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IN VITRO EVALUATION OF ANTIOXIDANT ACTIVITIES AND CHEMOPREVENTIVE POTENTIAL OF *DILLENIA INDICA LINN* FRUIT ON DMBA INDUCED SKIN PAPILLOMAGENESIS IN MICE

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ABSTRACT: *Dillenia indica* linn fruit, commonly known as elephant apple has indigenous use in various culinary dishes of Assam and it has a history of being used as traditional medicine. The present study was undertaken to evaluate its antioxidant and chemopreventive properties. Ethanolic extract of *Dillenia indica* fruit was used as experimental sample. Antioxidant activity was studied in vitro, at concentrations (20µg/ml, 40µg/ml ,60µg/ml, 80µg/ml, 100µg/ml) where it showed significant ($p<0.05$) inhibition of free radicals formation, hydroxyl radical and nitric oxide scavenging property and also reducing ability when compared with a standard antioxidant, ascorbic acid. Chemopreventive potential was evaluated in 8-10 weeks old swiss albino mice by giving a single tropical application of initiator- 7,12-dimethylbenza(α)anthracene (DMBA) at a concentration of 100µg/100µl acetone and promotion after 14 days by repeated application of croton oil (1% in acetone) thrice a week for 15 weeks. Oral application of *D.indica* at dose of 250 mg/kg body weight of mice selected after acute oral toxicity test, showed significant ($p<0.05$) reduction in DMBA induced mice skin papillomagenesis in (i) post-initiation group (oral dose given on the day of promotion) and (ii) pre+post initiation group (oral dose given 7 days before initiation and continued till the end of experiment). There was reduction in the total papilloma count, tumor yield, tumor burden and tumor incidence when compared with carcinogen control group. Inhibition of tumor formation upto 88.3% was found after 15 weeks of treatment which clearly shows the antitumor property of *Dillenia indica* fruit.

INTRODUCTION Cancer development is a multi-factorial, multi-staged and multi-mechanistic complex process [1]. After decades of multidisciplinary scientific investigations to combat cancer it still remains a major cause of mortality and it has been estimated in the year 2015 by American Cancer Society that everyday 1620 Americans die due to cancer [2].

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Exposure to various xenobiotic chemicals in air, water, soil and food, occupational exposure and lifestyle factors have resulted in increasing episodes of various health problems of which cancer is one major problem [3]. Cancer chemoprevention is a concept defined as the prevention of cancer by the administration of natural or synthesized pure chemicals or by daily food intake which are rich in cancer chemopreventive components.

Newer therapeutic strategy for cancer treatment involves molecular targeting of oncogenic signaling elements that are activated during cancer initiation and progression, thus getting rid of the

tumor initiating cells [4]. Inhibition of TGFβ signaling pathway is an emerging strategy for cancer therapy where prevention can be taken at early stage of tumor formation [5].

Naturally occurring chemopreventive agents such as antioxidants and phytochemicals in vegetables and fruits which are consumed by human population in their daily diets are gaining considerable attention [6]. This compounds are able to induce cellular detoxifying antioxidant enzymes and also apoptotic cell death in pre-neoplastic or neoplastic cells through different growth inhibitory mechanisms [7].

Combination of an effective phytochemical with chemotherapeutic agent enhances the efficacy by reducing cytotoxicity and thus enhances therapeutic efficacy [8]. Synergistic effects of antioxidant activity of phytochemicals present in fruits or vegetables may also contribute towards the chemopreventive potential of that plant [9, 10]. It has been reported that over 55-60% of drugs in clinical trials for anti-cancer activity were either isolated directly from natural sources or compounds derived from active natural products [11].

Dillenia indica L. belonging to the Dilleniaceae family is a common evergreen tree that grows widely in tropical forests of Assam, India. *Dillenia indica* fruit is sour in taste and is widely and favorably used in preparation of curries. The fruit had been used traditionally in relieving stomach pain, regulates heat in the body, tones up nervous system, removes fatigue, has cardiotonic effect [12] and its antidiarrhoel activity has also been reported [13]. The mixed juice of leaves, bark and fruits are taken orally for the treatment of cancer and diarrhea in some areas of Mizoram, India [14].

Previous studies reported *Dillenia indica* fruit to be antidiabetic, hypolipidemic [15], strong analgesic and has anti-inflammatory [16] effect but the chemopreventive property of the edible part of the plant i,e the fruit of *Dillenia indica* on DMBA induced skin papillomagenesis in mice model has not yet been reported. Natural compounds of dietary sources and of local origin is mostly preferred for chemoprevention because they are easily available to that locality, non-toxic, cost-effective and has no side-effects. Consumption of this compounds as food in daily diet will definitely delay the onset of cancer development [17].

Due to the presence of various medicinal properties and common use of *Dillenia indica* fruit as food in various communities of Assam, the present study was undertaken to investigate the antioxidant property of *Dillenia indica* fruit. Further the chemopreventive potential of the ethanolic extract of *Dillenia indica* fruit had ben studied *in vivo* on 7,12 dimethylbenz(α) anthracene (DMBA) induced skin papillomagenesis in swiss albino mice where croton oil had been used as promoting agent.

MATERIALS AND METHODS:

The experiments were performed in the year 2014-2015 at Department of Biotechnology, Gauhati University, Assam

The plant material:

The fruit of *Dillenia indica* was collected during the month of July and August from various locations around Guwahati, Assam, India. The plant was taxonomically identified by a plant taxonomist, Department of Botany, Gauhati University, Guwahati, Assam.

The extraction procedure:

The fruit of *Dillenia indica* were cut into pieces, washed, air and shade dried. It was then powdered by a mechanical grinder, passed through a sieve and stored in air tight container. The dried and powdered plant material was extracted with 80% ethanol in soxhlet apparatus at 60^0c and continued until the solvent run clear in the thimble. The solvent was then filtered in Whatman filter paper no 1. The solvent was then removed from the extract under vaccum rotary evaporator at 45^0c and a semi solid mass was obtained. This extract was stored at 4^0c for assessment of invitro antioxidant activity and mice skin papillomagenesis study. The percentage yield of the extract was determined by using the following formula.

$$\% \text{ yield} = \frac{\text{Weight of the extract (mg)}}{\text{Weight of the whole tissue powder (mg)}} \times 100$$

Chemicals:

Chemicals for *in vitro* antioxidant assay:

Ascorbic acid(used as standard), Potassium ferricyanide, Trichloroacetic acid, Ferric chloride, EDTA, Deoxyribose, Sodium hydroxide, Butylated hydroxyanisole, Sodium nitroprusside, Griess reagent (Sulphanilamide, Phosphoric acid, N-1-naphthylethylenediamine dihydro chloride). DPPH (2,2, diphenyl - 2 - picryl hydrazyl) and Thiobarbituric acid was purchased from Sigma Aldrich USA and the rest were purchased from SRL and Merck , India.

Chemicals for mice skin papillomagenesis

DMBA (7,12 dimethylbenz(a)anthracene) and Croton oil; both the chemicals were obtained from Sigma Chemicals Co. (St Louis, USA).

The test animals:

Randomly bred Swiss albino mice (*Mus musculus*) of both sexes were received from Assam College of Vetenary Sciences, Khanapara, Guwahati, Assam. The animals were maintained in the animal house, Department of Biotechnology, Gauhati University. The animals were housed in clean polypropylene cages under conventional laboratory condition with 12 hr light and dark cycle. The animals were provided with standard pellet feed and tap water under hygienic conditions. All the animals were handled as per the guidelines of Animal Ethical Committee of Gauhati University.

Experimental Design:

Both the experiments *in vitro* antioxidant assay and skin papillomagenesis in mice were carried out separately.

***In vitro* antioxidant assay**

DPPH test (Free Radical Scavenging Property):

Stock solution of DPPH was prepared in ethanol(33 mg in 1 ltr). 5 ml of DPPH solution was added to 1 ml of extract solution of different concentrations (0-100µg/ml) and incubated for 30 mins. Absorbance was measured at 517 nm and compared with standard of same concentration. Antioxidant activity is expressed as IC_{50}. IC_{50} is concentration in µg/ml of extract that inhibits formation of DPPH radical by 50%.

Reducing power assay:

Various concentration of *D.indica* extract was prepared in ethanol (0-100µg/ml) and mixed with 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50^0c in hot water bath for 20 mins. 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 mins. 2.5 ml of upper layer was taken and mixed with 2.5 ml of distilled water and then 0.5 ml of freshly prepared ferric chloride solution was added. Absorbance was measured at 700 nm and compared with the standard of same concentrations.

Hydroxyl radical scavenging property:

A reaction mixture was prepared by adding 0.1 ml of 1mM EDTA, 0.01 ml of 10mM $FeCl_3$, 0.1 ml of 10 mM H_2O_2, 0.36 ml of 10 mM Deoxyribose, 1 ml of extract solution of different concentration (0-100 µg/ml) mixed in ethanol, 0.33 ml of 50mM Phosphate buffer pH 7.4. The reaction was then incubated at 37^0c for 1 hr. 1 ml of 10% TCA and 1 ml of 0.5% TBA was added. The reaction mixture was then heated at 95^0c for 15 mins and then cooled. After that absorbance at 532 nm was taken. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation.

Nitric oxide scavenging property:

10mM Sodium nitroprusside in phosphate buffer saline was mixed with different concentration of extract solution (0-100µg/ml) in ethanol and incubated at 25^0c for 1hr and 30 mins. 1.5 ml of incubated solution was taken and to it 1.5 ml of Griess reagent was added. Absorbance was then taken at 546 nm.

Acute oral toxicity study of test material:

The acute oral toxicity of the ethanolic extract of *Dillenia indica* fruit was determined in Swiss albino mice. Mice were divided into five sets with three mice in each group. Each group of animal were given different doses of 100,200, 300, 400 and 500 mg/kg body weight respectively of ethanolic extract of *Dillenia indica* fruit orally. After administration the mice were kept under observation for 72 hrs to find their behavioural changes, locomotion, convulsion and mortality.

Skin papillomagenesis in mice:
Mice of both sexes and 8-10 weeks of age were taken for the experiment. Skin papillomagenesis in swiss albino mice was induced by single tropical application of initiator, DMBA (7,12 dimethylbenz (α)anthracene) (100µg/100µl of acetone) on dorsal intercapsular region of the mice where hairs were clipped off before three days. Croton oil (1% in acetone) as promoting agent was applied to that region after two weeks and was repeated thrice a week till the end of the experiment. The extract was diluted in normal saline to give a dose level of 250 mg/kg body weight of mice per week.

The animals were divided into six groups comprising of six mice in each group:

Group I (negative control): This group of animals received normal diet daily.

Group II (carcinogen control): A single dose of DMBA (100ug /100ul of acetone) was applied over the shaven area of the mice. After two weeks 100 ul of croton oil (1% w/v in acetone) was applied three times per week till the end of the experiment.

Group III (pre-initiation): This group of animals received application of DMBA and croton oil as in Group II and they were administered with *Dillenia indica* ethanolic extract orally at a dose of 250 mg/kg body weight; 14 days before application of DMBA and stopped after application of carcinogen.

Group IV (post-initiation): This group of animals received application of DMBA and croton oil as in Group II and they were administered with *Dillena indica* ethanolic extract orally at a dose of 250 mg/ kg body weight from the day of croton oil application till the end of experiment.

Group V (pre + post initiation): This group of animals received application of DMBA and croton oil as in Group II and they were administered with *Dillenia indica* ethanolic extract orally at a dose of 250 mg/kg body weight; 7 days before application of DMBA and continued till the end of the experiment.

Group VI (positive control): This group of animals were administered with *Dillenia indica* ethanolic extract orally at a dose of 250 mg/kg body weight and no carcinogen treatment given.

The experiment was carried out for 15 weeks after the application of croton oil. The number of papilloma appearing on the skin of mice were counted weekly for 15 weeks and the following parameters were observed to study the influence of *D.indica* on mice skin carcinogenesis.

1. Cumulative number of papillomas
2. Tumor yield. (average number of papillomas)
3. Tumor burden. (papillomas per papilloma bearing mice)
4. Tumor incidence. (percentage of papilloma bearing mice)
5. Percent inhibition of tumor multiplicity

Statistical analysis:
All the results of antioxidant assay was done in triplicates and were expressed as mean ± S.D. Two tailed unpaired Student's t-test was used to test the significance of differences between the results obtained for the extract and standard. A probability value of less than 0.05 was considered significant. In mice skin papillomagnesis study statistical difference between the experimental groups and the carcinogen control groups were determined by One Way Analysis of Variance (ANOVA) at 0.05 significance level. The analysis were done by using SPSS software, IBM Corporation, New York.

RESULTS AND DISCUSSION:

Percentage yield of the extract:
The percentage yield of the ethanolic extract of *Dillenia indica* fruit was found to be 5.6% (w/w)

***In vitro* antioxidant assay:**
DPPH test: The ethanolic extract of *Dillenia indica* showed significant inhibition ($p<0.05$) in formation of free radicals when compared with the standard ascorbic acid. At concentration of 100µg/ml the scavenging activity of the *D. indica*

extract on the DPPH radical was found to be 85.67% and the standard ascorbic acid was found to be 93.24% (**Fig. 1**). IC_{5O} value for *D. indica* extract was 46µg/ml and IC_{50} value of the standard ascorbic acid was 41µg/ml.

Reducing power assay:

The reductive property was measured by observing the ability of the antioxidant to transform potassium ferricyanide to potassium ferrocyanide which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. There was significant increase ($p<0.05$) in reductive capability of the *D.indica* extract as compared with the standard ascorbic acid (**Fig.2**).

Hydroxyl radical scavenging property:

The reaction generates hydroxyl radicals which degrade deoxyribose using Fe^{2+} salts as an important catalytic component. There was significant scavenging ($p<0.05$) of hydroxyl radical by the fruit extract of *D.indica*. With the increase in concentration of *D.indica* extract the hydroxyl radical scavenging property increased and at 100µg/ml the scavenging activity of *D.indica* extract was 71.18% and the standard ascorbic acid was 71.52% (**Fig. 3**).

Nitric oxide scavenging activity:

Sodium Nitroprusside at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. At 100µg/ml concentration the nitric oxide scavenging activity of *D. indica* was 34.54% and the standard ascorbic acid was 38.17%. (**Fig.4**). The scavenging ability of the *D.indica* extract increased significantly ($p<0.05$) with the increase in concentration and was comparable with the standard ascorbic acid.

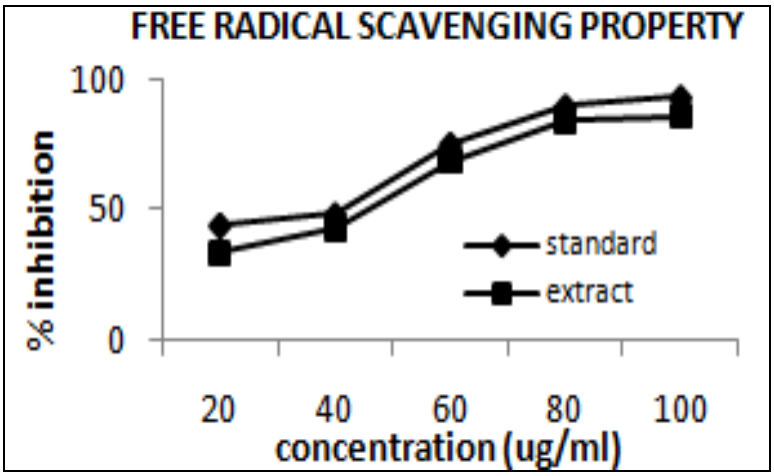

FIG. 1: FREE RADICAL SCAVENGING PROPERTY OF *DILLENIA INDICA* FRUIT EXTRACT AND STANDARD ASCORBIC ACID.

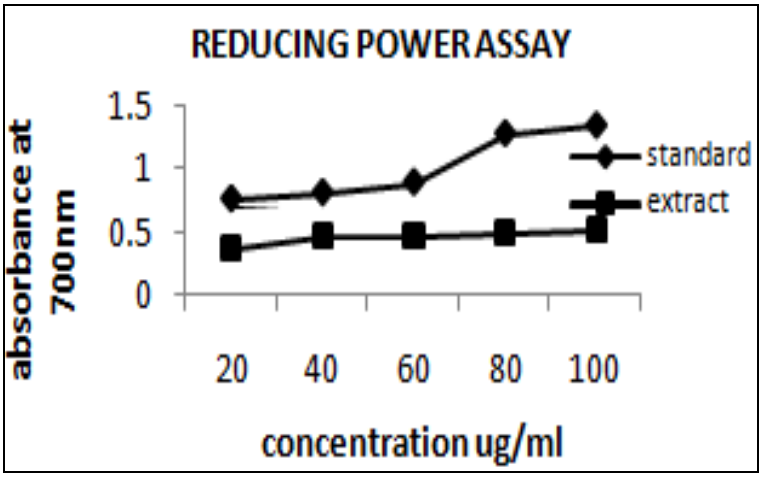

FIG. 2: REDUCING ABILITY OF *DILLENIA INDICA* FRUIT EXTRACT AND STANDARD ASCORBIC ACID. All Values Were Expressed as Mean ± S.D and $P<0.05$

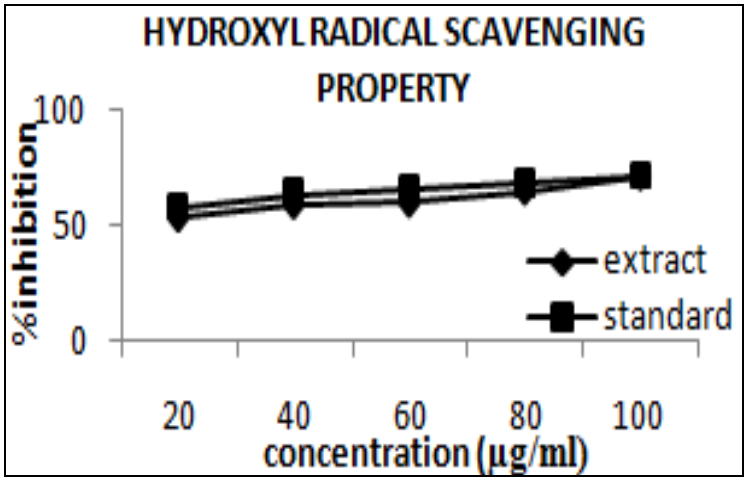

FIG. 3: HYDROXYL RADICAL SCAVENGING PROPERTY OF *DILLENIA* INDICA FRUIT EXTRACT AND THE STANDARD ASCORBIC ACID

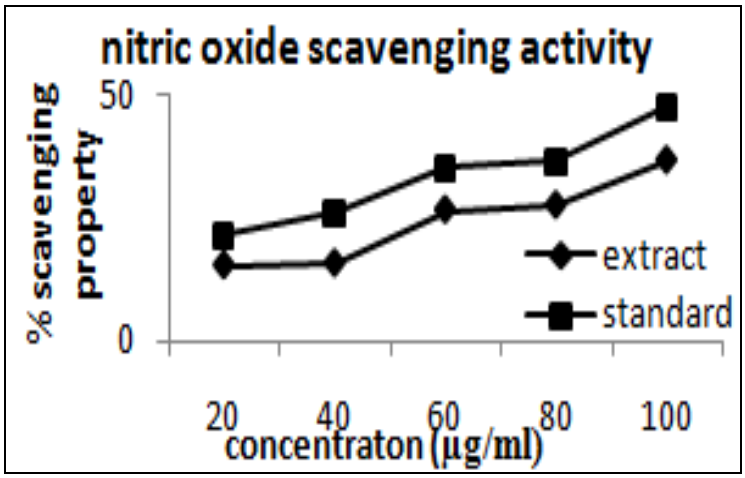

FIG. 4: NITRIC OXIDE SCAVENGING PROPERTY OF *DILLENIA INDICA* FRUIT EXTRACT AND OF THE STANDARD ASCORBIC ACID

Acute oral toxicity test

The acute oral toxicity of the ethanolic extract of *Dillenia indica* fruit was determined in Swiss albino mice for selection of working dose. No mortality, loss of body weight, change in locomotion, convulsion or any other behavioural change had been observed in the test animals.

After 72 hrs of observation it was found that the extract upto 500 mg/kg body weight of mice was non-toxic.

Skin papillomagenesis in mice:

The dose of 250 mg/kg body weight of mice had been selected for the carcinogenesis study Papillomas started appearing on the shaven intercapsular region of the mice from the 6th week after the application of croton oil. Papillomas whose size was more than 1mm in diameter and persisted for more than one week had been considered. The different treatment schedule and oral application of *D.indica* fruit extract did not alter the mortality rate and the average body weight gain of the six groups of mice during the experimental period.

The findings of the skin papllomagenesis study had been depicted from **Fig. (5-9)**. Oral application of *D.indica* fruit extract on GroupIV(post-initiation) and Group V (pre+post initiation) mice reduced the cumulative number of papillomas to 30 and 11 on the 15th week significantly ($p<0.05$) when compared with Group II (carcinogen control) whose value was 109 (**Fig. 5**). The tumor yield of Group IV and Group V reduced significantly ($p<0.05$) to 5 and 1.8 respectively when compared with Group II whose value was 18.2 (**Fig. 6**). The tumor burden of Group II was 18.2 however in Group IV and Group V tumor burden reduced to 5 and 2.7 respectively (**Fig. 7**).

In case of Group III(pre-initiation) and Group V (pre+post initiation) tumors started appearing from the 7th week. In Group V inhibition was seen throughout the treatment but in Group III inhibition in papilloma formation was there only upto the 9th week. This might be because of the application of the fruit extract for 14 days in Group III and stopped before tumor initiation. This indicates that the fruit of *D.*indica has strong chemopreventive potential. The total number of papillomas, tumor yield and tumor burden in Group III was found to be 103, 17.2 and 17.2 respectively on the 15th week which showed that there was no inhibition since the oral application of the fruit extract was stopped.

There was 100% tumor incidence in Group II, III and IV on the 15th week but in Group V the tumor incidence was 66.6% (**Fig. 8**). The percent inhibition of tumor multiplicity increased in the treated groups when compared with the carcinogen control group. Considering the inhibition to be 0 % in carcinogen control group the percentage of inhibition in Group III, Group IV, Group V was found to be 5.5, 72.4 and 88.3 respectively(**Fig.9**).

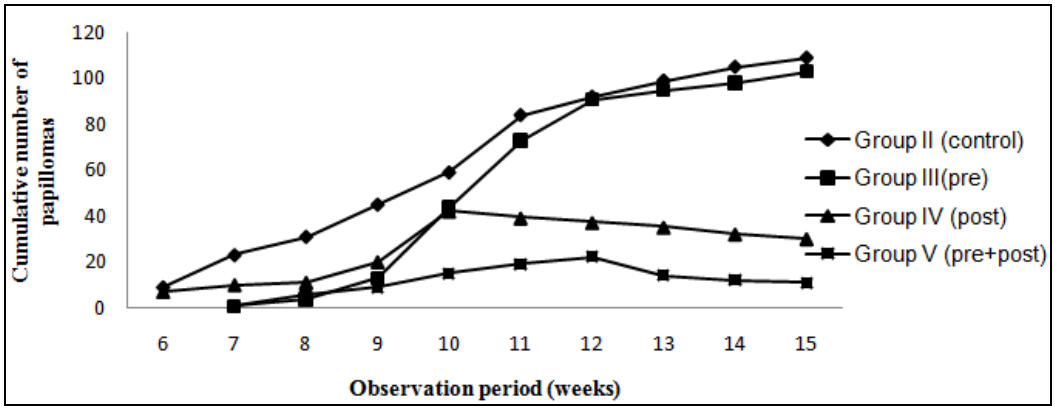

FIG 5: CUMULATIVE NUMBER OF PAPILLOMAS OF THE FOUR GROUPS (II, III, IVAND V) OF MICE ON WEEK BASIS.

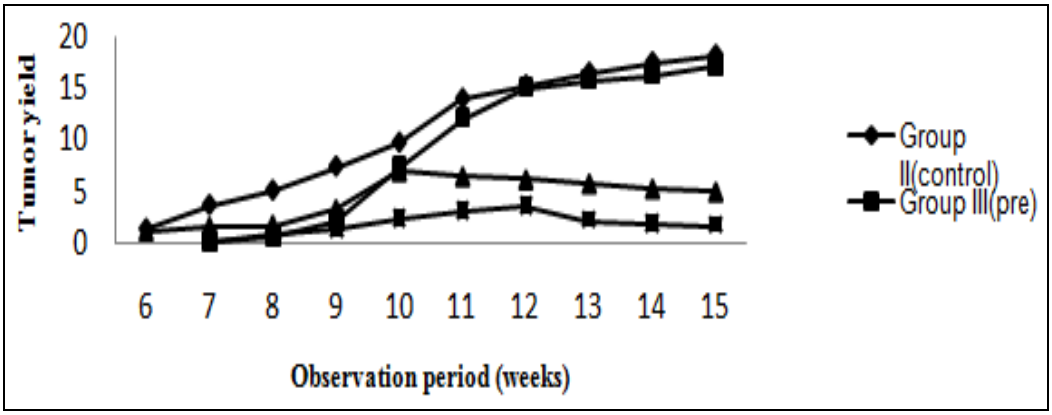

FIG 6: TUMOR YIELD OF THE FOUR GROUPS (II, III, IV AND V) OF MICE ON WEEK BASIS.

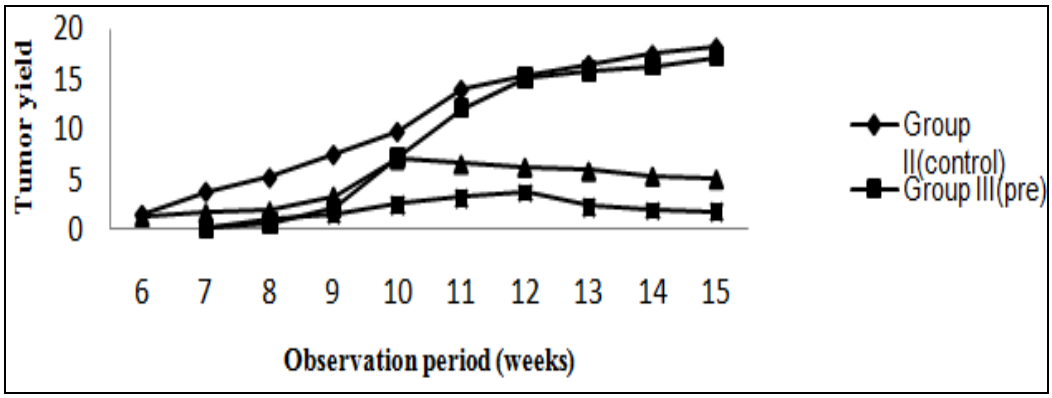

FIG 7: TUMOR BURDEN OF THE FOUR GROUPS (II, III, IV AND V) OF MICE ON WEEK BASIS

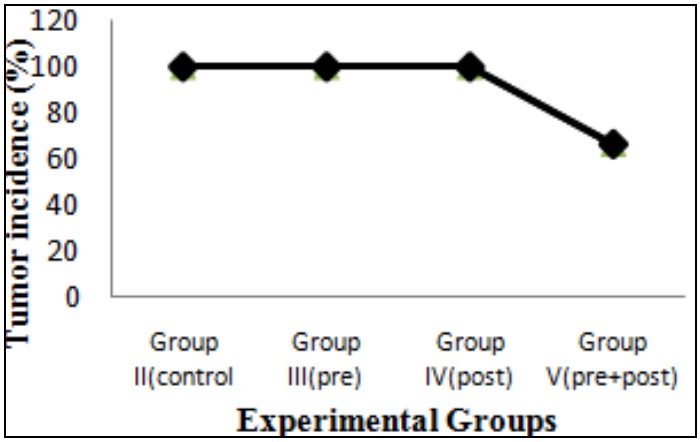

FIG 8: TUMOR INCIDENCE IN CONTROL AND TREATED GROUPS OF MICE ON THE 15TH WEEK

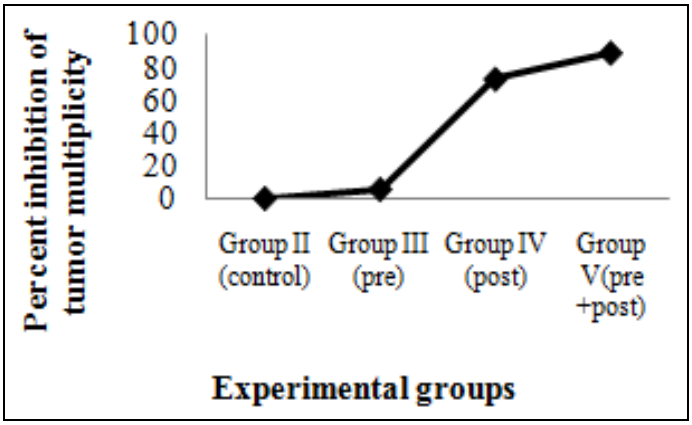

FIG 9: PERCENT INHIBITION OF TUMOR MULTIPLICITY OF TREATED AND CONTROL MICE ON THE 15TH WEEK

DISCUSSION: Free radicals are produced in the body during normal metabolic functions. Though free radicals are removed from the body via extensive protective mechanism normally present in the body but absence or defect in the proper removal of free radical species caninduce serious damage and if not repaired over a period of time can cause serious tissue injury ultimately inducing tumor formation[18]. Oxidation is one of the most important route for producing free radicals in food, drugs and even in living systems. The imbalance between oxidants and antioxidants is one of the main reason for many chronic diseases [19]. So the search for natural product which can either block or reverse the process of carcinogenesis has become very important as it can be developed as a promising anti-cancer agent [20].

The ethanolic extract of *D.indica* showed significant inhibition in formation of free radicals, scavenging of hydroxyl and nitric oxide radical increased along with the increase in concentration. The reducing ability was also found to increase with increasing concentration. Phytochemical analysis of fruit of *D.indica* showed the presence of about 34% of total phenolics in methanolic extract [21]. Also presence of flavanoids, sterols, glycosides, saponins, free acids and tannins were reported [22].

This phytochemicals may constitute towards the antioxidative ability of the fruit extract. The fruit has antioxidant properties as evident by our study which may contribute to its antitumor activity as considerable laboratory evidence from cell culture and animal studies indicate that antioxidant may slow or possibly prevent the development of cancer. In the present study we have demonstrated the chemopreventive potential of *D.indica* on

DMBA induced skin carcinogenesis in swiss albino mice. Skin Carcinogenesis in mice model involves two-stages- initiation and promotion where initiation is accomplished by a single application of sufficiently small dose of carcinogen and promotion requires repeated and prolonged exposure to promoter [23]. DMBA, used as initiating agent is carcinogenic and it has the ability to cause irreversible damage in DNA. It alters

procarcinogens into active carcinogens and induce DNA damage which eventually lead to carcinogenic response [24]. The promoter croton oil is the most potent promoting agent for mouse skin which act as skin irritant and cause inflammation ultimately leading to hyperplasia of the epithelium of skin but its activity is reversible and so repeated application is required [25].

The present study showed that the oral application of *Dillenia indica* fruit during the initiational and promotional stage of papillomagenesis significantly reduced the occurance of papillomas, tumor yield, tumor burden, tumor incidence in mice when compared with the carcinogen control group. There was inhibition of tumor formation upto 88.3% which clearly shows the antitumor property of *Dillenia indica* fruit. Crude drugs are more safe then synthetic drugs and this are mainly used as supplements. For chemoprevention it is important that this drugs are consumed in small quantities for a longer period of time [26].

For a potent chemopreventive agent, the presence of phytochemicals having strong antioxidant property which can quence reactive oxygen species, chelate metal ions and generate membrane bound antioxidants is necessary [27]. The antitumor property can be attributed to the presence of antioxidant properties and also to the phytochemical betulinic acid present in the fruit of *D.indica.* Previous studies reported the presence of betulinic acid in *D.indica* fruit which was responsible for anti-leukemic activity in human leukemic cell lines U937, HL60 and K562 [28]. Betulinic acid is a natural product that exhibit potent antitumor activities by triggering the mitochondrial pathway of apoptosis [29] and it has selective cytotoxicity towards cancer cell lines [30]. Betulinic acid in combination with irradiation under hypoxic condition showed increased cytotoxicity in human malignant glioma cells in a dose dependent manner [31]. Induction of apoptosis in human colon carcinoma HT-29 cells by a potent betulinic acid derivative obtained from *Dillenia indica* has also been reported [32].

Antitumor activity of an antioxidant is due to its ability to inhibit inflammatory responses, hyperproliferation, oxidative stress, and endogenous proinflammatory cytokines. Molecular mechanism of chemoprevention lies in down regulation of inflammatory mediators such as cyclooxygenase-2(COX-2), inducible nitric oxide synthase (iNOS), ornithine decarboxylase, Interleukin-6 expression which can be mediated directly by reduction of NF-κB activation[33]. Betulinic acid derived from birch tree *Betulla spp* has shown anti-fibrotic effect by inhibiting NF-κB signaling pathway [34]. This provide a strong evidence towards the molecular mechanism of chemoprevention by *D. indica* is due to the presence of betulinic acid which reduces NF-κB activation but this require further investigation.

D.indica had been used in various ailments for long time but its use as anticancerous agent can provide a new insight in the field of cancer prevention as chemoprevention seeks to eliminate precancerous cells in order to avoid the necessity of chemotherapy. Its ability to scavenge free radicals in vitro and its chemopreventive potential in DMBA induced mice skin papillomagenesis suggests that use of the fruit in our food will help the normal people to keep themselves protected from the onset of cancer to some extent. Nevertheless, extensive study is needed to elucidate the exact mechanism underlying this prevention effect.

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CONFLICT OF INTEREST: The authors have no potential conflict of interest.

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