

Research Article

Comparative Evaluation of Eye Irritation Potential of Aqueous Extracts of *Sapindus mukorossi*, *Phyllanthus emblica*, *Acacia concinna* by *In vitro* and *In vivo* Methods.

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ABSTRACT

The location of the eye and its anatomy predisposes it to exposure to a variety of environmental conditions and substances on a daily basis and they can be exposed to cosmetic products. Injury from ocular exposure to a variety of chemical agents can lead to a range of adverse effects with the most extreme being blindness. The evaluation of eye irritation potential for a cosmetic product and its ingredients is essential to provide reassurance that a product is safe for consumers. Aim: Present study is to evaluate the eye irritation potential and compare irritation index of aqueous extracts of fruits of *Sapindus mukorossi*, *Phyllanthus emblica* and pods of *Acacia concinna* by *in vitro*, Hen's Egg Chorioallantoic Membrane Test (HET-CAM) and *in vivo* acute eye irritation test in rabbits. Materials and Methods: HET-CAM assay uses the CAM of a 10-day-old fertilized hen's egg. The CAM, a membrane which surrounds the developing chick embryo is highly vascularized extracts of above plants were delivered to the CAM surface and end points were observed are hemorrhage, lysis, coagulation for a period of 300 seconds and irritation index is calculated. Acute eye irritation test is carried out in albino rabbits. 0.1mL of extract applied in conjunctival sac of one eye. Results: The irritation score of *S.mukorossi*, *P.emblica*, *A.concinna* were 8.56, 0.38, 3.29, respectively. Conclusion: *S.mukorossi* is categorized under 2B and both *P.emblica* and *A.concinna* doesn't come under either category 1 or 2 as per the harmonized integrated classification system.

Key words: *Sapindus mukorossi*, *Phyllanthus emblica*, *Acacia concinna*, Hen's Egg Chorioallantoic Membrane Test (HET CAM).

INTRODUCTION

The location of the eye and its anatomy predisposes it to exposure to a variety of environmental conditions (e.g., ozone, pollen) and substances on a daily basis. Injury from ocular exposure to a variety of chemical agents can lead to a range of adverse effects with the most extreme being blindness.¹

The eye can be exposed to cosmetic products and their ingredients either through use of products such as those meant to be used around the eyes (e.g. mascaras, eye creams) or through accidental exposure to products that may enter the eye in diluted form during normal use but are not meant to come in to contact with the eye undiluted e.g. Shampoos.² As such, the evaluation of eye irritation potential for a cosmetic product and its ingredients is essential to provide reassurance that a product is safe for consumers to use through intended and foreseeable.³ The conventional method for determination of the irritant or corrosive potential of chemicals is Acute eye irritation test and this has

become the international standard assay for acute eye irritation and corrosion. This test involves examination of cornea, conjunctiva and iris for three days after application of test item to one of the eyes of a rabbit.⁴ Concerns about animal welfare, the cost and time to conduct ocular irritation assessments, the reproducibility of the currently used *in vivo* rabbit eye test as well as scientific interest in understanding eye injury at tissue and cellular levels have led researchers to develop and evaluate alternative *in vitro* test methods. Recently, the EPA requested the evaluation of four *in vitro* test methods Isolated Chicken Eye (ICE), Isolated Rabbit Eye (IRE), Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) and Bovine Corneal Opacity and Permeability (BCOP) for their ability to identify ocular corrosives and severe irritants.¹

Sapindus mukorossi (fam: Sapindaceae), well known as soapnuts are used medicinally as an expectorant, emetic, contraceptive and for treatment of excessive

Table 1: Irritation Classification Based on IS

HET-CAM Score Range	Irritation Category
0-0.9	Nonirritant or Practically None
1-4.9	Weak or Slight Irritation
5-8.9 or 5-9.9	Moderate Irritation
9-21 or 10-21	Strong or Severe Irritation

Emblca officinalis Gaertn. Or *Phyllanthus emblica*

Table 2 : Irritation score, severity and effect classification in the *in vitro* HET-CAM assay.

Compound	Irritation score (mean)	Classification of effect
0.9% NaCl	0.07	Non irritant
1% SDS	12.80	Severe irritation
Extract of <i>S.mukorossi</i>	8.56	Moderate irritation
Extract of <i>P.emblica</i>	0.38	Non irritant
Extract of <i>A.concinna</i>	3.29	Weak irritation

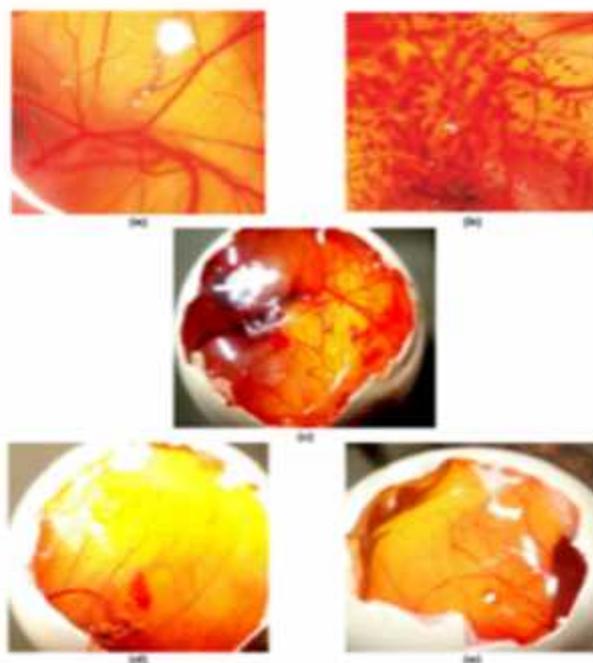


figure 1: a) Control, b) Positive control, c) Extract of *S.mukorossi*, d) Extract of *P.emblica*, e) Extract of *A.concinna*

Table 3 : Grading of ocular lesions by acute eye irritation test in rabbits (*Sapindus mukorossi*)

Animal	01				02				Mean
Hours	1	24	48	72	1	24	48	72	
Corneal opacity	1	24	48	72	1	24	48	0	1
Area of opacity	2	2	1	0	2	2	1	0	1
Iris	2	2	1	0	1	2	1	0	0.66
Conjunctivae	1	1	1	0	1	1	1	1	1.3
Chemosis	2	2	1	1	1	2	1	0	2

salivation, epilepsy, chlorosis and migrane.⁵ *Sapindus mukorossi* is a popular ingredient in Ayurvedic shampoos to remove dandruff, to reduce hair loss, skin cleaner and stain remover.⁶ Soap nuts are used for eczema and psoriasis. It is having anti-bacterial, anti-fungal, insecticidal properties. It is commonly found in the Western Ghats and plains of South India⁷. The major constituents of the fruits are saponins (10% - 11.5%), sugars (10%) and mucilage⁸.

Linn belonging to the family Euphorbiaceae. The fruit, also known as Indian gooseberry The fruit also forms an important constituent of nearly 300 Ayurvedic preparations of which the most popular formulations are *chyvanprash* and *triphala*⁹. The fruit is a very rich source of vitamin C. Its mineral and vitamin contents include calcium, phosphorous, iron, carotene, thiamine, riboflavin, and niacin. Fruit is high in pertin, phyllembin is there. Fresh amla contains about 20 times more vitamin C than orange juice and equal in antiscorbutic

Table 4 : Grading of ocular lesions by acute eye irritation test in rabbits (*Acacia concinna*)

Animal	01				02				Mean
Hours	1	24	48	72	1	24	48	72	
Corneal opacity	1	1	0	0	1	1		0	0
Area of opacity	1	1	0	0	2	1	0	0	0.33
Iris	1	0	0	0	1	0	0	0	0
Conjunctivae	2	1	1	0	1	1	0	0	0.66
Chemosis	2	1	0	0	2	1	0	0	0.33

Table 5 : Grading of ocular lesions by acute eye irritation test in rabbits (*Phyllanthus emblica*)

Animal	01				02				Mean
Hours	1	24	48	72	1	24	48	72	
Corneal opacity	1	0	0	0	0	0		0	0
Area of opacity	0	0	0	0	0	0	0	0	0
Iris	0	0	0	0	0	0	0	0	0
Conjunctivae	1	1	1	0	1	1	0	0	0.33
Chemosis	0	0	0	0	0	0	0	0	0

Figure 2: a) Control ,b) Extract of *S.mukorossi*, c) Extract of *P.emblica* d) Extract of *A.concinna*

value to 1-2 Oranges. Dried fruit have tannins and 3-4 colloidal complexes. Other components are phyllembic acid, lipids, gallic acid, emblicol, mucic acid, ellagic acid, glucose. Seeds contain a fixed oil, phosphatides, some essential oil with linolenic, linoleic, oleic, stearic, palmitic, myristic acids. Proteolytic and lipolytic enzymes are in seeds. Pharmacological actions include tonic erythrogenic, digestive laxative, rasayana, Refrigerant, diuretic, laxative, gastric acidity regulator, expectorant, anti-inflammatory, restorative tonic, regulates blood sugar, aphrodisiac, nervine, tonic, antitumour, anti-inflammatory, antibacterial properties¹⁰. *Acacia concinna* Linn (Leguminosae) is a medicinal plant that grows in tropical rainforests of southern Asia commonly known as shikakai¹¹. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds

fruits of this plants are used for washing hair, for promoting hair growth, as an expectorant, emetic, and purgative¹².

MATERIALS AND METHODS

Collection of plant material: Dried fruits of *Sapindus mukorossi*, *Emblica officinalis* and pods of *Acacia concinna* were collected from the local market and authenticated by Dr. Vatsavaya S. Raju, Botanist, Department of Botany, Kakatiya University, Andhra Pradesh

Preparation of aqueous extracts: One hundred grams of the dried fruit powder of *Sapindus mukorossi* were soaked in 400 ml of distilled water for 16 h. The percolate was then decanted, centrifuged and filtered through Whatman No. 1 filter paper to obtain clear extract (300 ml). This process of extraction was repeated again with same volume of distilled water.

The percolates were pooled and lyophilized to give a brown colored powder (68% yield).⁸

Fruits of *Phyllanthus emblica* were shade dried for a week, powdered mechanically, sieved (sieve no: 10/44) and stored in air tight containers. About 250g of the powdered material is boiled in 500ml distilled water for 30 minutes, kept for 3 days with intermittent shaking, filtered and concentrated completely dried aqueous extract.¹³

100g dry pods of *Acacia concinna* was soaked in 1000ml distilled water for 12h. The material was then filtered through Whatman filter paper and filtrate was concentrated by flash evaporation at 358 C to powder.¹⁴

Test systems

Hen's Egg Test Chorioallantoic Membrane: The HET-CAM protocol was first described by Luepke (1985) CAM, which is a vascular fetal membrane, composed of the fused chorion and adjacent wall of the allantois. According to the original test method, fertilized hen's eggs are incubated, under optimized conditions, for nine days. On the 10th day, the eggs are opened and the CAM exposed.¹

Fertile White Leghorn chicken eggs were obtained from commercial sources. Fresh (not older than seven days), fertile, clean eggs weighing between 50 and 60 grams were used and candled prior to use. Nonviable or defective eggs were discarded. Excessively misshapen eggs or eggs with cracked or thin shells were not used. Transport of eggs should occur under conditions that will not affect embryo viability or development.¹⁵ Place eggs in an incubator with a rotating tray. Incubate eggs at $38.3 \pm 0.2^\circ\text{C}$ and $58 \pm 2\%$ relative humidity when incubating in a still-air incubator or at $37.8 \pm 0.3^\circ\text{C}$ and $58 \pm 2\%$ relative humidity when incubating in a forced air incubator. Hand rotate eggs five times per day until day 8. Remove eggs from the incubator on day 9 for use in the assay. Candle eggs and discard any nonviable or defective eggs. Mark the air cell of the egg. Cut the section marked as the air cell with a rotating dentist saw blade and then pare it off. Care should be taken when removing the eggshell to ensure that the inner membrane is not injured. Finally, three eggs per group are used in the study.¹⁵

Negative Control: 0.3ml of 0.9% NaCl solution is directly applied on the chorioallantoic membrane to provide a baseline for the assay endpoints and to ensure that the assay conditions do not inappropriately result in an irritant response.¹⁵

Positive Control: 0.3mL of 1% SDS and 0.1N NaOH are applied on the chorioallantoic membrane a severe response in HET-CAM is expected.¹⁵

Treatment: 0.3mL of aqueous extracts of *Sapindus mukorossi*, *Phyllanthus emblica*, *Acacia concinna* applied on the chorioallantoic membrane on the 9th day. Observe the reactions on the CAM over a period of 300 seconds. The time for the appearance of each of the noted endpoints should be monitored and recorded, in seconds. Endpoints that should be observed are:

- Hemorrhage (bleeding from the vessels)
- Vascular lysis (blood vessel disintegration)
- Coagulation (intra and extra-vascular protein denaturation)

An irritation score (IS) is calculated, and the test item is classified with this score. The following formula is used to generate an irritation score (IS):¹

$$\left(\left(\frac{301 - \text{Hemorrhage time}}{300} \right) \times 5 \right) + \left(\left(\frac{301 - \text{Lysis time}}{300} \right) \times 7 \right) + \left(\left(\frac{301 - \text{Coagulation time}}{300} \right) \times 9 \right)$$

Hemorrhage: Time taken to start (in seconds) of hemorrhage reactions on CAM

lysis: Time taken to start (in seconds) of vessel lysis on CAM

coagulation: Time taken to start (in seconds) of coagulation formation on CAM.¹

Acute Eye Irritation Test: The healthy young albino rabbits are used for the study with prior examination of both eyes of each experimental animal 24 hours before starting the experiment, to avoid any animals showing ocular defects or preexisting corneal injury.

Animals should be individually housed. The temperature of the experimental animal room should be 20°C ($\pm 3^\circ\text{C}$) for rabbits. Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. Excessive light intensity should be avoided. For feeding, conventional laboratory diets may be used with an unrestricted supply of drinking water. Two animals were used in each group.⁴

Treatment: The test is carried out by applying 0.1mL containing each 100 mg of *Sapindus mukorossi*, *Phyllanthus emblica*, *Acacia concinna* aqueous extract in the conjunctival sac of one eye of rabbits after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second in order to prevent loss of the material. The other eye, which remains untreated, serves as a control.⁴

The eyes were examined at 1, 24, 48, and 72 hours after application. The grades of ocular reaction, that is conjunctivae, cornea and the iris are recorded at each examination as per OECD TG405. The cornea is visually observed both for the degree of corneal opacity and the area of the cornea in which opacity is involved. The iris is assessed for inflammation, iridal folds, congestion, swelling circumcorneal injection, reaction to light, hemorrhage and gross destruction. The conjunctiva is evaluated for the degree of redness, chemosis (swelling) and discharge.⁴

RESULTS

The experimental results regarding the eye irritation potential of aqueous extracts of *Sapindus mukorossi*, *Phyllanthus emblica*, *Acacia concinna* by in vitro method, that is, Hen's Egg Test Chorioallantoic Membrane (HET-CAM). Irritation score, severity, and effect classification in the in vitro HET-CAM assay

that is, Hen's Egg Test Chorioallantoic Membrane (HET-CAM), is presented in Table 2

The effects induced by the test compounds as well as the selected control were registered as macro photographs representing the surface of the chorioallantoic membranes after treatment for 5 minutes of contact (figure 1)

Acute Eye Irritation Test The induced effects after the single application of test compounds was noted by scoring the lesions of the conjunctiva, cornea and iris, at specific intervals. The mean score was calculated across three scoring times (24, 48, and 72 hours after treatment) for each animal for corneal opacity, iris, conjunctivae and chemosis. The obtained numerical scores were compared with the Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures, 14 August, 2001.

The effects induced by the test compounds as well as the selected control were registered as macro photographs representing the eye of the rabbit after 24 hours of treatment with test substances (figure 2)

DISCUSSION

In HET-CAM assay, SDS induced major damage at the vascular level of the chorioallantoic membrane a large area was affected by the formation of micro hemorrhages. When CAM treated with 0.3ml of extract of *Sapindus mukorossi* a medium to large area was affected by the formation of hemorrhage and lysis of blood vessels was also observed. Whereas on application with 0.3ml of extract of *Acacia concinna* very small area was affected by hemorrhage. On the other hand no effect of hemorrhage, lysis observed by application of *P.emblica*. From the IS scores obtained *Sapindus mukorossi* is categorized under moderate irritant and *Acacia concinna* as weak irritant and *Phyllanthus emblica* as nonirritant as per Luepke. In acute eye irritation test the animals treated with *S. mukorossi* extract showed a positive response of corneal opacity 1, iritis 1, conjunctival redness 2 (Chemosis) 2 are fully reversible within 7 days of observation is considered mildly irritating to eyes (Category 2B). And for both *A.concinna* and *P.emblica* the outcome of result did not qualify for category 1 and 2 of the classification criteria as per Harmonized Integrated Classification System For Human Health And Environmental Hazards Of Chemical Substances And Mixtures.¹⁶ The reason for causing irritation by *Sapindus mukorossi* may be due to the presence of saponins as major constituents.

CONCLUSION

This study showed good correlation between results obtained by HET-CAM and those of the acute eye irritation test. From present study we concluded that aqueous extract of *Sapindus mukorossi* is considered as mildly irritating to eyes (category 2B) and aqueous extracts of *Acacia concinna* and *Phyllanthus emblica* are non irritants.

Hence it is suggested that it is better to use shampoos containing *Acacia concinna* and *Phyllanthus emblica* than *Sapindus mukorossi* as it is mildly irritating to eyes.

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