International Journal of Mosquito Research

ISSN: 2348-5906
CODEN: IJMRK2
IJMR 2018; 5(5): 31-36

Received: 25-07-2018
Accepted: 26-08-2018

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Repellency of the essential oil extracted from the leaves of a local variety of *Artemisia scoparia* Waldst. & Kit. against *Aedes aegypti* L. using modified Y-tube olfactometer

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Abstract
Essential oil from *Artemisia scoparia* leaves was assayed for repellency against adult female *Aedes aegypti* L. using a modified Y-tube olfactometer. Three concentrations consisting of 10, 20, and 30 μl of the essential oil placed in 1x1 cm filter paper were separately assayed in the olfactometer against female *A. aegypti* adult mosquitoes. Results showed that 10 and 20 μl *A. scoparia* essential oil were effective repellent against *Aedes aegypti* as compared to control in one-way ANOVA (F_{cal} 13.57>F_{crit} 4.96, *P*, 0.004, *P*= 0.004219) (F_{cal}, 69.39>F_{crit},4.96, *P*= 8.25x10^{-6}) at 0.05% confidence level. Comparison of 30 μl *A. scoparia* essential oil repellency with a DEET-based commercial mosquito repellent showed comparable performance (f_{calc} 0.019<f_{crit} 4.30, *P*=0.89). On the basis of these results, it is concluded that *A. scoparia* essential oil is a potent repellent against *Aedes aegypti* and has comparable efficacy to the current DEET-based commercial mosquito repellent.

Keywords: Aedes, essential oil, repent, efficacy, olfactometer

1. Introduction

With increasing concerns of dengue, chikungunya and zika diseases, it is necessary to use all available means of protection against the mosquito vectors, *Aedes aegypti* and *Aedes albopictus* [1] such as the use of plant-based repellents [2] that contain secondary metabolites used as defense against insects herbivores. These repellents trigger behaviors that draws insects away thus are considered better than synthetic molecules because they are environment-friendly [2]. One of these plant species is the Oriental wormwood or Redstem wormwood, *A. scoparia* (Waldst et Kit). that is widely distributed across Eurasia and Africa [3]. This plant is popularly used as anticholesterolemic, antipyretic, antiseptic, cholagogue, diuretic and vasodilator [3].

Currently, tests of various substances in their effects on mosquito host-seeking has inspired a number of different *in vitro* methods of measuring mosquito response [4]. These *in vitro* methods are generally classifiable into three main categories, namely: olfactometers, alternative choice test systems, and in vitro blood-feeding test systems. Olfactometers, first fabricated in 1907 by Barrows, are instruments that detect and measure an organism's keenness of smell [5]. In mosquito research olfactometers are used to measure host-seeking as well as effects of repellent substances. In this study, the repellency of essential oil in *A. scoparia* against adult, female *Aedes aegypti* was tested using a modified version of the Y-tube olfactometer [6]. This is to determine whether the treatment with essential oil of *A. scoparia* and control have the same effect on the attraction of female *Aedes aegypti* to the volunteer's hands in the modified stimulus chamber thus making it a cheap alternative for the expensive olfactometer. Plant-based repellents has been exploited for thousands years by man most simply by hanging bruised plants in houses, a practice that is still widely used in developing countries [2]. In the Philippines, Filipinos normally sweep, gather, and burn dead leaves in their surroundings to smoke out unwanted biting insects like bees and mosquitoes. It is argued that the burning of plants releases insecticidal plants releases insecticidal and irritant chemicals that may repel hemophagous insects.

But this practice normally burn along dangerous materials with it such as discarded plastics and cellophanes that are left in the house premises, and thus has led to the passage of the "Philippine Clean Air Act of 1999" or Republic Act No. 8749 that prohibits burning and releasing fumes of smoke in house premises that may contribute to air pollution [7]. At present, Natural volatilization via hot plates or dermal convection has recently been favored over direct burning of plant materials because vapors resulting from thermal release have a richer compositional profile of volatiles than fumes resulting from direct burning [7]. Thermal volatilization method of releasing active chemicals has been found to release more repellent than burning. These active chemicals are classified as either repellents, feeding deterrents, toxins, or growth regulators and may also are effective repellents against mosquitoes [8]. These compounds are generally contained in essential oils, a complex mixtures of volatile organic compounds produced as secondary metabolites in plants that may be obtained by hydro distillation, steam distillation, dry distillation, or mechanical cold pressing of plants [9]. *A. scoparia* essential oil has been shown to possess insecticidal activity against the pesweevils [10] but has never been evaluated against mosquitoes, especially on *Aedes aegypti*, the mosquito vector of dengue, chikungunya and zika [1]. This study was therefore conducted to investigate the repellency of the essential oil of *A. scoparia* using a modified olfactometer designed by the senior author to determine whether it can be an effective repellent against dengue vector mosquitoes.

2. Methods

2.1 Collection and Steam Distillation of *A. scoparia*

Branches and sprigs of *A. scoparia* (Fig. 1A) were obtained from a hedge row along residence areas in La Buena Vida Subdivision, Gran Europa, Lumbia, Cagayan de Oro City and brought to MSU-IIT, Iligan City. Only leaves of *A. scoparia* were used. These were cut into tiny pieces prior to extracton (Fig. 1B) to obtain essential oil. Only about 1.2 kls of *A. scoparia* leaves were cut, immersed in about 0.8 liter of water, and were then subjected to steam distillation for 3 hours (1B). The steam distillation was conducted at the Chemistry Laboratory, College of Sciences and Mathematics, MSU-IIT. Thereafter, approximately 1.0 ml of essential oil from *A. scoparia* leaves (Fig. 1C) was obtained from the separator funnel, stored in a small, sealable plastic bottle and was set aside for subsequent repellent assay.

Fig 1 (A): A. *Scoparia* seedlings*:* (B) Steam Distillation set-up, and (C) Essential Oil

2.2 Collection and rearing of the test organisms, *Aedes aegypti* mosquitoes.

There were generally two sources for adult, female *Aedes aegypti* used in these experiments. On several occasions, live adult female *Aedes* mosquitoes were directly collected from water-logged tires in vulcanizing shops or truck parking lots scattered around Cagayan de Oro City, using prokopack aspirators (See Fig. 2). The mosquitoes collected were immediately transferred to Barraud cages and brought to the laboratory. In the lab, the adult mosquitoes were temporarily knocked down in a refrigerator and sorted into adult, female. All males were killed and discarded.

Fig 2: Collection of adult *Aedes* mosquitoes using Prokopack aspirator (J. Hock Co.)

The other source of adult mosquitoes were pupae of *Aedes* collected from the same water-logged tires in vulcanizing shops or nearby trucking parking lots, using kitchen baster. The pupae were then transferred and reared to adulthood in recycled mineral water bottles and capped with cut pvc pipes covered with 4 cm^2 muslin cloth at the top. Adult mosquitoes that emerged from pupae after 24-36 hours were immediately collected using handheld, battery operated vaccum cleaner and transferred to 1x1 ft cages prior to repellency experiments. Prior to experiment proper, the emerged mosquito adults were temporarily knocked down in cool temperature, identified, sexed and stored in Barraud cages. Identification of female *Aedes aegypti* were based on antennal hair, scale patterns at the scutum, palpi and ventral abdomen [11]. All live female *Aedes* mosquitoes were stored in stacks of Barraud cages in the laboratory for one day prior to repellent assay in modified Y-tube olfactometer.

2.3 Olfactometer assay for essential oil repellent.

The process of repellent assay for *A. scoparia* essential oil was conducted in a laboratory with a mosquito-rearing facility based in Cagayan de Oro City, using a modified Y-tube olfactometer. The Y-tube olfactometer is an *in vitro* method of testing repellents and attractants against insects [5]. It consists of fork-shaped set of three connected tubes with the stem as the point where the insects were introduced into the system. The stem then forks out into two tubes which presents two alternatives where the test insects can enter into or not at all. In one end of these "alternative" tubes, an experimental substance (attractant or repellent) was introduced which served as the "treatment" chamber, while in the other end of the second tube, none of the test substance was presented and may therefore serve as "control" treatment/chamber.

During experiment proper, adult female *Aedes* mosquitoes were introduced into the modified Y-tube olfactometer via "release" and "decision" chambers (See fig 2). Modifying from the protocol of Obemayer *et al* [6], a strip of 1 cm^2 filter paper were used as substrate for *A. scoparia* essential oil treatments and commercial repellent that were mounted on small aluminium foil squares so as not to contaminate the stimulus chamber and the volunteers' hands. The number of adult female *Aedes aegypti* that entered the test chambers from the decision chambers were counted at 5 minutes

interval for a stretch of 30 minutes. After 30 or so minutes, the treatments were removed from the stimulus chambers and the number of adults that entered the chambers after were counted as well. This data would confirm if the number of adults were indeed affected by the treatments.
The detailed parts of the modified Y-tube olfactometer, adapted from Geier and Boeckh, 1999 [12] consisted of the following parts. A *release chamber* (1) from which female *Aedes* mosquitoes were allowed to fly into a spacious *decision chamber* (2) [refer to fig 2 below]. Once inside the decision chamber, the *Aedes* mosquitoes were allowed to sense the skin volatiles emanating from a subject's left and right hands that were inserted into two stimulus source chambers (4). Prior to inserting the hands into the stimulus source, the subject was given time to exercise both hands by squeezing a silicon-made grip balls in order to trigger sweat gland activation and release sufficient amount of skin volatiles into the stimulus chamber to ensure attraction to the *Aedes* mosquitoes. One of the stimulus chamber contained a 1x1 cm filter paper soaked with a particular amount of *Artemisia* leaf essential oil and a volunteer's hand that is inserted into the chamber, this was the *treatment chamber*. And the other stimulus chamber contained only the volunteer's inserted hand as *control chamber*. To avoid bias, the treatment and control chambers were switched during replicate tests. Assay for repellency were replicated only twice. The stimulus chambers were also wiped clean with pure water after each run of the experiment to get rid of residual scents of both skin volatiles and essential oil. A single test run lasted only for 30 mins, after which the test substance was removed. The number of mosquitoes that entered the test chambers during the assay time and after assay time were counted and recorded.

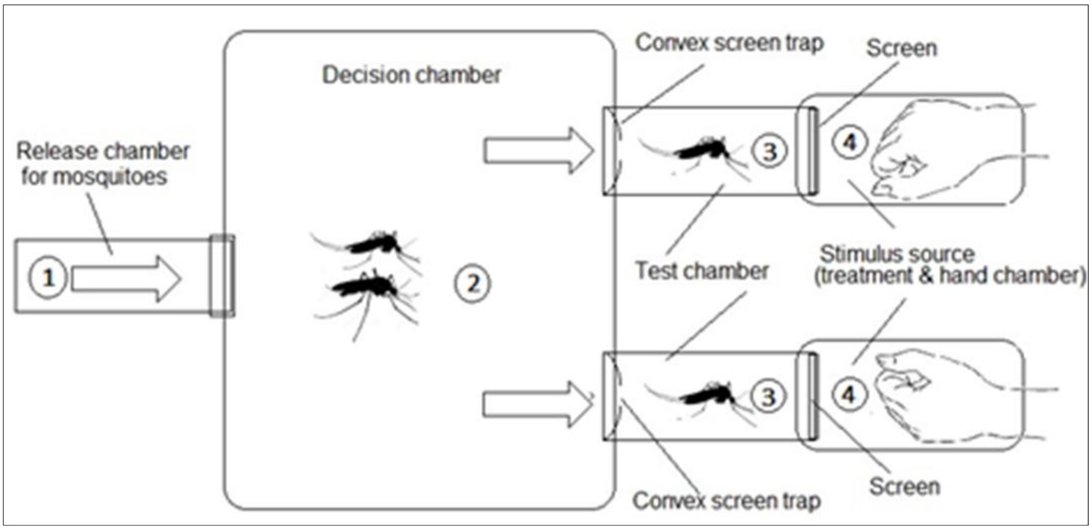

Fig 3: Top view of the modified Y-Tube Repellent Assay Set-up with their corresponding parts. Mosquito image sizes exaggerated but all other parts proportional in sizes.

During each run of the essential oil repellency tests, adult *Aedes* female mosquitoes were allowed to fly into either of the two trap/test chamber (3) which had a proximal end which is open to the decision chamber, thereby allowing test mosquitoes to fly in but closed by a 1 mm mesh screen at the distal end which held the same test mosquitoes from flying into to the stimulus chamber (4).
The novelty of this modified Y-tube olfactometer is that it does not require a volunteer's hand to be subjected to being bitten by *Aedes* mosquitoes because a screen is installed beween the capture chamber and the stimulus chamber. This design thereby avoid the dangers of vertical transmission of pathogenic arbovirus that might be passed on from parent mosquito to its offspring. It should be noted that the *Aedes* mosquitoes are wild-caught and therefore, has possibility of being an active vector. The set-up further prevents direct application of a topically untested chemical treatment, such as this essential oil of *A. scoparia* which has not yet been tested for topical application on humans. Other literatures and webpages, interestingly, has mentioned that *Artmesia* topical testing should be done with care because some of their chemical compounds may produce negative dermal effects on human skin.

3. Results
Repellent assay for 10, 20 and 30 microliter (μl) of *A. scoparia* essential oil in chamber 1 was compared to the control in chamber 2 (See Fig. 4). *Aedes* female mosquitoes almost immediately flew into the test chambers, most likely differentially attracted to the skin volatiles of the hands of the test volunteer. Results shown in Fig. 4 demonstrate that 10 μl (0.01 ml) of *A. scoparia* essential oil may not be sufficient to block the entry of *Aedes* mosquitoes into the *treatment* test chamber, but nonetheless reduce the attraction of *Aedes* mosquitoes to the subject's hand. One way-ANOVA calculation showed (Appendix 1) that there is significant difference between treatment with *A scoparia* essential oil and control (F_{cal} 13.57>F_{crit} 4.96, *P*, 0.004, *P*= 0.004219). Continued observation of the test chamber at the trials after thirty minutes appeared to show residual effect of *A. scoparia* essential oil volatiles. Less adult *Aedes* flew into the treatment chamber even after the trial ended.

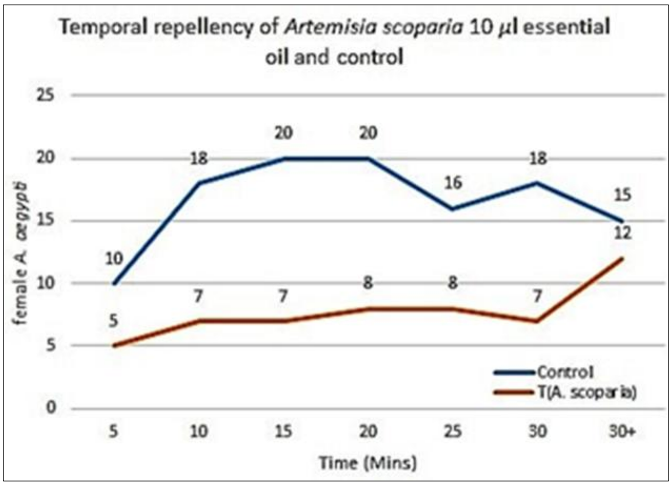

Fig 4: The count of adult female mosquitoes that entered the test chamber under *Artemisia scoparia* essential oil at *10* µl against control at 5-mins interval and after it is removal from the stimulus chamber, 30 mins after.

At application in another round of experiments, a mere 0.01 ml increase of *A. scoparia* essential oil yielded complete blockade of female *Aedes* mosquitoes across the test chamber for 20 mins (See fig 5). Only then at the 20th to the 30th minutes interval were adult female *Aedes* able to enter the test chamber. ANOVA calculations showed significant difference between treatment and control (F_{cal}, 69.39>F_{crit}, 4.96, P=8.25x10^{-6}) at 0.05% confidence level (See Appendix 1). The essential oil of *A. scoparia* appears to be highly potent, notwithstanding its strong lemony scent the permeates in lab after the meager drop of 10 or 20 μl essential oil is applied on the filter paper substrate. The same residual effect is shown after the removal of essential oil from the stimulus chamber.

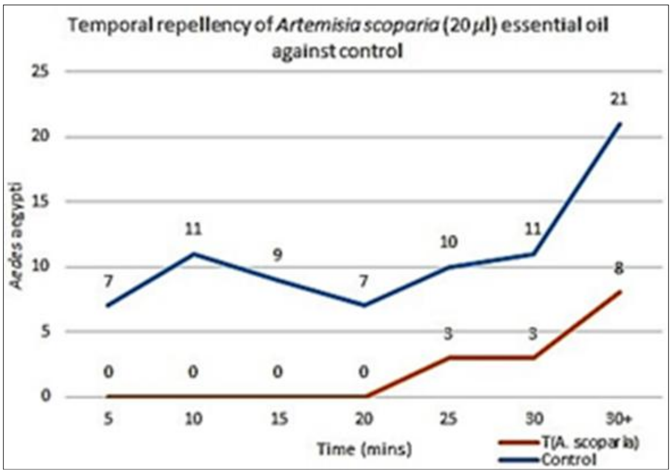

Fig 5: The count of adult female mosquitoes that entered the test chamber under Artemisia scoparia essential oil at.20 µl against control at 5-mins interval and after its removal from the stimulus chamber, 30 mins after.

Repellency performance was also done between DEET-based commercial repellent (T2) against "control" (T1). Most female *Aedes aegypti* were found to stay only outside the "treatment" capture chamber, while a number of *Aedes aegypti* freely entered the "control" capture chamber (see fig 6). Only one *Aedes aegypti* or none at all were able to fly into the "treatment" chamber. One-way ANOVA showed significant differences between treatment with commercial repellent and control (F_{calc}, 159.01>F_{crit}, 4.22, P=1.39E-12).

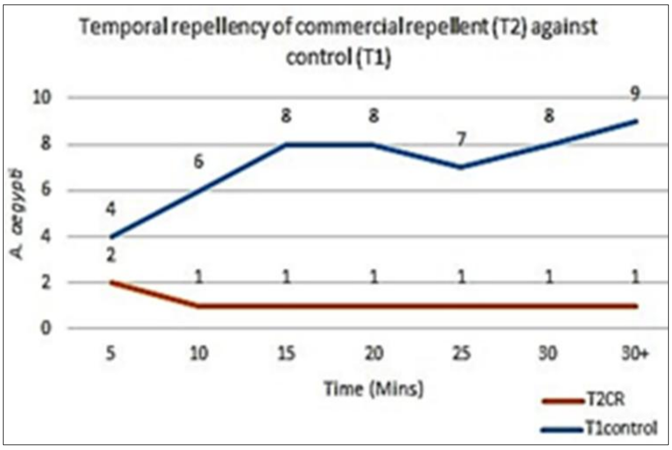

Fig 6: Comparison between commercial repellent against control

Two trials of comparative assay between *A. scoparia* essential oil and a commercial repellent was also done using the modified Y-tube. An amount of 30 µl of *A. scoparia* essential oil and about same amount of commercial repellent was applied on either of the 1 cm^2 filter paper substrate in each of the stimulus chambers.

In the first trial, essential oil treatment did not show a very promising repellant effect on the *Aedes* female mosquitoes against entering the test chambers (See fig 7). The commercial repellent appeared to perform better than the *A. scoparia* essential oil. ANOVA calculations yielded higher F calculated value than the F critical value (f_{cal}, 13.57>f_{crit}, 4.96, P=0.004) indicating that the commercial repellent outperformed the *A. scoparia* essential oil repellency.

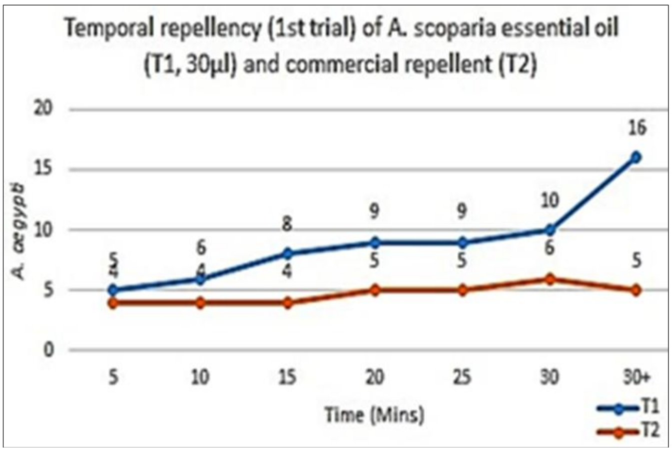

Fig 7: First trial of comparison between *A. scoparia* EO (blue) and commercial repellent (red)

In the second trial, however, *A. scoparia* essential oil yielded lower number of adult *Aedes* entering the test chamber compared to that of commercial repellent (See fig 8). ANOVA table showed significant differences between repellency rates between the two treatments (f_{calc}, 20.22>f_{crit}, 4.96, P=0.001).

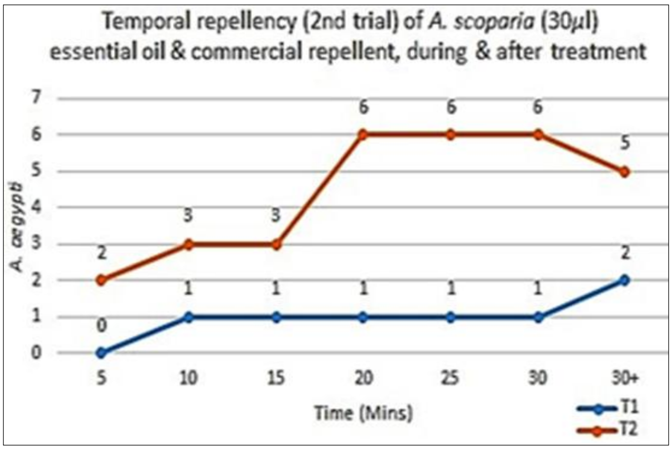

Fig 8: Second trial on comparing repellency of *A scoparia* essential oil (blue) and commercial repellent (red), and 30 mins post treatment effect

Upon analyzing the combined the values of the two trials, however, ANOVA showed no differences at all between the two repellents (f_{calc} 0.019<f_{crit} 4.30, P=0.89). ANOVA results imply that null hypothesis cannot be rejected (fig 9). This may therefore mean that the repellent property of *A. scoparia* essential oil may be as effective as the commercial repellent used in this study.

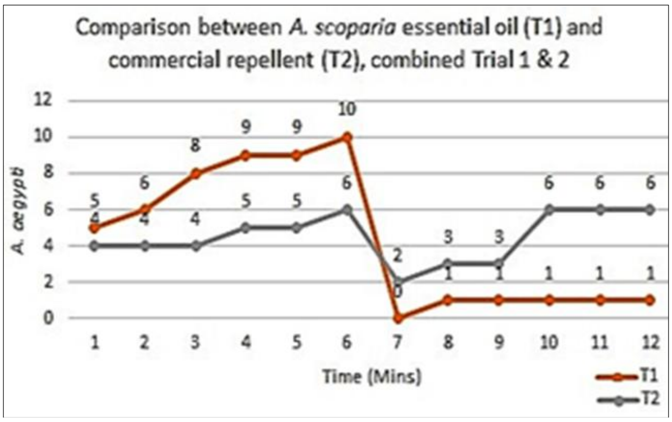

Fig 9: combined results of comparative trials between *A scoparia* EO and commercial repellent

It is interesting to mention here that the active ingredient in the commercial repellent used in this study was DEET. DEET (N, N-diethyl-3-methylbenzamide) is the gold-standard synthetic repellent that is effective across many insect species. The results of ANOVA mentioned in the previous paragraph suggests that *A. scoparia* essential oil is as effective dose-for-dose with DEET of the commercial repellent.

4. Discussion

The repellency assay of *A. scoparia* Waldst. & Kit. Essential oil against the dengue vector mosquito using the modified Y-tube olfactometer reported here is the first simple bioassay protocol to test the efficacy of the natural product. Based on literature review, no studies has been ever done on the mosquito repellent property of *A. scoparia* essential oil. Nor has its repellency been compared with a commercial repellent. Hence, despite of the preliminary and crude nature of the assay performed here, the data points out a promising fact that the locally-obtained *A. scoparia* leaf essential oil may indeed have mosquito repelling properties and that it works effectively against adult, female *Aedes aegypti*. At even a small amount of pure essential oil, at 20 µl (0.02 ml), the repellency against *Ae. Aegypti* is 100% for 20 mins. The results also suggests that It's repellent effect against *Ae. Aegypti* may be comparable to a commercially prepared mosquito repellent.

Essential oils of the genus *Artemisia* plants for example, have been documented to contain biologically active compounds that repel insects [2, 4, 8, 3, 14]. Some notable drug against malaria-vector mosquitoes have been discovered, particulary artemisinin, an antimalarial drug isolated from the relative Chinese herb *Artemisia annua* [15, 16, 17], and recently in *A. scoparia* [18], a species that is common in Asia and central Europe [3, 4]. *A scoparia* essential oil was also reported to contain compounds that repel *Aedes aegypti* mosquitoes[13] that include β-citronellol [19], ϒ-terpinene [9, 19, 20], α terpinene [8, 9, 21], citronellol [8, 9], eugenol [8, 10, 19, 22], *trans*-geraniol [19], geranyl acetate [19, 21], limonene [9, 19, 22], linalool [19, 21], β-myrcene [9, 10, 11, 19, 21, 22, 23], α-pinene [8, 9, 10, 11, 21, 22, 23], β-pinene [11, 22], phytol [23], β-terpeniol [8], α-thujone [23], β-thujone [23], coumarins [10]. Thus it is believed that based on chemical composition, *A. scoparia* essential oil is a very strong candidate for utilization as mosquito repellent. Considering that more commercial repellent products containing plant-based ingredients are becoming popular to consumers believing these are generally non-toxic and environmentally friendly, the utilization of the essential oil of *A. scopraria* as a component for the production of commercial repellent products can be considered a good idea. However, there is still a need to have a comparison with the standard of the World Health Organization (WHO) Pesticide Evaluation Scheme Guidelines for repellent testing. This is to make sure that the product developed really has no toxic effects. Likewise, there is also a need for a proper evaluation of the repellent compounds present in the plant by standardizing the protocol to better evaluate which of the compounds offer high repellency and good consumer safety.

5. Conclusion

This study have shown that the essential oil from *Artemisia scoparia* can be considered effective in repelling mosquitoes using the modified version of the olfactometer. The results can also be supported by other studies showing that the essential oil contain biological compounds that were considered to have repellency effects on insects.

6. Acknowledgement

The senior author would like to acknowledge the Commission on Higher Education for the scholarship support and the Premier Institute of Science and Technology (PRISM) of the College of Science and Mathematics of MSU-Iligan Institute of Technology for the support

7. Conflict of Interest

There are no conflict of interest in this study.

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