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Original Article

Modulation of DMBA- induced biochemical and histopathological changes by *Syzygium cumini* seed extract during skin carcinogenesis

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ABSTRACT

Aim & Method: The current study was designed to elucidate the protective effect of Syzygium cumini seed extract (SCE) on skin carcinogenesis induced by a single topical application of 7,12-dimethylbenz(a)anthracene (100 µg/100 µl of acetone) and 2 weeks later promoted by repeated application of croton oil (1% in acetone/three times a week) till the end of the experiment (16 weeks). Result: Oral administration of SCE at a dose of 125 mg/kg b.wt./day for 15 days at the peri-initiational stage (i.e., 7 days before & 7 days after DMBA application) and for 14 weeks at the promotional stage (i.e., from the time of croton oil application), revealed a significant reduction in lipid peroxidation ($p<0.05$-0.001) along with an elevation in the activities of enzymatic antioxidants (superoxide dismutase, $p<0.05$-0.001 & catalase, $p<0.05$-0.001), non-enzymatic antioxidant (reduced glutathione, $p<0.05$-0.01 & vitamin-C, $p<0.01$-0.001) and total proteins levels ($p<0.01$-0.001) when compared to the carcinogen treated control animals. Histopathological study revealed that dyskeratosis of the epidermis, deposition of keratinous pearl and epidermal hyperplasia in skin tumors of DMBA treated control and the same were found to be of lesser degree in both the SCE treated experimental animals. Conclusions: These results demonstrate that SCE ameliorate the DMBA/croton oil induced adverse biochemical and histopathological alterations during skin carcinogenesis in mice.

1. Introduction

Cancer is a major public health problem in the United States and many other parts of the world. Currently, 1 in 4 deaths in the United States is due to cancer. A total of 1,529,560 new cancer cases and 569,490 deaths from cancer are projected to occur in the United States in 2010. Public opinion considers cancer to be an increasingly threatening disease, affecting people of all ages. De flora et.al. (2001), proposed a detailed, updated classification of the points of intervention exploitable in the prevention of mutation and cancer. The general outline includes a variety of extracellular and cellular mechanisms modulating the genotoxic response and tumor initiation as well as tumor promotion, Polycyclic aromatic hydrocarbons (PAHs) are widespread genotoxic and tumorigenic environmental pollutants. It has long been known that PAHs require metabolic activation in order to exert their biological activities including carcinogenicity [1-4]. Upon metabolism, PAHs are either metabolized into biologically active metabolites, including diol epoxides and free radical intermediates, which bind to cellular DNA forming covalent DNA adducts responsible for mutagenicity and carcinogenicity [2-5].
The exact causes of most types of cancer are still not known, and there is not yet a cure for cancer. However, it is now known that the risk of developing many types of cancer can be reduced by adopting certain lifestyle changes, such as quitting smoking and eating a better diet.

Foods are complex mixtures of nutrients and nonnutritive substances that are difficult to measure accurately, and the effects of individual constituents as well as the possible interactions among these constituents are difficult to unravel. Differences among individuals, including inherited genetic susceptibility, also

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could contribute to inconsistent epidemiologic associations between dietary factors and specific cancers [6]. Free radicals are uncoupled electrons and are extremely active and unstable. Among the most important free radicals in the reactive oxygen species (ROS) are singlet oxygen, super oxide anions, hydrogen peroxide in the etiology of cancer [7]. Uncontrolled production of ROS results in the destruction of macromolecules such as DNA, lipids and proteins [8].

The use of medicinal plants in modern medicine suffers from the fact that although hundreds of plants are used in the world to prevent or to cure diseases, scientific evidence in terms of modern medicine is lacking in most cases.

The therapeutic value of *Syzygium cumini(Linn)* commonly known as "Jamun" has been recognized in different system of traditional medication for the treatment of various diseases and ailments of human beings. *Syzygium cumini* is a large evergreen tree found throughout India upto an altitude of 1,800 m [9].

It contains several phytoconstituents belonging to the category of alkaloids, glycosides, flavanoids and volatile oil. It has been reported as a digestive, astringent, blood purifier and anti-helminthic, antibacterial, analgesic, antiinflammatory, antioxidant, as well as gastro protective. It has been used for the treatment of bronchitis, asthma, thirst, biliousness, dysentery, ulcers and diabetes [10-12]. Looking towards the pharmaceuticals properties of this plant, the present study has been focused to find out it anti-carcinogenic and anti-oxidative activity against chemical induced skin carcinogenesis in mice.

2. Materials and Methods

The animal care and handling was approved by ethical committee of our institution and was done according to guidelines set by the World Health Organization, Geneva, Switzerland, and the Indian National Science Academy, New Delhi, India. The inhibition of tumor incidence by Syzygium cumini seed extract was evaluated on two-stage skin carcinogenesis, induced by a single application of DMBA (initiator), and two weeks later, promoted by repeated application of croton oil (promoter) thrice per week, following the protocol for 16 weeks [13].

2.1. Animals- 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 polypropylene cages in the animal house under controlled conditions of temperature (25°C ± 2°C) and light (14 light:10 dark). The animals were fed a standard mouse 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. For precaution against infections, tetracycline hydrochloride water was given to these animals once each fortnight. Chemicals - 7, 12-Dimethyl Benz (a) anthracene (DMBA) and croton oil was 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.

2.2. Plant material & Extract Preparation

The fruit of Syzygium cumini L. was collected locally after proper identification. The identification of the plant Syzygium cumini L. (Family: Myrtaceae) was done by a botanist (Voucher Specimen No: RUBL- 20425) from Herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India). The pulp was removed from the fruit and the seed were washed properly and shade dried, then after fruit was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) for 36 (12 x 3) hrs at 40ºC. The extract was cooled and concentrated by evaporating its liquid contents. The prepared Syzygium cumini extract (SCE) was stored at low temperature until its further use and it was redissolved in DDW prior for the oral administration in mice.

2.3. Experimental Design

The dorsal skin (2 cm diameter) of Swiss albino mice was shaved 2 days before chemical treatment and animals in the resting phase of growth cycle were selected for the experiment (Sasaki et al., 1995). Mice selected from inbreed colony were grouped into following five groups:

Group I: Vehicle treated Control

Animals of this group received topical application of acetone (100 µl/ mouse) on the shaven dorsal skin and double distilled water equivalent to SCE (100 µl/ mouse/ day) by orally for 16 weeks.

Group II: Drug (SCE) treated Control

These Animals received SCE (125 mg/kg/b. wt./animal/day) alone orally during the entire experimental period (i.e., 16 weeks).

Group III Carcinogen treated control

A single dose of 100 µg of DMBA in 100 µl of acetone was applied topically over the shaven area of the skin of the mice. Two weeks later 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).

Group IV SCE treated experimental-1 (Peri-initiational): Animals received hydro-alcoholic extract of S.cumini seed (125 mg/kg b.wt./day/animal) 7 days before and 7 days after the application of DMBA. Croton oil was given as in Group III.

Group V SCE treated experimental-2 (Post-initiational): Animals of this group were administered orally SCE (125 mg/kg b.wt./day/animal), starting from the time of croton oil application and continued till the end of the experiment (i.e., 16 weeks). DMBA was given as in Group III.

2.4.Biochemical Study

Biochemical alterations were measured in animals of all the above groups at the time of the termination of the experiment. At the end of the 16th week, the animals were killed by cervical dislocation. The dorsal skin affected by tumors and liver were quickly excised and washed thoroughly with chilled saline (pH

7.4), then weighed and blotted dry. A 10% tissue homogenate was prepared from the part of the sample (skin & liver) in 0.15 M Tris-KCl (pH 7.4), and centrifuged at 2000 rpm for 10 minutes. The supernatant thus obtained was taken for estimation of lipid peroxidation (LPO) and reduced glutathione (GSH). The following biochemical parameters were estimated in the liver and skin of mice.

2.4.1.Lipid Peroxidation (LPO)

The level of LPO was estimated spectrophotometrecally by thiobarbituric acid reactive substances (TBARS) method as described by Ohkhawa et al. (1979) [14]. Briefly, thiobarbituric acid (0.6%), sodium dodecyl sulphate (0.1%), and trichloroacetic acid (20%) were added to 200 µl of the tissue homogenate (10%) prepared as described above. This mixture was heated for 60 minutes, cooled, and extracted with N butanol- pyridine (15:1), the optical density (OD) was recorded at 532 nm and the contents were expressed as nmol/mg of tissue.

2.4.2.Reduced Glutathione (GSH)

The level of GSH was estimated as total nonprotein sulphahydryl group by the method of Moron et al. (1979) [15]. The homogenate was immediately precipitated with 100 µl of 25% trichloroacetic acid (TCA) and the precipitate was removed after centrifugation. Free endogenous-SH was assayed in a total volume of 3 ml by the addition of 200 µl of 0.6 mM 5, 5′ dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH 8.0) to 100 µL of the supernatant and the absorbance was recorded at 412 nm using a UV-VIS Systronics spectrophotometer. Reduced GSH was used as a standard and the levels of GSH were expressed as µmol/gm of tissue.

2.4.3.Catalase

The catalase activity was assayed by the method of Aebi [16]. The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H2O2 (30 mM) to 100 µL of the supernatant (10% of skin homogenate prepared in 50 mM phosphate buffer and centrifuged for 10 min.) in 50 mM phosphate buffer (pH 7).The activity of the enzyme is expressed as U/mg of tissue, where U is µmol of H2O2 disappearance/min.

2.4.4.Total Proteins

Total Proteins were estimated by the method of Lowery et al., [17] using bovine serum albumin as a standard and the level was expressed as mg/ gm.

2.4.5.Superoxide dismutase

SOD was determined by the method of Marklund and Marklund [18] by quantification of pyrogallol auto oxidation inhibition and the results are expressed as units/mg protein. Auto oxidation of pyrogallol in Tris-HCL buffer (50 mM, pH-7.5) was measured by increase in absorbance at 420 nm.

2.4.6.Vitamin-C

For this, tissue, the fresh organs were weighed, homogenized in acetate buffer (20 mg/ ml) extracted with cold 4 per cent trichloroacetic acid, centrifuged, and filtered. Ascorbic acid was determined by the method of Roe and Kuether [19].

2.5.Histopathological Study

Tumors and normal skin were removed from the sacrificed animals and immediately fixed in 10% formalin fixative for 24h. The tissues were then dehydrated in ascending series of alcohol, embedded in paraffin wax and 5 micron thick sections were cut and viewed under light microscope.

2.6.Statistical Analysis

Data from different experimental groups were analyzed and expressed as mean + SD. The significant level of difference between carcinogen treated control and SCE treated experimental groups were statistically analyzed using Student's t-test.

3.Results

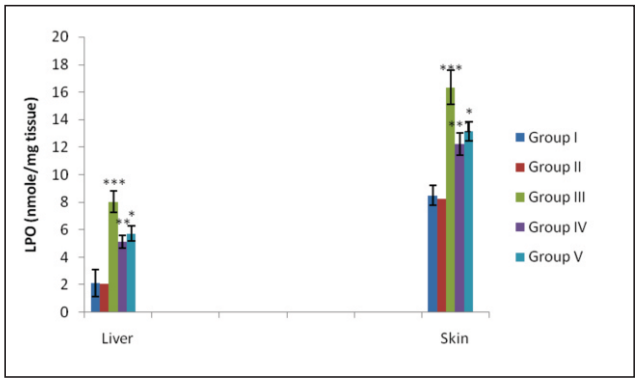
3.1.Biochemical Study

3.1.1.Lipid peroxidation

Relative to carcinogen treated control, the level of LPO in liver was recorded as significantly reduced in peri- and post-initiational Group IV & V from 3.769 ($p<0.001$) fold to 0.638 ($p<0.01$) and 0.71 ($p<0.05$) fold, respectively (Fig.1).

Whereas the same was found to be significantly increased in the skin of animals of carcinogen treated control group up to 1.926 ($p<0.001$) fold, as compared to vehicle treated control. A significant reduction in the level of LPO was observed in skin of mice of group IV & V to 0.747 ($p<0.05$) and 0.804 ($p<0.05$) fold then carcinogen treated control (Fig.1).

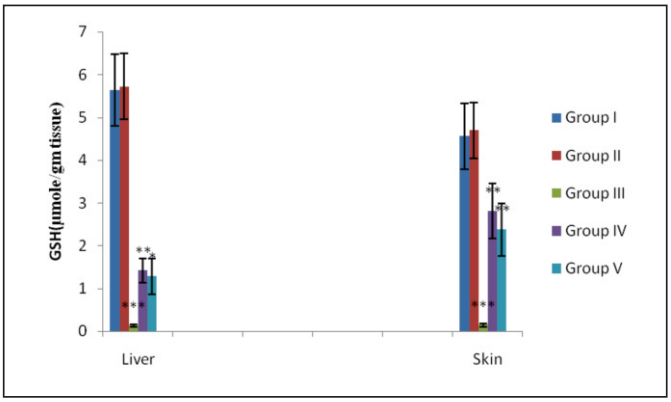
Fig. 1: Modulatory influence of the investigative dose (125 mg/kg. b.wt./day) of *Syzygium cumini* on tissue LPO in mice Statistical comparison Normal v/s Control; Control v/s Experimental. **Significance levels** **p ≤ 0.05, **p≤ 0.01, ***p≤ 0.001



3.1.2.Reduced Glutathione

No significant increase was observed in liver and skin of animals of Group II, which received SCE alone for 16 weeks as compared to vehicle treated control. The activity of GSH was found to be significantly increased by 10.92 ($p<0.01$) & 9.84 ($p<0.05$) fold in liver and 18.73 ($p<0.01$) & 15.86 ($p<0.01$) fold in skin of peri-& post-initiational group (Group IV& V) respectively, in comparison to carcinogen treated control (Fig.2).

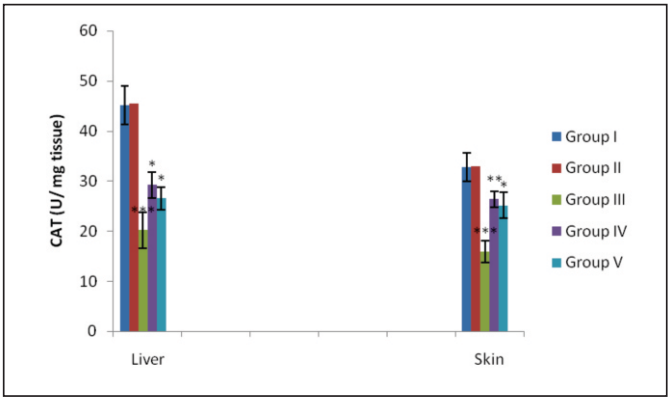
Fig. 2 Modulatory influence of the investigative dose (125 mg/kg. b.wt./day) of *Syzygium cumini* on tissue GSH in mice. Statistical comparison Normal v/s Control; Control v/s Experimental **Significance levels** **p ≤ 0.05, **p ≥ 0.01, ***p≤ 0.001



3.1.3.Catalase

Activity of catalase in liver was enhanced by 1.45(p<0.05) and 1.31(p<0.05) fold in peri- & post-initiational group respectively, while compared to carcinogen treated control (Fig.3). S.cumini extract administration increased the activity of catalase in skin of mice of group IV and V by 1.66 (p<0.01) and 1.58 (p<0.05) fold respectively; whereas on DMBA/croton oil application, the same was found to be significantly decreased in comparison to vehicle treated control (Fig.3).

Fig. 3: Modulatory influence of the investigative dose (125 mg/kg. b.wt./day) of *Syzygium cumini* on tissue catalase in mice Statistical comparison Normal v/s Control; Control v/s Experimental **Significance levels** *p < 0.05, **p 0.01, ***p 0.001

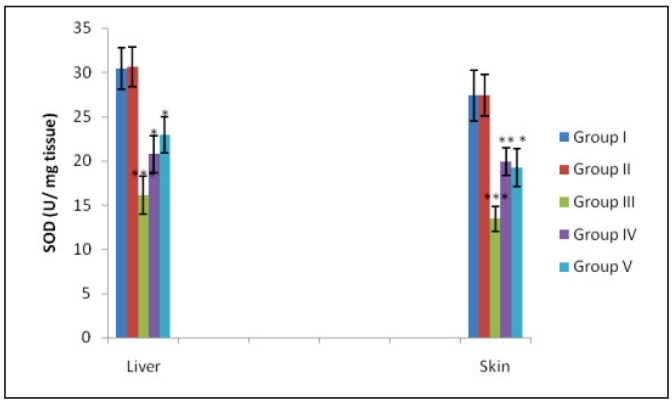


3.1.4.Superoxide dismutase

A decrease of 0.530 fold (P < 0.001) from normal in the activity of SOD was observed in liver of DMBA/croton oil-treated animals, whereas animals of group IV and V treated with SCE, exhibited 1.28 (p<0.05) & 1.42 (p<0.05) fold increase as compared with controls (Fig.4).

On topical application of DMBA/croton oil, the SOD activity in skin of these animals showed a 0.49 fold decrease (P < 0.001), whereas only 1.478 (p<0.001) & 1.428 (p<0.001) fold increase was observed in the SCE treated group IV and V, respectively animals as compared with controls (Fig.4).

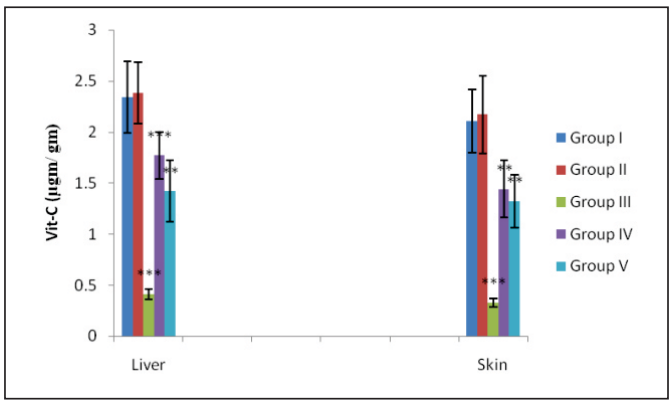
Fig. 4: Modulatory influence of the investigative dose (125 mg/kg. b.wt./day) of *Syzygium cumini* on tissue SOD in mice Statistical comparison Normal v/s Control; Control v/s Experimental **Significance levels *p ≤ 0.05, ***p≤ 0.001**



3.1.5.Vitamin-C

Vitamin-C content in liver and skin was drastically lowered i.e., 0.175 (p<0.001) & 0.156 (p<0.001) fold respectively, in the carcinogen treated control group. Administration of S.cumini extract could significantly enhance the Vit.-C content in liver to 4.31(p<0.001) & 3.46 (p<0.01) fold as well as in skin to 4.36 (p<0.01) & 4.00 (p<0.01) fold in Group IV and V respectively (Fig.5).

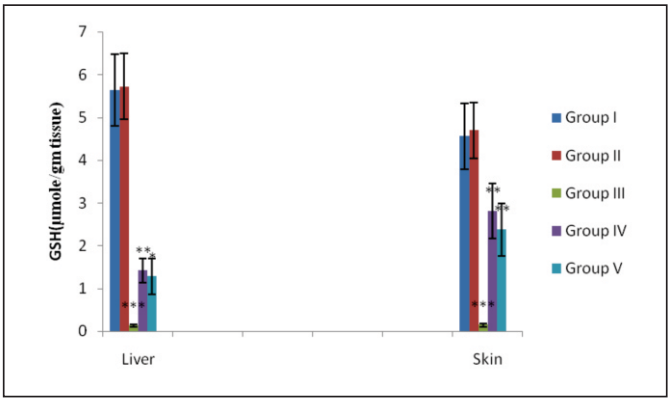
Fig. 5: Modulatory influence of the investigative dose (125 mg/kg. b.wt./day) of Syzygium cumini on tissue Vitamin-C in mice Statistical comparison Normal v/s Control; Control v/s Experimental **Significance levels** **p 0.01, ***p 0.001



3.1.6.Total Proteins

A significant reduction of 0.36 ($p<0.001$) and 0.37 ($p<0.001$) fold in the total protein level was observed in liver and skin of carcinogen treated Control (Group III) respectively, as compared to vehicle treated control. Contrary, SCE treated Group IV and V showed a significant elevation up to 2.188 ($p<0.01$) & 2.066 ($p<0.01$) fold in liver and 2.08 ($p<0.01$) & 1.98 ($p<0.01$) fold in skin respectively in comparison to carcinogen treated control (Fig.6).

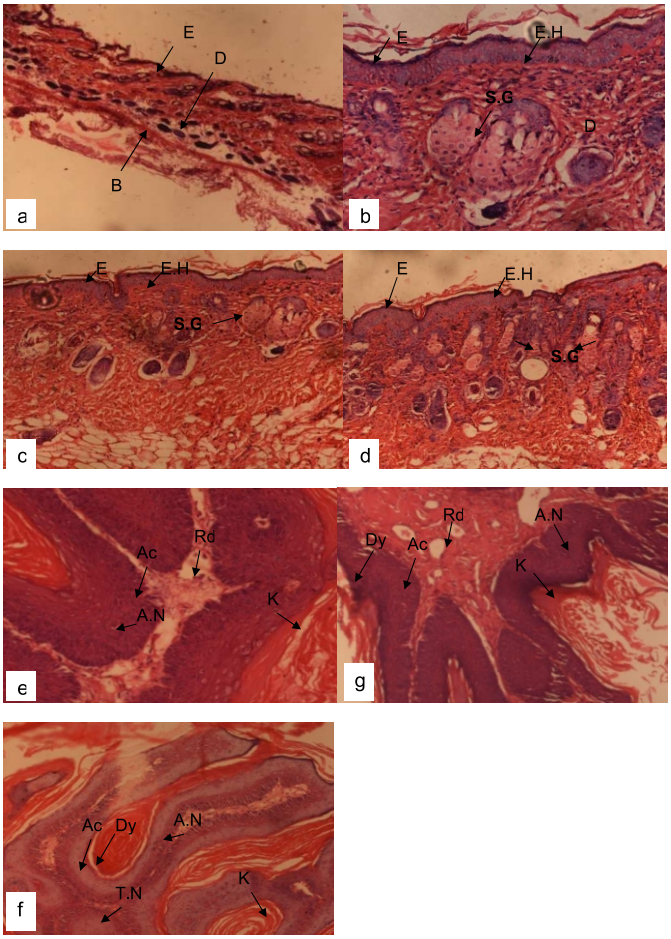
Fig. 6: Modulatory influence of the investigative dose (125 mg/kg. b.wt./day) of *Syzygium cumini* on total proteins in mice Statistical comparison Normal v/s Control; Control v/s Experimental **Significance levels** ***p≤ 0.01, ***p ≤ 0.001



3.2.Histopathological Study

Fig.7 (a-g) depicts the histology of the changes of effect of peri- and post- treatment with S.cumni extract on two-stage skin carcinogenesis in mice. The acetone treated mouse skin exhibited normal skin layer and appendages. The epidermis was seen with an approximately single layer of basal cells laid over by flattened squamous cells and the stratum corneum. DMBA and croton oil treated group (Group III) revealed squamous cell papillomas and keratoacanthomas type of tumors. There is marked epidermal thickening (acanthosis) with intradermal proliferation (rete ridges) and keratin pearls were commonly observed in this group. Skin of carcinogen treated control animals shows the cells with atypical (enlarged and hyperchromatic) nuclei at all the levels of the epidermis. Some of the cells break through the basement membrane, the process has become invasive. This invasive tumour cells exhibit enlarged nuclei with angulated contours and prominent nucleoli. Whilst, in case of SCE treated Group IV & V, the formation of squamous cell carcinoma was significantly reduced. All of these symptoms were found to be minimal in SCE treated groups of animals.

Fig.7 (a-g) Photomicrograph showing histological sections of skin and tumor of mice of different groups. E- epidermis, D- dermis, B- basal Lamina, E.H- epidermal hyperplasia, S.G- sebaceous gland Ac- Acnathosis, K- Keratinocyte Pearl, Dy- Dyskeratosis, Rd- reduced stroma with lymphocytes, A.N- Atypical nuclei, T.N.- Tumor Nest



4. Discussion

Carcinogenesis, caused by physical, chemical, or viral mechanisms, is a multistage process of coordinated acquisition of favorable genetic lesions and complex interactions between tumor and host tissues that ultimately leads to an aggressive metastatic phenotype [20].

The onset of tumor promotion is marked by numerous biochemical alterations [21]. Since the pathological development of tumors in mankind takes a long time to pass through the preneoplastic and premalignant stages to actually become malignant. Therefore, the opportunity to reverse the development of tumor is always present. Resultantly, in recent years extensive research on cancer prevention is being encouraged. The chemopreventive strategy encompasses the use of agents with certain effects that diminish the carcinogenic process.

In the present study, anti-oxidative potential of a plant extract was analyzed against the carcinogenic action of DMBA/ Croton oil in mouse skin. The present results show a significant increase in lipid peroxidation level with a significant decline in GSH, catalase, SOD and Vit- C level in liver and skin by application of DMBA/ Croton oil in Swiss albino mice.

Our previous study indicated that tumor incidence was found to be 100% in carcinogen treated control animals. Administration of S.cumini seed extract significantly reduced the tumor

administration of SCE enhanced the level of antioxidant molecule GSH. It is well established that GSH, the most important biomolecule protecting against chemically induced cytotoxicity, can participate in the elimination of reactive intermediates by conjugation or by direct free radical quenching.

Antioxidant enzymes such as catalase, superoxide dismutase and glutathione-s-transferase and glutathione peroxidase are present in oxygen handling cells which are the first line cellular defense against oxidative injury decomposing O2 and H2O2 before they interact to form more reactive radicals [28-29]. SOD detoxifies the superoxide radicals to H2O2, which has been eliminated by CAT. In the present study, activity of SOD and catalase was increased significantly in the liver and skin of animals of SCE treated experimental Group IV & V than carcinogen treated control (Group III) but no significant changes were observed in the activity of catalase and SOD in liver and skin of SCE treated group II as compared to vehicle treated.

The increase in the activities of the antioxidant enzymes in the SCE treated mice compared to DMBA/ Croton oil treated indicates its effective antioxidant activity. Ethyl acetate and methanol extracts of the seeds of S.cumini showed the presence of alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, tannins and triterpinoids [30]. All the above mentioned results suggest the protective role of seed of S.cumini, which could be due to the antioxidative effect of flavonoids [31] present in the seeds of such plant, which act as strong superoxide radical and singlet oxygen quenchers.

Free radicals which are generated by DMBA, decrease the total protein content in liver and skin of mice of group IV & V, which could be elevated by the administration of S. cumini seed extract in experimental groups. A number of hydrolysable tannins including ellagitannins and 1-o-galloyl castalagin and casuarinin have been shown to have activity against cell carcinomas and tumour cell lines [32-33]. Saponins also form complexes with proteins and could decrease protein degradability [34].

Mice treated topically with DMBA and croton oil showed a significant decrease in vit-c level in liver and skin, whereas such level could be elevated by the administration of hydro-alcoholic extract of S.cumini seed extract. Vitamin C has been known to stimulate immune function, inhibit nitrosamine formation, and block the metabolic activation of carcinogens [Ki Won Lee et al.], its cancer-preventive effects may be associated mainly with its protective effects against oxidative stress. ROIs are major molecules that can cause cancer through multiple mechanisms [35].

Skin of negative control mice (Group I) showed uniformly arranged epidermis and dermis layer resting upon basal lamina. DMBA/croton oil induced skin tumors of Group III had abnormally thickened epidermis with irregular proliferation and deposition of keratinocyte pearls in dermis and epidermis. Hyperkeratosis i.e. thickening of keratinized layer over the epidermis was also observed in skin tumors in group III. Also the invasive growth of keratin inward into the epidermis was observed in skin tumors of this group. Administration of SCE at peri- and post- initiation stages, showed the inhibition of cell proliferation, further growth and promotion of skin tumors induced by DMBA/Croton oil. The extent of above lesions was considerably lesser in group IV & V (DMBA/Croton oil+SCE). Dyskeratosis and keratinocyte pearls were observed to be of much lower degree in skin tumors as compared to carcinogen treated control tumors.

5. Conclusions

The present data suggest that S.cumini has potential to modulate the 7,12-dimethylbenz(a)anthracene induced biochemical and histopathological alterations during skin carcinogenesis. Therefore it seems to be a valuable plant source for use in modern chemopreventive drug discovery and development.

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