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ORIGINAL ARTICLE

Antitumoral Effect of *Lawsonia inermis* Extract on Melanoma Tumor-bearing C57BL/6 Mice

Wasim Raja[1,2], Sonam Pandey[1,3]

[1]Department of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, Madhya Pradesh, [2]Department of Central Laboratory Facility, Central Laboratory Facility, Chhattisgarh Council of Science and Technology, Raipur, Chhattisgarh, [3]Gujarat State Biotechnology Mission, Gandhinagar, Gujarat, India

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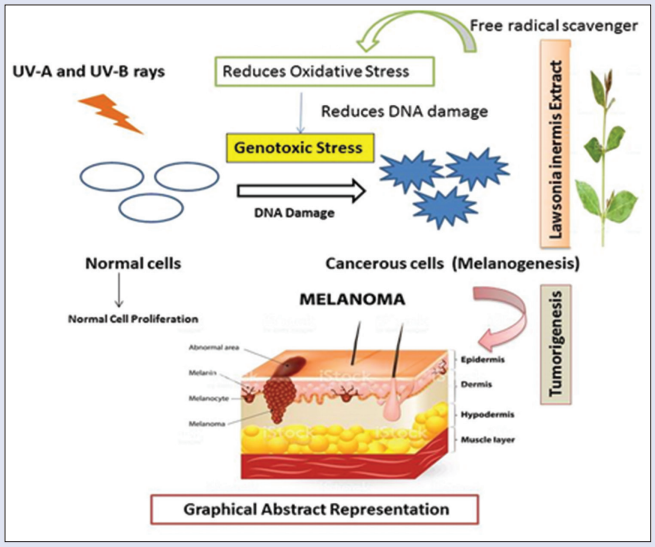
ABSTRACT

Background: Medicinal plants occupy an important position for being the paramount sources of drug discovery against cancer. Medicinal plants have been used as cancer chemopreventive nutraceuticals, and functional food is promising. Thus, there is a need for exploring drugs/agents which act as best chemopreventive agents. *Lawsonia inermis* L. is a well-known medicinal plant which has been used in Ayurvedic medicine as hepatoprotective, antiviral, antibacterial, anti-inflammatory, antipyretic, and cytotoxic. **Objectives:** The main objective of the study is to investigate the *in vivo* antitumoral effect of *L. inermis* extract on melanoma tumor-bearing C57BL/6 mice. **Materials and Methods:** In *in vivo* experiments, about 5 × 10^5 B16F10 cells in 50 µL of phosphate-buffered saline were subcutaneously injected into C57BL/6 mice that had received plain drinking water for 40 days. Tumor volume was measured using digital calipers. The behavior of the antioxidant defenses (superoxide dismutase, catalase, and glutathione peroxidase) was evaluated after the termination of treatments. **Results:** Preventive oral administration of *L. inermis* leave extracts (500 and 1000 mg/kg body weight) significantly suppressed the growth of B16F10 tumors in mice on day 40th, with an increased tumor necrosis area, increased infiltration of mononuclear cells at the site of the tumor. *L. inermis* L. leaf extract dose dependently reduced the amounts of free oxygen radicals (hydroxyl and superoxide anion radicals), generated in chemical systems. **Conclusion:** Our study suggests that *in vivo* antitumoral effect of *L. inermis* extracts is directly linked with enhanced antioxidant activity and depicted a significant reduction in tumor size against melanoma tumor. Hence, further elucidation of active components of *L. inermis* extract could lead to the development of potent antitumor agent or complementary and alternative medicine for the treatment against skin melanoma tumors.

Key words: B16F10 cell line, chemoprevention, *Lawsonia inermis*, mehndi/henna, melanomas

SUMMARY

- *Lawsonia inermis* extract combination among all the treatment studied and is found capable of reducing melanoma against reference drug cyclophosphamide (CP)
- Life span time of mice has also increased in each treatment groups. The life span was also increased in *L. inermis* extract + CP Group (LI_1 and LI_2) as compared to CP alone and *L. inermis* extract alone group (LI_3)
- Based on our observation, *L. inermis* along with CP (both doses) exhibited optimum antioxidant activity and rendered significant protection against oxidative stress induced by melanoma in liver tissues
- *In vivo* experiments highlight the effectiveness of *L. inermis* extract combination among all the treatment studied and is found capable of reducing melanoma against reference drug CP.



Abbreviations used: GSH: Glutathione; LI_0: *Lawsonia inermis* leaf extract alone without tumor-bearing mice (500 mg/kg); LI_1: *Lawsonia inermis* leaf extract with CP (500 mg/kg); LI_2: *Lawsonia inermis* leaf extract with CP (1000 mg/kg); LI_3: *Lawsonia inermis* leaf extract alone (500 mg/kg tumor-bearing); PBS: Phosphate buffered saline; CP: Cyclophosphamide; DTNB: 5,5-dithiobis (2-nitrobenzoic acid); H_2O_2: Hydrogen peroxide; NBT: Nitro blue tetrazolium; TBA: Thiobarbituric acid; TCA: Trichloroacetic acid; SOD: Superoxide dismutase; EDTA: Ethylenediaminetetraacetic acid; SC: Subcutaneous; I.P: Intraperitoneal; VDT: Volume doubling time; IR: Inhibition rate; ILS: Increase in life span; MST: Mean survival time; LTC: Live tumor cells; NC: Necrotic cells; TC: Tumor control; b.wt.: Body weight; H and E: Hematoxylin and eosin; B16-F10: B16 melanoma F10 subline.

Correspondence:

Dr. Sonam Pandey,
Gujarat State Biotechnology Mission, Gandhinagar, Gujarat, India. E-mail: sonam_research@ymail.com

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INTRODUCTION

Malignant melanoma is one of the most aggressive skin cancers with high metastatic potential and extraordinary resistance to anticancer agents.[1] Currently, 2–3 million nonmelanoma and 132,000 melanoma skin cancers occur globally each year.[2] If detected early and surgically excised, the 5-year survival rate is satisfactory; however, late-stage cancer is difficult to treat and long-term survival is low. Despite partial success

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gained by the use of platinum analogs, nitrosoureas, taxanes, vinca alkaloids, and cytokines,[3] at present, there is no effective chemotherapy against malignant melanoma. Among the drugs exerting anticancer activity against malignant melanoma, dacarbazine has been extensively used, though it could achieve only a response rate of 11%–25% and short survival time.[4] Therefore, it is necessary to develop anticancer agents with potent activity and weak side effects against melanoma. Out of the available anticancer drugs in the market, two-third is either of natural origin or a simple modification of the natural product.[5] *Lawsonia inermis* L. (*Lythraceae*), a monotypic genus, popularly known as "Mehndi" or "Henna" is renowned as a cosmetic as well as a medicinal agent in the oriental parts of the world since time immemorial. The plant extracts and a purified constituent of henna in folklore account for a variety of activities including antibacterial,[6] antifungal,[7] antioxidant and immunomodulatory,[8] hepatoprotective,[9] analgesic, anti-inflammatory and antipyretic,[10] and cytotoxic.[11] The bactericidal and fungicidal action of this plant has been attributed through its tanning effect,[12] and it has been confirmed that henna is neither an allergen nor a carcinogen.[13] The key coloring agent present in henna leaves is a red-orange pigment lawsone (2-hydroxy-1, 4-naphthoquinone),[14] which makes this plant useful for dying of hair, as well as to color palms, fingers, fingernails, and soles.[15,16] Lawsone is also a suitable reagent for the detection of latent fingermarks on paper, as contact evidence in criminology.[17] Lawsone (2-hydroxy-1, 4-naphthoquinone) is a starting material in the synthesis of many clinically useful anticancer compounds such as atovaquone, lapachol, and dichloroallyl lawsone.[18]

L. inermis is reported different types of photo component such as lawsone, esculetin, fraxetin, isoplumbagin, scopoletin, betulin, betulinic acid, hennadiol, lupeol, lacoumarin, laxanthone, flavone glycosides, and two pentacytic triterpenes.[19]

It is noticed by some researcher that lawsone itself has significant antioxidant and anti-inflammatory activity that could be a major characteristic of numerous anticancer phytochemicals. Ported for its pharmacological properties, but little attention has been paid to explore its anticancer potential. The present study evaluates the chemopreventive effect of different doses of *L. inermis* on B16F10 melanoma tumor on C57BL mice.

MATERIALS AND METHODS

Identification of pant

The identification of the plant *L. 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 *L. inermis* (Henna) leaves were collected and dried for a few days in the shade.

Preparation of *Lawsonia inermis* extract

Dried powdered plant material such as leaves (10 g) was extracted by continuous mixing in 100 ml 50% methanol, 24 h at room temperature. After filtration, methanol was evaporated until only water remained through evaporation on the water bath at 60°C–70°C temperature. The dried powder of Plant extract was kept in the air tied box.

Chemicals

The initiator cyclophosphamide (CP) and other chemicals were procured from Sigma Chemical Co (St Louis, MO).

Animals

The study was conducted on random bred, 6–7 weeks old, and 24–28 g body weight (b.wt.)-bearing, male C57BL mice. Animals were maintained under controlled conditions of temperature and light (light:dark, 10 h:14 h.). They were provided standard mice feed (procured from Hindustan Levers Ltd. and water *ad libitum*). The study protocol is approved by the Departmental Animal Ethical Committee.

Experimental design

Cell culture

B16F10 melanoma tumor cell line was purchased from the National Centre for Cell Science (NCCS, Pune, India). The cells were maintained in RPMI 1640 medium buffered with 2 g/l of hydroxyethyl piperazineethanesulfonic acid (HEPES) and sodium bicarbonate and supplemented with dextrose, penicillin, streptomycin, and 10% of fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. When needed for experiments the cells were harvested with trypsin; ethylenediaminetetraacetic acid (EDTA) (0.05:0.03 [w/v]) solution and then washed in phosphate-buffered saline (phosphate-buffered saline [PBS], pH 7.4). For the animal experiments, the recovered cells were adjusted to 5×10^5 cells/ml in PBS and then 200 µl of the suspension was injected subcutaneously (SC) into the dorsal side of C57BL/6 mice. After 8–10 days of injection, the tumor was found to develop into a budding state. When the tumor was developed to a palpable level, two doses of the plant extracts (leaf, stem bark, and floral bud) at 500 and 750 mg/kg b.wt. of mice were given orally and CP at 170 mg/kg was injected intraperitoneally (I.P.) every alternate day up to 40 days. During the treatment, the size of the implanted tumor was regularly measured at a given time interval, with a digital caliper and tumor volume was calculated.[20]

Treatment schedule

A total of 42 adult male C57BL 24–28 g mice aged 6–7 weeks were divided into seven groups, thus each group containing six animals.

The groups under treatment were designated as follows.

- Group 1 – Negative control (NC)- served as normal (saline)
- Group 2 – LI_0: *L. inermis* leaf extract alone (500 mg/kg b.wt.)
- Group 3 – Tumor control (TC)-served as tumor control (5 × 105 cells/ml) (tumor-bearing mice) receiving normal saline (10 mL/kg) treated as TC
- Group 4 – CP-served as standard, which received CP 170 mg/kg b.wt. (tumor-bearing mice)
- Group 5 and 6 – *L. inermis* leaf extract with CP (500 mg/kg) (LI_1) and *L. inermis* leaf extract with CP (1000 mg/kg) (LI_2) (tumor-bearing): Animals were received 500 and 1000 mg/kg b. wt. *L. inermis* leaf extracts, respectively, and after 30 min of *L. inermis* treatment, animals were treated with CP (170 mg/kg b.wt)
- Group 7 – *L. inermis* leaf extract alone (500 mg/kg tumor-bearing) (LI_3) (tumor-bearing): Animals were received orally with 500 mg/kg body weight of *L. inermis* leaf extract (tumor-bearing mice).

All treatment of *L. inermis* extract was administered orally through a metal oropharyngeal cannula and CP and normal saline was given I.P. by 1 ml syringe.

Whereas, NC: Negative control; TC: Tumor control; CP: Cyclophosphamide; LI_0, LI_1, LI_2, LI_3: *L. inermis* leaf extract; i. p.: Intraperitoneally.

The tumor response was assessed by the tumor growth kinetics:

Tumor regression studies

Tumor volume

During the treatment, the size of the implanted tumors was measured using Vernier calipers.[21,22] Tumor volume was calculated by the following formula:

Tumor volume = length × width^2/2

Volume doubling time

Time required for the tumor to attain double the treatment volume.

Inhibition rate

Tumor growth inhibition (%TGI) was determined twice weekly during the dosing period by the formula:[21,22]

%TGI = (1-{Tt/T0 / Ct/C0} / 1-{C0/Ct}) X 100

where Tt = median tumor volume of treated at time t, T0 = median tumor volume of treated at time 0, Ct = median tumor volume of control at time t, and C0 = median tumor volume of control at time 0.

TGI >50% is considered meaningful.

Calculation formula for Life Span Time

Mean survival time and median survival time (MST) were also calculated. The tumor response was assessed on the basis of the percentage increase in life span (% increase in life span [ILS]).[23,24]

$$\% \text{ ILS} = \frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \times 100$$

where MST = Mean survival time, ILS = Increase life span

Enhancement of life by 25% or more over that of control was considered an effective antitumor response.[24]

An antioxidant parameter was studied in all the groups at the time of termination of the experiment (i.e., after 41 days).

Evaluating enzymatic antioxidant activity

Enzymatic antioxidant activity was evaluated by sacrificing all the experimental mice were on day 41st by cervical dislocation and the liver was removed.

Preparation of liver homogenate

Ten percent liver cell homogenate was prepared by the homogenizing liver with buffer containing 0.25 M sucrose and 0.1M Tris-HCl buffer (pH 7.4), in Teflon pestle and glass homogenizer and centrifuged at 1000 rpm for 10 min. The decanted supernatant was separated and used to measure thiobarbituric acid reactive substance (TBARS). The supernatant was again centrifuged at 8000 rpm for 15 min and the level of antioxidant enzymes (superoxide dismutase [SOD], catalase [CAT], and glutathione-peroxidase [GSH]-px) was analyzed.[25,26]

Estimation of lipid peroxidation

Lipid peroxidation in the liver cell homogenate was estimated by treating 0.2 mL of cell homogenate with 1.5 mL of 20% acetic acid, 0.2 mL of 8.1% sodium dodecyl sulfate, and 1.5 mL of 0.8% thiobarbituric acid, used to estimate the concentration of malondialdehyde (MDA). The volume of the mixture was made up to 4.0 mL with distilled water and then heated at 95°C in a water bath for 60 min. After incubation, the tube was cooled at room temperature and the final volume was made up to 5.0 mL in each tube with distilled water. 5.0 mL n-butanol-pyridine (15:1) mixture was added and the content was vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min, the organic upper layer was decanted off and its optical density was measured at 532 nm against blank without liver cell homogenate. The level of lipid peroxidation was expressed as in moles of MDA/mg protein in liver cell homogenate.[27]

Assay of glutathione peroxidase

To 0.1 mL of the liver cell homogenate, 0.4 mL of buffer, 0.2 mL sodium azide, 0.2 mL EDTA, 0.2 mL of hydrogen peroxide (H_2O_2), and 0.2 mL of reduced GSH were added and volume was made up to 2 mL with distilled water. The tubes were incubated at 37°C for 10 min and 1 mL of trichloroacetic acid (TCA) was added to terminate the reaction. The reaction mixture was centrifuged at 3000 rpm for 10 min. To 1 mL of supernatant, 2 mL of disodium hydrogen phosphate and 1 mL of TCA was added and 1 mL of 5,5-dithiobis (2-nitrobenzoic acid) was added just prior to the estimation. The absorbance was read at 412 nm against a blank without liver homogenate. The concentration was expressed as units/mg of protein.[28,29]

Assay of catalase

The test tube containing reaction mixture of 0.1 mL of liver homogenate, 1 mL of phosphate buffer (0.1 M) and 0.4 mL of H_2O_2 was incubated at 37°C for 10 min. The reaction was stopped by the addition of 2 mL of 5% of dichromate-acetic acids reagent (5% potassium dichromate and glacial acetic acids in 1:3 ratio). The blank was treated out without liver cell homogenate. The absorbance was read at 620 nm. The CAT activity was expressed as μM H_2O_2 consumed/min/mg of protein. The enzyme activity was expressed as units/mg protein.[30,31]

Assay of superoxide dismutase

The assay based on the reduction of nitro blue tetrazolium (NBT) to water-insoluble blue formazan. To 0.5 mL of liver cell homogenate, 1 mL of 50 mM sodium carbonate, 0.4 mL of 24 μM NBT, and 0.2 mL of 0.8 mM EDTA were added. The reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. The control was simultaneously prepared without liver cell homogenate. The absorbance of reacting mixture was measured at 560 nm. Units of SOD activity were expressed as the amount of enzymes required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units/mg of protein.[32,33]

Histopathological study

After the completion of drug treatment (40 days), on day 41, mice were sacrificed by cervical dislocation. The tumor of three animals from each group was dissected out, fixed in 10% buffered formalin for 12 h, and processed for histopathological examination. Four micro meter-thick paraffin sections were cut and stained with hematoxylin and eosin and mounted in DPX (used as a synthetic resin-mounting media). Sections were qualitatively assessed under the light microscope for their architecture.[34]

Statistical analysis

Results of Statistical analysis are presented as mean ± standard deviation. Statistical evaluation of data was performed by using one-way analysis of variance followed by Dunnett's multiple comparison test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Our studies with the melanoma tumor model show the effect of CP alone, *L. inermis* alone and CP with *L. inermis* extract on B16F10 melanoma tumor-bearing mice. The preventive effect of *L. inermis* extract was calculated using the parameter of inhibition rate (IR), ILS, and volume of tumor doubling time volume doubling time (VDT).

Effect of *Lawsonia inermis* extract on melanoma tumor

The study of tumor volume of mice shown in Figure 2a, revealed that in the groups (LI_1 and LI_2) which received extract of *L. inermis* at 500 and 1000 mg/kg, b.wt., a reduction in the tumor volume was observed. On the day 40 of treatment, the reduction in the tumor volume was found to be statistically significant ($P < 0.05$) for both the doses compared to

the TC group, which received normal saline, 10 mL/kg, b.wt. as well as *L. inermis* alone group (LI_3).

The response of volume doubling time (VDT) and IR of LI_1 and LI_2 were also significantly higher with ($P < 0.05$) as compared to the TC group and test group (LI_3) [Figure 2b and c].

Percentage increase in the life span of mice in each treatment groups are provided in Figure 2d. The life span was also increased in *L. inermis* extract + CP group (LI_1 and LI_2) as compared to CP alone and *L. inermis* extract alone group (LI_3). The differences in the values of the results of experimental groups were statistically analyzed and found to be statistically significant as a comparison to the tumor control group and test groups ($P < 0.05$). The results are summarized in Figure 2.

Effect of *Lawsonia inermis* on enzymatic antioxidant

The percentage change on the level of lipid peroxidase, GSH-Px, SOD, and CAT per mg protein as a function of *L. inermis* alone or in combination against CP at 170 mg/kg on 40 days has been presented in Figure 3a-d.

Although the level of GSH-Px, SOD, and CAT are higher in case of individual *L. inermis*, interestingly, their combination at 500 and 1000 mg/kg (LI_1 and LI_2) concentration exhibited a further increase compared to CP-alone group. SC induction of $B_{16}F_{10}$ melanoma showed a significant lowering of GSH-Px, SOD and CAT in the liver (characteristic of antioxidants) compared to normal (NC) as well as treated groups and reduced the scavenging of reactive oxygen species. An increased concentration of MDA (per mg wet weight has also been observed compared to normal control (NC) and test groups (LI1 and LI2). Among the groups studied, the optimum value of MDA per mg wet weight is found to be in the order: CP>TC>LI_1>LI_2>LI_3>LI_0>NC. Based on our observation, *L. inermis* along with CP (both doses) exhibited optimum antioxidant activity and rendered significant protection against oxidative stress induced by melanoma in liver tissues.

Histopathological analysis

We have evaluated the effect of *L. inermis* extract on B16F10 mice melanoma tumors implanted SC in C57BL mice. Histology of the TC group revealed the presences of hypervascularization where the perimeter of blood vessels was large with the presence of hyperchromic tumor cells (vival cells) [Figure 4a-d].

The TC and CP groups showed SC tissue showing invasion by tumor cells with the presence of nuclear pyknosis (a degenerative state of the cell nucleus). The nucleus-to-cytoplasm ratio very disproportionate. Presence of necrosis, bleeding, and lymphocytes with extensive areas of melanin pigments [Figure 4a and b]. The group treated with *L. inermis* (LI_1, LI_2 and LI_3) showed the less number of enucleated tumor cells with apoptosis [Figure 4c and d].

DISCUSSION

Melanoma is a highly aggressive, therapy-resistant skin cancer, representing an increasing health problem due to its complex etiology, heterogeneity, and modest results obtained with the current treatment options. Development of targeted bioactive phytochemicals is targeted on the drug development process for melanoma tumor regression and enhancement in patient's survival. Therefore, the constant search for alternative drugs that are effective, specific, and non-toxic in the treatment of melanoma is required and encouraged.

Discovery of oncogene and apoptotic pathway represents a breakthrough for understanding the molecular and genetic basis of cancer and are considered as one of the most valuable targets for anticancer drug discovery program, endeavoring the selectivity of new drugs toward

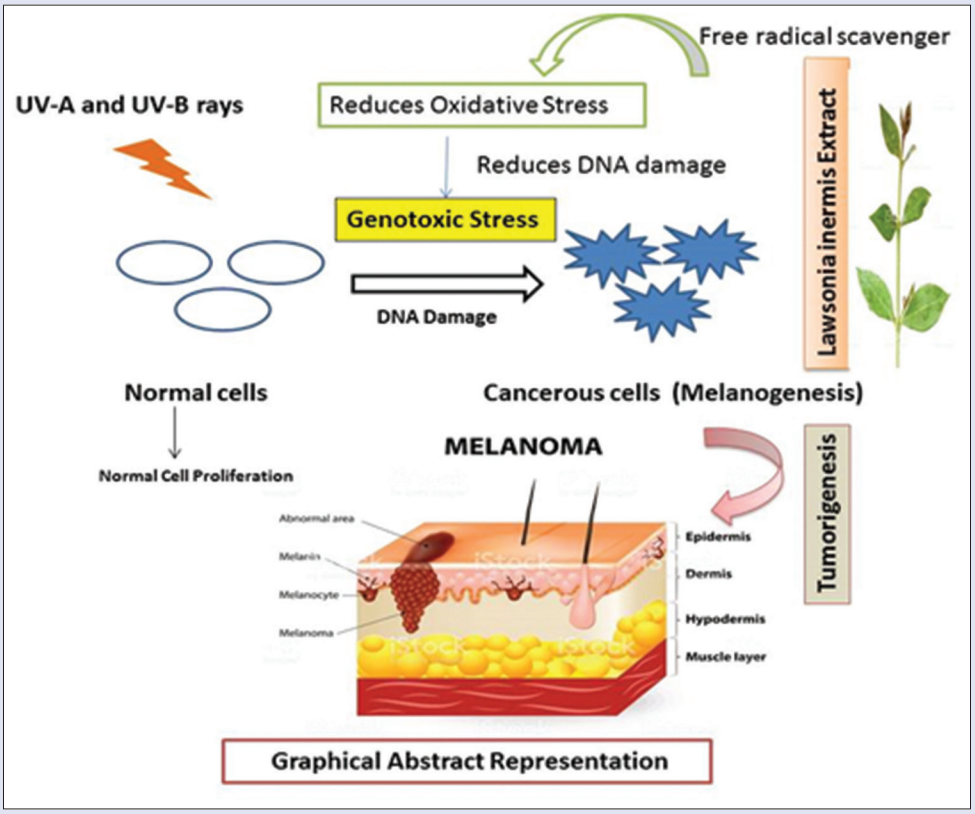

Figure 1: Showing the Graphical abstract representation of *Lawsonia inermis* leaf extract on Melanoma tumor with oxidative stress

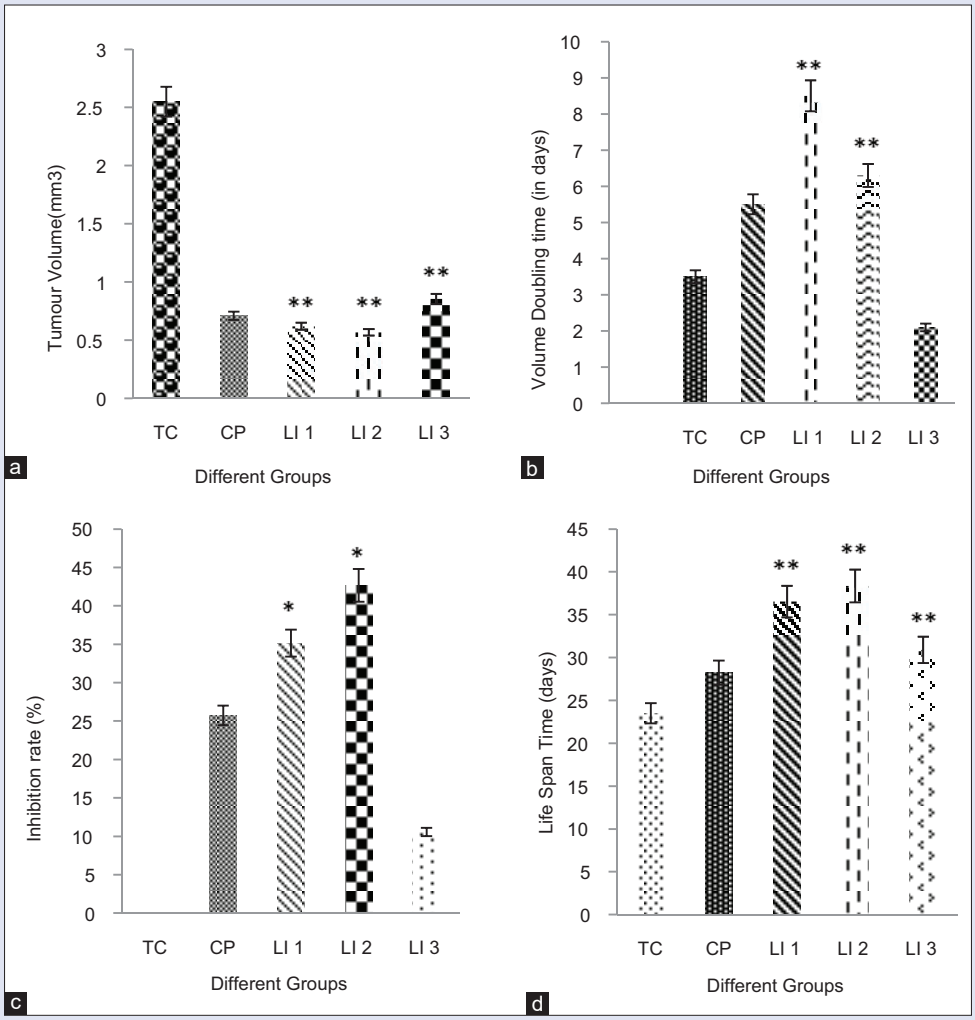

Figure 2: (a, b, c, d) Effect of *Lawsonia inermis* (Henna) extract, individually or in combination with cyclophosphamide, on the growth response of B16F10 melanoma tumor. (a) Changes in Tumour Volume of B16F10 tumour-bearing mice. The Tumour Volume was expressed in mili meter (mm3); (b) change in volume doubling time on the growth of induced melanoma tumour. The volume doubling time was expressed in days; (c) Inhibition Rate increase as per dose dependent manner; (d) Effect of *Lawsonia inermis* alone and single (low and high) dose of *Lawsonia inermis* (Henna) extract along with CP as combination therapy, on the response of B16F10 Melanoma tumour bearing mice and mean survival time of C57BL/6 mice. The data represented Means ± S.E.M. (*n*= 6 mice) from triplicate experiments. **Significantly different from Tumour control group (TC) ($P < 0.05$) *Significantly different from Cyclophosphamide group (CP) ($P < 0.05$)

cancer cell, and sparing the normal ones.[35] A study conducted on human liver cancer cell line (HepG2) demonstrated induction of the apoptotic phenomena by essential oil and leaves extract of Mehndi/Henna at a concentration of 20 and 30 mg/mL. The induction was evidenced by the number of apoptotic bodies, DNA fragmentation, and chromatin condensation in the treated groups.[36] An essential oil from the leaves of Mehndi/Henna also exhibited strong cytotoxicity on HepG2 with an 50% inhibitory concentration (IC_{50}) value of 24 µg/mL in 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromideassay. Likewise, chloroform extract of Mehndi leaves showed cytotoxicity on HepG2 and MCF-7 (hormone-dependent breast cancer cell line) with an IC_{50} value of 0.3 and 24.8 µg/mL, respectively. The effect of Mehndi/Henna extract on the expression of c-myc gene was also studied, and it was observed that the gene was not expressed in a cell (HepG2 and MCF-7) treated with 20 and 30 µg/mL of the crude extract.[37] The expression of the c-myc is indicative of early response during cell proliferation, and it has been found to be frequently overexpressed in a variety of tissues and cultured cancer cell lines.[38] From the study, it was concluded that cytotoxicity was mediated by the downregulation of c-myc expression, and it was also observed that the extracts did not exhibit any activity on normal, Caco-2 (colon cancer), and MDA-MB-231 (breast cancer) cell lines.[37]

The effect of *L. inermis* extract was calculated on the basis of the volume of the tumor, volume doubling time (VDT), IR, and survival of the animals. Hence, it seems that IR, the volume of tumor doubling time, and life span time of the animals were increased when CP known anticancer drug was used with *L. inermis* extract. The result suggests the anticarcinogenic potential of *L. inermis* extracts in the melanoma model too.

Melanoma is a disease that could spread to the lung, lymph nodes, or other organs.[39] In addition, the poor efficacy against melanoma could be attributed to the high metastatic potential of melanoma cells.[40]

Studies have been reported that several naturally-occurring compound exhibited antitumor promoting activity in $B_{16}F_{10}$ melanoma tumor model. *Solanum lycopersicum* fruit extracts have reported inhibiting the $B_{16}F_{10}$ melanoma tumor in C57BL mice[41] and *L. inermis* leaf extract has

been also reported anticarcinogenic property against papilloma tumor model.[42] The *Withania somnifera* and its bioactive fraction withanolide D were studied for their antimetastatic activity using B16F10 melanoma cells in C57BL/6 mice. Keishi-ka-kei-to is a traditional Chinese herbal medicine which is reported to inhibit pulmonary metastasis in mice-bearing B16F10 melanoma cells through the stimulation of CD8+ T cells.[43]

The mechanism of the anticarcinogenic activity of *L. inermis* has not yet been fully elucidated. *L. inermis* are reported to reduce activation of carcinogens and increase their detoxification finally exhibiting anticarcinogenic activity.[42]

The mechanism of the enhancement in the anticancer activity of *L. inermis* is not easy to predict owing to their complex pharmacological actions. However, the intrinsic properties of *L. inermis* in synergism play an important role toward enhancement in target bioefficacy. Based on the experimental findings and relevant available literature, a hypothesis is synthesized to explain observed synergistic action of *L. inermis* [Figure 1].

The liver of Ehrlich ascites carcinoma tumor-bearing mice, during the early phase after tumor implantation, has been found to be subjected to oxidative stress with increased steady-state levels of peroxyl radicals and reduced activities of antioxidant enzymes SOD and GSH peroxidase.[44] The same observation has been made in the present study as indicated by a significant reduction in SOD, CAT, and GSH levels of livers of melanoma tumor-bearing mice. The first-line defense component of the antioxidant system includes SOD and CAT. SOD mainly acts by scavenging superoxide, active oxygen radical, produced in different aerobic metabolism. CAT is a tetrameric enzyme present in most of the cells and acts by catalyzing the decomposition of H_2O_2 to water and oxygen. The second line of the defense comprises GSH, Vitamin C, Vitamin E, carotenoids, and flavonoid.[45] *L. inermis* administration inhibited the lowering of antioxidant levels in livers of melanoma-bearing mice demonstrating the potential of *L. inermis* extract in attenuation of oxidative stress through quenching of free radicals, thus maintaining normal levels of endogenous antioxidant markers.

The level of lipid peroxidation has been reported to increase during oxidative stress as well as in the malignant state.[46] The estimation of peroxidation of lipids is carried out by determining the levels of TBARS (MDA) in peroxidized lipid systems because of its simplicity and sensitivity.[47] The comparative lower levels of MDA in the *L. inermis* extract treated groups confirm the antioxidant activity of the extract.

The present piece of *in vivo* experiments highlights the effectiveness of *L. inermis* extract combination among all the treatment studied and is found capable of reducing melanoma against reference drug CP.

CONCLUSION

While cell culture models and *in vitro* studies are valuable and essential for testing potential therapies and understanding the biological processes, molecular physiology and pathology, as well as effects of gene alterations, it is impossible to fully recapitulate the complexity of the whole organism and the microenvironment, in which tumors develop. Thus, there is a

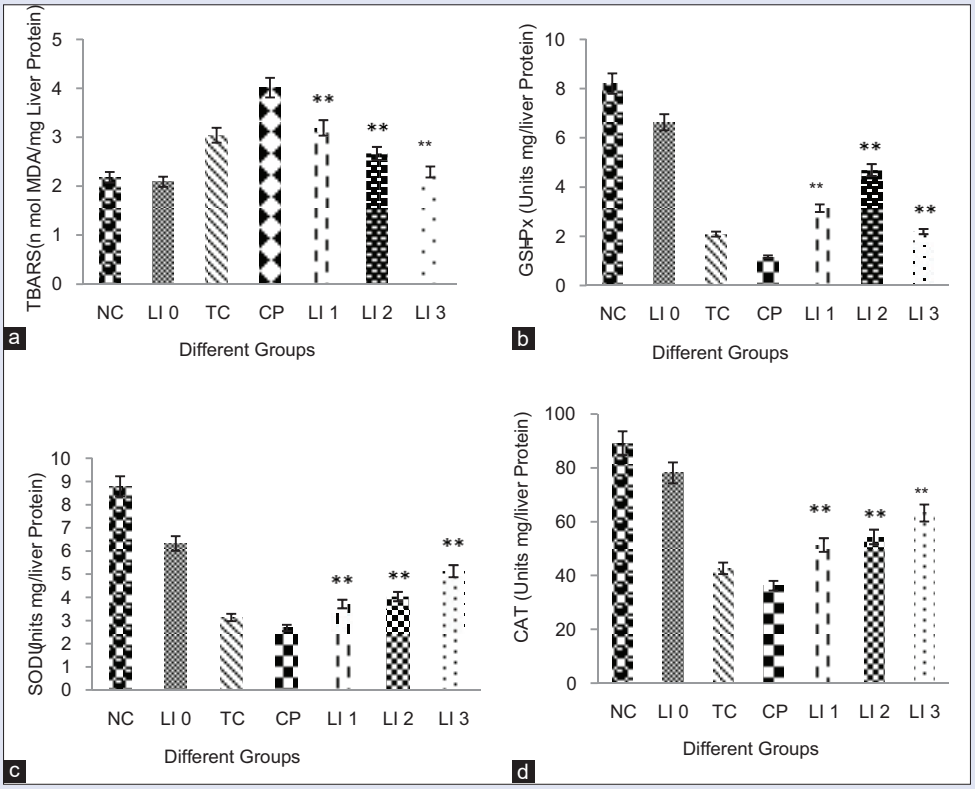

Figure 3: (a, b,c,d) Effect of *Lawsonia inermis* leaf extract on liver antioxidant status in B16F10 melanoma tumor -bearing mice. (a) Effect of tested extract on lipid peroxidase, expressed as TBARS (nmol MDA/mg Liver Protein). (b) Effect of tested extract on Glutathione peroxidase activity, expressed as GSH-Px (Units mg/liver Protein). (c) Effect of tested extract on Superoxide Dismutase activity, expressed as SOD (Units mg/liver Protein). (d) Effect of tested extract on catalase activity expressed as CAT (Units mg/liver Protein). Values are expressed as mean± S.E.M ($n = 6$) after 41 days of treatment $P<0.05$ as compared to either melanoma tumour control (TC) or negative control group (NC). The values of melanoma tumour group were compared with those of NC group (without tumour bearing mice). The values of treated group were compared with those of melanoma tumour groups. **Significantly different from Tumour control group (TC) as well as Cyclophosphamide group (CP) ($P < 0.05$)

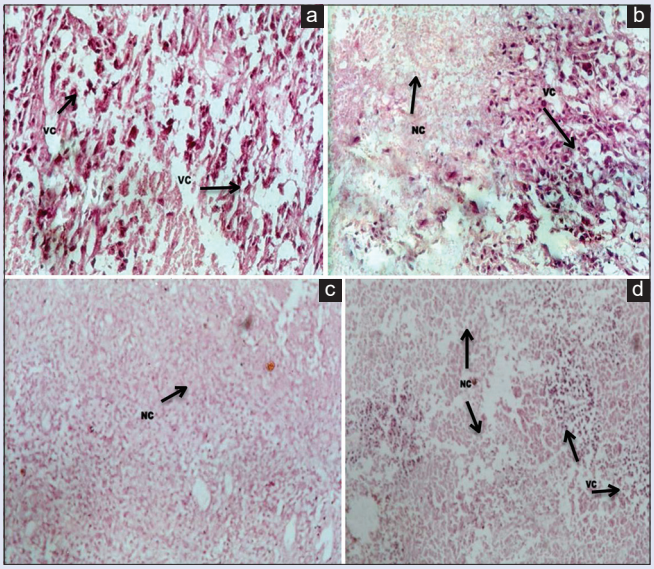

Figure 4: Photomicroscopy of the subcutaneous tissue of C57BL/6 mice bearing B16/F10 melanoma, after 40 days of treatment. After the end of the treatment period, the animals were euthanized and the tumour cells were collected for histological analysis. Tumour Control group (TC); (a) Showing marked subcutaneous tissue by tumour infiltrating mononuclear cells with vival cells. Subcutaneous tissue showing invasion by tumour cells (melanocytes). Tumour control group showed moderated number of apoptotic cells due to enhanced proliferation. Cyclophosphamide positive control group (CP): (b) showing vival cell with necrotic cells in equal ratio. *Lawsonia inermis* followed by Cyclophosphamide; (c and d) Showing skin healing process area with increasing fibroblast, collagen and mononuclear inflammatory cells. Focal areas of vival cells are present with necrotic cells

growing need for the development of effective and efficient *in vivo* model systems that share substantial similarities with the human melanomas so that it is as relevant as possible to increase and improve our understanding of the biology of this disease. There are many advantages to using mouse model systems to study melanoma development. In the last decade, numerous models have been developed that not only permit direct testing of new anti-melanoma therapies to determine efficiency and toxicity, but also allow control of gene expression or loss. With the help of several melanoma mouse models, there have been major advances in the diagnosis, treatment, and prevention of melanoma.

In the present study, the *in vivo* antitumor activity of *L. inermis* extract against mice melanoma ($B_{16}F_{10}$) cancer cells has been explored.

In conclusion, *L. inermis* extract could be regarded as promising drug for cancer therapy. The mechanism of the enhancement in the anticancer activity of *L. inermis* is not easy to predict owing to their complex pharmacological actions. However, the intrinsic properties of *L. inermis* in synergism play an important role toward enhancement in target bioefficacy. Based on the experimental findings and relevant available literature, a hypothesis is synthesized to explain observed synergistic action of *L. inermis*.

Phytochemistry study is a very useful method for the isolation of bioactive compounds from Henna, and it will helpful for pharmacological studies and established cancer biomarkers. It will also help to identify pioneering chemotherapeutic and/or chemopreventive agent. Further investigation is necessary on these aspects of Mehndi/Henna for the discovery of new anticancer agents from this miraculous plant.

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Conflicts of interest

There are no conflicts of interest.

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