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Acute Toxicity Study of Hexane Extract of *Sodhita Semecarpus anacardium* L. Drupe in Wistar Albino Rats and its Prediction Using *In-Silico* Tool

P. Gopinath and R. Arunadevi*

Department of Pharmacology, University of Madras, Captain Srinivasa Murthy Central Ayurveda Research Institute, Central Council for Research in Ayurvedic Sciences, Chennai - 600106, Tamil Nadu, India; 30aruna@gmail.com

Abstract

Semecarpus anacardium L. belongs to the family Anacardiaceae. Its drupe is widely used in Ayurvedic formulations after *sodhana* processing as it is listed under Schedule E1 of the Drugs and Cosmetics Act 1940. In this study, *sodhita Semecarpus anacardium* L. drupe was extracted with n-hexane and the yield was found to be 56%. The extract was subjected to GCMS (Gas Chromatography Mass Spectrometry) analysis and its characteristics were studied using an *in silico* tool. Acute oral and dermal toxicity studies were performed on the Wistar albino rats as per the OECD 425 and 402 guidelines respectively. The GCMS data revealed the presence of $C_{13}H_{18}O_3$ (m/z: 222.1) with an abundance of 93.63%. The compound was predicted as potentially hazardous with a probable mutagenic category of ICH M7 class 3. An oxirane functional group of the compound was predicted to cause potent irritation properties to the eyes and skin with positive scores of 0.76 and 0.57 respectively. The LD_{50} was found to be more than the limit dose of 2000mg/kg body weight upon oral administration. Acute dermal exposure at a limited dose of 2000mg/kg body weight did not cause mortality. However, inflammatory responses started appearing within 48 hours of exposure. Histopathology revealed mild dermal oedema, mild fibrosis, dilated follicles hyperplasia etc. No changes were found in haematological parameters. Inflammation and dermal allergic reactions were completely self-healed by the end of 14 days. The results suggest that the oil portion of the *sodhita* drupe possesses the irritant properties of the *Semecarpus anacardium*. It might be due to the presence of the compound $C_{13}H_{18}O_3$ with Oxirane as a functional group.

Keywords: GCMS, *In-Silico*, LD_{50}, *Semecarpus anacardium, Sodhana*, Toxicity

1. Introduction

Semecarpus anacardium L. is a deciduous tree that belongs to the family Anacardiaceae. The fruit is composed of two parts, a reddish-orange edible fruit when ripe and a black drupe (nut) that grows at the end[1]. The transverse section of the drupe reveals the presence of oil globules in lysigenous cavities between the layers of parenchymatous cells in the mesocarp and fixed oils in the endocarp[2]. The oil resin in the drupe causes allergic reactions when in contact with the skin. *Semecarpus anacardium* is listed in Schedule E1 of the Drugs and Cosmetics Act 1940[3]. The reported phytocompounds in this drupe are bioflavonoid, phenolic compounds, bhilwanol and catechol compounds etc[4]. A long hydrocarbon chain at the 3rd position of a catechol organic compound mixture called urushiol was

*Author for correspondence

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considered to cause contact dermatitis. Urushiol is a small molecule that absorbs through the skin and attaches to protein, leading to Type IV hypersensitivity reaction[5]. In the traditional practice of medicine, to avoid poisonous and untoward effects, a purification process has been followed in *Ayurveda* called *sodhana*. Different methods of *sodhana* process are referred to in classical texts, which are broadly grouped into two categories, such as *samanya sodhana* (common procedure) and *vishesha shodhana* (special procedure) described in *Rasashastra* (deals with drug formulation involving minerals and metals). It is believed that *sodhana* will remove the impurities, alter the physicochemical nature of the compounds, and reduce the particle size to improve bioavailability and improve therapeutic efficacy[6]. A few literature studies reported that the *sodhana-processed Semecarpus anacardium* produced significant cytotoxicity in a few cancerous cell lines[7] and absent/low allergic responses in small animals. In our study, the major portion of the oil content from the *sodhita* drupes was extracted in non-polar solvent hexane. The phytocompounds present in the extracted crude were studied by GCMS technique and the physicochemical characteristics of the compound were predicted using the *In-silico* tool. The extracted crude was used for evaluating its acute toxicity in Wistar albino rats in both intended and exposure routes by following the respective OECD guidelines 425 and 402.

2. Plant Material

Semecarpus anacardium drupe was procured in February 2022 from a local market in Madurai. It was verified and authenticated in the Department of Botany, CSMCARI (CCRAS) m/o AYUSH Government of India, Arumbakkam, Chennai. The voucher specimen no. *Semecarpus anacardium* L 00001, a sample was preserved in the Department of Pharmacology.

3. Materials and Methods

3.1 Detoxification *(Sodhana)* of Drupes

The drupes were subjected to purification (*Vallathagi Thooimai Murai*) as instructed in the classical text of *Anuboga Vaidhya Navaneedham*[8]. While processing, a double glove and a face mask were used. As a precaution the process was performed in an open space, using long tongs for mixing the boiling contents to avoid exposure. The whole drupes were soaked in cow dung and boiled for 24 hours, washed in water and dried under sunlight. This process was repeated seven times. The same process was repeated with tamarind (*Tamarindus indica* L) pulp juice, cow milk, Aloe vera (*Aloe barbadensis* miller) juice, and tender coconut *(Cocos nucifera* L) water respectively, by boiling for 24 hours each. Dried drupes were used for further studies.

3.2 Extraction Process

Soxhlet apparatus was used for the extraction process. *Sodhana* processed drupes were broken into pieces using a cutter and ground in a mixer to coarse material. About 150g of coarse material was packed in a thimble and placed in an extraction chamber which connected with a round bottom flask with added glass beads to avoid bumping. The pack was wetted using hexane solvent and poured sufficiently. The boiling temperature was set to 60-80 °C for circulation of solvent vapour till the colour of the solvents turned colourless. The collected crude with solvents was distilled and dried in the water bath. The resulting extract was weighed and packed for further study[9].

3.3 GC-MS Study

Gas chromatography (Make: Agilent Model: 6890 plus and 5973 MS) with DB-5MS capillary column, 60mX0.25mm, 0.25µm film thickness was used. The parameters programmed for this study were 30-1000 m/z at 1.6 scan/s, 230˚C of Ion source temperature, 280˚C of transfer line, 1.0 mL/min flow rate of carrier gas helium with a split ratio of 20:1. The temperature ramp for the oven was maintained for 60˚C at a hold time of 10min, followed by an increase to 250˚C at a ramp of 5˚C per minute and maintained at 250 ˚C for 10 min.

3.4 *In-Silico* Tool

From the GCMS data, the compound targeted was identified based on its abundance. Its canonical Simplified Molecular Input Line Entry system (SMILE) CC(=O) C1C=C2C(CC3C(C2(O1)C)O3)(C)C form was used for the prediction. A structure-based prediction was carried out to determine the physicochemical properties and toxicity using the Advanced Chemistry Development tool[10].

3.5 Acute Oral Toxicity Study

An acute oral toxicity study was performed following the procedure of OECD 425. This study was performed upon

approval of IAEC/CSMRADDI/02/2019. Animals were acclimatized to laboratory conditions. Five overnight fasted female Wistar albino rats weighing about 100-125 g were sequentially dosed. The dose was calculated according to the body weight of each animal and administered orally using an intubation canula. After test administration, food was withheld for about 3 hours. Animals were observed during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days for clinical signs and mortality. Body weight was taken at weekly intervals. On day 14 animals were sacrificed for gross necropsy.

3.6 Acute Dermal Toxicity

This study was performed upon approval of IAEC/CSMCARI/11/2023. Wistar rats weighing about 100-125 g were selected. Fur was removed (10% of total body surface area) 24 hours before the test. A dose of 2000 mg/kg b.w. of the undiluted extract was applied to the gauze patch and then applied on the skin, which was held in place with non-irritating tape for 24 hours. At the end of the 24-hour exposure period, the patch was removed, and the residual test sample was cleaned. Animals were observed during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 2 to 6 hours and daily thereafter, for a total of 14 days. Body weight was taken at weekly intervals. On day 14 animals were sacrificed for gross necropsy.

The same process was repeated for further animals to confirm the irritant responses on the skin and scored at 60 minutes, 24, 48 and 72 hours after patch removal. As the irritant responses were not reversed after 72 hours, the skin lesion was observed for 14 days.

4. Results and Discussion

Several literatures were discussed about the *Semecarpus anacardium* and reported that it tends to cause blisters or allergic responses on the skin upon exposure. The reason for the undesired responses was due to the presence of the irritant compounds in the oily portion of the drupe. It was reported that *Semecarpus anacardium* is purified by the processing of *sodhana* in various forms to convert the toxic urushiol into a non-toxic anacardol[11] derivative. Additionally, *sodhana* is reported to improve the yield and decrease total phenolic and flavonoid content. Some of the *sodhana* procedures use brick powder to remove the oily portion of the drupe, resulting in a significant change in ash values between *asodhita* and *sodhita Bhallataka*[12,13]. In our study, *Semecarpus anacardium* drupes were purified (*sodhana*) as per the instruction given in the classical text *Anubogha Vaidhya Navaneedham* that are represented in Figure 1.

Sodhita Semecarpus anacardium drupes were extracted with hexane and the yield was found to be 56%. The extract was submitted for gas chromatography analysis. The abundance of a phytocompound identified in GCMS analysis is represented in Table 1 and its mass spectrum in Figure 2.

The compound at the Retention Time (RT) of 18.11 minutes with m/z 222.1 was considered for further evaluation as the content of the analyte was found higher with an area percentage of 93.63%. Studies on *Semecarpus anacardium* drupes were reported on several compounds with molecular weights of 542.49 (Tetrahydrorobustaflavone/Tetrahydroflavone), 538.46 (Amentoflavone), 542.12 (Semecarpuflavanone), 674.65 (Nallaflavanone)[14] but no literature report could be correlated for the molecular weight of 222.1. Reports are available stating that the presence of catechol derivatives collectively called urushiol and anacardic acid (oxidized form of catechol) were responsible for the irritant properties of *Semecarpus anacardium*. In urushiol, each catechol-derived organic compound was substituted with 15 or 17 carbon hydrocarbon chains at carbon position 3. The hydroxyl group in the urushiol gets oxidized into double-bonded oxygen which reacts with protein nucleophiles within the skin[15] and causes dermatitis or blisters. Our observation of the results of GCMS did not reveal the presence of the above-reported compounds in the *sodhita Semecarpus anacardium*. Instead, we found the presence of Ethanone, 1-(1a,2,3,5,6a,6b-hexahydro-3,3,6a-trimethyloxireno[g]benzofuran-5-yl) (Figure 3) and further subjected it to *in-silico* analysis.

The three-member heterocyclic Oxirane functional group highlighted in Figure 3, as such, are found to be highly toxic, and a strong skin irritant without undergoing oxidation. To have a clear understanding of the chemical profile of urushiol and $C_{13}H_{18}O_3$, prediction was utilised, and the results were tabulated in Table 2.

The prediction shows that $C_{13}H_{18}O_3$ is a highly hazardous and stronger irritant for the eyes and skin than urushiol. Urushiol derivatives are predicted to have less hazardous and irritant properties. Interestingly, the drug profile of $C_{13}H_{18}O_3$ was found to be within Lipinski's acceptable limit, rather than urushiol, which

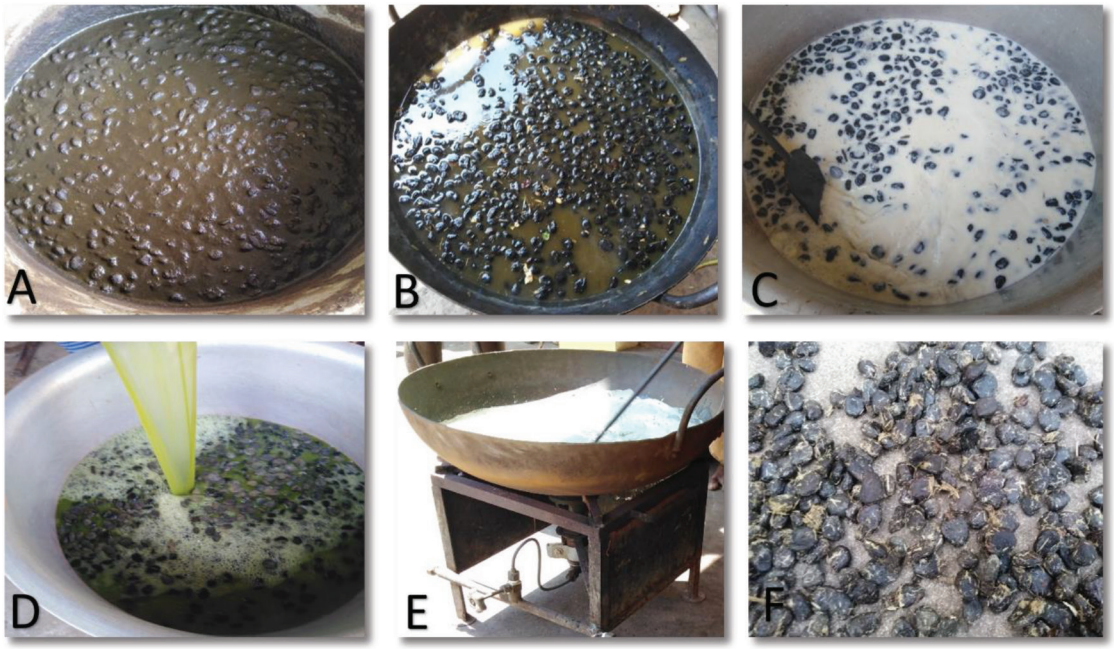


Figure 1. Sodhana processing of Semecarpus anacardium drupe. **(A)** Cow dung. **(B)** Tamarind pulp juice. **(C)** Milk. **(D)** Aloe vera Juice. **(E)** Boiling process. **(F)** Drying under sunlight.

Table 1. GCMS analysis of hexane extract of *sodhita Semecarpus anacardium* for identification of the compound of abundance

RT in minutes	Name	Area	Formula	Mass	Area%
18.11	Ethanone, 1-(1a,2,3,5,6a,6b-hexahydro-3,3,6atrimethyloxireno[g]benzofuran-5-yl)-	1471315285	$C_{13}H_{18}O_3$	222.1	93.63

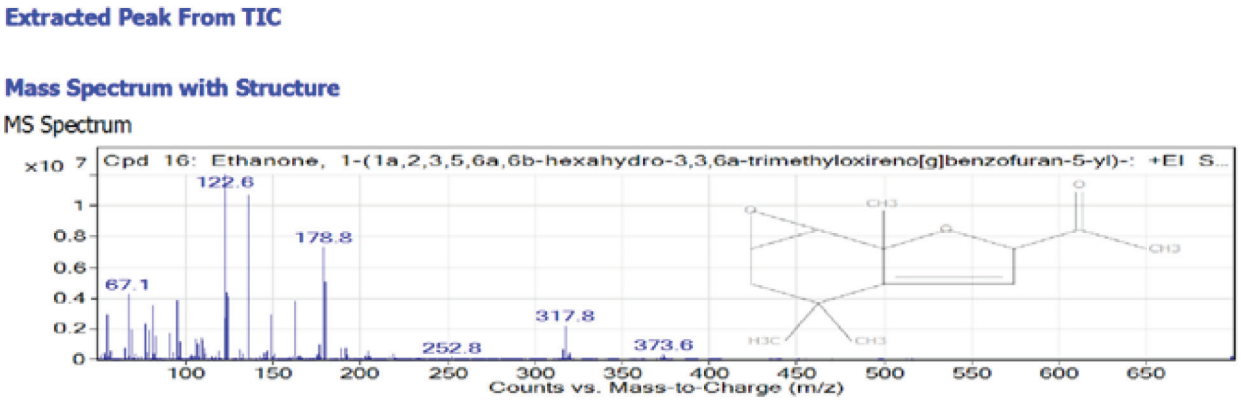


Figure 2. Mass spectrum of $C_{13}H_{18}O_3$.

might be beneficial for certain ailments that are to be discovered.

As per OECD 425, acute oral toxicity with a limited dose of 2000mg/kg body weight was administered to a female Wistar rat. An animal was observed for the first 30 minutes after dosing, periodically for 24 hours and daily thereafter, for a total of 14 days. No signs of toxicity were displayed, and no changes were found to the eyes, skin, and fur. Observations of somatomotor activity and behaviour patterns were found normal. Animals were more active,

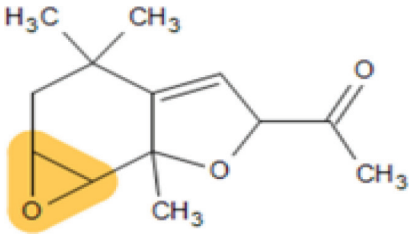
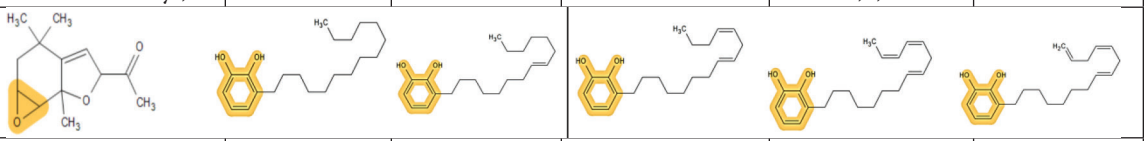


Figure 3. Molecular formula $C1_3H_{18}O_3$ with Heterocyclic Oxirane functional group.

and body weight was increased. Upon survival of the first animal, a further four animals were sequentially dosed. The same observations were noted on all the animals and no mortality occurred. The LD_{50} of the hexane extract was found to be more than 2000mg/kg b.w. Histopathology was not performed as no lesion was observed in a gross necropsy examination. The toxicity-related information on nut extracts was discussed as a supportive reference to our studies that the toxicity of chloroform soluble fraction

Table 2. Predicted chemical profile of irritants.

Parameters	$C_{13}H_{18}O_3$	Urushiol				
Chemical Name	Ethanone, 1-(1a,2,3,5,6a,6b-hexahydro-3,3,6a-trimethyloxireno[g] benzofuran-5-yl)-	(3-(8-Pentadecenyl)-1,2-benzenediol)	(3-Pentadecylcatechol)	(3-(8,11,13-Pentadecatrienyl) pyrocatechol)	(1,2-Benzenediol, 3-(8,11,14-pentadecatrienyl)-)	(Urushiol III)
Structure						
Irritant Molecule	Oxirane ring	Quinone Precursor	Quinone Precursor	Quinone Precursor	Quinone Precursor	Quinone Precursor
Probability Score for Acute Toxicity Category	74% Category 3,4	92% Category 5/ Non-toxic	81% Category 5/ Non-toxic	97% Category 4,5/ Non-toxic	98% Category 4,5/ Non-toxic	82% Category 4,5
Impurity Profile	Potentially Hazardous compound	Potentially safe compound	Questionable Mutagenicity potential	Potentially Hazardous compound	Potentially safe compound	Questionable Mutagenicity potential
ICH	M7 class 3	M7 class 5	M7, class-Inconclusive	M7 class 3	M7 class 5	M7, class-Inconclusive
Eye Irritation	0.76	0.17	0.18	0.18	0.18	0.18
Skin Irritation	0.57	0.27	0.27	0.27	0.28	0.28
Reproductive Toxicity	Negative	Negative	Negative	Negative	Negative	Negative
Genetic Toxicity	Outside AD	Negative	Negative	Negative	Negative	Negative
Log P	1.61 (optimal)	8.50 (very lipophilic)	7.38 (very lipophilic)	6.64 (very lipophilic)	6.39 (very lipophilic)	6.29 (very lipophilic)
Mol. Wt.	222.28	320.51	318.49	316.48	314.46	314.46
Lipinski Rule	0 violation	1 violation	1 violation	1 violation	1 violation	1 violation
Solubility	Soluble	Highly Insoluble	Highly Insoluble	Highly Insoluble	Highly Insoluble	Highly Insoluble

Note. These predictions scores are based on close structure similarities available with the database. Outside AD reflects not enough similar structure corresponding to genotoxic data

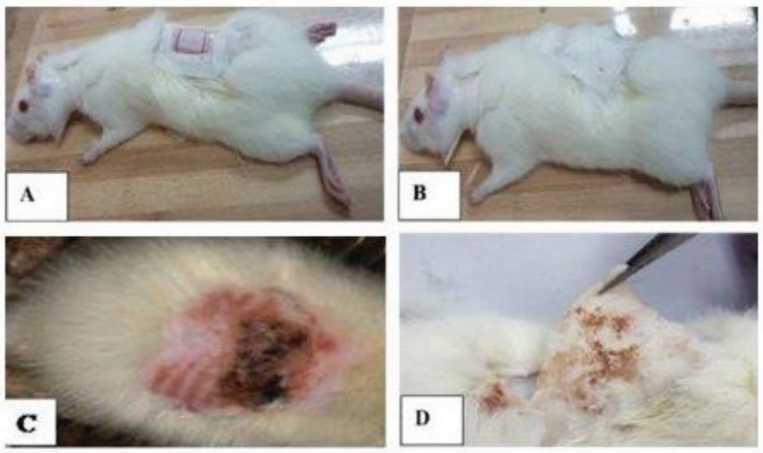


Figure 4. Acute dermal toxicity study of hexane extract of *sodhita Semecarpus anacardium*. **(A)** Animal preparation. **(B)** Gauze patch method. **(C)** Inflammation at 72 hours. **(D)** Recovery phase at 14 days.

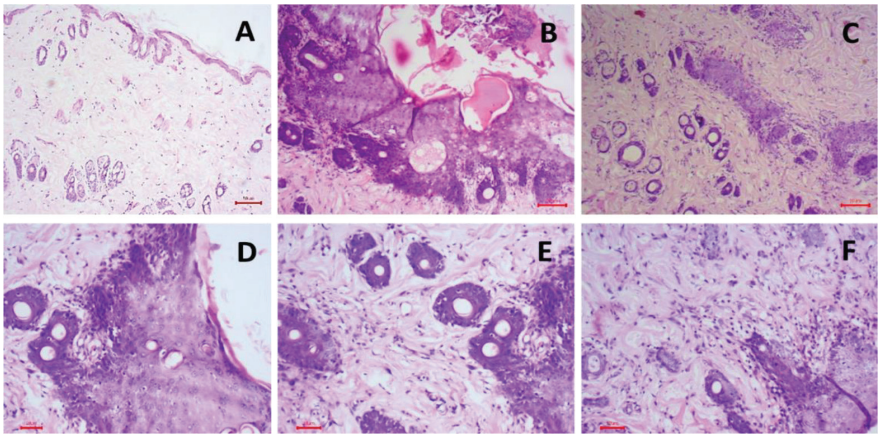


Figure 5. Histopathology images of hexane extract of *sodhita Semecarpus anacardium* treated dermal lesions. **(A)** normal skin. **(B-F)** Treated Skin. **(B)** Spontaneous vesicle Hyperplasia. **(C)** Hyper dilated follicles. **(D)** Apoptotic keratinocytes. **(E)** Mild dermal oedema and **(F)** fibrosis. Minimal mononuclear cell infiltration.

Table 3. Grading of skin reactions for the tested animals treated with hexane extract of *sodhita Semecarpus anacardium*

Time point	Grading of skin reactions	
	Erythema	Oedema
60 minutes	0	0
24 hours	0	0
48 hours	2	2
72 hours	2	2
7 days	1	1
14 days	1	1

Note. 0- No Erythema/No oedema, 1- very slight erythema/oedema and 2- well-defined erythema/slight oedema.

of *Semecarpus anacardium* nuts at the dose range of 50-400 mg/kg was reported[16]. Ramalingam Sundaram *et al.*,[17] reported no acute and sub-acute toxicity observed in catechol derivative of I-IV in Wistar albino rats at the dose of 800 and 300mg/kg, whereas LD_{50} was observed at doses of above 1250mg/kg. The traditional way of administration of *Semecarpus anacardium* extracts with peanut oil was reported to be safe and no mortality was observed up to 25 mg/kg/day for 9 days[18]. All these observations prove that the process of *sodhana* which was carried out by us was significantly reduced the toxic profile of *Semecarpus anacardium* drupe at the dose of 2000mg/kg which looks equivalent to the study reported by T. Vijayalakshmi *et al.*,[19] using milk extract of the nuts where no mortality found at the dose of 75-2000 mg/kg body weight.

Table 4. Body weight changes in Wistar female rats in acute toxicity studies of hexane extract of sodhita Semecarpus anacardium.

S. No.	Study Name	Drug	Route of Exposure	Dose (mg/kg bw)	Body Weight Change in Gram		
					Day 1	Day7	Day 14
1	Acute Oral Toxicity Study (OECD 425)	*Sodhita Semicarpus anacardium* drupe hexane extract	Oral	2000	117±6.10	130±6.40 (11.11)	145±5.20 (23.93)
2	Acute Dermal Toxicity Study (OECD 402)		Dermal	2000	114±4.40	125±5.60 (9.64)	137±4.8 (20.17)

Values are expressed as mean±SD; Values given in parenthesis indicate Percentage change in body weight with respect to day 1.

The acute dermal fixed dose toxicity study using hexane extract was studied in the Wistar rat. The fur on the dorsal side of the animal was shaved and marked the boundary measuring 10% of the total body surface area. A small plastic collar was held around the neck to avoid the removal of the gauze patch by a rat as shown in Figure 4(A). The hexane extract of about 2000mg/kg bw was applied on the gauze and placed within the marked boundary and it was held in place using non-irritant tape. (Figure 4(B)).

No allergic responses were observed at the end of 24 hours after the patch was removed. Later, at 48 hours, inflammation such as redness, and urticaria was observed on the skin with smudged liquid test material beyond the boundary made (Figure 4(C)) but itching and swelling were absent. On continued observation, the inflammatory condition was worsening with a grading score of 2 (well-defined erythema/slight oedema) and subsequently reduced to 1 (very slight erythema/oedema) at the end of 14 days (Figure 4(D)). Many authors reported that the cause of irritation was very minimal at the end of 14 days compared to *pre-sodhita* extracts but the response observed within the duration was not discussed[20] and the ayurvedic procedure claims conversion of a toxin into a safe drug using *sodhana* process where toxic urushiol was converted into non-toxic anacardol with decrease in phenolic and flavonoids content but no report was identified stating complete eradication of irritant capabilities of *sodhana* processed *Semecarpus anacardium*. At the end of the study, the animal was anaesthetised, and blood was collected on a retro-orbital sinus for measuring haematology parameters. A heparinized microhematocrit capillary tube was used for collecting the allowable amount of blood. To prevent excess bleeding, a slight pressure on the eyeball was applied[21]. Later, the animal was euthanized, and a portion of inflamed skin and normal skin (control) was dissected for histopathological evaluation (Figure 5). It was observed that the control skin showed normal architecture. Whereas the treated skin was showing the following changes namely epidermal, dermal and follicular epithelial cell hyperplasia, akeratinocytes, inter-epidermal vesicle, minimal mononuclear cell infiltration in the epidermal and dermal junction, dilated follicles, mild dermal oedema and mild fibrosis. The skin irritation scoring observations were tabulated as per draize criteria (Table 3) and changes in the body weight were tabulated in Table 4.

5. Conclusion

The results suggest that the oil portion of the *sodhita Semecarpus anacardium* drupe possesses skin irritant properties. It might be due to the presence of a phytocompound with an Oxirane functional group, which is further confirmed with a dermal toxicity study. However, both oral and dermal exposure at 2000mg/kg b.w did not cause any mortality. This study will be helpful for the safe handling of the formulations and labelling information.

6. Acknowledgment

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