ANTIVIRAL ACTIVITY OF MOZAMBICAN MEDICINAL PLANTS AGAINST HUMAN IMMUNODEFICIENCY VIRUS

Abstract

Seventeen plant species, which are widely used in the folk medicine in Mozambique, were investigated for their anti-HIV activity. Ethanol plant-extracts were evaluated for their ability to inhibit the enzymes glycohydrolase (α-glucosidase and β-glucuronidase) and reverse transcriptase. Glycohydrolase enzymes are found in the host cell Golgi apparatus of the endoplasmic reticulum of eukaryotic cells and are responsible for glycosylation of proteins. Inhibition of the glycohydrolase proteins has been found to decrease the infectivity of the HIV virion, as the HIV glycoproteins are highly glcosylated. Alpha-Glucosidase has been found to be partly responsible for the glycosylation of HIV gp120 (Collins *et al.* 1997). Reverse transcriptase (RT) is an essential enzyme for the survival of HIV-virus. Without Reverse transcriptase, the viral genome cannot be incorporated into the host cell; as a result a virus will not reproduce.

It was found that 8 plant species (*Cassia abbreviata, Elephantorrhiza elephantina*, *Rhoicissus tomentosa, Pseudolachnostylis maprouneifolia, Lippia javanica*, *Litogyne gariepina, Maerua junceae and Momordica balsamina*) showed inhibitory effects against α-glucosidase and β-glucuronidase at a concentration of 200 µg/ml. The results of the tests revealed that the plant extracts of *Melia azedarach* and *Rhoicissus tomentosa* appeared to be active, showing 49 and 40% inhibition of the enzyme activity respectively.

3.1 Introduction

Over 42 million adults and children are infected by HIV (UNAIDS/WHO, 2003). The global HIV epidemic has killed more than 3 million people in developing countries and 14 000 new infections occur daily (UNAIDS/WHO, 2003). In other words the epidemic in sub-Saharan Africa remains rampant. In 2003, an estimated 26.6 million people in this region were living with HIV/AIDS and approximately 2.3 million people succumbed to the disease (Table 3.1).

Table 3.1 Regional HIV/ AIDS statistics and features, end of 2003 (UNAIDS/WHO, 2003).

Region	**Adults and children living with HIV/ AIDS**	**Adults and children newly infected with HIV**	**Adults prevalence (%)***	**Adult & child deaths due to AIDS**
Sub- Saharan Africa	25.0-28.2 million	3.0-3.4 million	7.5-8.5 million	2.2-2.4 million
North Africa & Middle East	470 000 - 730 000	43000 – 67000	0.2 – 0.4	35 000- 50 000
South & South – East Asia	4.6-8.2 million	610000-1.1million	0.4-0.8	330 000- 590 000
East Asia & Pacific	700000-1.3 million	150000-270 000	0.1—0.1	32 000- 58.000
Latin America	1.3- 1.9 million	120 000- 180 000	0.5- 0.7	49 000- 70 000
Caribbean	350000-590000	45 000-80 000	1.9-3.1	30 000- 50 000
Eastern Europe & Central Asia	1.2-1.8 million	180 000-280 000	0.5- 0.9	23 000-37 000
Western Europe	520 000-680 000	30 000-40 000	0.3-0.3	2 600-3 400
North America	790000-1.2 million	36 000-54 000	0.5- 0.7	12 000- 18 000
Australia & New Zealand	12 000- 18 000	700-1 000	0.1- 0.1	<100
Total	**40 million (36-46 million)**	**5 million (4.2-5.8 million)**	**1.1 % (0.9-1.3 %)**	**2 million (2.5-3.5 million)**

In a belt of countries across southern Africa, HIV/AIDS prevalence is maintaining alarmingly high levels in the general population. Due to the enormity of the challenge, health services have been unable to provide communities with access to prevention and care. Whilst access to anti-retro viral (ARV) drugs is benefiting a larger fraction of people, there still remains a fundamental challenge which is to make prevention and care available to the poor (UNAIDS/WHO, 2003).

HIV (human immunodeficiency virus) is a member of the family of lentiviruses, a subfamily of retroviruses and was first known as human T-lymphocytotrophic virus type III or lymphadenopathy associated virus (Au *et al*., 2001). The virus (Figure 3.1) possesses a single-stranded RNA genome. Its structure consists of a lipoprotein surface studded by two viral- enveloping glycoproteins (Levy *et al*., 1994). Gp 120 is the surface protein (SU) and gp41 is the transmembrane protein (TM) (Levy *et al*., 1994). Just below the lipid bilayer is the matrix (MA) protein p17 and a cone-shaped nucleocapsid, built from a capsid protein (CA) p24. Inside this nucleocapsid are the nucleocapsid proteins (NU) p6, 9 as well as the polymerase enzyme with functions such as reverse transcription (RT) coded by p66, protease p11 and integrase p32**.**

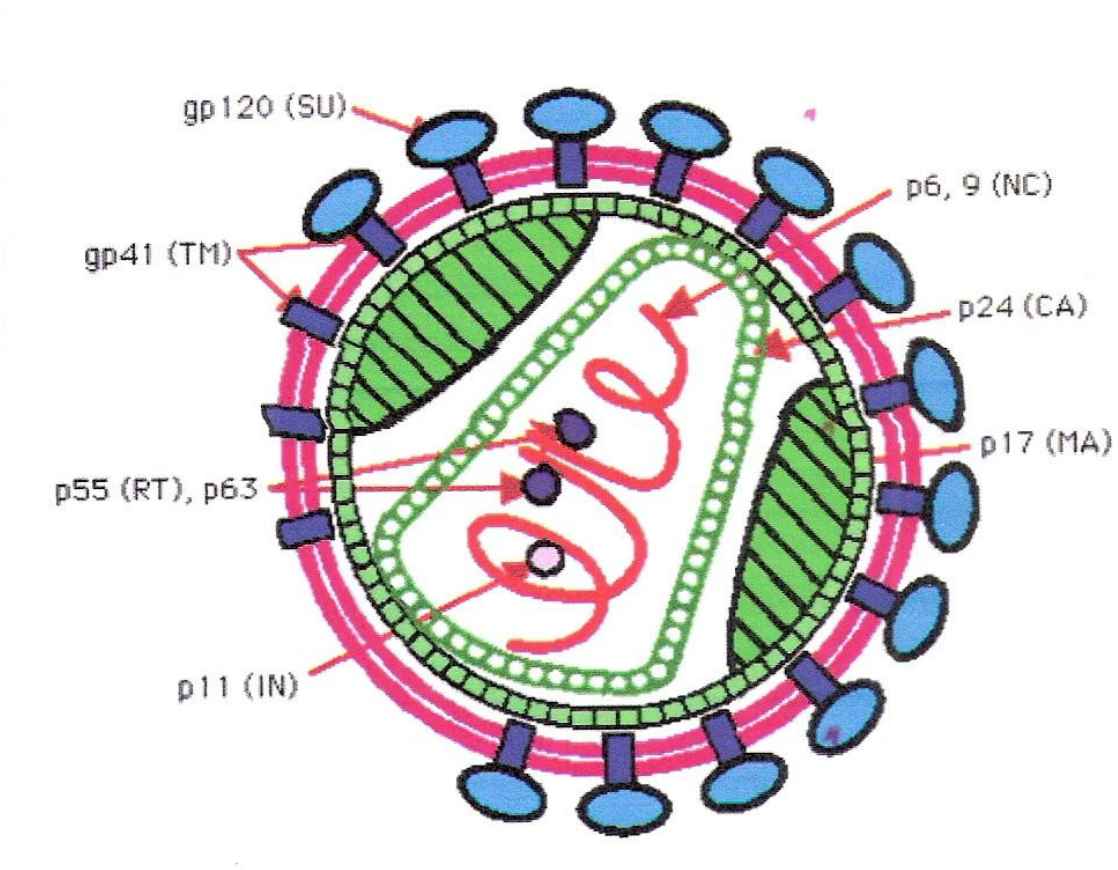

Figure 3.1 Human Immunodeficiency Virus (Da Cunha, 1999)

A number of laboratories are actively involved in the development of antiviral agents that interfere with HIV at different stages of viral replication (Balzarini *et al*., 1986; Sarin, 1988). A possible site of intervention is the inhibition of virus-specific RNA-dependent DNA polymerase (reverse transcriptase) (Vanden Berghe *et al.,* 1993)**.** If one can inhibit its reverse transcription catalytic activity, the viral RNA genome which encodes the viral genetic information would not be able to transcribe into a dsDNA strand encoding the cellular instructions to translate the viral proteins to form the provirus. When HIV infects a cell in a person′s body, it copies its own genetic code into the cell′s DNA. In this way, the cell is then "programmed" to create new copies of HIV. HIV's genetic material is in the

form RNA. In order to infect T-cells, it must first convert its RNA into DNA. HIV's reverse transcriptase enzyme is needed to perform this process (AIDSmeds, 2001). The first lines of the major class of drug therapy found useful in slowing HIV infections which were nucleoside RT inhibitors (nucleoside analogues). These include 3′-azido-3′-deoxythymidine or zidovudine (AZT), 2' deoxy-3'-thiacytidine or lamivudine, (3TC), 2', 3'-didehydro-3'-deoxythymidine or stavudine (d4T), 2', 3'-dideoxycytidine or zalcitabine (ddC) and 2', 3' dideoxyinosine or didanosine (ddI) that act by blocking the recording of viral RNA into DNA. On the other hand, specific enzymes called glycohydrolases contribute to the glycosylation of proteins (Collins *et al*., 1997). These glycohydrolase enzymes include α- glucosidase that is responsible for the glycosylation of HIV- gp120 (one of the membrane proteins that interacts with the CD4 receptor protein that is present on helper T cells of the immune system) and β-glucuronidase, all interfering with viral maturation. Inhibitors of glycosylation could have a potential therapeutic use.

3.2 Materials and methods

3.2.1 Plant material

Seventeen plants (Table 3.2) which are used to treat, HIV- infections in immunocompromised patients were collected from different areas in Mozambique.

3.2.2 Preparation of plant extracts

Dried powdered plant materials were extracted with acetone**.** Fifty grams of powdered plant material was extracted with 500 ml of solvent over two days under reflux. The extracts

were then filtered and concentrated to dryness under reduced pressure and the residues freshly dissolved in an appropriate solvent on the day that the bioassay was done.

3.2.3 Glycohydrolase enzyme assays

Determination of activity against HIV was based on the measure of inhibition of the glycohydrolase enzymes: α-glucosidase and β-glucuronidase. Two glycohydrolase enzymes (α- glucosidase and β- glucuronidase) and the substrates p-nitrophenyl- α-D-glucopyranoside and p-nitrophenyl-β -D-glucuronide were obtained from Sigma Chemical (MO, U.S.A). The glycohydrolase assay was performed in a colorimetric 96-well microtiter plate-based assay, determining the amount of p-nitrophenol released. The method described by Collins *et al*. (1997) was followed**.** The enzymes were diluted in 50mM of an appropriate buffer (sodium acetate, pH 5.0 for β-glucuronidase and Mes-NaOH, pH 6.5 for α- glucosidase). Appropriate substrates of the respective enzymes were added to microtiter wells**.** The assay was calibrated relative to enzyme concentration and ~ 0.25 µg enzyme was used per assay. After the addition of the enzymes, substrate and extracts, the plates were left at room temperature for 15 min. The reaction was stopped by the addition of 50 µl of 2 mM glycine-NaOH, pH 10, and measurement of absorbance undertaken at 412 nm. The extracts were tested at concentration of 200 µg/ml and the experiment was carried out in triplicate. The positive control Doxorubicin was tested at 100 µg/ml against both the enzymes.

3.2.4 HIV-1 Reverse transcriptase (RT) assay

The effect of plant extracts on RT activity *in vitro* was evaluated with a non-radioactive HIV-RT colorimetric ELISA kit (Roche, Germany). The assay was carried out in triplicate. Adriamycin, an anticancer drug and also an inhibitor of viral reverse transcriptase (Goud *et al*., 2003) was used as a positive control. In each test well, 20 µl of diluted recombinant HIV-1 reverse transcriptase (4-6 ng), 20 µl of diluted extract, and 20 µl of reaction mixture was dispensed. The final concentration of each extract in each well was 200 µg/ml. Since this part of the experiment was not conducted at the University of Pretoria, but at Nelson Mandela Metropolitan University; due to cost implications, only one concentration was selected. Negative control wells contained 40 µl of lysis buffer and 20 µl of reaction mixture. The concentration of positive drug control (Adriamycin) was 100 µg/ml. Positive control wells contained 20 µl diluted recombinant HIV-1 Reverse transcriptase (4-6 ng), 20 µl of lysis buffer containing 10 % DMSO, and 20 µl of reaction mixture. The wells of the microtiter plate modules were washed five times with 250 µl of washing buffer per well for 30 seconds each. The washing buffer was then carefully removed and 200 µl of anti-DIG-POD working solution was dispensed into each well. Incubation at 37^{o}C followed once again for 1 hour after the microtiter plate modules were covered with foil. The wells were then washed in the same manner as before, the washing buffer was carefully removed from the wells, and 200 µl of ABTS substrate was dispensed into the wells. Incubation then commenced for 10-30 min at room temperature (15-25^{o}C). The absorbencies of the samples were measured at 405 nm (reference wavelength: 492 nm). The percentage

inhibitory activity of the extracts samples were then calculated, with reference to the positive control.

3. 3 Results and discussion

The inhibition of α- glucosidase and β- glucuronidase by plant extracts is depicted in Table 3.2. It was found that 8 plant species (*Cassia abbreviata, Elephantorrhiza elephantina*, *Rhoicissus tomentosa, Pseudolachnostylis maprouneifolia, Lippia javanica*, *Litogyne gariepina, Maerua junceae and Momordica balsamina*) showed inhibitory effects against α-glucosidase and β-glucuronidase at 200 µg/ml.

Table 3.2 Inhibition of α- glucosidase and β- glucuronidase by the plant extracts.

Family	Botanical name	Plant part used	α- glucosidase % inhibition[a]	β- glucuronidase % inhibition[a]
Passifloraceae	*Adenia gummifera*	Root	34.9± 13.9	28.9 ± 38.3
Liliaceae	*Aloe marlothii*	Leaves	32.2 ± 3.6	62.8 ± 20.1
Liliaceae	*Aloe parvibracteata*	Leaves	2.1 ± 8.2	-9 ± 16.3
Apocynaceae	*Adenium multiflorum*	Root	-17 ± 18.3	25.7 ± 49.2
Fabaceae	*Cassia abbreviate*	Bark	89.9 ± 0.1	93.6 ±1.9
Apocynaceae	*Catharanthus roseus*	Leaves	43.9 ± 1.9	16.1 ± 19.1
Fabaceae	*Elephantorrhiza elephantina*	Root	80.6 ± 0.4	95.2 ± 0.1
Iridaceae	*Gladiolus dalenii*	Tuber	-35.9 ± 5.7	-24.9 ± 7.1
Lamiaceae	*Hoslundia opposita*	Leaves	70.2 ± 5.3	42.5± 8.6
Verbenaceae	*Lippia javanica*	Leaves	62.0 ± 0.9	73.2 ± 7.6
Asteraceae	*Litogyne gariepina*	Leaves	62.3 ± 15.0	91.2 ± 3.8
Meliaceae	*Melia azedarach*	Leaves	29.1 ± 4.6	23.1 ± 15.9
Capparaceae	*Maerua juncea*	Leaves	69.3 ± 0.8	90.4± 1.4
Cucurbitaceae	*Momordica balsamina*	Leaves	60.0 ± 1.5	67.3± 4.1
Euphorbiaceae	*Pseudolachnostylis maprouneifolia*	Bark	89.8 ± 0.1	95.4 ± 1.1
Vitaceae	*Rhoicissus tomentosa*	Root	72.8 ± 1.3	94.24 ± 0.6
	Coccinia rhemanii	Tuber	3.1 ± 3.7	-15 ± 3.4
Doxorubicin (positive control tested at 100 µg/ml)			98.2 ± 0.1	90.4 ± 0.4

[a] % inhibition are average ± standard deviation.

The most promising anti-HIV activity was found by the extracts of *Cassia abbreviata, Elephantorrhiza elephantina, Lippia javanica, Pseudolachnostylis maprouneifolia and Rhoicissus tomentosa*. Two of the most active extracts (*Cassia abbreviata* and *Elephantorrhiza elephantina*) were members of the same plant family (Fabaceae). The extracts from *Cassia abbreviata* inhibited α-glucucosidase and β-glucuronidase by 90 and 94%, respectively. *Elephantorrhiza elephantina* inhibited the activity of α-glucucosidase and β-glucuronidase by 80 and 95%, respectively. The extract of *Pseudolachnostylis maprouneifolia* (Euphorbiaceae) also inhibited α-glucucosidase and β -glucuronidase by 90 and 95%, respectively. *Aloe marlothii* showed only inhibition of β–glucuronidase, while *Hoslundia opposita* was only active against α- glucucosidase. *Adenia gummifera* and *Gladiolus dalenii* did not show any activity against α- glucucosidase at the highest concentration (200 µg/ml) tested.

Adenia gummifera, Cassia abbreviata, Elephantorrhiza elephantina, Gladiolus dalenii, Hemizygia bracteosa, Lippia javanica, Momordica balsamina, Pseudolachnostylis maprouneifolia, Rhoicissus tomentosa, *Melia azedarach* and *Maerua juncea* were also assayed for their ability to inhibit the enzyme HIV-1 Reverse transcriptase. These plants were selected based on their inhibitory activity against glycohydrolase enzyme and the availability of the extracts. Figure 3.2 shows the inhibitory effect of plant extracts on the enzyme RT. The results of the tests revealed that the plant extracts of *Melia azedarach* and *Rhoicissus tomentosa* appeared to be active, showing 49 and 40% inhibition of the enzyme activity respectively. The activity of the remaining plant extracts against RT was not

significant. Adriamycin, the positive control showed 80 % inhibitory activity at a 100 µg/ml concentration.

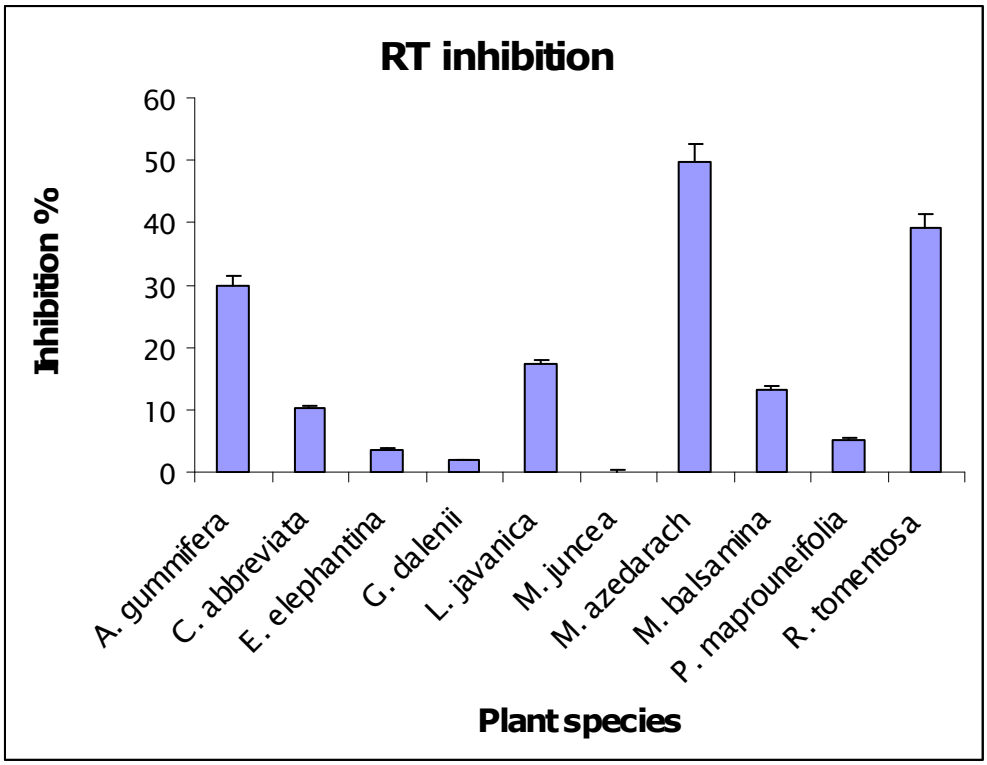

Figure 3.2 HIV Reverse transcriptase (RT) inhibition by the plant extracts

3.4 Conclusion

The results revealed that most of the plants tested, *Cassia abbreviata, Elephantorrhiza elephantina, Lippia javanica, Maerua juncea, Momordica balsamina, Rhoicissus tomentosa* and *Pseudolachnostylis maprouneifolia* showed good inhibitory activity against α- glucosidase and β- glucuronidase. Only two species (*Melia azedarach* and *Rhoicissus*

tomentosa) displayed activity against RT at 200 µg/ml. Despite the fact that the plant extracts were not pure compounds they could provide useful leads for the discovery of antiviral compounds.

3.5 References

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ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM *LIPPIA JAVANICA*

Abstract

Lippia javanica is an aromatic herb that occur all over in Mozambique and is well known for their medicinal properties. *Lippia javanica* was found to have the best activity exhibiting a minimum inhibitory concentration of 0.125 mg/ml against *B. cereus*, *B, pumilis, B, subtilis S. aureus* and *E. faecalis.* the extracts also showed positive activity against *Mycobacterium tuberculosis* at concentration of 0.5 mg/ml and HIV-enzyme glycohydrolase (α-glucosidase and β-glucuronidase) inhibited by 62 % and 73 % respectively. Considering its medicinal use local for HIV and various infections, it was therefore, selected for identifying its bioactive constituents. A Phytochemical investigation of *L. javanica* led to the isolation of eight compounds, 4-ethyl-nonacosane (**1**), (*E*)-2(3)-tagetenone epoxide (**2**), myrcenone (**3**), piperitenone (**4**), apigenin (**5**), cirsimaritin (**6**), 6-methoxyluteolin 4'-methyl ether (**7**), 6-methoxyluteolin and 3',4',7-trimethyl ether (**8**). This is the first report of compounds (**1**), (**2**), (**5-8)** from *L. javanica*.

4.1 Introduction

Twenty two plants were screened for bioactivity against Gram-positive and Gram negative bacteria.

A preliminary study indicated that extract of *Lippia javanica* was found to have the best activity against Gram-positive bacteria tested; *Mycobacterium tuberculosis* and HIV-enzyme glycohydrolase (α-glucosidase and β-glucuronidase) inhibited by 62 % and 73 % respectively. Considering its medicinal use local for HIV and various infections, it was therefore, selected for identifying its bioactive constituents.

4.1.1 Description and traditional use of *Lippia javanica*

The are about 200 species of *Lippia* include*s* herbs, shrubs and small trees (Terblanché & Kornelius, 1996). In general, the genus appears to present consistent profiles of chemical composition, pharmacological activities. The most common use of *Lippia* species is for the treatment of respiratory disorders (Pascual *et al*., 2001)**.** *Lippia javanica* (Burm.f.) Spreng (Figure 4.1) is an erect woody shrub up to two meters high, with strong aromatic leaves, which give off a lemon smell when crushed (Van Wyk & Gericke, 2000)**.**

The plant occurs in many parts of southern Africa and tropical Africa (Van Wyk & Gericke, 2000). Its infusion made from its leaves is commonly used in Africa as tea for various chest ailments, influenza, measles, rashes, malaria, stomach problems, fever, colds, cough and headaches (Smith, 1966; Watt & Breyer-Brandwijk, 1962; Hutchings, 1966 and Hutchings & van Staden, 1994). Hutchings (2003) reported the clinical use of *L. javanica* for the treatment of HIV in Ngwelezane Hospital, Kwazulu Natal (South Africa).

In Botswana it is used as a caffeine free tea and in Zimbabwe and Malawi as a nerve tonic (Manenzhe *et al*., 2004).

Figure 4.1 *Lippia javanica* (Plantzafrica.com)

4.1.1.2 Biological activity

Extracts of *Lippia javanica* displayed a reproducible inhibitory activity against the Gram-positive bacteria *Bacillus cereus*, *B. pumilis*, *B. subtilis*, *Staphylococcus aureus* and *Enterococcus faecali*s in the present study. The essential oil from *L. javanica* has also been extensively shown to exhibit bioactivity against many pathogenic microorganisms (Viljoen *et al*., 2005; Manenzhe *et al*., 2004). It has also been found with good insect repellent activity (Govere *et al.*, 2000), and antiplasmodial activity (Manenzhe *et al*. 2004, Mwangi *et al*. (1991).

4.1.1.3 Chemical constituents

Numerous monoterpenoids have been identified in the volatile extract of *Lippia javanica*, including mercyne, caryophyllene, linalool, *p*-cymene and ipsdionone (Neidlein and Staehle 1974; Mwangi *et al*., 1991). *Lippia javanica* contains various organic acids and alcohols (Neidlein and Staehle, 1973a, 1973b). Iridoid glycosides (Rimpler and Sauerbier, 1986) and toxic triterpenoids (icterogenins) have been detected in some *Lippia* species (Buckingham, 2006).

4.2 Materials and methods

4.2.1 Plant material

Leaves of *Lippia javanica* 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.

4. 2.2 Extraction and isolation

The air dried leaves of *L. javanica* (1.4 kg) were extracted with 4L ethanol for two days then filtered; the process was repeated two times. The extracts were combined and evaporated under reduced pressure to afford 47.5 g of crude ethanol extract. The total extract was subjected to a silica gel column (40 x 10 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. Eight fractions (300 ml), based on TLC profile were pooled and concentrated to dryness under reduced pressure. Fraction I (3.5 g) was chromatographed over silica gel using 100% hexane to afford compound (**1,** 437.6 mg). Fraction IV (10 g) was chromatographed on silica gel using hexane-EtOAc mixtures of increasing polarity which yielded compounds (**2,** 41.1 mg), (**3,** 18.3 mg), and (**4,** 568 mg). Fraction VII (4 g) was rechromatographed on silica gel column using gradient of EtOAc in hexane. The fraction eluted with EtOAc-hexane (4:6) was further chromatographed over Sephadex LH-20 using 100% methanol as eluent which yielded compounds (**5,** 5.3 mg), (**6,** 10 mg), (**7,** 8 mg), (**8,** 10 mg).

4.2.3 Bioautography of fractions obtained after the chromatographic purification of the ethanol extracts of *L. javanica*.

After each purification stage the antibacterial activity of fractions was tested using the direct bioautography. In this assay, an overnight culture of test bacteria in 20 ml MH broth was pelleted by centrifugation at 3000 rpm for 15 min and 10 ml fresh MH broth. This suspension was sprayed on a developed TLC plate and incubated at 37^{o}C overnight. A 2 mg/ml solution of INT (iodonitrotetrazolium violet) was then sprayed on the plate and incubated to detect the areas of bacterial inhibition. Antibacterial compounds on the TLC plate was visible as white spots against a deep red background, as bacterial growth reduces the tetrazolium salt to a red formazan product (Figures 4.2).

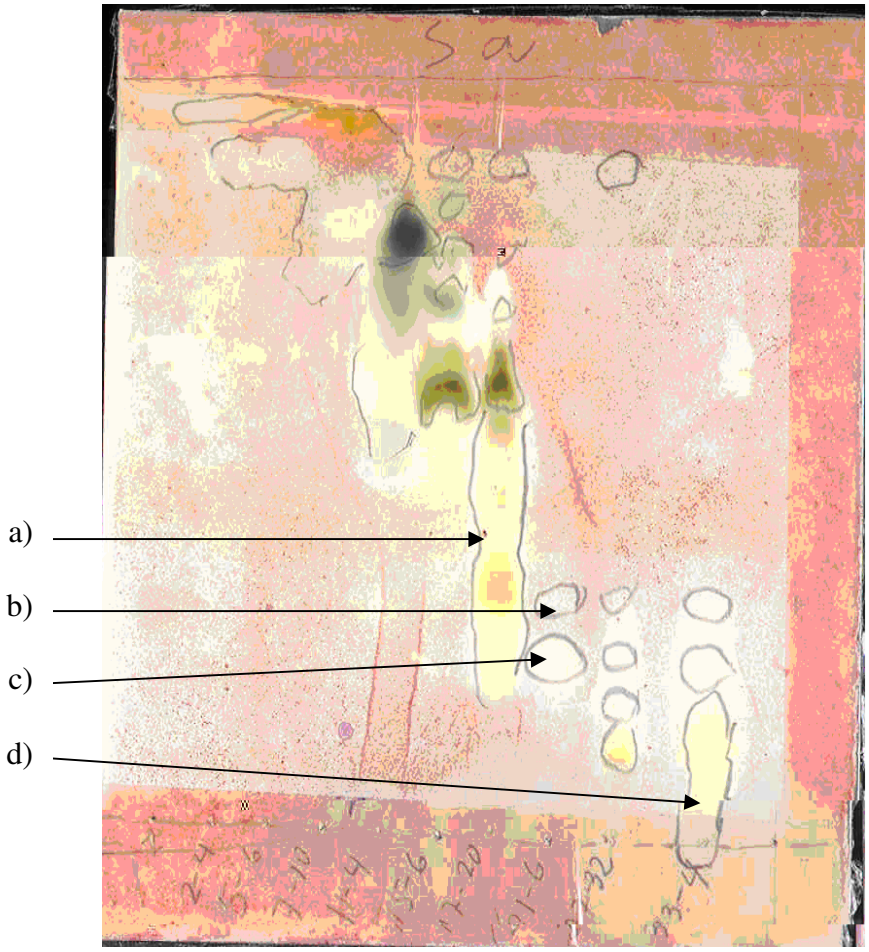

Figure 4.2 Fractions from silica column A tested for antibacterial activity **(Sa)** *Staphylococcus aureus* (ATCC 12600). Zones of inhibition (arrows, a-d)

4.2.4 Identification of purified 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

4.3 Results and discussion

4.3.1 Compound "4-ethyl-nonacosane"

The compound 4-Ethyl-nonacosane ($C_{31}H_{64}$) crystallized from fraction 1 in *n*-hexane and the structure was established based on electronic impact mass (EI-MS) (Figure 4.3) and ^{1}H-NMR spectra, which correspond to the T-branched hydrocarbon, 4-Ethyl-Nonacosane ($C_{31}H_{64}$, M_r = 436).

White crystals from hexane, $C_{31}H_{64}$, EI-Ms. m/z (%): 436(12.2%) [M] H^+, 408 (8.7%) $[M-C_2H_5+H]^+$, 393 (7%) $[M-C_3H_7 + H]^+$, 85 (57.8%) $[M-C_{25}H_{51}+H]^+$, 71 (70%) C_5H_{11} + , 57 (100 %, base peak) C_4H_9, 43 (9-) C_3H_7+, 29 (18) C2H5 +; ^{1}H- NMR δppm: 0.88 (9H, t3CH)

H-1, H29, 4-EtH-2, 1-26-29 (54H, m, -CH5-)

H- 2.3, H-5-28, 4-EtH-1, 1.53 (1 H, m, CH) H-4.

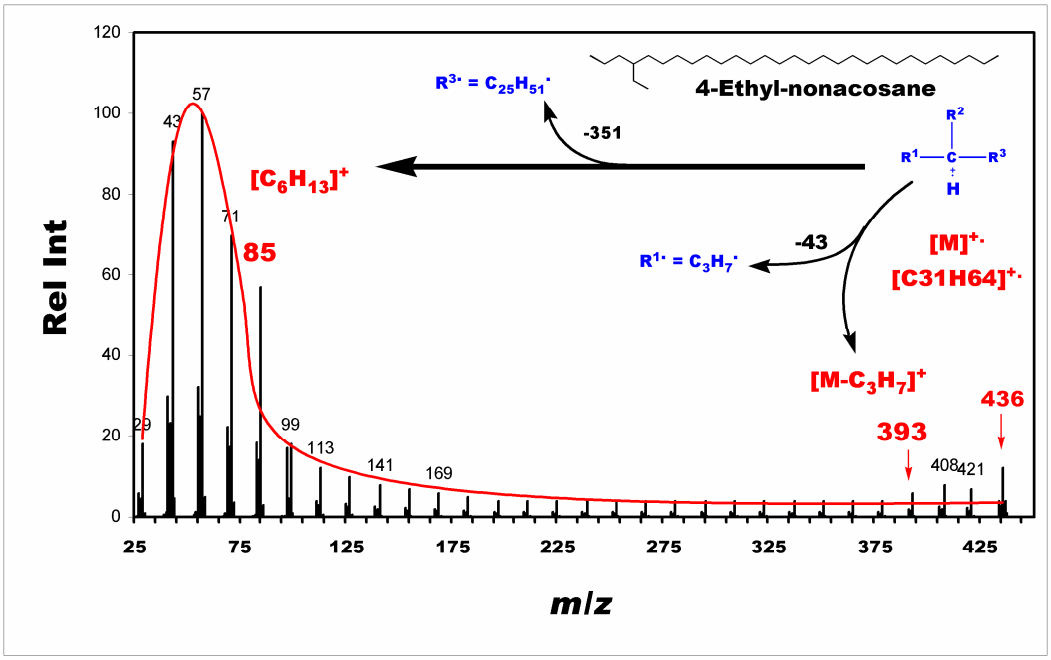

Figure 4.3 Electronic impact mass spectra (EI-MS) of 4-ethyl-nonacosane

4.3.2 Compound 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

This compound was isolated from the non-polar fraction of the ethanolic extract of *L. javanica,* and showed in NMR (^{1}H and ^{13}C) three singlet signals at δ_H 1.25 (δ_C 24.8), δ_H 1.40 (δ_C 18.6), and δ_H 2.25 (δ_C 13.8), two double bonds one of them vinylic with characteristic terminal CH_2 signals at δ_H 5.49 (d, *J*=10.9Hz), δ_H 5.67 (d, *J*=17.2 Hz) and proton signal at δ_H 6.39 (dd, *J*=10.9, 17.2Hz), the other double bond (δ_C 152.8s, 123.4d) and proton signal at δ_H 6.32 (s), in addition to a proton attached to oxygenated carbon at δ_H 3.35 (s) which form part of an oxirane ring (δ_C 61.1s, 66.4d) (Table 4.1). The above data correspond to the structure given in (Figure 4.5).

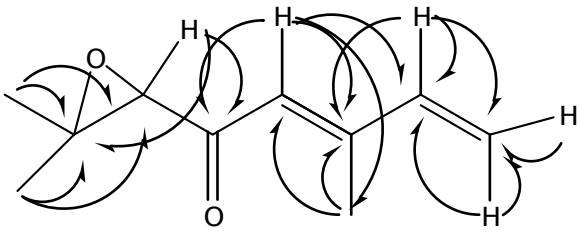

Figure 4.4 HMBC correlations of 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

The structure of this compound was further supported by HMBC (Figure 4.4) which showed cross peak connectivity between H-1/C-2, C-3; H-2/C-10, C-4, C-4; H-4/C-2, C-5, C-10, C-3; H-6/C-9, C-7, C-5, Me-8, 9/C-7, C-8; Me-10/C-4, C-2, C-3, C-5. NOESY experiment of compound 2 also showed cross peaks between H-6/H-8, H-4; Me-10/H-1 (trans), H-2/H-4, the correlations between H-2/ H-4 and H-4/H-6 indicated that all of the proton are in the same side, also the NOESY relation between, H-1 (trans)/Me-10 indicated the location of them on the other side. Compound 1-(3,3-dimethoxiranyl)-3-methyl- (2*E*), is a rare monoterpene identified in the Cameroonian *Clausena anista* (Rutaceae) essential oil (Ngassoum *et al*., 1999) and was not identified in *Lippia* species before, which indicates that the *L. javanica* collected from Mozambique as a new chemotype.

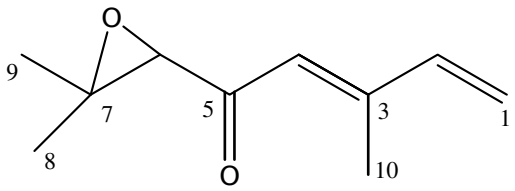

Figure 4.5 Structure of 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

Table 4.1 ^{1}H and ^{13}C NMR data of 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)* in $CDCl_3$

No.	Carbon	Proton
1	121.8 t	5.49 (d, 10.9), 5.67 (d, 17.2)
2	140.4d	6.39 (dd, 10.9, 17.2)
3	152.8 s	
4	123.4 d	6.32 s
5	196.7 s	
6	66.4 d	3.35 s
7	61.1 s	
8	18.6 q	1.40 s
9	24.8 q	1.25 s
10	13.8 q	2.25 s

4.3.3 Compound Myrcenone

Myrcenone was isolated from the non-polar fraction using a silica gel column. The compound showed in NMR three double bonds: one of them is vinylic and has two protons at δ_H 5.07 (d), 5.20 (d) attached to carbon at δ_C 119.9 (t), and proton at δ_H 6.44 (d), δ_C 138.2 (d), the other two double bonds contain an *exo* double bond at δ_C 140.6 (s), 114.9 (t), the later carbon attached to two singlet signals (one protons each) at δ_H 5.09 (s),

5.22 (s), the third double bond located at C-6 and attached to a singlet proton at δ_H 6.14. The remaining signals indicated the presence of two methyl groups over a double bond at δ_H 1.85 (δ_C 27.7), 2.12 (δ_C 20.8) in addition to conjugated carbonyl group at δ_C 198.0 (Figure 4.6, Table 4.2). The forgoing data are applicable only to myrcenone, the commonly found monoterpenes in *Lippia* volatile oils.

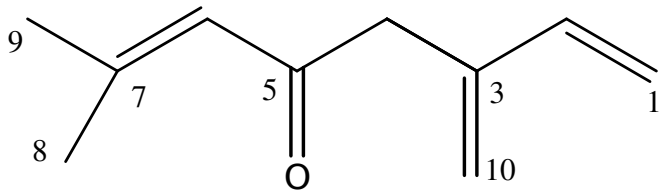

Figure 4.6 Structure of myrcenone

Table 4.2 ^{1}H and ^{13}C NMR data of myrcenone ($CDCl_3$)

No.	Carbon	Proton
1	119.9 t	5.07 (d, 8.8), 5.20 (d, 17.4)
2	138.2	6.44 (dd, 8.8, 17.4)
3	140.6 s	
4	47.9 t	3.27 (2H, s)
5	198.0 s	
6	122.4 d	6.14 s
7	143.5 s	
8	20.8 q	2.12 s
9	27.7 q	1.85 s
10	114.9 t	5.09, 5.22 (s, both)

4.3.4 Compound piperitenone

The compound was isolated from the non polar fractions. ^{13}C NMR gave 10 carbons, which indicated a monoterpene skeleton. ^{1}H NMR showed singlet olefinic proton at δ_H 5.67, two methylene groups at δ_H 2.46 (t, J=6.2 Hz), 2.10 (t, J=6.2 Hz) and three methyl singlets attached to double bonds at δ_H 1.89,1.73 and 1.66 (Table 4.3). The previous data

only can be accommodated in structure (Figure 4.7), piperitenone, which has been isolated before from the same source.

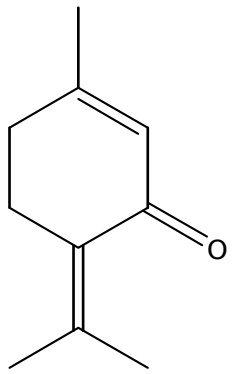

Figure 4.7 Structure of piperitenone

Table 4.3 ^{1}H and ^{13}C NMR data of piperitenone ($CDCl_3$)

No.	Carbon	Proton
1	191.0 s	
2	128.4 d	5.67 brs
3	141.9 s	
4,5	31.4 t, 27.5 t	2.46, 2.11 (2H each, t, J=6.2 Hz)
6	159.21 s	
7	128.51 s	
8,9	22.4 q, 22.1 q	1.89 s (9), 1.67 s (8)
10	23.3 q	1.74 s

4.3. 5 Compound β-sitosterol

The compound was identified as β-sitosterol based on the ^{1}H NMR and co-spotting with authentic sample.

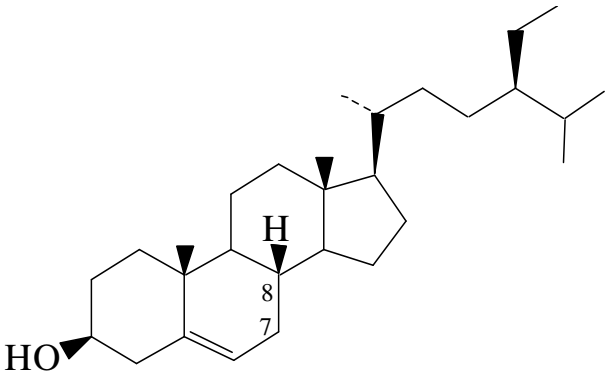

Figure 4.8 Structure of β-sitosterol

4.3.6 Compound Apigenin

The compound showed a yellow color on TLC plates when sprayed with $AlCl_3$ which indicating its flavonoidic nature. This was supported by ^{1}H NMR spectrum, which showed two proton doublets at δ_H 6.44 (d, J=2.2 Hz), 6.20 (d, J=2.2 Hz) corresponding to protons attached to positions 6 and 8 respectively of compound Myrcenone, another singlet at δ_H 6.59 corresponding to H-3, in addition to two doublets counted four protons at 7.84, 6.92 (2H/each J=8.8 Hz) corresponding to H-2`, 6` and H-3` and 5`. The given data is a typical NMR pattern of apeginin, the wide spread flavone aglycone in nature.

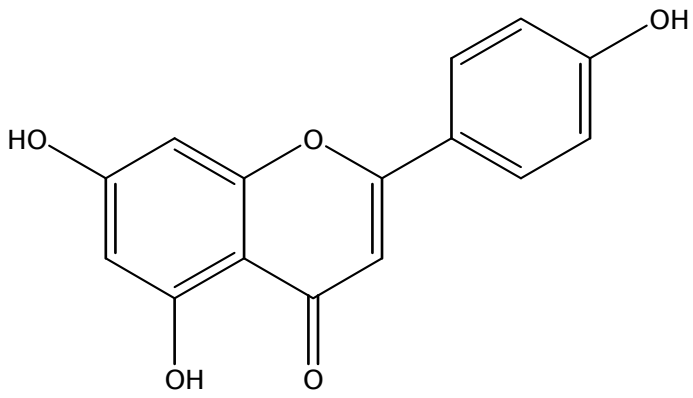

Figure 4.9 Structure of apigenin

4.3.7 Compound Cirsimaritin

The compound gives signals similar to compound apeginin (singlet at δ_H 6.59 corresponding to H-3, in addition to two doublets counted four protons at 7.84, 6.92 (2H/each J=8.8 Hz) corresponding to H-2`, 6` and H-3` and 5`), in addition to a singlet at 6.52 (H-8) and two singlets (3H each) at 3.94 and 3.90 of two methoxy groups. The previous data indicated the presence of 6-hydroxyapeginin. The two methoxy groups were positioned at C-6 and C-7 because the proton chemical shift of compound 4 is almost the same as the free aglycone apigenine (compound 3) except H-8 which shifted to a lower field from the corresponding value (δ_H 6.44), the other methoxy group was positioned at C-6 because the other signals in ring C were not affected and the 6-methoxy derivative is commonly found in labiatae.

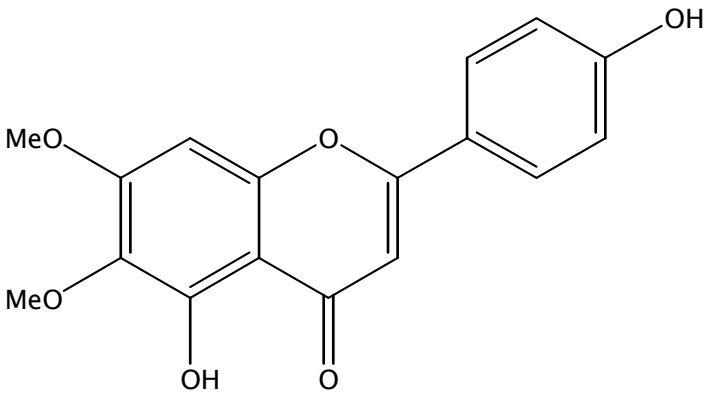

Figure 4.10 Structure of Cirsimaritin

4.3.8 Compound 6-Methoxyluteolin 4'-methyl ether

Compound **8** is flavonoidic in nature as indicated from the color reaction of the compound with $AlCl_3$. The NMR spectra showed similar signal to compound Cirsimartin, except that the presence of a hydroxyl group at C-3`, which indicated from the splitting of ring C signals to 1,3,5-trisubstituted pattern and gives signals attributed to H-2 (7.32, d, J=1.8 Hz), H-5 (7.01, d, J=8.4 Hz) and H-6 (7.48, dd, J=1.8, 8.4 Hz). In addition to two methoxy groups were present at 4.00, 4.04 (δ_C 60.9, 56.9). The two methoxy groups were positioned at C-6 and C-4 due to the fact that, the signal at δ_C at 60.9 indicated the connection of the methoxy groups should be between two oxygenated carbons i.e. C-6 and the other methoxy group positioned at C-4` due to the shift of H-5` from the basic skeleton (without methoxy groups, ~ 7.00).

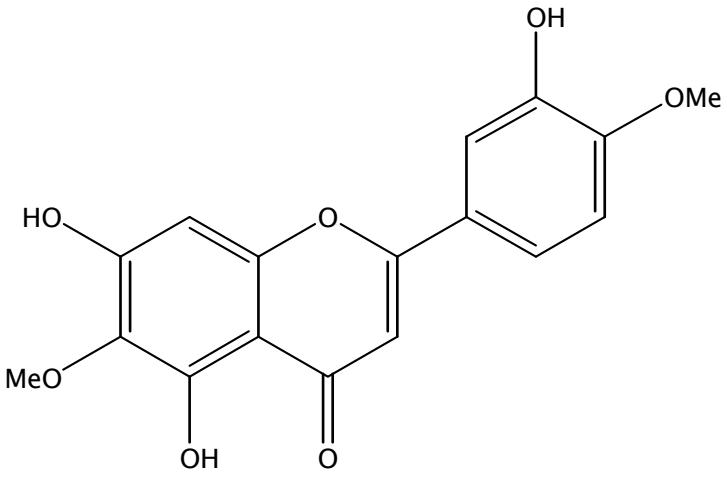

Figure 4.11 Structure of 6-Methoxyluteolin 4'-methyl ether

4.3.9 Compound 6-Methoxyluteolin 3',4',7-trimethyl ether

Compound 9 showed similar patterns in NMR as compound 6-Methoxyluteolin 4'-methyl ether, [H-2` (7.32, d, J=1.8 Hz), H-5` (7.01, d, J=8.4 Hz) and H-6` (7.48, dd, J=1.8, 8.4 Hz), and two singlets at 6.59 and 6.55 of H-3 and 6] except the presence of four methoxy groups in compound 9, accordingly the four methoxy groups were positioned at C-6,7,3` and 4`. Keeping in mind that the substitution at C-5 is eliminated due to the presence of the hydroxyl signal after 12.50 ppm.

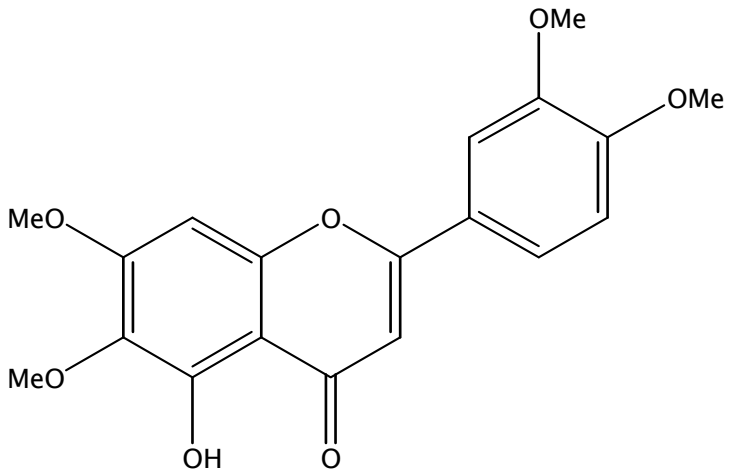

Figure 4.12 Structure of 6-Methoxyluteolin 3',4',7-trimethyl ether

4.4 Conclusion

A Phytochemical investigation of *L. javanica* led to the isolation of eight compounds, 4-ethyl-nonacosane (**1**), (*E*)-2(3)-tagetenone epoxide (**2**), myrcenone (**3**), piperitenone (**4**), apigenin (**5**), cirsimaritin (**6**), 6-methoxyluteolin 4'-methyl ether (**7**), 6-methoxyluteolin and 3',4',7-trimethyl ether (**8**). This is the first report of compounds (**1**), (**2**), (**5**-**8**) from *L. javanica*.

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