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Research Article

MOSQUITO REPELLANT MODELS USED IN DRUG DISCOVERY

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ABSTRACT

Vector-borne diseases are infections transmitted by the bite of infected arthropod species, such as mosquitoes, ticks, triatomine bugs, sandflies and blackflies. Mosquitoes are the most important insects which transmit a number of diseases such as dengue, chikungunya, filariasis, malaria and cause millions of deaths every year. An alarming increase in the range of mosquitoes is mainly due to deforestation, industrialized farming and stagnant water. Mosquito control and personal protection from mosquito bites are currently the most important measures to prevent these diseases. The use of mosquito repellent compounds dates back to antiquity. Insect repellents work by masking human scent. There are many types of mosquito repellents commercially available in market but these products have varying degrees of effectiveness. Purpose of this compilation is to bring all the methods at a single platform so as to enable scientists working on these repellents to know the scientific clues available in literature. The compilation reflects that there is no such method that is best for mosquito repellent as every method has drawbacks. So, there is a need to search such mosquito repellent product/ method for combating mosquitoes.

Keywords: Repellents, Mosquitoes, Vector-borne diseases

Introduction

Mosquitoes are relatively small insects belonging to the family *Culicidae.* Mosquitoes are among the most disturbing blood sucking insects afflicting human beings. Normally, both male and female mosquitoes use plant juice as food. Male mosquitoes do not bite human beings and female mosquitoes start biting human beings after mating with males as female mosquitoes require human blood protein for the maturation of their eggs [1]. Therefore, some of these female mosquitoes act as vectors for diseases. Typically, these mosquitoes transmit diseases from one human or animal to another by picking up a virus or parasite along with the blood while biting an infected human or animal. The mosquito and the virus do not harm one another but the virus reproduces inside the mosquito. Later, the mosquito passes the viruses to other humans while biting [2].

Several mosquito species belonging to genera *Anopheles*, *Culex* and *Aedes* are vectors of various diseases like Dengue fever, Malaria, Yellow fever, Japanese Encephalitis and several other infections [3]. Mosquitoes alone transmit diseases to more than 700 million people and over one million deaths are reported annually across the globe [4, 5]. Malaria caused by *Plasmodium* parasitie is transmitted through the bite of female *Anopheles* mosquitoes continues to impart a major disease burden on infants and young children in endemic regions [5, 6]. In 2012, there were about 207 million cases of Malaria and an estimated 627,000 deaths all around the globe [5]. Moreover, every year there are around 200,000 cases of illness and 30,000 deaths worldwide from Yellow fever which is transmitted by the *Haemagogus* and *Aedes* species of mosquitoes between monkeys and humans. The *Aedes aegypti* mosquito which spreads Dengue fever is responsible for more than 100 million infections worldwide every year, leading to thousands of deaths and more than 2.5 billion people or over 40% of the world's population

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are now at risk of Dengue [5]. The diseases caused by mosquito species are listed in table 1 [7-9].

Mosquito borne diseases currently represent a greater health problem. No part of the world is immune to their risks. They are a serious threat to public health transmitting several dangerous diseases. These diseases profoundly restrict socio-economic status and development in countries with the highest rates of infection, many of which are located in the tropics and subtropics. Mosquito control and personal protection from mosquito bites are currently the most important measures to prevent these diseases [10]. An alarming increase in the range of mosquitoes is mainly due to deforestation, industrialized farming and stagnant water. So there is a demand for search of such mosquito repellents for combating mosquitoes. A mosquito repellent is a substance applied to skin, clothing or other surfaces which discourages mosquitoes from landing or climbing on that surface. Usually, mosquito repellents work by masking human scent or by using a scent which mosquitoes naturally avoid. A mosquito can smell the carbon dioxide you exhale from about 60 to 75 feet away. A mosquito wing beats 300-600 times per second. Carbon dioxide and lactic acid present in sweat in warm-blooded animals act as an attractive substance for mosquitoes. The perception of the odour is through chemo-receptors which are present in the antennae of mosquitoes. The repellents block the lactic acid receptors and destroy upwind flight. Therefore, mosquitoes lose its contact with the host [11, 12].

Controlling mosquitoes is one of the important factors in the present day scenario with rising numbers of mosquito-borne diseases. On an individual level, people adopt numerous personal protective measures to decrease the risk of mosquito bites, it is important to know which repellent product can be relied on to provide predictable and prolonged protection from mosquito bites [13]. Repeated use of chemical repellents has resulted in the development of resistance against those repellent and toxic effects on human beings which include rashes, swelling, eye irritation, and worse problems, though unusual including brain swelling in children, anaphylactic shock, and low blood pressure [14]. Thus, there is an urgent need to develop new insecticides for controlling mosquitoes which are more environmentally safe, biodegradable and target specific against the vectors.

Different methods of repellents (Table 2):

Physical method: There are physical methods to repel mosquitoes such as mosquito nets which are considered as better protection from mosquitoes than coils and other repellents that cause health hazards. Sleeping under mosquito netting can guarantee the protection from mosquitoes. In addition, there are mosquito traps which mimic the different mosquito attractants such as exhaled carbon dioxide, human scents and body heat. Attracted by these chemicals, mosquito approaches and an impeller fan draws it in. It is then adhered to a sticky surface on the device and is eventually electrocuted. Moreover, there are mechanical methods such as Electric Mosquito Zappers that work by using ultraviolet light to lure in mosquitoes and then kill them upon contact with its lethal dose of electrical charge. There are also mosquito repellent products available based on sound productions, particularly ultrasound [12].

Chemical method: The first DEET product was introduced in 1956. DEET spray is still the most widely used mosquito repellent. It has generally been regarded as safe. Several other compounds have been evaluated for repellent activity, but none has had the commercial success of DEET. Various type of sprays are available that contain high concentration of DEET and is effective for several hours [6]. Creams and lotion repellents are also available in market that is applied to skin. Mosquito repellent clothing is also available which are designed in such a way that acts as mosquito barrier [15]. Mosquito repellent coils are widely used now a day the major active ingredient of the mosquito coils is Pyrethrins, accounting for about 0.3-0.4% of coil mass. When they are burnt as people feel that the coils maybe harmful for their health as it cause headache, nausea and dizziness [16]. Currently, mosquito repellent liquidizers are widely used all over the world. These mosquito repellent liquidizers contain synthetic Pyrethroids that produce

neurological toxicity on accidental ingestion. Recently, these are emerging as a source of hydrocarbon poisoning [17].

Biological method: The best biological method includes Growing of some fish species that feeds on mosquito larvae in water bodies. Another method includes use of essential oils of the leaves of *Cymbopogon nardus* (Citronella) [11], *Cymbopogon citratus* (Lemongrass) [3, 18], *Cymbopogon winterianus* (Citronella) [19, 20], *Ocimum basilicum* (Sweet Basil) [3], *Ocimum sanctum* (Tulsi) [21], *Ocimum americanum* (Hairy Basil) [19], *Eucalyptus citriodora* (Eucalyptus) [8], *Eucalyptus globules* (Eucalyptus) [22], *Rosmarinus officinalis* [21], *Melissa officinalis* [22], *Curcuma longa* (Turmeric) rhizomes [23], *Citrus sinensis* (Sweet Orange) peels [3], *Citrus hystrix* (Kaffir Lime) peels [19], *Citrus limonum* (Lemon) peels [22], *Syzygium aromaticum* (Clove) buds and *Pinus roxburghii* resins [20] have shown very high mosquito repellent activity. Moreover, the extracts of *Azadirachta indica* (Neem) seeds [20, 24], leaves of *Alpinia galangal* (Greater Galangale) [11], *Vitex negundo* (S. Nika) [25] and *Tribulus terrestris* (S. Gokatu) [4] also have been studied as possible mosquito repellents. The selection of these plants was based on their availability as raw materials, scientific evidence and folkloric use as mosquito repellents.

Screening Models/Methods for Mosquito Repellents

Human Bait method

Before application of the repellent, the forearms and legs of human volunteers are washed and rinsed thoroughly with distilled water. One forearm and leg are used for the treatment while the other forearm and leg of each volunteer are used for control. Different doses of the test substance are applied thinly to each of the three sets of volunteers' bare skin on the forearm and from knee to foot. The mosquito repellence is determined in triplicate experiments of three volunteers per experiment. After application, each volunteer is placed in a separate classroom in the same environment. The period of application of the test substance to the time of first landing or bite of mosquitoes on the treated parts as well as two subsequent bites or landings for each volunteer is noted and compared to the control bite [18, 26, 27].

Field Bioassay

Repellent action and relative efficacy of the sample is studied under field conditions. Test sample (1 ml) is applied on exposed hands, legs, neck and face of human volunteers at dusk time. They were allowed to sit on a chair or relax on a cot throughout the night, at a spacing of 5 m in a row. Untreated human volunteers are also allowed to sit or rest in a similar manner at 5 m distance. Mosquitoes landed on treated and untreated human volunteers are captured by trained insect collectors, with the help of an aspirator and flash light, continuously throughout the night. Insect collectors are rotated at an interval of 4 h to avoid slackness and bias. Mosquitoes collected on human volunteers are identified with the help of a hand lens and reconfirmed in the laboratory. Percent protection and average protection time is calculated [28, 29].

$$\% \text{ Protection} = \frac{\text{Control - Treated (Experimental)}}{\text{Control}} \times 100$$

Repellent Bioassay

In this, 3 and 6 days old hundred laboratory reared blood-starved adult female mosquitoes are introduced into separate laboratory cages (45 cm X 45 cm X 40 cm). Before each test, the forearms of a human subject are washed with unscented neutral soap, thoroughly rinsed, and allowed to dry before the application of the test solution. The test solution is applied on the right upper forearm and remaining regions are covered with gloves. The arm is left undisturbed. The left arm served as control. N-N Diethyl benzamide (12%, w/w) is used as negative control. The mosquito bites are observed for three full minute of every fifteen minutes. Protection time is recorded as the time elapsed between extract application and the observation period immediately preceding that in which a confirmed bite is obtained. The experiments are replicated five times in separate cages and in each replicate different volunteer is used to nullify any effect of skin differences on repellence. The protection time is calculated [30, 8].

Percentage and Contact Repellency Bioassay

A static-air choice-test apparatus consisting of a 9 x 60-cm section of glass tubing with a 2-cm hole in the mid for central introduction of mosquitoes is used. 1ml of the test solution is applied to one half of a 9-cm diameter round filter paper with an area of 63.6 cm^2 and then allowed to dry before testing. Treated filter papers are placed inside the lids of 9-cm glass petri dishes and placed over the ends of the glass tube. The position of the treated side, to the right or to the left, is selected by using a random-number table. Fifteen unmated adult female mosquitoes are anaesthetized with CO_2 and then introduced to the 9 x 60-cm glass cylinder through the centered 2-cm hole. Timing began 2 minutes after mosquito introduction, and mosquito distribution inside the static-air choice-test apparatus is observed over a 180-minute period for each treatment. Mosquito distribution (number of individuals on treated and untreated side) is recorded at 15, 30, 60, 90, 120, and 180-minute time points. Contact repellency is defined in this assay as 100% avoidance of the treated filter paper (no contact). 15, 30, 60, 90, 120, and 180-minute time-points are used to assess contact repellency for individual observations. Analysis of variance is performed to identify significant differences of percentage repellency due to treatment and concentration [31].

Experimental hut trial

A suite of six huts are used for the trial. The huts are sprayed with test and standard. Adult volunteers slept in the huts from 19:30–6:30 hours. Each morning, mosquitoes are collected from the verandah and window traps of huts. White plastic carpets are laid on the floor to make dead mosquitoes more easily visible. Live mosquitoes in the room are not collected in order to allow for natural resting times on treated surfaces, and were only collected after exiting to verandah or window traps. Collected mosquitoes are recorded as blood-fed or unfed and as dead or alive. Live mosquitoes are kept in paper cups with 10% glucose solution for 24 hours before scoring delayed mortality. Sleepers rotated between huts after each trial night to reduce any bias due to differences in individual attractiveness to mosquitoes. Treatments are rotated between huts every week. On the rotation day control and other treatment panels are dismantled and taken outside before cleaning the huts. After cleaning, the huts are left for 2 days for airing before resuming the trial to allow time for any vapour from previous treatments to dissipate [32].

Standard method of American Society for Testing and Material

The testing kit is made of Plexiglas cube at dimension of 4×5×18cm having four rectangular holes 4×3cm. Before starting the test the abdomen skins of rabbits are cleaned with alcohol and the kit is fixed on the abdomen. Each of 4 adjacent cells of kit is provided with 5 female 5–8 days mosquitoes that randomly selected from a cage containing 150 starved mosquitoes. Circles are drawn on the rabbit's skin. The drawn circles on the abdomen skin's of hold rabbit are treated with 50µl of test solution diluted with absolute ethanol. The treated circles are allowed to dry, and then test apparatus containing starved mosquitoes are fixed on the treated skin. The counts of probing and biting are recorded for 5 minutes. After each test, the mosquitoes are transferred to netted cups and the mortality of mosquitoes is recorded after 24 hours [33].

Adult Bioassay Method

Adult bioassays are carried out using 3—6 d-old male and female mosquitoes. Adults are lightly anesthetized with CO_2 then immobilized on a chill table for dosing with 5µl from a series of dilute solutions ranging from 0.05 to 10% using insecticide-grade acetone as the diluents. Solutions are applied to the thorax of 10 males and 10 females of each species using a Drummond digital micro-pipette (Fisher Scientific). Each extract dilution along with a control (acetone only application) group is replicated five times for each sex and species of mosquitoes. After treatment, mosquitoes are placed into a screened cage (30.5 by 30.5 by 30.5 cm) in an environmentally controlled room and provided a 10% sucrose solution and libitum. Adults are observed immediately and at 15, 30, and 60 min after application. Mortality was recorded at 24 h and tested by probit analysis [34].

Static-Air Olfactometer

For this test, an 8 x 1 x 1-foot Plexiglas apparatus is constructed. The tunnel is divided into four two-foot sections and the number of mosquitoes in each section is counted at five-minute intervals for 20 minutes. The data is analyzed by using a chi-square test to determine differences from a random distribution of insects. Approximately 50 female mosquitoes are released into the olfactometer and allowed to disperse for several minutes before the introduction of the treatments. Treatments are applied to a 25-cm piece of filter paper and placed in each end of the tube. The position of the treated side is randomized by using a random number table. The number of insects seen in each section is an average of three replications [35].

Y-Tube Olfactometer

The olfactometer consists of 13-cm diameter Plexiglas tubing in the shape of a Y, and air is pulled through from the arms to the stem by a small electric fan. The mosquitoes are introduced in the stem of the Y and allowed to acclimate for 15 minutes. The mosquitoes are then allowed to choose for 30 minutes between an arm of the olfactometer containing a test repellent and an arm containing a blank (solvent) treatment. For each treatment, 0.75 ml of the test solution is applied to a 7-cm piece of filter paper and the solvent allowed to dry for several minutes before being introduced to the apparatus. When carbon dioxide is used as an attractant odor source, it is introduced into the apparatus through plastic tubing that run in through the arms of the apparatus [35].

Pigeons as experimental animals:

Cages (30×30×30cm) are used to test repellent activity. Extract is dissolved in 2 ml (95% methanol or water with a drop of Tween 80) in to get different concentrations. Extract is then directly applied onto 5×6 cm of ventral abdomen of pigeon after removed feathers. After 10 min., pigeons are put for 3 h in cages of starved females. Control tests are carried out with water. Each test is repeated 3 times to get a mean value of repellent activity.

Post treatments number of fed and unfed females was counted and calculated.

Repellency % = (A% - B%/ 100 -B %) ×100 where A = treatment unfed females % & B = control unfed females % [36].

Repellent activity Test

Test dilutions of extract are prepared in DMSO. A special blood-containing feeding membrane, which is used to feed mosquitoes, is exposed to the test solution and then fitted in a 1-ft cage, with temperature kept at 37 °C through a 40–45 °C circulating water bath. Approximately 50 unfed 3–4-day-old mosquitoes are introduced in the aforementioned cage. The time it took for the first feeding in the cage where the repellent-treated membrane is fitted to be observed at 30-min intervals and each observation lasts for 60s. The experiment is repeated five times to confirm reproducible results. The time taken for feeding to complete is defined as protection time. The control is an identical test, where the feeding membrane is not treated with any repellent.

The percentage of repellency was calculated by the following formula:

Repellency %= Tb - Ta / Ta X 100

Where Ta is the number of mosquitoes in the control group and Tb is the number of in the treated group [37].

Contact Irritancy Assay

In this test, Whatman filter paper No. 1 is impregnated with different concentrations of the test, shade dried and finally cut into circles of cm diameter. The dried paper is placed on a glass plate and a perspex funnel with a hole on the top is kept inverted over the impregnated paper**.** Single female adult is released in the funnel and per-conditioned for 3min.Thereafter, the time at which first flight is taken was recorded. The experiment is continued for 15 min and the total number of flights undertaken by each female adult is scored. Three replicates are carried out for each treatment. Parallel control tests are performed with papers impregnated with acetone.

The relative irritability caused by the seed oil is calculated with respect to control by the following formula:

RI, Relative Irritability = Mean number of take-offs stimulated by the test /Mean number of take-offs stimulated by control [8].

Attraction Inhibition bioassay

This consists of a Y-tube in which a constant air current is produced by a computer fan that is placed at the bottom of the Y-tube. The air flow is adjusted to 0.4 m/s in the base leg (0.2 m/s in each port) by placing the probe of an anemometer within the different shafts of the Y-tube and moving the fan in relation to the tube opening until the correct air flow speed is achieved. One of the co-authors is selected as the attractant for Y-tube assays based on preliminary attraction studies. The volunteer is not allowed to wash her hands, wear perfume, or take a shower in the morning prior to the experiments.

One of the attractant's hands was sprayed with approximately 0.5 ml of liquid repellent; the other hand is covered with a nitrile glove. The hand is sprayed on both sides and allowed to air dry. Trap doors 1 and 2 are opened and the mosquitoes are placed in the holding chamber of the Y-tube. The treated hand is then placed in one of the decision ports (from here on referred to as the "hand port") the other, untreated gloved hand is inserted into the other port (control port). Alternating decision ports are used for the biological replicates to ensure there is no bias. The mosquitoes are given 30 s to acclimate to their environment while exposed to the odor on the hand before they were released from the holding chamber by opening trap door 3. The mosquitoes are given 2 min to relocate within the tube. After a 2-min period, all trap doors are closed. Three groups of mosquitoes are counted and recorded: the ones that stayed in the holding port, the ones that arrived in either decision port, and the ones that stayed in the shaft of the Y-tube. The mosquitoes that are not captured at either decision port or in the holding port are considered wandering. For each replicate, there are a total of 20 mosquitoes placed in the holding chamber. The efficacy of the repellent is evaluated over a 4-h time period at: 0 min, 30 min, 120 min, and 240 min post application.
Attraction rate (%) = Number of mosquitoes in the treated hand port/ Total number of mosquitoes in the replicate [38].

Repellent treated nets (tunnel test design)

The test materials are evaluated in an assay that involves the use of guinea pigs as mosquito attractants. Two animals are used to evaluate activity. A test device with three cages connected to each other is used. One animal is placed in a cage whose entrance (to the rest of the cage) is fitted with a plant extract treated net. The net, measuring 10×9 cm, is treated by applying 3 ml of extract using a pipette and left for 1 h under open air to allow for the solvent to evaporate before being placed onto the cages. The other animal is placed in a cage whose entrance is fitted with a net treated with ethanol only in the same manner as described for the plant extract and it served as the negative control. One hundred blood-starved mosquitoes are released into a space in between the two animal cages. The number of mosquitoes on each side of the cage is counted every 30 min for 3 h. Each test is repeated three times with new mosquitoes. Animals in this assay served solely as attractants but were not directly exposed to the mosquitoes and did not need to be anaesthetized or restrained. They are supplied with food and water while in the test equipment [39].

The repellency test

The test is carried out with the experimental cages containing mosquitoes in a room maintained at 27ºC and 70% relative humidity. These cages contain the plastic bowl along with cotton soaked in test solution, DEET 2% (positive control) in 10% sugar solution and 10% sugar solution (negative control) placed in four different corners and one in the centre of the cage. Five-minute landing counts are made at each hour for six hours (0, 1, 2, 3, 4, 5, and 6 hours). The bowls are removed from the cage after the five minute observation at each interval of time. The bowl is covered to avoid evaporation of the insecticide formulation and is placed in the refrigerator. For subsequent exposure the position of the bowls is inter changed to different corners [40].
% Repellency = Control-Treated/Control X100

CONCLUSION

Controlling mosquitoes is one of the important factors in the present day scenario with rising numbers of mosquito-borne diseases. On an individual level, people adopt numerous

personal protective measures to decrease the risk of mosquito bites, but no one method is effective. So, it is important to know which repellent product can be relied on to provide predictable and prolonged protection from mosquito bites. Thus, there is an urgent need to develop new method/product for controlling mosquitoes which are more environmentally safe, biodegradable and target specific against the vectors.

Table 1: Different diseases caused by mosquitoes

Table 2: Different methods of mosquito repellent

Table 1: Different diseases caused by mosquitoes

S.No.	Species of Mosquito	Type of Disease caused
1	*Anopheles*	Malaria, Filariasis, Arbovirus
2	*Culex*	Encephalitis or Meningitis, Bancroftian filariasis,Elephantiasis
3	*Aedes*	Dengue, chikungunya, yellow fever, haemorrhagic fever

Table 2: Different methods of mosquito repellent

Physical methods	Chemical methods	Biological methods
Physical method: Medicated net, Non medicated net, Mosquito traps. **Mechanical methods:** Electric mosquito zapper, Mosquito magnet	**Synthetic repellents:** DEET, Permethrin **Natural repellents:** Neem oil, Citronella Oil.	By growing some fish species that feeds on mosquito larvae in water bodies. Use of essential oils of the plant parts.

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