

Acute Toxicity Studies of Neem (*Azadirachta indica*) Seed Oil Cream Administered Topically in Albino Rat

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Abstract

This study was carried out to evaluate the toxicity profile of a formulation of Neem seed oil in a vanishing cream base on the skin of albino rats. Employing standard guidelines, Neem seed oil cream at concentrations of 0% w/w, 2.5% w/w, 5.0% w/w, 7.5% w/w and 10.0% w/w was applied to the dorsal part of the female albino rats' skin in a group of 5 animals per concentration level with the 0% w/w cream serving as control. One group of 5 male albino rats was topically administered with 10% w/w Neem seed oil cream to assess any sex related toxicity profile. The application was for fourteen days in all cases, after which two animals from each group were necropsied. The treated skin, distant skin, liver and kidney were harvested for histopathological examination. Two weeks post treatment, the remaining animals were sacrificed and the procedure above was repeated for histopathological examination of the designated organs.

The results show that Neem seed oil cream caused a form of dermal irritation in albino rats which did not resolve after fourteen days post treatment. No observable adverse effect on the liver and kidney morphology in the short term was demonstrated. Despite this, the dermatological irritation and structural changes observed need to be further investigated to ascertain their reversibility.

Key words: Neem, Animals, Irritation, skin, Liver, Kidney.

Introduction

Azadirachta indica A Juss, synonymous with *Melia azadirachta* and *Melia indica* (A Juss), belongs to the family meliaceae. This plant is indigenous to the Indo-Pakistan subcontinent (1), although it is now widely distributed in many countries of the world, it is believed that Indians migrating to Africa introduced it into the continent.

In a recent review Aremu, Femi-Oyewo & Adeyemi (2) cited recorded indications of Neem seed oil in the literature such as repellent action against *Aedes aegypti* mosquitoes (3), anti candidal activity (4), antipyretic effect (5) among others. In the past, there have been growing concerns on the usage of the insect repellent - N, N-diethyl-m-toluamide (DEET) - and this has necessitated the development of naturally occurring insect repellents such as Neem seed oil cream.

DEET is commonly used as a repellent against mosquitoes, flies, ticks and other insects (7,8). However, extensive and repeated topical DEET applications can cause human poisoning that can prove fatal (9,10). The symptoms associated with DEET poisoning include tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function and coma (7). DEET efficiently crosses the dermal barrier and may localize to fat deposits (11, 12). Although the exact mechanism of DEET toxicity has not been established, extremely high levels of DEET exposure causes demyelination and spongiform myelinopathy in the rat (13).

In the present study, Neem seed oil incorporated into a dermatological cream using a vanishing cream base, is being investigated for its local and systemic effects as it affects kidney and liver morphology, in particular.

Methods and Materials:

Experimental Animals

Healthy male and female (nulli parous and non-pregnant) albino rats weighing between 100-200g were obtained from the department of pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife. The animals were caged individually in cages with dimension of 22cm x 22cm x 22cm. The temperature of the animal house was kept at 25°C (±3°). The lighting of the animal house was maintained at 12 hours

light and 12 hours dark programme. The relative humidity was in the range of 30-70 per cent. The animals were fed growers mash obtained from Ladokun feeds, Ibadan, Nigeria. Unlimited clean drinking water was supplied to the animals throughout the entire period of the experiment. Subsequently, the animals were randomly selected into 5 groups of 5 animals each for females and the remaining 5 male animals was designated as the sixth group. The test animal groups were kept in labeled cages.

Plant species:

Ripe seeds of the Neem plant were collected from Osogbo, a town in South Western Nigeria. The plant species from which the seeds were collected was authenticated at Forest Research Institute of Nigeria (FRIN), Ibadan.

The seed kernels were sun dried until the moisture content was reduced to about 10% w/w. The seed kernels were later stored in air tight containers.

Extraction of the oil:

The dried seed kernels of the Neem seeds were comminuted using a blender (Model MX - 738, Nakai, Japan). The blended materials were stored in air tight containers. Using the method of Charmaine (4), Normal Hexane was used as solvent for extraction at seed weight: solvent ratio 1:10. The seeds were allowed to soak in the solvent for 8 days at room temperature. The solvent was then filtered through a whatman filter paper (No 1) to remove the coarse seed materials, into pre-weighed sterile containers. The containers were covered with filter paper and solvent was allowed to evaporate. The weight of the residue was determined and the extracts were kept at room temperature.

Preparation of the cream

Neem seed oil was incorporated into vanishing cream base to get the desired test concentrations of 0% w/w, 2.5% w/w, 5.0% w/w, 7.5% w/w and 10.0% w/w using the fusion method.

Performance of the Test

The animals were acclimatized to laboratory conditions for five days prior to the test. Approximately 24 hours before the test, fur was removed from the dorsal area of the trunk of the test animals by shaving 10 per cent of the body surface area. The shaving was done under anaesthesia using 0.5ml each of ketamine (1.0%w/v) and Diazepam (0.5%w/v).

Application of Neem Seed Oil Cream

Five concentrations of Neem seed oil in a vanishing cream base i.e. 0%w/w, 2.5%, 5.0%, 7.5%, and 10%w/w were administered topically at a fixed amount twice a day for fourteen days. Each test female group was assigned each test concentration accordingly with 0%w/w concentration serving as control. The sixth group which consisted of male rats was only administered with 10%w/w Neem seed oil cream.

Observation of the Animals

The animals were observed closely for 2 hours daily during the fourteen days of Neem seed oil cream application and for another fourteen days post treatment. Cage side observations made include changes in fur texture and appearance, eyes, mucous membrane, respiratory, circulatory, somatomotor activity, behaviour pattern, tremors, convulsions, salivation, lethargy, diarrhoea, skin eruptions, sleep and coma.

Necropsy of Animals

At the end of fourteen days application of test concentrations, two animals from each test group were sacrificed under anaesthesia using 0.5ml each of Diazepam(0.5%w/w) and Ketamine (1.0%w/w). The following organs were harvested,

local skin (site of application), distant skin (About 1 cm away from site of application), kidney, liver and lung. These organs were stored in formalin. This procedure was repeated for the remaining animals in all the test groups after fourteen days of recovery.

Histopathological Examination

The sections were dewaxed in xylene and taken to water through descending grades of alcohol (100%, 95%, 80%, 70%, water). Staining in Cole's heamatoxylin was done for 5 minutes. The sections were rinsed in water and differentiated in 1% HCL in 70% alcohol for 1 minute. The sections were then counter stained with 1% Eosin, rinsed in water and dehydrated in ascending grades of alcohol i.e. (70%, 80%, 90%, and 100%). The sections were once more cleared in xylene, mounted with DPX and covered with a cover slip and observed under a light microscope.

Statistical analysis was done using the one-way "ANOVA" for more than two samples.

Results

Observations of Animals Studied:-There were no changes in cage side observations made on fur texture and appearance, eyes, mucous membrane, respiratory system, gross autonomic and central nervous system activity, somatomotor activity, sleep and salivation of animals studied. These were observed to be normal throughout the period of the study before the animals were sacrificed. Also none of the animals in all the test groups had tremors or convulsions, diarrhoea, lethargy, skin eruptions or even fall into coma during this study.

Table 1: Weights of the Animals Studied over a five week period(g)

Animal	Week 1	Week 2	Week 3	Week 4	Week 5
F1	182.9	188.8	190.2	-	-
F2	102.1	125.1	130.1	-	-
F3	134.5	140	144.1	146.1	148.3
F4	122.3	134.4	134.9	135.1	136
F5	217.4	219.4	220.0	220.8	223.0
F6	150.8	157.0	160.0	-	-
F7	175.2	185.7	183.2	-	-
F8	192.0	195.3	195.7	196.1	197.0
F9	123.3	130.1	135.0	137.5	140.2
F10	180.6	188.8	189.0	191.1	193.8
F11	183.6	187.2	190.4	-	-
F12	140.9	145.8	147.6	-	-
F13	100.1	109.8	113.5	117.0	120.2
F14	150.0	151.2	152.0	155.3	160.3
F15	142.9	147.8	151.0	153.2	156.0
F16	137.9	141.1	118.9	-	-
F17	152.7	162.6	169.0	-	-
F18	122.1	123.8	125.9	130.0	132.3
F19	108.3	109.7	111.0	112.5	-
F20	168.1	173.1	175.1	177.9	180.0
F21	118.9	120.4	121.4	-	-
F22	143.7	151.6	152.2	-	-
F23	130.7	133.5	136.8	139.4	141.8
F24	143.3	146.0	149.7	150.7	154.3
F25	109.3	137.2	140.0	157.0	154.3
M1	110.0	113.6	115	-	-
M2	254.0	261.6	262.0	-	-
M3	152.7	154.7	156.0	158.9	159.8
M4	102.4	103.9	107.2	109.6	112.8
M5	285.9	291.8	294.1	295.6	298.3

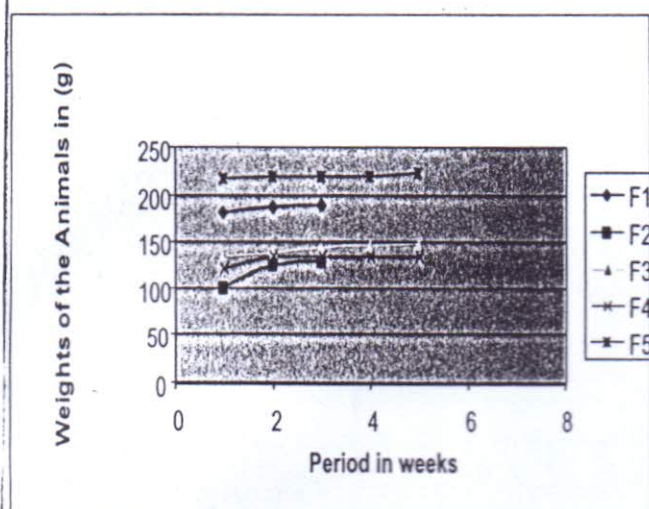


Fig 1: Weights of the Animals in First group (F1-F5) for a period of five weeks(g)

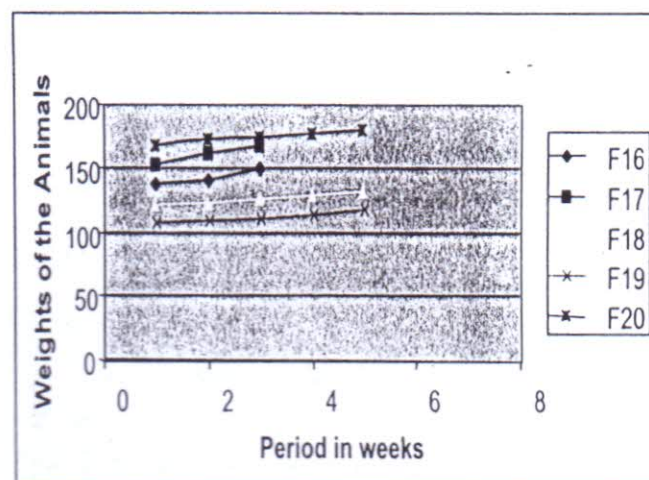


Fig 4: Weights of the Animals in Fourth group (F16-F20) over a period of five weeks in (g)

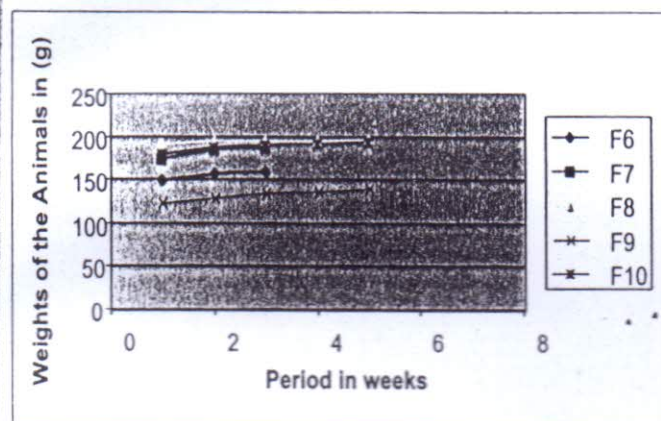


Fig 2: Weights of the Animals in second group (F6-F10) over a period of five weeks(g)

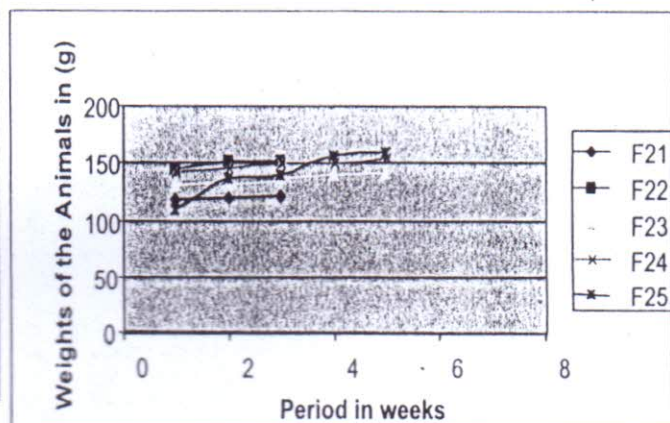


Fig 5: Weights of the Animals in fifth group (F21 - F25) over a period of five weeks in (g)

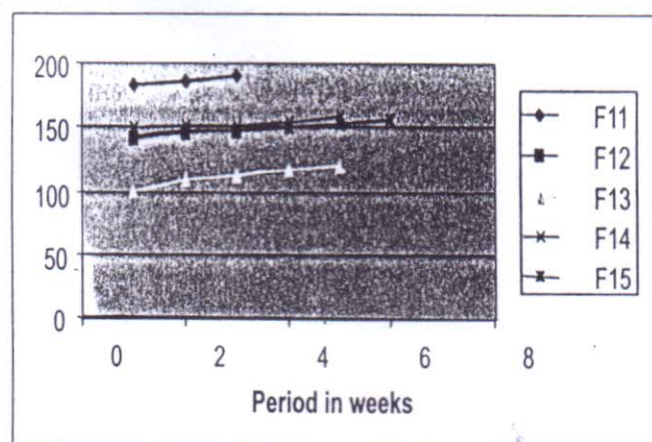


Fig 3: Weights of the Animals in third group (F11-F15) over a period of five weeks in (g)

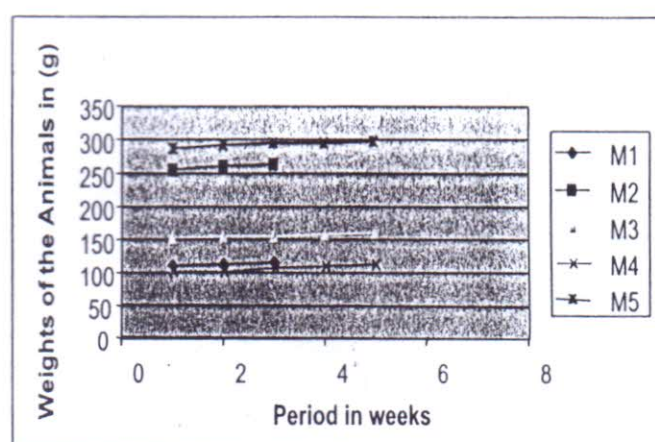


Fig 6: Weights of the Animals in sixth group (M1- M5) over a period of five weeks in (g)

Observations for casualty during and post application period:

At the end of fourteen days of Neem seed oil cream application, no casualty was recorded. During the two weeks post treatment recovery period, the fur on the shaved part of the skin of the animals remaining that had not been necropsied was observed to have started growing. All cage side conditions observed during the recovery period were found to be normal.

Result of Histopathological Examination

Histopathological examination revealed a dose dependent thickening of the epidermis and increase in melanin pigmentation in the location of Neem seed oil cream application (Plate 2, 3 & 4) not observed in the controls (Plate 1).



PLATE 1: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE SKIN OF RAT TREATED WITH 0% NEEM SEED OIL SHOWING NORMAL EPIDERMAL-DERMAL THICKNESS RATIO.
MAG. X400



PLATE 2: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE SKIN OF RAT TREATED WITH 2.5% OF NEEM SEED OIL SHOWING A DARK BROWN PIGMENT BENEATH THE KERATINISED SQUAMOUS CELL LAYER OF THE EPIDERMIS
MAG. X400



PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE SKIN OF RAT TREATED WITH 5% NEEM SEED OIL SHOWING THICKENED EPIDERMIS AND DARK PIGMENT BENEATH THE KERATINISED SQUAMOUS CELL LAYER
MAG. X400

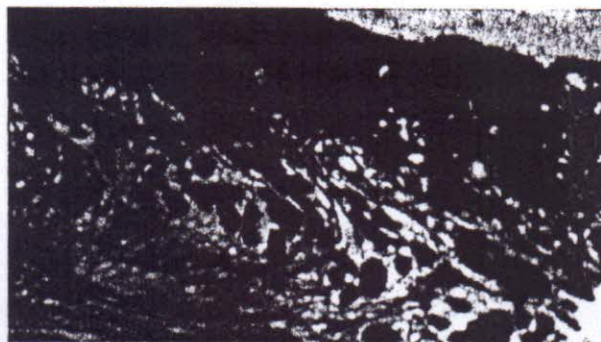


PLATE 4: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE SKIN OF RAT TREATED WITH 10% NEEM SEED OIL SHOWING DARK PIGMENTS EXTENDING FROM BENEATH THE KERATINISED SQUAMOUS LAYER TO THE DERMAL LAYER.
MAG. X400



PLATE 5: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE DISTANT SKIN OF RAT TREATED WITH 10% NEEM SEED OIL SHOWING NORMAL ARRANGEMENT OF THE EPIDERMIS AND DERMIS WITHOUT PROMINENT DARK PIGMENT BENEATH THE KERATINISED SQUAMOUS CELL LAYER
MAG. X400

There was also observed the presence of dark-brown pigment underneath the keratinized squamous epithelium together with a disruption of the ratio of the epidermis to the dermis which appeared to be dose dependent. No such presence of dark brown pigmentation and pronounced distortion of the epidermal-dermal ratio were observed in the distant skin locations at all concentration of Neem seed oil cream used (Plate 5). The observed pigmentation and structural changes were not reversed after 2 weeks post treatment (Plate 6).



PLATE 6: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE SKIN OF RAT TREATED WITH 2.5% NEEM SEED OIL HARVESTED 2 WEEKS AFTER TREATMENT SHOWING PERSISTENCE OF THE DARK PIGMENT LAYER.
MAG. X400

The examination of the liver and kidney did not show any apparent gross structural derangement (Plates 7, 8, 9 & 10).



PLATE 7: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF KIDNEY OF THE RAT TREATED WITH 10% NEEM SEED OIL SHOWING APPARENTLY NORMAL GLOMERULUS AND RENAL TUBULES
MAG. X400



PLATE 9: PHOTOMICROGRAPH OF THE LIVER OF RAT TREATED WITH 10% NEEM SEED OIL SHOWING NORMAL HEPATIC LOBULE, CENTRAL VEINS AND GENERAL HEPATIC HISTOARCHITECTURE.
Mag. X400

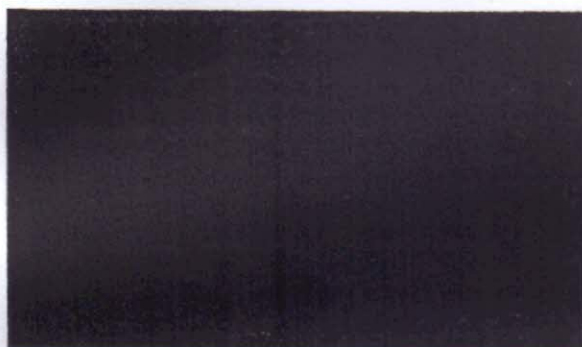


PLATE 8: PHOTOMICROGRAPH OF THE TRANSVERSE SECTION OF THE KIDNEY OF RAT THAT WERE TREATED WITH 0% NEEM SEED OIL SHOWING NORMAL GLOMERULUS AND TUBULES
MAG. X400



PLATE 10: PHOTOMICROGRAPH OF THE LIVER OF RAT TREATED WITH 0% NEEM SEED OIL SHOWING NORMAL HEPATIC LOBULES AND CENTRAL VEINS
MAG. X400

Discussion

The result of the cage side observations showed that the Neem seed oil cream formulated using Neem oil extract pressed from the seed of *Azadirachta indica* caused no observable changes. According to results obtained, the Neem seed oil cream used at concentrations 0 %w/w, 2.5%w/w, 5%w/w, 7.5%w/w and 10%w/w on female rats and 10% on male rats caused no changes in the activity of the test animals. There was no tremor, convulsion, diarrhoea or lethargy. No changes were observed in the fur, eyes or sleep pattern. The gross observable respiratory, autonomic and central nervous system activities were all normal. In addition, the skin was free of eruptions, blistering or reddening through out the period of the experiment. The observations made were in accordance with the specifications of the OECD guidelines on acute dermal toxicity studies, 1987.

A progressive increase in weight was observed in the weights of all the animals in the six groups as shown in Table 1 & Figs 1-6. The weight differences over a period of three weeks were statistically significant ($P < 0.05$). The increase in weight is most probably an indication of the nutritional status and general well being of the animals.

Joshua (14) reported that, triiodothyronine applied topically on hairless mice for 7 days caused a dose dependent increase

in epidermal thickening. The thickening of the epidermis offers additional protection to skin because it prevents the penetration of ultraviolet rays (15).

Azadirachtin when applied for 24 hours at a single dose of 2.0mg/kg to the shaved backs of the rabbits, caused dermal irritation which resolved by day nine. Azadirachtin has been classified as a mild dermal irritant (15). The histopathological examination done on the treated and distant skin of the animals at the end of fourteen days of cream application and two weeks post treatment revealed that the cream caused a dose dependent thickening of the epidermis and an increase in melanin pigmentation. This irritation observed did not resolve within 2 weeks post treatment period. On the other hand, application of 0%w/w Neem seed oil cream to the skin of albino rats (controls) did not show any apparent structural derangement of the skin as can be seen in Plate 1. This is an indication that the vanishing cream base did not contribute to the effects observed with the test concentrations of Neem seed oil cream.

The effects of topical application of different concentrations of Neem seed oil cream on the skin for a period of 14 days revealed the presence of dark-brown pigment underneath the keratinized squamous cell layer of the skin as well as the disruption of the ratio of the epidermis to the dermis. Com

parison between the plates showed that the stated effect of the Neem seed oil cream on the skin is apparently dose dependent. This is attested to by the increasing intensity of dark-brown pigment as the concentration of the Neem seed oil cream used increased, as well as a more pronounced epidermal-dermal thickness ratio distortion. The distortion of the epidermal-dermal thickness ratio as well as the dark-brown pigmentation is indicative of ongoing inflammatory process. A study using concentrations of Neem seed oil cream less than 2.5%w/w might help determine the minimal critical concentration required for the observed inflammatory effects.

The effect of the Neem seed oil cream is apparently localized since despite topical exposure to 10%w/w Neem seed oil cream, distant skin did not show any of the aforementioned indicators of inflammation. This is further corroborated by the fact that at application of the 10% Neem seed oil cream did not show any apparent gross morphological or structural derangement in both liver and kidneys. A systemic effect is expected to be reflected in the structure of distant skin as well as the kidney and liver histoarchitecture. The sparing of the kidney and liver is in consonance with previous reports (16), where rats were dosed with 600mg/kg/day and no adverse effects on the kidney and liver studied.

After topical exposure to 2.5%w/w Neem seed oil cream, the structural effect of the cream on the skin was not reversed within a 2 weeks period. This shows that a short term exposure is enough to result in structural derangement of the skin. Reversibility of the above stated effects might be possible after a longer period after cessation of exposure.

A possible explanation of the observations about the effect on the skin is that azadirachtin, the prominent constituent of Neem seed oil, might be reacting with some structural element of the skin, a situation that can be likened to antigen-antibody reactions.

Conclusion

It can be concluded that the topical application of Neem cream at the concentrations stated for 14 days in albino rats caused a form of dermal irritation. This irritation was however limited to the treated skin and was not observed in the distant skin. This irritation persisted for two weeks post treatment and further study would be necessary to ascertain its reversibility. The dermal irritation caused by Neem seed oil cream in the animals might be due to its azadirachtin content since this constituent when studied alone gave a similar reaction (16). The possible mechanism of dermal irritant effect of azadirachtin is probably due to its reaction with some of the components of the skin.

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