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RESEARCH ARTICLE

Anticarcinogenic Screening of Terminaliaarjuna in swiss albino mice

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Terminalia arjuna bark extract against 7, 12 - dimethylbenz (a) anthracene (DMBA) induced papillomagenesis in Swiss albino mice was studied. The ethanolic bark extract of *Terminalia arjuna* was analyzed for chemopreventive activity.Chemopreventive activity was evaluated by two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) followed by a promoter (croton oil) two times in a week were employed**.** A significant reduction in tumor incidence, tumor burden and cumulative number of papillomas was observed, along with a significant increase in average latent period in mice treated tropically with *Terminalia arjuna* bark extract as compared to the control group treated with DMBA and croton oil alone. Significant elevation in the level of reduced glutathione ($p<0.05$) was noted in the group treated *Terminalia arjuna* bark extract in comparison with the negative control group. Conversely, lipid peroxidation levels were significantly decreased ($P<0.05$). The above studies reveal information about the prevention of cancer. Therefore, the present study is immensely important in future drug development programs for the cancer treatment.

1 Introduction

Cancer is a major killer disease against which no unified treatment concept has emerged so far. Surgery, radiotherapy and chemotherapy often remain the methods of choice in the treatment of cancer. A major hurdle in treating cancer patients using chemotherapy is the severe side effects. Radiation produces reactive free radicals, which cause DNA damage leading to cell death and genomic damage in the stem cells. Antioxidants, which can scavenge the free radicals, are considered for chemo protector. The failure of research efforts to obtain more effective and low cost chemo protector drugs using the synthetic compounds has turned the focus of research towards the natural products in the past decade.Most cancer prevention research is based on the concept of multistage carcinogenesis (Fig. 1.): **initiation→promotion→progression**[(1,2)]. In contrast to both the initiation and progression stages, animal studies indicate that the promotion stage occurs over a long time period and may be reversible, at least early on. Therefore, the inhibition of tumor promotion is expected to be an efficient approach to cancer control [(3,4)]. Cancer chemoprevention is defined as the use of specific natural and synthetic chemical agents to reverse or suppress carcinogenesis and prevent the development of invasive cancers. There has been a growing awareness in recent years

that dietary non-nutrient compounds can have important effects as chemopreventive agents, and considerable work on the cancer chemopreventive effects of such compounds in animal models has been undertaken. A number of common medicinal plants have good antioxidant properties and therefore may act as chemoprotector and radioprotector.

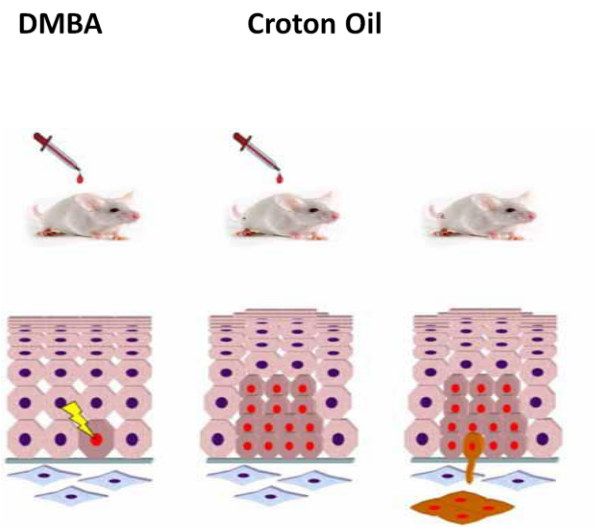


Figure 1: Showing initiation, promotion and progression

Scientists all over the world are concentrating on the herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body.
Terminalia arjuna is a medicinal plant of the genus *Terminalia* and Family Combretaceae, is widely used by Ayurvedic physicians for its curative properties in organic/functional heart problems including angina, hypertension and deposits in arteries. According to Ayurvedic texts it also very useful in the treatment of any sort of pain due a fall, ecchymosis, spermatorrhoea and sexually transmitted diseases such as gonorrhoea.Arjuna bark *(Terminallia arjuna)* is thought to be beneficial for the heart. The bark of *Terminalia arjuna* is one such Ayurvedic remedy that has been mentioned in many ancient Indian medicinal literature including CharakaSamhita and Astang Hridayam, to possess cardio protective property [(5).]clinical studies have proved that dried bark powder of this plant have potent hypolipidemic and cardioprotective activity [(6-10)]. The bark powder of *T. arjuna* has also been found to improve antioxidant status in the patients of coronary heart disease and these beneficial effects may be related to its high flavonide content[(11)].

2.Materials and Methods:

2.1Chemicals

7, 12 - Dimethylbenz (a) anthracene (DMBA), croton oil, reduced glutathione (GSH), 5.5/ - dithio-bis-2-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA) , TCA were obtained from Sigma Chemicals Co. (St. Louis, MO. USA). The other chemicals were obtained from local firms and were of the highest purity. DMBA was dissolved in acetone at a concentration of 104ug/100ul and croton oil was diluted in acetone to give a 1% dilution.

2.2Animals

Random bred male Swiss albino mice (7- 8 weeks old), weighing 24 ± 2 gm were used for the experiments. These animals were housed in polypropylene cages in the animal house at temperatures of 24 ± 30^{o}C. The animals were provided with standard mice feed and tap water ad libitum.

2.3 Preparation of *Terminaliaarjuna*extract

Plant material (*Terminalia arjuna*) was collected locally and identified and the specimen was authentified at Department of Botany, Safia college,Bhopal(MP), India. The voucher number is 500/bot/safia/2014. Bark was washed, air dried, powdered and extracted separately, with 95% ethanol and by refluxing for 36 hr (12 x 3) at 40^{0}C.

Extract thus obtained were vacuum evaporated to make it in powder form. These extract was again dissolved in DDW just before topically application.

2.4 Experimental design for Skin Carcinogenesis

The dorsal skin on the back area of the animals was shaven 1 day before the commencement of the experiment and only those animals in the resting phase of the hair cycle were chosen for the study. For induction of tumors a two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) followed by a promoter (croton oil) two times in a week were employed as per our previous modified method of Berenblum[12]reported elsewhere[13]. The animals were randomly allocated into 7 groups comprising six mice each. The treatment was provided topically on shaved area

2.4.1 Treatment Groups

Group 1 (Untreated control): No treatment

Group 2 (Vehicle control): 100 µl acetone 2 times /week up to 16 weeks

Group 3 (DMBA Alone): - 104 µg DMBA was dissolved in 100 µl acetone and single application was given.

Group 4 (Croton Oil Alone): - 1 % Croton oil was applied on skin 2 times a week up to 16 week.

Group 5 (*Terminalia arjunabark* Extract Alone): - was applied on skin 2 times a week up to 16 week.

Group 6 (DMBA + Croton Oil): - 104 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards 1 % Croton oil was applied on skin 2 times a week up to 16 week.

Group 7 (DMBA + *Terminaliaarjunabark* Extract + Croton Oil): -

104 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards the 100 µl dose of***Terminaliaarjunabark*** extract at the dose of 500 mg/kg b. wt.dose was given one hour before the each application of 1% croton oil 2 times a week up to 16 weeks.

The animals of all groups were kept under observation for gross and microscopic changes in skin. During the period of 16 weeks of experimentation, mice of all groups were weighed carefully examined once a week for skin papillomas and these were recorded. The following parameters were taken into consideration:

2.4.2 Tumor study:

Body weight: Change in mean body weight was measured weekly.

Tumor incidence: The number of mice carrying at least one tumor expressed as percent incidence.

Cumulative number of papillomas: Total number of tumors bearing mice.

Tumor yield: The average number of papillomas per mouse.

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

Average latent period: The lag between the application of the promoting agent and the appearance of 50% 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

Average latent period = Σfx/n

where f is the number of tumors appearing in each weeks , x is the numbers of weeks and n is the total number of tumors.

2.5 Biochemical Study:

Biochemical alterations were studied in all the groups at the time of termination of the experiment (i.e., at 16th week).

2.5.1 Preparation of Homogenates

Animals were killed by cervical dislocation and the entire liver was then perfused immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free of extraneous tissue. It was then weighed and blotted dry. For assaying reduced glutathione it was homogenized in ice-cold Tris- KCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. A 0.5 ml aliquot of this homogenate was used for assaying readuced glutathione. For assaying lipid peroxidation this tissue was homogenized in ice-cold 1.15% KCl to yield a 10% (w/v) homogenate. A 0.8 ml aliquot of this homogenate was used for assaying lipid peroxidation.

2.5.2Determination of Reduced Glutathione (GSH)

Hepatic level of reduced glutathione was determined by the method of Beutler[14]. Reduced glutathione was used as a standard to calculate µ mole GSH/100 gm tissue.

2.5.3 Estimation of Lipid Peroxidation (LPO)

The lipid peroxidation level was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkawa[15]and is expressed in terms of malondialdehyde (MDA) formed per mg protein.

2.6 Data Analysis: The differences in the incidence of tumors among different groups were considered to be significant at 5% significance level ($p<0.05$) when evaluated by Student's 't' test.

3. RESULTS:

3.1 Effect of *Terminalia arjun abark* extract on DMBA induced skin Papillomagenesis:

The findings of the present study are depicted in Tables I and Graphs I-V. Animals of Group- VI (control) in which a single topical application of DMBA, followed by croton oil produced skin papillomas, which started appearing from the 5th week onwards. The incidence in DMBA/croton oil treated mice (carcinogen control) reached 100% by the termination of the experiment (i.e. 16 weeks).

In the skin papilloma model, significant prevention of tumor incidences was observed in the *Terminalia arjuna bark* extract treated experimental groups (66.67% and 50 % in group VII) as compared to carcinogen control (100 %) group. The cumulative number of papillomas was also reduced in the *Terminalia arjuna bark* extract treated experimental groups (11 in group VII) as compared to carcinogen control (35) group. The tumor burden and tumor yield were significantly decreased (2.7 and 2.1) as compared to DMBA treated control (5.84) group.

Average latency period was significantly increased with *Terminaliaarjunabark* bothextract treatment (11.45±3.7)in compared to carcinogen control group (8.60±1.78).

3.2Antioxidant Enzymes:

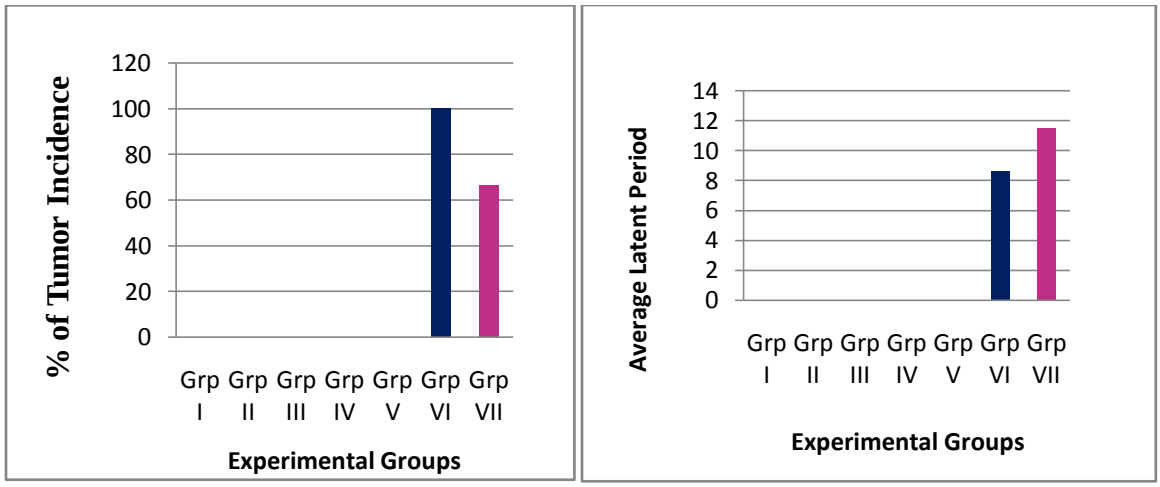
Significantly lower reduced glutathione (GSH) activity was noted in the skin of carcinogen control mice (group VI) as compared with *Terminalia arjuna bark* extract treated experimental animals (groups VII) at the time of termination of the experiment (ie,16 weeks). Treatment with *Terminalia arjuna bark* extract resulted in enhanced levels of GSH ($P<0.05$) in the group. A considerable elevation of LPO level was noted in the skin after DMBA and croton oil treatment, where as administration of *Terminalia arjuna bark* significantly reduced the level of LPO in all the *Terminalia arjuna* treated experimental groups (VII) in comparison with the carcinogen (control group VI).Results are depicted in Table **II and III** and Graph **VI – IX.**

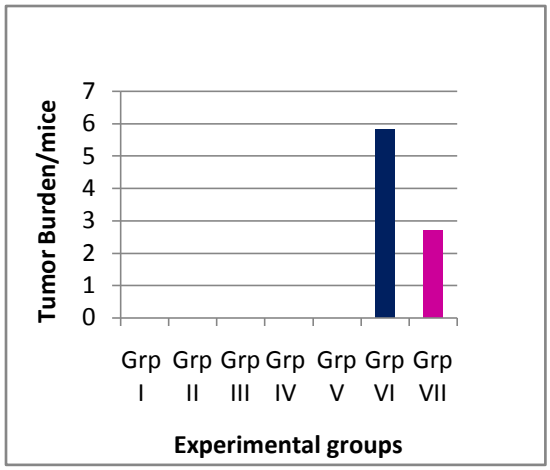
Table I: Chemopreventive Action of *Terminaliaarjunabark extract* on DMBA induced Skin Carcinogenesis in *Swiss albino* Mice

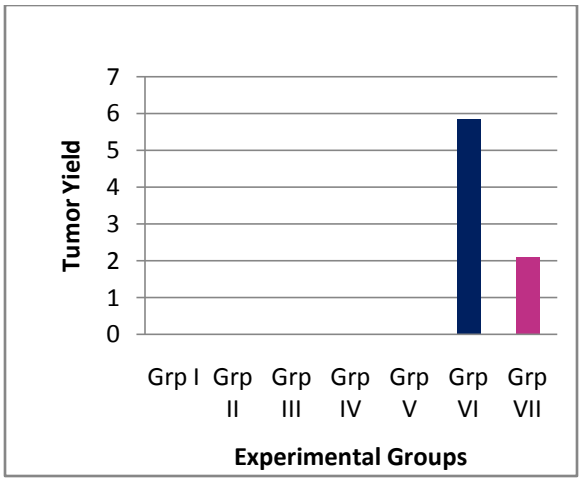
Groups	Treatment	Body weight (Mean±SEM)		Cumulative no. of Papilloma	Tumor Incidence (%)	Tumor Burden	Tumor Yield	Average Latent Period
		Initial	Final					
I(n=6)	Untreated Control	27.6±2.2	26.8±2.8	-	-	-	-	-
II(n=6)	Vehical alone	25.0±1.3	29.3±1.3	-	-	-	-	-
IIII(n=6)	DMBA alone (100µl acetone)	27.6±2.2	26.8±2.8	-	-	-	-	-
IV (n=6)	Croton oi alone (104µg/100µl acetone)	26.7±1.6	30.3±1.9	-	-	-	-	-

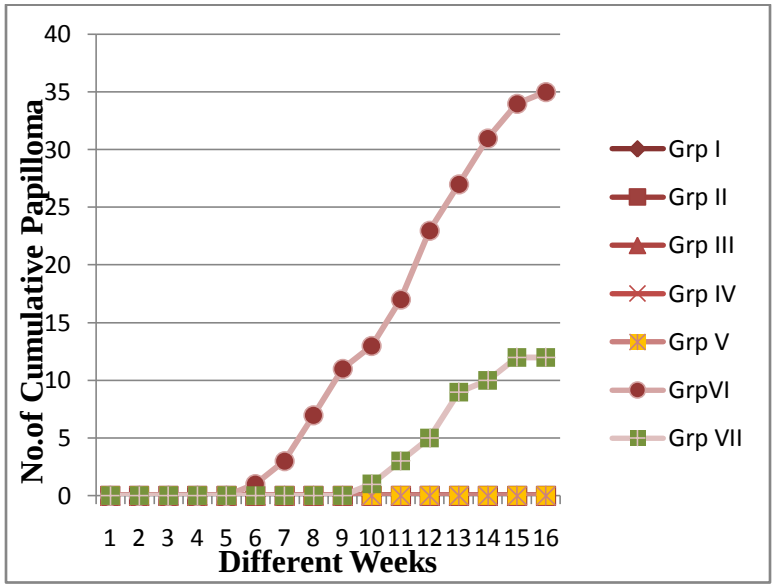
***V*(n=6)**	*T.arjuna* extract alone (500mg/kg b.wt.)	27.0±1.5	29.9±1.1					
VI(n=6)	DMBA(104µg/100µl acetone)+Croton oil(100µl of 1% concentration)	20.74±1.2	25.99±0.6	35	6/6 100%	5.84	5.84	8.60±1.78
VII(n=6)	DMBA + Croton oil + **TAE**(topical treatment)	27.35±0.39	28.9±0.5	11	4/6 66.6%	2.7*	2.1*	11.45±3.7

(*) denotes statistically significant value as compared to untreated group at $p<0.05$









Graph I,II,III,IV&V showing the tumour incidence, average latent period, tumour burden, tumour yield and cumulative no. of papillomas.

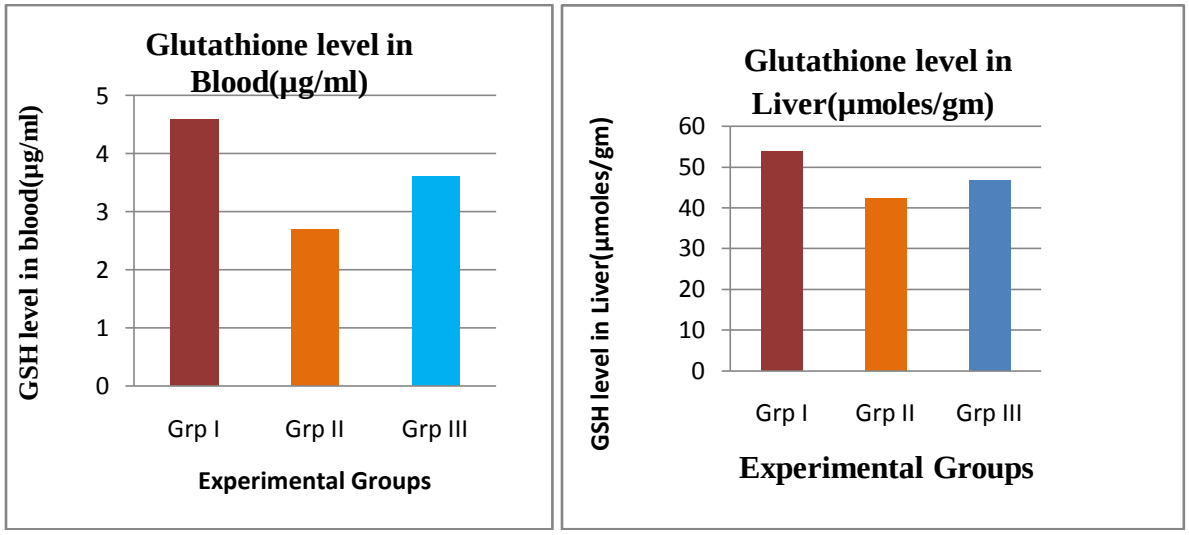
Table II: Effect of *Terminaliaarjuna*Extract on Glutathione Levels

S.No	Groups	Treatment Group	Glutathione level

			Blood (µg/ml)	Liver (µg/ml)
1	I.	Normal mice	4.58±.0.65	53.8±.3.34
2	II.	Carcinogen control (DMBA+CO)	2.7±0.06	42.3±1.3
3	III.	DMBA+TAE+CO	3.68±0.72	46.8±0.95

*Significance level among different groups at p< 0.05. Carcinogen control v/s *Terminaliaarjuna*extract experimental

Effect of *Terminaliaarjunabark* extracts on Glutathione Levels :

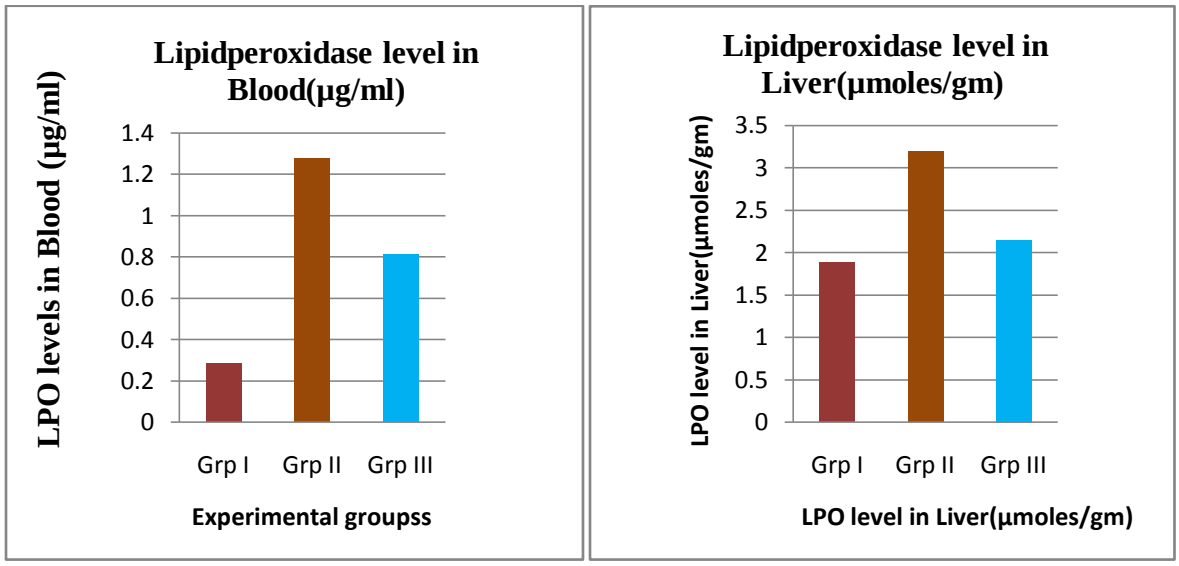


Graph VI ,VII showing the variation in the GSH levels and Lipid Peroxidation level during DMBA-induced skin carcinogenesis with/without *Terminalia arjuna*extract treatment

Table III. Showing the level of Lipidperoxidase (LPO) in Blood and Liver of Papilloma bearing Swiss albino mice receiving treatment of *Terminaliaarjuna* extract.

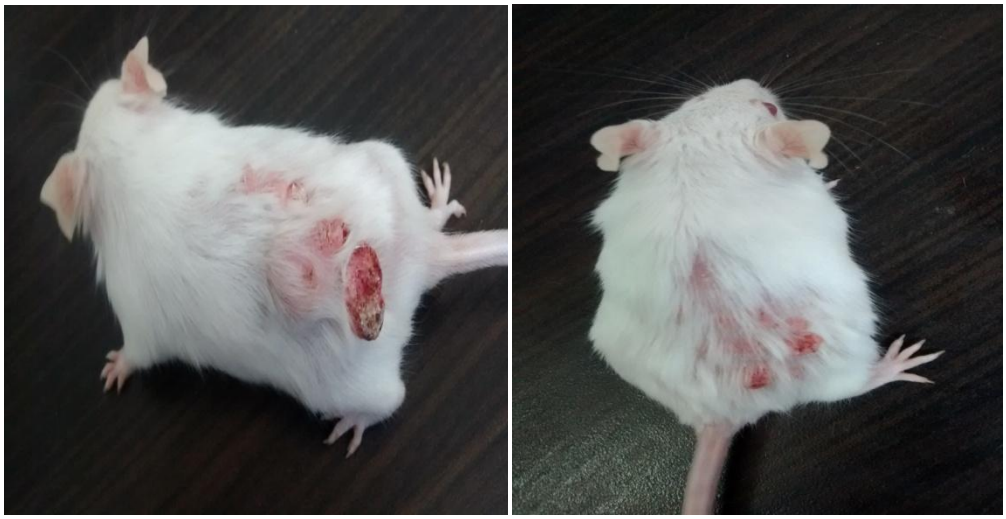
S.No	Groups	Treatment Group	Lipid Per oxidase

			Blood (µg/ml)	Liver (µmoles/gm)
1	I.	Normal mice	0.184±0.09	0.886±0.03
2	II.	Carcinogen control (DMBA+CO)	1.28±0.06	3.19±0.06
3	III.	DMBA+TAE+CO	0.813±0.02	2.15±0.08



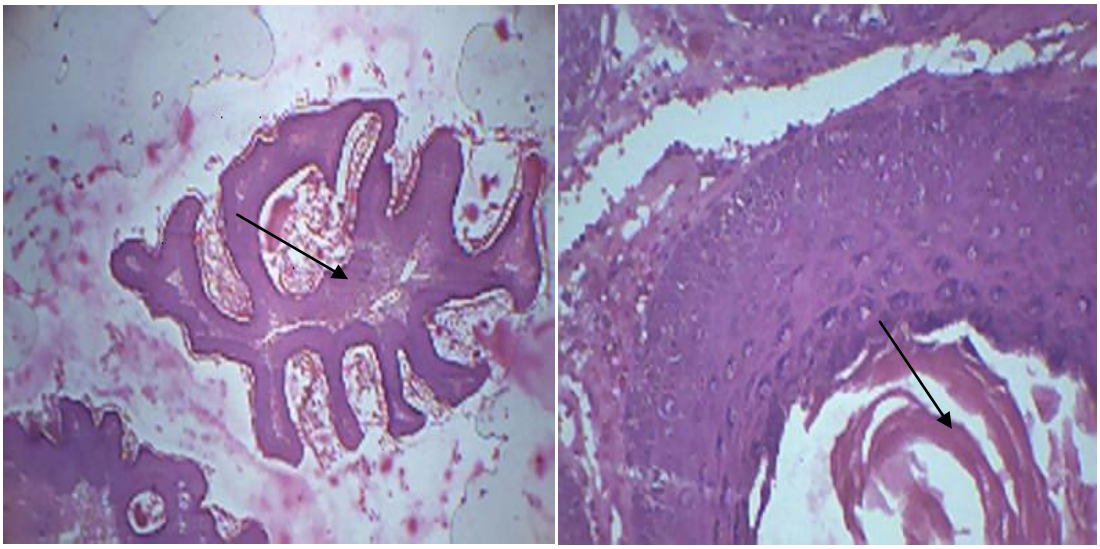
Graph VIII, IX showing the variation in the Lipid Peroxidation level during DMBA-induced skin carcinogenesis during *Terminalia arjuna*extract treatment

Photograph showing the skin tumour induced by DMBA + Croton oil for 16 weeks

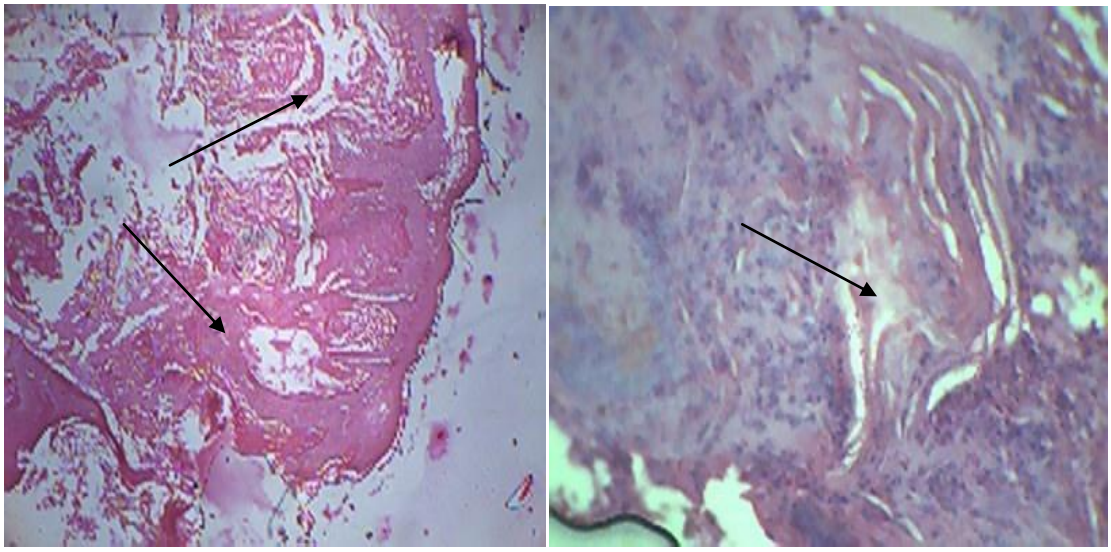


DMBA+ Croton oil **DMBA+TAE+Croton oil**

HISTOPATHOLOGY STUDY



(I) **(II)**



(III) **(IV)**

Section I shows Papillary projection with hyperplasia. (H &E stain, 10 X)
Section II shows Keratin Pearls. (H &E stain, 100 X)
Section III shows Papillary formation including Lesions & Projections. (H &E stain, 40 X)
Section IV Section D showing malignancy in skin tumor. (H &E stain, 40 X)

Photo 4.3.1.2.Histology of skin of mice.

4. Discussion:

The induction of cancer (carcinogenesis) is a multistage process and depends on inherited and acquired susceptibility factors, on exposure to initiation factors, i.e., exogenous and endogenous carcinogens, and on promotion and progression factors.Cancer chemoprevention can be defined as prevention by administration of chemical entities, either as individual drugs or as naturally occurring constituents of the diet. Chemoprevention has earned serious consideration as a mean of controlling cancer incidence, as it is no longer merely a theoretical strategy, but an approach yielding more impressive experimental and clinical results [(16)].

Tumor incidence, cumulative number of papillomas, tumor yield, tumor burden, tumor weight and tumor size were found to be decreased in all the experimental mice (groups VII) treated with*Terminalia arjuna* extract.This fall may be due to factors such as inhibition of DMBA metabolism to its active form or delay in the promotion phase of tumorigenesis via down regulation in the production of ROS and inhibitory effects on tumor promoter-induced epidermal ODC activity.Because reactive oxygen species have been implicated in premature skin aging, carcinogenesis, DNA damage, activation of signal transduction pathways related to growth differentiation and cell death, it is assumed that antioxidants could act as potential anticarcinogens at multiple stages of skin carcinogenesis [(17)]. Treatment of *arjuna* extracts increase reduced glutathione level and decrease malondialdehyde formation than treated animals of control group.Antioxidants such as GSH, cysteine and a tocopherol were shown to prevent the TPA- mediated decrease in the ratios of reduced to oxidized glutathione in mouse epidermal cells [(18)]. The increased glutathione reductase level plays a significant role in the reduction of oxidized glutathione to reduced glutathione at the expense of NADPH and regulates GSH-GSSG cycle in the cell [(19)]. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also detoxifies reactive oxygen species directly and/or neutralizes reactive intermediate species generated from exposure to xenobiotics including chemical

carcinogens [20]. GSH has been endowed with an important function in maintaining the reduced state of cellular environment, in addition to its conjugating ability owing to nucleophilic center and its involvement in detoxification of xenobiotics that cause toxicity and carcinogenicity. Such a mechanism would decrease the level of reactive electrophiles available to bind DNA, reducing the likelihood of DNA damage and possible induction of carcinogenic process [21]. Glutathione, often regarded as the first line of defense against oxidative stress, is the most important cellular thiol that acts as a substrate for several transferases, peroxidases and other enzymes that prevent the deleterious effects of oxygen free redicals[22]. The multiple physiological and metabolic function of GSH includes thiol transfer reactions that protect cell membranes and proteins. GSH participates in reactions that destroy hydrogen peroxide, organic peroxides, free radicals and certain foreign compounds. The apoptotic processes in cells are often associated with decreased levels of GSH due to increased efflux of this antioxidant from the cells [23]. Furthermore, the decreased lipid peroxidation which is measured by thiobarbituric acid reactive substances (TBARS) in the liver homogenate of *arjuna* treated mice, is correlated well with the induction of antioxidant enzymes above basal level. A wide range of plant products are source of antioxidants and act as modifiers of the carcinogenic process, appear to be the right approach for modifying cancer risk in the population [24]. The supplementation or topical application of synthetic agents viz. retinoids, vitamins, inhibitors of ornithine decarboxylase, cyclooxygenase, lipoxygenase and other antioxidant compounds including thiols and minerals have gained much attention on one hand while the use of natural agents like polyphenols, monoterpens, flavonoids, organosulfides, indoles, etc. have shown promise for their development as chemopreventive agent against skin cancer [25-27].

5. Conclussion:

Humans have used plants as foods and natural medicines since ancient times. Crude drugs, typically safer than synthetic drugs, have been used as both spices and supplements. Natural medicines have been used as anti-cancer agents by inhibiting the promotion process, and it is important that these are consumed in small quantities for extended periods of time. The study of cancer prevention using plants is generating vast amounts of information regarding their benefits. This paper provides, an outline of studies focusing on plant extracts. Several active components have been isolated, and their chemical structures have been and continue to be determined. In addition, structure-activity relationships, elucidation of physiological activities at the molecular level, and development of strategies that allow for the production of sufficient supplies of these agents are issues for further investigation. The continued search for natural medicines is necessary for finding additional sources of active components that are suitable for clinical application. For this purpose, we will harness the strength of researchers from various fields with the goal for cancer prevention.

From the present study, it is evident that *Terminalia arjuna* the Indian medicinal plant, is a source of many anti-carcinogenic agents and antioxidants, which may be useful for the prevention of chemical induced skin cancer in mice. This work demands further study to evaluate the exact mechanism of chemoprevention offered by ***Terminalia arjuna***constituents as well as its possible chemopreventive efficacy against other types of tumors in various models.

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