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Evaluation of Chemopreventive and Analgesic Activity of *Hibiscus cannabinus* L. Seed Oil in Mice

Rabindra N. Acharyya[1], Md. Arman Islam[1], Mahin Hossain[1], Susmita Paul[1], Shrabanti Dev[1], Mahtalat Ahmed[2], Md. Monirul Islam[2], Asish K. Das[1]*

[1]*Pharmacy Discipline, Khulna University, Khulna-9208, Bangladesh*
[2]*Agrotechnology Discipline, Khulna University, Khulna-9208, Bangladesh*

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ABSTRACT

Hibiscus cannabinus L., a useful edible plant from Malvaceae family is familiar as Kenaf. The study was aimed to evaluate the chemopreventive and analgesic potentials of Kenaf seeds oil (KSO) using skin carcinogenic mice where carcinogenesis was induced by 7, 12-dimethylbenz (a) anthracene (DMBA) and croton oil. KSO was administered orally at two different doses (250 and 500 mg/kg) from two weeks before the DMBA application and continued till the end of the experiment. Analgesic activity was assessed using the acetic acid-induced algesia in mice. Administration of KSO significantly suppressed the tumor incidence, tumor yield and tumor burden in comparison with carcinogen control group. The number of tumors, tumor diameter and tumor weight were comparatively lower in KSO-treated groups. Moreover, the biochemical analysis of tumor homogenate showed a significant elevation of reduced glutathione (GHS) levels in oil-treated mice in comparison with carcinogen control mice. These results support the cancer preventive potential of KSO. In addition, the seed oil showed 30.30% and 47.74% writhing suppression at the dose of 250 and 500 mg/kg respectively in acetic acid-treated mice whereas 76.13% suppression was observed with diclofenac sodium (25 mg/kg).

***Keywords*:** *Hibiscus cannabinus*, DMBA, Croton oil, Chemopreventive.

Introduction

Globally, cancer is one of the major cause of death.[1, 2] Worldwide, cancer patients' death is anticipated to rise from 7.1 million in 2002 to 11.5 million in 2030.[3] About 35% of the western population develop cancer at different stages during their life time. Although cancer treatment strategy developed a lot but still cancer remains beyond the control owing to the lack of effective drugs as well as high cost and extreme side effects of available chemotherapeutic agents. Therefore, researchers are making efforts to develop better drugs for the management of this disease. It is assessed that plant is the source of more than 50% of anticancer agents developed so far in one or other way.[4]

Reactive oxygen species (ROS) are considered as major causal factors for the development and promotion of cancer as well as many other diseases. They induce oxidative stress which ultimately causes mutagenesis and carcinogenesis. Several chemicals like DMBA induce cancer through ROS generation and compounds with antioxidant potentials have been proved to control cancer promotion.[5] As defined by Sporn, chemoprevention is the best among the strategies of cancer prevention.[6]

Edible natural antioxidants like vitamins, micronutrients etc. are nontoxic or lesser toxic than synthetic modern medicines.[7] So regular consumption of fruits and vegetables enriched with high antioxidant potentials could be helpful for cancer prevention.

Algesia (pain) is an unpleasant sensation associated with tissue injury. Although it is a primary indication as well as protective in nature but often causes a lot of discomforts. Most common side effects of established analgesics drugs are acidity, constipation, ulceration, gastrointestinal bleeding, drowsiness, nausea etc.[8] So it is also a dire need of the time to search for better analgesics with lesser adverse effects. Kenaf is cultivated worldwide for its fiber. Nowadays the plant is attracting increasing attention for its medicinal values. The plant contains various bioactive components and has long been prescribed in traditional folk medicine for various ailments.[9] Kenaf seeds oil is edible and contains vitamin E, β-sitosterol, and alpha-linolenic acid (ALA), which are reported to have very good chemopreventive activity. It is also reach in omega polyunsaturated fatty acids (PUFAs) which are well-known to limit oxidative stress and improve health status. The toxic effect of kenaf seed oil (KSO) on different cancer cells has been reported in recent years.[10] *In vitro* studies sometimes contradict with in vivo study as the biological system is completely different from the cell culturing medium. In these circumstances, the present study was designed to assess the anti-cancer as well as analgesic potentials of KSO through *in vivo* study using chemically-induced skin carcinogenic and acetic acid-induced algesia model mice respectively.

Materials and Methods

Collection and extraction

Kenaf seeds were collected from Khulna region, Bangladesh in July, 2017 and identified by Bangladesh Jute Research Institute, Monirumpur, Jessore, Bangladesh; Accession no.: BJRI-KU-AT-S08-2017. Seeds were cleaned and dried in shade. After grinding, seed powder (500 gm) was successively extracted with ethanol three times

*Corresponding author. E mail: dasasish03@yahoo.com
Tel: 88-01914066214

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at room temperature at a ratio of sample: solvent 1:5. The extract was concentrated using a rotary evaporator at 40°C. Ethanolic extract was then further extracted with petroleum ether to isolate the oil fraction and yield of KSO was 4% v/w. This fraction was used for phytochemical and pharmacological evaluation.

Phytochemical analysis
Different chemical tests were performed on KSO for the identification of various bioactive secondary metabolites following the established standard protocols.[11]

Experimental animals
Adult male Swiss albino mice weighing 20-25g were used as experimental animal. Animals were collected from International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B) and maintained under ideal environmental conditions in the animal house of Pharmacy Discipline, Khulna University. The standard guidelines of the Ethical Committee were followed strictly for caring and handling of experimental mice (approval number: KU/PHARM/AEC/15/06/029).

Chemicals
7, 12-dimethylbenz (α) anthracene (DMBA), Croton oil, Tris-HCl, BSA (Bovine Serum Albumin), Reduced glutathione (GSH), TCA (Trichloroacetic acid), DTNB [5, 5 dithiobis (2-nitrobenzoic acid)] were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Test materials
KSO was used at two different doses (250 and 500 mg/kg) for the evaluation of its anti-cancer and analgesic activity.

Experimental design
Assessment of chemopreventive potential
Evaluation of the chemopreventive effect of KSO on chemically induced skin carcinogenesis in Swiss albino mice was carried out as per the established procedure with some minor modification.[12] Forty experimental animals were randomly selected and skin of 3×3 cm² of the back area was shaven before the experiment. Animals were divided into four groups named as group-I, group-II, group-III, group-IV consisting of ten mice in each group. Group-I was served as control group which was administered 2% tween 80 in water (10 ml/kg) orally and 100 µL of acetone was topically applied on the shaven area. In group-II, mice were also administered 2% tween solution and received a single topical application of DMBA (100 µg in 50 µL of acetone per animal) and named as carcinogen control group. Two weeks after the initiation, 0.1 ml of 1% croton oil in acetone was topically applied on each alternative day until the termination of the experiment at 16 weeks. Group-III and IV were served as test groups and administered orally with KSO at a dose of 250 mg/kg and 500 mg/kg respectively for 15 days before initiation of carcinogenesis by DMBA and continued throughout the experimental tenure on daily basis. The croton oil treatment was continued to these animals like Group II.
Mice were closely monitored throughout the experimental period and weighed weekly. Appearing times of papilloma in each mouse of all groups were recorded on daily basis. Papillomas persisted for two weeks or more were considered for final evaluation. At the end 'Tumor Incidence' (the number of mice carrying at least one tumor); 'Tumor Yield' (the average number of papillomas per mouse); 'Tumor Burden' (The average number of tumors per tumor bearing mouse) were calculated.

Biochemical study
Homogenate of tumor tissue was prepared following the procedure as mentioned by Sharma with little modification.[13] In brief, the skin was removed from the selected dorsal area soon after the sacrifice of animals and washed properly with chilled 0.9% NaCl (pH 7.4) and used for biochemical study. A 10% tissue homogenate in 0.15 M Tris-HCL (pH 7.4) was prepared using these tissue samples. It was then centrifuged at 2500 rpm for 10 minutes. The supernatant was used for the assessment of total protein and reduced glutathione (GSH).

Estimation of total protein
The assay was performed using bovine serum albumin as a standard.[14] In brief, sample of 1.0 mL was diluted with 0.9 ml reagent A (2 gm sodium potassium tartrate.$4H_2O$ + 100 gm sodium carbonate + 500 mL 1N NaOH + H_2O to one liter) and incubated for 10 min at 50ºC and then cooled to room temperature. Reagent B of 0.1 mL (2 gm sodium potassium tartrate.4 H_20 + 1 gm copper sulfate.$5H_2O$ + 90 mL H_2O + 10 mL 1N NaOH) was then added to it and incubated for 10 min at room temperature. Finally, 3 mL reagent C (1 volume Folin-Ciocalteau reagent diluted with 15 volume water) was added, mixed and incubated for 10 min in the 50ºC bath. The mixture was then cooled to room temperature. The absorbance was taken at 650 nm using UV-VIS spectrophotometer.

GSH estimation
Following the method as described by Giustarini D, 2013, reduced glutathione (GSH) was estimated.[15] In brief, tissue homogenate of 0.1 ml was precipitated with 100 µl 5% TCA (trichloroacetic acid) and centrifuged. A mixer was made with 0.1 ml of supernatant, 2.0 ml of 0.6 mM DTNB [5, 5 dithiobis (2-nitrobenzoic acid)] reagent and 4.0 ml volume was ensured with 0.2 M phosphate buffer (pH 8.0). The absorbance was taken at 412 nm against a blank containing TCA (trichloroacetic acid).

Evaluation of analgesic activity
For evaluating this activity, the method of Devi was adopted with minor modification.[16] In brief, the experimental mice were randomly divided into four groups, consisting of five in each. Group I was considered as 'control group' which received 1% (v/v) Tween-80 in water at the dose of 10 mL/kg of body weight; group II was administered with standard drug diclofenac sodium (25 mg/kg); group III and group IV were considered as test groups and treated with the KSO at the doses of 250 and 500 mg/kg respectively. Intraperitoneal injection of 0.6% acetic acid to each mouse was done 30 min after oral administration of control vehicle, standard drug and extracts at two different doses. After 5 minutes of acetic acid injection, the number of writhes (squirms) was counted for 15 minutes of individual mouse.

Statistical analysis
Experimental results were expressed as the mean ± standard error of mean (SEM). Dunnett's t test was performed to calculate statistical significance by one-way analysis of variance (ANOVA). Pairwise comparisons among different treatment groups were done with Post-hoc Tukey test. SPSS software of IBM Corporation, New York, USA (version 16.0) was used for analyzing the data. $P<0.05$ was considered as statistically significant.

Results and Discussion

Phytochemical screening
Medicinal plants play important role in the healthcare system worldwide.[17] The plant-stored metabolites have been reported to possess anti-cancer potentials. Phytochemical screening of KSO ensured the presence of alkaloids, flavonoids, glycosides gums, steroids, tannins and terpenoid.

Chemopreventive activity
Chemoprevention plays a vital role to control carcinogenesis.[18] We have studied the chemopreventive effect of KSO in DMBA and croton oil-induced carcinogenesis model mice. Oral administration of KSO at both doses was found to be effective in suppressing both tumor incidence and tumor burden in carcinogenic mice (Table 1). The number, size and weight of tumors were also markedly decreased in KSO treated animals compared to group-II (Table 2 and Figure 1). The onset of tumor induction was also delayed in KSO administered mice. The result of this study authenticates the anti-cancer potentials of KSO claimed by other researchers.[19]

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Protein concentration determination

Oral administration of KSO resulted in a significant elevation of protein concentration level in group-III and IV compared with the group-II (Table 3). This finding revealed that DMBA/croton oil cause protein damage by oxidative stress generated through ROS. Oxidative stress arises due to the imbalance between pro-oxidants and antioxidants and leads to tissue damage. Thus, oxidative stress contributes to the process of carcinogenesis by enhancing or facilitating the metabolic activation and/or initiating effects of carcinogens.[20]

GSH estimation

Estimation of glutathione was done following the method of Giustarini.[15] Results revealed that the level of GSH was boosted in Group-III and Group-IV due to the oral administration of KSO compared with carcinogen control (Table 4). Glutathione is one of the important endogenous antioxidant enzymes that help to resist oxidative stress and act as the first line of defense. DMBA is a synthetic hydrocarbon which extensively induces carcinogenicity by DNA damage and cell death, especially in the skin and mammary gland of mice.[21]

Previous *in vitro* studies using several cancer cell lines and also the present *in vivo* studies in Swiss albino mice indicate that KSO has the potential to be of use in combating cancer. Seven phenolic compounds namely gallic acid, p-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, and p-coumaric and ferulic acids have been reported to be present in KSO.[22] Our studies also substantiate this claim showing the high content of phenolic acid in KSO. Several *in vivo* and *in vitro* studies indicated that polyphenols are the main phytochemicals having antioxidant and anti-proliferative activity. Due to these attributes, these molecules may suppress cancer promotion and progression.[23] From this discuss it may be claimed that the phenolic compounds in KSO are among the probable candidates responsible for the chemopreventive activity. However, more scientific investigations are needed to improve our understanding regarding its anti-cancer effects.

Analgesic activity

Aapplication of KSO at both doses showed significant analgesic effect in the animals under investigation. It exhibited inhibition of writhing reflex by 30.32 % and 47.74 % at the dose of 250 and 500 mg/kg respectively while the standard drug diclofenac sodium showed 76.13 % writhing inhibition at 25 mg/kg dose (Table: 5). It is well know that pain and localized inflammation generated by acetic acid is due to its production of PGE_2 and $PGE_2\alpha$ in the peritoneal fluid.[24] Several analgesic like ibuprofen, diclofenac sodium and indomethacin have been reported to inhibit writhing through inhibition of acetic acid induced prostaglandin synthesis in mice.

Prostaglandins are the major factors for peripheral pain induction and any agent which reduce the release of prostaglandins possess analgesic activity.[25] The reduction of pain by the kenaf seed oil might have occurred via inhibition of prostaglandin synthesis. Polyphenolic compounds, like flavonoids and tannins, have been reported to have multiple pharmacological effects, including analgesic activity.[26] So, flavonoids, alkaloids and tannins present in KSO might be responsible for its analgesic property.

Table 1: Anti-cancer activity of KSO in DMBA-induced skin carcinogenic mice

Group	Body weight (gm)		Tumor Incidence (%)	Tumor yield	Tumor burden	Average latent Period (weeks)
	Initial	Final				
Group-I (Control)	21.54 ± 0.57	35.58 ± 1.64$^{\#}$	0	0	0	0
Group-II (carcinogen control)	21.69 ± 0.56	30.0 ± 0.90*	100	3.75 ± 0.10*$^{\blacksquare\blacktriangle}$	3.75 ± 0.09*$^{\blacktriangle}$	7.43 ± 0.38*$^{\blacksquare}$
Group-III (250mg/kg)	21.42 ± 0.99	32.67 ± 0.38	75	2.2 ± 0.10*$^{\#\blacktriangle}$	3.5 ± 0.11*	9.93 ± 0.28*$^{\#\blacktriangle}$
Group-IV (500mg/kg)	22.10 ± 0.56	33.33 ± 0.49	50	1.75 ± 0.07*$^{\#\blacksquare}$	3.00 ± 0.29*$^{\#}$	7.99 ± 0.59*$^{\blacksquare}$

Values are expressed as Mean ± SEM (n = 6); *P < 0.05 versus control (Dunnett's t test); $^{\#}P$ < 0.05 vs carcinogen control; $^{\blacksquare}P$ < 0.05 vs KSO 250 mg/kg; $^{\blacktriangle}P$ < 0.05 vs KSO 500 mg/kg; (pair-wise comparison by Post Hoc Tukey test).

Table 2: Effect of KSO on tumor size and weight in DMBA-induced skin carcinogenic mice

Group	No. of tumor with size (mm) in diameter			Tumor weight (mg)
	< 2 mm	2-4 mm	> 4 mm	
Group-I (Control)	0	0	0	0
Group-II (carcinogen control)	30	19	6	625
Group-III (KSO 250 mg/kg)	12	13	1	235
Group-IV (KSO 500 mg/kg)	08	6	0	128

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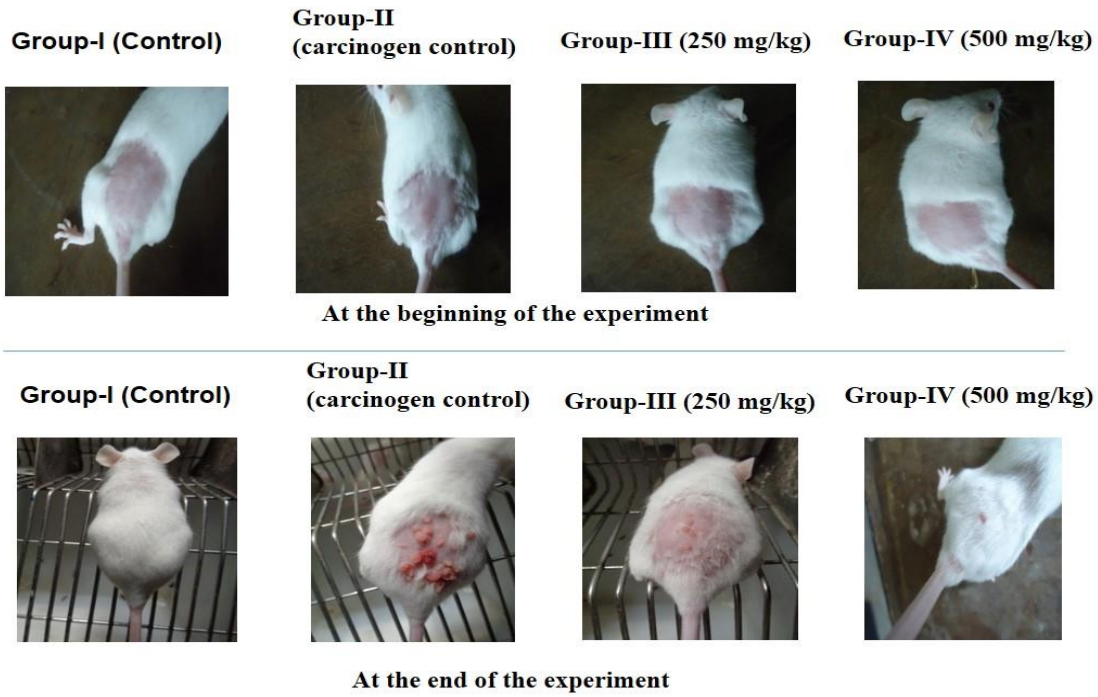

Figure 1: Pictorial presentation of chemopreventive effect of KSO in DMBA-induced skin carcinogenic mice

Table 3: Total protein concentration in tumor and liver tissue of experimental mice

Group	Total protein concentration (µg protein/mg tissue)	
	Tumor Tissue	Liver tissue
Group-I (Control)	9.45 ± 0.65$^{\#}$	11.04 ± 0.08
Group-II (Carcinogen control)	6.18 ± 0.30*$^{■▲}$	9.04 ± 0.70$^{\#}$
Group-III (KSO 250 mg/kg)	8.86 ± 0.62$^{\#}$	10.05 ± 0.57*
Group-IV (KSO 500 mg/kg)	9.20 ± 0.27$^{\#}$	10.63 ± 0.79*

Values are expressed as Mean ± SEM (n = 6); *P < 0.05 versus control (Dunnett's t test); $^{\#}P$ < 0.05 vs carcinogen control; $^{■}P$ < 0.05 vs KSO 250 mg/kg; $^{▲}P$ < 0.05 vs KSO 500 mg/kg; (pair-wise comparison by Post Hoc Tukey test).

Table 4: Variation in the reduced glutathione level in DMBA-induced skin carcinogenesis with/without KSO treatment

Group	Reduced GSH level (µg GSH/mg protein)	
	Tumor Tissue	Liver Tissue
Group-I (Control)	684.4 ± 34.49$^{\#}$	795.8 ± 42.59$^{\#■}$
Group-II (Carcinogen control)	473.2 ± 50.39*	279.6 ± 20.30*$^{■▲}$
Group-III (KSO 250 mg/kg)	573.6 ± 40.73	557.6 ± 52.97*$^{\#}$
Group-IV (KSO 500 mg/kg)	609.8 ± 64.58	677.8 ± 83.67$^{\#}$

Values are expressed as Mean ± SEM (n = 6); *P < 0.05 versus control (Dunnett's t test); $^{\#}P$ < 0.05 vs carcinogen control; $^{■}P$ < 0.05 vs KSO 250 mg/kg; $^{▲}P$ < 0.05 vs KSO 500 mg/kg; (pair-wise comparison by Post Hoc Tukey test).

Table 5: Effect of KSO on acetic acid-induced writhing in mice

Group	Mean writhing	Percent Writhing	Inhibition (%)
Control	31 ± 2.08$^{\#■▲}$	100	-
Standard (diclofenac sodium 25 mg/kg)	7.4 ± 0.49*$^{■}$	23.87	76.13
KSO (250 mg/kg)	46 ± 4.07*$^{\#▲}$	69.67	30.32
KSO (500 mg/kg)	16.2 ± 1.81*$^{■}$	52.25	47.74

Values are expressed as Mean ± SEM (n = 6); *P < 0.05 versus control (Dunnett's t test); $^{\#}P$ < 0.05 vs Standard (diclofenac sodium 25 mg/kg); $^{■}P$ < 0.05 vs KSO 250 mg/kg; $^{▲}P$ < 0.05 vs KSO 500 mg/kg; (pair-wise comparison by Post Hoc Tukey test).

Conclusion

The present study suggests that kenaf seeds oil has potential chemopreventive and analgesic activity. Results are quite promising and further work will help to explore the bioactive compounds responsible for these therapeutic benefits. Toxicological study should be done to ensure the safety of KSO for therapeutic usage.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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