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Original Research Paper

Dermal Toxicity of a Repellent Formulation Containing *Piper Aduncum* Linnaeus (Piperales: Piperaceae) Essential Oil

[1]Siti Nur Hanis Mamood, [1]Hidayatulfathi Othman, [1]Nurathirah Mat Nasir, [1]Ahmad Rohi Ghazali, [1]Siti Balkis Budin and [2]Mohd Hanif Zulfakar

[1]School of Diagnostics and Applied Health Sciences, Faculty of Health Sciences, National University of Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
[2]Centre for Drug Delivery Research, Faculty of Pharmacy, National University of Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia

Corresponding Author: Hidayatulfathi Othman School of Diagnostics and Applied Health Sciences, Faculty of Health Sciences, National University of Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
Phone: +60123736549/+603-92897693
Fax: +60326929032
Email: hida@ukm.edu.my

Abstract: It has been shown in previous research that *Piper aduncum* Linnaeus essential oil has the potential to be developed as an alternative mosquito repellent. When the essential oil was formulated into cream, it was able to provide >2h of protection against *Aedes aegypti* in the laboratory; thus, it can be commercialized as an alternative to synthetic repellent especially N, N-diethyl-3-methylbenzamide (DEET). In this study, the irritation and sensitization potential of a cream formulation containing *P. aduncum* essential oil was investigated to verify its safety for application purposes. The *P. aduncum* essential oil was formulated into a cream containing 10% of the essential oil for irritation and skin sensitization assays on New Zealand white rabbits and guinea pigs (Hartley strain), respectively, following the ISO10993-10:2010 (E) guidelines. The macroscopic and histological observations from both assays revealed that the cream formulation containing *P. aduncum* essential oil caused slight irritation on rabbit skin, with a Primary Irritation Index (PII) of 1.54; however, no positive response was detected in the skin sensitization assay. In conclusion, the cream formulation containing 10% *P. aduncum* essential oil was slightly irritating to rabbit skin but did not cause sensitization in the animals tested.

Keywords: *Piper Aduncum*, Repellent, Essential Oil, Irritation, Sensitization

Introduction

Repellent is a practical, economical substance that can be used to minimized the transmission of mosquito-borne diseases, which can be transmitted through a single mosquito bite (Keziah *et al*., 2015). Currently, most widely use mosquito repellents available in the market contains N,N-diethyl-3-methylbenzamide (DEET) as an active ingredient (Fradin, 1998; Kwon *et al*., 2011). DEET has been used for the past 55 years and is known as a gold-standard synthetic repellent (Bissinger *et al*., 2014). However, safety concerns related to DEET have led to the development of natural-based product as an alternative to the synthetic repellent (Choochote *et al*., 2007; Katz *et al*., 2008; Nerio *et al*., 2010). Furthermore, the associated odor and feel of DEET on the skin have made consumers reluctant to use DEET products; this has caused them to seek other alternatives (Adeniran and Fabiyi, 2012), especially plant-based repellents. The effectiveness, biodegradability, availability and environmental friendliness of such repellents have contributed to renewed consumer interest in them (Govindarajan, 2011; Govindarajan and Sivakumar, 2011).

According to Pohlit *et al*. (2006), *Piper aduncum* Linnaeus has been used traditionally in medicinal and culinary applications and it is well known for its insecticidal, molluscicidal and antibacterial activity. A previous study demonstrated that *P. aduncum* extract exhibit repellency activity against the adult *Aedes aegypti* Linn (Hidayatulfathi *et al*., 2004). Therefore, it can be developed as an alternative to synthetic repellents (Misni *et al*., 2008). The 10% *P. aduncum* Essentail Oil (EO) without formulation was only effective for <1h after application against *A. aegypti* in laboratory. However, when formulated into semisolid formulation especially cream, it could provide sufficient repellency effect (>2h) against *A. aegypti* thus can be developed and commercialized as an alternative insect repellent (Mamood *et al*., 2017).

According to Mehmood and Khan (2012), plant products usually cause skin reactions; most commonly, irritant and allergic contact dermatitis. Therefore, safety assessment evaluation is important to determine the substances potential to cause eye and skin irritation. The aim of such evaluation is to ensure the safety of the consumers that are exposed to the ingredients that contained in cosmetic, industrial and pharmaceutical products (Ngo and Maibach, 2010). The usage and acceptability of a product will be restricted if it has the potential to cause skin irritation (Patel *et al*., 2013). Since little is known about the skin toxicity of *P. aduncum* EO, this research was conducted to investigate the irritation and sensitization potential of a cream formulation containing *P. aduncum* EO in animals.

Materials and Methods

Chemicals

The chemical used in this study includes Cetostearyl alcohol and Cetomacrogol 1000 (R&M Chemicals, UK), Paraffin oil (Sigma-Aldrich, Germany) and 1,2-propanediol (Acros Organics, USA). Anhydrous magnesium sulfate was supplied by Fisher Scientific (UK). For the positive control materials, 1-chloro-2,4-dinitrobenzene (DCNB) was obtained from Sigma (USA) and Sodium Lauryl Sulfate (SLS) was obtained from ICN Biomedicals (Ohio). Chemicals for histological analysis included eosin (Leica, USA), Harris hematoxylin (Leica), 37% formaldehyde (Merck, Germany), absolute alcohol (VWR Chemicals, France), di-N-butyl phthalate in xylene (DPX; Ajax Finechem, Australia), paraffin wax (ICN Biomedicals, Germany) and xylene (Fisher Scientific).

Piper aduncum Essential Oil Extraction and Formulation

P. aduncum plants were obtained from Batu 13 Gombak, Selangor, Malaysia. The EO used in the experiments was extracted using a hydrodistillation method (FRIM, unpublished data) and dried over anhydrous magnesium sulfate. The plant species was confirmed by the Malaysian Forest Research Institute (FRIM) while the voucher number for this plant (UKM b 29778) was obtained from the National University of Malaysia (UKM). *P. aduncum* EO (10%) was then formulated into a cream that contains paraffin oil (20% w/w), 1,2-propanediol (30% w/w), emulsifying wax (30% w/w) and distilled water.

Test Animals

For irritation test, healthy male albino New Zealand white rabbits (n = 8, weight = 2-3 kg) were purchased from A Sapphire Enterprise (Malaysia) and kept individually in separate cages. Male and female albino Hartley-strain guinea pigs (n = 20, weight = 300-500 g) were supplied by Laboratory Animal Resource Unit, Medical Centre, UKM, were used for the skin sensitization test. All animals were acclimatized in the animal house for 1 week before the test was conducted and food and water was given (*ad libitum*). The animals were housed at room temperature with a 12-h light-dark cycle. This animal study was approved by the UKM Animal Ethics Committee (UKMAEC; FSK/BIOMED/2011/HIDAYATULFATHI/21-SEPT./391-NOV.-2011-APR.-2015).

Primary Irritation Assay

The test was conducted following ISO10993-10:2010 (E) guidelines for skin irritation and only animals with healthy, intact skin were used. The fur on the animals' backs (approximately 10×15 cm) was shaved prior to the test and special care was taken to prevent injury to the skin of the animals. Animals were divided into test and positive control groups (n = 4 for each group). In the test group animals, four areas of 2.5×2.5 cm were drawn on the fur-free skin; 0.5 g of cream repellent formulation was applied to two areas located across from one another, while the remaining areas were used as negative controls and treated with distilled water, as shown in Fig. 1. The areas were then covered with absorbent gauze and wrapped with elastic bandages and non irritating surgical tape. The same procedure was repeated for animals in the positive control group, but 0.5 g of 20% w/v SLS was applied instead of the cream repellent. The animals were exposed to the treatment and the patches were removed after a period of 6 h. The animals' skin was then washed with distilled water and carefully dried. Observations based on the scoring system were recorded after 24, 48 and 72 h. The animals were sacrificed after 72 h and the skin was collected for histological analysis. The Score for Primary Irritation (SPI) and the Primary Irritation Index (PII) were calculated (as shown below), with the degree of irritation classified according to PII categories in Table 1.

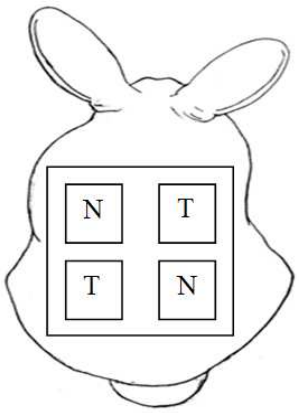


Fig.1. Location of the skin application sites (T: test, N: negative control)

Table 1. Erythema and edema formation scoring system based on ISO10993-10:2010 (E)

Erythema and edema reaction/PII categories	Score
Erythema reaction	
-No erythema	0
-Very slight erythema (barely perceptible)	1
-Well-defined erythema	2
-Moderate erythema	3
-Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Edema Reaction	
-No edema	0
-Very slight edema (barely perceptible)	1
-Well-defined edema (edges of area well-defined by definite raising)	2
-Moderate edema (raised approximately 1 mm)	3
-Severe edema (raised more than 1mm and extending beyond exposure area)	4
PII categories	
-Negligible	0-0.4
-Slight irritation	0.5-1.9
-Moderate irritation	2-4.9
-Severe irritation	5-8

Table 2. Scoring system based on the Magnusson and Kligman scale

Patch test reaction	Score
No visible change	0
Discrete or patchy erythema	1
Moderate or confluent erythema	2
Intense erythema and/or swelling	3

SPI calculation formula:

$$\left[\sum(a+b)_{t1}+(a+b)_{t2}+(a+b)_{t3}/n\right]_T -$$
$$\left[\sum(a+b)_{t1}+(a+b)_{t2}+(a+b)_{t3}/n\right]_C$$

a, erythema; b, edema; t_1, 24 h; t_2, 48 h; t_3, 72 h; n, number of observations (24 h, 48 h, 72 h) on animals (6); T, test group; C, control group.

$$PII = \frac{Mean\ of\ the\ SPI}{Number\ of\ animals\ in\ group}$$

Skin Sensitization Assay

This test consisted of an induction and a challenge phase. The animals were divided into three groups, namely a negative control (n = 5), test (n = 10) and positive control group (n = 5) comprising both males and females. The test was based on the ISO10993-10:2010 (E) guidelines for skin sensitization and only animals with healthy, intact skin were used.

The Induction Phase

Fur on the animals' right upper back region was shaved before the test and special care was taken to prevent injury to the skin of the animals. In the test group, 0.5 g of the cream repellent was applied to an area of 2.5×2.5 cm, covered with absorbent gauze and wrapped with elastic bandage and non irritating surgical tape. After an exposure period of 6 h, the dressings were removed and the skin was washed with distilled water and dried. The negative and positive controls were treated similarly using distilled water and 0.08% w/v DCNB, respectively. The above procedure was performed 3 days a week for 3 consecutive weeks. The animals were then allowed to rest for 14 days (rest phase) without patching.

The Challenge Phase

This phase started after 14-day of the last induction application (rest period) and the fur on the left upper back region of the animals was shaved before the test, with special care taken to prevent injury to the animals' skin. In this phase, the same procedure as in the induction phase was repeated for all animal groups, except that the patch was only applied once on the different sites. Any skin reactions were assessed and recorded after 24 and 48 h based on the Magnusson and Kligman scoring system (Table 2). Sensitization was interpreted as the number of animals that showed a positive response; a score of ≥1 in the test group indicating sensitization to the test materials. The animals were sacrificed after 48 h and the skin was collected for histological analysis. The mean score and sensitization percentage were calculated as follows:

$$Mean\ score = \frac{Total\ score\ for\ skin\ reaction}{Number\ of\ animals}$$

$$Sensitization\ percentage = \frac{Number\ of\ animals\ that\ showed\ positive\ response}{Number\ of\ animals} \times 100$$

Histological Analysis

The animals' skin was preserved in 10% formaldehyde for histological analysis. The skin samples were then processed using a tissue processor, embedded in paraffin wax, sectioned using a microtome (7 um) and stained (hematoxylin and eosin staining). Following this, examination of tissue was conducted using a light microscope (Olympus BX41, Japan).

Data Analysis

For irritation study, the data were presented as visual scores of erythema and edema. The SPI and PII values was calculated and the irritation potential was categorized based on the PII value. Meanwhile, the visual score for the sensitization study was determine based on the Magnusson and Kligman scoring system and presented as a mean score and sensitization percentage. Sensitization was interpreted as the number of animals that showed a positive response.

Results

Primary Irritation Assay

The rabbit skin showed signs of erythema with no edema 24 h after the patch was removed. The PII of the *P. aduncum* EO cream was 1.54 and it was classified as a slight irritant according to the ISO10993-10:2010 (E) guidelines. The erythema persisted until 72 h after the patch was removed; a reversible irritation effect was then observed in some rabbits as the symptoms of erythema started to fade. Meanwhile, rabbits in the positive control group showed severe signs of erythema and eschar formation when there was evidence of edema (Table 4). The results for the skin irritation test are shown in Table 3.

Histological observation results for the rabbit skin treated with *P. aduncum* EO cream are shown in Fig. 2. The epidermal layer remained intact as in the normal rabbit skin (negative control group), with slight hyperplasia, as there was a slight irritation effect observed on the skin. Inflammatory cells were present and these were restricted to the upper part of dermis. Meanwhile, the positive control rabbits showed histopathological signs of irritation, including acanthosis, hyperkeratosis (thickening of stratum corneum), inflammatory cells were present throughout the dermal layer and scabs (necroinflammatory debris).

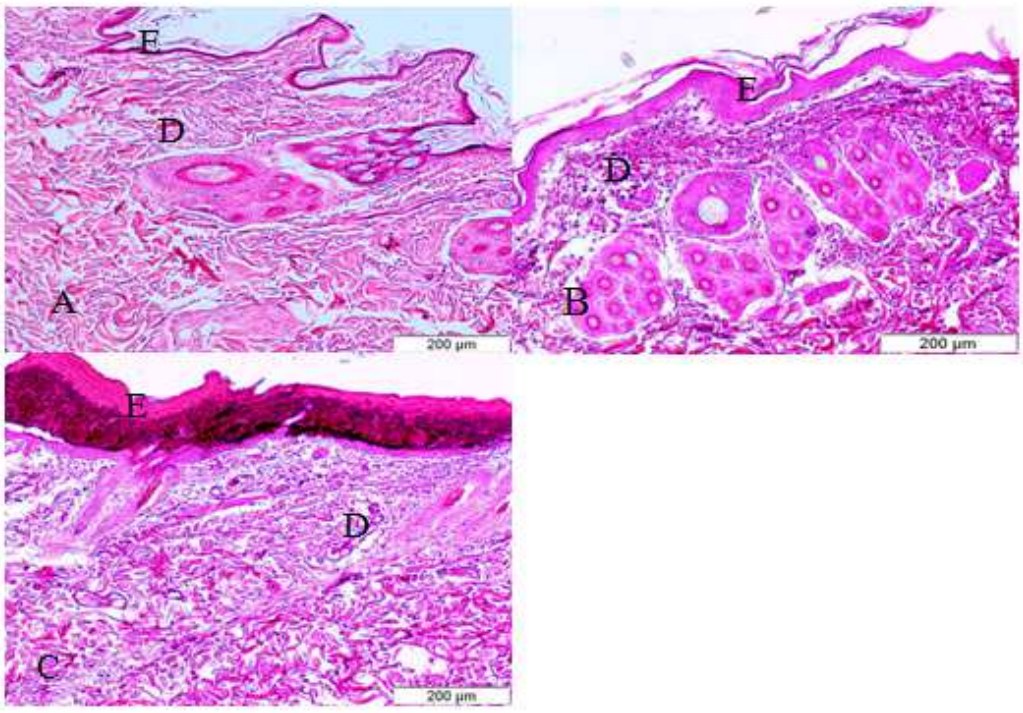


Fig. 2. Histopathology of rabbit skin. The epidermal layer remains intact with slight hyperplasia in skin treated with *Piper aduncum* Essential Oil (EO) cream. When inflammatory cells are present, they are restricted to the upper part of the dermis. The positive control rabbits showed histopathological symptoms of irritation, including acanthosis, hyperkeratosis (thickening of the stratum corneum), inflammatory cells throughout the dermis and scabs (necroinflammatory debris). A: Negative control group; B: Test group; C: Positive control group; E: epidermis; D: dermis; bar: 200 µm; magnification: ×100

Skin Sensitization Assay

The results of the skin sensitization assay for *P. aduncum* EO cream are shown in Table 5. The skin sensitization effect of *P. aduncum* EO cream was negligible and there was no sign of erythema or edema in guinea pigs treated with *P. aduncum* EO cream and distilled water (Table 6). However, the guinea pigs treated with 0.08% DCNB showed a positive dermal response. Histological analysis of guinea pig skin samples (Fig. 3) treated with the cream containing *P. aduncum* EO showed no sign of sensitization, with well-defined epidermal and dermal layers (Fig. 3). Skin samples exposed to 0.08% DCNB displayed evidence of hyperplasia of the epidermis, with the presence of inflammatory cells.

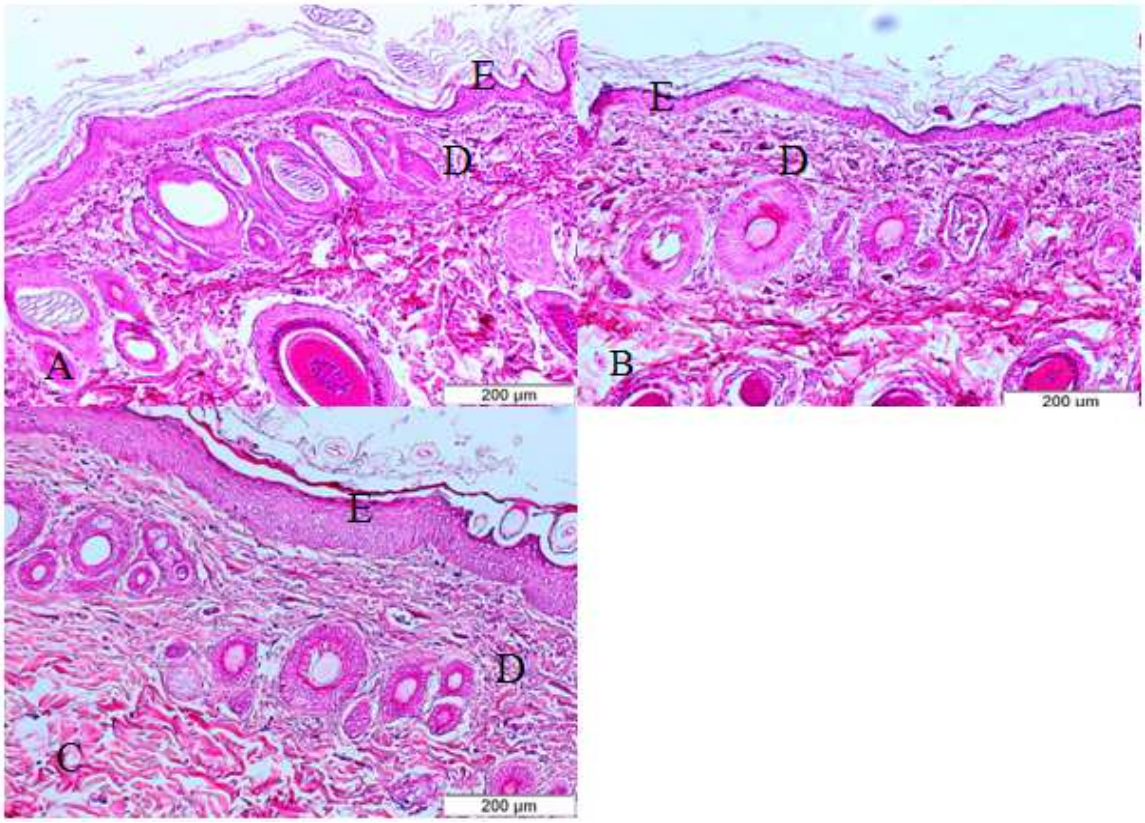


Fig. 3. Histopathology of the guinea pig skin. Guinea pigs treated with *Piper aduncum* Essential Oil (EO) cream and distilled water showed no signs of skin sensitization, with well-defined epidermal and dermal layers. The positive control group treated with 0.08% 1-chloro-2,4-Dinitrobenzene (DCNB) showed evidence of hyperplasia of the epidermis, with the presence of inflammatory cells. A: Negative control group; B: Test group; C: Positive control group; E: epidermis; D: dermis; bar: 200 µm; magnification: ×100

Table 3. Results of the rabbit skin irritation assay

Animal groups	Tested materials	Hours after challenge	Erythema score (Er)	Edema score (Ed)	Total score (Er + Ed)	SPI	PII
Negative control	Distilled water	24	0.00±0.00	0.00±0.00	0	0	0
		48	0.00±0.00	0.00±0.00	0		
		72	0.00±0.00	0.00±0.00	0		
Test	10% *P. aduncum*	24	3.00±0.58	0.00±0.00	12	6.17	1.54
	EO cream	48	3.75±0.25	0.00±0.00	15		
		72	2.50±0.50	0.00±0.00	10		
Positive control	SLS (20 w/v %)	24	4.25±0.63	2.25±0.48	26	16.83	4.21
		48	5.50±0.29	3.00±0.71	34		
		72	6.25±0.48	4.00±0.58	41		

SPI, Primary Irritation Score; PII, Primary Irritation Index

Table 4. Macroscopic evaluation of rabbit skin at 0, 24, 48 and 72 h in skin irritation assay

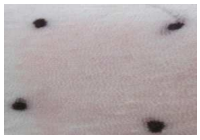
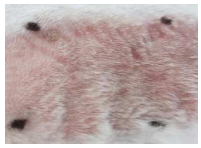
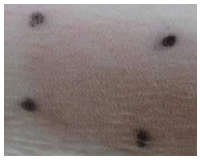
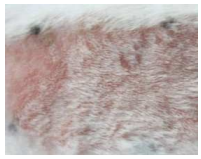
Hours after Challenge	Animal group: Negative control	Test	Positive control
0			
24			
48			
72			

Table 5. Results of guinea pig skin sensitization assay

Animal groups	Substance for assay	Hours after challenge	Mean score	Sensitization Percentage (%)
Negative control	Distilled water	24	0.00±0.00	0
		48	0.00±0.00	0
Test	10% *P. aduncum* EO cream	24	0.00±0.00	0
		48	0.00±0.00	0
Positive control	DCNB (0.08% w/v)	24	0.80±0.20	80
		48	0.40±0.24	40

Table 6. Macroscopic evaluation of guinea pig skin at 0, 24, 48 and 72 h in the skin sensitization assay

Hours after challenge	Animals group: Negative control	Test	Positive control
0			
24			
48			

Discussion

Repellents have been used widely as a personal protection method and as part of integrated vector control programs to minimized the incidence of mosquito-borne diseases, especially in areas where the risk of disease transmission is high. However, growing concerns regarding the side effects of synthetic repellents, especially DEET, has led to the emergence of several plant-based products in the market. To prevent any unwanted risk to the consumer, the safety evaluation of both the ingredients and formulations of new products is necessary before a product is released onto the market (Felter *et al*., 2003). As stated by Ema *et al*. (2012), irritation and sensitization data are a crucial part of this safety assessment. Therefore, this research was conducted to investigated the irritation and sensitization potential of *P. aduncum* EO formulated in a cream base, as the EO has the potential to be developed as an alternative mosquito repellents.

Skin irritation refers to a physiological reaction to a stimulus due to a local inflammatory response and such irritations can be visualized as erythema and edema (Clough *et al*., 2002). Rash, skin inflammation, swelling, scaling and abnormal tissue growth are commonly observed in the area affected by skin irritation reactions (Veronesi *et al*., 1995). Primary skin irritation assay using animals (the Draize assay) has been a method of choice for the past 60 years (Ngo and Maibach, 2010) and it is still currently used to evaluate the irritation potency of substances.

This study investigated the irritant potential of a cream repellent containing *P. aduncum* EO, revealing that the cream repellent can cause slight reversible irritation to rabbit skin, which decreases after 72 h. The *P. aduncum* EO cream was classified as a slight irritant according to the ISO10993-10:2010 (E) guidelines. The irritant reaction might occur due to the penetration of irritant agent through the stratum corneum layer where they bind covalently to the keratinocytes (Cavani and De Luca, 2010; Cohen and Heidary, 2004; El-Azhary and Yiannias, 2004). According to Greaves (2012), the epidermal layer will not erode but changes like hyperkeratosis and acanthosis can occur when the irritation intensity is mild or moderate. Miles *et al*. (2014) defined non irritant substances as those that induce slight or no damage to the skin, while irritants induce severe damage ranging from spongiosis to epidermolysis. In this study, the *P. aduncum* EO cream formulation only induced slight irritation to rabbit skin; thus, the epidermal layer was well defined, without the presence of hyperkeratosis or acanthosis.

The *P. aduncum* EO cream caused slight irritation to rabbit skin. However, according to Bronaugh *et al*. (1989), the rabbit skin is more sensitive than human skin, thus the results cannot be directly extrapolated to humans. Studies have shown that rabbits exhibit stronger irritation reactions compared to humans (Ishii *et al*., 2013; Roggeband *et al*., 2000). Indeed, Basketter *et al*. (2004) reported that 40% of irritants classified by animal testing were not irritants when tested on human skin. Furthermore, Jirova *et al*. (2007) found that out of 15 irritant chemicals reported in rabbits, only 33% caused irritation in humans. Nonetheless, the rabbit has been the animal of choice, since its sensitive skin makes it possible to identified substances that have the ability to cause extremely slight skin irritation (Kojic *et al*., 2009), thereby enabling steps to be taken to prevent injury in humans.

Substances or chemicals that can induce an allergic response upon contact with the skin are known as skin sensitizers and substances are classified as such if they test positive in human or animal tests (Chaudhry *et al*., 2010). The Buehler assay is suitable for testing formulated products, as the method involves the topical application of the test substances (Botham *et al*., 2005); the guinea pig is used as the standard animal for skin sensitization testing (Wiemann *et al*., 2002). In general, a score of 1 or higher on the Magnusson and Kligman scale is regarded as a positive response, provided that the score value in the control animals is less than 1 (ISO, 2002). In this study, it was found that the *P. aduncum* EO cream did not cause any sensitization effect in animals. A positive reaction was only detected in the animals treated with 0.08% DCNB.

Conclusion

This study demonstrated that *P. aduncum* EO cream was slightly irritating to rabbit skin but did not induce any sensitization effect in the test animals. Substances with a PII <5 are regarded as testing negative and they are not considered as primary irritants to skin (Aroonrerk and Kamkaen, 2009; Babul *et al*., 2012). If the PII value is 0-5, the substance is still within the acceptance criteria for safe use on humans. However, if the value is 6-8, the substance is considered as a primary irritant and it cannot be used on human skin (Betsabee *et al*., 2016). Further investigation is necessary to ensure the safety of the *P. aduncum* EO cream before commercialization.

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Author's Contributions

Siti Nur Hanis Mamood: Conducted the whole research, analyze the data and prepare the manuscript.

Hidayatulfathi Othman: Contribute in designing the research plan, organized the study and participate in the manuscript writing.

Nurathirah Mat Nasir: Participate in the research.

Ahmad Rohi Ghazali and Mohd Hanif Zulfakar: Contribute in designing the research plan and organized the study.

Siti Balkis Budin: Contribute in designing the research plan and manuscript writing.

Disclosure

No potential for conflicts of interest exists.

References

Adeniran, O. and E. Fabiyi, 2012. A cream formulation of an effective mosquito repellent: A topical product from lemongrass oil (*Cymbopogon citratus*) Stapf. J. Nat. Prod. Plant Resour., 2: 322-327.

Aroonrerk, N. and N. Kamkaen, 2009. Anti-inflammatory activity of *Quercus infectoria*, *Glycyrrhiza uralensis*, *Kaempferia galangal* and *Coptis chinensis*, the main components of Thai herbal remedies for aphthous ulcer. J. Health Res., 23: 17-22.

Babul, N., A. Rehni, A. Singh, H. Kao and A. Bajaj, 2012. Skin irritation potential of topical mepivacaine gel and cream dosage forms. J. Pain, 13: S87-S87. DOI: 10.1016/j.jpain.2012.01.361

Basketter, D.A., M. York, J.P. McFadden and M.K. Robinson, 2004. Determination of skin irritation potential in the human 4-h patch test. Contact Derm., 51: 1-4. DOI: 10.1111/j.0105-1873.2004.00385.x

Betsabee, O.S.I., S.S.J. Luis, R.S.J. Arturo and C.S. Montserrat, 2017. Evaluation of the toxicity and pathogenicity of biocontrol agents in murine models, chicken embryos and dermal irritation in rabbits. Toxicol. Res., 6: 188-198. DOI: 10.1039/C6TX00275G

Bissinger, B.W., J.P. Schmidt, J.J. Owens, S.M. Mitchell and M.K. Kennedy, 2014. Performance of the plant-based repellent TT-4302 against mosquitoes in the laboratory and field and comparative efficacy to 16 mosquito repellents against *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol., 51: 392-399. DOI: 10.1603/ME12240

Botham, P., M. Urtizberea, C. Wiemann, X. Manciaux and L. Tilbury *et al*., 2005. A comparative study of the sensitivity of the 3-induction and 9-induction Buehler test procedures for assessing skin sensitization potential. Food Chem. Toxicol., 43: 65-75. DOI: 10.1016/j.fct.2004.08.013

Bronaugh, R.L., R.F. Stewart and J.E. Storm, 1989. Extent of cutaneous metabolism during percutaneous absorption of xenobiotics. Toxicol. Appl. Pharmacol., 99: 534-543. DOI: 10.1016/0041-008X(89)90160-9

Cavani, A. and A. De Luca, 2010. Allergic contact dermatitis: Novel mechanisms and therapeutic perspectives. Curr Drug Metab., 11: 228-233. DOI: 10.2174/138920010791196300

Chaudhry, Q., N. Piclin, J. Cotterill, M. Pintore and N.R. Price *et al*., 2010. Global QSAR models of skin sensitisers for regulatory purposes. Chem. Cent. J. DOI: 10.1186/1752-153X-4-S1-S5

Choochote, W., U. Chaithong, K. Kamsuk, A. Jitpakdi and P. Tippawangkosol *et al*., 2007. Repellent activity of selected essential oils against *Aedes aegypti*. Fitoterapia, 78: 359-364. DOI: 10.1016/j.fitote.2007.02.006

Clough, G.F., P. Boutsiouki, M.K. Church and C.C. Michel, 2002. Effects of blood flow on the in vivo recovery of a small diffusible molecule by microdialysis in human skin. J. Pharmacol. Exp. Ther., 302: 681-686. DOI: 10.1124/jpet.102.035634

Cohen, D.E. and N. Heidary, 2004. Treatment of irritant and allergic contact dermatitis. Dermatol. Ther., 17: 334-40. DOI: 10.1111/j.1396-0296.2004.04031.x

El-Azhary, R.A. and J.A. Yiannias, 2004. A new patient education approach in contact allergic dermatitis: The Contact Allergen Replacement Database (CARD). Int. J. Dermatol., 43: 278-280. DOI: 10.1111/j.1365-4632.2004.01843.x

Ema, M., A. Matsuda, N. Kobayashi, M. Naya and J. Nakanishi, 2012. Dermal and ocular irritation and skin sensitization studies of fullerene C60 nanoparticles. Cutan. Ocul. Toxicol., 32: 128-134. DOI: 10.3109/15569527.2012.727937

Felter, S.P., C.A. Ryan, D.A. Basketter, N.J. Gilmour and G.F. Gerberick, 2003. Application of the risk assessment paradigm to the induction of allergic contact dermatitis. Regul. Toxicol. Pharmacol., 37: 1-10. DOI: 10.1016/S0273-2300(02)00021-1

Fradin, M.S., 1998. Mosquitoes and mosquito repellents: a clinician's guide. Ann. Intern. Med., 128: 931-940. DOI: 10.7326/0003-4819-128-11-199806010-00013

Govindarajan, M., 2011. Larvicidal and repellent properties of some essential oils against *Culex tritaeniorhynchus* Giles and *Anopheles subpictus* Grassi (Diptera: Culicidae). Asian Pac. J. Trop. Med., 4: 106-111. DOI: 10.1016/S1995-7645(11)60047-3

Govindarajan, M. and R. Sivakumar, 2011. Mosquito adulticidal and repellent activities of botanical extracts against malarial vector, *Anopheles stephensi* Liston (Diptera: Culicidae). Asian Pac. J. Trop. Med., 4: 941-947. DOI: 10.1016/S1995-7645(11)60223-x

Greaves, P., 2012. Integumentary system. Skin and Subcutaneous Tissue: Non-Neoplastic Changes. In: Histopathology of Preclinical Toxicity Studies: Interpretation and Relevance in Drug Safety Evaluation, Greaves, P. (Ed.). Academic Press, Amsterdam, ISBN-10: 0444538569, pp: 15-33.

Hidayatulfathi, O., S. Sallehuddin and J. Ibrahim, 2004. Adulticidal activity of some Malaysian plant extracts against *Aedes aegypti* Linnaeus. Trop. Biomed., 21: 61-67.

Ishii, S., K. Ishii, M. Nakadate and K. Yamasaki, 2013. Correlation study in skin and eye irritation between rabbits and humans based on published literatures. Food Chem. Toxicol., 55: 596-601. DOI: 10.1016/j.fct.2013.02.004

ISO, 2002. Biological evaluation of medical devices. Part 10: Tests for irritation and delayed type hypersensitivity (ISO 10993-10:2002).

Jirova, D., M. Liebsch, D. Basketter, E. Spiller and K. Kejlova *et al*., 2007. Comparison of human skin irritation and photo-irritation patch test data with cellular in vitro assays and animal in vivo data. Proceedings of the 6th World Congress on Alternatives and Animal Use in the Life Sciences, Aug. 21-25, AATEX 14, Special Issue, Japan, pp: 359-365.

Katz, T.M., J.H. Miller and A.A. Hebert, 2008. Insect repellents: Historical perspectives and new developments. J. Am. Acad. Dermatol., 58: 865-871. DOI: 10.1016/j.jaad.2007.10.005

Keziah, E.A., E.N. Nukenine, S.P.Y. Danga, L. Younoussa and C.O. Esimone, 2015. Creams formulated with *Ocimum gratissimum* L. and *Lantana camara* L. crude extracts and fractions as mosquito repellents against *Aedes aegypti* L. (Diptera: Culicidae). J. Insect Sci., 15: 45-45. DOI: 10.1093/jisesa/iev025

Kojic, Z., D. Stojanovic, S. Popadic, M. Jokanovic and D. Janackovic, 2009. The irritative property of α-tricalcium phosphate to the rabbit skin. Gen. Physiol. Biophys., 28: 168-173.

Kwon, H.W., S.I. Kim, K.S. Chang, J.M. Clark and Y.J. Ahn, 2011. Enhanced repellency of binary mixtures of *Zanthoxylum armatum* seed oil, vanillin and their aerosols to mosquitoes under laboratory and field conditions. J. Med. Entomol., 48: 61-66. DOI: 10.1603/ME10042

Mamood, S.N.H., O. Hidayatulfathi, S.B. Budin, G.A. Rohi and M.H. Zulfakar, 2017. The formulation of the essential oil of *Piper aduncum* Linnaeus (Piperales: Piperaceae) increases its efficacy as an insect repellent. Bull. Entomol. Res., 107: 49-57. DOI: 10.1017/S0007485316000614

Mehmood, F. and Z. Khan, 2012. Determination of skin irritancy by essential oils from some members of Family Rutaceae of Pakistan. Biologia (Pakistan), 58: 161-166.

Miles, A., A. Berthet, N.B. Hopf, M. Gilliet and W. Raffoul *et al*., 2014. A new alternative method for testing skin irritation using a human skin model: A pilot study. Toxicol. Vitr., 28: 240-247. DOI: 10.1016/j.tiv.2013.10.022

Misni, N., S. Sulaiman and O. Hidayatulfathi, 2008. The repellent activity of *Piper aduncum* Linn (Family: Piperaceae) essential oil against *Aedes aegypti* using human volunteers. J. Trop. Med. Parasitol., 31: 63-69.

Nerio, L.S., J. Olivero-Verbel and E. Stashenko, 2010. Repellent activity of essential oils: A review. Bioresour. Technol., 101: 372-378. DOI: 10.1016/j.biortech.2009.07.048

Ngo, M.A. and H.I. Maibach, 2010. Dermatotoxicology: Historical perspective and advances. Toxicol. Appl. Pharmacol., 243: 225-238. DOI: 10.1016/j.taap.2009.12.008

Patel, H.K., B.S. Barot, P.B. Parejiya, P.K. Shelat and A. Shukla, 2013. Topical delivery of clobetasol propionate loaded microemulsion based gel for effective treatment of vitiligo: *Ex vivo* permeation and skin irritation studies. Colloids Surf. B Biointerfaces, 102: 86-94. DOI: 10.1016/j.colsurfb.2012.08.011

Pohlit, A.M., A.C.S. Pinto and R. Mause, 2006. *Piper aduncum* L.: pluripotent plant and important phytochemical substance source. Rev. Fitos., 2: 7-18.

Roggeband, R., M. York, M. Pericoi and W. Braun, 2000. Eye irritation responses in rabbit and man after single applications of equal volumes of undiluted model liquid detergent products. Food Chem. Toxicol., 38: 727-734. DOI: 10.1016/S0278-6915(00)00057-0

Veronesi, B., D.M. Sailstad, D. Doerfler and M. Selgrade, 1995. Neuropeptide modulation of chemically induced skin irritation. Toxicol. Applid. Pharmacol., 135: 258-267. DOI: 10.1006/taap.1995.1232

Wiemann, C., K. Berthold, A. Heusener, N. Kruger and A. Seeberger *et al*., 2002. Joint positive control testing in guinea pig skin sensitization tests. A harmonized approach. Regul. Toxicol. Pharmacol., 35: 14-22. DOI: 10.1006/rtph.2001.1503