Chemopreventive action of *Lawsonia inermis* Leaf Extract on DMBA-induced skin papilloma and B16F10 Melanoma Tumour

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Summary

The present investigation was undertaken to explore the antitumour activity of *Lawsonia inermis* leaf extract on DMBA induced 2-stage skin carcinogenesis and B16F10 melanoma tumour model. Topical single application of 7,12-dimethylbenz(a)anthracene (DMBA) at the dose of 4 mg/kg b. wt. followed by 1% croton oil for 16 weeks in *Swiss albino* mice. Topical application of *L. inermis* leaf extract at a dose of 1000 mg/kg body weight was found to be effective in decreasing the number of the papillomas. A significant ($p<0.05$) reduction in tumor incidence 66% was observed in animals in the L. inermis leaf extract treated group compared with 100% tumor incidence in the control group. The cumulative number of papillomas during an observation period of 16 weeks was significantly reduced 10 in the *L. inermis* leaf extract treated group respectively as compared with a 21 cumulative number of papillomas in the control group. The tumor yield was significantly decreased 1.6 respectively as compared with the DMBA treated control group 3.5. Histopathological examination showed well and poorly differentiated squamous cell carcinoma in this group which received the DMBA + Croton oil treatment. When the *L. inermis* extract was applied along with the DMBA and Croton oil malignant tumours were not seen as compared to DMBA + Croton oil group. In the case of this histopathology report suggest in these animals were having papillomatous hyperplasia, papilloma, extracellular keratin and epithelial hyperplasia with mild displasia was reported and the remaining animals showed the normal skin. In a second experiment the effect of cyclophosphamide alone and in combination with *L. inermis* was studied in B16F10 melanoma tumour bearing mice. The Inhibition rate was 25.9% in the CP treated group but these increased to 35.14% with *L. inermis*. The life span time and volume of tumour doubling time were also increased. Thus in two models, *L. inermis* extract exerted protective potential against skin tumour.

Key Words: Skin papilloma; Melanoma; *L. inermis*; DMBA; Chemoprevention.

Introduction

***Lawsonia inermis* Linn (Henna)** is a plant which grows wild in abandoned area (1). This plant is a worldwide known cosmetic agent used to stain hair, skin and nails. However, it is not only relevant to cosmetics. The alcoholic extract of henna leaves reported mild antibacterial activity against *Micrococcus aureus* and *E.Coli* (2). *Lawsonia inermis* was also reported to have tuberculastatic activity (3). The leaves are used as a prophylactic against skin disease. They are used externally in the form of past or decoction against boils, burns, bruises and skin inflammations. A decoction as used as gurgle against sore throat (4). The roots of this plant are useful in burning sensation, loprosy, strangury and premature greying of hairs (5). The major phytochemical constituents of *Lawsonia inermis*, Lawson was found to possess significant anti-inflammatory, analgestic and antipyretic activities (6) Wound healing potential of different extracts of *Lawsonia inermis* leaves was also reported in the rat burn wound models. This compound has been reported to have the inhibitory growth effect against human colon carcinoma, HCT-16 cells (7). Modern pharmacological research on henna and its constituents has confirmed its antiinflammatory, antipyretic and analgesic effects (6). It can also be used to treat pediculosis (8). The active component of henna is lawsone (2-hydroxy-1,4-naphthoquinone, CAS 83-72-7), which is also the principal dye ingredient. Current research suggests that lawsone is non-problematic for external use because of its low toxicity and genotoxicity (9). However there is no study reported on the inhibitory effect of other skin cancer (papilloma) and melanoma tumour. Therefore we have planned to carry out this study to see the anticarcinogenic effects in *Swiss albino* mice.

Materials and Methods

Animals

The study was conducted on random bred, 6-7 weeks old and 24- 28 gm body weight bearing, male Swiss albino mice (Fig. 1). Animals were maintained under controlled conditions of temperature and light (Light: dark, 12 hrs: 12 hrs.). They were provided standard mice feed and water ad libitum. The study protocol is approved by the Departmental Animal Ethical Committee (IAEC Ref. No. 670/225.IACE/2008).

Chemicals

The chemicals, 7, 12-dimethylbenz (a) anthracine (DMBA) and croton oil were procured from Sigma Chemicals Co., St. Louis, USA. DMBA was dissolved at a concentration of 100 µg/100 µl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

Extract Preparation

The identification of the plant *Lawsonia inermis* (family*: Lythraceae*) was done by botanist Dr. S. S. Khan (Voucher Specimen No: WR/102/LGOB/2006), Department of Botany, Safia Science College, Bhopal, Madhya Pradesh (India). The *Lawsonia inermis* (Henna) leaves were collected and dried for few days in shade. . The leaves were washed, air dried, powdered and extracted with double distilled water by refluxing for 36 hrs. at 60˚C. On the day of experimentation, the desired amount of powder was disolved in double distilled water for the final administration.

Skin Bioassay Protocol

Procedure

Experiments were performed as per the method reported by Berenblum (10) A group of 6 mice (Shaved on dorsal skin two days earlier) were by the initiated single application of 104 µg of DMBA in acetone (100 µl), was began 1 week after initation *L. inermis* extract (100 µl) were applied 1 hrs before each croton oil treatment. The extract was applied to the shaved area using the micropipette. The experiment was continued for 16 weeks. Skin tumour formation was recorded weekly and the tumours greater than 1 mm in diameter were included in counting of total number of papillomas/mouse, tumor incidence and tumor yield if they persisted two weeks or more.

Group 1 (Untreated control) No treatment was given.

Group 2 (Vehicle control) 100 µl acetone 2 times /week up to 16 weeks

Group 3(DMBA alone) 104 µg DMBA was dissolved in 100 µl acetone and single application was given.

Group 4 (Croton oil alone) 1 % Croton oil was applied on skin 2 times a week up to 16 weeks.

Group 5 (DMBA + Croton Oil) 104 µg DMBA was dissolved in 100 µl acetone and Single application was given afterwards 1 % Croton oil was applied on skin 2 times a week up to 16 week.

Group 6 (DMBA + *L. inermis* ext. + Croton Oil) 104 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards the 100 µl dose of *L. inermis* ext. was given one hour before the each application of 1 % croton oil, 2 times a week up to 16 weeks.

Group 7 (*L. inermis* extract alone): - the 100 µl was given 3 times a week up to 16 weeks.

Each group's content 6 animals for skin model assay and the animals of all groups were kept under observation for gross and microscopic changes in skin.

Melanoma Skin Bioassay

Animals

The study was conducted on random bred, 6-7 weeks old and 25 ± 2 gm body weight of, C57BL hybrid mice of both sexes. These were maintained under controlled conditions of temperature (25±2˚C) and light (12 light: 12 dark). They were housed in good laboratory condition and were given standard mouse pellet diet and water *ad Libitum.* 4-6 animals were housed in polypropylene plastic cages.

Procedure

Melanoma cell line was obtained from National Cell science centre, Pune and maintained in our laboratory. The C57BL mice of both sexes of the mean weight of 25 gm and 6-7 weeks old were obtained from the animal colony of our institute. They were housed in good laboratory condition and have given standard mouse pellet diet and water *ad Libitum.* All the mice were kept at controlled light and temperature condition. Cell suspensions having about 5 Lacks cell were injected to every mouse by S. C. route.

After implantation of the melanoma cell line, animal kept in observation and experiment was started after 10 days when the tumours were seen and the treatment was given for 30 days and tumour volume and survival time was recorded. The following groups were maintained.

Group 1 (Positive Control Group)
This group consisted of four animals bearing the melanoma cell line (B16F10). The dose of 300 mg/ Kg body weight of Cyclophosphamide dissolved in double distilled water was given by intraperetonally route. During the treatment, the size of the implanted tumors was measured by Vernier calipers.

Group 2 (No Treatment Group)
This group consisted of four mice having the melanoma cell line (B16F10).

Group 3 (*L. inermis* extract alone Group)
This group consisted of four animals bearing the melanoma cell line (B16F10). The mouse was given aqueous *L. inermis* extract orally at the dose of 1000 mg/kg body weight during the treatment; the size of the implanted tumors was measured by Vernier calipers.

Group 4 (*L. inermis* extract + Cyclophosphamide Group)
This group consisted of four animals and the melanoma cell line (B16F10). The mice was given aqueous extract of *L. inermis* extract orally at the dose of 1000 mg/ Kg body weight and after 30 min. 0.2 ml cyclophosphamide was inject intraperetonally during the treatment, the size of the implanted tumors was measured by Vernier calipers.

Results

The results of the present investigation have been summarized in Tables 1 and 2. Topical single application of DMBA at the dose of 4 mg/kg b. wt. followed by 1 % croton oil produced skin papilomas the incidence of tumors reached 100% animals and the value of cumulative number of papillomas in these animals were recorded 21 and the average number of papillomas per mouse (tumor yield) was found to be 3.5.
The mice which received *L. inermis* extract showed a significant decrease in the number and incidence of tumor as compared with that of the DMBA + croton oil group. When *L. inermis* extract was topically applied at the dose of 0.6 g/kg b. wt. for 16 weeks to the skin of animals the tumor incidence was found to be 66% (Grp.6), and the value of cumulative number of papillomas in these animals were recorded 10 and the average number of papillomas per mouse (tumor yield) in this group was found to be 1.6 respectively, The differences in the values of the results of experimental groups were statistically analyzed and found to be significant in comparison to the control group (Grp. 5) ($p< 0.05$). Vehicle Control, No treatment, *L. inermis* extract alone, Croton oil alone and DMBA alone groups did not show any tumor incidence.

Histopathology of skin tumour
The animals which received the treatment of DMBA + Croton oil for 16 weeks showed the infiltration nests of neoplastic squamous epithelium were observed. Tumour cells exhibited a high nuclear cytoplasmic ratio. Moderate cytoplasm and dense clumped chromatin were also seen. Adjacent epithelium showed marked hyperkeratosis.

This is suggestive of kerating squamous cell carcinoma grade II. When the *L. inermis* extract was applied along with the DMBA and Croton oil malignant tumours were not seen as compared to DMBA + Croton oil group. In the case of this group only four mice having tumours and the histopathology report suggest in these animals were having papillomatous hyperplasia, papilloma, extracellular keratin and epithelial hyperplasia with mild displasia was reported and the remaining animals showed the normal skin.

The present studies with the melanoma tumour model shows the effect of cyclophosphamide alone*, L. inermis* alone and cyclophosphamide with *L. inermis* extract on B16F10 melanoma tumour bearing mice.

The preventive effect of *L. inermis* extract was calculated using the parameter of inhibition rate (IR), Increase the life span (ILS), and Volume of tumour doubling time (VDT).The inhibition rate was 10.56% and 25.73% in *L. inermis* extract and cyclophosphamide (CP) treated group as compared to 35.14% in *L. inermis* + cyclophosphamide group. The life span was also increase in *L. inermis* extract + cyclophosphamide group (50.53%) as compared to cyclophosphamide alone (28.23%) and 30.89% in *L. inermis* extract alone group. The volume of tumour doubling time of CP alone group was 5.50 ± 2.08 (days), *L. inermis* extract alone group 2.10 ± 0.05 (days) and *L. inermis* extract + CP group volume of tumour doubling time was recorded 8.50 ± 0.57 (days) respectively. The differences in the values of the results of experimental groups were statistically analyzed and found to be significant as comparison to the control group ($p< 0.05$).

Table 1 Effect of *L. inermis* (henna) leaf extract on DMBA induced skin papillomas in *Swiss albino* mice.

S.N.	Groups	Body weight (Mean ± SE) Initial	Final	Cumulative Number of Papillomas	Tumour Yield	Tumour Incidence
1.	No Treatment	25.8±1.2	28.9±1.1	00	00	0/6
2.	Vehicle alone	27.7±1.6	29.3±1.9	00	00	0/6
3.	DMBA alone (1 application)	28.0±1.3	30.0±1.3	00	00	0/6
4.	Croton oil alone	26.7±2.2	29.8±1.8	00	00	0/6
5.	DMBA+ Croton oil	27.0±1.3	29.9±1.4	21	3.5	6/6 (100%)
6.	DMBA+ *L. inermis* +Croton oil	27.7±2.1	29.5±1.6	10	1.6*	4/6 (66%)
7.	*L. inermis* alone	28.1±1.6	30.1±1.4	00	00	0/6

* Level significance as compared to carcinogen control group at $p<0.05$.
Carcinogen control v/s *L. inermis* experimental

Table 2 Effect of *L. inermis* (henna) extract on B16F10 Melanoma tumors

S.N.	Group	Tumour Volume	VDT (days)	I R %	ILS%
1.	Control (Untreated)	2.550 ± 0.49	3.5 ±1.19	-	23.50
2.	Cyclophosphamide alone (Positive control)	0.710 ± 0.045*	5.50 ±2.08*	25.73	28.23
3.	*L. inermis* ext. + CP	0.620 ± 0.273*	8.50 ±0.57*	35.14	50.53
4.	*L. inermis* ext. alone	0.855 ± 0.078*	2.10 ±0.05*	10.56	30.89

* denotes statistically significant as compared to untreated group at $p<0.05$.
VDT - Volume doubling time, IR - Inhibition rate, ILS - Increase in life span.

Discussion

The present study demonstrates that when *L. inermis* extract was given one hour before the each application of croton oil, the incidence and the number of skin papillomas was significantly decreased. The appearance time of papillomas was also prolonged in the *L. inermis* experimental group in comparison to the carcinogen treated group. The reduction in tumour count may be due to effect in the promotional phase of tumourgenesis which prevent the reduction of free radicals (11). This compound has been reported to have the inhibitory growth effect against human colon carcinoma, HCT-16 cells (7). Active constituent of *L. inermis*, lawsone is non-problematic for external use because of its low toxicity and genotoxicity (9). Topical application of TPA (active constituent of croton oil) has been reported to increase production of free radicals (11). This is perhaps due to the free radical oxidative stress that has been implicated in the pathogenesis of a wide variety of clinical disorders (12). Many antioxidants and anticarcinogenic compounds appear to have major effect on the detoxification of the carcinogens by the induction of Phase II detoxification enzymes since these enzymes divert carcinogens to react with critical cellular macromolecules (13).The exact mechanism of anticarcinogenecity of *L. inermis* extract is not fully understood but it may be possible the free radicals scavenging activity. The anticarcinogenic effect of *L. inermis* (henna) leaf extract suggest its role in chemoprevention of skin cancer. These results are important because this plant is a worldwide known cosmetic agent used to stain hair, skin and nails. It may an important drug for chemotherapeutic treatment of cancer.

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