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Laboratory study to determine the efficacy of repellents against mosquitoes, *Culex quinquefasciatus*

i2LResearch Ltd
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November 2013

- Chemicals Regulation Directorate Accredited
- GLP (Good Laboratory Practice) Compliant

Study Information

Laboratory study to determine the efficacy of repellents against mosquitoes, *Culex quinquefasciatus*

i. Testing facility: i2LResearch Ltd
Capital Business Park
Wentloog
Cardiff CF3 2PX
UK

ii. Sponsor: Hebe Botanicals Ltd
34 Riverbank Road
Otaki 5512
New Zealand

iii. i2LResearch Ltd Study code: 13/396

iv. Study start date: 16.09.13
Experiment start date: 04.11.13
Experiment end date: 25.11.13

v. Study Director: Pavel Foltan

vi. Primary personnel: Jitka Zelova, Dylan Gibson

vii. Certification: ORETO Number 266

viii. Test chemicals:

Test Substance	Active ingredient	Lot Number	Physical description of test substance	Storage conditions	Expiry date	i2LResearch Ltd code number
Repellent spray	unknown	N/a	Liquid in white atomiser	Ambient	06.09.15*	13090601
Jungle Formula Medium	20% DEET	W0018903	Liquid in spray bottle	Ambient	25.11.15*	620
Repel (tropical strength pump spray)	30% DEET, 3.75% IR3535	08115	Liquid in spray bottle	Ambient	01.08.16	13110906

*i2L default expiry date

Summary

A laboratory bioassay was conducted to assess the efficacy of three repellents (Repellent spray, Jungle formula and Repel) against mosquitoes, *Culex quinquefasciatus*, in terms of repellency. The products were applied onto the skin of the forearm of a human volunteer. The forearm was then placed inside a cage of mosquitoes and the number of mosquitoes landing on the skin was assessed at hourly intervals over a maximum of 8 hours.

Exposure to the repellent spray resulted in 100% repellency for up to 8 hours post treatment against *Culex quinquefasciatus*. In terms of complete protection time (CPT), application of the repellent spray resulted in a mean CPT of 495 minutes against *Culex quinquefasciatus.*

In comparison, exposure to Jungle formula and Repel resulted in over 95% repellency for up to 4 hours post treatment in a mean CPT of 360 and 410 minutes against *Culex quinquefasciatus*, respectively.

It can be concluded that the repellent spray was highly effective against mosquitoes, *Culex quinquefasciatus*, offering complete protection for over 8 hours post application.

Aim

A laboratory bioassay was conducted to evaluate a repellent spray for efficacy against mosquitoes, *Culex quinquefasciatus,* in terms of repellency.

The method for testing the repellency generally follows the EPA Product Performance Test Guidelines OPPTS 810.3700 Insect Repellents For Human Skin and Outdoor Premises (http://www.regulations.gov/oldLinks.jsp?url=contentStreamer?disposition=attachment&objectId=0900006480b1f1d0&contentType=pdf), WHO Guidelines for efficacy testing of mosquito repellents for human skin, Ref: WHO/CDS/NTD/WHOPES/2009.4 (http://whqlibdoc.who.int/hq/2009/WHO_HTM_NTD_WHOPES_2009.4_eng.pdf) and Technical Notes for Guidance of European Commission: Insecticides, acaricides and products to control other arthropods (PT 18) And Repellents and attractants (only concerning arthropods) (PT19) Draft guidance document to replace part of Appendices to chapter 7 (page 187 to 200) of the TNsG on Product evaluation (http://ec.europa.eu/environment/biocides/pdf/guidance_efficacy_pt18_19.pdf).

Methodology

Test systems. Mosquitoes, *Culex quinquefasciatus*, were obtained from a laboratory culture maintained at i2LResearch Central Europe and the London School of Hygiene and Tropical Medicine (London, UK). Adult female mosquitoes, aged 5-10 days old were used in the experiments. The insects were provided with sugar water prior to use but had not received a blood meal. Test insects were starved for 12 hours immediately before the experiment. Test insects were used for only one test and were destroyed after the trial.

Test treatments and application.

One product was assessed (a repellent spray), provided by the sponsor. Two comparison products, Jungle formula and Repel) were also assessed, at the sponsor's request. Three replicates were conducted for each product.

Test cages

Experiments were performed in transparent acrylic framed cages measuring approximately 40 cm long x 25 cm wide x 40 cm high, with a sleeved fabric opening for access at the front (Czech Republic) and metal framed netted cages measuring approximately 40 cm long x 40 cm wide x 40 cm high (UK). The cages were placed in a controlled environment room and the lighting was kept at normal daylight levels throughout the trial. Temperature for the mosquito testing was between 24.6 and 26.5 ºC and relative humidity ranged from 26.4% to 85%, for the duration of the experimental period. One hundred female mosquitoes were released into each cage.

Human volunteers

Recruitment of human volunteers conformed with Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Section 12(a)(2)(P). Informed consent was obtained from every volunteer used during this study.

Skin area preparation

In preparation for the laboratory study, the test area of the volunteer's skin was washed with unscented soap and rinsed with water, then rinsed with a solution of 70% ethanol or isopropyl alcohol in water and dried with an uncontaminated towel.

Product application

The product testing was carried out using a rate of 1 g per 600 cm^2 of the exposed area of the volunteer forearm. The exposed area of each volunteer's forearm was calculated (by measuring the circumference of the wrist, elbow and three equally spaced places in between) to ensure the quantity per unit area applied to each volunteer did not vary by more than 5%. The test product was then spread evenly over the exposed forearm. The forearm was bordered by proboscis impenetrable material (ie. Gloves, fabric and plastic tape). Volunteers avoided wearing dark coloured clothing on their arms.

Experimental design

Before testing commenced, the repellent spray was applied to one arm of a volunteer. After 5 minutes, the untreated arm was inserted into the cage and was exposed for 5 minutes and the

number of mosquitoes landing and/or probing on the skin was assessed. The treated arm of the same volunteer was then exposed for a further 5 minute period in the same cage to determine landing and/or probing activity, in the same manner as for the untreated arm. This procedure was repeated at 1 hourly intervals for a maximum of 8 hours. Please note that insects were not allowed to bite for health and safety reasons.

Three replicates were performed for each product giving a total of 3 tests. Each replicate consisted of one cage of 100 mosquitoes. A different volunteer was used for each replicate.

Statistical analyses

For each assessment of each replicate, the percentage repellency was calculated as % protection = (C – T) x 100 / C, where

C = number of probes received on the untreated control arm.

T = number of probes received on the treated arm.

The mean percentage repellency for each assessment of the test product was then calculated. 95% repellency was calculated to reflect the duration of repellent protection where there is a 95% reduction in bites for each volunteer. The length of protection was calculated for the complete protection time (CPT) and 95% repellency. CPT was calculated as the number of minutes elapsed between the time of repellent application and the first mosquito landing and/or probing, confirmed by a second landing and/or probing incident within the same 5 minute time interval. In the event that no confirmed landing incident occurred, the final observation with a further 15 minutes added was used for calculation purposes. 95% repellency was calculated to reflect the duration of repellent protection where there is a 95% reduction in bites for each volunteer.

Results and Conclusions

Exposure to the repellent spray resulted in 100% repellency for up to 8 hours post treatment against *Culex quinquefasciatus*. In terms of complete protection time (CPT), application of the repellent spray resulted in a mean CPT of 495 minutes against *Culex quinquefasciatus.*

In comparison, exposure to Jungle formula and Repel resulted in over 95% repellency for up to 4 hours post treatment in a mean CPT of 360 and 410 minutes against *Culex quinquefasciatus*, respectively.

Results are summarised in Tables 1 and 2.

It can be concluded that the repellent spray was highly effective against mosquitoes, *Culex quinquefasciatus*, offering complete protection for over 8 hours post application.

Table 1. Percentage repellency against *C. quinquefasciatus*, exposed to products (means ± standard errors, n = 3)

Hours post treatment application	*Culex quinquefasciatus*		
	Repellent Spray	Jungle Formula	Repel
0	100 (± 0)	100 (± 0)	100 (± 0)
1	100 (± 0)	100 (± 0)	100 (± 0)
2	100 (± 0)	99.7 (± 0.3)	100 (± 0)
3	100 (± 0)	100 (± 0)	97.0 (± 0)
4	100 (± 0)	98.6 (± 1.4)	97.1 (± 0)
5	100 (± 0)	100 (± 0)*	100 (± 0)*
6	100 (± 0)	94.6 (± 5.4)*	98.8 (± 0)*
7	100 (± 0)	95.2**	99.4 (± 0)*
8	100 (± 0)	87.5**	99.2 (± 0)*

* n = 2

** n = 1

Table 2. Complete protection time (CPT) in minutes against *C. quinquefasciatus*, exposed to products (means ± standard errors, n = 3)

Repellent Spray	Jungle Formula	Repel
495 (± 0)	360 (± 69.3)	410 (± 85)

Aim

A laboratory study is required to determine efficacy of two repellents against *Culex quinquefasciatus*.

The method for testing the repellency generally follows the EPA Product Performance Test Guidelines OPPTS 810.3700 Insect Repellents For Human Skin and Outdoor Premises (http://www.regulations.gov/oldLinks.jsp?url=contentStreamer?disposition=attachment&objectId=0900006480b1f1d0&contentType=pdf), WHO Guidelines for efficacy testing of mosquito repellents for human skin, Ref: WHO/CDS/NTD/WHOPES/2009.4 (http://whqlibdoc.who.int/hq/2009/WHO_HTM_NTD_WHOPES_2009.4_eng.pdf) and Technical Notes for Guidance of European Commission: Insecticides, acaricides and products to control other arthropods (PT 18) And Repellents and attractants (only concerning arthropods) (PT19) Draft guidance document to replace part of Appendices to chapter 7 (page 187 to 200) of the TNsG on Product evaluation (http://ec.europa.eu/environment/biocides/pdf/guidance_efficacy_pt18_19.pdf), however the number of replicates conducted is reduced.

Test systems - insects

Adult *Culex quinquefasciatus* mosquitoes will be obtained from a specified laboratory culture. Five to ten day old post-emergence female mosquitoes will be collected from a stock population cage in which both sexes have been maintained to allow mating to occur. Active host-seeking females will be selected using an aspirator or an appropriate airflow apparatus to ensure a good response from the test mosquitoes. One batch of 100 adult female mosquitoes will be released into each of the test cages. Prior to the testing, the mosquitoes will be provided with approximately 10% sucrose solution, but no blood meal, and mosquitoes will be starved for 12 hours.

Test items - products

Efficacy of one natural repellent spray will be compared with an untreated control. A DEET standard reference product will also be assessed in the same manner. The products will be provided by the sponsor.

Test cages and test conditions

The testing will be conducted in transparent acrylic cages, measuring approximately 40 cm x 40 cm x 25 cm (40,000 cm^3), with a sleeved fabric opening for access from the front (Figure 1).

During the testing, cages will be held at 27 ± 2 °C, > 60 % relative humidity and decontaminated each time before use of a different product. The tests will take place within the premises of i2LResearch Ltd in the Czech Republic.

Figure 1 Exper*imental transparent acrylic cage, measuring approximately 40 cm x 40 cm x 25 cm (40,000 cm^3), with a sleeved fabric opening for access from the front*

Volunteers

The study will be undertaken in accordance with the applicable national ethical regulations, on mix-sexed volunteers (subjects), on the basis of their written informed consent, written in their own language. Signed consent forms will be submitted with the study report. All test subjects (volunteers) will freely volunteer to participate in the test.

The volunteers will be fully informed of the nature and purposes of the test and of any physical and mental health consequences which are reasonably foreseeable there from.

Volunteers will not be identified by name in data collection forms or study reports. Volunteer names unavoidably will appear on consent forms and on administrative documents, but

volunteers will be identified only by an arbitrary code on other study documents. The key linking identifying codes to subjects will be stored securely, away from other study records.

If photographs or videos are made to document the research, care will be taken to minimize making recognizable images of the volunteers. If faces or identifying marks cannot be excluded from a photograph or video image, the image will be altered to protect the identity of the volunteer(s).

The volunteers will be allowed to stop the procedure at any point without any penalty. Volunteers will be recruited from populations in the area where testing will be conducted as an offering of distant travel may unduly influence a candidate's choice to enrol, and a subject who has accepted long-distance transportation may feel less than free to withdraw from a study.

The participants/volunteers in the study will be compensated for their time and trouble. The level of compensation will not be so high as to constitute an undue influence in the choice to participate, nor will it be so low as to make participation in the research attractive only to the economically disadvantaged. Compensation will not be used or administered so as to compromise the freedom guaranteed to subjects to withdraw from participation at any time for any reason, without sacrificing benefits to which they are entitled.

Any unsuitable volunteers will be excluded from the study. The main exclusion criteria include usage of fragrance and repellent products or alcohol for 12 hours before and during testing. Volunteers should preferably not be tobacco users, or at least have refrained from tobacco use for 12 hours prior to and during testing. Under no circumstances, will pregnant or nursing women and people of age under 18 or over 55 years be used as volunteers for the study.

Members of certain vulnerable populations, including people of limited mental capacity, those not in good health or with compromised immune systems, those sensitive to chemicals, sensitive to repellents or to insect bites, will be excluded.

Sufficient number of sub-investigators will be present at all times during the testing, so that the Principal Investigator can, if needed, attend to the safety of a subject without compromising the integrity of the research or endangering other subjects.

Skin area preparation

In preparation for the laboratory study, the test area of the volunteer's skin will be washed with unscented soap and rinsed with water, then rinsed with a solution of 70% ethanol or isopropyl alcohol in water and dried with an uncontaminated towel.

Product application and experimental design

The product will be applied at a rate of 1 g per 600 cm^2 on the exposed area of the volunteer forearm, or as instructed by the sponsor. The forearm will be bordered by proboscis impenetrable material (ie. Gloves, fabric and plastic tape). Volunteers will avoid wearing dark coloured clothing on their arms.

Before testing commences, the tested repellent product will be applied to one arm of a volunteer. After 5 minutes, from the product application, the untreated arm will be inserted into the cage and will be exposed for 5 minutes to determine landing and/or probing activity. All such activity will be recorded. Next, the treated arm of the same volunteer will be exposed for a further 5 minute period in the same cage to determine landing and/or probing activity.

This procedure will be repeated at hourly intervals for a maximum of 8 hours or until at least two attempts to bite are recorded on the treated arm within one 5 minute test interval.

The initial biting pressure must exceed 10 landings/probings during 30 seconds starting 1 minute after the hand insertion into the test cage, otherwise the particular replicate must be discarded and new batch of mosquitoes used.

Please note that insects will not be allowed to bite for health and safety reasons.

Number of replicates, volunteers and insect specimens used

Three replicates will be conducted for each of the two products giving a total of 6 tests, each replicate using different mixed sexed volunteers.

Statistical analyses

Length of protection will be calculated for complete protection time (CPT) and 95% repellency. CPT will be calculated as the number of minutes elapsed between the time of repellent application and the first mosquito landing and/or probing, as confirmed by second landing and/or probing incident within the same 5 min time interval. 95% repellency is calculated to reflect the duration of repellent protection where there is a 95% reduction in bites for each volunteer. The mean protection time and standard error based on a 95% reduction in landings/probings will be reported.

The product efficacy results will be analysed by appropriate statistical methods.

Analyses will be performed by the Study Director and will depend on the outcome of the testing at the discretion of the Study Director. Statistical analyses performed will be fully documented in the report.

Protocol amendments and deviations

Any protocol amendments and/or deviations will be documented, fully justified and maintained with the protocol. All protocol amendments will be approved by the Study director and sent to the sponsor.

Archiving records

The original raw data, final report and any amendments will be archived at i2LResearch Ltd for a period of five years. The final report, including any protocol amendments or deviations, will be forwarded to the Sponsor. Any unused test substances will be either be returned to the Sponsor or disposed of with the Sponsor's consent.