INTRODUCTION

Cancer is one of the most important causes of death. In this disease, considerable suffering, mutilation and loss of many physiological processes occur. Cancer is not a single disease, but is a group of many different diseases that all share common biological and pathological characteristics. Cancer prevalence is still increasing, particularly in developing countries. It is the second leading cause of death in developed countries (Parkin, 2001). Chemoprevention is regarded most auspicious and realistic approach with enormous potential to prevent, suppresses, inhibit or reverse of initiation and progression phases of carcinogenesis. (Goldsmith, 2000; Manoharan, 2009). Cancer prevention is carried out by use of chemopreventive agent that blocks induction of the neoplastic process, or transformed cells from progressing to malignant phenotypes (Hong and Sporn, 1997; Ramakrishnan et al. 2007). Epidemiologic studies indicate that consumption of fruits and/or vegetables cause inverse association as well as prevention of carcinogenic process. Diets in general contain antinutmutagenic/anticarcinogenic compounds as well as mutagenic/carcinogenic agents. Nutritional substances work as antinutmutagens, chemical inactivators, enzymatic inducers, antioxidants and anticarcinogenic (Shukla and Pal, 2004).

Poorer intake of vitamin E, vitamin C, vitamin D, β-carotene, retinal and long-chain n-3 fatty acids and higher consumption of total fat have been related with increased skin squamous cell carcinogenesis in animal studies. Cancer-inducing oxidative damage might be prevented or limited by dietary antioxidants found in fruits and vegetables (Dam et al. 2000; Sun et al. 2002; Sarah et al. 2005; Vaid et al. 2014). Carissa carandas L. (Apocynaceae), popularly known as karaunda is an important medicinal plant of tropical and subtropical areas as well as an important crop of arid zones in India. Numerous therapeutic and biological activities as demonstrated include anti-diabetic, antimicrobial, cytotoxicity, anticonvulsant, hepatoprotective, anti-hyperlipidemic, cardiac depressant, analgesic, anti-inflammatory, antipyretic and antiviral have been reported in this plant (Hegde et al. 2009; Hegde and Joshi, 2009; Sumbul and Ahmed, 2012).

MATERIALS AND METHODS

The animal care and handling were done according to guidelines set by the Indian National Science Academy (INSA), New Delhi (India). The inhibition of tumor incidence by Carissa carandas fruit extract was evaluated on two-stage skin carcinogenesis, induced by a single application of DMBA (initiator) and two weeks later promoted by the repeated application of croton oil (promoter) thrice per week, following the protocol for 16 weeks.
Animals

The study was conducted on random-breed male Swiss albino mice with 7-8 weeks age and 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). These mice were fed a standard mouse feed procured from Aushirwad Industries, Chandigarh (India) and water ad libitum.

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 g/ 100 l 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 L. were collected locally after the competent proper identification (Voucher Specimen No: RUBL- 211416) from Herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan (India). The fruits were washed properly, shade dried and then after fruit was powdered in a mixture, and a hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) for 36 (12 x 3) hrs at 40°C. The extract was cooled and concentrated by evaporating its liquid contents. The prepared Carissa carandas extract (CCE) was stored at low temperature until its further use and it was redissolved in DDW prior to the oral administration in mice.

Experimental Design

The dorsal skin from interscapular region (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. Mice selected from inbred 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 CCE (100 µl/ mouse/ day) by orally for 16 weeks.

Group II: CCE treated Control

These Animals received CCE (50 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 g of DMBA in 100 l of acetone was applied topically over the shaven skin of the mice of these groups. Two weeks later croton oil (100 1 of 1% croton oil in acetone) was applied three times per week until the end of the experiment (i.e. 16 weeks).

Group IV: CCE treated Experimental-I (Peri-initiation)

Animals received hydro-alcoholic extract of Carissa carandas (50 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: CCE treated Experimental-2 (Post-initiational)

Animals of this group were administered orally CCE (50 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.

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 following biochemical parameters were estimated in the liver and skin of mice.

Lipid peroxidation (LPO)

The level of LPO was estimated Spectrophotometricaly by thiobarbituric acid reactive substances (TBARS) method as described by Ohkawa et al. (1979) in the liver and skin and the contents were expressed as nmol/mg of tissue.

Reduced Glutathione (GSH)

The level of GSH was estimated as total non protein sulphahydryl group by the method of Moron et al. (1979) in the liver and skin. Reduced GSH was used as a standard and the levels of GSH were expressed as mol/gm of tissue.

Catalase

The catalase activity was assayed by the method of Aebi (1984) in the liver and skin. The activity of the enzyme was expressed as U/mg of tissue, where U is mol of H2O2 disappearance/min.

Total Proteins

Total proteins were estimated by the method of Lowery et al. [1951] in the liver and skin using bovine serum albumin as a standard and the level was expressed as mg/ gm.

Superoxide dismutase

SOD was determined as the method of Marklund and Marklund [1974] in the liver and skin and its activity was expressed as units/min/mg protein.

RESULTS

Morphological study

Table 1 represents the results obtained from the present study. Mice administered the different doses of Carissa carandas extract survived throughout the experiment and did not show did not affect average body weight gain of mice in different groups. Carcinogen treated group showed considerably higher tumor appearance at different weeks of the experiment as compared to CCE treated experimental groups. Mice of vehicle treated control group did not show any incidence and appearance of tumors. In the positive control Group III, where a single topical application of DMBA was followed 2 weeks later by repeated application of croton oil, showed 100% tumor incidence; whereas mice belonging to experimental groups IV– V revealed skin tumor incidence as 80% and 70% respectively.
Oral administration of CCE during the peri-initiation stage (Group IV) and Post-initiation stage (Group V) of DMBA-induced tumorigenesis demonstrated reduced tumor yield to 3.80 and 2.90 (positive control value 6.50), tumor burden to 4.7 and 4.1 (positive control value 6.50) and the cumulative number of papillomas to 38 and 29 (positive control value 65) and higher average latent period 10.1 and 10.48 weeks (positive control value 8.72 weeks), respectively. The maximum inhibition of multiplicity of tumors was evident in Group V (55.38%) and followed by Group IV (41.5%) (Fig.1-6).

### Table 1. Chemopreventive effect of Carissa carandas extract (CCE) against DMBA-induced skin tumorigenesis in mice*

<table>
<thead>
<tr>
<th>Treatment Group*</th>
<th>Body weight (gm) (Mean ± S.E)</th>
<th>Tumor Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Initial 25.58±1.83 Final 34.23±1.23</td>
<td>2-5 6-9</td>
</tr>
<tr>
<td>Drug Control</td>
<td>26.29±1.19</td>
<td>- -</td>
</tr>
<tr>
<td>Positive control (Carcinogen treated)</td>
<td>25.46±2.40 Final 32.81±1.46</td>
<td>39 26</td>
</tr>
<tr>
<td>Exp. Group I (Peri-initiation)</td>
<td>25.30±1.3</td>
<td>23 15</td>
</tr>
<tr>
<td>Exp. Group II (Post initiation)</td>
<td>24.80±1.95</td>
<td>18 11</td>
</tr>
</tbody>
</table>

*Treatment schedule of the groups is specified in materials and methods.

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Figure 1. Variation in the cumulative no. of papillomas during DMBA-induced skin carcinogenesis with or without *Carissa carandas* extract (CCE) administration

Figure 2. Variation in the tumor incidence during DMBA-induced skin carcinogenesis with or without *Carissa carandas* (CCE) administration

Figure 3: Variation in the tumor Burden during DMBA-induced skin carcinogenesis with or without *Carissa carandas* extract (CCE) administration

Figure 4. Variation in the tumor yield during DMBA-induced skin carcinogenesis with or without *Carissa carandas* extract (CCE) administration
Biochemical Study

Relative to carcinogen treated control, the formation of malondialdehyde measured as index of lipid peroxidation in liver revealed a significant decrease in CCE experimental peri- and post initiational group (Group IV and V) from 2.88 fold \((p< .001)\) to 1.58 fold \((p<0.01)\) and 1.78 fold \((p<0.001)\), respectively. On contrary the level of MDA was found to be significantly increased in the skin of animals of carcinogen treated control mice (Group III) to 3.01 fold \((p<0.001)\), as compared to vehicle treated control (Group II). A significant decrease in the level of LPO was observed in the skin of CCE treated mice (Group IV and V) as 1.24 fold \((p<0.01)\) and 1.36 fold \((p<0.01)\) than the carcinogen treated control (Fig.7).

No significant increase in GSH was observed in the liver and skin of animals of Group II, which received CCE alone for 16 weeks as compared to vehicle treated control. The level of GSH was found to be significantly increased to 1.74 fold \((p<0.01)\) and 2.27 fold \((p<0.01)\) in liver of peri and post-initiation group (Group IV and V) respectively, in comparison to carcinogen treated control \((p<.001)\). A significant elevation in the activity of GSH was found in the skin of peri and post initiational group (Group IV and V) as 2.024 fold \((p<0.01)\) and 2.929 fold \((p<0.001)\) respectively while compared to carcinogen treated control (Fig.8).

The superoxide dismutase activity was significantly enhanced in the liver of peri and post initiation group (Group IV and V) as 1.25 fold \((p \leq 0.05)\) and 1.44 fold \((p \leq 0.01)\) respectively, as compared to carcinogen treated control. Whereas skin of CCE treated mice (Group IV and V) showed about 1.19 fold \((p \leq 0.05)\) and 1.33 fold \((p \leq 0.01)\) elevation in SOD levels as compared to Group III \((p<.001)\). There was no statistical significant difference was noticed in CCE treated Group II as compared to vehicle treated control (Fig.9). Peri and post-initiation (Group IV & V) showed a significant elevation in the activity of catalase in the liver by 1.26 fold \((p<0.01)\) and 1.38 fold \((p<0.01)\) respectively, on the contrary, increased the activity of catalase was noted in skin of mice of group IV and V by 1.27 fold \((p<0.05)\) and 1.49 fold \((p<0.01)\) respectively; whereas in carcinogen treated group, the same was found to be significantly decline in comparison to vehicle treated control \((p<0.001)\) (Fig.10). A significant decrease of 2.15 \((p<0.001)\) and 1.80 \((p<0.001)\) fold in the total proteins level was observed in the liver and skin of carcinogen treated control (Group III) respectively, as compared to vehicle treated control. However same was found to be significantly increased by 1.37 fold \((p<0.01)\) and 1.54 fold \((p<0.001)\) in the liver and 1.18 fold \((p<0.01)\) and 1.32 fold \((p<0.001)\) in skin of peri and post-initiation (Group IV & V), in comparison to carcinogen treated control (Fig.11).

**Figure 5.** Variation in the inhibition of tumor multiplicity during DMBA-induced skin carcinogenesis with or without *Carissa carandas* extract (CCE) administration

**Figure 6.** Variation in the average latent period during DMBA-induced skin carcinogenesis with or without *Carissa carandas* extract (CCE) administration

**Figure 7.** Variation in the Lipid per oxidation (LPO) during DMBA induced skin carcinogenesis with or without *Carissa carandas* extract (CCE) administration
DISCUSSION

One of the foremost causes of morbidity and mortality throughout the globe is Cancer. Carcinogenesis is a multistep molecular process induced by genetic and epigenetic, during this cell proliferation, apoptosis, differentiation, and senescence pathways interruption occurs (Jaffe, 2003; Lopez, 2010). 7,12-Dimethylbenz[a]anthracene (DMBA) a PAH, a tumor initiating chemical, which undergoes metabolic activation to become an ultimate carcinogen (Manoharan et al. 2012).

Figure 8. Variation in the reduced Glutathione (GSH) during DMBA induced skin carcinogenesis with or without Carissa carandas extract (CCE) administration

Figure 9. Variation in the Super Oxide Dismutase (SOD) during DMBA induced skin carcinogenesis with or without Carissa carandas extract (CCE) administration

Figure 10. Variation in the Catalase activity during DMB induced skin carcinogenesis with or without Carissa carandas extract (CCE) administration

Figure 11. Variation in the total proteins level during DMBA induced skin carcinogenesis with or without Carissa carandas extract (CCE) administration
Many enzymes concerned in DNA repair are destroyed by induction of DMBA and several type of cancer, which may be induced by DMBA in experimental animal models (Agrawal et al. 2010; Parmar et al. 2010; Chaudhary et al. 2011; Sharma et al. 2011; Sharma et al. 2013). Formations of free radicals are enhanced by TPA (active constituent of croton oil) which is applied tropically during chemical carcinogenesis (Huachen and Krystyna, 1991). Reactive oxygen species (ROS) has been concerned in the pathogenesis of several chronic diseases including cancer (Li et al. 2013).

Many studies have shown that lipid peroxidation induces disturbances and alterations of biological membranes (Niki, 2009). Therefore, it is an essential task for radical scavenging antioxidants to suppress lipid peroxidation. In the present study, MDA level was found to be a significant increase in liver and skin of DMBA/Croton oil induced mice, indicating that oxidative stress increased in the carcinogen treated liver and skin, while this level was markedly decrease in CCE administration mice. DMBA induced (Group III) mice showed significant depletion in both enzymatic antioxidants such as SOD, CAT, and non-enzymatic antioxidants such as GSH when compared to Vehicle treated Control (Group I) mice.

 Xenobiotics is detoxify by GSH through inactivation of chemical carcinogens into a less toxic form (Kaur and Arora, 2013). In the present study, GSH level was noted as significantly elevated in liver and skin of CCE treated mice (Group IV & V) with compared to DMBA/Croton oil treated mice. The lower amount of reduced glutathione with enhancement of lipid per oxidation in DMBA treated mice indicates that an increased consumption of antioxidant to counteract the elevated level of reactive oxygen species suggesting that the cells lack thiol group undergo fast lipid per oxidation. Such depletion of MDA level and non enzymatic antioxidants as GSH due to similar factors and in various plants has been investigated by the others (Chaudhary et al. 2007, Sharma et al. 2010; Kumar et al. 2010). In antioxidant defense system, SOD performs an essential role by converting superoxide radical into hydrogen peroxide by inhibiting reactive oxygen species (Li, 2000). Decreased level of CAT, SOD and Total proteins was observed in some tumorgenesis study (Sancheti et al. 2007; Jahan et al. 2009; Goyal et al. 2010). The present study also confirms such observations. Pharmacological studies on fruits of C. carandas revealed its potential use as anti-scorbutic agent and as a remedy for biliousness, anticonvulsant activity, hepatoprotective effect, cardiotonic effect and anticancer (Hegde et al. 2009; Hegde and Joshi 2009; Karunakar et al. 2009).

C. carandas have been used as a medicinal food or dietary supplement for centuries (Rai and Misra, 2005). Numerous biological active compounds have been reported from the fruit of such plant i.e. carisol, epimer of α-aminyl, linalool, β-caryophyllene, carisone, carassic acid, carindone, ursolic acid, carinol, ascorbic acid, lupeol and β-sitosterol (Devmurari et al. 2009; Shailajan et al. 2013). The present study established that C. carandas fruit extract has antioxidant and anticancer properties by enhancing antioxidant enzymes, so activate the defense system. It has potent free radical scavenging activity that could scavenge the superoxide and hydroxide radical and inhibit lipid per oxidation.

In conclusion, the results of the present investigation, it could be suggested that the ethanol extract of Carissa carandas fruit part exhibit potent anticancer and antioxidative activities. These facts indicate the scientific basis of Carissa carandas being used as a traditional medicine. However, further experiments may help to determine the pharmaceutical potentialities of the plant as a preventative medicine against cancer.

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REFERENCES


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