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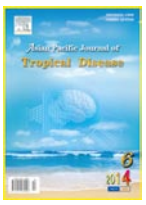

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Composition of the non-polar extracts and antimicrobial activity of *Chorisia insignis* HBK. leaves

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PEER REVIEW

Peer reviewer

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Comments

The article is valuable, and the comment is scientific and fair.

Reda Sayed Mohammed, Department of Pharmacognosy, National Research Centre, 12622, Dokki, Cairo, Egypt.

Comments

This is a valuable study in which the authors isolated and identified the constituents of the non polar fraction and the article contains the spectroscopic data which lead to identification of these compounds.

Details on Page 479

ABSTRACT

Objective: To investigate the chemical constituents of the petroleum ether extract and the ether fraction of the 70% ethanol extract of *Chorisia insignis* HBK. leaves, as well as screen its antimicrobial activity.

Methods: Different chromatographic methods were applied to investigate the non-polar extracts and the diffusion assay method was applied to study the antimicrobial activity.

Results: A total of 50 compounds from the unsaponifiable matter and 20 fatty acid methyl esters were identified from the petroleum ether extract by GC/MS analysis. *n*-Hentriacontane, *n*-tritriacontane, stigmastanol, 3-methoxy-5, 6-dihydrostigmasterol, 7,8-dihydroergosterol, 4-methylcholesterol, cholestanol, multiflorenol, cholest-5-en-3-one, cholest-6-one, 5,6-dihydroergosterol, stigmasterol, dihydroalbigenin and 11-methyl-$\Delta^{5,7,9,15,17,23}$-triacont-hex-ene were isolated from the petroleum ether extract. Methyl heptacosanoate and quinic acid ester of rhamnose were isolated from the ether fraction of the 70% ethanol extract. Antimicrobial activity of the total alcohol extract and the successive fractions showed that the ether and the ethyl acetate fractions have potent antibacterial activity against *Bacillus subtilis* and *Bacillus cereus*.

Conclusions: The ether and the ethyl acetate fractions could be used in pharmaceutical formulations as antibacterial agents against *Bacillus subtilis* and *Bacillus cereus*, and further clinical trials should be performed in order to support the above investigations.

KEYWORDS

Antimicrobial activity, *Chorisia insignis*, Fatty acid, GC/MS, Methyl heptacosanoate, Quinic acid ester of rhamnose

1. Introduction

Chorisia insignis (C. insignis) HBK., known as white floss silk tree, belongs to family Bombacaceae. It is native to South America, Peru, Brazil and Argentina[1,2].

Chorisia was named in honor of the botanical artist and traveler Ludwig I. Choris (1795-1828, 19th century)[1]. *C. insignis* is mainly cultivated for its ornamental brilliant flowers. It is also cultivated for the silky white fibre (or floss) that is obtained from the ripened fruits. This floss has been used to form cushions and vests which explain the common name of this tree, "floss silk tree"[1].

Phytochemical investigations of *C. insignis* remained confined to the isolation of one flavonoid (rhoifolin glycoside) from fresh leaves[3]. Thus, it was deemed of interest to investigate the phytoconstituents as well as the biological activities of the plant.

In our previous work, the flavonoid content and the pharmacological actions of the leaves were investigated, three flavonoids were isolated and it was found that the plant possesses significant anti-inflammatory, antihyperglycemic, antioxidant and hepatoprotective activities[4]. It was also found that the polar extracts contain several phenolic compounds and that the plant has

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significant cytotoxic activity against larynx carcinoma[5]. The polysaccharide content of the leaves and stems were studied[6]. Moreover, El Alfy *et al.* confirmed the identity and the purity of *C. insignis* through studying its macro and micromorphological features, determination of its pharmacopoeial constants, DNA fingerprinting as well as protein electrophoresis to monitor the seed storage protein and determination of the total protein and amino acids contents[7].

The present work was carried out to study the chemical constituents of the petroleum ether extract and the ether fraction of the 70% ethanol extract and the antimicrobial activity of *C. insignis* leaves cultivated in Egypt.

2. Materials and methods

2.1. Plant material

Samples of the leaves of *C. insignis* were collected from National Research Centre (NRC) garden, Dokki, Cairo, Egypt in June, and were kindly authenticated by Dr. Mohamed Gibali, senior botanist and by Agricultural Engineer Therese Labib, consultant of plant taxonomy at the Ministry of Agriculture and ex-director of Orman Botanical Garden, Giza, Egypt. A voucher specimen (No. 23569) is kept at NRC Herbarium. Samples of the plant under investigation were separately air-dried, powdered and kept in tightly closed amber coloured glass containers.

2.2. Solvents

Petroleum ether (40-60 °C), diethyl ether, chloroform, ethyl acetate and ethanol were used in the present study. All solvents used were of analytical grade (RFCL Limited, New Delhi, India).

2.3. Adsorbents for chromatography

- Silica gel 60 F254 for thin layer chromatography (TLC) (Fluka Chemie AG, Switzerland).
- Silica gel 60 for column chromatography (CC) (E. Merck, Darmstadt, Germany).
- Sheets of Whatman filter paper No. 1 for paper chromatography (PC) (Whatman Ltd., Maidstone, England).
- Sheets of Whatman filter paper No. 3 for paper partition chromatography.
- Sephadex LH-20 for CC (Fluka Chemie AG, Switzerland).

2.4. Solvent systems (v/v)

S1: benzene: ethyl acetate	(9:1)
S2: *n*-BuOH: acetic acid: H_2O	(4:1:5)
S3: acetic acid: H_2O	(15:85)
S4: MeOH	(100%)

2.5. Tested microorganisms (MO)

Bacteria, fungi and yeast strains were kindly obtained from the Microbial Chemistry Department, NRC, Egypt. *Bacillus subtilis* (M1), *Bacillus cereus* (M2), *Staphylococcus aureus* (M3) and *Streptococcus pyogenes* (M4) were the Gram-positive bacterial strains used, while *Escherichia coli* (M5) was the Gram-negative strain. Also, four fungi *Aspergillus niger* (M6), *Fusarium oxysporum* (M7), *Botrytis allii* (M8) and *Trichoderma viride* (M9), and one yeast *Saccharomyces cervisiae* (M10) were used.

2.6. Culture media

All the chemicals used in the preparation of the media were of the analytical grade. Distilled water was used. Routine sterilization was carried out by autoclaving for 20 min at 15 psi (121 °C). The following media including Lauria-Bertani medium[8], potato dextrose agar growth medium[9], and yeast extract peptone dextrose medium[10] were used.

2.7. Standard drugs

Ampicillin (ADWIC, El Nasr Pharmaceutical Chemicals Co.) was used as a standard antibacterial drug, while clotrimazole (Locasten, Alexandria Company for Pharmaceuticals) was used as a standard antifungal drug.

2.8. Apparatus

- Mass spectrometer: Finnigan Model 3200 Mass spectrometer at 70 eV.
- Gas chromatograph coupled with a mass spectrometer (GC/MS): Finnigan Mat SSQ 7000, Digital DEC EL, 70 eV for GC/MS analysis of unsaponifiable matter and fatty acid methyl esters.
- UV-visible spectrophotometer: UV-vis double beam UVD-3500 spectrophotometer, Labomed, Inc.
- Electrospray ionization mass spectrometer (ESI-MS): Thermo Finnigan (ion trap).
- NMR: Joel ECA 500 (^{1}H-NMR at 500 MHz).

2.9. Experimental

2.9.1. Preparation of crude extracts

2.9.1.1. Successive extracts

A total of 550 g of air-dried powdered leaves were exhaustively defatted using petroleum ether (40-60 °C) (E1) in a Soxhlet apparatus. The defatted powder was refluxed with 70% ethanol till complete exhaustion. The combined ethanol extract was evaporated to dryness to give 145 g, then suspended in water (600 mL) and partitioned successively with ether (E2) (10×100 mL) followed by chloroform (E3) (15×100 mL), ethyl acetate (E4) (15×100 mL) and *n*-butanol (E5) (12×100 mL). The solvents were evaporated to dryness under reduced pressure at 40 °C.

2.9.1.2. Total alcohol extract (E6)

About 100 g of air-dried powdered leaves were exhaustively extracted by refluxing with 95% ethanol. The combined extract was evaporated under reduced pressure at 40 °C to give 14 g total alcohol extract.

2.9.2. Saponification of E1

A total of 0.5 g of the residue of E1 was saponified

according to the method described by Tsuda *et al.* to give 0.30 g unsaponifiable matter (UNSAP) and 0.08 g fatty acids[11]. Preparation of the fatty acid methyl esters (FAME) was carried out according to Finar[12].

GC/MS analysis for UNSAP and FAME were performed using capillary column of fused silica, 30 m length, 0.32 mm inner diameter and 0.25 mm thickness, and helium at 1 mL/min, 13 psi as the carrier gas. The ion source temperature adjusted at 180 °C and the ionization voltage 70 eV using MS detector. DB-WAX was the stationary phase for FAME and DB-5 for UNSAP; the temperature programming was 50-260 °C at a rate of 4 °C/min and 70-290 °C at a rate of 4 °C/min respectively. Identification of the constituents was carried out by comparison of their spectral fragmentation patterns with those of the available database libraries: Wiley, USA and National Institute of Standards and Technology and/or published data[13,14].

2.9.3. Isolation and identification of the major constituents of E1

A total of 15 g of E1 was applied on 300 g silica gel (150 mm×5 mm) column using petroleum ether (40-60 °C) then increasing the polarity by adding ether gradually till 100% ether then chloroform then methanol. Fractions were screened by TLC silica gel using S1. Compound T1 was obtained from 100% petroleum ether fraction, compounds T2-T4 from 80% $CHCl_3$/Ether fraction, compounds T5-T9 from 90% $CHCl_3$/Ether fraction and compounds T10-T12 from 2% MeOH/$CHCl_3$ fraction. All compounds were isolated from the corresponding fraction by preparative TLC using S1 as the developing system.

2.9.4. Investigation of fraction E2

Fraction E2 was subjected to PC examination using Whatman No. 1 sheets with S2 and S3 for developing. The chromatograms were examined under UV light before and after exposure to ammonia vapour or spraying with $AlCl_3$ solution; two spots were detected. About 8 g of E2 were chromatographed by paper partition chromatography using Whatman No. 3 sheets and developed using S3 to give compound A1 and band A21, the latter purified on column Sephadex LH-20 using S4 as eluant to give compound A2.

2.9.5. Antimicrobial activity

The antimicrobial test was carried out according to the diffusion assay method[15].

3. Results

3.1. GC/MS analysis

Analysis of UNSAP of E1 (Table 1) resulted in the identification of 50 compounds constituting 95.81% of the total peak area. The non-oxygenated compounds constitute 37.97% while the oxygenated constitute 57.84%. *n*-Triacontane (21.45%) was the major constituent. The oxygenated compounds classified into eight classes; ketones (27.31%) and the major constituent was 4-hydroxy-4-methyl-2-pentanone (14.65%), followed by hydroxylated compounds (19.83%) and its major constituent was butylated hydroxytoluene (16.52%), then terpenoids (9.11%) and the major constituent was Δ^{12}-lupan-3-ol (8.49%), anhydrides (0.56%), ether compounds (0.37%), steroidal compounds (0.36%), aldehydes (0.19%) and finally miscellaneous compounds (0.11%).

Table 1

GC/MS analysis of the unsaponifiable matter of the petroleum ether extract of *C. insignis* leaves.

Rt	MWt	BP	Area (%)	MF	Compound
4.64	116	43	14.65	$C_6H_{12}O_2$	4-hydroxy-4-methyl-2-pentanone
5.58	98	55	4.48	$C_6H_{10}O$	Cyclohexanone
5.79	98	43	5.99	$C_6H_{10}O$	2-methyl cyclopentanone
7.13	154	43	0.42	$C_{10}H_{18}O$	Yomogi alcohol (2,5,5-trimethyl-3,6-heptadien-2-ol)
7.57	158	73	0.58	$C_{10}H_{22}O$	Linalool tetrahydride
9.51	156	57	8.44	$C_{11}H_{24}$	*n*-undecane
11.25	156	43	0.69	$C_{11}H_{24}$	2,8-dimethyl nonane
12.97	154	43	0.34	$C_{10}H_{18}O$	Cis-*p*-menth-2-en-1-ol
13.91	138	83	0.25	$C_9H_{14}O$	Nopinone
14.16	170	57	1.24	$C_{12}H_{26}$	*n*-dodecane
14.50	184	57	0.15	$C_{13}H_{28}$	*n*-tridecane
14.73	144	73	0.10	$C_9H_{20}O$	2-methoxy-2,4,4-trimethyl pentane
17.00	198	57	0.15	$C_{14}H_30$	*n*-tetradecane
20.35	192	43	1.06	$C_{13}H_{20}O$	β-ionone
20.66	234	177	0.13	$C_{16}H_{26}O$	Propyl homologue of β-ionone
22.18	220	205	16.52	$C_{15}H_{24}O$	Butylated hydroxytoluene
23.38	236	41	0.18	$C_{16}H_{28}O$	Methyl bulnesol
24.33	220	73	0.20	$C_{15}H_{24}O$	Dehydrofarnesol
24.57	240	57	0.20	$C_{17}H_{36}$	3-Methylhexadecane
24.70	256	57	0.27	$C_{17}H_{36}O$	2,10,12-trimethyl-7-methoxy tridecane
25.08		73	0.23		Unknown
26.89	240	57	0.54	$C_{17}H_{36}$	*n*-heptadecane
27.65	202	41	0.13	$C_{14}H_{18}O$	Hydroxy-dehydrochamazulene
29.00	254	57	0.61	$C_{18}H_{38}$	*n*-octadecane
29.83	268	43	0.49	$C_{18}H_{36}O$	6,10,14-trimethyl-2-pentadecanone (Hexahydrofarnesyl acetone)
30.36	278	82	1.49	$C_{20}H_{38}$	Neophytadiene
31.01	268	57	0.29	$C_{19}H_{40}$	*n*-nonadecane
31.30	254	41	0.13	$C_{16}H_{30}O_2$	Cyclohexadecanolide
31.92	296	71	0.30	$C_{20}H_{40}O$	Isophytol
32.62	214	99	0.21	$C_{12}H_{22}O_3$	Hexanoic anhydride (Caproic anhydride)
32.95		43	0.54		Unknown
34.83		43	0.13		Unknown
35.06	242	99	0.35	$C_{14}H_{26}O_3$	Heptanoic anhydride
35.56	296	71	0.86	$C_{20}H_{40}O$	Phytol
36.10	266	55	0.19	$C_{18}H_{34}O$	13-Octadecenal
36.26	282	97	0.13	$C_{18}H_{34}O_2$	3-Hexadecenyl acetate
36.62	310	57	0.25	$C_{22}H_{46}$	*n*-docosane
38.08	308	41	0.11	$C_{20}H_{36}O_2$	Labd-13E-8,15-diol
38.86	436	97	0.11	$C_{26}H_{44}O_5$	Ethyl iso-allocholate
40.00	394	71	0.16	$C_{28}H_{58}$	2,2-dimethyl hexacosane
41.33	436	71	0.89	$C_{31}H_{64}$	2,2,4,4-tetramethyl heptacosane
41.57	306	41	0.15	$C_{20}H_{34}O_2$	Δ1,13-labddien-8,15-diol
42.84		96	0.49		Unknown
43.09	396	41	0.49	$C_{29}H_{48}$	10-demethyl squalene
44.30	422	57	21.45	$C_{30}H_{62}$	*n*-triacontane
45.59	428	70	0.23	$C_{30}H_{52}O$	Dehydrolupan-3-ol
46.18	410	69	0.31	$C_{30}H_{50}$	Squalene
47.31	424	207	8.47	$C_{30}H_{48}O$	Δ^{12}-lupan-3-ol
47.58	436	57	0.27	$C_{31}H_{64}$	15-methyl triacontane
47.91	380	133	0.23	$C_{28}H_{44}$	4-methyl-$\Delta^{1,3,5}$-cholesttriene
48.08	428	70	0.11	$C_{30}H_{52}O$	Dehydrolupan-3-ol isomer
48.41	426	207	0.28	$C_{30}H_{50}O$	3-lupanol
51.77	450	57	0.35	$C_{32}H_{66}$	2,2-dimethyl triacontane
53.97	370	124	0.13	$C_{27}H_{46}$	Δ^{25}-cholestene
54.53		87	0.42		Unknown

Rt: retention time, MWt: molecular weight, BP: base peak, MF: molecular formula.

Analysis of FAME (Table 2) resulted in the identification

Table 2

GC/MS analysis of the fatty acid methyl ester of *C. insignis* leaves.

Rt	MWt	BP	Area (%)	MF	Compound
6.50	220	205	19.87	$C_{14}H_{20}O_2$	Methyl-2-(3′,3′-dimethyl-1′-butyn-1′-yl)-1-cyclohexene carboxylate
8.15	220	41	0.16	$C_{14}H_{20}O_2$	Methyl-2-(4′-methylene-1′-buten-1′-yl)-1-cyclohexene carboxylate
11.24	242	74	0.67	$C_{15}H_{30}O_2$	Methyl tetradecanoate (Methyl myristate)
15.63	256	74	0.02	$C_{16}H_{32}O_2$	Methyl pentadecanoate
15.93	270	74	11.05	$C_{17}H_{34}O_2$	Methyl hexadecanoate (Methyl palmitate)
18.27	270	74	0.02	$C_{17}H_{34}O_2$	Methyl-14-methyl penadecanoate
18.49	284	74	0.42	$C_{18}H_{36}O_2$	Methyl-14-methyl hexadecanoate
19.77	294	67	2.14	$C_{19}H_{34}O_2$	Methyl-9,12-octadecadienoate
19.91	296	55	5.11	$C_{19}H_{36}O_2$	Methyl-9-octadecenoate (Methyl oleate)
20.77	298	74	7.53	$C_{19}H_{38}O_2$	Methyl octadecanoate (Methyl stearate)
21.19	312	55	0.02	$C_{19}H_{36}O_3$	Methyl-4-hydroxy-9-octadecenoate
21.41	324	55	0.49	$C_{21}H_{40}O_2$	Methyl-3-methyl-8-nonadecenoate
25.18	326	74	7.74	$C_{21}H_{42}O_2$	Methyl eicosanoate (Arachidic acid methyl ester)
26.91	338	43	0.39	$C_{22}H_{42}O_2$	Methyl-11-heneicosenoate
28.88	354	74	4.16	$C_{23}H_{46}O_2$	Methyl docosanoate (Methyl behenate)
29.72	354	71	0.06	$C_{23}H_{46}O_2$	Methyl-19,19-dimethyl eicosanoate
30.44	378	207	0.07	$C_{25}H_{46}O_2$	Methyl-19,21-dimethyl-15,19-docosadienoate
30.94	378	41	0.35	$C_{25}H_{46}O_2$	Methyl-17,23-tetracosadienoate
32.20	380	55	28.22	$C_{25}H_{48}O_2$	Methyl-22-tetracosenoate
33.68	406	43	2.32	$C_{27}H_{50}O_2$	Methyl-16,22-hexacosadienoate

Rt: retention time, MWt: molecular weight, BP: base peak, MF: molecular formula.

of 20 compounds constituting 90.81%. The unsaturated FA constitute 59.14% while the saturated constitute 31.67%. Methyl-22-tetracosenoate (28.22%) was the major monounsaturated FA, followed by a polyunsaturated compound, methyl-2-(3′,3′-dimethyl-1′-butyn-1′-yl)-1-cyclohexene carboxylate (19.87%), and a saturated compound, methyl palmitate (11.05%). It was the first time to study the lipoidal matter of *C. insignis* leaves.

3.2. Isolation and identification of the major constituents of E1

Upon chromatographing E1 on silica gel column, the following compounds were isolated and identified for the first time from the plant (Figure 1).

T1: white crystals (500 mg), appeared as one spot in TLC (R_f= 0.98 S1). Two peaks (P1 and P2) appeared in the mass spectrum. On running the mass of each peak, P1 and P2 showed a molecular ion peak (MIP) at m/z 436 [M^+, 8%] and m/z 464 [M^+, 5.8%] calculated for the molecular formula (MF) $C_{31}H_{64}$ and $C_{33}H_{68}$, respectively. Both compounds have mass fragmentation pattern of typical straight chain alkane with the base peak (BP) at m/z 57. Therefore, P1 and P2 were identified as *n*-hentriacontane and *n*-tritriacontane, respectively.

T2: steroid (2 mg) appeared as one spot in TLC (R_f=0.37 S1). Two peaks (P3 and P4) appeared in the mass spectrum. On running the mass of each peak, P3 showed MIP at m/z 416 [M^+, 30.5%], BP at m/z 415 [M^+-H, 100%] calculated for MF $C_{29}H_{52}O$, in addition to the following characteristic peaks at 401 [M^+-CH_3, 17%], 373 [M^+-43, 7.5%], 303 [M^+-113, 19%], 288 [M^+-113-CH_3,12%], 279 [M^+-H-CH_3-85-CH_3-H_2O-3H, 30%] and 212 [34.5%]. Therefore, P3 was identified as 5,6-dihydrositosterol (stigmastanol).

P4 showed MIP at m/z 428 [M^+, 4%], BP at m/z 59 ($^+CH_2CH_2OCH_3$) calculated for MF $C_{30}H_{52}O$, in addition to the following characteristic peaks at 413 [M^+-CH_3, 9%], 398 [M^+-2CH_3, 7.5%], 239 [M^+-side chain-CH_3OH-CH_3-3H, 55%], 111 [31.5%], 83 [111-2CH_2, 35.5%] and 57 [58%]. Therefore, P4 was identified as 3-methoxy-5, 6-dihydrostigmasterol.

Compound T3: (1 mg) showed MIP at m/z 398 [M^+, 6%], BP at m/z 69 (side chain-43-CH) calculated for MF $C_{28}H_{46}O$ and the following characteristic peaks at 381 [M^+-OH, 7%], 256 [M^+-side chain-OH, 21%], 225 [M^+-side chain-2CH_3-H_2O, 14%] and 95 [side chain-2CH_3, 24%]. Therefore, T3 was identified as 7,8-dihydroergosterol.

Compound T4: (4 mg) showed MIP at m/z 400 [M^+, indistinct], BP at m/z 83 calculated for MF $C_{28}H_{48}O$. In addition, the following characteristic peaks at 385 [M^+-CH_3, 20.5%], 382 [M^+-H_2O, 16.5%], 323 [M^+-OH-4CH_3, 19%] and 266 [M^+-H_2O-3CH_3-43-2CH_2, 41%] were observed. Therefore, T4 was identified as 4-methylcholesterol.

Compound T5: (2.4 mg) showed MIP at m/z 388 [M^+, 17.54%], BP at m/z 293 [M^+-H_2O-CH_3-2H-CH_3(CH)CH_3CH_2-3H, 100%] calculated for MF $C_{27}H_{48}O$, in addition to the characteristic peaks at 373 [M^+-CH_3, 17.64%], 355 [M^+-H_2O-CH_3, 14.25%], 352 [M^+-H_2O-CH_3-3H, 43.88%], 303 [M^+-85, 19.39%], 275 [M^+-side chain, 31.31%], 245 [M^+-side chain-2CH_3, 46.37%] and 227 [M^+-side chain-2CH_3-H_2O, 32.59%]. From the above data, T5 was identified as cholestanol.

Compound T6: (3.1 mg) showed MIP at m/z 426 [M^+, 33.19%], BP at m/z 257 calculated for MF $C_{30}H_{50}O$ and the characteristic peaks at 411 [M^+-CH_3, 19.56%], 396 [M^+-2CH_3, 21.47%] and 288 [M^+-H_2O-8CH_3, 13.80%]. From the above data and by comparison with published data[16], T6 was identified as multiflorenol.

Compound T7: (1.5 mg) showed MIP at m/z 384 [M^+, indistinct], BP at m/z 285 [M^+-(CH_2)3CH(CH_3)2-CH_2, 100%] calculated for MF $C_{27}H_{44}O$ and the peaks at 342 [M^+-CH_2=C=O, 47.46%], 341 [M^+-43, 47.46%] and 284 [M^+-43-3CH_2-CH_3, 59.5%]. From the above data, T7 was identified as cholest-5-en-3-one.

Compound T8: (5 mg) showed MIP at m/z 386 [M^+, indistinct], BP at m/z 279 [M^+-4CH_2-2CH_3-18-3H, 100%] calculated for MF $C_{27}H_{46}O$, in addition, the following characteristic peaks at 341 [M^+-3CH_3, 11%], 329 [M^+-(CH_3)2CH-CH_2, 9%] and 273 [M^+-side

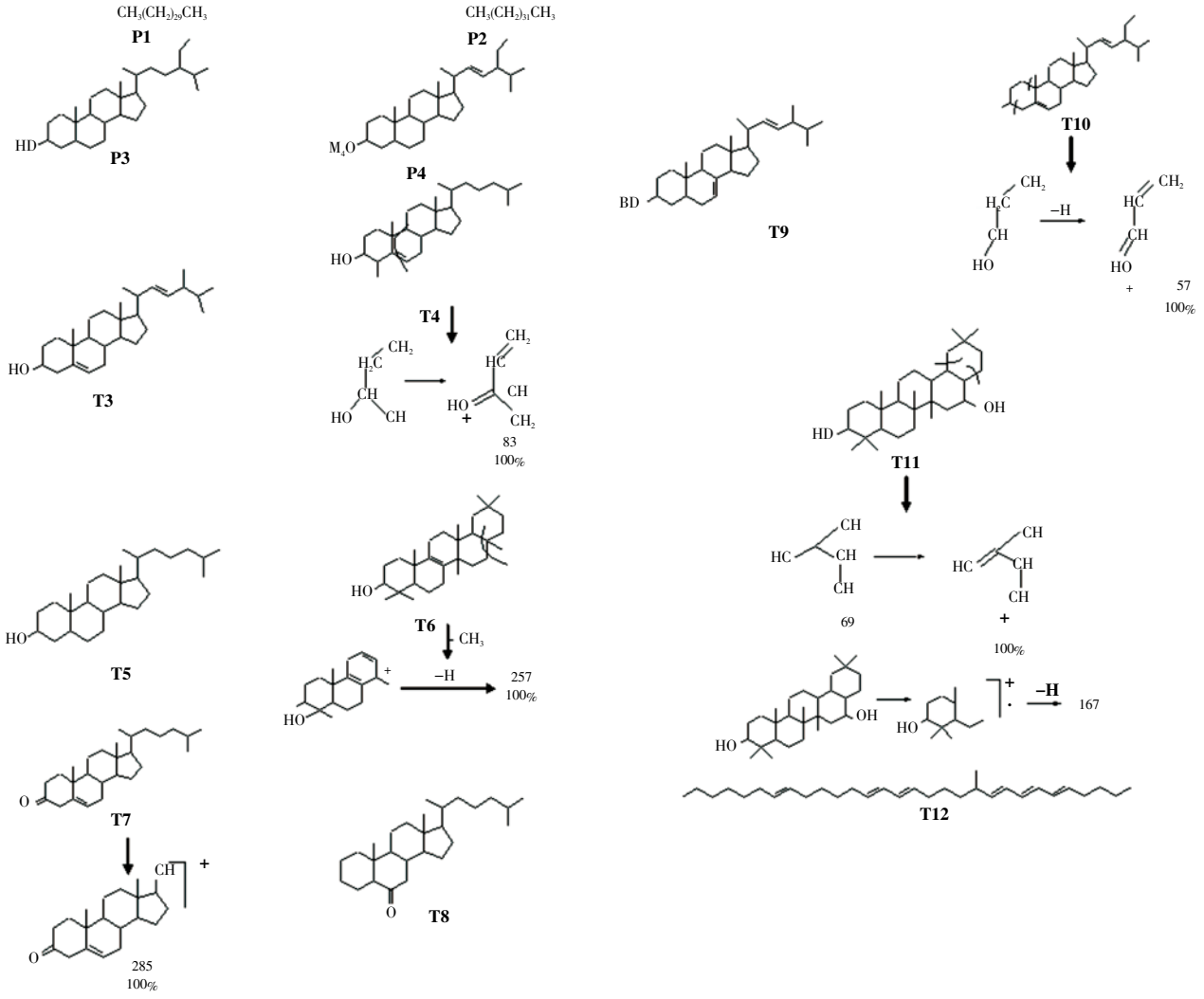

Figure 1. Structure of the compounds isolated from the petroleum ether extract of *C. insignis* leaves.

chain, 21%] were observed. Therefore, T8 was identified as cholest-6-one.

Compound T9: (1.3 mg) showed MIP at m/z 398 [M^+, indistinct], BP at m/z 239 [M^+-side chain-H_2O-CH_3-H, 100%] calculated for MF $C_{28}H_{46}O$, and the following characteristic peaks 273 [M^+-side chain, 16%], 243 [M^+-side chain-$2CH_3$, 54.5%] and 225 [M^+-side chain-$2CH_3$-H_2O, 47%] were observed. From the above, T9 was identified as 5,6-dihydroergosterol.

Compound T10: (2 mg) showed MIP at m/z 412 [M^+, 21.5%], BP at m/z 57 calculated for MF $C_{29}H_{48}O$, and the following characteristic peaks 397 [M^+-CH_3, 7.5%], 369 [M^+-43, 16%], 340 [M^+-43-CH_2CH_3, 6%], 327 [M^+-85, 4.5%], 279 [M^+-H_2O-$2CH_3$-85, 12.5%], 258 [M^+-side chain-CH_3, 14.5%] and 111 [49%] were observed. From the above data, T10 was identified as stigmasterol.

Compound T11: (1 mg) showed MIP at m/z 430 [M^+, 25%], BP at m/z 69 calculated for MF $C_{29}H_{50}O_2$ and the characteristic peaks at 322 [M^+-$6CH_3$-H_2O, 43.52%], 280 [M^+-$7CH_3$-OH-$2CH_2$, 64.45%] and 167 [98.64%]. From the above data and by comparison with published data[17], T11 was identified as dihydroalbigenin.

Compound T12: (1.7 mg) showed MIP at m/z 424 [M^+, 18%], BP at m/z 57 calculated for MF $C_{31}H_{52}$ and the characteristic peaks at 341 [M^+-83, 15%], 111 [M^+-83-122-108, 18%] and 83 [M^+-341, 73%]. Therefore, T12 was expected to be 11-methyl-$\Delta^{5,7,9,15,17,23}$-triacont-hex-ene.

3.3. Investigation of fraction E2

Fraction E2 was subjected to PC investigation and two compounds were isolated and identified for the first time from the plant (Figure 2).

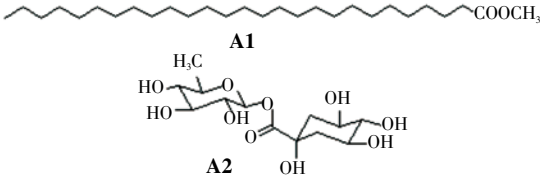

Figure 2. Structure of the compounds isolated from the ether fraction of the 70% ethanol extract of *C. insignis* leaves.
A1: methyl heptacosanoate, A2: quinic acid ester of rhamnose.

Compound A1 isolated as yellow amorphous powder (45 mg), R_f= 0.82 and 0 in S2 and S3, respectively. It appeared as a yellow spot (2 mg) under UV light unchanged on exposure to ammonia vapour or spraying with $AlCl_3$. The UV spectral data in MeOH showed one main band at 279 which did not give shift by addition of NaOMe. The EI-MS spectrum showed MIP at m/z 424 [M^+, 86.20%], BP at m/z 74 calculated for MF $C_{28}H_{56}O_2$. The mass fragmentation pattern was typical FAME with the characteristic peaks at 143 [M^+-CH_3-$19CH_2$, 18.68%], 87 [M^+-CH_3-$23CH_2$, 70.90%], and 71 [M^+-$COOCH_3$-$21CH_2$, 28.32%]. The structure was confirmed by ^{1}H-NMR spectrum which exhibited a singlet at δ 3.12 assigned to the acetoxy group,

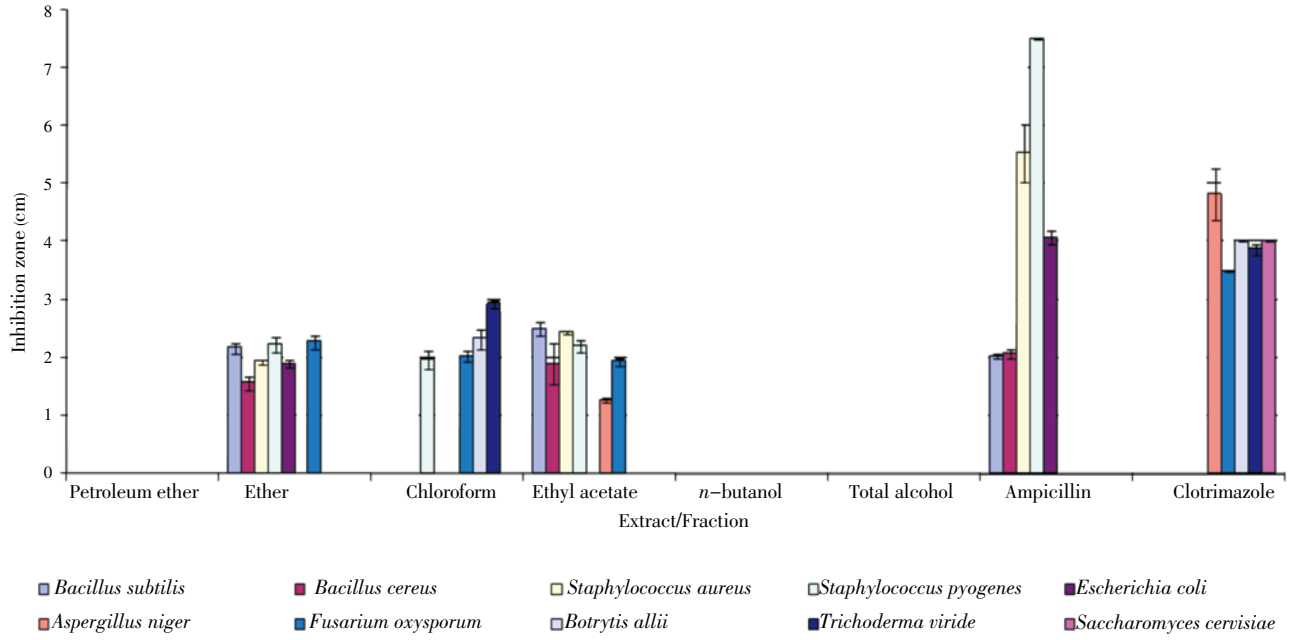

Figure 3. Antimicrobial activity of the total alcohol and petroleum ether extracts and fractions of the 70% ethanol extract of *C. insignis* leaves. Data are expressed as mean±SE.

a singlet at δ 2.47 assigned to the CH_2 group α to $COOCH_3$, a singlet at δ 2.11 assigned to the CH_2 group β to $COOCH_3$, a multiplet at δ 1.18 assigned to the methylene groups of C4-C26 and a multiplet at δ 0.80 assigned to the terminal methyl group. Based on the previous discussion, A1 was identified as methyl heptacosanoate.

Compound A2 isolated as white amorphous powder (10 mg), R_f= 0.78 and 0.52 in S2 and S3, respectively. It appeared as a blue fluorescent spot under UV light unchanged on exposure to ammonia vapour or spraying with $AlCl_3$. UV spectral data in MeOH showed one main band at 271. The ^{1}H-NMR spectrum exhibited signals for three oxymethine protons at δ 4.10 (m, 2H) and 3.64 (m, 1H) and two sets of methylene protons at δ 1.16 (d, *J*=2.5 Hz, 2H) and 1.24 (d, *J*=2.5 Hz, 2H) assigned to H-2 and H-6, suggesting a quinic acid moiety in the molecule. An anomeric proton signal at δ 5.40 (d, *J*=2.5 Hz, 1H) together with a broad singlet of three protons at δ 0.85 for Me-6′ indicating the presence of rhamnose. The structure was confirmed by determination of positive electrospray ionization mass spectrometry (ESI-MS): m/z 339 [M^++H] (which is the molecular weight of quinic acid ester of rhamnose+H). Therefore, A2 was identified as quinic acid ester of rhamnose.

3.4. Antimicrobial screening

Reviewing current literature, nothing was reported concerning the antimicrobial activity of *C. insignis*. E6 and the successive fractions were tested against representative MO; results are shown in Figure 3.

There was no significant difference between the effect of E2 and the standard antibiotic, ampicillin, on M1 and M2. It showed high activity against both bacteria. It had moderate activities on M3, M4, M5 and M7. E3 showed moderate activities against M4, M7, M8 and M9. The E4 showed high activity on M1 and M2. It had moderate activities on M3, M4, M6 and M7. All the extracts were inactive against M10. E1, E5 and E6 were inactive against all MO.

4. Discussion

It was reported that the ether and ethyl acetate extracts of several plants possess antimicrobial activities against different MO, for example, the ether extract of *Artemisia nilagirica* leaf showed antibacterial activity against one or more of 12 tested bacterial strains[18]. Extracts of *Ficus racemosa* Linn. leaves produced significant antibacterial potential against M1, M3, M5, *Bacillus pumilis* and *Pseudomonas aeruginosa*[19]. The ether extract of the lichen *Cladonia foliacea* showed antimicrobial activities against nine bacteria and fungi[20]. The diethyl ether extract of *Plantago major* L. displayed activity on *Escherichia coli*[21]. Quinic acid derivatives isolated from *Ageratina adenophora* Spreng showed antibacterial activity toward M3, *Bacillus thuringiensis*, M5, *Salmonella enterica* and *Shigella dysenteria*[22].

In addition, several flavonoids were reported to possess antimicrobial activity[23]. For instance, the ethyl acetate extract of *Bryophyllum pinnatum* Lank. and its isolated kampferol derivatives have interesting antimicrobial properties[24]. The ethyl acetate extract of *Marsilea quadrifolia* showed excellent antibacterial activities against 5 Gram-positive and 11 Gram-negative human pathogenic bacteria[25]. Previously, El Sawi *et al.* found that the ethyl acetate extract of *C. insignis* contains flavonoids and specially kampferol derivatives which may be responsible for the antimicrobial activity[5].

From this study it can be concluded that the ether and the ethyl acetate fractions have potent antibacterial activity against *Bacillus subtilis* and *Bacillus cereus*, so they could be used in pharmaceutical formulations. Further clinical trials should be performed in order to support the above investigations and to facilitate their pharmaceutical formulations.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The literature reported the importance of *C. insignis* plant which were found to exhibit variable biological activities and contain different constituents as reported previously by the author, so it is very important to test for its antimicrobial activity.

Research frontiers

This study was carried out to complete the chemical and biological investigation of *C. insignis* and to add another importance of the plant as antimicrobial. The chemical constituents of the petroleum ether extract and the ether fraction of the 70% ethanol extract were studied and their activities as antimicrobial were screened.

Related reports

The plant was reported to possess different biological activities as published before by the authors. The study completes the research on the plant as antimicrobial which is not reported before.

Innovations & breakthroughs

The authors studied in this work the antimicrobial activity of *C. insignis* which was not studied before and it appeared that two fractions of the plant possess significant activity against two microorganisms. Also, this is the first report for the isolation and identification of the chemical constituents of the non-polar fractions of the plant.

Applications

It is interesting to know that fractionation of inactive antimicrobial extract lead to different fractions which possess variable antimicrobial activities, the synergism and the antagonism properties appear in this study in the total ethanol extract which is inactive against all MO while the ether and the ethyl acetate fractions have potent antibacterial activity specially against *Bacillus subtilis* and *Bacillus cereus*.

Peer review

This is a valuable study in which the authors isolated and identified the constituents of the non polar fraction and the article contains the spectroscopic data which lead to identification of these compounds.

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