Original Article

Acute Dermal and Ocular Irritation Testing of Herbal Shampoos in New Zealand White Rabbits

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ABSTRACT

Aim: The objective of the presented study is to evaluate the acute dermal and ocular irritation of herbal shampoos consisting of a mixture of plant extracts. **Materials and Methods:** Both studies performed using New Zealand white rabbits in accordance with HICS and OECD guidelines respectively. In the cutaneous skin irritation test, erythema, eschar or edema evaluated. In the ocular irritation study, corneal opacity, area of opacity, iris, chemosis and conjunctival redness were evaluated. **Results:** In the cutaneous skin irritation test, all three herbal shampoos (PS9, PS20, and LS17) showed no significant erythema, eschar, or oedema formation after 72 hr of exposure and did not meet any of the irritation classification requirements. According to the Harmonized Integrated Classification System (HICS), all three herbal shampoos are classified as "Non Irritant" based on acute cutaneous testing. In the ocular irritation study, after 1, 24, 48, and 72 hr of observation, the treated eye appeared normal. There were no symptoms of systemic toxicity in any of the animals during the acclimatization and post-treatment periods. The results did not meet the irritation classification requirements for categories 1, 2A, or 2B because the treated animals had corneal opacity of 0.00, iris lesions of 0.00, conjunctival redness of 0.00, and chemosis of 0.00. Based on ocular irritation studies, all three herbal shampoos are classified as "Not Irritant" by HICS. The body weights of all the animals were within the typical range of variation for this species, strain, and age. All of the animals gained weight towards the end of the experiments. **Conclusion:** The acute cutaneous and ocular irritation studies indicated that three herbal shampoos (PS9, PS20, and LS17) were not irritant to the rabbit skin or eyes, indicating that they can be used in humans without irritation to the skin or eye.

Key words: Herbal shampoo, Ocular irritation, Acute dermal, New Zealand white rabbits, Erythema.

INTRODUCTION

Shampoos are certainly the most widely used cosmetic items for cleansing our hair and scalp in our daily life.[1,2] A shampoo is primarily a detergent solution that has been supplemented with ingredients for hair conditioning, lubrication, and medicine. More number of medicated, Non-medicated, synthetic and herbal shampoos are already available on the market, out of this shampoos containing herbal products gaining high popularity among customers due to their view that natural products are good and devoid of side effects.[3,4] Shampoo contains synthetic surfactants for foaming and cleaning, but their chronic use causes hair dryness, hair loss, scalp inflammation, and eye irritation.[5]

Herbal shampoos are seen as a viable alternative to synthetic shampoos, however making cosmetics from entirely natural raw materials is a challenging undertaking.[6,7] Many medicinal plants have been believed to have beneficial benefits on hair and are commonly included in shampoo formulations.[8,9] The powdered, crude, refined extracts, and derivative forms of these plant components can all be employed.[10] It's exceedingly difficult to make an herbal shampoo with only one natural component that's milder and safer than

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synthetics while yet competing favourably in terms of foaming, detergency, and solid content. As a result, we contemplated creating a pure herbal shampoo with traditional and popular plant materials.[11-13]

In continuation to our previous work; Acute Dermal and Ocular irritation evaluations were performed on optimized formulations. This study was designed to evaluate the acute dermal and ocular irritation testing of herbal shampoos in New Zealand White rabbits. During the study, three herbal shampoos with different compositions were evaluated. Two Solid herbal shampoos and one Liquid herbal shampoos were prepared using combination of plant extracts with different concentrations as part of our research work. Solid herbal shampoos prepared with compositions of Reetha (15%), Aloe (10% - 12.5%) and Henna (2 - 3%),[14] and second solid herbal shampoo prepared with compositions of Peppermint (15%), Lemon (1% - 3%) and Hibiscus (12.5% – 14.5%).[15] Based on physicochemical properties, liquid herbal shampoo,[16] formulations including Amla (1% – 3%), Pomegranate (6% – 10%), Reetha (10% – 15%), and Hibiscus (11.25% – 15%) were chosen as optimum formulations.

Presence of Polyphenols in the Pomegranate peels powder extract reduces hair loss, dandruff and darkens the hair colour. Pomegranate extracts stimulate hair growth and strengthens hair follicles by improving blood flow to the scalp.[17] The presence of high saponin content in Reetha provide hair cleansing property and used as foaming agent.[18] Hibiscus helps to boost hair growth, conditioning behaviour to reduce split ends and strength the hair. Acidic nature presence in Amla promotes healthier hair growth, tighten hair follicles and prevent hair fall.[19] Peppermint's vasodilator action improves new hair growth and minimizes hair loss by improving blood circulation. Lemon's acidic nature strengthens hair follicles, prevents hair loss, and promotes better hair development.[20-22]

MATERIALS AND METHODS

Herbal shampoos prepared during research work were used for acute dermal and ocular irritation studies. All solutions and reagents used are either analytical grade or pharmaceutical grade. All solutions were made using deionized water that was ultra-pure (Millipore, Billerica, MA, USA). This study will be conducted in a CPCSEA-approved laboratory using all ethical principles outlined in the animal care guidelines. The test facility's Institutional Animals Ethics Committee (IAEC) has given its approval to this study. Approximately 1.5 to 2.5 Kg weight New Zealand white rabbits procured from the approved laboratory. For each study three animals were used. Nutrilab rabbit feed used for feeding of rabbits. Heine ophthalmoscope (Model; HEINE/Beta 200) and fluorescein ophthalmic strip used for estimation of Ocular irritation.

Test Animals

Animals used in research the rabbits utilised in the investigation were white both males and females from New Zealand. After IAEC permission, they were obtained from in-house bred animals as part of project SLL/PCT/IAEC/113-20. The animals were kept individually in stainless steel wire mesh cages at a temperature of 22°C and a standard relative humidity of 30-70%, with a 12-hr fluorescent light and 12-hr dark cycle. The animals were fed a laboratory diet for a minimum of seven days. Throughout the acclimation and testing phase, clean and potable water was available at all times.

Acute Dermal toxicity test of Herbal shampoos[23]

The aim of the Acute Dermal Irritation Test in Rabbits was to determine the potential skin irritation effects that might result from a single application of the herbal shampoo formulation to the skin. This research provides a reasonable framework for human risk assessment.

Preparation of animals

Three animals were employed in the experiment. Each animal's patch was assigned to one of two locations on the dorsal portion of the trunk. One patch will be used to apply the positive control and the other will be used to apply the test shampoo formulation.

Hair was carefully removed from the dorsal part of the trunk of all test animals by cutting without abrading the skin around 24 hr before study started. Only animals with a healthy undamaged epidermis were chosen for the experiment based on their appearance.

Dosage Preparation

Solid herbal shampoos were prepared for application by diluting them with distilled water to a test concentration of 10% - 20% (equivalent to the concentration used for humans). Liquid herbal shampoo Un-diluted form used for application.

Herbal shampoo application

Both flanks were clipped with a clipper around 24 (± 2) hr before treatment, exposing an area of roughly 100 cm^2 (10 cm × 10 cm). In the study, animals with no visible evidence of skin damage or irritation were employed. First, a single rabbit was treated. The test was completed using the remaining two rabbits for a 4-hr exposure

period after no serious skin responses were noticed after the 4-hr exposure and for the next 48 hr.

0.5 ml of the diluted test shampoo formulation was applied to the anterior patch on the day of treatment. A total of 100 cm^2 (10 × 10 cm) of 12-ply cotton gauze was used to cover each patch. The test patches were secured in place with non-irritating adhesive tape and a cotton bandage wrapped over the whole trunk. For four hours, the test shampoo formulation was in touch with the skin. The protective covering and patches were removed at the conclusion of the contact period, and the treated area was cleansed with distilled water and dried with tissue paper, and local skin responses were recorded. The irritation ratings for erythema and oedema for each individual animal at all measurement intervals following patch removal were presented in tabular form.

The mean score for each animal was determined over three scoring intervals (24, 48, and 72 hr following patch removal) for erythema/eschar grades and oedema grades, independently, to assess the test item's irritating potential.

Interpretation of results

After four hours of post experiment patch removal and 48 hr the scores for erythema and odema were totalled for the three test rabbits, then divided by 3 and multiplied by 2 (number of observations) to get the principal irritant index of the test shampoo formulation, which was 3/6.

The following approach would be used to categorise the test shampoo formulation:

The Primary Irritation Index (PII) is a metric that measures how Irritancy is divided into several categories.

Primary Irritation Index	Classification of Irritancy
0	Non - Irritant
Greater than 0 - 2	Mild Irritant
Greater than 2 - 5	Moderate Irritant
Greater than 5	Severe Irritant

Ocular Irritation test of Herbal shampoos[24]

The aim of the rabbit ocular irritation test was to assess the eye irritancy potential of herbal shampoo formulations based on their capacity to produce harm to the cornea, iris, and Conjunctival when applied to the eye.

Use of Analgesic and Anaesthetic Agents

Pre-treatment

Buprenorphine 0.01 mg/kg was subcutaneously delivered 60 min before test item instillation, and two drops of topical ocular anaesthetic (0.5 percent Proparacaine hydrochloride) were injected in each eye 5 min before test item instillation. The control eye (right eye) was not treated with the test item but was given topical anaesthetics.

Post-treatment

Subcutaneous injections of Buprenorphine 0.01 mg/kg and Meloxicam 0.5 mg/kg were given eight hours after the test item was instilled. After the initial 8-hr post-treatment period, the animals were given Buprenorphine 0.01 mg/kg by subcutaneous injection every 12 hr, along with Meloxicam 0.5 mg/kg every 24 hr for the next 72 hr.

Treatment Process

Three animals were employed in the experiment. Before starting treatment, both eyes of each experimental animal were checked for 24 hr. Only those animals were chosen who showed no evidence of eye irritation, inflammatory changes, or ocular abnormalities. A substance's ocular irritancy potential was determined by its ability to produce harm to the cornea, iris, and conjunctivae when it was applied to the eye.

On treatment day, each animal's left eye was infused with 0.1 ml of 1 percent herbal shampoo formulations in distilled water after gently removing the lower lid away from the eyeball. The reference control was the right eye, which was left untreated. During the application, the rabbits were held in a rabbit-holder. 2 sec following instillation of the test shampoo formulation, the treated eyes were rinsed with 20 ml of lukewarm water (37 ± 1°C). Before the study started, the pH of the test item was determined. The average pH was discovered to be close to that of the skin.

The first rabbit to be treated was a single female rabbit. The test was finished using the remaining two female rabbits because no ocular reactions were seen up to 48 hr after the test item instillation. At 24 hr after the test item instillation, the treated eyes were rinsed with distilled water.

The ocular reactions were graded using the OECD[13] regulations No. 405, "Grading of Ocular Lesions," which included a numerical score system. Animals Nos. 01, 02, and 03 had their eyes checked at 1, 24, 48, and 72 hr following instillation. The eye lesions were scored using a Heine ophthalmoscope and a fluorescein ophthalmic strip.

Fluorescein ophthalmic strips

Approximately 24 hr after instillation, the treated eyes were rinsed with distilled water. Following the wash, a fluorescein ophthalmic strip was placed for around 1 min in the treated eye before being cleansed with

distilled water. An ophthalmoscope was used to inspect the eye around 10 min after it was washed.

Interpretation of results

On the basis of total area and density of opacity of cornea scoring was considered. The extent of chemosis, redness, and discharge were scored on the iris, whereas the extent of chemosis, redness, and discharge were scored on the conjunctiva.

A.	Conjunctiva	Score
	Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris only)	
	Normal vessels	0
	Vessels injected far more than usual.	1
	More dispersed Individual vessels are difficult to distinguish with a richer crimson colour.	2
	Reddish-brown meaty splotch	3
B.	**Chemosis**	
	There is no swelling.	0
	Any edema that is more than typical (includes nictitating membrane)	1
	Swelling is visible, and the lids are somewhat everted.	2
	Swelling with nearly half-closed lids	3
	With lids half-closed to totally closed, swelling occurs.	4
C.	**Iris**	
	No Change	0
	Folds above normal, congestion, edema (any one or all of these, or a combination of any of these), iris still responding to light are all typical (sluggish reaction is positive)	1
	There was no reaction to light, there was bleeding, and there was a lot of devastation (any one or all of these)	2
D.	**Cornea**	
	Opacity – Degree of density (area taken for reading)	
	There is no opacity	0
	Iris features are evident in a scattered or diffused region.	1
	Translucent parts are easily identifiable, however iris features are slightly concealed.	2
	There are opalescent patches, no iris characteristics apparent, and the size of the pupil is scarcely recognisable.	3
	Iris is not seen since it is opaque.	4
	Area of Cornea Involved	
	Not zero, but a quarter (or less).	1
	less than one-quarter - more than one-half	2
	less than three-quarters but greater than one-half	3
	More than three-quarters of the way up to the entire area	4

Category	Description
Category 1	When a substance classified as Category 1 (irretrievable effects on the eye): a) Effects on the cornea, iris, or conjunctiva that are not expected to reverse or have not fully reverted during an observation period in at least one tested animal. b) A positive reaction of i) Corneal opacity ≥3; and/or ii) Iris >1.5 in at least two of the three examined animals; calculated as the mean scores following grading at 24, 48, and 72 hr after herbal shampoo application.
Category 2A	When a substance classified as Category 2A (irritating to eyes): i) Corneal opacity ≥1; and/or ii) Iris >1; and/or iii) Conjunctival redness ≥ 2 and/or iv) Conjunctival edema ≥ 2 in at least 2 of 3 examined animals. Calculated as the average of the scores obtained after grading at 24, 48, and 72 hr after using herbal shampoo.
Category 2B	When the symptoms described for Category 2A above are totally reversible within 72 hr of observation, an eye irritant is classified as slightly irritating to eyes (Category 2B).

RESULTS AND DISCUSSION

Acute Dermal and Ocular irritation studies performed using three different formulations of optimized herbal shampoo compositions. Two solid herbal shampoo compositions containing formulation Code: PS9 [Reetha (15% w/w), Aloe (10% w/w) and Henna (2% w/w)] and Second formulation Code: PS20 [Peppermint (15% w/w), Lemon (2% w/w) and Hibiscus (13.5% w/w)]. The third composition is a Liquid herbal shampoo formulation with code: LS17 [Amla (3% w/w), Reetha (10% w/w), Pomegranate (6% w/w) and Hibiscus (11.25% w/w)].

Above optimized herbal shampoo formulations were evaluated for acute dermal and ocular irritation study and the results and observations are summarized below

Acute Dermal irritation/Corrosion study

The OECD test guideline Number 404 was used to study the acute cutaneous irritation/corrosion of optimized herbal shampoos – PS9, PS20, and LS17 in rabbits. The test was conducted utilizing the remaining two Male rabbits for a 4-hr exposure session because no serious erythema / oedema was found 72 hr after exposure.

During the trial, no death or aberrant clinical symptoms were noticed in any of the animals.

At 1, 24, 48, and 72 hr following exposure, the skin response was scored (after removal of the dressing,

gauze patches and test item). For all three animals, the mean score for erythema / eschar development at 24, 48, and 72 hr was 0.00, 0.00, and 0.00, respectively. The mean oedema score for all three animals was 0.00, 0.00, and 0.00 at 24, 48, and 72 hr summarized in Table 1.

None of the treated animals developed erythema or oedema Refer Table 1. At 1, 24, 48, and 72 hr following treatment, no aberrant results were seen on any animal's control skin (removal of the dressing, gauze patch and test item) Refer Figure 1.

On the treated skin of any rabbit, the herbal shampoo formulation did not cause any coloring. On the skin, there were no corrosive effects. All of the rabbits' body weights were found to be within the usual range of variation seen in this strain and age group Refer Table 2.

Selected herbal shampoos are categorized as 'Non-irritant' to the rabbit skin according to the Harmonized Integrated Classification System since the mean score for erythema and oedema did not qualify for any irritation category of the classification criteria.

Ocular irritation/Corrosion study[25]

The OECD test guideline 405,[26] was used to assess the acute ocular irritation/corrosion of herbal shampoos in rabbits. Herbal shampoos were injected into the conjunctival sac of a single female rabbit's left eye. The test was finished using the remaining two female rabbits because no ocular responses were seen up to 72 hr after treatment.

For animal No. 01, 02, and 03 posttest item instillation, ocular responses were scored at 1, 24, 48, and 72 hr. For each animal, the mean score for corneal opacity, iris, conjunctival redness, and chemosis was computed during three scoring intervals (24, 48, and 72 hr after treatment). For Animal Nos. 01, 02, and 03, the individual mean scores for opacity, iris, conjunctivae, and chemosis were 0.00, 0.00, 0.00, and 0.00, respectively Refer Table 3.

A B

Figure 1: Photograph of skin of rabbit before application herbal shampoo (A) and 1hr after removal of herbal shampoo formulation (B).

Table 1: Skin irritation scores - Mean values at 24, 48 and 72 hr – left flank (treated – site).

	PS9 [Reetha (15% w/w), Aloe (10% w/w) and Henna (2% w/w)] *(mean value)*			PS20 [Peppermint (15% w/w), Lemon (2% w/w) and Hibiscus (13.5% w/w)] *(mean value)*			LS17 [Amla (3% w/w), Reetha (10% w/w), Pomegranate (6% w/w) and Hibiscus (11.25% w/w)] *(mean value)*		
Hours → / *Activity ↓*	24	48	72	24	48	72	24	48	72
Erythema	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oedema	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 2: Body weights – individual.

Body weight in Kg									
Animal identification Numbers	Sex	PS9			PS20			LS17		
		First Day of Acclimatization	Day of Treatment	Last Day of Observation	First Day of Acclimatization	Day of Treatment	Last Day of Observation	First Day of Acclimatization	Day of Treatment	Last Day of Observation
01	Male	1.5208	1.8546	1.9021	1.6148	1.7924	1.8457	1.5842	1.6651	1.8156
02	Male	1.5196	1.8823	1.9145	1.7241	1.8056	1.8751	1.5739	1.6894	1.8372
03	Male	1.5198	1.9012	1.9224	1.6547	1.7845	1.8387	1.6088	1.7059	1.8972

Kg = Kilogram

Instillation of the test item in the left eye of rabbits in Animal Nos. 01, 02, and 03 caused no response. Corneal opacity, region of opacity, iris, chemosis, and conjunctival redness were all assessed in the treated eye. At 1, 24, 48, and 72 hr after treatment, the treated eye appeared normal. During the acclimatization and post-treatment periods, no clinical symptoms of systemic toxicity were found in any of the animals Refer Figure 2. All of the animal's body weights were found to be within the usual range of variation seen in this species and age group. By the end of the trial, all of the animals had gained body weight Refer Table 4.

Since the treated animals corneal opacity was 0.00, iris lesions were 0.00, conjunctival redness was 0.00, and chemosis was 0.00, the results did not qualify for HICS irritation categories 1, 2A, or 2B of the ocular irritation classification criteria, so PS9, PS20, and LS17 were classified as 'Not Irritant' to rabbit eyes by the Harmonized Integrated Classification System.

Table 3: Eye irritation scores - mean values after 24, 48 and 72 hr (treated – left eye) for Individual animal.

Animal identification Number	Sex	Eye reaction											
		PS9				PS20				LS17			
		Corneal Opacity	Iris	Conjunctivae	Chemosis	Corneal Opacity	Iris	Conjunctivae	Chemosis	Corneal Opacity	Iris	Conjunctivae	Chemosis
01	Females	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
02		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
03		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 4: Body weights – Individual.

Body weight in Kg										
Animal identification Numbers	Sex	PS9			PS20			LS17		
		First Day of Acclimatization	Day of Treatment	Last Day of Observation	First Day of Acclimatization	Day of Treatment	Last Day of Observation	First Day of Acclimatization	Day of Treatment	Last Day of Observation
01	Female	1.5584	1.6852	1.8856	1.5987	1.6651	1.8366	1.6184	1.7141	1.8723
02	Female	1.6124	1.6724	1.8763	1.6083	1.6734	1.8547	1.6237	1.7082	1.8881
03	Female	1.5955	1.6983	1.8827	1.5899	1.6937	1.8485	1.6168	1.7106	1.8766

Kg = Kilogram

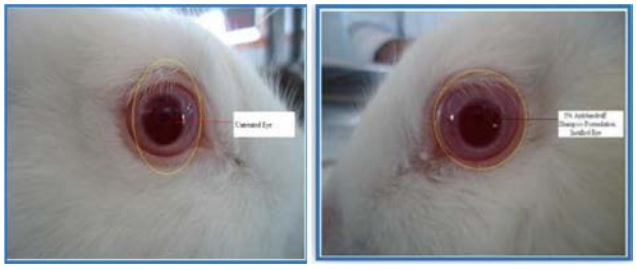

Figure 2: Ocular irritation/Corrosion study.

CONCLUSION

In conclusion, an ocular irritation study in New Zealand white rabbits using herbal shampoos PS9, PS20, and LS17 revealed corneal opacity ≤ 0.00, iris lesions ≤ 0.00, conjunctival redness ≤ 0.00, and chemosis of 0.00. The results did not qualify for HICS irritation categorizes 1, 2A, or 2B. As a result, several herbal shampoos are labelled as "Not Irritant to Rabbit Eyes" by the Harmonized Integrated Classification System.

According to the findings of the Acute Dermal Irritation research, the mean score for erythema and oedema in treated animals did not meet any of the irritation

categorization criteria, hence the herbal shampoos PS9, PS20, and LS17 are declared "non-irritant" to rabbits.

The skin and ocular sensitization levels of selected herbal shampoos utilised in this rabbit investigation were classified as non-irritant. The results of the Acute Dermal and Ocular Irritation research show that some herbal shampoos did not irritate the skin or eyes of rabbits, implying that they may be used on people without causing skin or eye irritation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HICS: Harmonized Integrated Classification System; **OECD:** Organisation for Economic Co-operation and Development; **Hr:** Hour; **IAEC:** Institutional Animals Ethics Committee; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **PII:** Primary Irritation Index.

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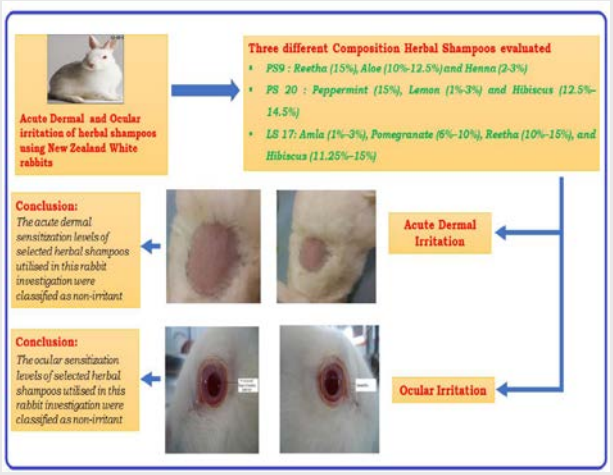
PICTORIAL ABSTRACT



SUMMARY

In New Zealand white rabbits, we evaluated the acute dermal and ocular irritation potentials of herbal shampoos consisting of a mixture of plant extracts. In the cutaneous skin irritation test, all three herbal shampoos showed no significant erythema, eschar, or oedema formation after 72 hr of exposure and did not meet any of the irritation classification requirements. According to the Harmonized Integrated Classification System (HICS), all three herbal shampoos are classified as "Non-irritant" based on acute cutaneous testing. In the ocular irritation study performed according to OECD test requirements. The corneal opacity, area of opacity, iris, chemosis, and conjunctival redness of the treated rabbit eye were evaluated. After 1, 24, 48, and 72 hr of observation, the treated eye appeared normal. There were no symptoms of systemic toxicity in any of the animals during the acclimatization and post-treatment periods. The results did not meet the irritation classification requirements for categories 1, 2A, or 2B because the treated animals had corneal opacity of 0.00, iris lesions of 0.00, conjunctival redness of 0.00, and chemosis of 0.00. Based on ocular irritation studies, all three herbal shampoos are classified as "Not Irritant" by HICS. The body weights of all the animals were within the typical range of variation for this species, strain, and age. All of the animals gained weight towards the end of the experiments. The outcomes of acute cutaneous and ocular irritation studies indicated that three herbal shampoos were not irritant to the rabbit skin or eyes, indicating that they can be used in humans without irritation to the skin or eye.

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