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PREVENTIVE EFFECTS OF *CARISSA CARANDAS* FRUIT EXTRACT AGAINST DMBA INDUCED SKIN CARCINOGENESIS STUDIES IN SKIN OF SWISS ALBINO MICE: MORPHOLOGICAL AND HISTOPATHOLOGICAL STUDY

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Keywords:

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ABSTRACT: Antitumor and antioxidative efficacy of *Carissa carandas* extract (CCE) was investigated in this study against skin carcinogenesis which was initiated by a single topical application of 7, 12-dimethyl-benz(a)anthracene (100 mg / 100 ml acetone in 1% acetone) and two weeks, later promoted by repeated treatment of TPA (Croton oil) for 16 weeks (in 1% acetone) in Swiss albino mice. Oral administration of *Carissa carandas* hydroalcholic extract (100 mg/kg/b.wt/day) during the peri-initiational (*i.e.* 7 days before and 7 days after the application of DMBA) and post-initiational (*i.e.* from the day of croton oil treatment and continued till the end of the experiment) stage and exhibited a reduced number of tumor burden, tumor yield, number of tumors and percent incidence of mice bearing skin tumors as compared to DMBA-TPA treatment group. Average latent period and inhibition of tumor multiplicity were increased when compared to positive control values (only DMBA-TPA treated mice). After oral administration of CCE histopathological changes found less as compared to carcinogen treated mice in both the Groups. Thus, it can be concluded that the administration of CCE in mice reduces skin tumors and histopathological changes indicate anticarcinogenic potential of *Carissa carandas*.

INTRODUCTION: Non-communicable diseases like cancer, lung and cardiovascular diseases are basis of foremost health difficulties and morbidity in developed and developing countries, affected millions of people yearly. Chief factors for increasing risk of cancer are imbalance in the lifestyle, incorrect diet, genetic inclination and environment. It is estimated that 30-40% of cancers can be directly linked to dietary habits [1, 2].

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During carcinogenesis process epigenetic alterations occur which represent early stage of developing cancer [3]. Melanoma and non-melanoma (Basal and squamous call carcinoma) skin cancer cases growing rapidly in an endemic proportion between the populations of the world. Several agents contribute to causing skin cancer are viruses, transformations in environmental composition, mutagen in food and chemicals, genetic susceptibility and depletion of ozone layer due to UV radiation [4].

Lower incidence of developing cancer can be achieved by escaping of cancer causing carcinogens and mutagens and an increased intake of diet which have chemopreventive activity. Now-a-days according to research over past decades

have been investigated many diet rich plant derivatives and herbal extracts as dietary supplementation with vitamin A, E, C, minerals, or essential fatty acids, fruits, vegetables and phyto-chemicals for reducing incidences and prevention of certain types of cancers [5, 6, 7, 8, 9].

Carissa carandas belongs to family apocynaceae, known as 'karaunda', used as a prophylactic agent due to its medicinal properties *i.e.* anti-inflammatory and antibacterial [10] antioxidant activity [11] antinociceptive, anthelmintic and cytotoxicity [12] antipyretic, anti-constipation and antidiarrheal [13]. It contains several active phyto-constituents such as flavinoids, terpenoids, phenolic acid and tannins. Therefore, the present study was designed to test *in-vivo* anticancer potential of *C. carandas* extract against chemical induced two stage skin carcinogenesis.

MATERIALS AND METHODS:

Animal: The animal care and handling was done as per approval of institution and ethical committee according to guidelines set by the World Health Organization, Geneva (Switzerland) and the Indian National Science Academy, New Delhi (India). The study was conducted on random-breed male Swiss albino mice of 7-8 weeks old with 24 ± 2 g body weight. These animals were housed in poly-propylene cages in the animal house under controlled conditions of temperature (25 °C ± 2 °C) and light (14 light:10 dark). The animals were fed standard mice feed procured from Aashirwad Industries, Chandigarh (India) and water *ad libitum*. Eight animals were housed in one polypropylene plastic cage containing saw dust (procured locally) as bedding material. Institutional animal ethics committee (IAEC) approval number is 1678/Go/Re/S/12/CPCSEA dated 16.06.2017. As precaution against infections, tetracycline hydro-chloride water was given to these animals once each fortnight.

Chemicals: 7, 12-Dimethyl Benz (a) anthracene (DMBA) and croton oil were procured from Sigma Chemical Co. (USA). DMBA was dissolved at a concentration of 100 µg/ 100µl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

Plant Material and Extract Preparation: The fruits of *Carissa carandas* were collected locally after the competent proper identification (Voucher specimen no: RUBL- 211416) by expert Botanist from Herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India).

The whole fruits were washed properly, shade dried and then were powdered in a hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) for 36 (12×3) h at 40 ºC. The extract was cooled and concentrated by evaporating its liquid contents and stored at low temperature for its further use. The required dose for the treatment was prepared by dissolving the extract in double distilled water at the dose level of 100 mg/ kg.b.wt.

Experimental Protocol: A total of 50 animals were randomly divided into the following 5 groups to evaluate the anticarcinogenic potential of *Carissa carandas* extract against DMBA-induced skin papillomagenesis in mice. For this purpose, the dorsal skin of the animals in the back area was shaven 3 days before the commencement of the experiment, and only those animals having the resting phase of hair cycle were chosen for the study. Animals for this study were divided into following groups:

Group I: Vehicle Treated Control / Normal (n=10): Animals of this group received topical application of acetone (100 µl / mouse) on the shaven dorsal skin of mice and double distilled water equivalent to CCE (100 µl / mouse / day) orally for 16 weeks. This group of animals was negative control.

Group II: CCE Treated Control (n=10): Animals belonging to this group were administered CCE (100 mg/kg/b.wt. /animal/day) orally during the entire experimental period (*i.e.* 16 weeks). Mice of this group were drug treated control.

Group III: Carcinogen Treated Control (n=10): Animals of this group treated topically with carcinogenic dose of DMBA (100 µg/100 µl acetone). After two weeks, croton oil (100 µl of 1% croton oil in acetone) was applied three times per week until the end of the experiment (*i.e.* 16 weeks) and it served as the positive control.

Group IV: CCE Treated Experimental-1 (Peri-initiational) (n=10): Animals belonging to this group received the same treatment as in Group-III

but they also received CCE (100 mg/kg/b.wt./ animal/day) orally, for 7 days before and 7 days after DMBA application.

Group V: CCE Treated Experimental-2 (Post-initiational) (n=10): Animals of this group were administered orally CCE (100 mg/kg b.wt./day /animal), starting from the day of croton oil application and continued till the end of the experiment (*i.e.* 16 weeks). DMBA was applied as in Group III.

Induction of Tumor: For the induction of skin tumors, dorsal hair between the cervical and caudal portions of the animals of Group III to V were removed using a surgical clipper, two days prior to the initiation of the experiment, and 100 µl DMBA (100 µg / 100 µl acetone) was applied. After 14 days, the tumor initiation by DMBA was promoted with the topical application of 100 µl croton oil (1% w/v in acetone), thrice a week for the next 14 weeks.

Tumor Study: During the 16 weeks of experimentation, mice were observed daily and weighed weekly. Tumors appearing on the shaven area of the skin were examined and recorded at weekly intervals. Only those tumors which persisted for two weeks or more, with a diameter greater than 2 mm, have been taken into consideration for the final evaluation of the data. Skin tumors, which regressed after one observation, were not considered for the counting.

Dose Selection of *Carissa carandas* Extract: Different doses (10, 25, 50, 100, 150, 200 mg/kg. b. w/animal/day) of *Carissa carandas* extract (CCE) were administered orally to the mice for 15 days, and the alterations in morphological parameters like body weight, mortality, morbidity, food and water consumption, general behavior, gait etc were observed till 30 days. The biochemical parameters such as GSH, LPO and total proteins, were studied in liver and skin on 16^{th} and 31^{st} day. The optimum dose of CCE was selected on the basis of level of GSH, total proteins and LPO. The required dose for treatment was prepared by dissolving the extract in double-distilled water at the dose rate of 100 mg/kg/b.wt.

Following parameters were taken into consideration for the study:

A. Morphological Analysis:

Body Weight: The weight of each mouse was measured weekly.

Average Latent Period: The time lag between the application of the promoting agent and the appearance of 50% of tumors was determined. The average latent period was calculated by multiplying the number of tumors appearing each week by the time in weeks after the application of the promoting agent and dividing the sum by total number of tumors.

$$\text{Average latent period} = \sum FX / N$$

Where F is the number of tumors appearing each week, X is the numbers of weeks, and n is the total number of tumors.

Tumor Incidence: The number of mice carrying at least one tumor expressed as a percentage incidence.

Tumor Yield: The average number of tumors per mouse.

Tumor Burdon: The average number of tumors per tumor bearing mouse.

Cumulative No. of Tumors: The total number of skin tumors appeared till the termination of the experiment.

Inhibition of Tumor Multiplicity:

Total no. of tumors in carcinogen control - Total no. tumors in CCE treated experiment / Total no of tumors in carcinogen treated control × 100

B. Histopathological Studies: A part Skin of sacrificed animals were removed after end of the experiment (16 week) and fixed in 10% formalin fixative for 24 h. Dehydration of the tissue was done in ascending series of alcohol, embedded in paraffin wax, and 4-µm thick sections were prepared and studied using a light microscope.

Statistical Analysis: Data obtained from different experimental groups were analyzed and expressed as mean ± SE.

RESULTS:

A. Morphological Study: Body Weight: Mean body weight of Group I (Vehicle treated mice) after tropical application of acetone and oral

administration of DDW for 16 week, was 34.23 ± 1.23 g, meanwhile mice which received only CCE (Group II) did not show any alteration in the body weight during the entire experiment and it was measured near to the normal. Mean body weight of animals related to Group III reduced to 95.85 % (32.81 ± 1.46 g) as compared to Group I, whereas mice of Group IV at peri-initiation phase and Group V at post-initiation phase improved their respective body weight after administration of CCE as 102.89% (33.76 ± 1.16 g) and 106.06 % (34.80 ± 1.46 g) as compared to Group III **Table 1**.

TABLE 1: VARIATION IN THE BODY WEIGHT, DURING DMBA- INDUCED SKIN CARCINOGENESIS IN MICE

Groups		Treatment	Body Weight (g) Mean ± S.E.	
			Initial	Initial
Vehicle treated	I	Acetone	25.58 ± 1.83	34.23 ± 1.23
CCE treated Control	II	100 mg /animal/day	25.38 ± 1.03	34.36 ± 1.46
Carcinogen treated control	III	DMBA + Croton oil	25.46 ± 2.40	32.81 ± 1.46
CCE treated Experimental I	IV	(DMBA + Croton oil) + CCE	24.43 ± 1.18	33.76 ± 1.16
CCE treated Experimental II	V	CCE (DMBA + Croton oil) + CCE	25.23 ± 2.44	34.80 ± 1.46

Average Latent Period: The average latent period (*i.e.* time lag between the application of the promoter and the appearance of 50% of tumors) was considerably longer in Group IV (Peri-initiation) and V (Post-initiation) *i.e.* 10.46 weeks and 11.47 weeks, respectively, while it was found much lower in the carcinogen treated control (Group III) *i.e.* 8.72 weeks **Fig. 1**.

Tumor Incidence, Tumor Yield and Tumor Burden: The incidence of skin tumors was 100% in DMBA-TPA treated animals (Group III); whereas in CCE treated mice, it was found to be significantly declined as noticed in Group IV and V, 60% (6 mice out of 10) and 50% (5 mice out of 10), respectively at the end of the experiment **Fig. 2**. Similarly, mice belonging to Group IV and V with CCE administration revealed comparative reduction in tumor yield to 2.8 and 2.1 (Positive Control value 6.5) and tumor burden to 4.6 and 4.2 (Positive Control value 6.5), respectively **Fig. 3**, **4**.

Cumulative No. of Papillomas: Morphological appearance of tumors in carcinogen treated control group was scaly, larger and darker while in CCE administered groups tumors were soft, smaller and lighter in color **Fig. 7**.

Oral administration of CCE in Group IV (Peri-initiation) and Group V (Post-initiation) significantly reduced the cumulative number of tumors (total number of papillomas till the end of the experiment) *i.e.* 28 and 21, respectively as compared to the positive control Group III **Fig. 5**.

Inhibition of Tumor Multiplicity: Tumors appeared in all the mice of the Group III. Therefore, inhibition in tumor multiplicity was considered zero percent for carcinogen treated control.

Maximum inhibition in tumor multiplicity was observed in Group V (Post-initiation; received CCE before and after one week of DMBA application) as 56.92% followed by Group IV (Peri-initiation; received CCE after two weeks of DMBA application and continued till the end of experiment) as 67.69% **Fig. 6**.

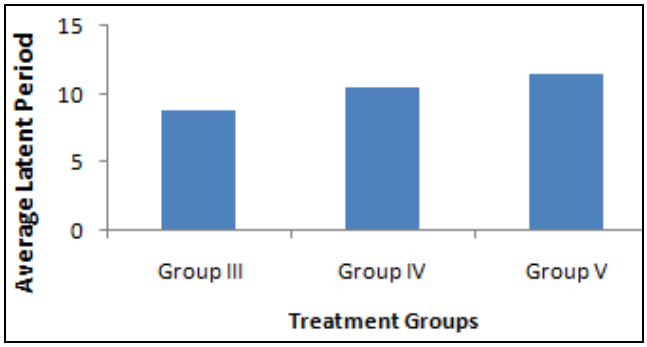


FIG. 1: MODULATORY EFFECT OF *C. CARANDAS* EXTRACT ON AVERAGE LATENT PERIOD DURING THE DMBA INDUCED SKIN CARCI-NOGENESIS IN TREATED MICE (GROUP IV AND GROUP V) IN CONTRAST TO THE POSITIVE CONTROL (GROUP III)

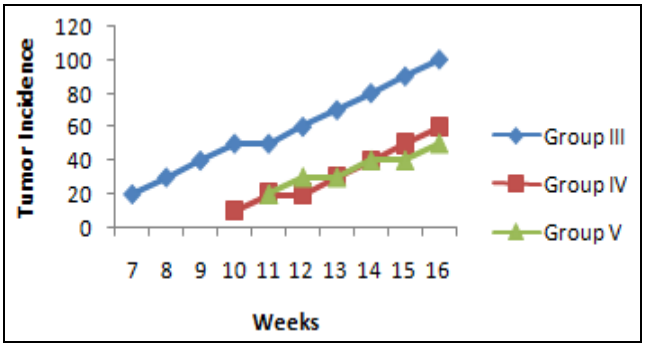


FIG. 2: MODULATORY EFFECT OF *C. CARANDAS* EXTRACT ON TUMOR INCIDENCE DURING THE DMBA INDUCED SKIN CARCINOGENESIS IN TREATED MICE (GROUP IV AND GROUP V) IN CONTRAST TO THE POSITIVE CONTROL (GROUP III)

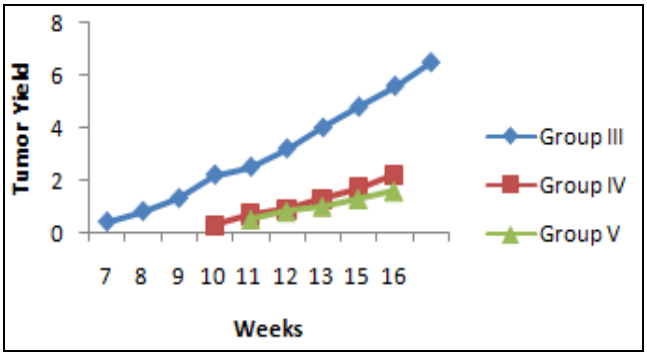


FIG. 3: MODULATORY EFFECT OF *C. CARANDAS* EXTRACT ON TUMOR YIELD DURING THE DMBA INDUCED SKIN CARCINOGENESIS IN TREATED MICE (GROUP IV AND GROUP V) IN CONTRAST TO THE POSITIVE CONTROL (GROUP III)

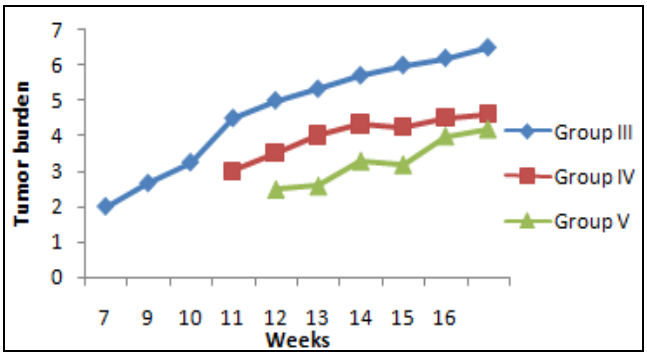


FIG. 4: MODULATORY EFFECT OF *C. CARANDAS* EXTRACT ON TUMOR BURDEN DURING THE DMBA INDUCED SKIN CARCINOGENESIS IN TREATED MICE (GROUP IV AND GROUP V) IN CONTRAST TO THE POSITIVE CONTROL (GROUP III)

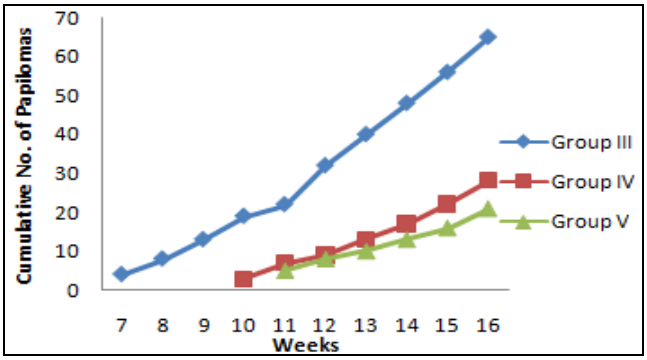


FIG. 5: MODULATORY EFFECT OF *C. CARANDAS* EXTRACT ON CUMULATIVE NUMBER OF PAPILLOMAS DURING THE DMBA INDUCED SKIN CARCINOGENESIS IN TREATED MICE (GROUP IV & GROUP V) IN CONTRAST TO THE POSITIVE CONTROL (GROUP III)

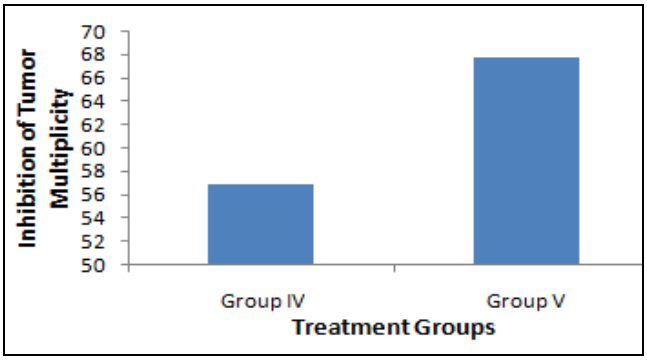
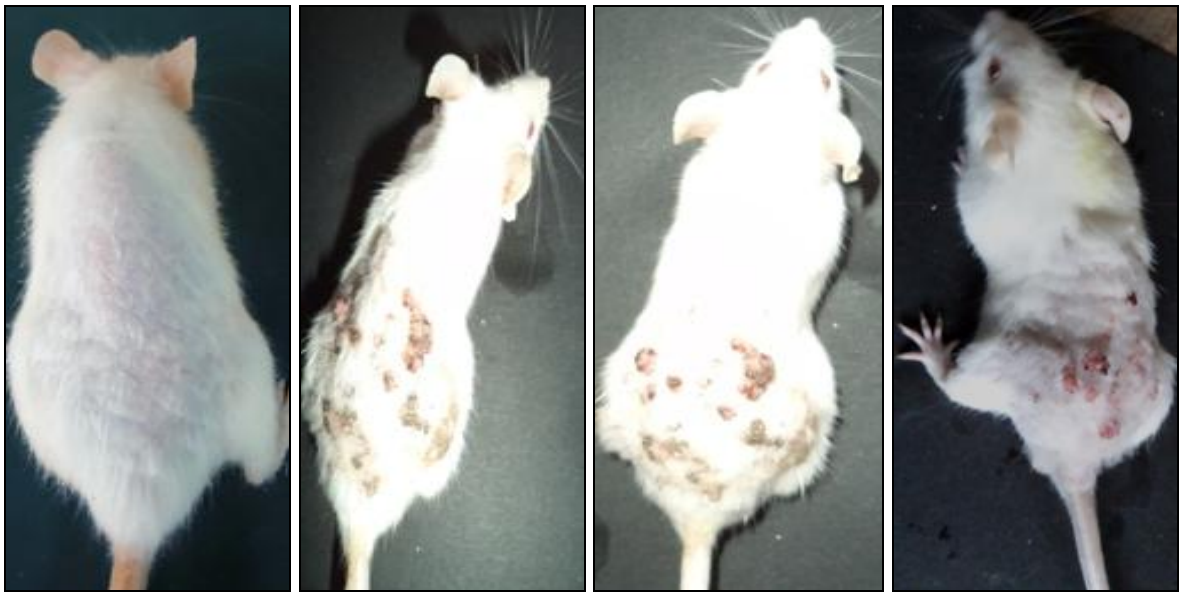


FIG. 6: MODULATORY EFFECT OF *C. CARANDAS* EXTRACT ON INHIBITION OF TUMOR MULTI-PLICITY DURING THE DMBA INDUCED SKIN CARCINOGENESIS IN TREATED MICE (GROUP IV & GROUP V) IN CONTRAST TO THE POSITIVE CONTROL (GROUP III)



Group I **Group III** **Group IV** **Group V**

FIG. 7: THE GROSS APPEARANCE OF SKIN TUMOR IN MICE OF DIFFERENT GROUPS DURING CHEMICAL INDUCED SKIN CARCINOGENESIS WITH OR WITHOUT *C. CARANDAS* EXTRACT

B. Histopathological Study: Vehicle treated mice (Group I) exhibited normal skin histology that is epidermis layer, dermis layer, hair follicle and sebaceous gland **Fig. 8A**. Histopathological examination of skin of DMBA-TPA treated mice had epidermal hyperplasia (thickening of

epidermis), thickening of keratinized layer over the epidermis (hyperkeratosis), and invasion of epidermal layer in dermis (dermal invasion), epidermal erosion and lymphocyte infiltration (Group III) **Fig. 8B**. Administration of CCE at a dose of 100 mg/kg/b.wt /amimal/d in peri initiation phase (Group IV) **Fig. 8C** and post initiation phase (Group V) **Fig. 8D** had lesser hyperkeratosis, hyperplasia and erosion in skin as compared to carcinogen treated mice.

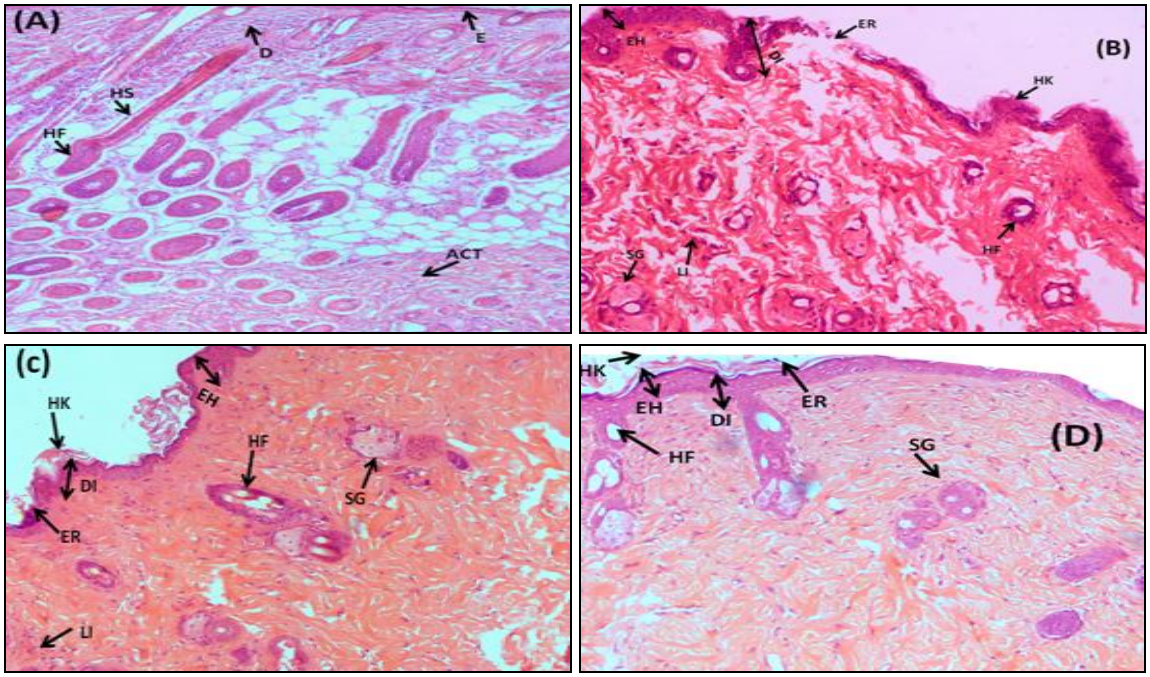


FIG. 8: PHOTOMICROGRAPH INDICATING HISTOLOGICAL SECTIONS IN THE SKIN TISSUE OF MICE OF DIFFERENT GROUPS. (A) NORMAL GROUP (B) GROUP- III SKIN, (C) GROUP- IV SKIN, (D) GROUP-V SKIN. E- Epidermis, D- Dermis, HK- Hyperkyratosis, ER-Epidermal erosion, EH- Epidermal hyperplasia, DI- Dermal invasion, SG- Sebaceous gland, HF-Hair follicle

DISCUSSION: The findings of present studies of reveal that *Carissa carandas* plant extract suppress DMBA/TPA induced two stage skin carcinogenesis due to its anticarcinogenic effect. Application of 7,12 dimethylbenz(a) anthracene (DMBA) and 12- O-tetra decanoylphorbol- 13-acetate (TPA) constitute of croton oil induced two stage skin carcinogenesis through three sequential steps of tumor initiation, promotion, and progression, also helpful for study of genetic and biochemical alterations caused by them. In the present investigation DMBA + TPA (Group III) used as topical application because it was reported that fastest way of absorption of DMBA carcinogen was found in skin tissue. In present experiment carcinogen treated animals exhibited 100% tumor incidence and highest cumulative number of tumors. However, treatment with CCE (Group IV and V) showed regressed of tumor incidence and cumulative number of tumor.

In addition, DMBA also cause formation of cytokines such IL-α, which act in the similar way to TPA mediated inflammation and oxidative stress which contribute to cause carcinogenesis, because of chronic inflammation and oxidant formation. In this study due to carcinogenic effect of DMBA and TPA, higher number of tumors and their rapid growth of tumor were recorded in Group III (DMBA/TPA treated). DMBA and TPA cause carcinogenesis mediated by generation of reactive oxygen species and hydroperoxides in keratino-cytes which exaggerates normal cell transformation into cancerous cell [14, 15, 16].

Carcinogenesis is multistep process in which normal cell transforms into neoplastic cell that involves tumor initiation (rapid and irreversible stage), promotion (highly proliferative and reversible) and progression (invasive and metastasis of tumor). DMBA cause biochemical and physiological impairment in the balance of the free radicals production (reactive oxygen species, primarily superoxide and H_2O_2) and antioxidant defense, known as oxidative damage [17]. Naturally, free radicals are generated in the body and play an important role in many normal cellular processes [18, 19].

However, this protective mechanism can be disrupted by carcinogen such as DMBA and TPA, so as a of result elevated concentrations of free radicals, they cause oxidative damage to bio-molecules, cellular growth and tissue injury, and also involve in the pathogenesis of several diseases including cancer [20, 21]. In the current study, mice treated with DMBA and TPA developed detectable tumor size, higher tumor burden (The average number of tumors per tumor bearing mouse) and tumor yield (The average number of tumors per mouse) in carcinogen treated animal (Group III) because of DMBA and TPA increases free radicals including reactive oxygen species.

Anti-oxidants neutralize free radicals by donating one of their own electrons, ending the electron-"stealing" reaction and preventing them from causing damage and finally covert dangerous H_2O_2 into H_2O. Thus, availability of free radicals is normally controlled by scavenging through different anti-oxidative defense components and through chemoprevention approach. Animals treated with the CCE (100 mg/kg/b.wt. /animal/ day) at either the peri- (*i.e*. 7 days before & 7 days after the application of DMBA) or post-initiational (*i.e.* from the day of croton oil treatment till the end of the experiment) phases demonstrated significant reduction in the cumulative numbers of papillomas and tumor incidence, lowering down tumor burden and tumor yield.

It is correlated by antioxidants present in *Carissa carandas* fruit. The consumption of anti-oxidants exogenously as well as endogenously improves antioxidant defense system. Antioxidants are natural guard system against free radicals. Various studies have shown that low risk of cancer is more strongly interrelated to antioxidant rich diet [22, 23]. Many phyto-constituents present in CCE such as carbohydrates, flavonoids, alkaloids, tannins, phenolic components, proteins, amino acids and saponin [24] might have worked significantly declined tumor size and numbers in CCE administered groups (IV and V) than the carcinogen treated group (Group III).

In results of the present study, normal mice histopathological study showed uniformly arranged epidermis and dermis layer (Group I), were observed while severe damages in histology of skin in the Group III were observed and signs of squamous cell carcinoma (SCC) development, such as abnormally thickened epidermis (hyperplasia) with higher proliferation of epidermis (dermal invasion) were also present. Thickening of keratinized layer *i.e*. hyperkeratosis over epidermis also noted in carcinogen treated mice (Group III). Moderation in epidermal and dermal layer of skin in Group IV and group V was recorded after oral administration of *Carissa carandas* extract at the rated 100 mg/kg/b.wt /amimal/d dose, in contrary to carcinogen treated mice. Hyperplasia, dermal invasion and hyperkeratosis were also recorded in CCE and DMBA/TPA treated mice at peri-initiation and post-initiation phase but of reduced size in contrast to only DMBA/TPA treated mice *i.e.* Group III. This may be due to presence of phytoconstituents in CCE drug [24]. Thease results are supported by several reports which show chemomodulatory effect of several medicinal plants such as *Gymnema sylvestre, Dillenia indica*, *Panax ginseng* and flaxseed oil [25, 26, 27, 28].

Other chemical components in the genus *Carissa* are steroids, terpenes, benzenoids, phenyl-propanoid, lignans, sesquiterpenes, and coumarins [13]. The antioxidant potential of these compounds might have removed the free reactive oxygen species and hazardous consequences through significant delayed average latent period in tumor appearance and enhanced inhibition of tumor multiplicity in CCE administered mice was recorded in Group IV and V which appears to be due to CCE extract.

CONCLUSION: Findings of the present study indicate that naturally occurring antioxidants in CCE significantly regress skin tumor growth induced by DMBA-TPA in the skin of Swiss albino mice and which is accompanied by tumor inhibition. Outcomes of the current study suggest that CCE formulation may be developed further into a chemopreventive agent.

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CONFLICT OF INTEREST: There is no potential conflict of interest.

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