

Original Article

Potential anticancer effect of red spinach (*Amaranthus gangeticus*) extract

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The objective of this study was to determine the anti cancer effects of red spinach (*Amaranthus gangeticus* Linn) *in vitro* and *in vivo*. For *in vitro* study, microtitration cytotoxic assay was done using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit assay. Results showed that aqueous extract of *A. gangeticus* inhibited the proliferation of liver cancer cell line (HepG2) and breast cancer cell line (MCF-7). The IC₅₀ values were 93.8 µg/ml and 98.8 µg/ml for HepG2 and MCF-7, respectively. The inhibitory effect was also observed in colon cancer cell line (Caco-2), but a lower percentage compared to HepG2 and MCF-7. For normal cell line (Chang Liver), there was no inhibitory effect. In the *in vivo* study, hepatocarcinogenesis was monitored in rats according to Solt and Farber (1976) without partial hepatectomy. Assay of tumour marker enzymes such as glutathione S-transferase (GST), gamma-glutamyl transpeptidase (GGT), uridyl diphosphoglucuronyl transferase (UDPGT) and alkaline phosphatase (ALP) were carried out to determine the severity of hepatocarcinogenesis. The result found that supplementation of 5%, 7.5% and 10% of *A. gangeticus* aqueous extract to normal rats did not show any significant difference towards normal control ($P < 0.05$). The exposure of the rats to chemical carcinogens diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) showed a significant increase in specific enzyme activity of GGT, GST, UDPGT and ALP compared to normal control ($P < 0.05$). However, it was found that the supplementation of *A. gangeticus* aqueous extract in 5%, 7.5% and 10% to cancer-induced rats could inhibit the activity of all tumour marker enzymes especially at 10% ($P < 0.05$). Supplementation of anti cancer drug glycyrrhizin at suggested dose (0.005%) did not show any suppressive effect towards cancer control ($P < 0.05$). In conclusion, *A. gangeticus* showed anticancer potential in *in vitro* and *in vivo* studies.

Key Words: red spinach, anticancer effect, *in vitro* and *in vivo* studies.

Introduction

Cancer is believed to be the result of external factors combined with a hereditary disposition for cancer. It is a neoplasm characterized by the uncontrolled growth of the anaplastic cells that tends to invade surrounding tissue and to metastasize to distant body sites.¹ In Malaysia, cancer is one of the leading causes for morbidity and mortality. It was estimated to be 30,000 cases annually.² Cancers are complicated diseases. Although epidemiological data on populations may help identify exogenous agents, the probability of identifying the agent is not enough unless there are good dose-response data for humans or animal models. A group of vegetables with considerable anti-carcinogenic properties are the cruciferous vegetables. In epidemiological studies, it was shown that intake of cruciferous plants is inversely associated with kidney, prostate, bladder, colon, rectum and lung cancer risk.³⁻⁸

Malaysia has a variety of natural resources. Previous studies showed that low consumption of vegetables is found to be associated with the increased risk of cancer.⁹ Antioxidant activity present in these vegetables perhaps may have some benefits in cancer. Epidemiological studies

suggest that vitamin E and other antioxidants may reduce cancer incidence. It has been observed that people who eat diets rich in fruits and vegetables, which are rich in antioxidants, have lower incidences of cancer.¹⁰

Amaranthus tender (Red spinach: *Amaranthus gangeticus*) is a carotene-rich food available in Malaysia that has potential as a dietary source of chemopreventive phytochemicals. Consumption pattern of beta-carotene rich foods from 500 household of Coimbatore District in India was studied.¹¹ Results indicated that greens mainly were purchased from market and were consumed 2–3 times per week.

In-vitro and *in-vivo* experiments have been analysed from red spinach for possible anticancer agents. The cytotoxic effect of *Amaranthus gangeticus* aqueous and ethanolic

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extracts were used to determine the IC_{50} -value against several cancer cell lines such as non-estrogen dependent breast cancer cell lines (MDA-MB-231), estrogen-dependent breast cancer cell lines (MCF-7), liver cancer cell lines (HepG2), colon cancer cell lines (Caco-2) and transformed liver cell lines (Chang Liver). *In-vivo* studies were focused primarily for identifying the crude extract against hepatocarcinogenesis in rats induced by Diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF). We report the effect of red spinach on the enzyme tumour markers activity including Glutathione S-Transferase (GST, EC 2.5.1.18), γ -Glutamyl Transpeptidase (GGT, EC 2.3.2.2), Alkaline Phosphatase (ALP, EC 3.1.3.1) and Uridyl Diphospho Glucoronoyl Transpeptidase (UDPGT, EC 2.4.1.18) from liver of rats.

Materials and Methods

In-vitro studies

The *in-vitro* studies were designed to determine cytotoxic effect of the leaves aqueous and ethanolic extracts against several cancer cell lines such as MDA-MB-231, MCF-7, HepG2, Caco-2 and Chang Liver.

The leaves of *Amaranthus gangeticus* were obtained from a supplier at Seri Serdang, Selangor. Ethanol extraction method was used according to Ali *et al* (1996).¹² One hundred grams of fresh leaves were ground and soaked in water or 90% ethanol at room temperature overnight. The extracts were then filtered and evaporated with a rotary evaporator. After that, the dried residue was stored at $-80^{\circ}C$ and freeze-dried. The extract was ready for the treatment (*in vitro*).

MTT Assay (Boehringer Mannheim)

HepG2, Caco2, MDA-MB-231, MCF-7 and Chang liver cell lines culture were obtained from American Type Culture Collection (ATCC). HepG2 cells were cultured in Earl's Minimum Essential Medium; MCF-7, MDA-MB-231 and Caco2 cells were cultured in Dullbecco's Modified Eagle's Medium; and Chang liver cells were cultured in Roswell Park Memorial Institute 1640 supplemented with 10% of fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μ g/ml of Streptomycin using 25-cm² flasks, in 5% CO₂ incubator at 37°C. The viability of cells was determined with trypan blue reagent. Exponentially growing cells were harvested, counted with haemocytometer and diluted with a particular medium. Cell culture with the concentration of 1×10^5 cells/ml was prepared and was plated (100 μ l/well) onto 96-well plates (NUNCTM, Denmark). The diluted ranges of extracts were added to each well and the final concentrations of the test extracts were 5, 10, 20, 40, 60, 80, and 100 μ g/ml. The proliferative activity was determined using the MTT assay (3-[4, 5 - dimethylthiazol - 2-yl]-2,5-diphenyl tetrazolium bromide). The incubation period used was 72 hours. After solubilization of the purple formazan crystals were completed, the spectrophotometrical absorbance of the plants extract was measured using an ELISA reader at a wavelength of 550 nm. The cytotoxicity was recorded as the drug concentration causing 50% growth inhibition of the tumour cells (IC_{50} value).

$$\% \text{ cell viability} = \frac{\text{OD sample (mean)}}{\text{OD control (mean)}} \times 100\%$$

After the determination of the cytotoxicity percentage, graphs were plotted with the percentage of cytotoxicity against its respective concentrations.

In-vivo studies

The *in-vivo* studies were conducted to determine the effect of three different doses of red spinach juice against hepatocarcinogenesis. A total of 64 male Sprague-dawley rats, each initially weighing between 120 – 150 g were housed individually at 27°C and were maintained on normal or treated rat chow. The rats were divided into nine groups i.e. group I: control (basal diet) (N), group II-IV: AG-supplemented diet (5%, 7.5% and 10%) in drinking water, group V: cancer (DEN/AAF) with basal diet after week 4 (C), group VI- IX: cancer (DEN/AAF)-AG supplemented diet (C 5, C 7.5 and C 10), group XI cancer (DEN/AAF)-treated with Glycyrrhizin). The crude extract was prepared from the modification of a previous method.¹³ A 100 g of *A. gangeticus* leaves were ground in 1000 ml of distilled water (10%) and filtered. The filtrate was diluted with distilled water to obtain the concentration that was used (5, 7 and 10%). The extract was stored at 4°C.

Hepatocarcinogenesis was induced according to the method of Solt and Farber (1976),¹⁴ but without partial hepatectomy. Animals in the groups 2, 7-14 were intra-peritoneally given a single injection of DEN (200 mg/kg body weight) dissolved in corn oil at the beginning of the experiment to initiate hepatocarcinogenesis. After 2 weeks of feeding with standard basal diet, promotion of hepatocarcinogenesis was done with administration of AAF (0.02% in basal diet) for 2 weeks without partial hepatectomy. Treatment with AG (at different concentration) was given as a substitute to distilled water in Groups II - IV and glycyrrhizin in group XI. A summary of the protocol is presented in (Fig. 1).

Groups	Treatments				
NC	Basal diet + water				
N 5.0	Basal diet + AG 5.0				
N 7.5	Basal diet + AG 7.5				
N 10	Basal diet + AG 10				
CC	Basal diet + water	AAF + water	Basal diet + water		
C 5.0	Basal diet + AG 5.0	AAF + AG 5.0	Basal diet + AG 5.0		
C 7.5	Basal diet + AG 7.5	AAF + AG 7.5	Basal diet + AG 7.5		
C 10	Basal diet + AG 10	AAF + AG 10	Basal diet + AG 10		
C 5.0	Basal diet + GL	AAF + GL	Basal diet		
Weeks	DEN	0	2	4	14

Figure 1. Study protocol for *in-vivo* studies. DEN, 200 mg/kg diethylnitrosamine (ip); AAF, 0.02% 2-acetylaminofluorene; NC=control; C=DEN/AAF; AG 5=*Amaranthus gangeticus* extract 5%; N=Normal, AG 7.5=*Amaranthus gangeticus* 7.5%; AG 10=*Amaranthus gangeticus* extract 10%; GL=Glycyrrhizin; CC=Cancer control

Table 1. IC₅₀ values of aqueous and ethanolic extracts from *Amaranthus gangeticus* extracts

No.	Types of cell lines	IC ₅₀ (µg/ml)	
		Ethanolic extract	Aqueous extract
1.	HepG2	27.75	93.8
2.	MCF-7	12.5	98.8
3.	MDA-MB-231	27.75	>110
4.	Caco-2	>100	>100
5.	Chang Liver	>100	>100

Determination of glutathione, γ -glutamyl transpeptidase, GST, UDPGT and alkaline phosphatase.

Blood was taken immediately from the orbital sinus vein and plasma was separated by centrifugation at 3000 rpm 4°C and used for GGT and ALP assays. The rats were sacrificed by cervical dislocation at 14 weeks from the DEN injection. The livers were weighed and stored at -70°C before use. The microsomal fraction of the liver was prepared according to the method of Speir and Wattenberg (1975).¹⁵ GGT assay was determined in the microsomal fraction while ALP activity and the level of GSH were determined in the homogenate of the liver.

Plasma and liver GGT activities were assayed following the method of Jacobs (1971)¹⁶ and the activities were expressed as units per litre and units per gram protein, respectively. The microsomal pellet was first resuspended in 5 volume of 0.1 M Tris-HCl buffer pH 8.2 containing 1 mM MgCl₂. Alkaline phosphatase activity was assayed by the method of Jahan and Butterworth (1986).¹⁷ One unit of activity was expressed as the amount of enzyme required to catalyse the release of 1 µmol p-nitrophenol/min under the condition stated. Liver ALP and GGT were expressed as units per litre per

milligram protein, respectively. Protein concentration was determined by using the method of Bradford (1976).¹⁸ The activity of GST in the liver cytosol was assayed according to the method of Habig *et al.*, (1974)¹⁹ using CDNB and DCNB as the substrates. Specific activity was defined as µmol/min/mg protein in the cytosol. UDPGT activity in the liver microsome was assayed by the method of Vassey and Zakim (1972)²⁰ using p-nitrophenol as substrate and uridyl diphosphoglucuronyl acid (UDPGA) as glucuronic acid source. Specific activity of UDPGT was expressed as µmol/min/mg protein.

Statistical analysis

Statistical comparisons were carried out using student's t-test. Probability level of $P < 0.05$ was chosen as the criterion of statistical significance. Values reported were mean \pm SD.

Results

In vitro studies

The IC₅₀ from aqueous and ethanolic extracts of *A. gangeticus* are shown in (Table 1). The ethanolic extract of *A. gangeticus* was observed to inhibit the proliferative of HepG2 cells (IC₅₀ 27.75µg/ml), MDA-MB 231 cells (IC₅₀ 27.75µg/ml) and MCF-7 cells (IC₅₀ 12.50 µg/ml) whereas the aqueous extract inhibited the proliferation of HepG2 and MCF-7 cells (IC₅₀ 93.8 and 98.8µg/ml respectively).

In vivo studies

The result showed that supplementation of 5%, 7.5% and 10% of *A. gangeticus* aqueous extract to normal rats did not show any significant difference towards normal control ($P < 0.05$). The exposure of the rats to chemical carcinogens DEN/AAF showed a significant increase in specific enzyme activities of GGT, UDPGT, GST and ALP compared to normal control ($P < 0.05$). However, it was found that the supplementation of *A. gangeticus* aqueous extract to the DEN/AAF-treated rats decreased all tumour marker enzymes, especially at 10% ($P < 0.05$).

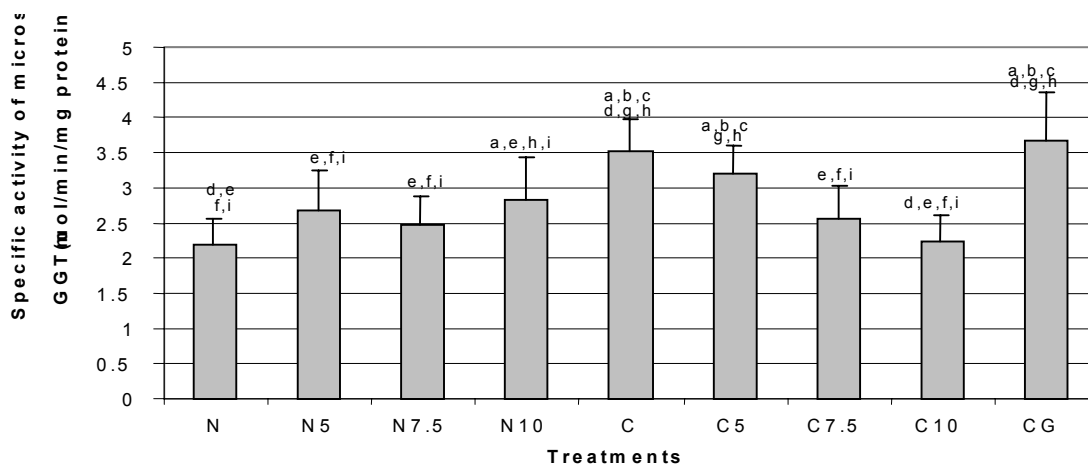


Figure 2. Effect on specific activity of microsomal GGT in 3 months. Mean \pm S.D. (N = 8). N - Normal, N5 - Normal + 5% dose of *A. gangeticus*, N7.5 - Normal + 7.5% dose of *A. gangeticus*, N10 - Normal + 10% dose of *A. gangeticus*, C - Cancer induced, C5 - Cancer induced + 5% dose of *A. gangeticus*, C7.5 - Cancer induced + 7.5% dose of *A. gangeticus*, C10 - Cancer induced + 10% dose of *A. gangeticus*, CG - Cancer induced + Glycrrhizin. a: $P < 0.05$ compared to normal; b: $P < 0.05$ compared to normal + 5% dose of *A. gangeticus*; c: $P < 0.05$ compared to normal + 7.5% dose of *A. gangeticus*; d: $P < 0.05$ compared to normal + 10% dose of *A. gangeticus*; e: $P < 0.05$ compared to cancer induced; f: $P < 0.05$ compared to cancer induced + 5% dose of *A. gangeticus*; g: $P < 0.05$ compared to cancer induced + 7.5% dose of *A. gangeticus*; h: $P < 0.05$ compared to cancer induced + 10% dose of *A. gangeticus*; i: $P < 0.05$ compared to cancer induced + Glycrrhizin

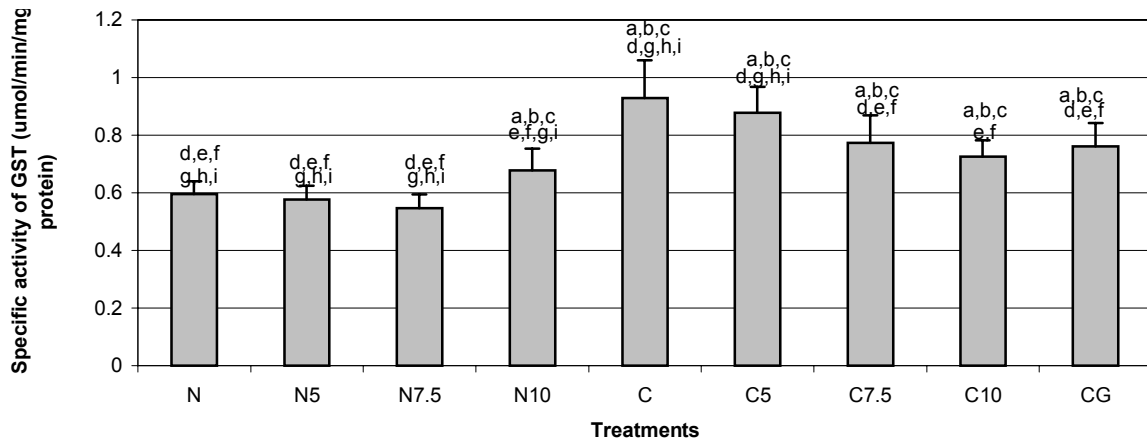


Figure 3. Effect on specific activity of GST in 3 months. Mean \pm S.D. ($N = 6-8$). N - Normal, N5 - Normal + 5% dose of *A. gangeticus*, N7.5 - Normal + 7.5% dose of *A. gangeticus*, N10 - Normal + 10% dose of *A. gangeticus*, C - Cancer induced, C5 - Cancer induced + 5% dose of *A. gangeticus*, C7.5 - Cancer induced + 7.5% dose of *A. gangeticus*, C10 - Cancer induced + 10% dose of *A. gangeticus*, CG - Cancer induced + Glycrrhizin. a: $P < 0.05$ compared to normal; b: $P < 0.05$ compared to normal + 5% dose of *A. gangeticus*; c: $P < 0.05$ compared to normal + 7.5% dose of *A. gangeticus*; d: $P < 0.05$ compared to normal + 10% dose of *A. gangeticus*; e: $P < 0.05$ compared to cancer induced; f: $P < 0.05$ compared to cancer induced + 5% dose of *A. gangeticus*; g: $P < 0.05$ compared to cancer induced + 7.5% dose of *A. gangeticus*; h: $P < 0.05$ compared to cancer induced + 10% dose of *A. gangeticus*; i: $P < 0.05$ compared to cancer induced + Glycrrhizin

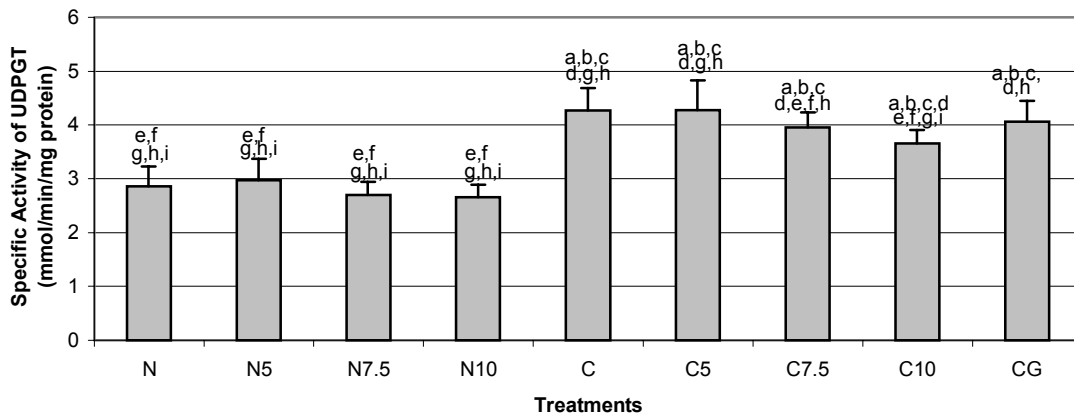


Figure 4. Effect on specific activity of UDPGT in 3 months. Mean \pm S.D. ($N = 6-8$). N - Normal, N5 - Normal + 5% dose of *A. gangeticus*, N7.5 - Normal + 7.5% dose of *A. gangeticus*, N10 - Normal + 10% dose of *A. gangeticus*, C - Cancer induced, C5 - Cancer induced + 5% dose of *A. gangeticus*, C7.5 - Cancer induced + 7.5% dose of *A. gangeticus*, C10 - Cancer induced + 10% dose of *A. gangeticus*, CG - Cancer induced + Glycrrhizin. a: $P < 0.05$ compared to normal; b: $P < 0.05$ compared to normal + 5% dose of *A. gangeticus*; c: $P < 0.05$ compared to normal + 7.5% dose of *A. gangeticus*; d: $P < 0.05$ compared to normal + 10% dose of *A. gangeticus*; e: $P < 0.05$ compared to cancer induced; f: $P < 0.05$ compared to cancer induced + 5% dose of *A. gangeticus*; g: $P < 0.05$ compared to cancer induced + 7.5% dose of *A. gangeticus*; h: $P < 0.05$ compared to cancer induced + 10% dose of *A. gangeticus*; i: $P < 0.05$ compared to cancer induced + Glycrrhizin

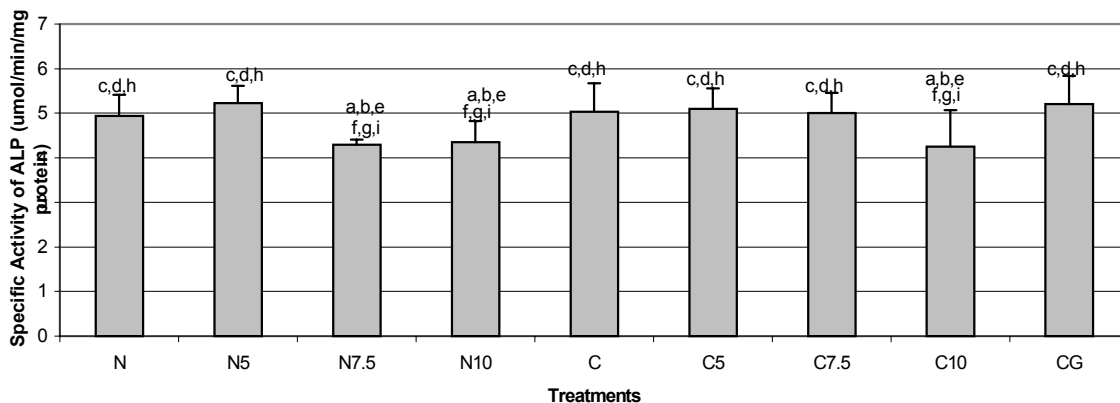


Figure 5. Effect on specific activity of ALP in 3 months. Mean \pm S.D. ($N = 6-8$). N - Normal, N5 - Normal + 5% dose of *A. gangeticus*, N7.5 - Normal + 7.5% dose of *A. gangeticus*, N10 - Normal + 10% dose of *A. gangeticus*, C - Cancer induced, C5 - Cancer induced + 5% dose of *A. gangeticus*, C7.5 - Cancer induced + 7.5% dose of *A. gangeticus*, C10 - Cancer induced + 10% dose of *A. gangeticus*, CG - Cancer induced + Glycrrhizin. a: $P < 0.05$ compared to normal; b: $P < 0.05$ compared to normal + 5% dose of *A. gangeticus*; c: $P < 0.05$ compared to normal + 7.5% dose of *A. gangeticus*; d: $P < 0.05$ compared to normal + 10% dose of *A. gangeticus*; e: $P < 0.05$ compared to cancer induced; f: $P < 0.05$ compared to cancer induced + 5% dose of *A. gangeticus*; g: $P < 0.05$ compared to cancer induced + 7.5% dose of *A. gangeticus*; h: $P < 0.05$ compared to cancer induced + 10% dose of *A. gangeticus*; i: $P < 0.05$ compared to cancer induced + Glycrrhizin

Discussion

GGT, GST and ALP have been recognized as a positive marker for hepatocytes, which have undergone malignant transformation.²¹ Our previous studies on tocotrienol supplementation, administered over the short or long-term, attenuated the impact of carcinogens in the rats.²¹⁻²³ In rats treated with DEN/AAF, vitamin E supplementation attenuated GGT and ALP activities and blood GSH levels. The optimum dose required for highest attenuation of the tumour marker enzyme activities was 34mg/kg diet for α -tocopherol and 30mg/kg diet for γ -tocotrienol. Higher doses of the vitamin did not show further attenuation in the level of the tumour marker enzyme activities. The IC₅₀-values of ethanolic extracts of *A.gangeticus* from *in-vitro* studies were very much lower when compared to the aqueous extracts. This may be due to the high antioxidant activities that offer protection against damage due to free radicals.²⁴ Carotenoids, vitamin E and fibres in plants have been implicated as anticarcinogenic agents.²⁵⁻²⁶ Based on the low IC₅₀ values, ethanolic extract was used for isolation of cytotoxic (bioactive) compounds. The mechanism of the cytotoxic effect of these bioactive compounds obtained from this plant is being studied.

Further study is needed since they have pronounced effect on some of the tumour biomarkers, which encourages the utility in human studies. Moreover, there was no evidence suggesting side effects of the extracts towards normal cells, indicating a potent preventive agent for cancer.

Conclusion

These results indicate that *A.gangeticus* possess hepatoprotective properties against chemical carcinogenesis.

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