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SKIN RENEWAL EFFECT OF DIFFERENT EXTRACTS OF LEAVES OF *AZADIRACHTA INDICA*

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Abstract: Different extracts of *Azadirachta indica* (neem) leaves were tested for their epidermal turnover activity. Amongst the different investigational neem leaves extracts, absolute alcohol extracts, hydro alcoholic extracts and hydro alcoholic macerates showed a significant effect on skin renewal rate while petroleum ether extracts, propylene glycol, benzene extracts chlorbutanol extracts and acetone extracts were found to be ineffective. The dansyl chloride fluoresce method was used to measure skin renewal effect of various dried neem leaves extracts.

Key words: *Azadirachta indica,* extracts, skin renewal, dansyl chloride.

Introduction

Azadirachta indica (Neem) is one of the popular auspicious and well known trees in India, which is more extensively studied for its pharmaceutical and clinical properties. In Ayurvedic literature neem is describe as NIMB[1]. A variety of biological activity of *Azadirachta indica* has been reported in literature. Literature revealed antifertility[2], htpoglycemic[3], typanocidal[4], analgesic[5], insecticidal[6], activity.

In present work, we report the skin renewal effect of various dried neem leaves extracts. Absolute alcohol extracts, hydro alcoholic extracts, hydro alcoholic macerate extracts showed a significant effects. The student t test shows that increase in skin renewal by alcohol extracts, hydro alcohol macerate are significant at $p< 0.005$ level of significance while alcohol extract is significant at $p<0.05$ level of significance.

Materials and Methods

Dansyl chloride was purchased from sigma chemicals Japan. An occlusive adhesive patch was purchased from Johnson and Johnson, India. All the other chemicals used were of AR grade. Spectrofluorometer Jobin Yvou, Japan was used for analysis**.**

Fresh healthy neem leaves were collected from vicinity of Nagpur University campus, Nagpur; India and allowed to dry in a shade under normal environmental condition for about one week and then crushed it into small pieces by hand. These coarsely grounded neem leaves were extracted exhaustively in soxlet apparatus successively with different solution in the increasing order of polarity.

Cold maceration of dried neem leaves:

About 100 g of coarsely grounded leaves were steeped in hydroalcohol (1:1) for one week at ambient temperature and then filtered. This extraction was repeated with another fresh portion of hydroalcohol (1:1) for about one week. The hydro alcohol macerate extracts were combined and evaporated up to dryness, dark brown coloured product was obtained**.**

Effect of neem leaves extracts on skin renewal:

The dansyl chloride fluorescence method [7] was used to measure skin renewal effect of various dried neem leaves extracts. Dansyl chloride (5-dimethyl-amino-1-naphthlene sulfonyl chloride) is a fluorescent due which stains the skin. It combines avidly with amino group and this is useful for fluorescent tagging of proteins.

a) Preparation of dansyl chloride base:
Dansyl chloride dispersion (5%w/w) was prepared using white soft paraffin as a base, using a glass mortar and pastel. Since dansyl chloride is a light sensitive dye, preparation of the base was carried out in a dark room with subdued red light.

b) Preparation of standard for comparison of fluorescence intensity:
A fresh piece of forearm skin surface of about (10 x 2 cm) was obtained from the Government medical college, Nagpur. It was immediately put in to ice to avoid deterioration and brought to laboratory. Subcutaneous fats adhering to skin were removed with forceps and scissors and the skin was made as clean as possible. The epidermis was then separated by heat-trypsinization method of Klingman [8]. It was cut in to pieces of 1 x 1 cm and stored in a desiccator which was then placed in a refrigerator. Each concentration of dansyl chloride base was liberally and uniformly smeared on the pieces of skin with the help of index finger in dark. The skin pieces were than sandwiched between two glass slides tied with rubber band and preserved in refrigerator below $0^{O}C$. This way the slides of different concentration (0.5-5.0% w/w) of dansyl chloride were prepared. After 24 h, the fluorescent intensity of these stained pieces was measured at 340 nm by spectroflurometer. Fluorescence intensity of these standards was checked over a two months period to ascertain that the fluoresces does not decline.

c) Development of fluorescence test patches on human volunteer:
Ten healthy human volunteer, five male and five female, between ages 18–24 years were participated in the study. Informed consent was taken from the participants. Both the inner forearms of each volunteer were used as site for development of patches. On each forearm three marking having dimension of 5 % dansyl chloride base was liberally and uniformly smeared over the marked area on each forearm in dark room with red subdued light. These areas were then covered with occlusive adhesive bandages (Handyplast, India). Volunteers were advised to prevent patches from water contact for at least 24 h. After 24 h the bandages were removed and sites were examined under U.V. radiation to ascertain the development of fluorescence stain.

d) Application of investigational neem leaves extracts on the test patches:
All the dried extracts were dispersed in propylene glycol to prepare 1% w/v. Each volunteer had six patches stained with dansyl chloride, three on each forearm. Out of these six patches; four were treated with investigational extracts once a day. The fifth patch was treated with propylene glycol to ensure whether the solvent had any effect on the skin renewal rate or not. The sixth patch was untreated and used as a control. The patches were marked with blue pen by viewing in ultraviolet light chamber. Each extract was uniformly and liberally applied on the respective patch site with rubbing finger to ensure maximum absorption. Volunteer were advised not to apply any cosmetics preparation on these test patches.
Fluorescent intensity of patches were measured by comparing it with standard, prior to application of extracts, volunteers feedback report was prepared subsequently which involved observation for skin irritation, if any.

e) Fluorescence intensity determination:
Fluorescence intensity of test patches was measured by keeping forearm in UV radiation chamber, in which patches give fluorescence. The intensity of fluorescence of test patches was compared with that of standard preparation. By visual comparison between standard patches having measured fluorescence intensity and test patches, the test patches were assigned appropriate fluorometric values. These observations were taken on alternate days.

f) Application of varied concentration of selected neem leaves extracts:
In another set of volunteer study, ten volunteer of either six, participated and six patches were developed on inner aspect of the forearm by method described earlier.

g) Interaction of neem leaves extracts with dansyl chloride stained patches:
To insure that no interaction occurs between dansyl chloride and investigational extracts, 5 % dansyl chloride dispersion was applied on the ten 1 x 1cm patches of stratum corneum prepared by the method of Kligman for twenty four h these were kept in cool and dark place. After twenty four h intensities these patches were determined by comparing with standards in UV radiation chamber. Each extracts was applied on two patches and stored in refrigerator. The patches were observed at a definite interval for one month to see whether there is any change in fluorescence or not.
Dansyl chloride staining technique was used for determing the effect of neem leaves extracts on skin renewal rate.
The fluorescence intensities of these patches were measured on alternate days by comparison with standard patches in U V radiation chamber till the fluorescence disappeared completely. Time taken for complete disappearance of fluorescence was noted.
The fluorescence intensities were measured at 340 nm by spectrofluorometer. The fluorescence intensities of these standards are tabulated in Table 1, which indicates that fluorescence intensity is directly proportional to concentration of dansyl chloride applied. The decline in fluorescence intensities of the dansyl chloride stained patches due to daily application of various extracts of neem leaves of the two groups of volunteers are depicted in Table 2.

The % increase in renewal rate of skin due to topical application of various extracts was calculated by using following formula 1.

$$\% \text{ increase in renewal} = \frac{\text{Mean No. of renewal} - \text{Mean No. of days for renewal of patch}}{\text{Mean No. of days for removal of control patch}} \times 100 \quad \ldots(1)$$

Discussion and Conclusion

Table 3 gives the % increase in renewal rate of stain due to topical application of various extracts. The hydro alcoholic macerate extracts shows maximum % increase in renewal 27.5 %, followed by hydro alcoholic and alcoholic extracts as 23.75 % and 23.12 % respectively.

Stastical calculation were carried out for finding significance of the result obtained student 't' test was carried out for comparison between the mean number of days for removal of control patches treated with various neem leaves extracts (Table 4).

The student t test shows that increase in skin renewal by alcohol extracts, hydro alcohol macerate are significant at $p < 0.005$ level of significance while alcohol extract is significant at $p<0.05$ level of significance .

TABLE 1: FLUORESCENCE INTENSITIES OF STANDARD DANSYL CHLORIDE STAINED SLIDES

Sr. No	Dansyl chloride (%)	Fluorescence intensity (%)
1	5.0	99
2	4.5	91
3	4.0	72
4	3.5	65
5	3.0	52
6	2.5	41
7	2.0	30
8	1.5	22
9	1.0	17
10	0.5	10

TABLE 2:COMPARATIVE DECLINE IN FLUORESCENCE INTENSITIES WITH APPLICATION OF DIFFERENT EXTRACTS.

Days	Control	Petroleum ether extract	Benzene extract	Chloroform extract	Acetone extract	Propylene glycol	Alcohol extract	Hydroalcohol extract	Hydroalcohol macerate extract
1	98.2	88.8	91.2	90.4	93.9	93.9	91.5	90.4	92.0
3	69.9	63.8	67.3	63.9	62.9	66.5	57.3	59.7	52.4
5	51.5	43.3	47.2	42.3	47.0	46.7	31.5	36.4	29
7	35.0	29.3	27.4	27.4	23.9	30.9	17.7	19.3	12.3
9	23.2	19.9	16.9	17.4	15.0	19.4	9.6	8.4	5.5
12	16.0	14.6	9.2	9.2	9.0	11.6	2.1	1	0.5
14	9.4	4.7	3.0	3.0	1.0	5.5	-	-	-
16	2.0	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-

TABLE 3: PERCENTAGE IN INCREASE IN SKIN RENEWAL RATE DUE TO APPLICATION OF VARIOUS INVESTIGATIONAL NEEM LEAVES EXTRACTS

Neem leaves extracts	% increase in renewal rate
Petroleum ether	7.9
Benzene extract	9.2
Chloroform extract	9.8
Acetone extract	11.6
Alcohol extract	23.12
Hydro alcohol extract	23.75
Hydro alcohol macerate extract	27.5
Propylene glycol	2.78

TABLE 4: STUDENT T TEST FOR COMPARISON OF CONTROL AND INVESTIGATIONAL NEEM LEAVES EXTRACTS

Treatment compared	t value	Level of Significance
Petroleum ether extract Vs. control	1.1383	* Not Significant
Benzene extract Vs. control	1.0210	* Not Significant
Chloroform extract Vs. control	1.5840	* Not Significant
Acetone extract Vs. control	2.0740	* Not Significant
Alcohol extract Vs. control	2.7130	P <0.05
Hydro alcohol extract Vs. control	3.2390	P <0.005
Hydro alcohol (macerate) extract Vs. Control	3.5690	P <0.005
Propylene glycol	0.3410	* Not Significant

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