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# Evolution of Anticarcinogenic Potential of Cassia fistula against 7, 12 Dimethylbenzene(a) anthracene-induced Skin Papillomagenesis in Mus musculus

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## ABSTRACT

Cancer is the second leading cause of death globally. *Cassia fistula* (CF), a member of *Leguminosae* family, is Native to India, Amazon and Sri Lanka, is widely used for its medicinal properties in Indian system of medicine for various ailment from the ancient time. The present investigation was undertaken to investigate the anticarcinogenic action of *Cassia fistula* **L.** extract on 2-stage skin carcinogenesis model in Swiss albino mice. The papilloma were produced on mice skin by DMBA and promoted by croton oil and one weeks later, promoted by repeated application of croton oil (1% in acetone/twice in a week) till the end of the experiment (16 weeks). After 16 weeks at the time of termination of the experiment estimation of GSH from the liver and blood tissue of the animals were also done by the method of Beutler *et. al*. (1963). the outcomes of the papilloma study and GSH estimation was correlated and it was found to be preventive for the formation as well as persistence of papilloma. 21 no. of papilloma were found in CF treated mice as compared to (35) untreated control mice. Our results suggest that *Cassia fistula* can be a cost effective future herbal alternative medicine for cancer treatment.

**Keywords:** *Cassia fistula*, *Leguminosae*, Papilloma, Carcinogenesis, DMBA, Croton oil, Chemoprevention.

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# INTRODUCTION

Medicinal plants play a vital role for the development of new drugs. Indian medicinal plants are now well recognized to have great potential for preparing clinically useful drugs that could even be used by allopathic physicians. *Cassia fistula* Linn. (*Leguminosae)* is a very common plant and is widely known for its medicinal properties. *Cassia fistula* Linn. Commonly known as Amaltas, Fistula, Laburnum, Purging Fistula, and golden Shower. "Indian Laburnum is dispersed in deciduous and mixed monsoon forests throughout greater parts of India, ascending to 1300 m in outer Himalaya. The vegetative parts of *Cassia fistula* contain phenolic derivatives including anthraquinones, xanthones, phenolic acids, phenolic diterpenes, flavonoids, catechins, proanthocyanidins and anthocyanins. These substances have also been reported to exhibit antioxidant activity (Jothy *et. al.*, 2011, Middleton *et. al.*, 2000; Carlo *et. al.*, 1999; Ramma *et. al.*, 2002)[1-4]. Aqueous extract of *C. fistula* root is reported for the antioxidant properties, both *in vitro* and *in vivo*. Chaminda *et. al*., (2001) but there is no sufficient data published on the anticarcinogenic activities of the *Cassia fistula* (CF) plant. Hence, an attempt has been made in the present study to investigate the possible anticarcinogenic activity of the methanolic extract of CF using mice models.

# MATERIALS AND METHOD

**Collection & Identification of Plant**

The pods of *C. fistula* were collected from local areas of Bhopal, M.P. (India). Authenticated by the Botanist Dr. Z. Hasan, Department of Botany, Safia Science College, Bhopal, Madhya Pradesh (India), where the herbarium was deposited. Voucher Specimen No: 294/bio/Safia/ 2011.

**Project Approval:**

Project approved by Institutional Animal Ethical Committee (IAEC), Project no. 1695/PO/C/B/CPCSEA/1.

**Extraction of *Cassia fistula* seeds**

Seeds were collected and washed under running tap water and dried in oven at 50°C. The dried seeds were grinded to fine powder and stored in airtight bottles. 50gm of dried powder was defatted by treating with petroleum ether for 1-2 hour. Defatted powder was then packed in separating funnel extracted with 250 ml of 50% methanol (v/v). After 24 hr. solution was collected in a beaker and this process was repeated until transparent solution appeared. The extract was filtered using whatman filter paper (No.1) and then concentrated in a vacuum and dried at 45-50

°C in Water bath for elimination of Solvents. The extracts were collected in a sterile airtight bottle under refrigerator of about 2-8°C for further use.

**Animals:**

The study was conducted on random bred, 6-7 weeks old and 24- 28 gm body weight bearing, male female *Swiss albino* mice (*Mus musculus*). Animals were maintained under controlled conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). They were provided standard mice feed (procured from Hindustan Levers Ltd. India) and water *ad libitum*. The study protocol is approved by the Departmental Animal Ethical Committee and confirms to the guidelines set by World Health Organization, Geneva, Switzerland and Indian National Science Academy (INSA), New Delhi (India) (Project no 1695/PO/C/B/CPCSEA/1).

**Chemicals**

DMBA, croton oil, acetone were procured from Sigma Chemical Co (St Louis, MO). EDTA, $Na_2HPo_4$, DTNB (5, 5'-dithiobis-2-nitrobenzoic acid), DDW, Glacial meta phosphoric, EDTA, NaCl. Ellman's Reagents. were procured from Sigma Chemicals Co., St. Louis, USA.

**Chemopreventive activity:**

This assay has been done according to the method proposed by Berenblum, (1975)[5] in skin carcinogenesis assay and standardized by us.The animals (Swiss Albino mice) have been divided randomly into different groups comprises of 6 animals in each group. two day before hair were removed with the help of hair removing cream from the dorsal region with proper care in an area of 2cm² in all the groups. The treatment has been provided topically on the shaved area using the protocol for the testing of chemo preventive effects of the drug.

**EXPERIMENTAL PROTOCOL**

Three days before the commencement of the experiment, hair on the dorsal region of the mice were shaved. Only the mice showing no hair growth were selected for the study. The animals were randomly allocated into 6 groups comprising six mice each. The treatment was provided topically on shaved area using the following protocol (Berenblum, 1975)[5].

**Experimental Design for *Cassia fistula* (CF) extract**

Swiss Albino mice were divided into 6 groups for the present study.

**Dose**: CF hydromethanolic seed extract 5000mg/kg bd. wt.100 µl/ animal on dorsal area of skin.

**Route of administration**: Topical

**Experiment duration:** 16 week

**I Group: (Vehicle Alone):**

This group served as controls and received (1%) acetone (100 µl/ mouse) on the shaved dorsal area of skin 2 times in a week.

**II Group: (DMBA Alone):**

This group received single application (100 µl/mouse) of DMBA (104 µg in 100 µl acetone) on the shaven dorsal area of skin.

**III Group: (Croton Oil Alone (CO)):**

This group received application of Croton oil (1% in 100 µl of acetone) (100 µl/mouse) 2 times in a week up to 16 week.

**IV Group: (CF extract alone):**

This group received application of CF extract (dose 5000 mg/kg bd. wt.) (100 µl/mouse) 2 times in a week up to 16 week.

**V Group: (DMBA+ Croton oil):**

This group served as positive controls group and were received single application of DMBA (104 µg in 100 µl acetone) (100 µl/mouse). 2 weeks later, Croton oil (1% in 100 µl of acetone) (100µl/mouse) was applied as a promoter 2 times in a week for 16 week.

**VI Group: (DMBA+ CF extract + CO):**

This group treated as group V mice along with topical application of 100µl of CF extract (dose of 5000 mg/kg body wt.), one hour before each application of 1% croton oil 2 times in a week for 16 week.

**Tumor study**

Mice were observed daily and weighed weekly up to the 16 weeks of experimentation. Papilloma appeared on the shaven area of the skin were examined and recorded at weekly intervals of all groups. Papilloma which persisted for two weeks or more, with a diameter greater than 2mm, only have been taken into consideration for final evaluation.

**Study parameter**

- Body Weight
- Cumulative no. of papilloma
- Tumor Incidence
- Tumor Burden
- Tumor Yield
- Average latent period

**Statistically Analysis**

The difference in the incidences of tumor among different groups were considered to be Significant at 5% significance level ($p < 0.05$) in Student 't' test.

**BIOCHEMICAL ASSAY**

**Estimation of Glutathione (GSH) in blood sample**

**Requirements:**

**Precipitating solution:** Glacial metaphosphoric acid, $Na_2EDTA$, NaCl, EDTA, NaCl, DTNB (5, 5'-dithiobis-2-nitrobenzoic acid), PBS-0.423g in 100 ml (1 tablet of pH 7.6 in 100 ml DDW).

**Ellman's Reagents-** DTNB (200 mg) in 100 ml of 1% Sodium citrate solution.

**Experimental protocol:**

Blood GSH was determined by the method of Beutler *et. al*. (1963)[14]. Almost All non-protein sulfahydryl of red blood cells is in form of GSH. DTNB is a disulphide compound which is readily reduced by sulfahydryl compounds forming a highly colored yellow anoin.

- 0.2 ml of mice blood was collected and mixed with 1.8 ml DDW.
- 3 ml of precipitating solution was added and allowed to stand for 5 min. So that a precipitate is formed.
- The test tube was centrifuged with solution at 3000 rpm for 10 min.
- After centrifugation 8 ml phosphate buffer was added in filtrate.
- 0.5 ml DTNB was added to the above solution and kept in dark for 30 min. After 30 min. absorbance was taken.
- Both the blank and sample reaction mixtures were read against water at 412 nm in a UV-spectrophotometer.
- GSH concentration was calculated on the basis of a millimolar extinction Coefficient of 13.6 and a molecular weight of 307. (Ellman, 1959).

**Glutathione (GSH) determined in homogenate tissue (liver) *in vivo.***

**Requirements:**

EDTA, $Na_2HPo_4$, DTNB (5, 5'-dithiobis-2-nitrobenzoic acid), DDW, Precipitating Solution

**Stock solution:**

**Precipitating solution -** Glacial meta phosphoric, EDTA, NaCl.

**Ellman's Reagents -** DTNB (200 mg) in 100 ml of 1% Sodium citrate solution

**Experimental protocol:**

- Animals were sacrificed by cervical dislocation & the liver was excised and immediately fixed with 0.9% NaCl.

- The liver was blotted dry, weight quickly & homogenized in ice-cold saline EDTA (pH 4.7) to make a 5% homogenate.
- 0.2 ml homogenate was taken & 1.8 ml EDTA solution was added.
- 3ml precipitating solution was added after 5 min. and mixture was centrifuged at 3000- 4000 rpm for 15 min.
- 2 ml filtrate was taken and 4ml phosphate buffer ($Na_2HPo_4$) was added.
- 1 ml of DTNB reagent then added in solution & incubated it for 30 min. at room temperature.
- After incubation absorbance was taken at 412 nm in a UV-spectrophotometer.
- GSH concentration was calculated as per the formula.

## RESULTS AND DISCUSSION

Single topical application of DMBA followed by 2 weeks, by repeated application of croton oil (twice a week) induced skin papillomas in all animals which started to appear from $6^{th}$ week onwards till the experiment was terminated ($16^{th}$ week). No tumor incidences were observed in animals of vehicle treated (Group I); DMBA alone (Group II), Croton oil alone (Group III) and *Cassia fistula* (CF) extract alone (Group IV). In carcinogen control group (Group V), which received single topical application of DMBA followed by repeated application of croton oil, showed the 100% tumour incidence and 35 cumulative no. of skin papillomas. Tumor burden and tumor yield was 5.8 ± 0.33. The average latent period was observed 26.2 days. while In group VI in which Hydromethanolic extract of CF seed at the dose of 5000 mg/kg along with DMBA and croton oil was given by topical application in alternative day, total 21 no. of papillomas were observed and incidence of papillomas was 83.3 %, tumor yield (papillomas bearing mice) was found 4.2 ± 0.2, while tumor burden (papillomas/mice) was 3.5 ± 0.2. There was not much difference in average latent period of this group which was 26.74 in comparison to that of the group V (DMBA +CO). A gradual increase in body weight was noted in all animal groups that were nearly the same in the animals of all groups. Average and total number of papilloma appearing in different treated groups showed statistically significant difference compared to untreated control, at all time periods of observation namely $6t^h$, $8^{th}$ , $12^{th}$ and $16^{th}$ weeks.

**Table 1. Effect of *Cassia fistula* Hydromethanolic seed extract on DMBA induced Skin carcinogenesis in Swiss albino mice.**

| Treatment | Body weight (Mean ± SEM) | | Cumulative no of papillomas | Tumor incidence (%) | Tumor Burden | Tumor yield | Average latent period (days) |
|---|---|---|---|---|---|---|---|
| | Initial | Final | | | | | |
| Vehicle control (acetone 100 µl/animal) | 25.46 ± 0.27 | 32.30 ± 0.36 | 0 | 0 | 0 | 0 | 0 |
| DMBA alone (104 µg in 100 µl acetone) | 25.71 ± 0.57 | 31.40 ± 0.26 | 0 | 0 | 0 | 0 | 0 |
| Croton oil alone(1% in100 µl acetone) | 26.75 ± 0.55 | 34.20 ± 0.46 | 0 | 0 | 0 | 0 | 0 |
| CF extract alone (5000 mg/kg) | 26.46 ± 0.27 | 32.40 ± 0.44 | 0 | 0 | 0 | 0 | 0 |
| DMBA(104 µg in 100µl acetone) + (1%Croton Oil In 100 µl of acetone | 26.56 ± 0.37 | 32.39 ± 0.33 | 35 | 6/6 (100%) | 5.8 ±0.33 | 5.8 ±0.33 | 26.2 |
| DMBA (104 µg in 100 µl acetone )+ (C F (5000 mg/kg) + (Croton Oil 1% in 100 µl of acetone) | 25.66 ± 0.29 | 31.60 ± 0.49 | 21 | 4/6 83.3 (%) | 3.5 ±0.2* | 4.2±0.2* | 26.74 |

(*) denotes the level of significance as compared to Control (No treatment) group at p <0.05 in Student "t" test. Each group comprised of 6 animals.

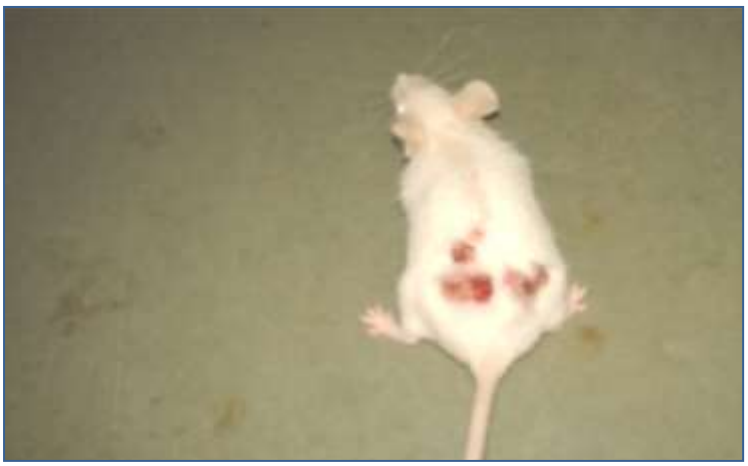
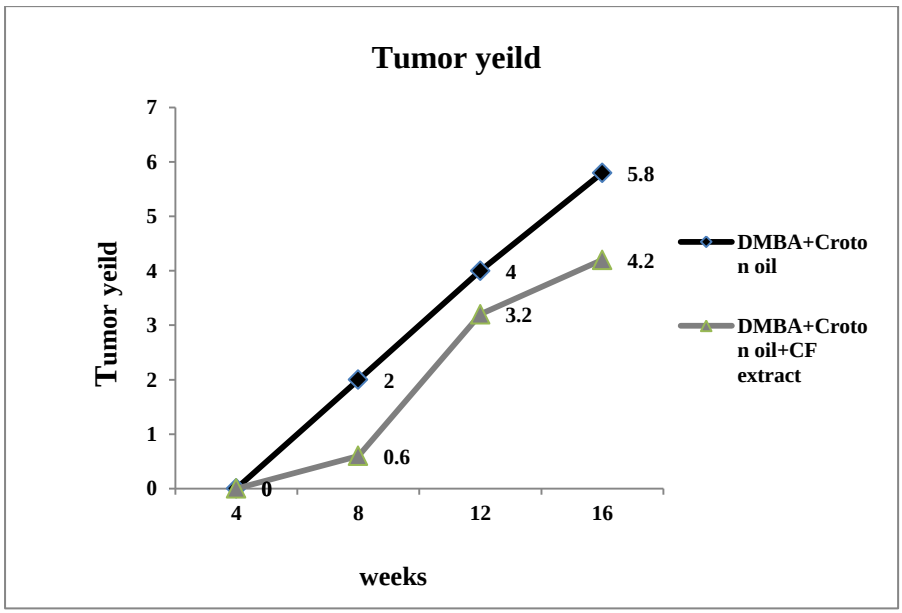
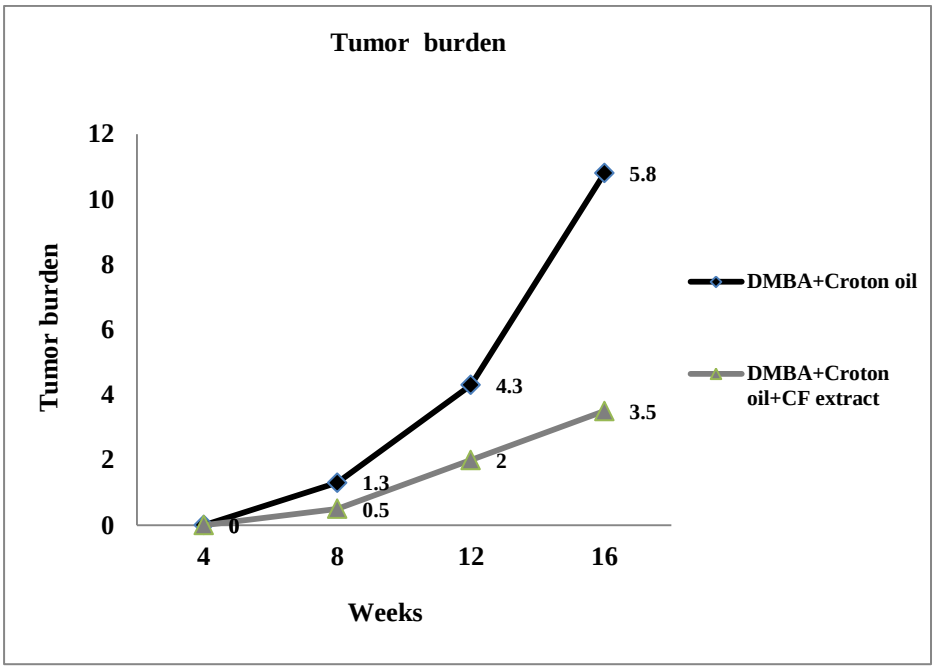


**Figure (a): Showing the Skin tumor papillomas induced by DMBA+ Croton oil for 16 weeks**

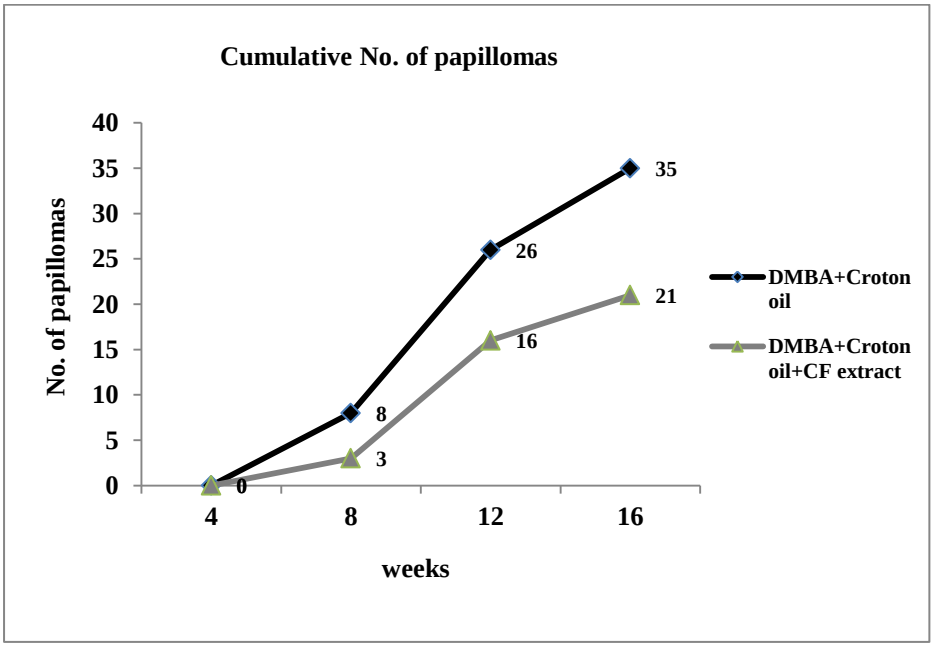
**Figure (b): Showing reduced Skin papillomas which received *Cassia fistula* treatment.**



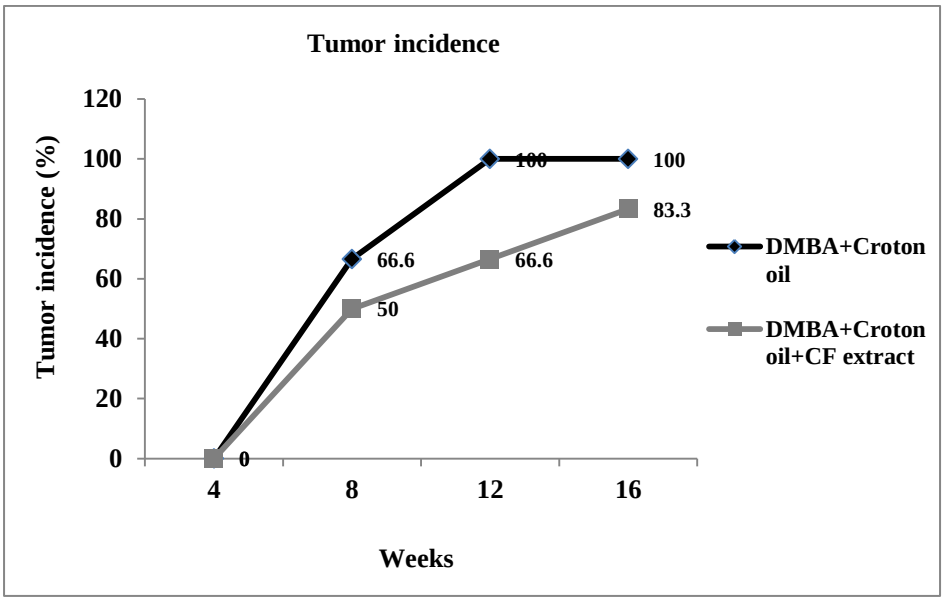

**Graph 1: Showing the effect of Hydromethanolic seeds extract of *C. fistula* on Tumour yield in CF treated mice as compared to carcinogen control group.**




**Graph 2: Showing the effect of Hydromethanolic seeds extract of *C. fistula* on Tumour burden in CF treated mice as compared to carcinogen control group.**




**Graph 3: Showing the effect of Hydromethanolic seeds extract of *C.fistula* on cumulative no. of papillomas in CF treated mice as compared to carcinogen control group.**

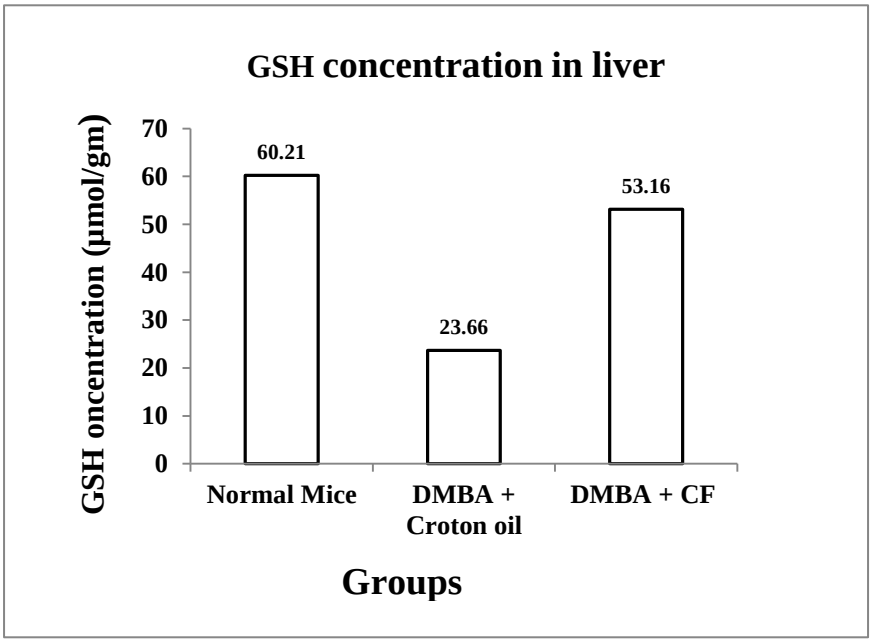



**Graph 4: Showing the effect of Hydromethanolic seeds extract of *C .fistula* on tumor incidence of papillomas in CF treated mice as compared to carcinogen control group.**

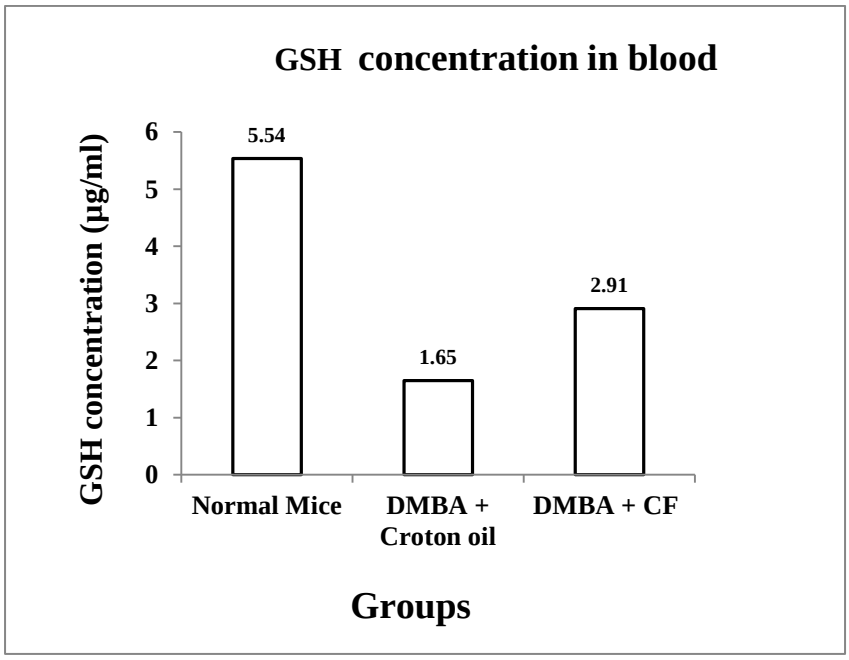
**Biochemical study**

**Table 2: Effect of hydromethanolic extract of *Cassia fistula* seeds on GSH level in blood (µg/ml) and liver (µmol/gm) in DMBA induced papilloma model.**

| S. No | Groups | Glutathione level | |
|---|---|---|---|
| | | Blood (µg/ml) | liver (µmol/gm) |
| 1 | Normal Mice | 5.54 ± 0.04 | 60.21 ± 0.48 |
| 2 | Carcinogen (DMBA + Croton oil) | 1.65± 0.04 | 23.66 ± 2.07 |
| 3 | DMBA + CF (5000 mg/kg body wt.) + Croton oil | 2.91 ± 0.06* | 53.16 ± 0.31* |




**Graph 5: Showing liver Glutathione level in different groups in papilloma model**




**Graph 6: Showing blood Glutathione level in different groups in papilloma model.**

The chemical induction of tumors in mouse skin has been used to study mechanisms of epithelial carcinogenesis and evaluate modifying factors for more than 60 years (Erika *et. al*., 2009)[6]. Multi-stage carcinogenesis starts with the development of initiated cells after interactions of a carcinogenic agent with normal (target) cells. In the mouse skin tumour promotion model, tumourigenesis is initiated by one sub minimal dose of carcinogen and treatment with croton oil as promoter (Berenblum and Shubik, 1949)[5].The exact mechanism of Anticarcinogenic effect of *Cassia fistula* seed extract is not yet clearly known however it is believed that Free radicals are highly reactive chemicals that have the potential to harm cells when a molecule either gains or losses an electron then they are formed. Glutathione (L-*γ*-glutamyl-L-cysteinylglycine) is ubiquitous in eukaryotic cell and is implicated in all cellular functions. In normal conditions, the GSH concentrations in mammalian cells can range between 1 to 10 mM, with the reduced GSH predominating over the oxidized form (Meister, 1988; Hassan and Fridovich, 1980).[9,10] GSH plays a crucial role in many biological processes, Harmful hydrogen peroxide are minimized by the enzyme glutathione using it as a reluctant (Meister, 1994)[7]. Glutathione is one of the antioxidant enzymes that act as the first line of defence against oxidative stress. Its central role in maintaining the cell's redox state (Meister and Anderson, 1983)[8]. Increase of ROS in cancer cells probably part of the initiation and progression of cancer. As excessive levels of ROS stress can also be toxic to the cancer cells and cells are likely to be more susceptible to damage by further ROS induced by exogenous drugs and make them more responsive to ROS producing cancer treatments. Therefore,

changes in level of ROS by GSH variation is an approach to selectively kill cancer cells without causing toxicity to normal cells (Behrend *et. al.*, 2003; Makiya 2008; Trachootham *et. al.*, 2009)[11-13.] In some other studies Rhein (an anthraquinone), is isolated from the flowers and leaves of CF. Rhein has reported to activate the P21 and P53 path way with in Rhein induced apoptosis of human lung cancer. Seeds extract of CF contains Anthraqunion so it may be Rehine responsible for anticancerous activity for the present study.

## CONCLUSION

The treatment of human diseases has been attempted with varying degrees of success with the use of herbs by various peoples of the world through the history of humankind. Medicinal plants are having promising pharmacological activities which are nontoxic in nature and can be utilized in treating various kinds of diseases. In the present investigation seed extract of *Cassia fistula* has restored the level of GSH in the treated mice and the application of CF prevented tumour initiation and tumour promotion in treated groups. The ability of the CF treatment is suggestive of its ability to reduce tumour promotion by reducing the colonel extension. *C. fistula* should be promoted for the development of modern drug for the control of various diseases including cancer. However there is need to isolate the active component and formulation of the drug to treat the cancer by performing clinical trial.

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