



***In vitro* and *In vivo* Effects of the Leaf Extracts of *Cassia tora* and *Cassia sophera* in Reducing the Cytotoxicity and Angiogenesis**

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Authors' contributions

This work was carried out in collaboration between the two authors. Author CS designed the study, performed the statistical analysis, made the final draft of the manuscript. Author SR managed the literature searches, carried out the study under the supervision of author CS and wrote the protocol. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: Consumption of *C. tora* and *C. sophera* of family caesalpinaceae as leafy vegetables by local folklore has initiated this study to investigate the cytotoxic effect and antiangiogenic effect of their leaf extracts on human cancer cell lines and chick embryo respectively.

Study Design: For *in vitro* studies colon and hepatocarcinoma cell lines were used. For *in vivo* studies chicken egg chorioallantoic membrane (CAM) assay is used.

Methodology: Methanol, ethyl acetate and petroleum ether extracts of leaves were tested for antiproliferative activity using MTT assay, on HCT15 and Hep G2 cell lines. *In vivo* CAM model was used to study anti-angiogenesis activity of extracts using filter paper discs.

Results: All extracts of both plants were toxic to tested cell lines with increasing concentrations and time. Ethyl extract of *Cassia tora* was found to be most toxic on Hep G2 cell line, though ethyl extract and methanol extracts of both plants were found to give strong antiproliferative activity. Hep G2 was found to be most sensitive to all extracts than HCT15 as inhibited by low IC₅₀ values. Ethyl acetate extracts of both plants exhibited

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greater anti-angiogenesis activity than other extracts.

Keywords: C. tora; C. sophera; HCT15; Hep G2; antiproliferative; MTT assay; CAM model; anti-angiogenesis.

1. INTRODUCTION

Fruits and vegetables have been examined extensively in nutritional research in relation to colorectal cancer and researchers found that consumption of vegetables is associated with decreased risk of three cancers in different parts of the bowel. They also found that consumption of whole leafy vegetable or fruit decreased the occurrence of cancer [1].

Angiogenesis is a multistep process leading to the formation of new capillaries emerging from pre-existing blood vessel systems. Recruitment of new blood vessels plays an important role in tumor survival and growth. In 1971, a concept of anti-tumor strategy by controlling angiogenesis was hypothesized by Folkman, as tumor growth is angiogenesis dependent and every aspect of tumor growth requires increment in vascular growth. [2,3] Highly vascularized chorioallantoic membrane of the chicken embryo is used as in vivo model and widely used for determination of anti-angiogenesis activity. Due to the increasing interest in anti-angiogenic therapy for cancer, several agents are investigated which act as angiogenesis inhibitors [4].

Cancer being one of the leading causes of deaths worldwide has many therapeutic strategies including chemotherapy. But high systemic toxicity and drug resistance limit the successful outcomes in most cases.[5] Plants have been used in the treatment of cancer since ancient period. Natural products and related drugs are used to treat 87% of all human diseases including cancer. About 25% of prescribed drugs in the world are obtained from various plants. Over 3000 species of different medicinal plants have been reported to have anticancer properties.[6] Ayurvedic system of medicine reveals that there are several herbs with anticancer and anti-inflammatory activity [7].

Herbal medicine for cancer therapy is popular in complementary and alternative medicine. Vegetables and herbs used in folk and traditional medicine have been accepted as one of the main sources of chemo preventive drugs. Herbal medicine as potent anticancer drug candidates is supported by scientific studies.[8]. Majority of phytochemicals are phenolic compounds. Dietary polyphenols can modulate the process of carcinogenesis by several mechanisms. Anticancer activity has been shown to be associated with a different phytochemicals like polyphenols, flavonoids and catechins [6,7,8]. As cancer is an ailment that affects more or less 200 types of cells, whose major characteristic is the lack of control of the cell proliferation, differentiation and health, invading organs and tissues. The prognosis for a patient with metastatic carcinoma of the lung, colon, hepatic or prostate remains a concern and accounts for more than half of all cancer deaths [9].

Though problems of cancer control have long been waiting for the best solution, challenges of drug discovery still remain. [10] Currently over 60% of used anticancer agents are derived in one or the other way from natural sources, including plants, marine organisms and microorganisms. There are worldwide efforts to discover new anticancer agents from plants. [11] The rich and diverse plant resources of India are likely to provide effective anticancer agents.

Some members of family caesalpinaceae are known for their antimicrobial, antioxidant and anti-inflammatory activity. *C.tora* is a wild, seasonal, edible herb (family caesalpinaceae) is widely distributed in tropical and Asian countries. It is shown to have various pharmacological activities including antihepatotoxic, antiallergic, antifungal, antibacterial, radical scavenging and is used as laxative, diuretic, antiasthmatic agent. Several chemical components like anthraquinones, chrysofenol, emodin, Obtusifolia, obtusin, chrysoobtusin, and naphthopyrones have been reported in *C. tora* [12,13,14].

C.sophera is also edible in Indian subcontinent, used as folk medicine for treatment of many diseases. It is effective in the treatment of pityriasis, psoriasis, asthma, acute bronchitis, cough, diabetes, piles, jaundice and palpitation. The chemical analysis revealed the presence of ascorbic acid, dihydroascorbic acid and β -sistosterol [15,16].

Studies carried out by researchers revealed that low consumption of vegetables is associated with an increased risk of cancer.[17] *C. tora* and *C.sophera* are not so well-known leafy vegetables which have good antioxidant activities.[18] This activity may have some benefit in preventing cancer by regular use of these plants. As both the above plants are edible, the objective of this study is to investigate cytotoxic activity of crude extracts from leaves on colon cancer (HCT15) and hepatocarcinoma cell lines (Hep G2) using MTT assay. As there is no report on anti-angiogenic activity, extracts were also tested for their anti-angiogenic activity by CAM assay.

2. MATERIALS AND METHODS

2.1 Collection and Identification of *C.tora* and *C. sophera*

The plants were collected in June-July 2011 from different localities of Warangal, AP, Mangalore, Karnataka, and identified by Dr. V. Thirupathiah, Retd Prof, KU, Warangal and specimen is deposited in the laboratory.

2.2 Organic Extraction

Fresh leaves were collected, washed, shade dried and powdered. For every 100g powder 350mL of solvent was added for soxhlet extraction, successively using solvents of increasing polarity beginning with petroleum ether, ethyl acetate and methanol. After soxhlet extraction solvents were removed by evaporation and further dried in oven. Thick dark paste like extracts were preserved in glass vials in refrigerator. The portion of crude extracts was dissolved in DMSO for further use.

2.3 Preliminary Phytochemical Estimation

Phytochemical analysis, total phenols and flavonoids, DPPH assay were conducted using standard protocols [18].

2.4 Antioxidant Assays

Enzymatic and non-enzymatic antioxidant assays were performed using standard protocols. Quantitative estimations of GPX, SOD, CAT, GSH and VIT C were done. SOD was estimated according to the procedure of Marklund and Marklund (1974). The Catalase activity was measured according to the method of Arbi (1983). GPX was estimated by

coupled assay procedure of Pagila and Valentine (1967). Reduced glutathione was estimated according to the method of Hissin and Hiff (1976) Ascorbic acid was determined according to the spectrophotometric method of Zannoni et al (1974) [19].

2.5 Cell Lines and Cultural Conditions

Colon cancer (HCT15) and hepatocarcinoma (Hep G2) cell lines from NCCS, Pune were cultured in RPM 1 1640 medium with 20% FBS, 2mM L-glutamine, 1% penicillin /streptomycin under a fully humidified atmosphere 5% CO₂ at 37°C.

2.6 Proliferation Assay

The effect of leaf extracts of *C.tora* and *C.sophora* on the viability of human cancer cells was determined by MTT (3-[4, 5-dimethyl thiozole-2-yl]-2-5-diphenyl tetrazolium bromide) assay.[20] Briefly, cells of HCT15 and Hep G2 cell lines were harvested. 99µl of cell suspensions in growth medium were plated in 99-well microtitre plates at concentrations of 1x10⁴cells/well and incubated for 48h at 37⁰C in a humidified incubator.

The plates were then microscopically examined for confluent monolayer and turbidity. The growth medium was removed carefully. The cell monolayer was washed with RPM medium. To the washed cell sheet 100µL of test sample was added. Test samples were prepared by dissolving extracts in DMSO (stock 1-10mg extract/mL DMSO) followed by diluting with RPM1, 1640 medium (stock 2- 0.5mL of stock 1 and 0.5 mL of growth medium) to give final concentration at 5, 10, 25, 50,100µg/mL. Different concentrations of extracts were added to the respective wells in such a way that final volume in each well is 100µL. each concentration was placed in triplicate. The plate was incubated for 72 h. MTT solution at 5mg/mL was dissolved in 1ml phosphate buffer solution, mixed gently; 100µL of MTT was added to each well of the plate. The plate was incubated for 5h at 37⁰C. The solution in each well including MTT was aspirated and 100µL of buffered DMSO was added to dissolve formazone. The plates were shaken for 5min. optical density was measured on a microplate ELISA reader at 540nm with DMSO (20µL DMSO+80µL growth medium) as control. The cytotoxicity was obtained by comparing the absorbance between the samples and control. The percentage viability was calculated as follows:

$$\% \text{ viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

$$\% \text{ inhibition} = 100 - \% \text{ viability.}$$

IC₅₀ concentration was observed after incubation period, and calculated from dose-response curves.

2.7 Chicken Egg Chorioallantoic Membrane Assay

Anti- angiogenic activity of crude leaf extracts of *C. tora* and *C. sophora* was conducted on fertilized eggs by modified CAM assay method [21,22,23].

Fertile white Leghorn chicken eggs (*Gallus domesticus*) were obtained from a local hatchery with 3 days incubation. The eggs were incubated at 37°C in humidified incubator for 48 h, placed in horizontal position and rotated several times. The eggs were grouped as per type

of extracts and sprayed with 70% ethanol and air-dried to reduce contamination from the egg surface. On day 6, 26-gauge needle was used to puncture a small hole in the air sac of the egg, and 2-3 mL of albumen was sucked and sealed. This allows separation of vascularized CAM from the vitelline membrane and the shell. A window was then cut in the shell using a sterile blade and shell was removed with sterile forceps, under Laminar air flow. The window is closed with a cellophane tape after capturing the photographs of the embryo. The eggs were returned to the incubator after the filter paper discs (100micrograms of extract) of various extracts are placed on blood vessels of embryo using sterile forceps. After 48 h of incubation on 8th day photographs of embryos were taken to obtain the image of CAM after treatment with various extracts or standard drug (celecoxib). At least six eggs were used for each extract dose.

The percentage inhibition was calculated using the following equation, [24] % inhibition = [(vessel number of untreated CAM - vessel number of CAM treated with herbal extract) / vessel number of untreated CAM] x 100.

2.8 Statistical Analysis

All experiments were performed in triplicates and data were presented as mean \pm SD. Statistical data was conducted using SAS 9.

3. RESULTS AND DISCUSSIONS

3.1 Phytochemical Screening

Phytochemical screening of both plants revealed the presence of carbohydrates, proteins, glycosides, phenolics, alkaloids, tannins, saponins, flavonoids and phytosterols. *Cassia tora* methanol extract exhibited 128 μ g/mL phenols with Gallic acid standard and 175 μ g/mL flavonoids with Quercetin standard. *C. sophera* ethyl acetate extract showed high phenols and flavonoids [18].

3.2 Antioxidant Assays

Both plants exhibited different levels of GPX, SOD, GSH, CAT, VIT C and also radical scavenging activity. Though both plants exhibited the presence of antioxidant activity, proteins and SOD activity are high in *C.tora*, while CAT, GPX GSH and VIT C are more in *C. sophera* extracts.

Cassia tora -SOD=51.70 \pm 1.6 U/mg pr/ min. GPX= 0.45 \pm 0.01U/mg, CAT=1.105 \pm 0.09U/mg pr/min, GSH=187.5 μ g/mg pr, VIT C= 30 \pm 1mg, PROTEIN=0.64mg/mL.

Cassia sophera-SOD=17.41 \pm 0.6U/mgpr/min.GPX= 52 \pm 0.10U/mg, CAT=1.778 \pm 0.21U/mg pr/min, GSH=230.0 μ g /mg pr, VIT C= 27 \pm 0mg, PROTEIN=0.68mg/mL [19].

3.3 MTT Assay

The results of antiproliferative assay reveal that all the different extracts used of both plants exert antiproliferative action on HCT15 and Hep G2 cell lines (Fig.1 and Fig. 2). The dying cells exhibited ultra structural and biochemical features that characterize loss of viability. The morphological changes were inspected by microscopy. Some cells were beginning to detach

from the plate and become rounded after 5-6 h of treatment (50-100µg/mL). All the samples used exhibited different levels of cytotoxicity like cell rounding, shrinkage, aggregation and cell death, depending on the concentration of the extract. (Figs 3. A-F). At a concentration of 100 µg/mL, all the tested samples produced cytotoxic effect as evidenced by the increased number of dead cells. Reducing the concentration of extracts to 50, 25 and 10µg/mL reduced the cytotoxicity of the extract.

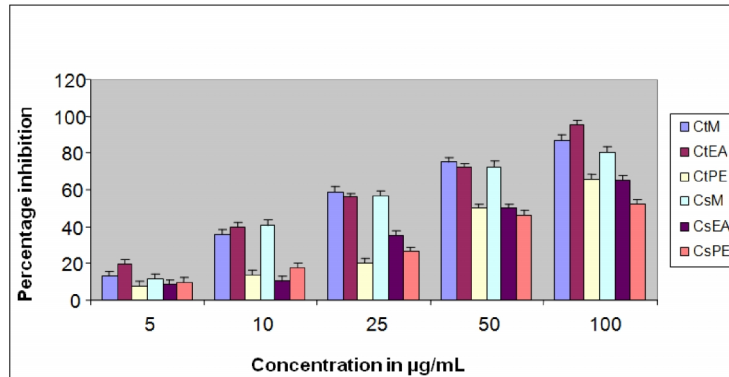


Fig. 1. Graph showing differences in percentage inhibition of cell viability (HCT 15) by Cassia tora and Cassia sophora leaf extracts

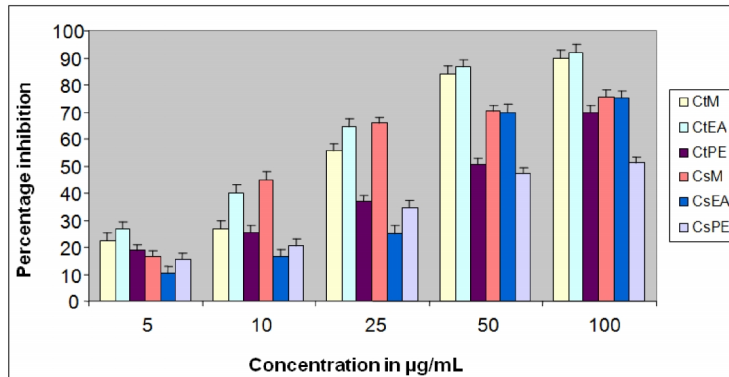
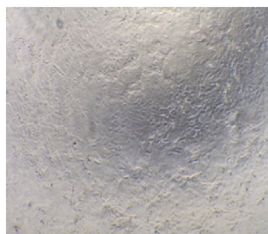
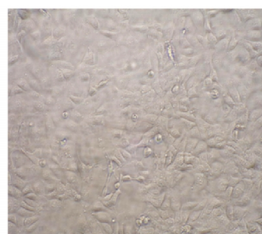


Fig. 2. Graph showing differences in percentage inhibition of cell viability (Hep G2) by Cassia tora and Cassia sophora leaf extracts

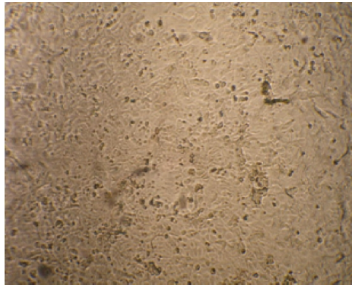
Where, Ct=Cassia tora, Cs= Cassia sophora, M= methanol extract, EA=Ethyl acetate, PE= Petroleum ether



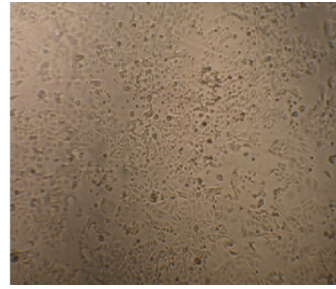
3.A. control well showing HCT 15 cell line



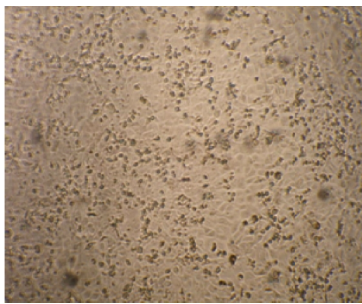
3.B. control well for Hep G2 cell line



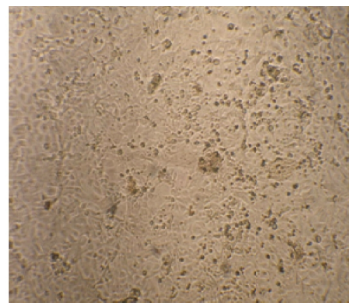
3.C. HCT 15 cell line after treatment of 50 µg/mL of extract *C. tora*



3.D. HCT 15 cell line after treatment of 50 µg/mL of extract *C. sophera*



3.E. HCT 15 cell line after treatment of 100 µg/mL of extract *C.tora*



3.F. HCT 15 cell line after treatment of 100 µg/mL of extract *C. sophera*

Fig. 3. Photographs showing varying degrees of inhibition of cell lines

Both the cell lines used were inhibited by all the extracts of two plants used. Hep G2 cell lines were more sensitive to extracts of both plants compared to HCT15. *C. tora* Ethyl acetate extract is most inhibitory to both cell lines used as exhibited by least IC₅₀ values (Table 1). Of all the extracts used, methanol and ethyl extracts of both plants exhibited strong activity. Petroleum ether extracts of both plants showed mild activity. Of the cell lines used Hep G2 was highly sensitive to extracts than HCT 15. Ethyl acetate extract of *C. tora* showed most potent activity to inhibit cell proliferation with IC₅₀ of 15.46 ± 1.25µg/mL, against Hep G2 cell lines. Reference compounds were Vinblastin and Mitomycin with IC₅₀ of 23µg/mL and 10µg/mL respectively for Hep G2.

Table 1. IC₅₀ values for *C.tora* and *C. sophera* extracts against HCT15 and Hep G2 cell lines. (Presented as Mean±SD)

EXTRACTS	HCT 15 (IC ₅₀ in µg/mL)	Hep G2 (IC ₅₀ in µg/mL)
CtM	35.16 ± 0.49	25.6 ± 0.1
CtEA	32.5 ± 0.78	15.46 ± 1.25
CtPE	68.53 ± 0.15	58.76 ± 0.37
CsM	40.7 ± 0.34	42.76 ± 2.6
CsEA	65.26 ± 0.97	34.76 ± 0.55
CsPE	81.2 ± 1.0	78.2 ± 0.26

Screening for anticancer substances is commonly conducted using viability assays.[25] Cell viability was assessed using MTT colorimetric assay. MTT is taken into cells by endocytosis and reduced, mainly by mitochondrial enzymes, to yield a purple formazan product and accumulates within living cells. Purple product can be detected using a colorimetric measurement. The ability of cells to reduce MTT provides a measure of cell number / proliferation / viability / toxicity [26].

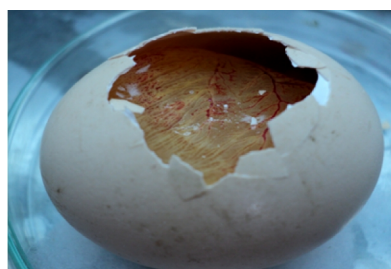
3.4 CAM Assay

Anti-angiogenesis activity of crude methanol, ethyl acetate and petroleum ether extracts was tested in vivo CAM model. We examined the 8th day old embryo after treatment for number of vessels and their reduction. The blood vessel analysis was based on evaluation of angiogenesis by three independent expert observers within 100cm² area surrounding the applied disc. Percentage inhibition is shown in table 2. Ethyl acetate and methanol extracts of both plants showed higher anti-angiogenesis than petroleum ether extracts. However *C.tora* ethyl acetate extract exhibited greater inhibition of blood vessels compared to any other extract (Figs. 4A-C).

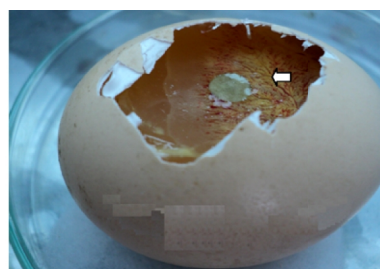
Table 2. Antiangiogenic activity- percentage inhibition of blood vessels by different extracts of *C. tora* and *C. sophora* (presented as Mean±SD)

Extracts	% inhibition
CtM	13.37 ± 0.89
CtEA	30.55 ± 4.80
CtPE	6.66 ± 0.44
CsM	15.13 ± 3.12
CsEA	20.83 ± 3.60
CsPE	6.36 ± 0.20

Different cytotoxicity of the tested plant extracts on HCT 15 and Hep G2 cell lines is based on their chemical composition and relative content of biologically active substances. The studies prove that the plants exhibit the presence of flavonoids, steroids, saccharides, proteins, terpenes, phenolic acids, saponins etc, of which polyphenols and flavonoids may contribute to their cytotoxic activity. The presence of phenolic compounds emphasizes the antioxidant and antiproliferative potential of the two plants used. The antiproliferative activity of *C. tora* methanolic extract was reported [27] which prompted us to evaluate its anticancer potential with other extracts. Moreover as these two herbs are edible, they are good sources of dietary anticancer agents.



Before



After

Fig. 4A. CAM treated with extract CtEA

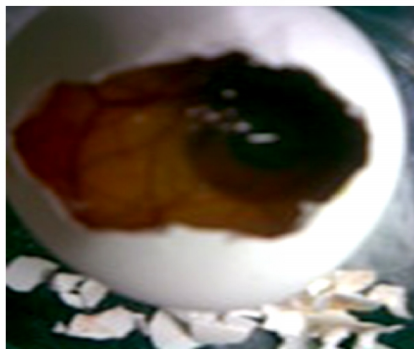


Before

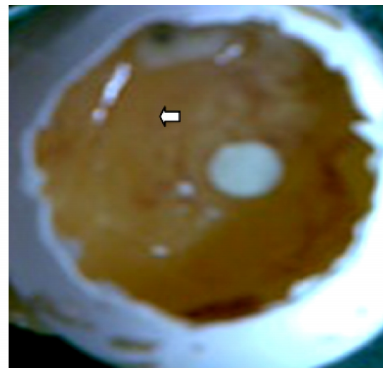


After

Fig. 4B. CAM treated with extract Cs EA



Before



After

Fig. 4C. CAM treated with CtM extract

Fig. 4. CAM assay- Photographs showing inhibition of blood vessels by plant extracts of *Cassia tora* and *Cassia sophera*

Several reports have shown that crude plant extracts are more effective pharmacologically than isolated active compounds. This may be due to the synergistic effects of various components present in the extracts [28].

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Medicinal plants represent a vast potential resource for anticancer compounds. The anticancer activity of medicinal plant derived compounds may result from a number of mechanisms, including effects on cytoskeletal proteins that play a key role in cell division, inhibition of DNA topoisomerase enzymes, antiprotease or antioxidant activity, stimulation of the immune system etc. the value of medicinal plants lies in the potential access to extremely complex molecular structures that would be difficult to synthesize in the laboratory [29].

Colorectal cancer is the third most common cancer in both males and females and accounts for about 9% of cancer deaths each year. Due to high incidence of colon cancer and an increased trend of natural product utilization, scientists are promoting medicinal plants as potential source of new remedies, as they are the most exclusive source of life saving drugs for the majority of the world's population [29].

HepG2 cell line was selected as hepatocellular carcinoma is the fifth most common cancer worldwide and accounts for approximately 54000 deaths every year. As liver is the major site that metabolize ingested material, it is prone to carcinogenic insult, but often faces poor diagnosis [30]. Several species of *Cassia* were shown to have hepato-protective activity. *Cassia auriculata* was shown to have strong hepato-protective effect in Wister Albino rats [31].

Literature reveals that *Cassia tora* is consumed by folklore as it is sweet tasting and in traditional medicine, leaves are used as laxative in the form of decoction, paste is used for skin ailments, also used as gelling agent in air fresheners, and in pet food preparations, seeds are roasted and used as coffee substitutes [32]. *Cassia sophera* leaves are used as raw, dried powder, in the form of juice and as decoctions. Roots and bark is also used in treating snake bite, respiratory problems, as diuretic and even for diabetes [33]. In this regard two edible plants of genus *Cassia* were chosen for this study.

4. CONCLUSION

In our study each extract inhibited colon cancer and hepatocarcinoma cell proliferation differently. There are reports that free radicals in the cell play an important role in carcinogenesis. Thus we conclude that the antiproliferative activity of the extracts of these two plants may arise from the antioxidative action they possess. Various anti-angiogenic agents are shown to decrease tumor growth and metastatic dissemination. Extracts of *C.tora* and *C. sophera* were elucidated for anti-angiogenic activity. As these plants are edible, they act as excellent source of herbal, dietary antioxidants, when consumed as whole may prevent occurrence of cancers. But a lot of lacunae need to be filled by further research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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