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Lippia javanica (Burm F) Spreng: its general constituents and bioactivity on mosquitoes

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Abstract. Mosquito repellent plants are used in the rural areas of Zimbabwe despite the fact that very few of them have been biologically evaluated. Leaves of the plant *Lippia javanica*, were collected from Mumurwi village, Zimbabwe and evaluated for repellency against laboratory reared *Aedes aegypti* mosquitoes. Major plant compounds were identified using Thin Layer Chromatography (TLC) and Mass Spectrometry (MS). Fraction 'A' contained coumarins, flavonoids and essential oils and offered a protection time of 8 and 5.5hrs in choice and non-choice experiments respectively. Fraction 'B_1' contained flavonoids with a protection time of 1 and 0.5hrs in choice and non choice experiments respectively. Fraction 'B_2' contained coumarins and essential oils and offered a protection time of 2hrs in either test. No major compounds were identified from the following fractions: 'C_1', 'C_2', 'D_1', 'E_1', 'E_2', 'F_1' and 'F_2' and all of them failed to give 100% repellence. The 'C_1' supernatant fraction contained coumarins and provided protection from mosquito bites for 1 and 0.5 hrs in choice and non-choice experiments respectively. Fraction 'C_2' did not have the major compounds but gave a protection time of 1 hr in either experiment. Fraction 'D_2' contained essential oils only and it provided a protection time of 2.5hrs in choice experiments. Analysis by MS showed the presence of alpha pinene, 1,3-5 cycloheptatriene, beta phellandrene, (+)-2-carene, 3-carene, eucalyptol and caryophyllene oxide. *L. javanica* offered protection from mosquito bites for 8hrs (choice) and 5.5hrs (non choice experiments). The combined presence of coumarins, flavonoids and essential oils have an additive effect compared with individual plant fractions.

INTRODUCTION

Plants have to survive by all means in the vicinity of predators and threats. Some plants have thorns in order to deter herbivores, which feed on them to ensure species survival or they emit odours with a deterrent effect on insects (Reichardt *et al.*, 1990). These defence mechanisms have been utilised by man for the development of plant – based drugs and chemicals. Watt & Brandiwjk (1962) listed plant remedies and pharmacological products. Other plants of importance in traditional medicine are now being sold as herbal medicines e.g. valerian *(Valeriana officinalis)*, cammone *(Chamomilla recutita)* and garlic (*Allium salivum*) (Tunon, 1995).

Lippia javanica is known as fever tea/ lemon bush and has dense creamy white, flower heads. It grows in open veld, in the bush, grassland on hillsides and stream banks, and as a constituent of the scrub on the fringes of forest. The plant is widely distributed in Zimbabwe, Ethiopia, East Africa and South Africa. Manenzhe *et al.* (2004) studied the chemistry of the volatile oil of *L. javanica* and concluded that it contains several terpenoids of which 3-methyl-6-(1-methyl-ethylidene)-cyclohex-2-en-1-one (1) was the major component. Results suggested that the oil was effective

in inhibiting cultures of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (Manenzhe *et al.*, 2004). Mokoka (2006) carried out a chemical analysis *L. javanica* by using Gas Liquid Chromatography (GLC) and Mass Spectrometry (MS) and concluded that the essential oils were alpha pinene, sebinen, myrcene, 1.8 myrcene, ipsenone, ipsedienone, beta caryophyllene and germacrene D. The leaves of *Lippia origanoides* are widely used as a spice in Brazil and analysis by GLC and MS showed a high content of oxygenated mono-terpenes, carvacrol and thymol (Oliveira *et al.*, 2007). Loy *et al.* (2001) studied the chemical composition of the essential oil of *Calycotome villosa* leaves and isolated falcarinol and some alcohols, terpenes, furan derivatives, and paraffins. A total of 13 alkaloids and falcarinol were identified in the chloroform fraction of the basic methanol extract (Loy *et al.*, 2001). A total of 6 flavonoids and 4 anthra-quinones were isolated in the chloroform fraction after acidification of the basic methanol extract (Loy *et al.*, 2001).

The use of repellents to avoid man/mosquito contact is not a new concept and is well documented (Batchelor, 1930). People use mosquito control methods in order to avoid mosquito bites and disease transmission. Ethno - botanical studies were conducted in order to collect information on traditional uses of plants against mosquitoes and other pests (Curtis *et al.*, 1990; Lukwa *et al.*, 1999). A wide range of plants is used against mosquitoes even though a few of them have been biologically evaluated. Such information is useful for planning conservation programs targeted at natural resources of medicinal value. Thus, people could be discouraged from cutting down plants perceived to be effective against mosquitoes but proved biologically not to be so. Plants are used to supplement several disease control programs in Africa and elsewhere because they are relatively cheap and locally available.

Mosquito repellence has been ascribed to the presence of essential oils from camphor, citronella, lemongrass, clove, thyme, geranium, bergamot, pine, wintergreen, pennyroyal and eucalyptus (Fradin, 1998). *Lippia javanica* and *Ocimum canum* ethanolic extracts repelled mosquitoes for 5hrs (Lukwa *et al.*, 1996). On the other hand, steam distilled essential oils from *Artemesia afra*, *Lantana angiolensis* and *Syzygium huillense* failed to inhibit mosquitoes from biting (Lukwa *et al.*, 2000).

MATERIALS AND METHODS

Mosquitoes

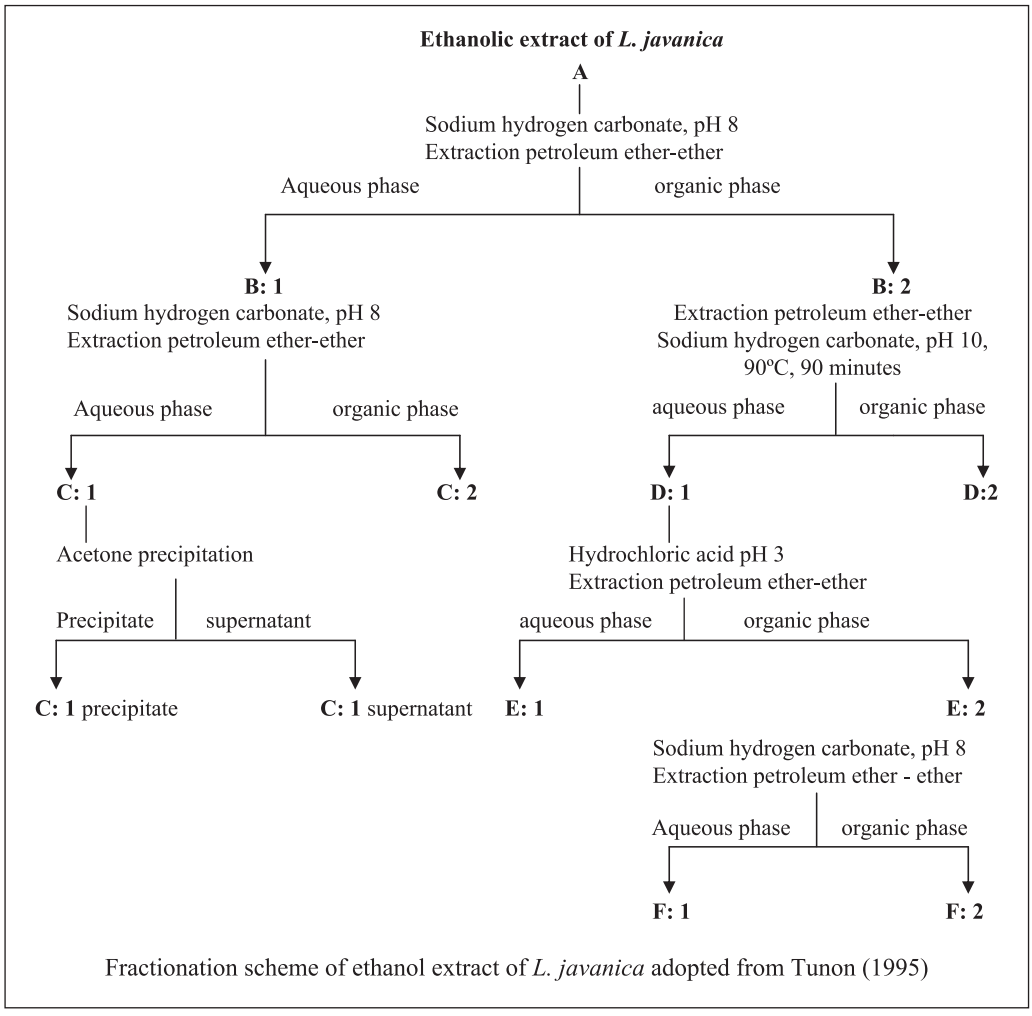
Three to five day old laboratory reared female *Aedes aegypti* mosquitoes (colony from the Danish Bilharziasis Laboratory) were used in all the experiments. The mosquitoes were reared in the insectary at 70-80% Relative Humidity (RH), 25ºC±2ºC and a photo period of 12:12hrs light; darkness. Ground dog biscuits that were supplemented with yeast were used to rear the larvae and female adult mosquitoes were fed on a guinea pig. The mosquitoes were starved for 24 hours before commencing the studies.

Collection of plant material

Lippia javanica (Burm.f.) Spreng (Verbenaceae) was collected from Musana Communal Lands (17º 31'S, 22º 31'E) and forwarded to the Zimbabwe National Botanical Garden for the confirmation of the plant species. A specimen was deposited as a reference.

Preparation of plant material

The leaves of *L. javanica* were dried in the shed by placing them in a single layer for 4 days before grinding them to powder. The powder was placed in absolute ethanol by weighing 100 grams of it into 1 litre of ethanol (extraction was repeated 5 times) and the preparation was placed for sonication overnight. The extraction proceeded as shown in Figure 1 using 50ml of ether. When the 'A' fraction was extracted in ether-ether at pH 8, the aqueous solution gave 5 fractions as follows: 'B_1', 'C_2' and 'C_2', 'C_1' supernatant and 'C_2' precipitate. The organic phase of fraction 'A' gave 7 fractions namely 'B_2', 'D_1', 'D_2', 'E_1', 'E_2', 'F_1' and 'F_2'. According to the



Fractionation scheme of ethanol extract of *L. javanica* adopted from Tunon (1995)

Figure 1. Preparation of plant fractions.

fractionation scheme in Figure 1, 'C_1' fraction ought to contain mostly hydrophilic neutral and / or charged compounds with a low vapour pressure. Acetone precipitation was done in order to remove carbohydrates. The 'C_2' fraction was expected to contain mainly neutral / or acidic compounds. The 'F_2' fraction was expected to contain lipophilic substances with boiling points above 200ºC that should be phenolic in nature and contain fatty acid esters.

Distillation of essential oils

Essential oils from the leaves of *L. javanica* were steam distilled by measuring 10 grams of the plant and placing them into an Erhlenmayer conical flask containing 100ml distilled water. The flask was heated to boiling at 120ºC – 140ºC. About 300*ul* of toluene was used to wash the wall of the collecting funnel and the resultant oil was made up to 5ml toluene.

Mass spectrometry (MS)

The following parameters were used: air (50kPa), hydrogen (60kPa), primary gas (400kPa), carrier 2 gas (300kPa), make up gas (65kPa), detector and injector temperature (220ºC), column initial temperature (60ºC), initial time (10 minutes), programme rate (5ºC/min), maximum temperature (185ºC), running time (35 minutes) and mass detection range (30-300). A total of 0.3*ul* of the essential oil was injected into the MS.

Identification of major plant compounds
A total of 10*ul* of each fraction was applied to a silica gel 60 F_{254} Thin Layer Chromatography (TLC) plate measuring 5cm by 7.5cm. For the determination of coumarins, the toluene-ethyl acetate solvent system was used with methanolic Potassium hydroxide as the spraying reagent. The standards were coumarin, scopelelin and bergapten. Flavonoids were detected in an ethyl acetate-formic acid-glacial acetic acid-water solvent system and detection was done using NST Natural products. The standards were chlorogenic acid, caffeic acid, rutin and hyperoside. Essential oils were detected using the toluene-ethyl acetate solvent system and Vanillin sulphuric acid reagent was used for detection. Carvon, geranyl acetate, menthol and geranol were used as standards.

Choice experiments
A special glove with an opening measuring 5cm by 5cm was used for all experiments. The area was cut out and the edges lined with masking tape. Plant extracts were prepared to give a concentration of 5mg/cm^2. Mosquito cages made from perspex were used and these had an open sleeve for introducing and retrieving mosquitoes. A total of 50 starved female *Aedes aegypti* mosquitoes were released in the mosquito cage and left to acclimatise for 1 hour. Two hands of the same person were placed in the cage at the same time (one was treated and the other one was not for choice experiments and one hand only for the non choice experiments) for one minute. The number of mosquitoes probing to bite was recorded, excluding mosquitoes that took off before biting. Re-testing was done every 30 minutes until repellence was lost. This was done for all fractions, including the control that had ethanol only.

Repellence tests were done following the test method previously described by Curtis *et al.* (1990) and Tunon (1995). Percentage mosquito repellence was calculated following the method previously described by Mehr *et al.* (1985). The calculation was carried out as follows:

$$\left(\frac{\bar{B}c - \bar{B}t}{\bar{B}c}\right) 100$$

Where $\bar{B}_c$ = mean number of bites on control and $\bar{B}_t$ = mean number of bites on treated.

Data analysis
Data was analysed using the Analysis Of Variance (ANOVA) method at 95% confidence limit.

RESULTS

The major constituents of *L. javanica* extract are shown in Table 1. MS was done on steam distilled essential oils from *L. javanica* and the results are shown in Figure 2. 1,3,5 Cycloheptatriene was the most abundant and it constituted 41.97% of all the constituents as compared to alpha pinene (6.77%). Beta phellandrene constituted 1.22% of all the constituents and (+)-2-Carene was 0.54%. Eucalyptol constituted 5.77% of the compounds and 3-Carene was 1.11%. Caryophyllene oxide and Alloparinol constituted 0.97% and 0.33% respectively.

In experiments where mosquitoes were not given a choice, fraction 'A' had a protection time of 5.5 hours from mosquito bites (Table 1). The mean protection times in choice experiments were 1.19 hours and those in no choice experiments were 0.73 hours and the results were not significantly different using t-test (p=0.543698).

In choice experiments, fractions containing flavonoids and coumarins offered protection from mosquito bites for 1 hour and the results are shown in Figure 3. The longest protection time was realised with fractions containing essential oils, coumarins and flavonoids. When non-choice experiments were conducted, protection times were less than those obtained in choice experiments (Table 1).

DISCUSSION

The process of fractionation applied to *L. javanica* produced different fractions that were analysed. Our results showed that the 'A' fraction contained coumarins, flavonoids and essential oils as compared to the basified

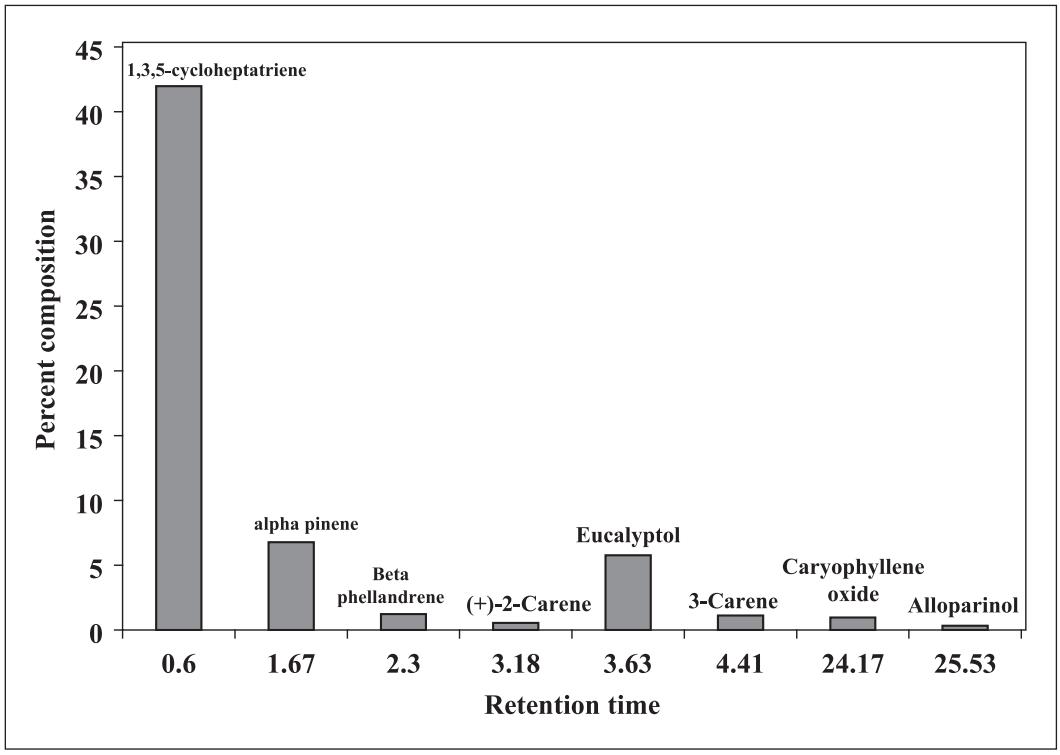


Figure 2. Compounds detected by mass spectrometry.

Table 1. Protection times of *L. javanica* on *Ae. aegypti* mosquitoes

Fraction	Major plant compounds	Non choice experiments	Choice experiments
A	Coumarins, flavonoids and essential oils	5.5 hours	8 hours
B_1	Flavonoids	0.5 hour	1 hour
B_2	Coumarins and essential oils	2 hours	2 hours
C_1	—	—	—
C_1 supernatant	Coumarins	0.5 hour	1 hour
C_1 precipitate	Coumarins and flavonoids	—	—
C_2	—	1 hour	1 hour
D_1	—	—	—
D_2	Essential oils	—	2.5 hours
E_1	—	—	—
E_2	—	—	—
F_1	—	—	—
F_2	—	—	—

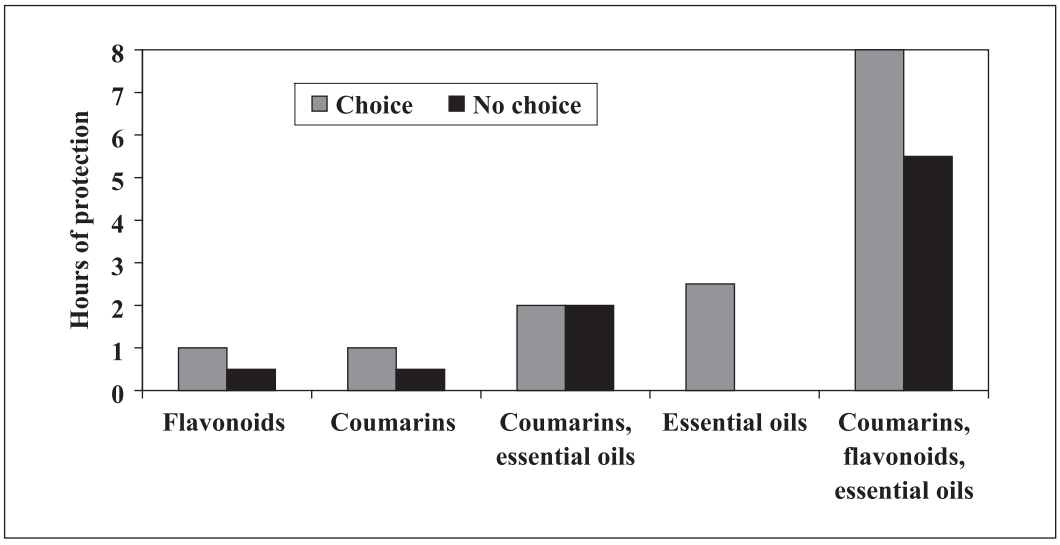


Figure 3. Mosquito repellence in choice and no choice experiments (n=5; SD=2.37641)

aqueous solution of ' B_1' that had flavonoids only. Loy *et al.* (2001) detected flavonoids after acidification of the basic methanol extract of *C. villosa* leaves but we failed to detect any flavonoids after undergoing the same process in fractions 'E_1', 'E_2', 'F_1' and 'F_2' of *L. javanica*. The resultant aqueous solution from 'B_1' (C_1) did not have the major compounds and this might be due to the basic nature of the extract (pH 8). However, after precipitation, the supernatant contained coumarins and the precipitate contained coumarins and flavonoids. Our results suggest that the process of precipitation is required in order to make the detection of major compounds easy. Flavonoids and essential oils could not be detected in fractions 'C_1', 'C_2', 'D_1', 'E_1', 'E_1', 'F_1' and 'F_2' and only coumarins were detected in 'B_1' and 'C_1' supernatant. Our results suggest that individual fractions do not contain all major compounds found in the parent plant. Our results on MS revealed the presence of 1,3,5 cycloheptatriene, alpha pinene, beta phellandrene, (+)-2-carene, eucalyptol, 3-carene, caryophyllene oxide and alloparinol. Studies by Mokoka (2006) showed the presence of alpha pinene and caryophyllene in *L. javanica*.

Experiments were conducted in situations were mosquitoes were either given a choice of biting one treated hand only or a combination of a treated and untreated hand at the same time. Our results suggest that more protection from mosquito bites is obtained when mosquitoes have a choice than when there is no choice at all. The results also indicate that an 8-hour protection time can be obtained with the main preparation (fraction 'A') when there is a choice than when there is no choice (5.5). Our results compare well with studies done on DEET as reported by studies performed by Tunon (1995) and Curtis *et al.* (1990). Lukwa *et al.* (1996) recorded a repellent activity of *O. canum* of 5 hours and our results on choice experiments appear superior. Curtis *et al.* (1990) ascribed mosquito repellence properties to the presence of essential oils but our results suggest that the oils alone are not very effective since they give a short repellent period and this has been observed by Lukwa *et al.* (2000).

The level of protection from mosquito bites becomes less important from fraction 'A' to E_2, suggesting that all the compounds in a plant have a synergistic effect. It was also interesting to note that fractions where no major compounds were detected did not offer any protection from mosquito bites.

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