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ORIGINAL ARTICLE

Primary Skin Irritation and Dermal Sensitization Assay: *In vivo* Evaluation of the Essential Oil from *Piper sarmentosum* Roxb.

Nor Zafirah Abu Bakar, Hidayatulfathi Othman, Nor Fadilah Rajab, Siti Balkis Budin, Ahmad Fuad Shamsuddin[1], Nor Azwani Mohamed Nor

Biomedical Science Program, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur, [1]Faculty of Pharmacy and Health Sciences, Universiti Kuala Lumpur Royal College of Medicine Perak, Ipoh, Perak, Malaysia

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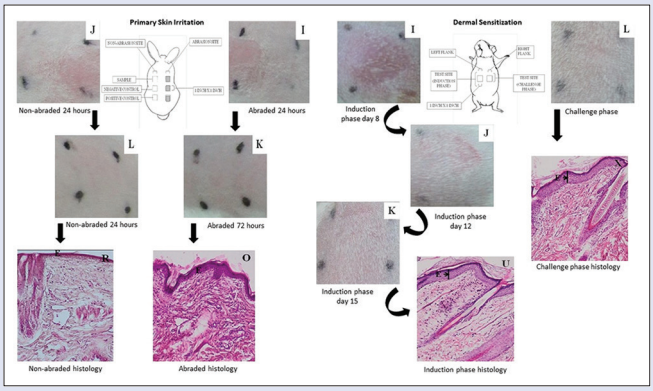
ABSTRACT

Background: *Piper sarmentosum* Roxb. is a traditional medicine which can also be consumed as a vegetable. Despite the availability of a variety of toxicological data on extracts of this plant, to our knowledge, until now, no dermal toxicological tests have been conducted. The aim of the present study was, therefore, to carry out *in vivo* assays to verify the safety of application of *P. sarmentosum* extract gel on the skin. **Materials and Methods:** The essential oils were extracted from the dried leaves of *P. sarmentosum* by hydrodistillation and then formulated into a gel. An *in vivo* skin irritation test of the *P. sarmentosum* extract gel was then conducted on albino rabbits, and an *in vivo* sensitization test was carried out on albino guinea pigs. **Results:** For both abraded and non-abraded sites, the calculated primary irritation index value for *P. sarmentosum* was 2.55, indicating that topical use of a gel with *P. sarmentosum* (1.55%) is nonirritative. However, a dermal sensitization assay revealed mild sensitization effects in 1 out of 10 guinea pigs in response to the application. Histological analysis revealed a slight thickening of the stratum corneum epidermis layer in the guinea pigs' skin. **Conclusion:** Together, these results indicate that the gel with *P. sarmentosum* is safe for application on the skin, but may lead to sensitization upon repeated application.

Key words: Dermal sensitization assay, essential oil, *Piper sarmentosum*, primary skin irritation, topical gel

SUMMARY

- *Piper sarmentosum* possesses pesticide, larvicide, and repellent properties. Most repellents containing N, N-diethyl-3-methylbenzamide (DEET) as an active ingredient have toxic effects on human beings. The essential oil of *P. sarmentosum* in gel form was developed because gel-based repellents are almost non-existent in the market. However, dermal toxicity assessments are essential before production and commercialization of repellent. The essential oil of *P. sarmentosum* was obtained from hydrodistillation. New Zealand albino rabbits and guinea pigs were used as test subjects for dermal assessment. For primary skin irritation, the score for *P. sarmentosum* was lower than the score for DEET on both abraded and non-abraded sites. Macroscopic observation showed that DEET caused more redness on abraded and nonabraded sites compared to *P. sarmentosum*. Primary irritation index for *P. sarmentosum* was 2.55, which is considered nonirritation. Microscopic observation revealed no change in the epidermal layer of the abraded site treated with *P. sarmentosum* compared to the non-abraded site. Erythema score and redness for *P. sarmentosum* were decreased from day 8 to day 15 after application. The results obtained indicated *P. sarmentosum* as mild sensitization (Grade II) agent and DEET as moderate sensitization (Grade III) agent.



Abbreviations used: LD_{50}: Lethal dose 50; DEET: N, N-diethyl-3-methylbenzamide; Roxb.: Roxburgh, a botanist who wrote about plants; OECD: Organisation for Economic Co-Operation and Development; PSI: Primary skin irritation; PII: Primary irritation index; WT/BALB: Wild-type mice.

Correspondence:

Prof. Hidayatulfathi Othman,
Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, Kuala Lumpur 50300, Malaysia.
E-mail: hida@ukm.edu.my

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INTRODUCTION

Piper sarmentosum Roxb. (Piperaceae), better known in Malaysia as Daun Kaduk, is commonly used in traditional medicine and cooking in Southeast Asian countries.[1] The aerial parts of the plant are consumed as vegetables in various forms. The whole or parts of the plant are also used in folk remedies, alone or in combination with other herbs, to treat various ailments.[2] Previous research has shown that *P. sarmentosum* extract possesses antibacterial,[3-6] antifungal,[6,7] antiprotozoal,[8,9] antiviral,[10] anti-inflammatory and antipyretic,[5,11,12] and antioxidant[13,14] activities, as well as functioning as a pesticide,[15] a larvicide,[16-19] and a repellant.[20]

It also shows significant acute oral toxicity,[11,21,22] but only at relatively high doses. *P. sarmentosum* has edible uses, in which the aerial parts of the plant are consumed after cooking or boiling in water as a food. The cooked food is termed as *Ulam* in Malaysia and Indonesia. Certain traditional cuisines are wrapped in the leaves of the plant to impart aroma

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and enhance taste. Hussain *et al.*[22] found no acute oral toxicity from the ethanol extracts of the leaves and fruits at the maximum dose tested, 2000 mg/kg; the LD_{50} of both extracts is, therefore, much higher than 2000 mg/kg. Whereas the aqueous extracts of the plant have been reported to be safe below a 10 g/kg dose, a number of deaths have occurred in rats at doses ≥10 g/kg.[21] Finally, Ridtitid *et al.*[11] reported that the methanol extracts of the leaves at a dose of 5 g/kg cause no mortality in mice.

The present study examined the dermal effects of using a *P. sarmentosum* extract as a natural, alternative insect repellent. Most repellents use N, N-diethyl-3-methylbenzamide (DEET), commonly known as DEET, as the active ingredient. However, studies have shown that DEET can exert toxic effects on infants, children, and some adults.[23] The essential oils of the plant, often used as spices and flavoring agents in foods, have been recommended as possible active ingredients in insect repellents, and some have been reported to be effective in repelling insects.[24] For example, Tawatsin *et al.*[25] reported the repellency of volatile oils extracted by steam distillation from four plant species: turmeric (*Curcuma longa*), kaffir/makrut lime (*Citrus hystrix*), citronella grass (*Cymbopogon winterianus*), and hairy basil (*Ocimum americanum*). All of them were evaluated in the laboratory and found to be effective repellents against three mosquito vectors: *Aedes aegypti*, *Anopheles dirus*, and *Culex quinquefasciatus*.

Insect repellents are produced in many forms, for example, lotions, creams, aerosols, patches, and wrist bands. Gel-based repellents, however, are almost non-existent in the market, even though topical medication in gel form is common. Gel is a transparent or translucent semisolid two-component system that is rich in liquid.[26] The process of manufacturing gel is economical and its structure is well suited for repellent application. To be an effective repellent, it should fulfill the fundamental criterion of being not easily absorbed into the skin.[27] The essential oil of *P. sarmentosum* in gel form fulfills this criterion, but before any production and commercialization of *P. sarmentosum*-based repellent, dermal toxicity assessments are essential to determine any adverse effects and the seriousness of any such effects. Two important types of dermal toxicity were considered in the present study: skin irritation and skin sensitization. Skin irritation is a localized inflammatory reaction induced by a stimulus or agent. Clinical signs of irritation include erythema (redness), edema (swelling), itching, and pain. Skin sensitization refers to an allergic reaction to a particular irritant that results in the development of skin inflammation and itchiness. Unlike skin irritation, in the case of skin sensitization, the skin becomes increasingly reactive to the substance as a result of subsequent exposure.[28] Thus, this study was designed to assess skin irritation and dermal sensitization on experimental animals after *P. sarmentosum* repellent application.

MATERIALS AND METHODS

The leaves of *P. sarmentosum* Roxb. were collected from Jubli Perak Sultan Haji Ahmad Shah Agricultural Park in Kuantan, Pahang, Malaysia. The *P. sarmentosum* plants had been certified and tagged as UKMB29779 by the Herbarium Department, Universiti Kebangsaan, Malaysia. A hydrodistillation process using a Clevenger-type apparatus was used to extract the essential oil of *P. sarmentosum* (100% concentration). The distillate was dried using anhydrous magnesium sulfate before the oil was extracted and formulated into gel form.

On previous study, the gel with *P. sarmentosum* was tested and showed the repellency effect toward *A. aegypti* mosquitoes in laboratory (Hidayatulfathi *et al.* 2017). Because the gel with the essential oil of *P. sarmentosum* showed the repellent effect, DEET as a gold standard repellent was employed and formulated in gel as well for this study.

New Zealand albino rabbits and guinea pigs were used as test subjects for these dermal assessments. The standard ethical principles confirmed the animal subjects and methods of this study and was approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (approval code: FSK/BIOMED/2011/HIDAYATULFATHI/21-SEPTEMBER/309-SEPT.-2011-SEPT.-2013).

Primary skin irritation assay

Test materials and animals

Primary skin irritation (PSI) studies were conducted due to commercialization purpose. It is to ascertain that the active ingredients in the formulation of the sample do not cause any harm to the skin and as a guarantee for the product are safe to be apply.

Six adult New Zealand albino rabbits, weighing between 2.5 kg and 3.5 kg, were used for the experiment. Three substances were tested on each rabbit: 1.55% *P. sarmentosum* in gel, 25% DEET in gel (positive control; DEET has been previously shown to cause PSI in rabbits), and normal saline in gel (negative control). The gel itself has been shown to have extremely low-irritant properties and is non sensitizing with repeated usage.[29-31] The test animals were acclimatized at least 5 days before the tests. Each rabbit was housed in an individual cage in a temperature-controlled (20°C–25°C) and humidity-monitored (45%–65%) environment. The dorsal areas of the albino rabbits measuring 11 cm × 18 cm were shaved, and six test areas (three on the right side and three on the left side), each measuring 1 inch × 1 inch, were marked. The three right dorsal test areas were abraded to the stratum corneum with a sterile needle. (To obviate the need for any topical anesthetic, a mild procedure, in which the abrasion was sufficiently deep to penetrate the epidermis, but not deep enough to induce bleeding, was used.) The three left dorsal test areas were not abraded [Figure 1].

Procedure

About 0.5 ml of each sample (gel with *P. sarmentosum*, gel with 25% DEET, and gel with saline) was placed on the two pieces of filter paper, each measuring 1 inch × 1 inch. The filter paper was then placed on the test area as shown in Figure 1, covered with a gauze pad, and attached using a surgical tape. The entire dorsal area was wrapped with rubberized cloth and micropore tape for 24 h. The scores for erythema and edema were evaluated according to the Organisation for Economic Co-Operation and Development[32] [Table 1]. The scores were also evaluated 72 h after removal of the filter paper for each abraded and intact skin test area.

The albino rabbits tested were sacrificed by giving excessive phenobarbital intraperitoneally. The albino rabbit was placed in the strainer and the fur

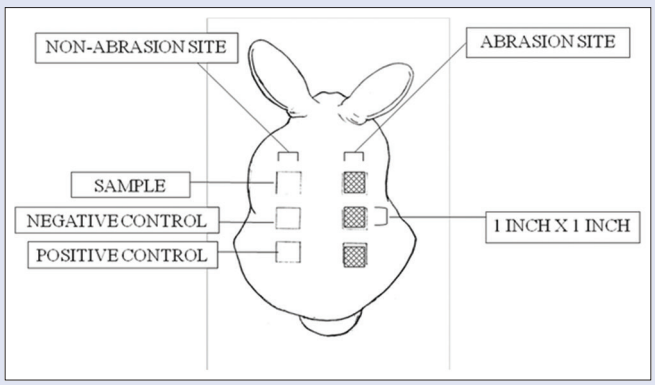

Figure 1: Experimental design for dermal primary irritation testing: rabbit dorsal area

at the ear was shaved to reveal the vein. Then, the skin on the ear was sterilized by swapped with alcohol and the vein was injected with the phenobarbital. The albino rabbit that had sacrificed was biopsied to get the tested skin area. The skin-biopsied specimens were then soaked in 10% formalin solution and were processed for histological identification.

Dermal sensitization assay

Test materials and animals

Dermal sensitization assay was conducted to observe the potential of the product to cause irritation on the skin when being applied repeatedly.

The experiments were conducted according to the Buehler method.[33] The guinea pigs were divided into experimental (1.55% *P. sarmentosum* in gel, $n = 10$), positive control (25% DEET in gel, $n = 10$), and negative control (saline in gel, $n = 5$) groups. The guinea pigs were placed in plastic cages and acclimatized to the environment before the test. The anterior, bilateral, and dorsal areas of the test animals, each measuring 5 inch × 3 inch, were shaved, and the test areas measuring one square inch each were carefully marked [Figure 2].

Procedure

The test was divided into three phases: induction, rest, and challenge. In the induction phase, 0.5 ml of the sample was applied on the left test site of the backs of the guinea pigs as described above for the PSI test on the rabbits. The sample patch was attached to the guinea pigs using gauze and wrapped with an elastic bandage. After 6 h, the gauze was removed and the skin was observed for any sign of erythema/edema based on the Magnusson and Kligman scale[34] [Table 2]. This procedure was repeated 3 times/week for 3 weeks. During the resting phase, no formulation was applied to the skin of the test animals.

On day 27 post application, the start of the challenge phase, the same volume of sample as in the induction phase was applied on the right test site of the backs of the guinea pigs. The patch was removed after 6 h, and the presence of any erythema/edema on the challenged skin was recorded 24 h and 48 h after application. The same procedure was used for the positive and negative control groups. Rating on sensitization response was classified according to the grade summarized in Table 3.

The guinea pigs were sacrificed by giving excessive phenobarbital intraperitoneally. This procedure was made by injecting underneath the abdominal skin of the guinea pigs. The sacrificed guinea pigs were then biopsied to get the tested skin areas. The skin-biopsied specimens were then soaked in 10% formalin solution and were processed for histological identification.

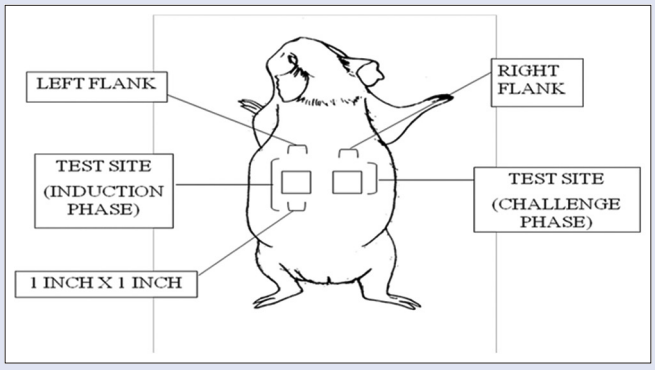

Figure 2: Experimental design for dermal sensitization testing: guinea pig dorsal area

Histology

The skin-biopsied specimens were obtained from the sites exposed to gels with *P. sarmentosum* extract, DEET (positive control), or saline only (negative control). The animals were sacrificed after 72 h of observation, and histological analysis was then undertaken. The biopsied specimens were fixed in buffered 10% formalin, processed, and embedded in paraffin. Each sample was cut and sectioned for hematoxylin–eosin staining.

Statistical analysis

For the toxicological assays, the experimental designs were performed using ISO10993:2010 and Buehler method, but not necessarily analyzed statistically.

RESULTS

Skin Irritation Score

PSI was scored from 0 to 4 for erythema and edema effects. Table 4 shows the skin scores for abraded and non-abraded sites. After 24

Table 1: Primary skin irritation scoring (Organisation for Economic Co-Operation and Development 2002)

Skin reaction	Value
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate-to-severe erythema	3
Severe erythema (beet redness) to slight eschar formations (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised >1 mm and extending beyond the exposed area)	4

Table 2: Magnusson and Kligman (1969) scale for skin sensitization

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

Table 3: Rating of sensitization response

Percentage sensitized	Grades	Classification
0-8	I	No difference from control
9-28	II	Mild
28-64	III	Moderate
65-80	IV	Strong
81-100	V	Extreme

Table 4: Skin irritation score

Observation Site	24 h		72 h	
	Abraded	Non-abraded	Abraded	Non-abraded
Negative control (saline)	0±0.0	0±0.0	0±0.0	0±0.0
Positive control (DEET)	6.17±0.87	4.5±0.85	5.33±0.99	4.17±0.87
Piper sarmentosum extract	3.6±0.6	3.6±0.81	1.8±0.58	1.6±0.4

DEET: N, N-diethyl-3-methylbenzamide

h of observation, the PSI scores were equal for abraded and non-abraded sites treated with the *P. sarmentosum* gel: 3.6 ± 0.6 and 3.6 ± 1.8, respectively. The PSI scores for the areas treated with 25% DEET were 6.17 ± 0.87 for the abraded site and 4.5 ± 0.85 for the non-abraded site. The macroscopic appearance of the abraded (I) and non-abraded (J) test areas treated with *P. sarmentosum* extract and the abraded (E) and non-abraded (F) test areas treated with DEET is shown in Figure 3. The scores decreased after 72 h for the areas treated with *P. sarmentosum*: 1.8 ± 0.58 and 1.6 ± 0.4 for the abraded and non-abraded sites, respectively. Similar results were observed for the area treated with DEET; the PSI scores were 5.33 ± 0.99 for the abraded site and 4.17 ± 0.87 for the non-abraded site. The macroscopic observation also showed more redness on the abraded (G) and non-abraded (H) sites treated with DEET than on the abraded (K) and non-abraded (L) sites treated with *P. sarmentosum*.

Table 5: Primary irritation index

	Concentration (%)	Values referenced to		Sum	PII
		Erythema	Edema		
Saline only	-	0	0	0	0
DEET	25	10.49	8.99	19.48	4.87
Piper sarmentosum	1.55	6	4.2	10.2	2.55

PII: Primary irritation index; DEET: N, N-diethyl-3-methylbenzamide

Skin irritation assay classification

Primary irritation index (PII) values are shown in Table 5. The PII for *P. sarmentosum* was 2.55, whereas the PII for DEET was 4.87. Thus, both of the results were lower than 5, which indicates that the formulations are considered not irritative. However, the index for DEET was close to 5. Microscopic observation revealed no change in the epidermal layer of the abraded site (N) treated with *P. sarmentosum*, compared with the non-abraded site (Q). In both of these sites, the epidermis was slightly thickened [Figure 4].

Skin Sensitization Score

Sensitization scoring results are shown in Table 6, where weak erythema and edema effects can be seen. The erythema and edema scores for the guinea pigs treated with *P. sarmentosum* gel were 1.7 ± 0.26 and 1.1 ± 0.1 at day 8, respectively. However, the scores decreased to 0.7 ± 0.21 and 0.7 ± 0.21 at day 15 after application, respectively. Macroscopically, Figure 5 shows that redness can be clearly seen on the skin (I), and that

Table 6: Skin sensitization score

Group	Day 8		Day 12		Day 15		Challenge phase	
	E	O	E	O	E	O	E	O
Saline only	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
DEET	1.0±0.0	0.9±0.1	2.1±0.28	1.4±0.22	3.0±0.37	1.2±0.13	0.4±0.16	0±0.0
Piper sarmentosum	1.7±0.26	1.1±0.1	1.1±0.28	0.7±0.21	0.7±0.21	0.6±0.16	0.1±0.1	0±0.0

DEET: N, N-diethyl-3-methylbenzamide

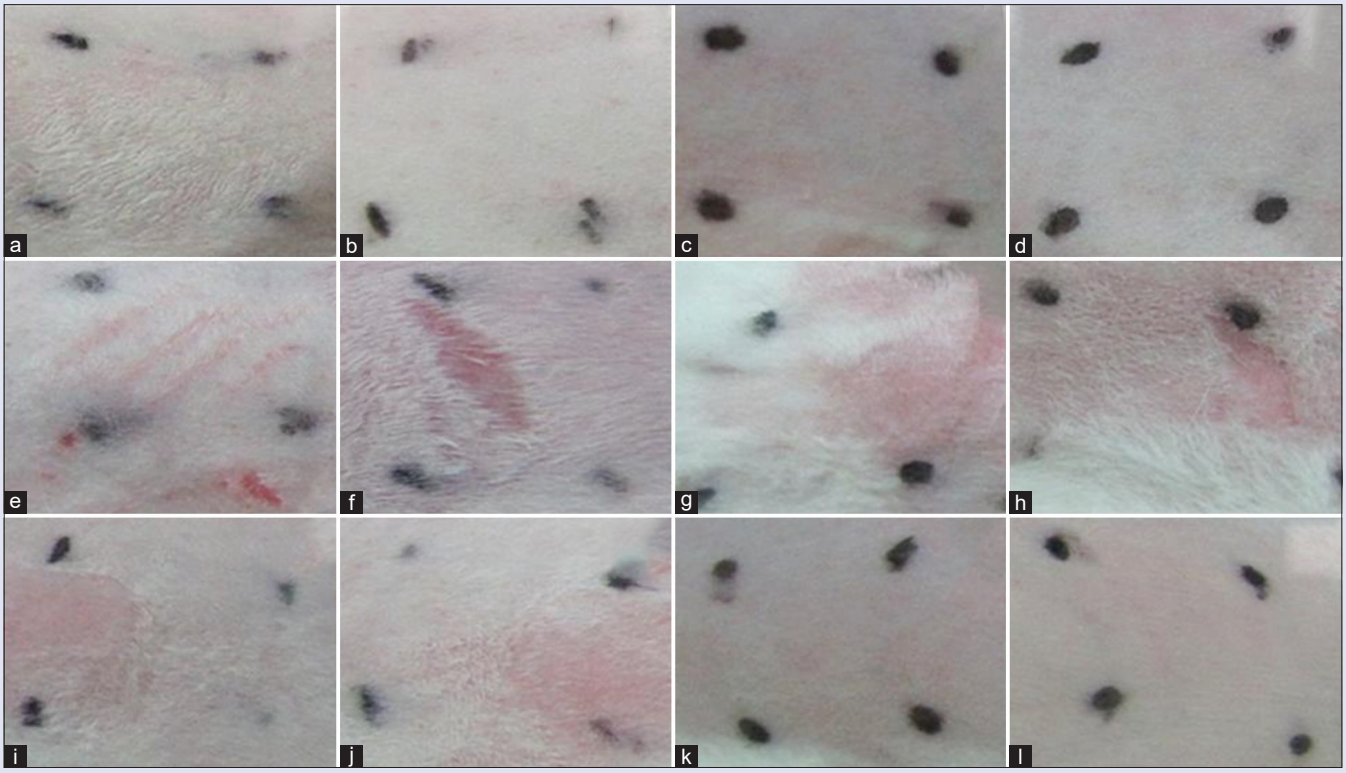

Figure 3: Macroscopic results. Abraded sites (a, e, i, c, g, and k); non-abraded sites (b, f, j, d, h, and l); treatment with normal saline (a-d); treatment with N, N-diethyl-3-methylbenzamide (e-h); treatment with *Piper sarmentosum* gel (i-l); observation after 24 h (a, b, e, f, i, and j); observation after 72 h (c, d, g, h, k, and l)

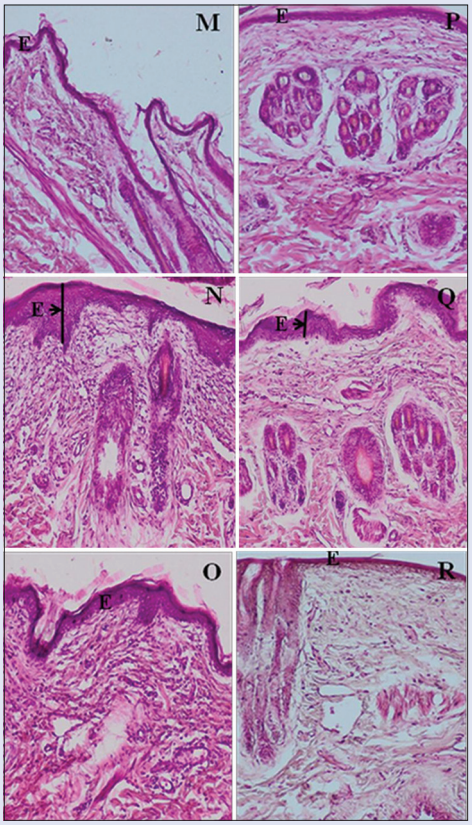

Figure 4: Medium magnification of histology of abraded areas treated with normal saline (M, P), N, N-diethyl-3-methylbenzamide 25% (N, Q), or *Piper sarmentosum* gel (O, R). Epidermal thickness is clearly evident in the N, N-diethyl-3-methylbenzamide Group. E, epidermis

this redness decreased at day 15 (K). In the group treated with DEET, the erythema and edema scores were 1.0 ± 0.0 and 0.9 ± 0.1 on day 8 post application, 2.1 ± 0.28 and 1.4 ± 0.22 on day 12 post application, and 3.0 ± 0.37 and 1.2 ± 0.13 on day 15 post application, respectively. These high scores were due to severe erythema with slight eschar formations clearly seen starting at day 12 after application [Figure 5f] that worsened at day 15 [Figure 5g].

Skin sensitization assay classification

Based on the challenge phase results [Table 7], 1 out of 10 guinea pigs exhibited a response to the applied *P. sarmentosum*, whereas 4 out of 10 guinea pigs exhibited a response to the DEET. These results indicated that *P. sarmentosum* can be classified as a mild sensitization (Grade II) agent, and DEET as a moderate sensitization (Grade III) agent based on Table 3. Figure 6 shows the histology of the left side (induction phase) test sites and the right side (challenge phase) test sites of the skin. Thickening of the epidermis layer was seen in the skin of the guinea pigs treated with *P. sarmentosum* and DEET (U, T, X, and W).

DISCUSSION

Skin irritation is defined as the production of 'reversible damage of the skin following the application of a test substance for up to 4 hours'. It has generally been assessed by the potential of a certain substance/product to cause erythema/eschar and/or oedema after a single topical

Table 7: Dermal sensitization assay

Test material	Challenge phase response*	Classification
Saline gel	0/10	Negative
DEET gel, 25%	4/10	Moderate
Piper sarmentosum gel, 1.55%	1/10	Mild

*Number of positives/number of tested. DEET: N, N-diethyl-3-methylbenzamide

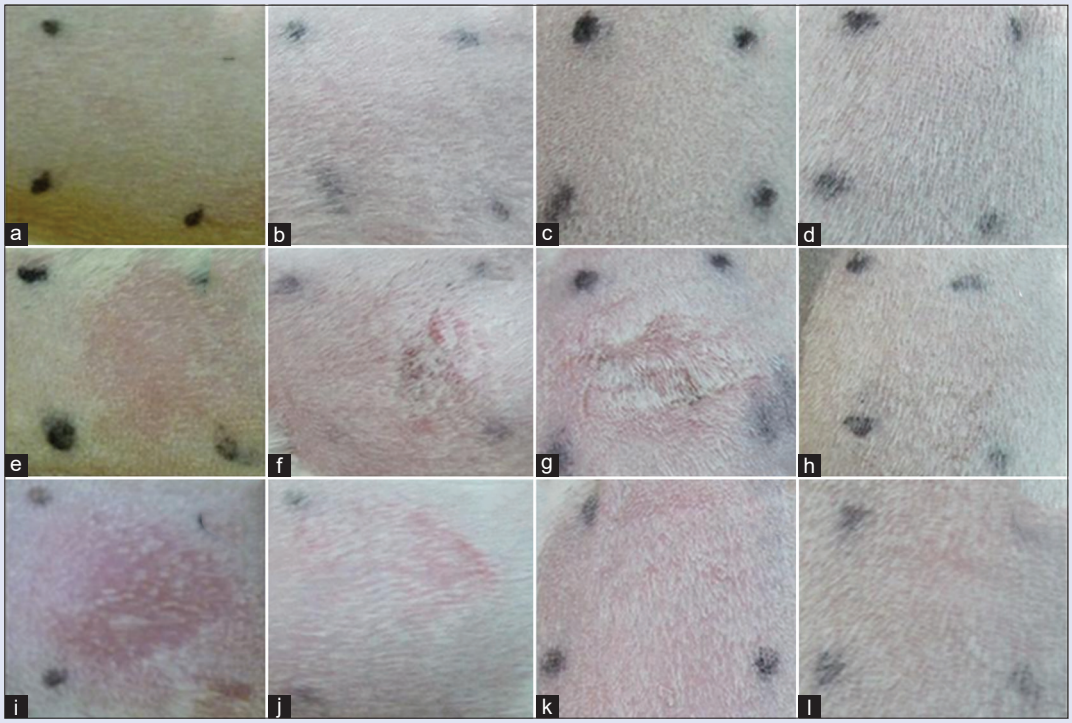

Figure 5: Macroscopic results. Treatment with normal saline (a-d); treatment with N, N-diethyl-3-methylbenzamide (e-h); treatment with *Piper sarmentosum* gel (i-l); induction phase (day 8: a, e, and i; day 12: b, f, and j; and day 15: c, g, and k); challenge phase (d, h, and l)

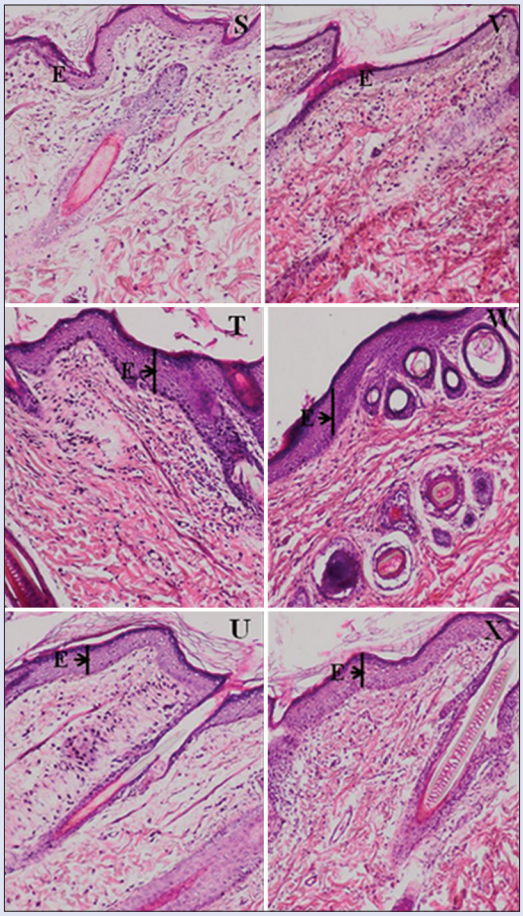

Figure 6: Medium magnification histology of the left side (induction phase) and the right side (challenge phase): treatment with normal saline (S, V), treatment with N, N-diethyl-3-methylbenzamide 25% (T, W), and treatment with *Piper sarmentosum* gel (U, X)

application on rabbit skin and based on the Draize score.[32] Whereas, skin sensitization means allergic reaction toward certain irritation that may contribute to skin irritation and itchiness. Contrary to skin irritation, the skin will be more reactive effect toward substance as if repeatedly applied.[28]

Craig *et al.*[35] studied the acute dermal toxicity of pure essential oils extracted from *Juniperus occidentalis* and *Chamaecyparis lawsoniana* plants at concentrations of 0.5%, 5%, and 50% in albino New Zealand rabbits. Roy *et al.*[28] studied sensitization by hydrogels in guinea pigs. Based on these precedents, we chose to use these same models in the present study. In a retrospective analysis of 224 dermal toxicity studies that used six rabbits per experimental group, it was noted that reducing the number to five or four resulted in a loss of statistical power, with agreement declining to slightly below 90%, and when three animals were used, agreement declined to nearly 70%.[36] Thus, the number of animals (six) used in the present trials can be considered to be optimal.

Queiroz *et al.* (2009) studied the toxicity test on albino rabbits using the chosen carbopol gel based on the stability of formulation after 90 days of observation. In the study, the gel was applied on the nonabrasion skin and showed no irritation effect. However, the gel form showed mild irritation toward the tested animals with abrasion skin. Queiroz *et al.* (2009) also found that the effect of erythema and edema was no longer after 72 h of observation on all the tested skins. The study also found that all the effects of edema and irritation erythema were due to the formulation as the mild irritation.

Histological assessment allowed us to quantify the damage caused to the epidermis and stratum corneum, such as intracellular edema.[37] Skin irritation and cell proliferation were the findings, and their incidence and severity correlated with the dose applied to the animals. Acanthosis (epidermal thickening) also correlated with the increased rate of cell proliferation. Jibry and Murdan[37] observed that sodium lauryl sulfate caused significant damage, including destruction of the epidermis, pronounced hyperkeratosis, parakeratosis (persistence of nuclei in the subcutaneous layer), spongiosis, and hyperemia (increase in blood vessels) in the dermis, as well as other dermal effects (such as changes in the nature of the collagen). Spergel *et al.*[38] reported that the skin sites of WT/BALB mice repeatedly sensitized with ovalbumin showed induced inflammation characterized by epidermal thickening. Our results found significantly less epidermal thickening from the *P. sarmentosum* extract gel than from the DEET gel. A study Siti Nur Hanis *et al.*[39] has revealed the same result where the epidermis layer had thickened coincidently with the edema responses when a newly developed natural product which contained *Piper aduncum* was applied in the same manner.

The use of animal models is essential to determine the allergy-inducing properties of chemicals. Modjtahedi *et al.*[40] reported that the use of animals as models with these methods caused minimal harm to the animals while allowing for efficient screening for possible allergens. The guinea pig is believed to be the most-sensitive animal model for this type of study. Using guinea pigs to test topical treatments facilitates the testing of formulations as well as assaying cross-reactivity of chemicals and various formulations during the elicitation phase.[41] The use of animal assays in the present study indicated that gel containing *P. sarmentosum* does not induce significant effects in terms of irritation, but may elicit allergic reactions after repeated use.

Thus, the results interpreted as the sample been classified as mild sensitization (Grade II) agent and DEET as moderate sensitization (Grade III) agent. The result of this study was similar to previous studies conducted,[42-43] and in agreement to the United States Environmental Protection Agency studies on 1999 and 2005. It is also support the result of a study by Agency for Toxic Substances and Disease Registry Division of Toxicology and Environmental Medicine (2004) which had classified DEET could cause primer skin irritation and acute dermal as Grade III with mild toxicity.

CONCLUSION

PSI studies indicated that topical use of a gel with *P. sarmentosum* (1.55%) is nonirritative, but repeated use showed mild sensitization effects. Histological analysis revealed a slight thickening of the stratum corneum epidermis layer in the shin of the guinea pigs. Together, these results indicate that the gel with *P. sarmentosum* is safe for application on the skin, but may lead to sensitization upon repeated application.

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Conflicts of interest

There are no conflicts of interest.

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