many countries of Southeast Asia [1, 2]. The juice of the leaves is used orally against bronchitis in India [3]. In Vietnam, this plant is part of a traditional medication (Tiêu Phong Nhuân Gan), used against hepatic disorders, skin diseases, allergies, and as detoxifier, anti-inflammatory and laxative. Several biological activities of leaves extracts were measured showing for example, that the polar extracts possessed a high antiradical activity [2] and that the aqueous methanol extract exhibited an inhibitory effect against rat intestinal maltase [4]. Literature also reports that a methanol extract from the leaves exhibited a strong growth inhibition of six strains of methicillin-resistant *Staphylococcus aureus* (MRSA) [5]. The same article demonstrated, in a preliminary approach, the detection of flavonoids, saponins, triterpenes, and tannins in the leaves but without any structural characterization [5]. In the present paper, we report the isolation and characterization of

phytochemical constituents from leaves of *D. spathacea*, and the evaluation of antimicrobial activities of extracts and isolated compounds against different microorganisms.

Results and Discussion

The leaves of *D. spathacea* were extracted by successive percolations with petroleum ether, chloroform, ethyl acetate, methanol, and 80 % aqueous methanol. The methanolic extract was purified by using a combination of liquid chromatographic techniques to yield 37 compounds. Among these, five were new compounds: three iridoid glucosides (**1**-**3**) and two triterpenoid saponins (**4** and **5**) (▶ **Fig. 1**). The 32 known isolated compounds were: 6-O-p-E-coumaroyl-ajugol [6], 6-O-E-caffeoyl-ajugol and 6-O-E-isoferuloyl-ajugol [7], nemorososide [8], 6-O-(E)-cinnamoyl-catalpol [9], specioside [10], 6-(E)-p-methoxycinnamoyl-catalpol [11], a mixture of (E)- and (Z)-6-p-methoxycinnamoyl-catalpol [12], verminoside and minecoside [13], nemoroside and 6''(Z)-nemoroside [14], ixoside [15], arjunglucoside I [16], decaffeoylacteoside [17], verbascoside (or acteoside) [18], isoverbascoside (or isoacteoside) [19], luteolin, luteolin-7-O-β-D-glucopyranoside, luteolin-7-O-β-D-glucuronide and luteolin-7-O-rutinoside [20–23], (2E,6E)-8-hydroxy-2,6-dimethyl-2,6-octadienoic acid [24], (2E,6Z)-8-hydroxy-2,6-dimethyl-2,6-octadienoic acid [25], 6 R- and 6 S-(2E)-8-hydroxy-2,6-dimethyl-2-octenoic acid in mixture [26, 27], p-hydroxybenzoic acid [28], vanillic acid [29], p-hydroxycinnamic and p-methoxycinnamic acids [30], isoferulic acid [31], and 6 S,9 S-roseoside [32, 33].

The new compounds (**1**-**5**) possessed the same sugar residue in their structures (▶ **Table 1** and **2**), which was determined as D-glucose after acid hydrolysis and chiral HPLC analysis. NMR showed that it was in the β-D-pyranosyl configuration.

The ^{1}H-NMR data (▶ **Table 1**) of **1** and **2** revealed the characteristic signals of an ajugol part [6] with an acetal proton at δ_H 5.53, two cis olefinic protons at δ_H 6.24 and 5.00, one methyl singlet at δ_H 1.41, and a deshielded hydroxymethine proton at δ_H 4.96. The ^{13}C-NMR (DEPT and HSQC) data confirmed the presence of the ajugol moiety (▶ **Table 1**) [6], and the relative stereochemistry was confirmed by observation of ROE effects between H-5β/H-9β, H-1α/H-10α, H-10α/H-7α, H-7α/H-6α, and the absence of an ROE effect between H-5β/H-1α and H-6α /H-9β.

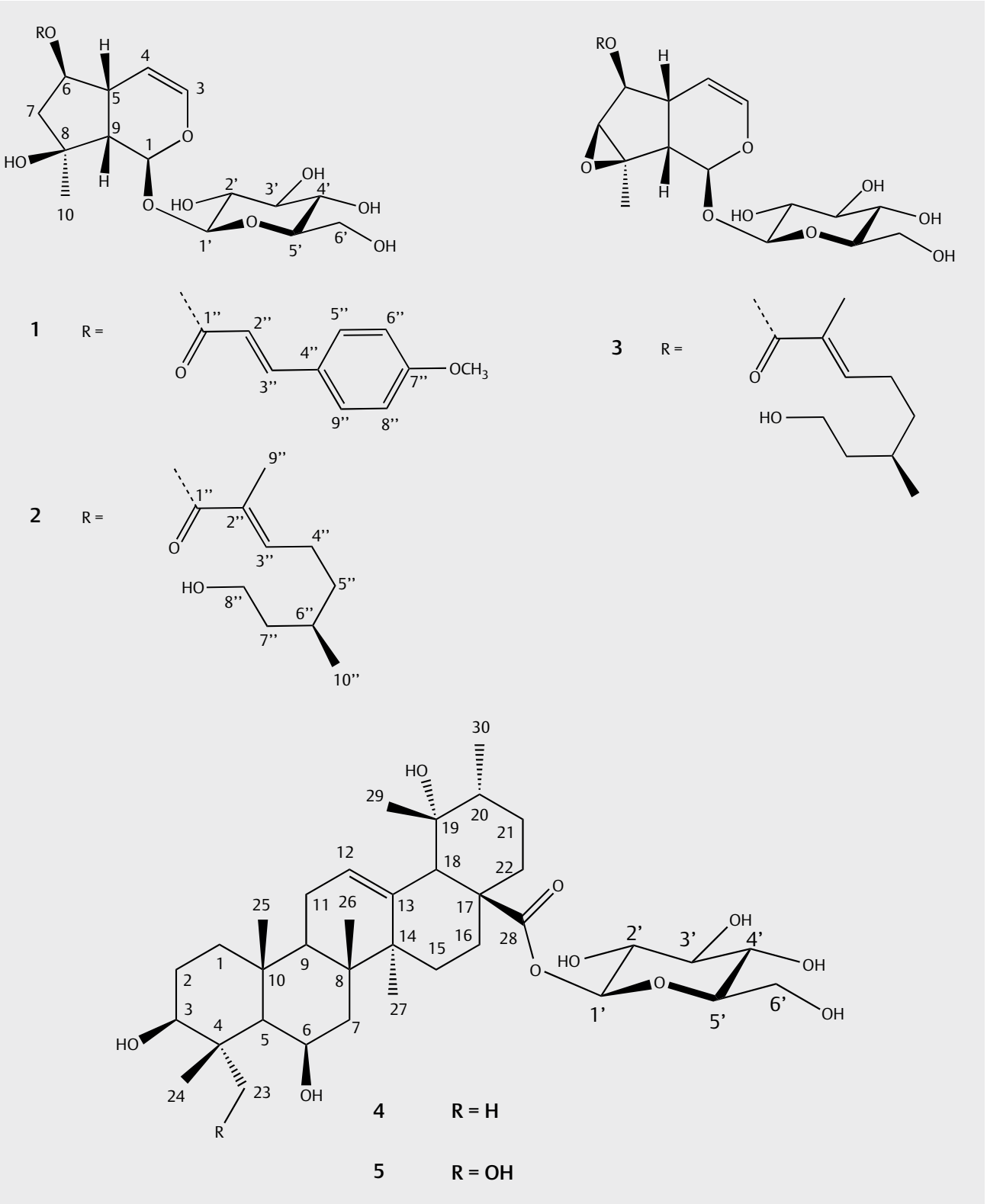
The molecular formula of **1** was determined to be $C_{25}H_{32}O_{11}$ by HR-ESI-MS analysis [molecular ion at m/z 531.1848 $[M + Na]^+$ (calcd. for $C_{25}H_{32}O_{11}Na$, 531.1842)]. By subtracting the ajugol part, there remained a $C_{10}H_{24}O_9$ (161 uma) residue. It was assigned to a p-O-methoxycinnamoyl group by NMR: AA'BB' system of four aromatic protons at δ_H 7.56 (2 H) and 6.97 (2 H), one E-double bond at δ_H 7.68 and 6.43 (J = 16.0 Hz), and one methoxy group at δ_H 3.85. The ester carbonyl carbon at δ_C 167.4 (C-9'') showed an HMBC correlation with H-6 of ajugol moiety and therefore, compound **1** was determined as 6-O-(p-methoxy-E-cinnamoyl)-ajugol.

Iridoid glucoside (**2**) showed a molecular ion peak $[M + Na]^+$ at m/z 539.2462 (calcd. for $C_{25}H_{40}O_{11}Na$, 539.2468) in agreement with a $C_{25}H_{40}O_{11}$ formula. Its ^{1}H- and ^{13}C-NMR spectra (▶ **Table 1**) were similar to those of **1** with characteristic signals for a 6-esterified derivative of ajugol. The ^{13}C-NMR and DEPT spectra of **2** exhibited ten carbons for a monoterpenic acid elucidated as 8-hydroxy-2,6-dimethyl-2-octenoic acid which was isolated as pure known compound (see below) [26, 27] (▶ **Table 1**). An E-configuration could be assigned to the trisubstituted double bond according to the shielding of CH_3-9'' at δ_C 12.4 in comparison with δ_C 22-25 for Z-configuration [34]. To determine the absolute configuration of the CH-6'', we carried out the alkaline hydrolysis of **2** and then the monoterpenic acid was purified by semi-preparative HPLC [18]. The positive sign of the optical rotation of the isolated acid indicated that it was 6 R-(2E)-8-hydroxy-2,6-dimethyl-2-octenoic acid. The absence of any supplementary peak in the ^{13}C as well as ^{1}H NMR spectra demonstrated that **2** was diastereoisomerically pure despite the fact that the optical rotation value of the acid ($[\alpha]_D$ = + 3.7) was lower than those found in the literature [26, 27]. This discrepancy is most probably due to difficulties in measuring rotations on tiny amounts of material. We concluded that **2** was 6''R-O-(2E) -8-hydroxy-2,6-dimethyl-2-octenoyl-ajugol.

The HR-ESI-MS analysis of the third new iridoid glucoside (**3**) revealed a molecular formula of $C_{25}H_{38}O_{12}$ with the molecular ion at m/z 553.2256 $[M + Na]^+$ (calcd. for $C_{25}H_{38}O_{12}Na$, 553.2261). The comparison of ^{1}H- and ^{13}C-NMR spectral data (▶ **Table 1**) of compounds **2** and **3** showed great similarities for the ester monoterpene parts. The iridoid skeleton of **3** was identified as catalpol [14] with only one deshielded proton H-7 at δ_H 3.70 (brs) and two oxygenated carbons for CH_2OH-10 at δ_C 61.3 and –CHO–7 at δ_C 60.2 [35]. The relative stereochemistry of the six asymmetric centers was confirmed by analysis of ROE effects. The stereochemistry of the carbon C-6" of the monoterpenic acid was determined by the same method as that employed for hydrolysis of **2**. The same optical rotation ($[\alpha]_D$ = + 3.7) was measured and compound **3** was identified as 6''R-O-(2E)-8-hydroxy-2,6-dimethyl-2-octenoyl)-catalpol.

The positive HR-ESI-MS of saponin (**4**) gave a molecular ion peak $[M + Na]^+$ at m/z 673.3934 (calcd. for $C_{36}H_{58}O_{10}Na$ 673.3928) corresponding to a molecular formula $C_{36}H_{58}O_{10}$. The presence of 36 carbons was observed in the ^{13}C-NMR spectrum including 6 carbons for a β-D-glucopyranose and 30 carbons for a Δ^{12} ursene skeleton with δ_C 130.0 (CH-12) and 138.9 (C-13; ▶ **Table 2**). The glucose unit was linked to the triterpene moiety at position C-28 since an HMBC correlation was observed between the anomeric proton H-1' and the carbonyl C-28 at δ_C 178.5 [36]. The aglycone was determined to be triterpene uncaric acid (or 3β,6β,19α-trihydroxyurs-12-en-28-oic acid) [37]. The 3β–OH configuration was confirmed by the large coupling constant $^3J_{H\text{-}3ax/H\text{-}2ax}$ = 11.7 Hz suitable with an (α) axial H-3, the hydroxyl group being in equatorial position (β). The ROE effects observed between H-6 and H-5α, and between H-6 and H-23α indicated a 6β–OH configuration. The third hydroxyl group was localized in axial position (19α-OH) due to the observation of ROE between the axial proton H-18β and the equatorial methyl CH_3-29β. Thus, compound **4** was identified as 28-O-β-D-glucopyranosyl-3β, 6β, 19α-trihydroxyurs-12-en-28-oic acid, named 28-O-β-D-glucopyranosyl uncaric acid.

The positive HR-ESI-MS of saponin (**5**) displayed a molecular ion peak $[M + Na]^+$ at m/z 689.3869 (calcd. for $C_{36}H_{58}O_1Na$, 689.3877) consistent with the molecular formula $C_{36}H_{58}O_{11}$, therefore a hydroxylated derivative of saponin **4**. Comparison of the NMR spectral data of **4** and **5** revealed significant similarities (▶ **Table 2**); the difference was the existence of only six methyl groups and an additional hydroxyl group located at position C-23 or C-24 according



▶ **Fig. 1** Chemical structures of iridoids (**1**, **2**, **3**) and saponins (**4**, **5**).

to HMBC correlations between the supplementary protons CH_2-OH and carbons C-3, C-4, and C-5. The ROE effects observed between H-6α/H-5α, and between H-6α and CH_2-OH confirmed the α-equatorial position of 23-CH_2-OH. The expected effects due to 24-hydroxylation were well observed on C-23 (Δα = + 38.4), C-4 (Δβ = + 3.5), C-24 (Δγ = -3.5), C-5 (Δγ = -6.7), and C-3 (Δγ = -6.3; ▶ **Table 2**). Thus, the aglycone of **5** was 3β, 6β, 19α, 23-tetrahydroxyurs-12-en-28-oic acid [38], and saponin **5** was identified as

▶ **Table 1** ^{1}H- and ^{13}C-NMR spectroscopic data (CD_3OD) of iridoids (**1**), (**2**) and (**3**).

	(1)		(2)		(3)	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	5.53, d (2.5)	92.0	5.56, d (2.4)	93.4	5.18, d (9.6)	95.1
3	6.24, dd (6.3, 2.2)	139.7	6.23, dd (6.3, 2.2)	141.1	6.39, d (6.0)	142.4
4	5.00, dd (6.3, 2.6)	103.2	4.97, dd (6.3, 2.7)	104.6	4.98, dd (5.6, 4.1)	102.9
5	2.95, dd (9.1, 2.3)	38.0	2.90, dq (9.2, 2.2)	39.3	2.61-2.56, m	36.7
6	4.96, ddd (6.5, 4.1, 2.7)	78.9	4.89, m	80.5	4.98, d (7.7)	81.6
7	2.02, dd (14.2, 4.0) β 2.27, dd (14.2, 6.4) α	46.5	2.23, dd (14.3, 6.5) α 1.98, dd (14.3, 4.0) β	47.8	3.70, brs	60.2
8		77.7		79.0		66.8
9	2.60, dd (9.3, 2.3)	50.2	2.59, dd (9.2, 2.0)	51.6	2.63, dd (9.3, 7.9)	43.2
10	1.41, s	24.7	1.39, s	26.1	4.18, d (13.2) 3.85, d (13.1)	61.3
β-D-glucopyranose (C-1)						
1’	4.69, d (8.0)	98.0	4.68, d (7.9)	99.4	4.81, d (7.9)	99.7
2’	3.22, dd (9.2, 7.9)	73.4	3.22, dd (9.2, 8.1)	74.8	3.30, dd (8.8, 8.3)	74.9
3’	3.39, dd (9.2, 8.7)	76.6	3.59, t (8.9)	78.0	3.43, t (9.0)	77.7
4’	3.31, dd (9.2, 8.6)	71.8	3.29, dd (9.7, 8.5)	71.7	3.28, t (9.3)	71.8
5’	3.32-3.35, m	76.8	3.32-3.35, m	78.2	3.35, ddd (9.4, 6.3, 1.5)	78.7
6’	3.68, dd (12.0, 6.0) 3.92, dd (12.0, 2.2)	61.5	3.68, dd (12.0, 5.9) 3.92, dd (12.0, 3.1)	62.9	3.67, dd (12.2, 6.1) 3.95, brd (11.9)	62.9
6-Acyl ester						
1’’		167.4		169.5		169.5
2’’	6.43, d (16.0)	115.0		128.8		128.3
3’’	7.68, d (16.0)	144.8	6.83, td (7.5, 1.3)	144.3	6.88, t (7.3)	145.0
4’’		127.0	2.20-2.32, m	27.2	2.22-2.35, m	27.3
5’’	7.56, d (8.6)	129.6	1.47-1.54, m 1.29-1.34, m	36.9	1.49-1.56, m 1.30-1.37, m	36.9
6’’	6.97, d (8.7)	114.0	1.64-1.66, m	30.5	1.60-1.67, m	30.5
7’’		161.9	1.59-1.64, m 1.35-1.40, m	40.6	1.60-1.65, m 1.41.37-1.43, m	40.6
8’’	7.56, d (8.6)	114.0	3.62-3.67, m 3.58-3.3.62, m	60.9	3.58-3.66, m	60.9
9’’	6.97, d (8.7)	129.6	1.85, s	12.4	1.89, s	12.5
10’’			0.97, d (6.5)	19.8	0.97, d (6.4)	19.8
OCH_3	3.85 (s)	54.5				

28-O-β-D-glucopyranosyl-3β, 6β, 19α 23-tetrahydroxyurs-12-en-28-oic acid, or 28-O-β-D-glucopyranosyl-23-hydroxy-uncaric acid.

MIC were determined for the five extracts prepared from the leaves of *D. spathacea* against 8 Gram-positive bacterial strains, 9 Gram-negative bacterial strains, and 5 yeast strains (▶ **Table 3**). The results demonstrated stronger antibacterial activity of all extracts against Gram-positive bacteria, and against two pathogenic bacteria, *Streptococcus pyogenes* and *Shigella sonnei* (MIC ≤ 0.3 mg/mL). The AcOEt extract was the most active against all tested microorganisms. The antimicrobial activity of the isolated compounds was first evaluated with an immersion bioautography method [39] against *Staphylococcus aureus* CIP 53.154 (▶ **Table 4**). Five compounds, among them decaffeoylacteoside and verbascoside that showed growth inhibition areas close to the ones observed with the antibiotic controls, as well as luteolin, p-methoxycinnamic acid and 6-O-E-caffeoyl-ajugol, seemed promising antimicrobial candidates. Our results were in agreement with the literature on antibacterial activity of verbascoside [40], luteolin [41], and p-methoxycinnamic acid [42].

A serial dilution technique in 96-well plates was used to determine the MIC of the active compounds against five bacteria (▶ **Table 4**); the best inhibitory activity was found for the two phenylethanoid diglycosidic compounds, decaffeoylacteoside and verbascoside (MIC = 31 µg/mL), and for p-methoxycinnamic acid (MIC = 62 µg/mL).

In summary, it may be concluded from this study that iridoid glucosides could be considered as chemical markers of Bignoniaceae, represented here by sixteen compounds amongst which three were never described before. The 6-O-esterified iridoids like minecoside and 6-O-caffeoyl-ajugol had weaker antibacterial activity than reported for the non-esterified aucubin [43]. Six compounds of the methanol extract of *D. spathacea* showed a strong inhibitory activity against resistant bacterial strains, particularly against

▶ **Table 2** ^{1}H- and ^{13}C-NMR spectroscopic data (CD_3OD) of saponins (**4**) and (**5**).

	(4)		(5)	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	0.98-1.03 m 1.58-1.66, m	42.1	0.94-1.04, m 1.58-1.65, m	41.7
2	1.58-1.66, m 1.70-1.78, m	28.0	1.58-1.65, m 1.71-1.79, m	27.6
3	3.10, dd (11.7, 4.1)	80.2	3.58, dd (11.7, 3.8)	73.9
4		40.7		44.2
5	0.75, brs	57.2	1.19, brs	49.5
6	4.50, brs ($w_{1/2}$ = 7.5)	69.0	4.41, brs ($w_{1/2}$ = 7.8)	68.9
7	1.55, dd (14.5, 2.0) eq 1.70-1.78, m ax	41.8	1.51, brd (14.2) 1.78-1.85, m	41.4
8		40.3		40.3
9	1.70-1.76, m	49.0	1.71-1.79, m	48.9
10		37.6		37.3
11	2.00-2.12, m	24.7	2.01-2.13, m	24.7
12	5.36, t (3.4)	130.0	5.36, brt (3.4)	130.0
13		138.9		138.9
14		43.1		43.1
15	1.91, td (14.0, 4.4) ax 1.03, m eq	29.7	1.91, td (13.9, 3.9) ax 1.01-1.04, m eq	29.7
16	2.62, td (13.3, 4.3) ax 1.64-1.68, m eq	26.6	2.62, td (13.3, 4.3) ax 1.66, brd (13.0) eq	26.6
17		49.3		48.6
18	2.55, brs	55.0	2.25, s	55.0
19		73.7		73.7
20	1.34-1.40, m	43.0	1.35-1.40, m	43.0
21	1.29-1.24, m 1.73-1.77, m	27.2	1.24-1.29, m 1.71-1.79, m	27.2
22	1.64-1.68, m 1.78-1.83, m	38.3	1.66, brd (12.6) 1.78-1.85, m	38.3
23	1.07, s	28.4	3.50, d (10.9) 3.62, d (11.0)	66.8
24	1.18, s	17.6	1.09, s	14.1
25	1.33, s	17.4	1.35, s	17.7
26	1.07, s	18.7	1.07, s	18.7
27	1.32, s	24.7	1.33, s	24.7
28		178.5		178.5
29	1.23, s	27.1	1.23, s	27.1
30	0.95, d (6.7)	16.6	0.95, d (6.7)	16.6
β-D-glucopyranose (C-28)				
1’	5.33, d (8.1)	95.9	5.33, d (8.2)	95.9
2’	3.34, brt (8.2)	73.9	3.35, t (8.2)	73.9
3’	3.43, t (8.9)	78.3	3.43, t (8.8)	78.3
4’	3.37, t (9.0)	71.2	3.38, dd (9.4, 8.5)	71.2
5’	3.32-3.36, m	78.6	3.32-3.36, m	78.6
6’	3.82, dd (11.8, 2.0) 3.70, dd (11.9, 4.6)	62.4	3.83, dd (12.0, 1.9) 3.71, dd (12.0, 4.6)	62.4

P. aeruginosa and *E. faecalis*. Dicaffeoylacteoside, verbascoside, and p-methoxycinnamic acid, the major compounds of the methanol extract, possessed strong antibacterial activity against most of the strains tested (MIC < 100 µg/mL). This result may explain the use of this species in folk medicine to treat oral thrush, bronchitis, and eye infection as well as cutaneous antiseptic. The large number and wide diversity of isolated compounds from leaves of *D. spathacea* supported the other traditional uses of this plant in gastrointestinal diseases as depurative, carminative, and against liver disease.

▶ **Table 3** Minimal inhibitory concentration of extracts from *Dolichandrone spathacea.*

		MIC of extract (mg/mL)							
	Microbial strain	**Petroleum ether**	**$CHCl_3$**	**AcOEt**	**MeOH**	**Aqueous MeOH**	**Vancomycine**	**Gentamicine**	**Amphotericin B**
Gram-positive bacteria	*Bacillus subtilis* ATCC 6633	5	1.2	5	10	10	S	S	NT
	Enterococcus faecalis ATCC 1034	5	2.5	10	10	10	R	R	NT
	Staphylococcus aureus CIP 8325-4	5	2.5	5	NA	10	S	S	NT
	Staphylococcus aureus CIP 53.154	5	2.5	5	2.5	10	S	S	NT
	Micrococcus luteus (lab. collection)	5	1.2	5	5	10	S	S	NT
	Listeria innocua (lab. collection)	5	2.5	5	NA	10	S	S	NT
	Staphylococcus epidermidis (lab. collection)	≤ 0.3	1.2	2.5	10	10	S	S	NT
	Streptococcus pyogenes (lab. collection)	≤ 0.3	≤ 0.3	≤ 0.3	≤ 0.3	≤ 0.3	S	S	NT
Gram-negative bacteria	*Providencia stuartii* (lab. collection)	5	1.2	5	NA	10	R	S	NT
	Pseudomonas aeruginosa ATCC 9027	10	NA	10	10	10	R	S	NT
	Shigella sonnei (lab. collection)	≤ 0.3	≤ 0.3	≤ 0.3	≤ 0.3	≤ 0.3	I	S	NT
	Proteus vulgaris (lab. collection)	NA	NA	10	NA	10	R	S	NT
	Klebsiella pneumoniae (lab. collection)	NA	NA	10	NA	NA	R	R	NT
	Serratia marcescens (lab. collection)	NA	NA	10	NA	10	R	S	NT
	Escherichia coli CIP 54.127	NA	NA	10	NA	NA	R	S	NT
	Enterobacter cloacae (lab. collection)	NA	NA	10	NA	NA	R	S	NT
	Salmonella enterica (lab. collection)	NA	NA	10	NA	10	R	S	NT
Yeast	*Candida glabrata* (lab. collection)	2.5	1.2	5	NA	10	R	R	S
	Candida tropicalis (lab. collection)	5	10	5	NA	10	R	R	S
	Candida kefyr (lab. collection)	5	10	10	NA	NA	R	R	S
	Cryptococcus neoformans (lab. collection)	10	NA	10	NA	NA	R	R	S
	Candida albicans ATCC 2091	5	10	5	NA	10	R	R	S

NA = not active; S = sensitive; I = intermediate sensitivity; R = resistant.

MIC (µg/mL) of positive controls: Gentamicin, S: ≤ 4, R: > 8; Vancomycin, S: ≤ 4, R: > 16; Amphotericin B, S: ≤ 1, R: > 4.

Material and Methods

General experimental procedures

Optical rotations were determined with a Perkin-Elmer 341 polarimeter. UV-Vis spectra were measured with a Shimadzu UV/Vis U-2450 spectrophotometer. IR spectra were recorded on a Nicolet Impact 410 FTIR spectrometer. HR-ESI-MS experiments were performed using a Micromass Q-TOF micro instrument. NMR spectra were acquired in CD_3OD on a Bruker Avance DRX-600 instrument using standard pulse sequences and parameters. TLC was performed on pre-coated silica-gel 60 A: Alugram UV_{254} Macherey-Nagel in normal phase, and Silicagel 60 RP-18 F_{254S} Merck in inversed phase. CC and vacuum liquid chromatography (VLC) were realized on Kieselgel 60 (63-200 mesh), Merck. Flash chromatography (FC) was carried out on a Grace Reveleris apparatus equipped with a detector ELSD, a detector UV/vis and Reveleris Flash System software. HPLC was performed on a Dionex apparatus equipped

▶ **Table 4** Bioautography and Minimal Inhibitory Concentration of antimicrobial compounds of *Dolichandrone spathacea*.

	Bioautography	MIC (µg/mL)				
Compound	**S. aureus CIP 53.154**	**S. aureus CIP 53.154**	**E. faecalis ATCC 1034**	**S. pyogenes (lab. collection)**	**P. aeruginosa ATCC 9027**	**S. sonnei (lab. collection)**
decaffeoylacteoside	+++	62.5	31.2	62.5	250	31.2
verbascoside	+++	125	31.2	125	250	31.2
luteolin	++	250	250	125	500	125
p-methoxycinnamic acid	++	62.5	125	62.5	125	62.5
minecoside	NA	NA	500	250	500	250
6-O-*E*-caffeoylajugol	++	125	250	125	500	125
Vancomycin	+++	3.9	62.5	≤ 1.9	62.5	7.8
Gentamicin	+++	≤ 1.9	15.6	1.9	7.8	≤ 1.9

(+): Anti-staphylococcal effect, rated from + (0.5 cm inhibition zone) to ++++ (> 1.5 cm inhibition zone); NA: no activity.

with a LPG 3400AB pump, an ASI-100 auto-sampler, a diode array detector UVD 340 S, an oven STH 585, and a Chromeleon software. Phenomenex Luna C18 columns (5µ, 100 Å; 250 × 10 mm: column 1; 250 × 15 mm: column 2) and Interchrom Uptisphere Strategy C18 column (2–5 µ, 250 × 10 mm: column 3) were used for semi-preparative HPLC with gradient eluent [solvent A, H_2O or H_2O (pH 2.4, 0.025 % TFA); solvent B, MeCN or MeOH], and the chromatogram was monitored at 205 and 254 nm. Preparative HPLC chain comprises a Merck column of 200 × 50 mm packed with C-18 silica gel, an Armen AP 250/500 pump, an ACC 250/500 injector, and an UV Merck K-2501 Knauer detector; fractions were collected using a Büchi C-660 collector. Sugars were purified on a Waters chromatographic chain with a Rezex ROA column (250 × 21.2 mm: column 4), Empower software, a 600 E pump, a 717 plus auto-sampler, and a refractive index (RI) detector (flow rate = 3.5 mL/min, pressure = 1400 psi); Chiralpak IC column (5 µm, 250 × 4.6mm: column 5) was used for the chiral identification of sugars (flow rate = 0.5 mL/min, pressure = 300 psi).

Plant material

The leaves of *D. spathacea* were collected in Vinh Long Province, Vietnam in December 2011. The plant was identified by Professor Nghia Thin Nguyen (Faculty of Biology, University of Sciences Hanoi, Vietnam) and a voucher specimen (CTU-CD001) was deposited at the Department of Biology, Faculty of Pedagogy, Can Tho University, Vietnam.

Extraction and isolation

Dried and powdered leaves (150 g) were macerated for 7 h with petroleum ether (1.5 L) to furnish 0.98 g of extract (yield 0.65 %), and treated in the same way successively with 1.5 L $CHCl_3$ for 20 h (5 g; yield 3.4 %), 1.5 L EtOAc (2 g; yield 1.4 %), 1.5 L MeOH (23 g; yield 15.3 %), and 1.5 L of 80 % aqueous MeOH (17 g; yield 12 %).

The MeOH extract (2 × 10 g) was subjected to silica gel VLC (7 × 9.5 cm) using a gradient of solvent $CHCl_3$ – MeOH – H_2O (100:0:0 – 0:100:5) to give 20 fractions. The p-methoxycinnamic acid (480 mg) was obtained in VLC-fraction 4 eluted with $CHCl_3$ – MeOH (90:10).

VLC-fraction 5 [620 mg, eluted with $CHCl_3$ – MeOH (85:15)] was separated by C-18 FC using a gradient of MeOH – H_2O (10:90 – 100:0); sub-fraction 5.9 [53 mg, eluted with MeOH – H_2O (45:55)] was purified by semi-preparative HPLC (column 2; 6 mL/min) using an isocratic solvent of 25 % MeCN (pH = 2.4) during 30 min to yield (2E,6E)-8-hydroxy-2,6-dimethyl-2,6-octadienoic acid (6.2 mg; R_t = 10.66 min) and (2E,6Z)-8-hydroxy-2,6-dimethyl-2,6-octadienoic acid (3.5 mg; R_t = 11.31 min); sub-fraction 5.11 (40 mg) was purified by the same way using an isocratic solvent of 28 % MeCN to furnish a mixture of 6 R- and 6 S-(2E)-8-hydroxy-2,6-dimethyl-2-octenoic acids (5.8 mg; R_t = 14.62 min).

VLC-fraction 6 [2.3 g, eluted with $CHCl_3$ – MeOH (80:20)] was chromatographed by silica gel FC using a gradient of $CHCl_3$ – MeOH (100:0 – 0:100) to obtain p-hydroxycinnamic acid (22 mg) and 6-(E)-p-methoxycinnamoyl-catalpol (240 mg); sub-fraction 6.3 [31 mg, eluted with $CHCl_3$ – MeOH (93:7)] was purified by semi-preparative HPLC (column 1; 3 mL/min) using an isocratic mode of 23 % MeCN (pH = 2.4) to give vanillic acid (1 mg; R_t = 7.13 min) and isoferulic acid (2.3 mg; R_t = 12.39 min); the purification of sub-fraction 6.5 [28 mg, eluted with $CHCl_3$ – MeOH (91:9)] by semi-preparative HPLC (column 2; 6 mL/min) using a gradient solvent of 20-45 % MeCN (pH = 2.4) gave p-hydroxybenzoic acid (3 mg; R_t = 8.69 min) and luteolin (4.5 mg; R_t = 16.18 min); sub-fraction 6.7 (470 mg) eluted with $CHCl_3$ – MeOH (90:10) was separated by C-18 FC using a gradient solvent of MeOH – H_2O (10:90 – 100:0), then fractions eluted with MeOH – H_2O (60:40) were subjected to silica gel CC using a gradient system of $CHCl_3$ – MeOH (100:0 – 0:100) to obtain 6-O-(p-methoxy-E-cinnamoyl)-ajugol (**1**) (50 mg) eluted with 5 % MeOH and a mixture of (E)- and (Z)-6-p-methoxycinnamoyl-catalpol (2 mg; R_t = 25.46 min), finally purified by semi-preparative HPLC (column 1, 4 mL/min) using an isocratic mode of 28 % MeCN.

VLC-fraction 7 [2.6 g, eluted with $CHCl_3$ – MeOH (75:25)] was chromatographed by preparative HPLC using a gradient of MeOH – H_2O (25:75 – 100:0); sub-fraction 7.5 [32 mg, eluted with MeOH – H_2O (35:65)] was separated by semi-preparative HPLC (column 3, 4 mL/min) using an isocratic of 28 % MeCN (pH = 2.4) to obtain a mixture (R_t = 10.43 min) which was purified by a semi-preparative HPLC using an isocratic of 17 % MeOH during 55 min to yield 6 S,9 S-roseoside (6 mg; R_t = 46.98 min); the purification of sub-fraction 7.9 [90 mg, eluted with MeOH – H_2O (35:65)] by semi-preparative HPLC (column 3; 4 mL/min) using an isocratic mode of 23 % MeCN gave minecoside (5 mg; R_t = 14.49 min) and 6-O-E-isoferuloyl-ajugol (4 mg; Rt = 16.15 min); in the same way, sub-fraction 7.10 [87 mg, eluted with MeOH – H_2O (35:65)] yielded nemorososide (4 mg;

R_t = 14.70 min) and 6''(Z)-nemoroside (7.5 mg; Rt = 19.02 min); the 6-O-p-E-coumaroyl-ajugol (23 mg; R_t = 25.19 min) was obtained from sub-fraction 7.12 [50 mg, eluted with MeOH – H_2O (40:60)] by a semi-preparative HPLC (column 1; 4 mL/min) using an isocratic of 20 % MeCN; semi-preparative HPLC (column 3; 3.5 mL/min) of sub-fraction 7.15 [62 mg, eluted with MeOH – H_2O (60:40)] using an isocratic of 25 % MeCN furnished mixture of 6''R-(2E) -8-hydroxy-2,6-dimethyl-2-octenoyl)-ajugol (**2**) (7.5 mg; R_t = 22.54 min) and mixture of 6''R-(2E)-8-hydroxy-2,6-dimethyl-2-octenoyl)-catalpol (**3**; 10 mg; R_t = 25.91 min); in the same way, sub-fraction 7.17 (52 mg, eluted with MeOH – H_2O (65:35)) gave 6-O-(E) -cinnamoyl-catalpol (14 mg; R_t = 20.72 min); the 28-O-β-D-glucopyranosyl uncaric acid (**4**; 7.3 mg; R_f = 0.28) was obtained from sub-fraction 7.21 (27 mg, eluted with MeOH – H_2O (100:0)) by using C-18 preparative TLC eluted with MeOH – H_2O (70:30).

VLC-fraction 8 [2.2 g, eluted with $CHCl_3$ – MeOH (70:30)] was separated by preparative HPLC using a gradient mode of MeOH – H_2O (25:75 – 100:0) to yield verbascoside [397 mg, eluted with MeOH – H_2O (25:75)]; the purification of sub-fraction 8.4 [397 mg, eluted with MeOH – H_2O (35:65)] by semi-preparative HPLC (column 3; 4 mL/min) using an isocratic of 10 % MeCN gave decaffeoyl-acteoside (8 mg; R_t = 8.61 min); semi-preparative HPLC (column 1; 4 mL/min) applied to sub-fraction 8.12 [33 mg, eluted with MeOH – H_2O (40:60)] was carried out by using an isocratic mode of 20 % MeCN to give verminoside (7 mg; R_t = 11.33 min); semi-preparative HPLC (column 3, 3.5 mL/min) of sub-fraction 8.14 [40 mg, eluted with MeOH – H_2O (45:55)] yield isoverbascoside (14 mg; R_t = 18.41 min) by using an isocratic mode of 19 % MeCN; sub-fraction 8.15 [12 mg, eluted with MeOH – H_2O (45:55)] was purified by semi-preparative HPLC (column 3; 4 mL/min) using an isocratic mode of 18 % MeCN to furnish luteolin-7-O-β-D-glucopyranoside (7.5 mg; R_t = 15.81 min); 6-O-E-caffeoyl-ajugol (7 mg; R_t = 24 min) was obtained from sub-fraction 8.16 [40 mg, eluted with MeOH – H_2O (50:50)] by semi-preparative HPLC (column 1; 4 mL/min) using an isocratic mode of 18 % MeCN; the same protocol applied to sub-fraction 8.18 [33 mg, eluted with MeOH – H_2O (55:45)] gave specioside (9 mg; R_t = 12.87 min) using an isocratic mode of 23 %MeCN; the purification of sub-fraction 8.21 [90 mg, eluted with MeOH – H_2O (65:35)] yielded arjunglucoside I (6 mg; R_t = 13.13 min) and nemoroside (9.5 mg; R_t = 17.16 min) by semi-preparative HPLC (column 3; 4 mL/min) using an isocratic mode of 25 % MeCN; sub-fraction 8.23 [20 mg, eluted with MeOH – H_2O (65:35)] was purified by silica gel preparative TLC eluted with $CHCl_3$ – MeOH – H_2O (80:20/2) to yield 28-O-β-D-glucopyranosyl-23-hydroxy-uncaric acid (**5**; 7.5 mg; R_f = 0.33).

VLC-fraction 13 [617 mg, eluted with $CHCl_3$ – MeOH – H_2O (70:30:5)] was separated by C-18 FC using a gradient of MeOH – H_2O (10:90 – 100:0) to give 17 sub-fractions; luteolin-7-O-rutinoside (6 mg; R_t = 14.85 min) was obtained by semi-preparative HPLC (column 1; 3.5 mL/min) of sub-fraction 13.9 [18 mg, eluted with MeOH – H_2O (45:55)] using an isocratic solvent of 18 % MeCN (pH = 2.4).

Purification of VLC-fraction 17 [90 mg, eluted with $CHCl_3$ – MeOH – H_2O (30:70:5)] by semi-preparative HPLC (column 1; 4 mL/min) using a gradient mode of 10 – 35 % MeCN (pH = 2.4) furnished ixoside (6 mg; R_t = 5.79 min) and luteolin-7-O-glucuronide (4.5 mg; R_t = 15.89 min).

The purities of all isolated compounds were ≥ 95 %, as determined by HPLC and ^{1}H NMR.

Compound **1**: amorphous powder; $[\alpha]_D$ -143 (c 0.39, MeOH); UV (MeOH) λ_{max} nm (log ε) 202 (2.88), 226 (2.82), 308 (3.09); IR (KBr) ν_{max} cm^{-1}: 3428, 2931, 1706, 1662, 1632, 1575, 1514, 1170, 1002, 825; ^{1}H- and ^{13}C-NMR: see ▶ **Table 1**; HR-ESI-MS m/z 531.1848 $[M + Na]^+$ (calcd. for $C_{25}H_{32}O_{11}Na$ 531.1842), 483.2442 $[M + Na\text{-}MeO\text{-}OH]^+$, 185.2039 $[Ajugol\text{-}Glc]^+$.

Compound **2**: amorphous powder; $[\alpha]_D$ -94 (c 0.25, MeOH); UV (MeOH) λ_{max} nm (log ε) 224 (3.34), 308 (2.33); ^{1}H- and ^{13}C-NMR: see ▶ **Table 1**; HR-ESI-MS m/z 539.2462 $[M + Na]^+$ (calcd. for $C_{25}H_{40}O_{11}Na$ 539.2468).

Compound **3**: amorphous powder; $[\alpha]_D$ -107 (c 0.22, MeOH); UV (MeOH) λ_{max} nm (log ε) 224 (3.33), 308 (2.33); ^{1}H- and ^{13}C-NMR: see ▶ **Table 1**; HR-ESI-MS m/z 553.2256 $[M + Na]^+$ (calcd. for $C_{25}H_{38}O_{12}Na$ 553.2261), 385.1032 $[M + Na\text{-}Ester]^+$, 351.2457 $[M\text{-}Glc]^+$.

Compound **4**: white powder; $[\alpha]_D$ -18 (c 0.12, MeOH); UV (MeOH) λ_{max} nm (log ε) 210 (3.37), 308 (3.05); IR (KBr) ν_{max} cm^{-1}: 3423, 2929, 1734, 1653, 1458, 1383, 1074; ^{1}H- and ^{13}C-NMR: see ▶ **Table 2**; HR-ESI-MS m/z 673.3934 $[M + Na]^+$ (calcd. for $C_{36}H_{58}O_{10}Na$ 673.3928), 457.2697 $[M\text{-}Glc\text{-}OH]^+$.

Compound **5**: white powder; $[\alpha]_D$ -7 (c 0.19, MeOH); UV (MeOH) λ_{max} nm (log ε) 214 (3.43), 298 (2.83); IR (KBr) ν_{max} cm^{-1}: 3422, 2927, 1732, 1598, 1455, 1384, 1072; ^{1}H- and ^{13}C-NMR: see ▶ **Table 2**; HR-ESI-MS m/z 689.3869 $[M + Na]^+$ (calcd. for $C_{36}H_{58}O_{11}Na$ 689.3877), 503.3242 $[M\text{-}Glc]^+$.

Acid hydrolysis

The MeOH extract (1 g) was refluxed in 25 mL of 2 N HCl in 4 h. After extraction with EtOAc (3 × 25 mL), the aqueous layer was neutralized with 0.5 M NaOH and freeze-dried. The glucose was identified by comparison with an authentic sample by TLC using MeCOEt – i-PrOH – MeCOMe – H_2O (20/10/7/6). It was purified by semi-preparative HPLC (column 4) using 2.5 µM H_2SO_4 as solvent, and identified by chiral analytic HPLC (column 5) using an isocratic mixture of n-hexane/EtOH/TFA (80/20/1) in comparison with the authentic samples of D-glucose (R_t = 19.44 min) and L-glucose (R_t = 18.73 min).

Alkaline hydrolysis

4 mg of (**2** or **3**) were dissolved in 1 mL of 2 N NaOH and stirred overnight. The solution was treated with Et_2O (3 × 1 mL) to remove neutral material. Acidification with dilute HCl was followed by extraction with Et_2O (3 × 1 mL). The Et_2O extract were evaporated in vacuo and residue was purified by semi-preparative HPLC to give 1.2 mg (or 1.5 mg) of monoterpenic acid.

Antimicrobial assays

After an overnight culture at 37 °C in Mueller-Hinton (MH) medium, the bacteria (ATCC or CIP collections or clinical strains) and yeasts (clinical strains) were resuspended in fresh MH medium up to the working solution of 105 microorganisms/mL. The clinical strains were part of the internal laboratory of microbiology or parasitology collections. All the antibiotics were used in serial dilutions with the higher working concentration of 64 mg/L.

To determine the minimum inhibitory concentration (MIC) of the extracts, agar plates containing six different concentrations (10, 5, 2.5, 1.2, 0.6, and 0.3 mg/mL) of each extract were loaded with the different microorganism using a multiple inoculator (Steers). After 24 h of incubation at 37 °C, the activity was estimated looking at the presence or absence of colonies. Solvents used to prepare extracts and MH agar medium were checked for absence of antibacterial activity (negative controls). Positive active controls were gentamicin (≥ 99 % of purity) and vancomycin (≥ 99 % of purity) for antibacterial assays, and amphotericin B (≥ 99 % of purity) for anti-yeast assays.

The immersion bioautography method was used to identify the active compounds against *S. aureus* CIP 53.154. 10 µL of each compound (2 mg/mL of methanol) or gentamicin (50 µg/mL, positive control) were spotted onto TLC plates (10 × 10 cm). The plates were than dried, sterilized and covered by the MH agar medium containing *S. aureus* (10^5 bacteria/mL) in square Petri dishes. After incubation for 24 h at 37 °C, bacterial growth was revealed by a 2 mg/mL solution of thiazolylbluetetrazolium bromide (MTT) and growth inhibition zones were measured [39].

The broth microdilution method in 96-well plates was used to determine MIC of active compounds against five bacteria sensitive to the extracts. Nine concentrations of each compound, from 500 µg/mL to 1.9 µg/mL in a serially twofold dilution, were tested against 0.5×10^5 bacteria/mL, in a total volume of 200 µL, and incubated overnight at 37 °C. Positive control wells with bacteria in MH medium, gentamicin or vancomycin, as well as negative controls such as medium alone or medium with methanol were systematically added to the test. Bacterial growth was followed visually and by spraying MTT incubating at 37 °C for at least 10 min. The MIC value was determined as the lowest concentration of a compound leading to a clear well. This test was performed in triplicate.

Supporting information

Tables with ^{1}H- and ^{13}C-NMR data of the 30 known compounds, and ^{1}H- and ^{13}C-NMR Spectra of compounds (**1**-**5**) are available as Supporting Information.

Acknowledgments

The authors wish to thank Dominique Harakat (ICMR) for recording mass spectra, Prof. Nghia Thin Nguyen for the plant identification, Dien Trung Nguyen and Hoang Viet Ho (Can Tho University) for the plant collection, Dr. Georges Massiot (ICMR) for stimulating discussions, Prof. Christophe De Champs and Janick Madoux (Laboratory of Bacteriology, CHU Reims) for access to multiple inoculators material, and Prof. Jerome Depaquit (Laboratory of parasitology-mycology, University of Reims) for providing yeasts.

Conflict of Interest

The authors declare no conflict of interest.

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