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

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

*School of Diagnostics and Applied Health Sciences, Faculty of Health Sciences, National University of Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

*Centre for Drug Delivery Research, Faculty of Pharmacy, National University of Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia*

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

<table>
<thead>
<tr>
<th>Erythema reaction/PII categories</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>-No erythema</td>
<td>0</td>
</tr>
<tr>
<td>-Very slight erythema (barely perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>-Well-defined erythema</td>
<td>2</td>
</tr>
<tr>
<td>-Moderate erythema</td>
<td>3</td>
</tr>
<tr>
<td>-Severe erythema (beet-redness) to eschar formation preventing grading of erythema</td>
<td>4</td>
</tr>
<tr>
<td>Edema reaction</td>
<td></td>
</tr>
<tr>
<td>-No edema</td>
<td>0</td>
</tr>
<tr>
<td>-Very slight edema (barely perceptible)</td>
<td>1</td>
</tr>
<tr>
<td>-Well-defined edema (edges of area well-defined by definite raising)</td>
<td>2</td>
</tr>
<tr>
<td>-Moderate edema (raised approximately 1 mm)</td>
<td>3</td>
</tr>
<tr>
<td>-Severe edema (raised more than 1 mm and extending beyond exposure area)</td>
<td>4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Patch test reaction</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No visible change</td>
<td>0</td>
</tr>
<tr>
<td>Discrete or patchy erythema</td>
<td>1</td>
</tr>
<tr>
<td>Moderate or confluent erythema</td>
<td>2</td>
</tr>
<tr>
<td>Intense erythema and/or swelling</td>
<td>3</td>
</tr>
</tbody>
</table>

SPI calculation formula:

\[
\left[ \frac{\Sigma (a+b)_{t_1} + (a+b)_{t_2} + (a+b)_{t_3}}{n} \right]_{T} - \left[ \frac{\Sigma (a+b)_{t_1} + (a+b)_{t_2} + (a+b)_{t_3}}{n} \right]_{C}
\]

Where:

- \(a\), 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:

\[Mean\ score = \frac{Total\ score\ for\ skin\ reaction}{Number\ of\ animals}\]
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 determined 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.

![Histopathology of the guinea pig skin](image)

**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>
<thead>
<tr>
<th>Table 3. Results of the rabbit skin irritation assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal groups</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>Negative control</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Test</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Positive control</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Hours after Challenge</th>
<th>Negative control</th>
<th>Test</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td><img src="Image1" alt="Image" /></td>
<td><img src="Image2" alt="Image" /></td>
<td><img src="Image3" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td><img src="Image4" alt="Image" /></td>
<td><img src="Image5" alt="Image" /></td>
<td><img src="Image6" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td><img src="Image7" alt="Image" /></td>
<td><img src="Image8" alt="Image" /></td>
<td><img src="Image9" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td><img src="Image10" alt="Image" /></td>
<td><img src="Image11" alt="Image" /></td>
<td><img src="Image12" alt="Image" /></td>
</tr>
</tbody>
</table>

Table 5. Results of guinea pig skin sensitization assay

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

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

<table>
<thead>
<tr>
<th>Animals group</th>
<th>Hours after challenge</th>
<th>Negative control</th>
<th>Test</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td><img src="Image13" alt="Image" /></td>
<td><img src="Image14" alt="Image" /></td>
<td><img src="Image15" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td><img src="Image16" alt="Image" /></td>
<td><img src="Image17" alt="Image" /></td>
<td><img src="Image18" alt="Image" /></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td><img src="Image19" alt="Image" /></td>
<td><img src="Image20" alt="Image" /></td>
<td><img src="Image21" alt="Image" /></td>
</tr>
</tbody>
</table>
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 (Felten 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 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 (Aroonnerk 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.

Acknowledgement

We would like to thank the School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, National University of Malaysia, for providing research facilities to conduct this study. We would also like to thank the Faculty of Pharmacy, National University of Malaysia, for providing the facilities to develop the P. aduncum EO cream formulation.
Funding Information
This work was funded by The National University of Malaysia, Research University Grant (GUP) [grant number GUP-2012-064].

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


