Original Research Article

"Evaluation of *In-Vivo* Anticancer Activity *Choerospondias Axillaris* in Swiss Albino Mice Models"

ABSTRACT

Background and Objective: The fruits of the plant, *Choerospondias axillaris* are one of the richest sources of flavonoids phenol and vitamin C that have been shown to possess a variety of biological activities. The present study was aimed to study the potential anti-cancer activity of the methanolic extract of fruit of *Choerospondias axillaris* using the *in-vivo* model to scientifically validate the folkloric use of the *Choerospondias axillaris*.
Study design: *In-vivo* model
Place and duration of the study: Department of pharmacology, Karnataka College of pharmacy, Bangalore India, between October 2021 to June 2022.
Methods: Anti-cancer property of *Choerospondias axillaris* was evaluated against DMBA/croton oil induced skin tumorigenesis in Swiss albino mice. A single topical application of DMBA (100g/100l of acetone), followed 2 weeks later by repeated application of croton oil (1% in acetone three times a week) for 16 weeks. In contrast, animals treated orally with *Choerospondias axillaris* 200mg/kg/b.w. (group IV) and orally with *Choerospondias axillaris* 400mg/kg/b.w. (group V) and 5 Flu 10mg/kg (group III). The following parameters like; body weight, tumor incidence, cumulative number of tumors, tumor yield, average latency period, number of papillomas, haematological parameters, Serum Zinc and C-Reactive Protein, anti-oxidants enzyme, pro-inflammatory cytokines & Histopathological of Tumor skin studies were observed.
Results: 100 percent tumor incidence exhibited in group II (DMBA/croton oil) whereas the group IV (*Choerospondias axillaris* 200mg/kg) and group V (*Choerospondias axillaris* 400mg/kg) and group III (5 Flu 10mg/kg) exhibited 50, 33.7 and 42.5% tumor incidence, which significantly lesser when compared to than group II (Toxic control). The cumulative number of papillomas during the observation period of 16 weeks was significantly decreased in the *Choerospondias axillaris* treated groups IV, V and III (9, 4 and 6 no's tumor respectively) in comparison to 18 cumulative numbers of papillomas in carcinogen control group. The average latent period significantly increased in the Toxic control group to 3.2, 4.3, 6.5 and 5.4 in group II, IV, V and III respectively. Tumor yield were significantly lesser (1.5, 0.66 and 1.0) as compared to DMBA/Croton oil treated control. Furthermore, the level of LPO was significantly increased whereas, the activity of CAT level were significantly decreased in skin tissue of DMBA/Croton oil treated animals compared *Choerospondias axillaris* treated animals. Similarly, NLR (< 3.0), ESR, Serum Zinc and CRP have got improved in treated with *Choerospondias axillaris.* Protein expression of TGF-beta, IFN-G, TNF-alpha and IL-6 have shown Improvement in markers indicating a reduction in inflammation and immune imbalance in treated with *Choerospondias axillaris* compared with DMBA/Croton oil where shown moderate immune suppression.
Conclusion: Based on the results it can be concluded that the test drug *Choerospondias axillaris* could be a potential candidate for the treatment of skin cancer.

Keywords: Skin cancer, DMBA, Croton oil and *Choerospondias axillaris*.

1. INTRODUCTION

Cancer is a major health problem that can debilitate and destroy human lives. The development of cancer involves multiple steps, which occurs over several years after the first exposure from normal to hyperplasia, mild, moderate, and severe dysplasia.[1] Different cancer prevention strategies such as behavioural modification, vaccines, surgical manipulation, and chemoprevention have evolved with tremendous research efforts.[2] Many investigations have proven that healthy lifestyles involving balanced diets, regular exercise, smoking cessation, alcohol reduction, weight control, and stress management are beneficial for decreasing cancer risk and can never be overemphasized.[2-6] Cancer is one of the leading causes of mortality worldwide and the failure of conventional chemotherapy to affect major reduction in the mortality indicates that new approaches are critically needed.[7] Over the past few years, cancer has remained a major cause of death and the number of individuals affected with cancer is continuing to expand. Hence a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets. Naturally occurring antioxidants such as vitamins, micronutrients and other plant products are nontoxic or markedly lesser toxic than synthetic chemo preventive agents and are constituent of human diet. Herbal medicines are the oldest remedies known to mankind. Herbs had been used by all cultures throughout history but India has one of the oldest, richest and most diverse cultural living traditions associated with the use of medicinal plant.[8]

Lapsi (*Choerospondias axillaris*) is a large, deciduous, edible native fruit tree of the family Anacardiaceae. Native to hilly region in Nepal (850–1900 m), the tree has also been reported from India, China, laos, japan and East Asia. *C. axillaris* is also known as Hog plum that can grow up to 30 meters tall. Lapsi is a wild, indigenous fruit tree of multiple benefits. Different researches have reported that plants with constituents like quercetin, protocatechuic acid, gallic acid, beta- sitosterol and octacosanol have potent to enhance the immunity and neutralize free radicals formed in the body. Fruits of *C. axillaries* have been reported to possess several properties for treatment of myocardial ischemia, calming nerves, ameliorating blood circulation. Several studies have described the antioxidant properties of medicinal plants, foods, and beverages which are rich in phenolic compounds. The plant has the functions of anti- inflammation, detoxification, haemostasis and many more.[9]

As the nature provides best of the cure provided with examples above, much attention should be gathered to formulate them for a better cancer free world. This rising interest in the anticancer and antioxidant activities of the existing natural drugs has leaded us to investigate the DMBA/Croton oil induced skin cancer study of extract of *Choerospondias axillaris*.

2. MATERIALS AND METHOD:

2.1 Collection and Preparation of plant material:

The fruits of the plant *Choerospondias axillaris* were collected from the hilly area of Nepal, in the month of October 2021. This plant species were authenticated by Prof Dr. Hari Datta Bhattarai, Department of Botany, Tribhuvan University, Nepal and the reference number was 382/078/079. The collected plant material (Fruits) was washed thoroughly with water to remove the adhering soil, mud and debris. All old insect damage or fungus infected fruits were removed. The plant material was dried in the shade at room temperature to a constant mass. The plant material was coarsely powdered using blender. The powder was stored in an air tight container and was protected from light.

2.2 Extraction of the plant material and sample preparation:

The coarse ground powder of Choerospondias axillaris was transferred into the extraction glass and the plant material was loaded into the main chamber of the Soxhlet extractor. Then this part of the extractor is connected into the round bottom flask containing extraction solvent. The grinded coarse powder was packed tightly in the Soxhlet extractor and methanol solvent was used for the extraction of the Choerospondias axillaris fruits powder. In this extraction process, each 100gm powder was subjected to extraction with 1000ml methanol in a reflux condenser for 3 cycles of 7hrs. The extract was again re-extracted under the same conditions to ensure complete extraction. The methanol was filled into the

solvent vessel and extracted at a temperature of 75□C for 6 hrs. The solvent was drained into a beaker by opening the spigot on the Soxhlet extractor. The solvent was removed from the extractor and dried. The extract was then stored in dry airtight bottles for the pharmacological studies. The portion of the extract which is non- soluble remains in the thimble and it was discarded.[10-14]

2.3 Experimental animals:

Adult 7-8 weeks old male Swiss albino mice weighing 24±2 g were used for conducting this study. They were acclimatized for one week prior to experiment. Animals were caged in fully ventilated room, were maintained in 12:12 h light and dark cycle and were housed at temperature of 25 ± 2°C. They had free access to a standard chow diet and water ad libitum. All the experiments conducted on the animals were in accordance with the standards set for the use of the laboratory animal use and the experimental protocols were duly approved by the IAEC (Institutional Animal Ethical Committee) of Karnataka College of pharmacy, Bangalore. (IAEC Reg. Number: KCP/IAEC/09/21-22/14/18-12-21).

2.4 Experimental Design:

2.4.1 Acute toxicity studies for Dose Fixation:

Previously reported and the acute oral toxicity study was performed according to the OECD guidelines no. 425.[15]

2.4.2 Preparation of Dose:

Dose: Selection of dose was done on the basis of acute toxicity OCED guideline 425. 200mg/kg and 400mg/kg were selected and the further study was carried out.

2.4.3 Induction of skin cancer

Skin of 3 x 3 cm^2 back area of animals was shaven three days before the commencement of experiment, and only those animals in the resting phase of hair cycle were selected for the study. A total of 50 selected animals were randomly divided into six groups (I, II, III, IV, V & VI) to evaluate chemo preventive role of Plant Extract against DMBA/croton oil induced skin papilloma genesis.

Group-1 [DDW treated mice (Normal control)]:

Animals [N=6] of this group were given DDW (10ml/kg body weight), a normal diet and tap water ad libitum daily. After 16 weeks, mice were autopsied and the skin of dorsal area (3 x 3 cm2) was taken for the biochemical and Histopathological studies.

Group-2:-[DMBA/Croton oil (Toxic control)]:

Animal (N=6) in this group receives a single dose of DMBA (100g / 100l of acetone)[16] over 3 x 3 cm^2 shaven area of the mice skin as initiator. Two weeks later, croton oil (1% in 100l of acetone) was applied, as a promoter three times in a week for 16 weeks.

Group-3:-[5-Fluorouracil (10mg/kg body weight)]:

Animals were received 5-Fluorouracil (10mg/kg body weight, S.C.)[17] for 2 weeks before DMBA application. After 2 weeks, DMBA (100µg/100µl of acetone) was applied 12 followed by topical application of croton oil (1% in 100µl of acetone) 3 times a week until the end of the experimental period up to 16 weeks.

Group-4:- [Choerospondias axillaris orally (200mg/kg.b.w)]:

Animals were received Choerospondias axillaris (200mg/kg.b.w/day)[15] orally for 2 weeks before DMBA application. After 2 weeks, DMBA (100µg/100µl of acetone) was applied and followed by topical application of croton oil (1% in 100µl of acetone) 3 times a week until the end of the experimental period up to 16 weeks. Choerospondias axillaris was given orally after 2 hrs. of croton oil application until the end of experimental period up to 16 weeks.

Group-5:- [Choerospondias axillaris orally (400mg/kg.b.w.)]:

Animals were received Choerospondias axillaris (400mg/kg.b.w./day)[15] for 2 weeks before DMBA application. After 2 weeks, DMBA (100µg/100µl of acetone) was applied and followed by topical application of croton oil (1% in 100µl of acetone) 3 times a week until the end of the experimental period up to 16 weeks. *Choerospondias axillaris* was given orally after 2 hrs. of croton oil application until the end of experimental period up to 16 weeks.

During the period of 16 weeks of experimentation, mice of all groups were weighed carefully examined once a week for skin tumors and these were recorded. The following parameters were taken into consideration;

2.5 Morphological estimation

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 papilloma's: The total number of Tumors appeared till termination of the experiment, was recorded
Tumor yield: The average number of papilloma's per mouse.
Average latent period:

"Average latent period = Σfx/n"

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

At the end of the experimental period, the blood was collected by cardiac puncture after mild anesthesia with pentobarbitone sodium, the next day after an over-night fast and used for the estimation CBC; Red blood cell count (RBC),[18] White blood cell count (WBC),[19] Neutrophils and lymphocyte Ratio (NLR), ESR and Serum Zinc and C-Reactive Protein (CRP).

2.6 Histopathological Studies - Skin:

The portion of skin was collected from all groups of animal and fixed in 10 per cent formalin (10 ml of 40% formaldehyde added to 90 ml of water). The tissue was fixed for 48 hr and washed for 1 hr in running tap water. Then dehydration of the tissue was performed with increasing concentrations of ethanol (70, 90 and 100 per cent; each for 1 hr). Then the tissues were cleared in xylene for 1 hr for two changes. Paraffin embedding was carried by keeping the tissues in melted paraffin at 56º C for three changes. Longitudinal and transverse sections (5µm) were prepared with semiautomatic microtome and placed on glass slide coated with Meyer's egg albumin. Tissue sections were dried by incubating them for 2 hr at 40ºC. Rehydration of fixed sections was carried in decreasing grades of alcohol (100, 90, 70 and 50 percent; each for 1 hr) and then water. The sections were stained with haematoxylin and eosin stains (Luna, 1968) with some modifications. Then the sections were covered with DPX (SRL, India) mounting medium with cover glass and observed under light microscope (Nikon, Japan) to study the Histopathological changes.[20]

2.7 Biochemical Estimation:

Biochemical estimations were carried out in skin tissues of experimental animals in each group. Biochemical parameters related to carcinogenic process were analysed. Skin tissues from mice (DMBA treated, untreated) or all groups were dissected, blotted dry, weighed and homogenized using appropriate buffer in a homogenizer with Teflon glass pestle.

2.7.1 Lipid peroxidation assay:

Levels of lipid peroxides were estimated using the method of Ohkawa et al.[21].Briefly, thiobarbituric acid (0.8%), sodium dodecyl sulphate (0.1%) and acetic acid (20%) were added to 100 ml of the tissue homogenate (10%) prepared as described above. This mixture was heated for 30 min, cooled, extracted with Nbutanol-pyridine, and the OD of MDA recorded at 532 nm. The content of MDA is expressed as nmol/mg protein.

2.7.2 Catalase (CAT) assay:

This was assayed by the method of Aebi.[22] The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H2O2 (30 mM) to 100 ml of the supernatant (of 10% tissue homogenate obtained as described above) in 50 mM phosphate buffer (pH 7). The activity of the enzyme is expressed as U/mg of protein, where 1 U is equivalent to 1 mol. of H2O2/mg/min/mg protein.

2.8 Pro-Inflammatory Cytokines: INF-g, TNF-Alpha, TGF-Beta, IL-6, Protein, from Plasma.

2.8.1 Determination of total protein:[23-24]

Total protein was determined in plasma according to the method described by Lowry et al. as modified by Hartree. 3 solutions were prepared for the procedure. Solution A: 2 gm potassium sodium tartrate and 100 gm Na2CO3 are dissolved in 500 ml 1 N NaOH and diluted with water to 1 liter. Solution B: 2 gm potassium sodium tartrate and 1 gm CuSO4.5H2O are dissolved in 90 ml water and 10 ml 1 N NaOH is added. Solution C: 1 vol. Folin-Ciocalteu reagent is diluted with 15 vol water.

Protein samples were diluted to 1 ml with water and treated with 0.9 ml solution A. A blank and a standard are set up in the same way. The tubes were placed in a water bath at 50º for 10 min, cooled to room temperature (2l-25º), and treated with 0.1 ml solution B. The solutions were left at room temperature for at least 10 min, then 3 ml solution C was forced in rapidly to ensure mixing within 1 sec. The tubes were again heated at 50º for 10 min and cooled to room temperature. Absorbencies were recorded at 650 nm. The results were expressed as µg/ml protein.

2.8.2 Measurement of Pro-Inflammatory Cytokines:

Markers of disease severity; Il6, IL-1beta, and TNF-alpha by Sandwich ELISA Assay (Commercial Available kit, Mercodia, Sweden).[25-26] Plasma was normalized for protein content by Lowry et al. method. Concentrations of pro-inflammatory cytokines tumor necrosis factor (TNF)-alpha, interferon (IFN)-g, interleukin (IL) 6, and as well as of TGF-b were determined by using commercial kits. The Plasma were used for the estimation of the cytokines viz, IFN-g, IL-6, TGF beta and TNF-α was done using antigen capture ELISA and the A450nm was measured by using ELISA reader.

2.9 Statistical analysis:

The results are expressed as Mean ± SEM from n=6 mice in each group. Data were analysed using statistical software Graph Pad Prism version 5. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test compared between Normal control (Untreated) vs. all groups $p<0.05$ were considered significant.

3. RESULTS

3.1 *In-Vivo* data in DMBA/Croton Oil Induced Skin Cancer Model

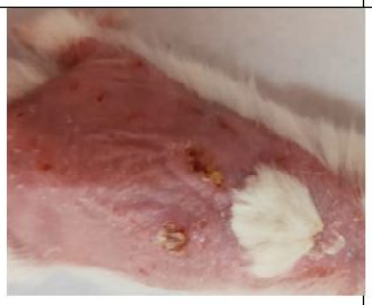
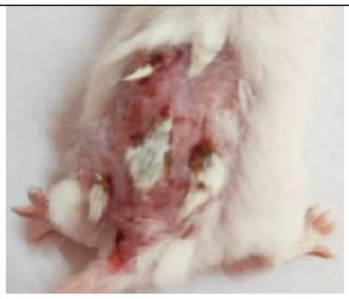
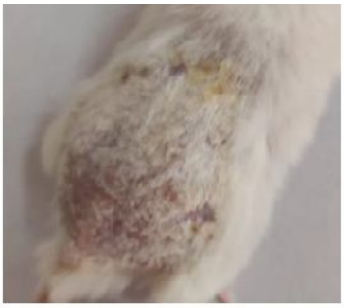
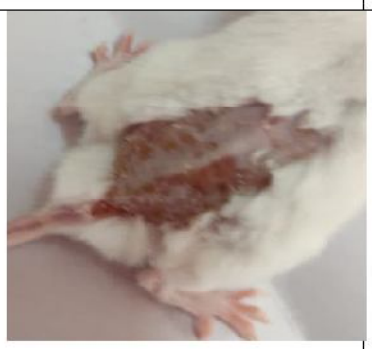
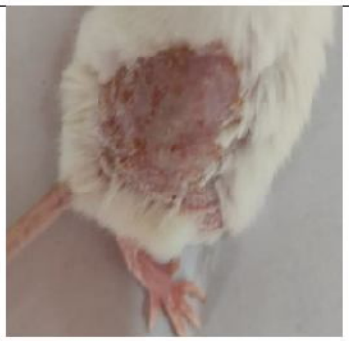
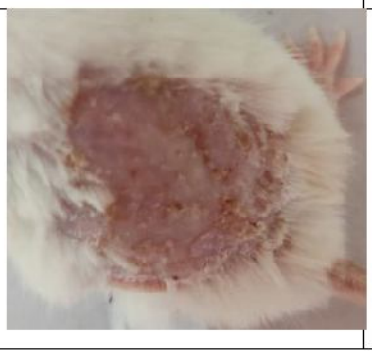
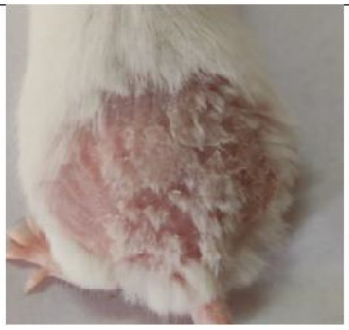
Effect of *Choerospondias axillaris* Extract on various parameters of skin cancer induced by DMBA (100g/100l of acetone)/Croton oil (1 % in 100 l of acetone).

Table 01: In-Vivo study parameters for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer

Group		Body Weight (gm)		Tumor Incidence (%)	Cumulative of no's Tumours	Tumor Yield	ALP	No. of Papilloma with Tumor size (mm)		
		Initial	Final					<2	2-4	4>
1	DDW treated mice (Normal control; 10ml/kg.b.w)	26.96 ± 0.57	30.00 ± 0.58	-	-	-	-	-	-	-
2	DMBA/Croton oil (Toxic control)	25.73 ± 1.06	27.17 ± 0.74	100	18	3	3.2	9	5	4
3	5-Fluorouracil (10mg/kg body weight) - STD	27.09 ± 0.57	29.17 ± 0.60	42.5	6	1	5.4	4	2	-
4	*Choerospondias axillaris* orally (200mg/kg.b.w)	25.26 ± 1.38	28.00 ± 0.577	50	9	1.5	4.3	6	3	-
5	*Choerospondias axillaris* orally (400mg/kg.b.w)	26.24 ± 0.54	28.50 ± 0.76	33.7	4	0.66	6.5	4	-	-

Values are expressed as Mean ± SEM, (n=6 mice in each group)

Figure no 01: Pictograph showing the anticancer effect of Choerospondias axillaris against DMBA/Croton oil Induced Skin cancer in mice

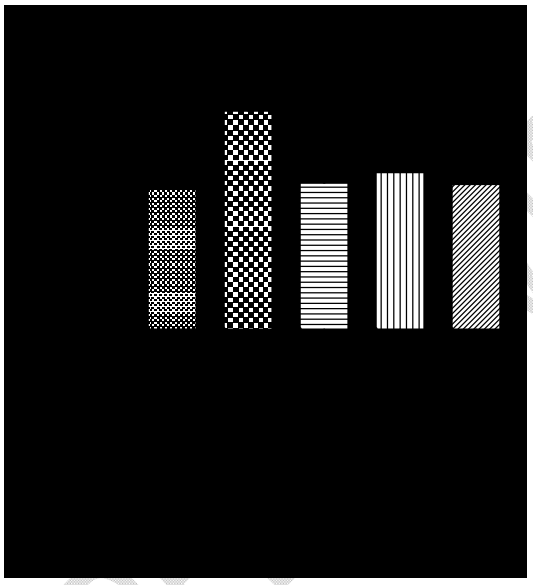
Group	8th Week	16th Week
DMBA/Croton oil (Toxic control) DMBA (100g / 100l of acetone), croton oil (1% in 100l of acetone)		
5-Fluorouracil (10mg/kg Body weight S.C.) - STD		
Choerospondias axillaris orally (200mg/kg.b.w)		
Choerospondias axillaris orally (400mg/kg.b.w)		

3.2 Effect on Haematological Parameters:

Haematological parameters for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer.

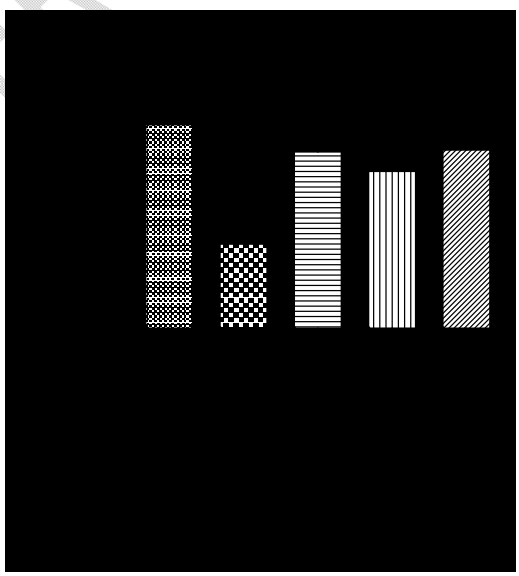
INTERPRETATION: WBC is generally considered high if it is greater than 11,000 cells per microliter. A high WBC count may result from an infection. Increased lymphocytes also occur with other conditions, such as infections and inflammatory diseases. Lymphocyte reduction is correlated with good response of the varieties of disease. High ESR has been found to correlate with overall poor prognosis for various types of cancer. NLR > 3 count indicates that the body is fighting an infection.

Figure no 02: Effect on WBC after treatment in their respective groups



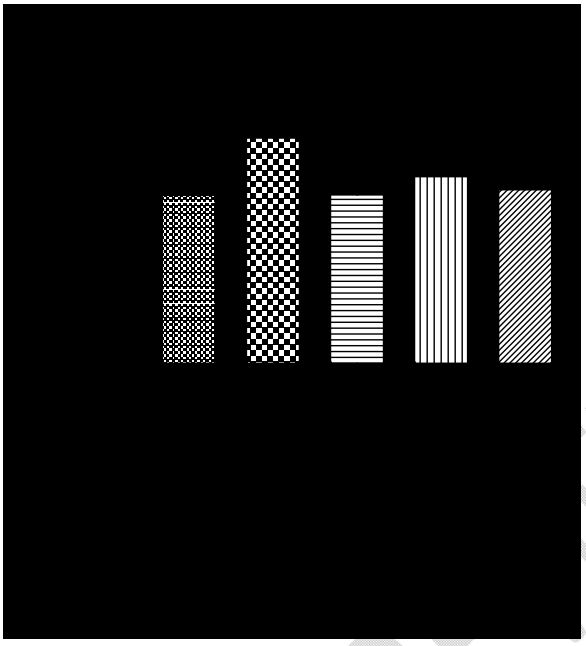
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 03: Effect on RBC after treatment in their respective groups



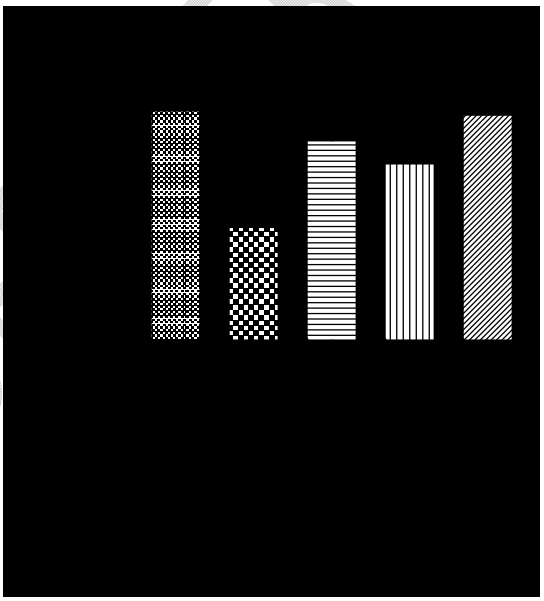
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 04: Effect on Neutrophil after treatment in their respective groups



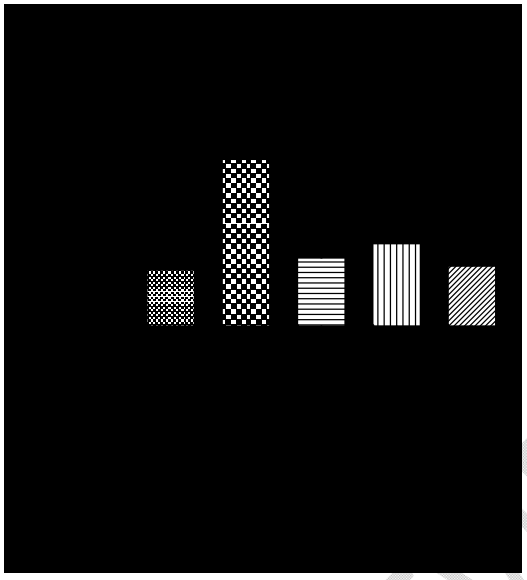
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 05: Effect on Lymphocyte after treatment in their respective groups



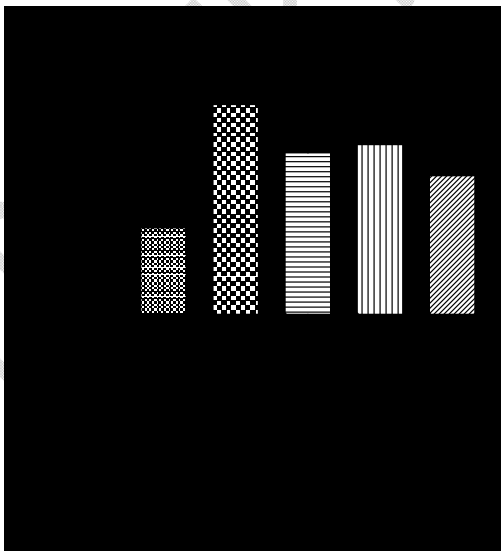
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 06: Effect on NLR after treatment in their respective groups



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 07: Effect on ESR after treatment in their respective groups



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Table 02: Statistical Comparison Test on WBC between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	-4.583	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-0.3283	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-0.9433	Yes	*
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-0.2517	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	4.255	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	3.640	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	4.332	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-0.6150	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	0.07667	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	0.6917	No	ns

Table 03: Statistical Comparison Test on RBC between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	4.798	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	1.083	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	1.873	Yes	**
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	1.015	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	-3.715	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	-2.925	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	-3.783	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	0.7900	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-0.06833	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	-0.8583	No	ns

Table 04: Statistical Comparison Test on Neutrophil between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	-20.36	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-0.1500	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-6.617	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-1.950	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	20.21	Yes	***

DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	13.74	Yes	**
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	18.41	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-6.467	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-1.800	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	4.667	No	ns

Table 05: Statistical Comparison Test on Lymphocyte between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	13.38	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	3.433	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	6.083	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	0.4917	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	-9.947	Yes	**
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	-7.297	Yes	*
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	-12.89	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	2.650	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-2.942	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	-5.592	No	ns

Table 6: Statistical Comparison Test on NLR between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	-4.397	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-0.4767	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-1.043	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-0.1533	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	3.920	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	3.353	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	4.243	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-0.5667	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	0.3233	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	0.8900	No	ns

Table 07: Statistical Comparison Test on ESR between All the Groups

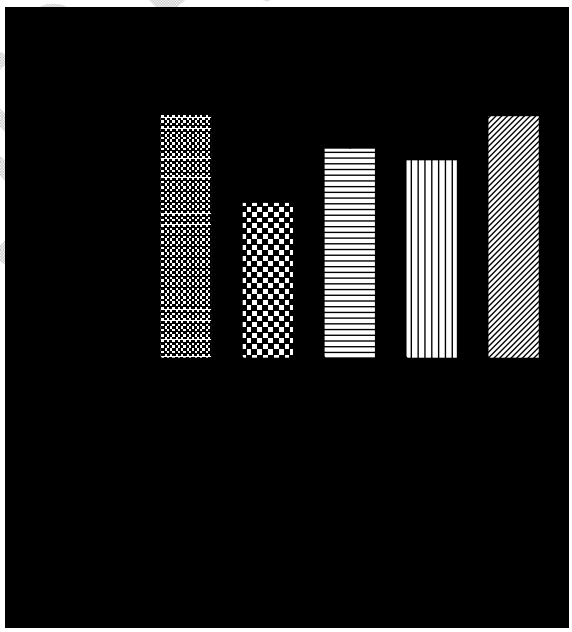
Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	-20.50	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-12.50	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-13.83	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-8.667	Yes	**
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	8.000	Yes	*
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	6.667	Yes	*
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	11.83	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-1.333	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	3.833	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	5.167	No	ns

3.3 Effect on Serum Zinc and CRP for anticancer effect of *Choerospondias axillaris* against DMBA/Croton oil induced Skin Cancer

INTERPRATION:

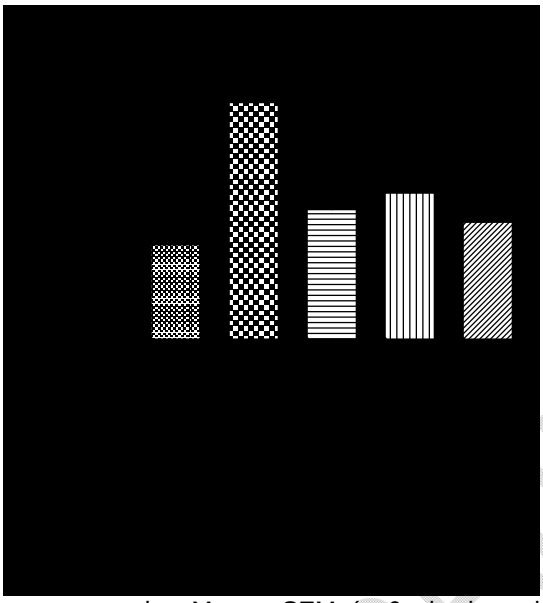
Elevated CPR level can be diagnostic of complicating pathologies(e.g.infctions).
Serum zinc has ant-i inflammatory properties and is useful for inflammatory skin conditons.

Figure no 08: Effect on Serum Zinc for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 09: Effect on Serum CRP for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Table 08: Statistical Comparison Test on Zinc and CRP between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary	Mean Diff.	Significant? P < 0.05?	Summary
	Zinc			CRP		
NC - DW (10ml/kg/b.w.) vs DC-DMBA/Croton oil	4.858	Yes	***	-1.363	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	1.885	No	ns	-0.3383	Yes	*
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	2.525	Yes	*	-0.4950	Yes	**
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	0.1083	No	ns	-0.2167	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	-2.973	Yes	**	1.025	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	-2.333	Yes	*	0.8683	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	-4.750	Yes	***	1.147	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	0.6400	No	ns	-0.1567	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-1.777	No	ns	0.1217	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	-2.417	Yes	*	0.2783	No	ns

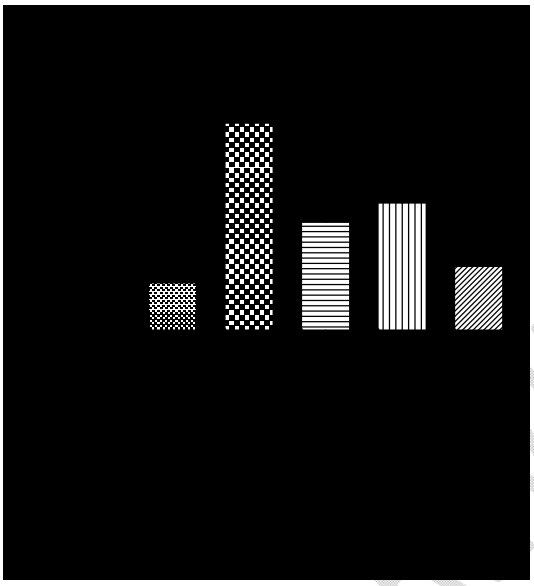
3.4 Effect on Antioxidant Enzyme Study:

INTERPRETATION

Generation of ROS in the skin develops oxidative stress and mostly linked to the development of skin cancer.

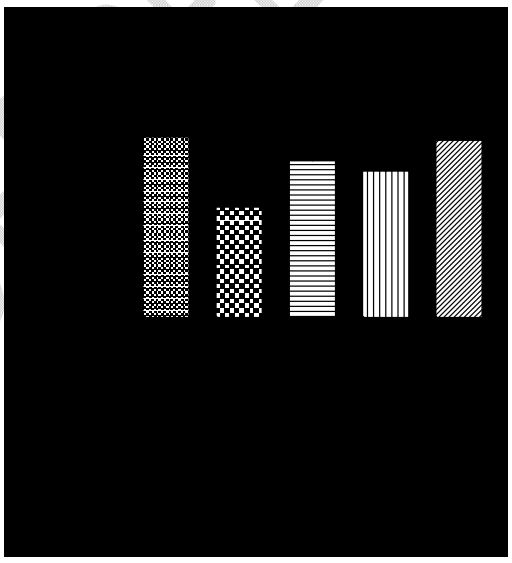
Finding lower skin catalase activity may be responsible for the increase the exposure of oxidative stress.

Figure no 10: Effect on LPO study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 11: Effect on CAT study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Table 09: Statistical Comparison Test on LPO and CAT between All the Groups

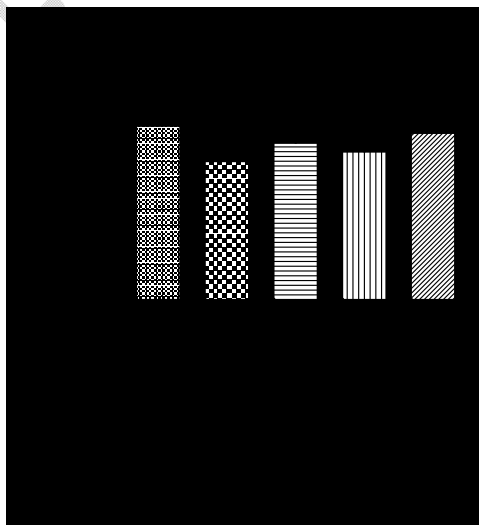
Tukey's Multiple Comparison Test	Mean Diff.	Significant ? P < 0.05?	Summary	Mean Diff.	Significant? P < 0.05?	Summary
	LPO			**CAT**		
NC - DW (10ml/kg/b.w.) vs DC-DMBA/Croton oil	-3.767	Yes	***	17.30	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-1.428	Yes	***	5.822	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-1.880	Yes	***	8.398	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-0.3900	No	ns	0.7600	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	2.338	Yes	***	-11.48	Yes	*
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	1.887	Yes	***	-8.900	No	ns
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	3.377	Yes	***	-16.54	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-0.4517	No	ns	2.577	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	1.038	Yes	*	-5.062	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	1.490	Yes	***	-7.638	No	ns

3.5 Effect on Inflammatory Cytokine Analysis:

INTERPRETATION

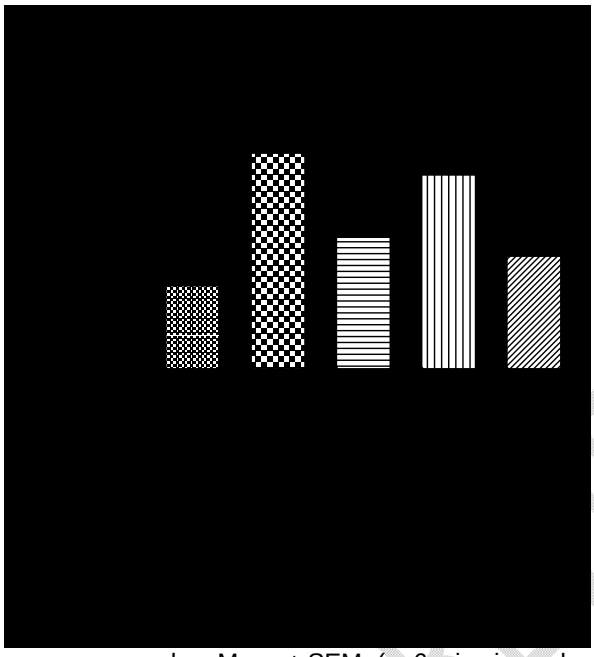
Increase in TGF levels is indicative of an immunosuppressive environment. Increase in INF-Gamma levels is indicative of an Immunoprotective and better outcomes. Elevated levels of circulating TNF-α have been linked to a wide variety of diseases, including Inflammation. Elevated IL 6 levels are associated with excessive inflammation and poorer outcomes

Figure no 12: Effect on Protein study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



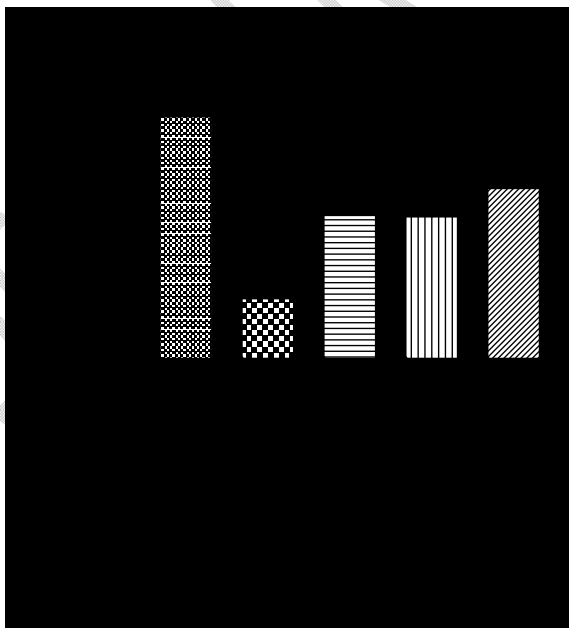
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 13: Effect on TGF-B study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



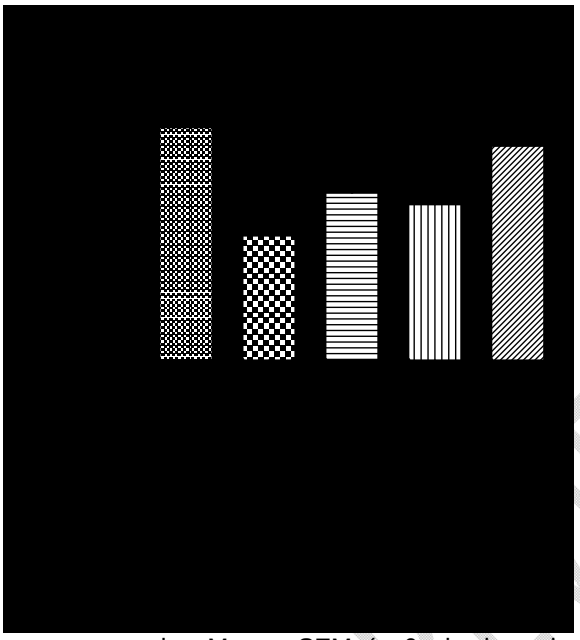
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 14: Effect on IFN-G study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



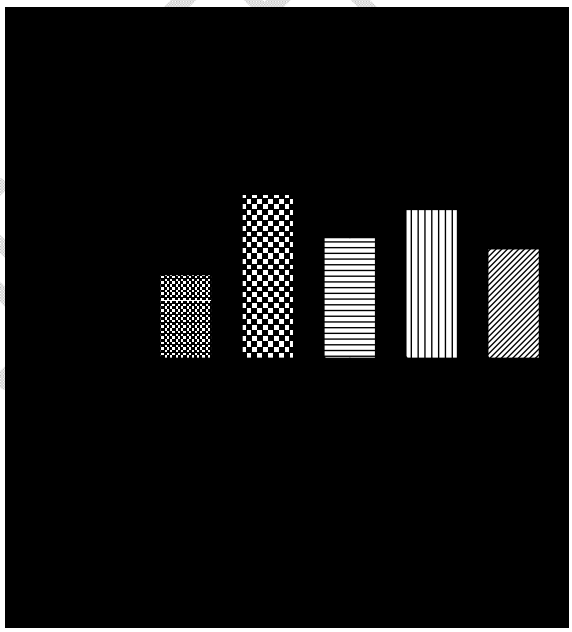
Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 15: Effect on TNF-A study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Figure no 16: Effect on IL-6 study for anticancer effect of Choerospondias axillaris against DMBA/Croton oil induced Skin Cancer



Values are expressed as Mean ± SEM, (n=6 mice in each group).

Table 10: Statistical Comparison Test on Protein between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	123.2	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	59.43	Yes	**
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	89.58	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	24.69	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	-63.76	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	-33.61	No	ns
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	-98.50	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	30.15	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-34.74	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	-64.89	Yes	***

Table 11: Statistical Comparison Test on TGF-B between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	-372.8	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-136.2	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-312.0	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-82.92	Yes	***
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	236.7	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	60.86	Yes	**
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	289.9	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-175.8	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	53.26	Yes	**
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	229.1	Yes	***

Table 12: Statistical Comparison Test on IFN-G between All the Groups

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	16.56	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	8.980	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	9.110	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	6.515	Yes	***
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	-7.583	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	-7.453	Yes	***
DC- DMBA/Croton oil vs Test Drug - CA	-10.05	Yes	***

(400mg/kg.b.w)			
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	0.1300	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-2.465	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	-2.595	No	ns

Table 13: Statistical Comparison Test on TNF-A between All the Groups

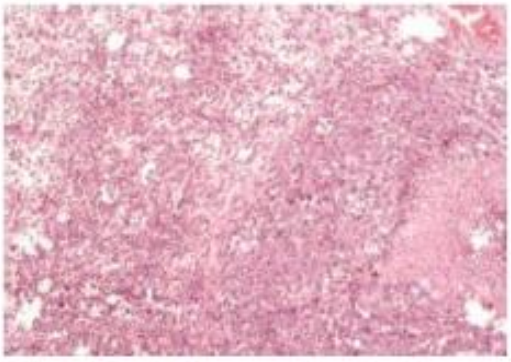
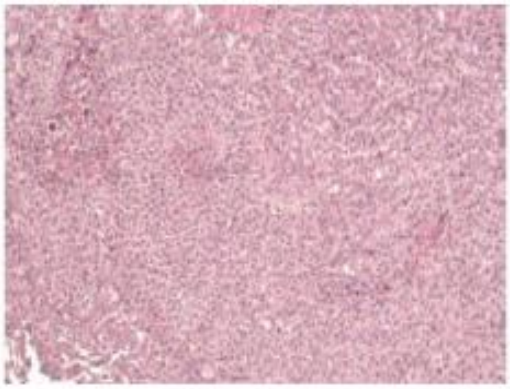
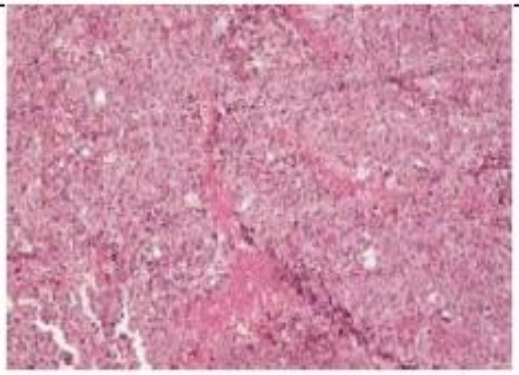
Tukey's Multiple Comparison Test	**Mean Diff.**	**Significant? P < 0.05?**	**Summary**
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	38.93	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	23.54	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	27.69	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	6.602	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	-15.39	Yes	*
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	-11.24	No	ns
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	-32.33	Yes	***
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	4.147	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	-16.94	Yes	*
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	-21.09	Yes	**

Table 14: Statistical Comparison Test on IL-6 between All the Groups

Tukey's Multiple Comparison Test	**Mean Diff.**	**Significant? P < 0.05?**	**Summary**
NC - DW (10ml/kg/b.w.) vs DC- DMBA/Croton oil	-4.395	Yes	***
NC - DW (10ml/kg/b.w.) vs STD - 5-Flu (10mg/kg/b.w)	-2.025	No	ns
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (200mg/kg.b.w)	-3.567	Yes	***
NC - DW (10ml/kg/b.w.) vs Test Drug - CA (400mg/kg.b.w)	-1.415	No	ns
DC- DMBA/Croton oil vs STD - 5-Flu (10mg/kg/b.w)	2.370	Yes	*
DC- DMBA/Croton oil vs Test Drug - CA (200mg/kg.b.w)	0.8283	No	ns
DC- DMBA/Croton oil vs Test Drug - CA (400mg/kg.b.w)	2.980	Yes	**
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (200mg/kg.b.w)	-1.542	No	ns
STD - 5-Flu (10mg/kg/b.w) vs Test Drug - CA (400mg/kg.b.w)	0.6100	No	ns
Test Drug - CA (200mg/kg.b.w) vs Test Drug - CA (400mg/kg.b.w)	2.152	Yes	*

3.6 Histopathological Study

Fig 17: Image of Skin showing the anticancer effect of *Choerospondias axillaris* against DMBA/Croton oil Induced Skin cancer in mice

DMBA/Croton oil (Toxic control)		**INTERPRETATION** Found irregular architecture, more necrosis, cell death and progressive cells are negative, reflecting the less invasive character. **Transverse section, H&E, X200s**
5-Fluorouracil (10mg/kg body weight) – STD Drug		**INTERPRETATION** Found hemorrhagic changes, increased areas of necrosis and less inflammation, increased cell death, decreased the progression of growth. **Transverse section, H&E, X200s**
Choerospondias axillaris orally (400mg/kg.b.w) – Test Drug		**INTERPRETATION** There is a certain decline in progressive feature of the cells due to cell death and necrosis leading to an abnormal result. But decreased the progression of cell growth **Transverse section, H&E, X200s**

4. DISCUSSION

Plants and natural products play an important role in medicine and provide important prototypes for the development of novel drugs.[27] They offer a valuable source of compounds with a wide variety of biological activities and chemical structures. Many anticancer agents have been derived from natural sources; directly as pure native compounds, or as semisynthetic analogues.

In *In vivo* studies, the reliable criteria for judging the value of anticancer drugs are of gain in average body weight and decrease of WBC from blood. Histopathological studies are

considered as indicative of significant drug activity. Tumor incidence was recorded as % and the average number of tumor incidence was scored 18, whereas average latent period was recorded as 3.2 in mice belonging to group II (DMBA/Croton oil). 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 III, IV & V) but maximum reduction in all such parameters was evident in *Choerospondias axillaris* orally 400mg/kg treated mice (group V). According to *R Sharmila & Manoharan*[28] Anti-tumor activity of rosmarinic acid in DMBA induced skin carcinogenesis in Swiss albino mice shows the biochemical parameter TBARS increased and CAT, SOD, GPx, GSH decreased in cancer induced animals. In the other hand, *Choerospondias axillaris treated* animal data decreased LPO and increased CAT antioxidant levels. High dose of *Choerospondias axillaris* 400mg/kg administration produced better results compare to low dose of *Choerospondias axillaris* 200mg/kg and standard group drug. *Sharmila et al* have further shown the body weight of animals in diseased control group will decrease due to papilloma formation and in my experiment .the body weight of DMBA and DMBA/Croton oil treated animals was found to decrease. Test drug treated decreased the body weight near normal values, oral + topical drug administration produced more better results compare to only oral and topical, only oral produced better results than only topical drug administration. After 1 month of cancer induction treatment with oral + topical drug administration also has shown significant results. In normal control skin was observed normal and smooth with fur but in diseased control presence of papilloma on skin and skin become hard. According to *Huseyin TURKER at* al[29] a significant decrease in Haemoglobin (Hb), Haematocrit (Ht) and Neutrophil values; and a significant increase in White Blood Cell (WBC) counts, Lymphocyte and Eosinophil values during the experiment.in other hand In DNBA control and DMBA/Croton oil treated group the total WBC count was found to increase with a reduction in the haemoglobin content and the RBC count. On the other hand, *Choerospondias axillaris treatment* changed these altered parameters to recover near normal values in a dose dependent manner, and the *Choerospondias axillaris* drug administration produced a significant effect. Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anaemia. The anaemia encountered in tumour bearing mice is mainly due to reduction in RBC or Haemoglobin percentage, and this may occur due to iron deficiency or due to haemolytic or myelopathic conditions. The analysis of the haematological parameters showed minimum toxic effect in mice treated with the *Choerospondias axillaris*. After 16 weeks of treatment, the *Choerospondias axillaris* was able to reverse the changes in the haematological parameters, Serum zinc and CRP consequent to tumour inoculation. Zinc has anti-inflammatory properties and is useful for inflammatory skin conditions. The immune environment, both at a systemic level and at the level of the tumour microenvironment plays a major role in the cancer development and progression. Cytokines as Prognostic Indicators for Skin Cancer and Cytokines are mediators and are crucial for facilitating the recruitment and activation of specific subsets of leukocytes within the microenvironment of skin cancers. Cytokines including IL6, IFN-γ, TNF-α, and TGF- β has been monitored throughout the course of disease and treatment to help understand the evolving immune environment. High IL-6 levels showed significantly poorer survival than with low IL-6 levels. TNF-α can act as an endogenous tumour promoter. The risk o. High pre-treatment serum TGF-β levels were associated with poor prognoses cancer is increased in chronic infections and inflammatory diseases. An increased IFNγ gene signature is correlated with higher overall response rates and longer median progression-free survival. Treatment of *Choerospondias axillaris* was able to decrease the level of these pro-cytokines levels and increase the levels of IFN-γ. Increase in INF-Gamma levels is indicative of an Immunoprotective and better outcomes. Overall impression was significant improvement in systemic immune suppression and inflammation.

5. CONCLUSION

In-Vivo studies showed that the exhibited anticancer activity against DMBA/Croton oil induced skin cancer. The activity was confirmed by significant increment of gain in average body weight, decrease of WBC count, Serum Zinc and CRP, Level of LPO, CAT enzymatic antioxidants and histopathology in skin tissue of control and experimental animals were observed. In the study of anticancer activity of in skin cancer model showed significant decrease NLR and ESR Count It also showed to increase the RBC value compared to disease control group. Tumor incidence, cumulative number of Papilloma's, Tumor yield,

was found to be decreased in all the experimental mice but maximum reduction in all such parameters was evident in group V. On the other hand treatment of *Choerospondias axillaris* was able to decrease the level of pro-cytokines levels and increase the levels of IFN-γ and Improvement in markers indicating a reduction in inflammation and immune imbalance. In-Vivo studies points to the potential anticancer activity of and might be a promising anti cancerous agent against skin cancer induced by DMBA alone and DMBA/Croton oil. With the antitumor activity the observations suggest that the oral administration of has a potent anticancer activity against skin cancer. Due to presence of various-phytochemicals it is suspect that *Choerospondias axillaris* is showing anticancer effect and also due to this it may be used traditionally as an anticancer drug.

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