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Evaluation of skin corrosive property of ethanolic leaf extract of *Acyranthus aspera* by *in vitro* Transcutaneous Electrical Resistance Test and Human skin Model Test.

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ABSTRACT

Objective: The present article is an attempt to investigate the corrosive property of ethanolic leaf extract of *Achyranthus aspera* (Amaranthaceae) by in vitro transcutaneous electrical resistance (TER) test and in vitro human skin model test before conducting wound healing experiments on rats to know instead of wound healing activity, whether it is having skin corrosion property or not. **Methods:** In TER test, application of extract for up to 24 hours to the epidermal surfaces of skin discs in a two-compartment test system in which the skin discs function as the separation between the compartments. Corrosive substances will be identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which was measured as a reduction in the TER below a threshold level (5 KΩ). In human skin model test, the extract is applied topically to a three-dimensional human skin model, comprising reconstructed epidermis with a functional stratum corneum for 1 hour. Corrosive substances are identified by their ability to penetrate the stratum corneum by diffusion or erosion, and cytotoxic to the underlying cell layers to produce a decrease in cell viability below threshold levels (15 %). **Results:** The ethanolic extract of *Achyranthus aspera* does not show corrosive property in both transcutaneous electrical resistance and human skin model test. **Conclusion:** The results obtained in these studies suggest a significant scope for the isolation of biological active components for further studies.

KEY WORDS: Epidermis, EpiSkin, MTT, Stratum corneum, Transcutaneous electrical resistance.

INTRODUCTION

Skin corrosion refers to the production of irreversible tissue damage in the skin following the application of a test substance [1, 2]. The use of laboratory animals in the assessment of skin corrosivity is very common. This involves pain and suffering to the animals, hence, to determine the corrosive property of the chemicals various alternatives *in vitro* methods were used [3-5]. An important consideration in cosmetic innovation and toxicology is the growing concern about the ethics of testing final/finished products on animals, and it is gradually being discouraged and alternative methods are being designed [6].

Achyranthes aspera is an important medicinal herb known as a weed throughout India. However, in traditional systems of medicines almost all parts of it are used. Seeds, roots and shoots are the most important parts, which are used by traditional healers for the treatment of fever, dysentery and diabetes [7-9]. The present article is an attempt to evaluate the corrosive property of ethanolic leaf extract of *Achyranthus aspera* by *in vitro* skin corrosion assay using rat skin and reconstituted human epidermis skin model.

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MATERIALS AND METHODS

Plant material & Preparation of leaf extract

The leaves of *Achyranthes aspera* were freshly collected during January-March in and around the Yenugonda village (Mahabubnagar District, Andhra Pradesh, India) and were cleaned with distilled water and shade dried at room temperature. The dried leaves were powdered (100 g) and were extracted separately to exhaustion in a soxhlet apparatus using ethanol as a solvent system. The extract was filtered through Whatman filter paper No.1 and then concentrated by evaporating at the low temperature (40-50 °C) to get a 2.59 g yield. The extract was preserved in an airtight container at 4±2 °C until further use.

Preliminary qualitative analysis of phytochemicals in leaf extract

A preliminary qualitative phytochemical analysis of leaf extract of *Achyranthes aspera* was carried out [10].

Sodium hydroxide Test:

Small quantity of the extract was treated with sodium hydroxide solution; Formation of yellow color indicates the presence of Flavonoids.

Foam Test:

The extract was diluted with 20 ml of distilled water and agitated in the graduated cylinder for 15 minutes. A one-centimeter layer of foam indicates the presence of saponins.

Haemolysis Test:
About 2 ml of blood was taken in two test tubes separately. In one test tube equal quantity of water and in another test tube, an equal quantity of extract dissolved in water was added. Presence of clear red liquid in the first tube, indicates haemolysis, if same is the case with second test tube (with extract) indicates the presence of saponin.

Mayer's Test:
To a few ml of extract, two drops of Mayer's reagent was added by the sides of the test tube. A white or creamy precipitate indicates the presence of alkaloids.

Wagner's Test:
To a few ml of extract, few drops of Wagner's reagent (Iodine 1.278 g and potassium iodide 2 g dissolved in 100 ml of distilled water) was added by the side of the test tube. The presence of a reddish brown precipitate indicates the presence of alkaloids.

Hager's Test:
To a few ml of extract, 2 ml of Hager's reagent (Saturated aqueous solution of picric acid) was added. A prominent yellow precipitate indicates the presence of alkaloids.

Molish's Test:
To two ml of extract, Two drops of the alcoholic solution of a-naphthol was added, the mixture was shaken well and 1 ml of concentrated sulphuric acid is added slowly along the sides of the test tube and allowed to stand. A violet ring indicates the presence of carbohydrates.

Ferric Chloride Test:
Two grams of extract were boiled with 5 ml of 45 % ethanol for 5 minutes. The mixture was cooled and filtered. 1 ml of filtrate was diluted with distilled water and added 2 drops ferric chloride. A transient greenish to black color indicates the presence of tannins.

Test System
The transcutaneous electrical resistance test and human skin model test were performed by using (a) rat skin and (b) EpiSkin (reconstituted human epidermis) respectively.

(a) Rat skin disc preparation
Transcutaneous electrical resistance test using rat skin has been approved by the IAEC on 10th February 2010.

Normal, young and healthy inbred female Wistar rats of 20±2-day-old were obtained from Central animal house, Aptus Biosciences Private Limited (CPCSEA Registration Number 1312/c/09/CPCSEA). Hairs from the dorsal and flank region of female rats were removed carefully with small clippers. The animals have given a wash with antibiotic solution twice with an interval of three days to inhibit bacterial growth, before initiation of the experiment [2]. Animals were humanely sacrificed and the dorso-lateral skin of each animal was removed. The excess subcutaneous fat stripped off carefully by peeling it away from the skin. Circular skin discs, with a diameter of approximately 20 mm each was removed from the skin. Each skin disc was placed over one of the ends of a tube (internal diameter of the tube approx. 10 mm) ensuring that the epidermal surface was in contact with the tube. The skin disc was tied to hold in place with suturing thread. Excess tissue was trimmed and sealed with petroleum jelly.

(b) Human Skin Units
EpiSkin, the human skin units were obtained from EpiSkin SNC, France, which was used in this study. Lot number 10-EKIN-028.

EpiSkin is a three-dimensional reconstituted human epidermis model comprising the main basal, supra basal, spinosus and granular layers and a functional stratum corneum.

EXPERIMENTAL PROCEDURES

1.Transcutaneous Electrical Resistance Test
In this test, the transcutaneous electrical resistance was measured with Millicell®ERS-2, Epithelial Volt-Ohm meter, MILLIPORE, USA. Prior to the test, the electrical resistance of two prepared skin discs was measured as a quality control procedure for each animal skin. Skin discs with resistance values greater than 10 kΩ (10000Ω) were only used for the study.

Three skin discs per group were used. In group 1 (G1), the skin disks were treated with distilled water as a negative control. The skin discs from group 2 and 3 (G2 & G3) treated with 10M hydrochloric acid and acrylic acid respectively as positive control. While, the skin discs from group 4 (G4) were treated with extract. The controls and extract were applied to the epidermal surface of the skin disc at 20 – 23 ^{0}C for 24 hours and then washed with distilled water. TER was measured by pouring a 154mM solution of $MgSO_4$ into the tube and lower reservoir (surrounding the tube). The data bridge electrodes were placed on either side of the skin disc to measure the resistance. The inner electrode was placed inside the tube during resistance measurement to ensure that a constant length of the electrode was submerged in the $MgSO_4$ solution. The outer electrode was positioned inside the receptor chamber so that it rests on the bottom of the chamber. The TER values of negative control, positive control and treated skin discs were recorded.

2. Human Skin Model Test
In this test, after receiving of skin units from the EpiSkin SNC, France, quality check was performed to see the suitability of the skin units for the study. When found suitable the skin units transferred to fresh maintenance medium and kept in incubation at 37 ^{0}C, 5 % CO_2 and 95 % humidity for 24 hours.

Liquid test items 12 µl directly or 12 ± 2 mg of solid materials with 5 µl of distilled water were applied to the epidermal surface in order to improve further contact with gentle spreading to cover all surface areas.

Three skin units per group were used. Group 1 (G1) the skin units was treated with sodium chloride 0.9 % as negative control. However, the skin units from group 2 and 3 treated with 8 N potassium hydroxide and acrylic acid respectively as positive controls and group 4 (G4) skin units were treated with extract. The epidermis were allowed to be in contact with the test items for 60 minutes at room temperature (19 to 28 ^{0}C) and then rinsed with 25 ml phosphate buffered saline. After washing, the skin units were transferred to fresh maintenance medium under sterile condition and incubated at 37 ^{0}C, 5 % CO_2 and 95 %

humidity for 42 hours. After incubation, the skin units were transferred to the next wells of same 12 well plate with 2 ml of MTT solution (0.3 mg / ml) in the assay medium. The plate was incubated for 3 hours at 5 % CO_2 and 95 % humidity. After the final incubation, the skin units were removed and punched biopsy was carried out, the biopsy material was transferred in prelabeled tubes containing 500 µl of acidic isopropanol. These tubes were kept overnight for formazan extraction at 4 ^{0}C. After extraction, the tubes were centrifuged at 500 RPM for 10 minutes. 200 µl solution from each tube was transferred to two different wells in 96 well flat-bottom microtiter plate as aliquot 1 and aliquot 2. The optical density of each sample was measured by 570 nm using a multimode microplate reader (SYNERGY - 4). The percent cell viability of each skin unit was calculated by a formula

$$\text{Percent cell viability} = \frac{\text{Mean OD of respective skin units}}{\text{Mean OD of negative control}} \times 100$$

BASIC HISTOLOGY

Skin specimens used in the transcutaneous electrical resistance were removed from the tubes after measuring TER for histological examination. The tissues were processed routinely for light microscopy (fixation in 10% buffered formaldehyde, dehydration, paraffin embedding, sectioning [5 micrometer], and staining). Hematoxylin-eosin was used for basic staining. The tissue samples were evaluated for the following histological criteria; Integrity of stratum corneum, organization of the epidermal squamous cells. All the treated groups were assessed blindly by the pathologist and the results were compared with the negative control and positive control groups.

RESULTS

Preliminary qualitative phytochemical analysis of ethanolic extract of *Achyranthus aspera* revealed positive for Flavonoids, Saponins, Alkaloids, Carbohydrates **(Table I)**.

Transcutaneous electrical resistance was measured for *Achyranthus aspera* extract, Positive controls and negative control. Mean TER values of negative control (distilled water) and extract treated skin discs were observed more than 10 kΩ i.e. 12235.67 and 11461 with no obvious damage and hence considered non-corrosive. However, TER value of positive control 10 M hydrochloric acid & acrylic acid treated skin discs were observed less than 5 kΩ i.e 327.33 and 355 with obvious damage, hence considered as corrosive **(Table II)**.

In the human skin model test, the OD values of negative control, positive controls and extract were recorded and the percent cell viability was calculated. The percent cell viability of negative control and extract treated skin units were observed more than 50 percent i.e 100.00 and 94.71 at 60 minutes of exposure time, hence considered non-corrosive. However, percent cell viability of positive control (8 N potassium hydroxide and Acrylic acid) was less than 15 percent i.e 3.81 and 2.97. Hence, considered as corrosive **(Table III)**.

Table I: Results of preliminary phytochemical analysis of Ethanolic extract of *Achyranthus aspera.*

Test	Observation	Inference
Sodium Hydroxide Test	Formation of yellow color	Presence of flavonoids
Foam Test	Formation of 1 cm of foam	Presence of saponins
Haemolysis Test	Clear red liquid indicates haemolysis	Presence of saponins
Mayer's Test	No white or creamy precipitate	Presence of alkaloids
Wagner's Test	No reddish brown precipitate	Presence of alkaloids
Hager's Test	Formation of yellow precipitate	Presence of alkaloids
Molish's Test	Formation of violet ring	Presence of carbohydrates
Ferric Chloride Test	No transient greenish to black color	Absence of tannins

Table II: Transcutaneous electrical resistance values of skin disc in each group.

Group	Skin disc No.	Electrical Resistance (Ω)	Mean± SD	Visual damage
G1	1	11425	12235.67 ± 1185.28	No
	2	13596		
	3	11686		
G2	1	350	327.33** ± 32.57	Yes
	2	342		
	3	290		
G3	1	325	355** ± 56.34	Yes
	2	420		
	3	320		
G4	1	11564	11461^{ns} ± 125.65	No
	2	11321		
	3	11498		

 # The values in the table are rounded off to the nearest digit value.G1- Distilled Water (Negative Control); G2 – 10 M HCl (Positive Control-1); G3 - Acrylic acid (Positive Control-2); G4 - Extract treated. One-way ANOVA with Dunnett's post-test was performed P< 0.05 was considered statistically significant.

** = P < 0.01, ^{ns}= P > 0.05.

Table III : Mean percent cell viability of skin units of each group.

Group	Skin Unit No.	Optical Density Aliquot 1	Optical Density Aliquot 2	Blank corrected OD Aliquot 1	Blank corrected OD Aliquot 2	Mean of corrected OD	% Cell Viability Relative	% Cell Viability Mean
Blank	1	0.033	0.032	0	0	0.032	-	-
	2	0.033	0.033	0	0	0.033	-	
	3	0.032	0.032	0	0	0.032	-	
G1	1	0.748	0.534	0.716	0.502	0.609	98.54	100.00
	2	0.714	0.575	0.682	0.543	0.612	99.11	
	3	0.654	0.675	0.622	0.643	0.632	102.35	
G2	1	0.055	0.06	0.023	0.028	0.025	4.05	3.81**
	2	0.054	0.054	0.022	0.022	0.022	3.48	
	3	0.056	0.057	0.024	0.025	0.024	3.89	
G3	1	0.048	0.049	0.015	0.017	0.016	2.59	2.97**
	2	0.058	0.052	0.025	0.02	0.022	3.64	
	3	0.047	0.051	0.014	0.019	0.016	2.67	
G4	1	0.612	0.662	0.547	0.587	0.567	91.82	94.71*
	2	0.587	0.562	0.625	0.598	0.612	99.03	
	3	0.551	0.565	0.551	0.601	0.576	93.28	

 # The values in the table are rounded off to the nearest digit value.Blank (Acidic isopropanol); G1 - Negative control (0.9 % NaCl); G2 - Positive control (8 N KOH); G3 - Positive control (Acrylic Acid); G4 - Extract treated.One-way ANOVA with Dunnett's post-test was performed P< 0.05 was considered statistically significant.

** = P < 0.01, ^{ns}= P > 0.05.

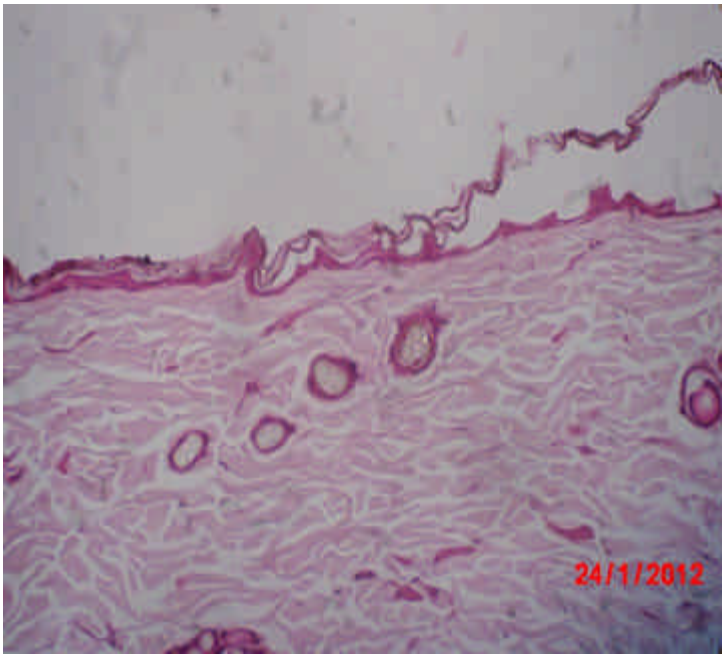
Histopathological findings

Histopathology was performed to skin discs used in the transcutaneous electrical resistance test. All the groups were subjected to histopathology, No histopathological findings were observed in negative control and extract treated skin discs **(fig I)**, whereas destruction of stratum corneum, stratum granulosum, stratum spinosum and stratum basale was observed both the group skin discs treated with positive controls these are the four layers present in the epidermis. Stratum basale is the bottom layer of epidermis these cells are a kind of stem cells, which generates proliferating keratinocytes that migrates towards stratum corneum in the process of cell shedding. During this process these cells undergo some modifications like accumulation in the stratum spinosum, enucleated and undergo death in stratum granulosum and finally reaches stratum corneum and acts as a barrier to the internal tissues. From the microphotographs **(fig II & III)** treated with positive controls we can clearly see the destruction and necrosis of these cells in the stratum basale and stratum spinosum, which created irreversible damage, is corrosive to skin.

STATISTICAL ANALYSIS

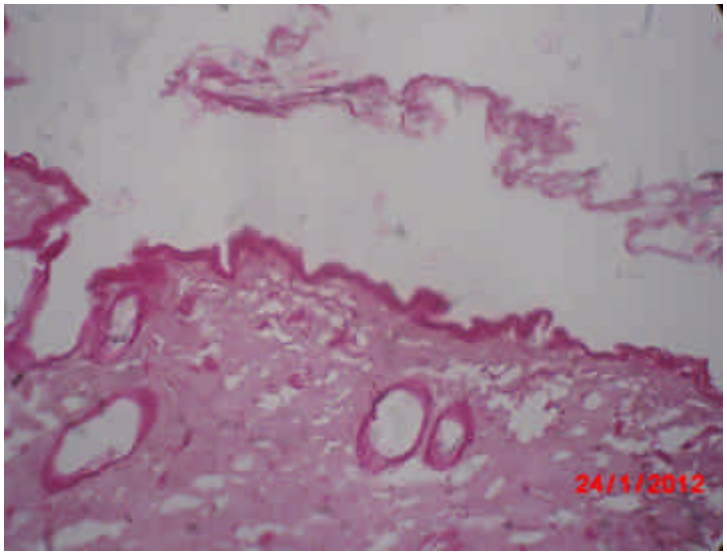
Statistical analysis was performed by using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com. The electrical resistance in transcutaneous electrical resistance test and % cell viability in human skin model test was compared to the results of the negative control group by one-way ANOVA with Dunnett's post-test was applied to determine the statistical significance of the results, and a value of $P < 0.05$ was considered significant.

Figure I: Skin disc treated with ethanolic extract of *Achyranthus aspera*



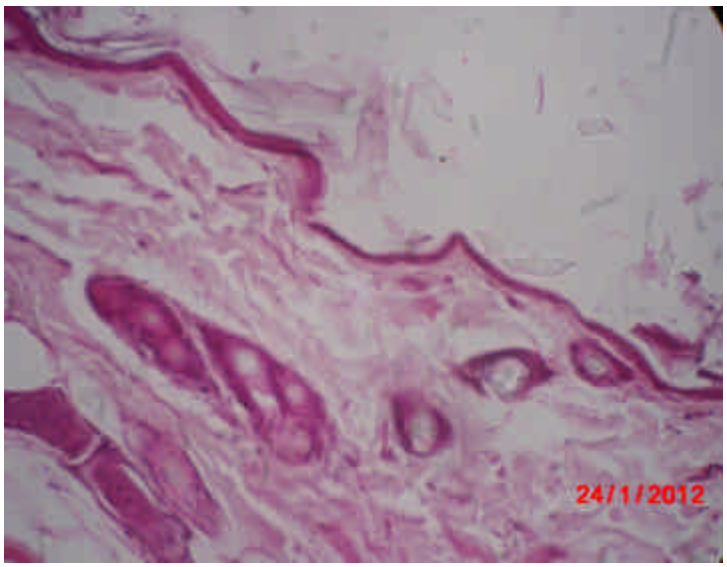
Legend : Figure I treated with ethanolic extract of Achyranthus aspera showing the normalstratum corneum, Stratum granulosum, Stratum spinosum and Stratum basale which clearly indicate the extract is non-corrosive to the skin.

Figure II: Skin disc treated with 10 M Hydrochloric acid



Legend : Figure II treated with 10M HCl showing the erosion stratum corneum and the destruction of Stratum granulosum, Stratum spinosum and Stratum basale which is clear indication of irreversible damage to the skin.

Figure III: Skin disc treated with Acrylic acid



Legend : Figure III treated with Acrylic acid showing the erosion stratum corneum and the destruction of Stratum granulosum, Stratum spinosum and Stratum basale which is clear indication of irreversible damage to the skin.

CONCLUSION

From the present study, the *in vitro* skin corrosion assays by transcutaneous electrical resistance test; ethanolic leaf extracts of *Achyranthus aspera* showed TER values greater than 10 kΩ with no visual damage to the skin. Similarly, in human skin model test, the mean percent cell viability of the extract treated skin units was greater than 50 %. Hence, it can be concluded that the ethanolic leaf extract

of *Achyranthus aspera* has no skin corrosive property, which could be beneficial in different skin preparations and are a subject for future studies.

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