ISOLATION AND IDENTIFICATION OF THREE COMPOUNDS FROM *HOSLUNDIA OPPOSITA* VAHL

Abstract

Hoslundia opposita is an aromatic herb that occur all over in Mozambique and is well known for its medicinal properties. In the initial screening of plants used in Mozambique for antimycobacterial activity, *Hoslundia opposita* demonstrated good antitubercular activity (Chapters 2). It was therefore selected to identify its bioactive constituents. A Phytochemical investigation of *H. opposita* led to the isolation of three known compounds, 5,7-dimethoxy-6-methylflavone (1), hoslunddiol (2) and euscaphic acid (3). This is the first report of the isolation of "5, 7- dimethoxy-6-methylflavone" from *Hoslundia opposita*.

5.1 Introduction

5.1.1 *Hoslundia opposita*: biological activity and chemical constituents

Hoslundia opposita Vahl (Figure 5.1) is an herbaceous perennial shrub (1-2m tall) belonging to the Lamiaceae.

It is widely distributed in tropical and subtropical open lands of Africa (Morton, 1981). Various parts of *Hoslundia opposita* are popular remedies in Africa to treat gonorrhea, cystitis, cough, wounds, sores, snake bites, conjunctivitis, epilepsy, chest

Figure 5.1 *Hoslundia opposita* (Plantzafrica.com)

pain, stomach trouble, and mental disorders (Ayensu & De Filipps, 1978, Watt and Breyer-Brandwijk, 1962). Infusions of its leaves are widely used in traditional medicine as a purgative, diuretic, febrifuge, antibiotic, and antiseptic (Onayade *et al*.1989).

The crude extracts of the entire plant have been found to exhibit strong antibacterial activity (Khan *et al*., 1993) and volatile constituents have been identified (Onayade *et al*.1989). A recent study had reported that leaves of this plant could be potentially used in treatment of epilepsy and convulsions (Risa *et al*., 2004). There have been no reports on the antitubercular or antiviral biological activity. In the initial screening of plants used in Mozambique *Hoslundia opposita* demonstrated good antitubercular activity (Chapter 2). It was therefore selected to identify its bioactive constituents.

5.2 Materials and methods

5.2.1 Plant material

Leaves of *Hoslundia opposita* were collected at Matola- Gare, Mozambique in June 2004.

The voucher specimens have been deposited at H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

5.2.2 Extraction and isolation

Leaves of *H. opposite* (130 g) were extracted with 1.5 L of ethanol for two days then filtered, the process was repeated two times. The extracts were combined and evaporated under reduced pressure to afford 21 g of crude ethanol extract. as described above. The total extracts (21 g) were subjected to a silica gel column (30 x 5 cm). Solvent system ethyl acetate: hexane with increasing polarity (EtOAc %, volume; 0 %, 1L; 10%, 2 L; 30%, 2 L; 50%, 2 L; 70%, 2 L; 100%, 1 L) followed by 10% of methanol in ethyl acetate (2L) was used as an eluent. Ten fractions based on their TLC profile were combined and concentrated to dryness under reduced pressure. Fraction IX (3.7 g) was chromatographed on silica gel which was followed by Sephadex LH-20 columns to yield 5,7-dimethoxy-6-methylflavone (1, 216 mg) and hoslunddiol (2, 36.4 mg). Fraction V (786 mg) was chromatographed over a silica gel column using $CHCl_3$–MeOH (98:2) to yield euscaphic acid (3, 80 mg).

5.2.3 Identification of isolated compounds

UV spectra were recorded using a Pharmacia LKB-ultraspec 111 UV spectrophotometer. NMR spectra were recorded using a Bruker ARX 300 or a Bruker Avance DRX 500 MHz. Mass spectra were obtained with a JEOL JMS-AX505 W mass spectrometer. The recorded spectral data of the isolated compounds were compared with those published in literature.

5.3 Results and discussion

5.3.1 Compound 1: 5, 7- dimethoxy-6-methylflavone

The Compound 5,7- dimethoxy-6-methylflavone, (Figure 5.1), showed in ^{1}H-NMR two singlets δ_H 6.67 and 6.57 typical to H-3, H-8 of flavone , two multiplet signals integrated to two and three protons respectively at 7.90 and 7.51 of unsubstituted B ring in addition to two singlets, three protons. Signals at δ_H 3.89, 3.85 of two methoxy groups and an aromatic methyl group signal at 2.35.The previous data indicated the presence of the known compound 5,7- dimethox-6 methylflavone which is reported here for the first time from *Hoslundia opposite* (Häberlein and Tschiersch, 1994).

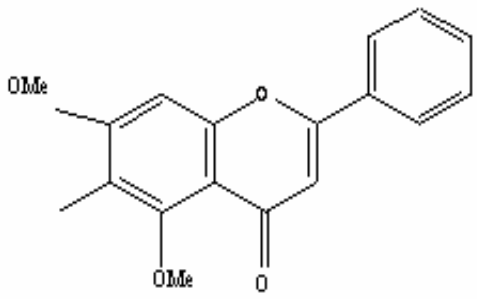

Figure 5.1 Structure of 5,7- dimethoxy-6-methylflavone

5.3.2 Compound 2: Hoslunddiol

UV spectral data λmax 252, 275 and 312 nm suggested a flavone with OH at C-5. ^{1}H-NMR showed singlets at position 6.58 (H-3) and 6.41 (H-8) integrated 7.78 (2H) and

7.46 (3H) of unsubstituted ring B, anomeric proton at 5.4 (H-1``, *J*= 8.0 Hz) attached to carbon resonating at δ_C 105.5, aromatic methoxy group at 3.85, in addition glycosy signal typical to β-digitoxopyranose. The above data indicated the presence of 6-C-β-digitoxopyranosyltectochrysin, hoslunddiol (Figure 5.2) which was isolated before from the same species (Ngadjui *et al*., 1991).

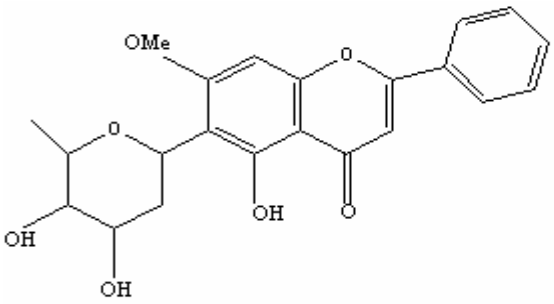

Figure 5.2 Structure of Hoslunddiol

5.3.3 Compound 3: Jacarandic acid or euscaphic acid

The compound jacarandi**c** acid or euscaphic acid (Figure 5.3) showed in ^{1}H-NMR four methyl singlets at δ_H 0.69, 0.91, 1.06, 1.27, one doublet signal of a methyl group at δ_H 0.83 (*J*=5.8 Hz), two protons attached to hydroxyl bearing carbons at δ_H 3.34 (obscured by H_2O signal) and broadening doublet at 4.34 and an olefinic proton at δ_H 5.15.

The previous data in addition to careful analysis of Dept-135 data, confirmed the presence of uresane type triterpene with carboxylic group at C-28, two vicinal axial – equatorial oriented two protons at C-2, C-3, double bond at C-11 and a methyl attached to hydroxyl bearing carbon at C-19 (δ_H 1.27, s). The NMR data published by (Ogura *et*

al., 1977; Chandel and Rastogi, 1977 & Takahashi *et al*., 1974) verified that the isolated compound is jacarandic acid. The review of literature on the species indicated that these data is typical with that of Jacarandic acid isolated before from the same source (Ogura *et al*., 1977; Chandel and Rastogi, 1977 and Takahashi *et al*., 1974).

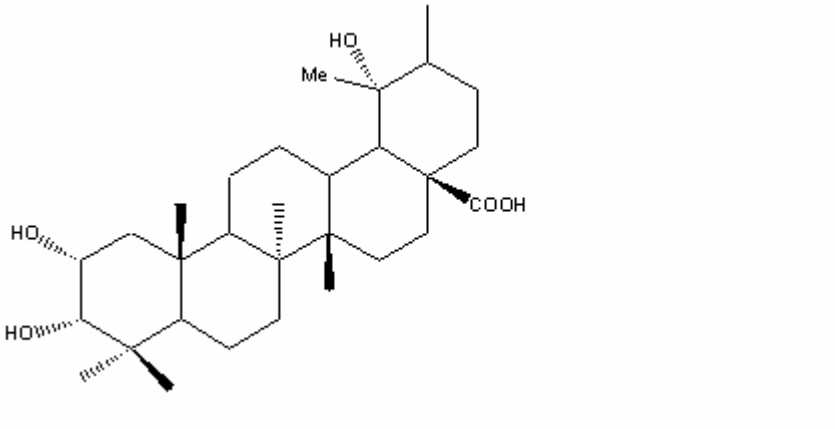

Figure 5.3 Structure of Jacarandic acid

5.4 Conclusion

Phytochemical investigation of *H. opposita* led to the isolation of three known compounds, 5,7-dimethoxy-6-methylflavone (1), hoslunddiol (2) and euscaphic acid (3). This is the first report of the isolation of "5,7- dimethox-6 methylflavone" from *Hoslundia opposita*.

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ANTIBACTERIAL ACTIVITY OF THE COMPOUNDS ISOLATED FROM *LIPPIA JAVANICA* AND *HOSLUNDIA OPPOSITA*

Abstract

The isolated compounds from *Lippia javanica* and *Hoslundia opposita* were investigated for their *in vitro* antimicrobial proprieties against two bacterial strains, one Gram-positive *Staphylococcus aureus* (ATCC 12600) and one Gram-negative *Escherichia coli* (ATCC 11775). A bioautographic assay, using *Staphylococcus aureus* (ATCC 12600), was used to detect the presence of the antibacterial compound 4-ethyl-nonacosane . The compound showed notable effects against *S. aureus*. No inhibitory effect was found in the compounds tested against Gram-positive and Gram-negative bacteria strains at a concentration of 200µg/ml by microdilution technique using 96-well microtitre plates.

6.1 Introduction

The antimicrobial activity of medicinal plants has been evaluated previously using various methods, which are classified into three groups: The disc-diffusion, dilution and bio-autographic methods. In this study bio-autography and dilution methods were used. The dilution assays are those, which require a homogeneous dispersion of the sample in water (Rio et al., 1988). These methods are mainly used to determine the Minimum Inhibitory Concentration (MIC) values of an extract or pure compound. These values are

taken as the lowest concentration of the extract or pure compound that completely inhibits bacterial growth after incubation for 24 h. In the liquid dilution method, turbidity is taken as an indication of bacterial growth, so where the sample is inactive against the micro organism tested, the liquid will appear turbid (Rio *et al*., 1988). The advantages of this are its simplicity and speed, and the possibility of using it in the antimicrobial study of water-soluble or insoluble samples such as essential oils (Rio *et al*. 1988). Eloff (1998) developed a microdilution technique using 96-well microtitre plates. A two-fold serial dilution of the extract, pure compound/ drug is prepared in the wells of the microplate, and bacterial culture is added. After incubation p-iodonitrotetrazolium violet (INT) is added, and in the wells where bacterial growth occurs, a deep red colour develops. Wells containing antibacterial compounds remain clear.

The bioautographic method is an important detection for new or unidentified antimicrobial compounds (Rio *et al*., 1988**).** In the direct bio-autography assay, a suspension of micro-organisms in liquid medium is sprayed on a developed TLC plate and incubated overnight. A solution of tetrazolium salt is then sprayed on the plate and incubated to detect the areas of bacterial growth inhibition. According to Hamburger & Cordell (1987) an advantage of the bioautograhy is that it allows the localization of activity, even in complex mixtures.

6.2 Material and methods

6.2.1 Bioautographic bioassay

The antibacterial activity of the isolated compound **1** (4-ethyl-nonacosane) was evaluated against *Staphylococcus aureus* (ATCC 12600) by direct bioautography technique in a TLC bioassay (Hamburger & Cordell, 1987) because of its low solubility. Compound quantities ranging from 50 µg to 1.56 µg were applied to percolated TLC plates. The TLC was observed under ultra violet (UV) light (254 and 366 nm) after development, left overnight for the solvent to evaporate completely and sprayed with the bacterial suspension. These plates were then re-incubated at 25°C for 24 h (Lund & Lyon, 1975). The results were stained with an aqueous solution of INT.

6.2.2 Microdilution assay

The Minimal Inhibitory Concentration (MIC) values of the compounds were determined against the Gram-positive *Staphylococcus aureus* (ATCC 12600) and Gram-negative *Escherichia coli* (ATCC 11775) bacterial strains. The microplate dilution method of Eloff (1998) was used. The bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37°C and diluted 1:100 with fresh MH prior to use in the microdilution assay. A two-fold serial dilution of the compound (100µl) was prepared in 96-well microtitre plates, and 100µl bacterial culture was added to each well. The pure

compounds were dissolved in 10 % DMSO. The antibiotic Streptomycin was used as a standard in each assay, as well as a DMSO solvent control. The covered microplates were incubated overnight at 37°C. As an indicator of bacterial growth, 40 µl *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells and incubated at 37°C. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product in biologically active organisms (Eloff, 1988). Where bacterial growth is inhibited, the solution in the well will remain clear after incubation. Only two bacteria strains were used to test the activity of the isolated compounds, since we isolated little amount these compounds.

6.3 Results

6.3.1 Bioautography results

The compound 4-ethyl-nonacosane displayed good bactericidal activity against *Staphylococcus aureus* (ATCC 12600). Zones of bacterial growth inhibition could be seen on TLC plates sprayed with *S. aureus* (ATCC 12600) as white spots on a red background (Figure 6.1). The white areas indicate the presence of antibacterial compounds, as the lack of bacterial growth cannot convert the indicator tetrazolium salt to a red product. Metabolically active bacteria convert the tetrazolium salt into the corresponding intensely coloured formazan. The activity of 4-ethyl-nonacosane may be

attributed to the presence of the toxicity. 4-ethyl-nonacosane is an alkane. Alkanes are organic compounds which are found to be useful as anaesthetic and toxic agents (Di Paolo, 1978a; Di Paolo, 1978b).

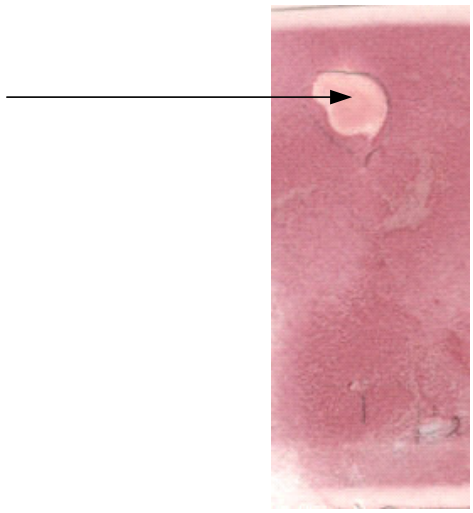

Figure 6.1 Inhibition of *Staphylococcus aureus* (ATCC 12600) by 4-ethyl-nonacosane.

6.3.2 Bioassay results

All isolated compounds from *Lippia javanica* and *Hoslundia opposita* did not show activity against the bacteria on the microdilution assay at the tested concentration of 200µg/ml, as is shown in Figures 6.2 and 6.3.

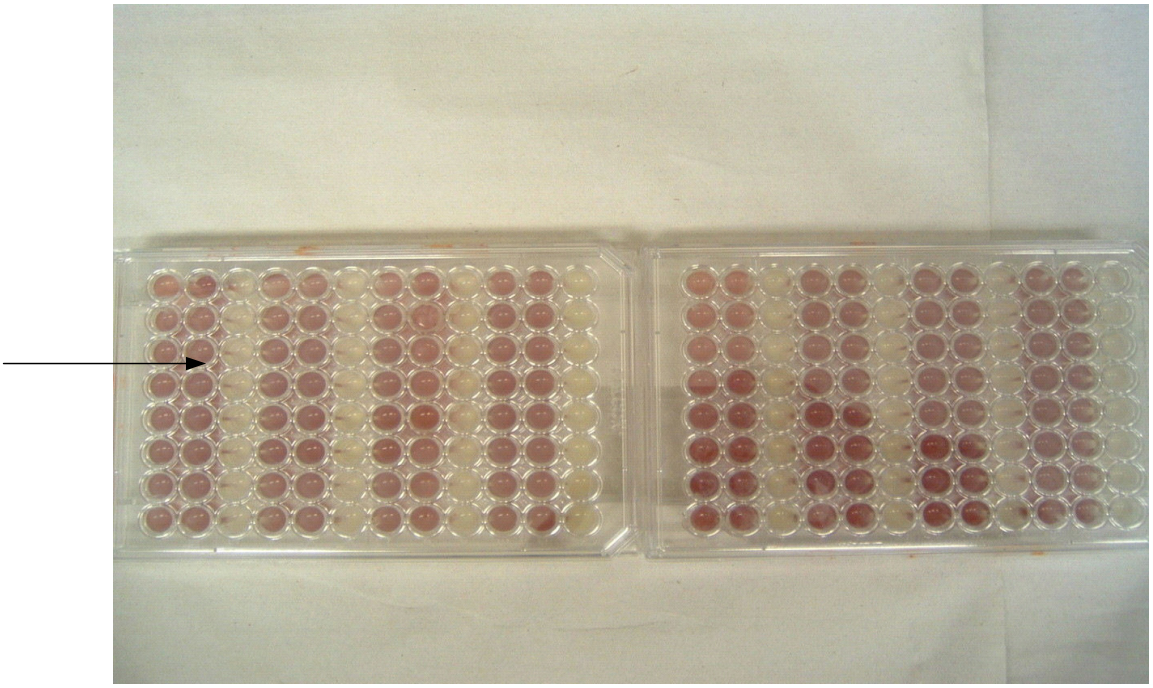

Figure 6.2 Antibacterial test of isolated compounds against *Escherichia coli* (ATCC 11775). Dark coloured wells (arrow) indicate normal bacteria growth.

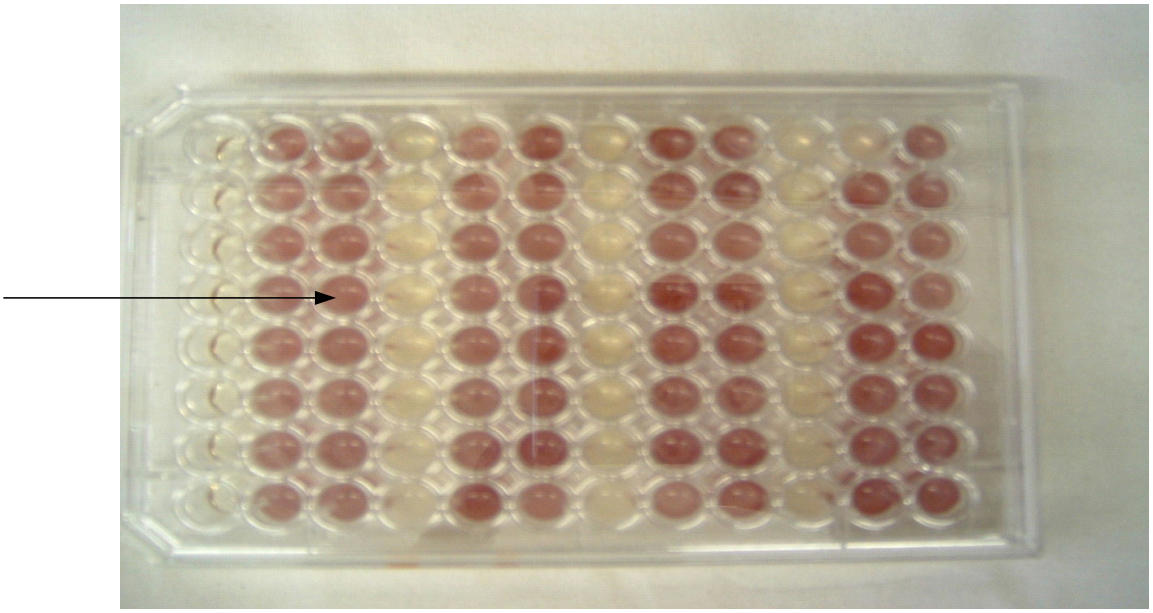

Figure 6.3 Antibacteria test of isolated compounds against *S. aureus*. Dark coloured wells (arrow) indicate normal bacteria growth.

Although the compounds had no activity at the highest tested concentration, the antifungal proprieties of many of those compounds are well known (El-Gammal and Mansour 1986; Aziz *et al*., 1998).

6.4 Conclusion

The reported antibacterial activity of *Lippia javanica* and *Hoslundia opposita* can be attributed to the synergistic combinations of compounds (Viljoen *et al*., 2005, *Mujovo* et al*., 2003a; 2003b; Khan* et al*., 1980),* and it may also be possible that some of the active compounds were not isolated.

Lack of biological activity in the compounds tests does not necesarily indicate lack of effectiveness of the remedies.. They may act in other ways to effect a cure, Such as by stimulating the immune system of the patient, or by manufacturing internal conditions unfavourable for the multiplication of bacteria. For another hand, if plants are used as part of a mixture, the synergistic effects of principles in more than one plant may cause relief from the ailment

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