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Isolation of Two Bioactive Compounds from the Stem Bark of *Lycium inerme* and their Biological Activities against *H. influenzae* and *S. pneumoniae* in Mthatha, Eastern Cape, South Africa

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Abstract

Lycium inerme is a plant used by traditional healers in the Eastern Cape Province, South African against respiratory illness. Different solvent extracts of *Lycium inerme* was screened against clinical isolates of *Haemophilus influenzae* and *S. pneumoniae* pathogen. The dichloromethane extract was the most active against these two pathogens. Bioassay-guided column chromatography of dichloromethane extract led to the isolation of three compounds which were very active against *H. influenzae* and *S. pneumoniae*. Three active compounds:a coumarin (7-hydroxy-6-methoxy-coumarin) and two triterpenoids, Ursolic acid (3-oxo-19-hydroxylpomolic acid) and Moronic acid (3-oxoolean-12-en-28-oic acid) were isolated from the dichloromethane fraction. The study highlights the characterization, structural elucidation and biological activities of the two compounds against *H. influenzae* and *S. pneumoniae*.

Keywords: Medicinal plants; Antimicrobial activity; Dichloromethane; Coumarin; Triterpenoids

Introduction

Medicinal plants are useful sources of drugs and the majority of individuals in developing countries rely on their uses [1]. It has been reported [2] that crude extracts of medicinal plants justified their use in folklore medicine in the treatment of wounds and ulcers. In view of this, the World Health Organization is encouraging all countries to preserve and use medicinal plants in their national health systems [3].

Lycium inerme, belongs to the family Solanaceae. It is a spiny or spicky tree or shrub that is also often known as the Matrimony vine [4]. Leafy spine-like branches are borne in opposite pairs, the leaves are light green, glossy and hairless with a soft, leathery texture and cream coloured to green yellow flowers are produced from august to February [5]. The antioxidant radical (Fructuslycii) from *Lycium* family has been used as medicine in many countries, particularly in China where the herb is been used as emenagogue, diuretic, antipyretic, tonic, aphrodisiac, hypnotic and hepatoprotective agent [4].

The plant has been extensively studied in China, India and South Korea. *L. inerme* is a relatively common species with a wide distribution range in South Africa, extending from the Cape Peninsula in the Western Cape, into Eastern Cape, through Kwazulu-Natal to Swaziland, Mpumalanga and Limpopo. *Lycium* species is commonly used for decoration of gardens, about 50 species is grows in Africa, of which around 15 species are found in Southern Africa [6]. Several species of this genus have been used in folk medicine as sedatives and for the treatment of digestive disorders, rheumatism, headache, arthritis, diabetes, toothache, coughs and chest pains [7]. *Lycium* roots or extracts are used to bring down or sooth the blood temperature and thus help in lessening fever, irritability, sweating, and thirst. The cooling properties of Lycium are also effectual in impeding nosebleeds; diminish vomiting of blood and also relief coughs and wheezing caused by excessive heat prototypes [8].

In the literature, there are many studies about antibacterial, antifungal, antiulcer, anticancer, antiviral, antifertility and antioxidants activity and the chemical composition of *Lycium* species [8,9]. *Lycium* fruits and berries are highly useful for safeguarding the liver from injuries caused by exposure to toxins. Roots are useful for safeguarding against parasympathetic nervous system. They also, lower blood pressure, loosen the muscles, lower fever and reduce excess body temperature in malaria patients [8]. *Lycium* stem bark contains betaine, beta-sitosterol, the berries contain physalien, carotene, vitamin B1, B2 and C. The root contains enclosed cennamic acid and psyllic acid. Some of the commonly found compounds of *L. inerme* include, Coumarin (Scopoletin). In South Africa, there are a paucity of information concerning the chemical and the biological studies of *Lycium inerme*.

The aim of the current study was to determine the active compounds in different extracts of *Lycium inerme*, based on their antimicrobial activities against isolates of *Haemophilus influenzae* and *S. pneumoniae* in South Africa.

Materials and Methods

Collection of plant materials

The stem bark of *Lycium inerme* was collected from Lusikisiki in the Eastern Cape Province, South Africa, from June 2010 to December 2011, based on its ethnomedical application in the treatment of respiratory tract infections. The plant was identified and authenticated by Taxonomist (Immelman K.L.) in the Department of Botany, Walter Sisulu University, South Africa. Voucher specimen MI 008 was deposited at the University herbarium.

Preparation of plant crude extracts

Plant materials were washed with sterile distilled water, air-dried at room temperature. The dried material (179.4 g) was ground into a coarse powder using Macsalab mill (Model 200 LAB), Eriez, Bramley [3].

Solvent extractions

The ground plant material was soaked in different solvent systems (n-Hexane, Dichloromthane, Ethylacetate, Methanol and Water) for 72 h each with frequent shakings, as previously described [2,3]. The samples were suction filtered through Whatman No 1 filter paper. The filtrate was evaporated to dryness under reduced pressure using a rotary evaporator. The crude extracts were left to air-dry at room temperature until it was completely dry and then stored in the dark for subsequent steps. After plant extracts had dried, the percentage yield of each extract Hexane (65 g), Dichloromethan (56 g), Ethylacetate (45 g), and Methanol (12.8 g) [2,3].

Bioassay

Antimicrobial assay of plant extracts Test organisms: The following organisms, *S. pneumoniae* and *H. influenza* were clinical isolates from patients from selected hospitals in the geographical areas of O.R Tambo Municipality, Eastern Cape Province, South Africa.

Detection of zone of inhibition against microbial agents on TLC bioautography: The agar overlay or immersion bioautography was used as previously described [9,10]. This is where chromatograph plates (TLC plates of 0.25 mm thickness) were placed into a beaker containing a known solvent system and the inoculum (agar seeded with pathogens, for example, *H. influenzae*, *S. pneumoniae*, and *C. albicans* were rapidly distributed over the TLC plate with a sterile Pasteur pipette. After solidification of the medium, TLC plates were incubated overnight at 37°C in a plastic-bowels lined with moist cotton wool. The bioautograms were sprayed with an aqueous solution (2.5 mg/ml) of tetrazolium salt (3-4, 5-Dimethylthiazole-2-yl-2,5-diphenyl-tetrazolium bromide (Aldrich, Gillingham, England) and incubated at 37°C for 2 h. Active compounds appear as clear spots against a purple coloured background due to formation of formazen dye [11].

Culture media

Microorganisms are media sensitive thus the bacterial strains were maintained on tryptic soya agar slants. All the cultures were introduced aseptically (using a heat-sterilized wire loop) into 250 ml Elernmeyer flasks containing tryptic soya broth. To achieve a homogenous distribution of microorganism in the nutrients, the cultures were shaken during incubation at 37°C for 24 h using a LAB-LINE ORBIT shaker. All media were autoclaved at 121°C for 15 minutes prior to inoculation [12].

Inoculums for the assay

Tryptic soya agar (TSA) was used as a solid media for the bioautographic overlays. The molten media were maintained in water bath at approximately 42°C and seeded with microorganisms from the tryptic soya broth; 10 ml of the broth culture was introduced into 100 ml of tryptic soya agar. The optical density at 540 nm (OD_{540}) of the agar was measured with spectronic 21D (Milton Roy) UV/VIS spectrophotometer and OD_{540} equal to 1 corresponds to approximately 10^7 cells/mL [13].

Isolation of bioactive compounds using column chromatography

After they were weighed, mixed with equal amounts of silica gel and loaded into a column packed with silica gel using n-Hexane. The column was eluted using a gradient solvent system staring with a mixture of % n-hexane, n-hexane/chloroform, % chloroform, chloroform/methanol and % methanol to give 126 fractions of 40 mL each. With the guide of TLC observations, the concentrated fractions with similar TLC profiles were combined and labeled as A (% $CHCl_3$) [fraction 2-3], B ($CHCl_3$/MeoH; 9.5:0.5) [fr.4-9], C ($CHCl_3$ /MeoH; 9:1) [fr.10-14], D ($CHCl_3$:MeoH; 9:1) [fr.15-33], E ($CHCl_3$/MeoH; 8:2) [fr.34-36], F ($CHCl_3$/MeoH; 7:3) [fr.37-47], G ($CHCl_3$/MeoH; 6:4) [fr. 48-52], H ($CHCl_3$/MeoH;1:1) [fr.53-64] and I (% MeoH; 65-126) [10,11-16].

TLC autographic assay of crude extracts and compounds

Crude extracts and compounds were developed in duplicate on separate TLC plate using an appropriate solvent system. One plate was sprayed with Vanillin-Sulphuric acid spray reagent and the other with the DPPH spray reagent. The plates were placed side by side and components showing antioxidants activity were identified, examined and recorded after 30 minutes (Figure 1). Yellow spots against a purple background when sprayed with DPPH showed a positive activity [16].

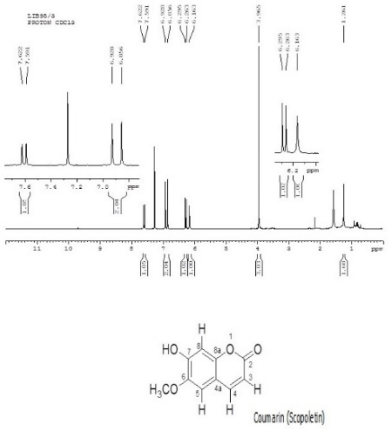

Figure 1: Structure of coumarin (SCOPOLETIN=7-hydroxy-6-methoxychromen-2-one): Compound 1.

Results

Percentage yields of crude extracts

Ethylacetate crude extract produced the highest percentage yield (45.0%), followed by methanol (12.8%), water (11.0%), hexane (6.5%) and while dichloromethane was the least with (5.6%).

Compounds isolated from *Lysium inerme* stem bark

Coumarin (Scopoletin), is a glycoside (Scopolin) formed by the action of the enzyme Scopoletin glucosyltransferase and commonly found in the roots of plants in the genus *Scopolia* like *Scopolia carniolica* or *Scopalia japonica* in chicory, in *Artemisia scoparia*, in the passion flower, in *Brunfelsia*, in *Viburnum prunifolium*. It can also be found in vinegar, some whiskies or in dandelion coffee (Table 1).

The chemical shifts in the ^{13}CNMR spectrum were typical for pentacylic triterpenes. The presence of a set of vinylic carbons occurring in the ^{13}CNMR at σ 143.1 and 129.1 was characteristic of a 3-oxo-19-hydroxylpomolic acid. Compound 2 was thus characterized as a triterpene and was identified as ursolic acid by the close match of the ^{13}CNMR data (Table 2) with that compiled [17] (Figure 2).

Position	δC	Δh
1	-	
2	161.4	-
3	113.5	6.28, d, J=9.6 Hz, IH
4	143.3	7.61, d, J=9.3 Hz, IH
4a	111.5	-
5	107.5	6.93, s,^{1}H
6	150.3	-
7	149.7	-
8	103.2	6.86, s,^{1}H
8a	144.0	-
-OCH_3	56.5	3.96, s,^{3}H

Table 1: NMR Spectroscopic Data (300 MHZ) for compound 1 and 'H positions.

Position	δc	δ^{1}H
1	36.1(t)	1.50 m,1.50 m
2	30.1(t)	127 m
3	76.5(s)	3.35 brs
4	37.6(s)	
5	49.2(d)	1.25 m
6	18.6(t)	0.99 m, 1.46 m
7	32.7(t)	1.55 m
8	40.3(s)	
9	47.6(d)	1.70 m
10	37.3(s)	
11	23.7(t)	1.9 m
12	129.1(d)	5.16, d(3.3)
13	143.1(s)	
14	42.0(s)	
15	27.2(t)	1.05 m, 2.02 m
16	25.5(t)	0.99 m, 1.82m
17	42.8(s)	
18	46.2(d)	2.00 m
19	40.6(t)	1.38 m, 2.18 m
20	32.8(s)	
21	33.3(t)	1.21 m, 1.35 m
22	29.2(t)	1.92 m, 1.48 m
23	28.4(t)	0.88 s
24	22.6(q)	0.86 s
25	15.6(q)	0.98 s
26	17.1(q)	0.99 s
27	26.4(q)	1.18 s
28	184.5(s)	
29	28.5(q)	0.99 s
30	19.4(q)	1.25 s

Table 2: NMR Spectroscopic data (300 MHz) for compound 2 and 'H positions.

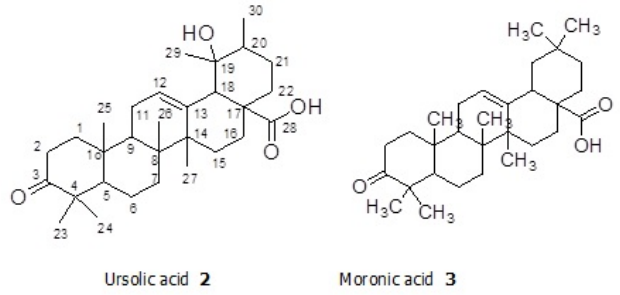

Figure 2: Structures of ursolic acid and moronic acid.

Bioassay

Antimicrobial assay of extracts:

Detection of zone of inhibition against microbial agents on TLC bioautography: The minimum inhibition quantinty (MIQ) from the

semi quantitative analysis showed antimicrobial activity in both organisms (Table 3). However, the activity between the two organisms was different for extracts and isolated compounds, for instance, Table 4. Showed the highest activity for *H. influenzae* than *S. pneumoniae*, whereas in Table 4. *S. pneumoniae* showed the highest activity than *H. influenzae*. The antioxidant activity bioautograms showed the highest activity in methanolic extracts and compounds followed by Hexane and Dichloromethane. Ethylacetate showed the least activity after 24 h. This was reversal of the activity trend observed in the antimicrobial activity tests.

Microorganism and MIQ of the extract (µg)[A]		
Extract code	***H. influenzae***	***S. pneumoniae***
Hex	9	9
DCM	10	6
EtoAc	3	4
MeoH	11	12
Ampicillin	0.01	0.01
Chloramphenicol	0.001	0.01
Hex, DCM, EtoAc, MeoH:Hexane, Dichloromethane, Ethylacetate and Methanol respectively. A=Zone of inhibition (mm).		

Table 3: Minimum Inhibiting quantities (MIQ) of the *L. inerme* crude extracts.

Microorganism and MIQ of the extract (µg)[A]		
Compound code	***H. influenzae***	***S. pneumoniae***
C1	5	11
C2	5	5
C3	3	3
C4	11	11
Ampicillin	0.001	0.001
Chloramphenicol	0.001	0.001
C1, C2, C3, C4: Compound 1, 2, 3 and 4, A=Zone of inhibition (mm).		

Table 4: Minimum inhibiting quantities (MIQ) of isolated compounds.

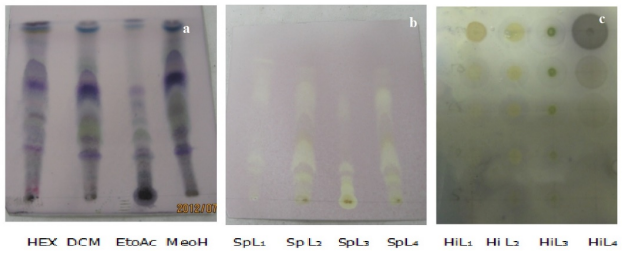

Figure 3: Vanillin sulphuric acid TLC bioautogram of *L. inerme* extracts and combined fractions from the column before final isolation.

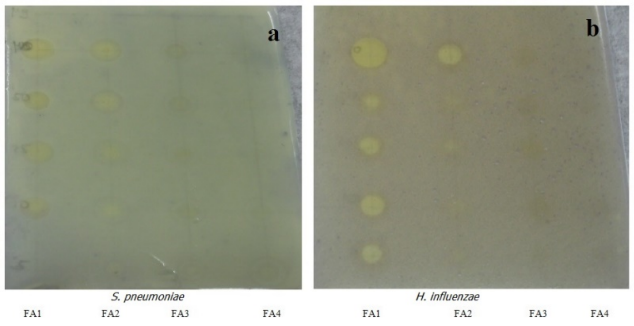

Figure 4: 3-(4,5 dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) TLC autobiograms showing antimicrobial activity of compounds against *S. pneumoniae* and *H. influenzae*.

Discussion

In this study two compounds were isolated, namely; Coumarine-scopoletin (1) and a pentacylic triterpene (2). Compound 1 has a formula $C_{10}H_8O_4$, molecular weight of 192.17, yellow to beige crystalline powder, a melting point of 200-207°C. Its synonyms are 7-hydroxy-6-methxycoumarin. Coumarin derivatives are used widely as anticoagulants (such as warfarin -OH group is attached at position 4) for the treatment of disorders in which there is excessive or undesirable clotting, such as thrombophlebitis, pulmonary embolism and certain cardiac conditions. It was reported [18-20] that the pharmacological properties of scopoletin (SCT) molecules might be useful in the treatment of bain disturbances associated with dementia. Other beneficial properties of SCT are its antioxidant radical scavenger and anti-inflammatory activities. [18] Several studies suggest that appropriate antioxidants can prevent neurode-generation as reviewed [18].

The phytochemical investigations of the dichloromethane extracts of *L. inerme* stem bark yielded compound 2, which was identified as 3-oxo-19-hydroxylpomolic acid. 1-hy:Compound 2. The ^{1}HNMR spectrum showed signals mainly located at the high field region between δH 0.6-2.1. There are signals for two tertiary methyl protons at δH 0.72 (3H, s) and 1.03 (3H, s) two doublets at δH 0.79 (3H, J=6.9Hz) and δH 0.82 (3H, J=6.3 Hz) were attributed to an isopropyl group methyl and the presence of another secondary methyl protons at δH 1.04 (3H, d, J=6.6 Hz). The signal at δH 0.82 (3H, s) was attributed to a primary methyl group [21].

The ^{1}HNMR spectrum exhibited signals for three protons, the carbonilic proton resonated at δH 3.54 (^{1}H, m). The ^{13}CNMR showed signals for 29 carbons and the signals proton resonated at Δh 3.54. The 13CNMR showed signals for 29 carbons and the signals were assigned based on DEPT, COSY, HMQC and HMBC spectral data analysis. Based on the data above and literature values [17] Compound 2 was identified as carboxylic acid and has been reported to have antirheumatic properties [19]. The ^{1}H and ^{13}CNMR assignments are given in Table 2.

Of the independent sample extracts, dichloromethane ethyl acetate and methanolic extracts showed good antibacterial activity against both bacterial strains (Tables 3 and 4). On the other hand good antibacterial activity was recorded in *L. inerme* against *S. pneumonia* than *H. influenzae*. Studies have shown that DCM/EtoAc extracts of *L. inerme* are rich in antimicrobial agents that could be targeted and isolated in a bioassay-guided fraction [16]. The TLC agar overlay method was observed to be a highly effective method for providing a quick visual analysis of the location and level of antimicrobial agents in the plant extracts. *L. inerme* extracts in this study leads to the conclusion that active constituents in these crude extracts are among the lipophilic (non-polar) group of compounds. Although hexane extracts are highly non-polar compounds than DCM, the two solvents generally extract non-polar classes of compounds compared to methanolic extracts, which are mostly polar compounds (Figures 3 and 4).

Conclusion

L. inerme stem bark screened in this study may have potential medicinal and antimicrobial activity against *H. influenzae* and *S. pneumoniae*. Bioassay showed that compounds with R_f-values of 0.67 to 0.80 were very active against *H. influenzae* and *S. pneumoniae*. Two active compounds:a coumarin (7-hydroxy-6-methoxy-coumarin) and two triterpenoids:ursolic acid (3-oxo-19-hydroxylpomolic acid) and moronic acid (3-oxoolean-12-en-28-oic acid) were isolated from the dichloromethane fraction. These compounds showed some antimicrobial activity, therefore *L. inerme* stem bark can be used as a source of alternative medicine, new pharmaceutical and health care product that can be used as a therapeutic agent, a starting material for synthesis of drugs or as a model for pharmacologically active compounds.

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