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Mosquito Repellency of Whole Extracts and Volatile oils of *Ocimum americanum, Jatropha curcas* and *Citrus limon*

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ABSTRACT

The study was carried out to evaluate the efficacies of the crude extracts and of the essential oils from three plants which are used as mosquito repellents. The crude extracts were obtained from the leaves of Ocimum americanum and Jatropha curcas, and from fruit peels of Citrus limon by dry distillation and the volatile oils were isolated from the crude extracts using separating funnels. The mosquito repellency of the crude extracts and of the volatile oils were evaluated using the human-bait technique, whereby hands treated with a pre-determined minimum dose of the repellent were exposed to mosquitoes and the extent of repellency determined, using DEET as standard, revealing that the oils were much more repellent than the crude extracts. The mixtures of crude extracts and the mixtures of volatile oils generally gave higher protection than their respective single repellents. Thus, the components of the different repellents appeared to reinforce each other.
Key words: *Mosquito repellents; crude extracts; volatile oils; Ocimum americanum;Jatropha curcas; Citrus limon*

INTRODUCTION

The global incidence of malaria has been estimated at 300 - 500 million clinical cases annually, causing 1.5 - 2.5 million deaths each year. More than 90% of these occur in sub-Saharan Africa where severe malaria and death affect mainly children of rural areas with little access to health care services [1]. Malaria causes substantial losses to national economies in terms of lost productivity, costs of treatment, school and work absenteeism, and funeral costs [2, 3]. Economists believe that malaria is responsible for a 'growth penalty' of up to 1.3% per year in some African countries, worsening the poverty burden [3, 4].

Conditions that favour mosquito breeding, for example the natural disasters like cyclone Eline which promoted the breeding of mosquitoes, have led to increases in malaria cases in Zimbabwe, increasing drug resistance of *Plasmodium falciparum* to chloroquine and fansidar, and increasing mosquito resistance to DDT-based mosquitocides [5].

The intensity of the malaria burden varies substantially even between regions of the same country [6]. In Zimbabwe, approximately 5.5 million people out of a population of 12.5 million live in malaria endemic areas [7]. The inhabitants of these areas could guard against mosquito bites by spraying their houses/huts with mosquito repellents or with mosquitocides [8], or by using personal protection measures [9]. Personal protection has the added advantage of giving protection in outdoor areas but it is becoming increasingly difficult due to increasing resistance of mosquitoes to pesticides and repellents [9]. DEET, a commercial repellent which has been in worldwide use since 1957, has been reported to have disadvantages associated with its activity as a solvent of paints, varnishes, and some plastic products such as watch crystals, frames of spectacles, and certain synthetic fabrics. There have also been concerns over its toxicity. Its continuous application has been reported to cause in-folding of the epidermis and a thickened dermis [10-12].

Thus, there is need to evaluate plant-based repellents in order to supplement conventional control methods. Mosquito repellent properties of plants were well known before the advent of synthetic chemicals [13]. A wide range of plants have been used for centuries for repelling mosquitoes and other insects using varying techniques such as burning plant materials to generate smoke that repels mosquitoes [14], hanging bruised fresh plants in houses [15], and placing potted plants inside houses [16].

Reports from clinics and non-governmental organizations in Africa, where 80%of the world's malaria burden exists, indicate that the poorer members of society are now using traditional medicine at least for economic reasons [17, 18]. Mosquitocidal and mosquito repellent properties of plants have been investigated over the years, revealing that people use plants because they are cheap, effective, and locally available [8]. The repellency of plants may differ dependent on the region or cultivar of growth [19].

MATERIAL AND METHODS

This naturalistic inquiry involves evaluation of the efficacy of crude extracts of some selected plants and oils extracted from the crude extracts as mosquito repellents against laboratory-reared *Aedes aegypti* mosquitoes, using the human-bait technique [20, 21]. Hands treated with a pre-determined minimum dose for both the whole extract and the extracted volatile oils were exposed to mosquitoes and the extent of repellency determined based on the relationship:

% repellency = $[(N_c - N_t) / N_c] \times 100$, where N_c is the number of mosquitoes landing on the control subject, and N_t is the number of mosquitoes landing on the treated subject (Oshaghi et al., 2003). Mosquitoes, which had been starved for one hour prior to repellency tests, were then released into a 5-liter cage where they found the host (treated hand). The untreated hand was used as a negative control while the one treated with DEET was used as a standard or positive control. Experiments to determine the landing time, the exposure time, and the dose finding experiments were carried out.

The repellency experiments were conducted using each of the whole extracts, and using ethanol as control and DEET as standard, performing each test three times, replacing the mosquitoes and rotating the volunteers. The number of mosquitoes landing on the control, on the treated hands, and on the one treated with DEET were recorded. The procedure was repeated using mixtures of whole extracts and further repeated using the extracted volatile extracts, and finally using mixtures of the volatile oils.

Citrus limon, *Jatropha curcas*, and *Ocimum americanum* were chosen for this study because of their historical medicinal use as well as their use in mosquito control by the rural people of Zimbabwe do not appear to have been studied in Zimbabwe although similar plants growing in other regions of the world have been reported. It is desirable to establish if the Zimbabwean plants will show the efficacy reported for similar plants from other regions, since the growing environment of a plant affects its chemical composition.

Citrus limon (Rutaceae)

Citrus limon is thought to have originated from India and introduced into Italy toward the end of 5th century. Lemons have been used to control malaria in the form of mosquito repellents over the centuries all over the world and have been used as food and food additives [19, 22, 23].

Jatropha curcas (Euphobiaceae)

The origins of *Jatropha curcas* is still uncertain but it is believed that the plants originated from Mexico and Central America [24] and is now cultivated worldwide for the latex from its bark, and oil from its seeds [25]. The oil is used to treat skin ailments and as cosmetic cream and the leaves are used to repel mosquitoes in some parts of Zimbabwe by hanging them in houses or by burning them during the day to control mosquitoes during the evening (personal observation).

Ocimum americanum (Lamiaceae)

Ocimum americanum is commonly known as Lime Basil, an annual plant which grows under full sun to partly shady conditions in average soils. The plant, as well the as oils from it, have received considerable attention for their potential medicinal properties. The leaves have been shown to have antibacterial essential oils [26]. The seeds germinate easily, producing the plant which is used for culinary purposes as a fragrance accent for soups, salads, and fish and is good a tea as well [27]. The juice of the leaves of the plant is used for the treatment of catarrh and the volatile oils from the plant have been shown to be mosquito repellent and to have larvicidal activity [28].

About 1.0 kg of each of the leaves of *O. americanum and J. curcas,* and about 1.0 kg of fruits of *C. limon* were gathered in Mt Darwin in February 2007 and transported to the Department of Chemistry, Bimdura University of Science Education, Bindura, Zimbabwe, for processing. The samples of leaves *O. americanum* and *J. curcas* were crushed (mortar and pestle) and spread on the laboratory bench overnight. The crushed leaves of each plant (300 g) were heated at about 120^{o}C in a round-bottomed flask of the distillation apparatus until the dry distillation was complete, cooling the condenser with cold water and collecting the distillate in 100 cm^3 conical flasks. The procedure was repeated with 300 g of *C. limon* fruit peels which had been cut to small pieces. The distillates (the whole extracts) consisted of the volatile compounds, including water, which were released from the raw material by the heat. These were allowed to cool to room temperature, their volumes and masses recorded, and then kept at 4^oC until required for further experiments.

Extraction of volatile oils from the whole plant extracts

The distillate from each plant sample (50 cm^3) was separated into the aqueous and oil phases using a separating funnel, draining off the aqueous phase until only the oil remained, yielding *O. americanum* (4.4 cm^3), *C. limon* (5.1 cm^3), and *J. curcas* (4.0 cm^3) oils. These oils were kept at 4^oC in stoppered 10 cm^3 plastic sample tubes until they were tested for mosquito repellency [28].

Preparation of mixtures for mosquitoe repellency tests

The extract mixtures were prepared on the basis of the minimum doses established through 'dose finding experiments'.

Thirty-five laboratory- reared 5-8 day old mosquitoes which had been starved for an hour were paled in a 5-litre cage. The starving reduced the time needed for the mosquitoes to start landing on the host in search of a meal. Limiting the number of mosquitoes to 35 made it easier to count the mosquitoes landing on the host. The mosquito cage had a mosquito-netting on top and a muslin sleeve on the side for introducing and removing the mosquitoes.

Repellency experimental design

A special glove with an opening measuring 6 cm by 6 cm, giving an open area of 36 cm^2, was used in each of the experiments. The edges of the opening were lined out with a masking tape. Using a dropper to apply each test repellent, a 0.5 cm^3 dose was applied on to the open area of the hand and the % repellence during the 3-minute exposure time was determined. The dose was increased by 0.5 cm^3 for each succeeding experiment, calculating the % repellence after each dose, until a dose that gave 100 % repellence during the 3-minute exposure time was achieved (Figure 1). This dose was the minimum amount of extract that gave complete protection from mosquito landing and was used in all experiments. The % repellency (% protection) was calculated basing on the relationship: % repellency = $[(N_c\text{-}N_t)/N_c]$ x100 where N_c is the mean number of mosquitoes landing on the control subject, and N_t is the mean number of mosquitoes landing on the treated subject [19]. Percentage protection is defined as the average number of bites received by the subjects in the treated trial relative to that of the control [19].

For each repellent, the minimum dose established in the dose finding experiment was applied to the exposed area of the hand. The hands of the respective volunteers were placed for 3 minutes in the cage containing mosquitoes which had been starved for an hour. This was repeated at 30 minute intervals until repellency was completely lost. Three replicates were run for each repellent and in each replicate different volunteers were used to nullify any effect of skin differences on repellency. Before each test, the skin of volunteers was washed using unscented soap and the repellent being tested was applied to the exposed skin. After the application, the hand was not allowed to be rubbed, touched, or wetted. An untreated hand was used as control and the one with DEET was used as a standard. All experiments were run at ambient temperature (27 ± 2^o C) and relative humidity of 80 ± 10 %. The numbers of mosquitoes landing were recorded and the mean percentage protection was calculated [19].

Data analysis

The mean protection time from replicate tests was used as the standard measure of repellency for all test repellents against *Aedes aegypti* mosquitoes. Two-way analysis of variance (ANOVA) was used to determine the significance of the differences in repellency at 95 % confidence level between different test samples. All P values are two sided, and a P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

The minimum volumes that gave 100 % protection from *Aedes aegypti* mosquitoes were established from the dose finding experiments and these were applied on to the exposed areas of the hand in all experiments. The results show that repellent activity was dose dependent with oils requiring lower doses than whole extracts, and mixtures requiring lower doses than single test repellents.

The results indicated that the minimum dose for *O. americanum* oil was 1 cm^3, that for *C. limon* oil was 1.5 cm^3, and that for *J. curcas* oil and *O. americanum* extract was 2 cm^3, that for *C. limon* extract was 2.5 cm^3 and that for *J. curcas* extract was 3 cm^3. Hence in all experiments involving *O. americanum oil*, the dose used was 1 cm^3 and in those involving *C. limon* oil the dose was 1.5 cm^3. In all experiments involving *J. curcas* oil and *O. americanum* extract the dose was 2 cm^3, and in those involving *C. limon* extract the dose was 2.5 cm^3 and 3 cm^3 in those involving *J. curcas* extract.

The minimum dose for *O. americanum / C. limon* extracts mixture was 1.5 cm^3 and that for *O. americanum / J. curcas* extracts mixture and for *J. curcas / C. limon* extracts mixture was 2 cm^3. Hence in all experiments involving *O. americanum / C. limon* extracts mixture, the dose used was 1.5 cm^3. In all experimrnts involving *americanum / J. curcas* extracts mixture and for *J. curcas / C. limon* extracts mixtures the dose of 2 cm^3 was used. **T**he minimum dose for the mixtures *O. americanum / C. limon, O. americanum / J. curcas, and J. curcas / C. limon* oils was 1.5 cm^3. Hence in all experiments involving these mixtures of oils, the dose used was 1.5 cm^3.

Table 1: Percentage mean repellency of whole plant extracts against Aedes aegypti mosquitoes

Plant extract	100% repellence (hours)	Repellence below 70% (hours)
O.americanum	1.5	3.5
C. limon	1.0	2.5
J. curcas	0.5	2.0
O.a /C.l	1.5	5.0
C.l /J.c	0.5	4.5
O. a / J.c	1.0	4.0
O.a /J.c /C.l	2.0	6.0

Key

O. a = O. americanum C. = C. limon J. c = J. curcas
O. a /C. l = O. americanum / C. limon mixture
C. l /J .c = C. limon / J. curcas mixture
O. a /J. c / C. l = O. americanum / J. curcas / C. limon mixture

Table 2. Percentage mean repellency of volatile oils and their mixtures against Aedes aegypti mosquitoes

Time in Hours	Time spent giving 100% repellence (hours)	Time for repellence to fall below 70%
O.americanum	1.5	5.5
C. limon	1.5	5.0
J. curcas	1.0	4.0
O.a /C.l	2.0	4.0
C.l /J.c	1.0	4.5
J. c / O. a	1.0	6.0
O.a /J.c /C.l	2.0	8.0

Key

O. a = O. americanum C. = C. limon J. c = J. curcas
O. a /C. l = O. americanum / C. limon mixture
C. l /J .c = C. limon / J. curcas mixture
O. a /J. c / C. l = O. americanum / J. curcas / C. limon mixture

DISCUSSION

The ideal mosquito repellent would be the one that gives 100 % protection against mosquito bites over the desired time. Obtaining such a plant based repellent that gives such a high protection over long periods of time is not easy. A cut-off point for acceptable percentage biting protection needs to be chosen for discussions of effective repellencies of plant-based repellents. Citrus limon which gave 71 % protection against *A. stephensi* has been described as giving acceptable percentage biting protection, hence effective, and *Melissa* oil which gave 60 % protection was described as not effective [19]. For our discussion, we chose 70 % protection as effective and acceptable percentage biting protection, and describe levels below 70% as ineffective.

2 (a) Mean % repellency of whole plant extracts

O. americanum whole extract gave 100 % protection for 1.5 hours whilst that of *C. limon* and *J. curcas* gave 100 % protection for 1.0 hour and 0.5 hour respectively. The effectiveness of *J. curcas* fell rapidly and approached the cut-off point after 2.0 hours, when it became 74 %, whilst that of *O. americanum* and *C. limon* were 94 % and 80 %, respectively. The effectiveness of *C. limon* fell to 73 % after 2.5 hours compared to that of *O. americanum* which fell to 73 % after 3.5 hours (Table 1).

2 (b) Mean % repellency of extracted volatile oils

O. americanum and *C.limon* oils gave 100 % protection for 1.5 hours whilst *J. curcas* gave 100 % protection for 1.0 hour. The effectiveness of *O. americanum* and *C. limon* fell more or less at equal rates, *C. limon* falling to 71 % after 5.0 hours and *O. americanum* reaching 71 % after 5.5 hours, compared to *J. curcas* which reached 70 % 4.0 hours post application (Table 2). Thus, the effective protection times of oils were roughly double those of the whole extracts.

2 (c) Mean repellency of mixtures of whole extracts

O. The mean repellencies of whole extracts of *americanum, C. limon,* and their mixtures

The mixture was a more effective repellent than either of the single extracts. It would appear that the mixtures acted in synergism, improving the effectiveness to 5.0 hours post application for the mixture, compared to 2.5 hours for *C. limon* and 3.5 hours for *O. americanum* (Table 1).

(ii) Mean repellency of whole extracts of *O. americanum, J. curcas* and their mixtures

That the mixture gave 100% protection for 1.0 hour post application compared to *O. americanum* and *J. curcas* which gave 100 % protection up to 1.5 and 0.5 hours, respectively, would suggest that the components of the two extracts interacted in a way that lowered the repellency of the components of *O. americanum,* resulting in depressed repellent effects for the mixture. However, the overall effectiveness of the repellents was improved from 3.5 hours for *O. americanum* and 2.0 hours for *J. curcas* to 4.0 hours for the mixture. Thus, overall, the components of the extracts appear to act in way that enhances the effects of one another (Table 1).

(iii) Mean repellents of whole extracts of *C. limon, J. curcas*, and their mixtures

The mixture gave 100 % protection for 0.5 hour compared to *J. curcas* and *C. limon* which gave 0.5 hour and 1.0 hour, respectively. Thus, the components of *J. curcas* appeared to interact with the components of *C. limon* in a way that suppressed the components of *C. limon.* But 2.0 hours post application they appeared to reinforce each other resulting in the mixture giving better protection than either of the single extracts, raising the effectiveness of the mixture to 4.5 hours post application, compared to 2.0 hours for *J. curcas* and 2.5 hours for *C. limon* (Table 1).

2 (c) Mean repellency of mixtures of oils of test repellents

The mixture of oils of *O. americanum, C. limon* gave 100 % protection for 2.0 hours compared to *O. americanum* and *C. limon* which gave 100 % protection for 1.5 hours each. In general the effectiveness of the mixture was higher than that of either of the single volatile oils. The effectiveness of the mixture persisted for 8.0 hours post application compared to that of *O. americanum* which fell off after 5.5 hours and that of *C. limon* which fell off after 5.0 hours. Thus the components of the oils reinforced each other to give a better repellent (Table 2).

(ii) Mean repellency of oils of curcas, C. limon, and their mixture

The mixture gave 100 % protection for 1.0 hour compared to 1.5 hours for *J. curcas* and 0.5 hour for *C. limon.* However, the effectiveness of the mixture was generally higher, 76 % at 4.5 hours post application and falling to 64 % at 5.0 hours, compared to *J. curcas* which gave 70 % at 4.0 hours post application falling to 68 % at 4.5, and *C. limon* which gave 71% at 5.0 hours post application and falling to 68 % at 5.5 hours.

(iii) Mean repellency of oils of J. curcas, O. americanum, and their mixtures

The effectiveness of the mixture persisted for 6.0 hours post application, giving 71 % protection compared to *O. americanum* oil which gave 71 % protection at 5.5 hours and *J. curcas* which gave 70 % protection at 4.0 hours post application. The oils of *J. curcas* appeared to suppress the repellency of the oils of *O. americanum* at higher concentration in the first two hours post application, but as the concentrations fell off due to evaporation the two oils appear to reinforce each other and raise the overall effectiveness of the mixture. Results showed that extracted volatile oils were significantly more effective than whole plant extracts. This might be due to the greater concentration of bioactive components that repel mosquitoes in oils than in the bulkier solutions of whole extracts. Hence *O. americanum* oils with protection time of 5.5 hours were better than *C. limon* and *J. curcas oils* with protection times of 5.0 hours and 4.0 hours respectively. Their respective whole extracts had protection times of 3.5, 2.5, and 2.0 hours, respectively. Results of statistical analysis revealed significant differences between whole extracts and extracted volatile oils (P less than 0.05) against *Aedes aegypti* mosquitoes for all the plants evaluated. Thus, extracted oils were more effective mosquito repellents than their respective whole extracts; mixtures of oils were more effective repellents than their single oils, and their repellencies were not significantly different from Deet over the first two or three hours.

CONCLUSIONS

Extracts from different mosquito repellent plants could be prepared and tested and produce effective mosquito repellents which could compete or supplement repellents which are currently on the market.

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