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Effect of *Strobilanthes crispus* on Tumour Marker Enzymes and Glutathione During Chemical Hepatocarcinogenesis in the Rat

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Abstract: The administration effect of *Strobilanthes crispus* extracts (SC) during hepatocarcinogenesis in rats was studied to investigate the possible cancer suppressive effect of the component existed in the leaves. Hepatocarcinogenesis was induced using Diethylnitrosamine (DEN) (200 mg kg^{-1}) and 2-acetylaminoflourence (AAF) (0.02% w/w). Glycyrrhizin (G), the commercial anticancer drug, was used for comparison. A total of 84 male *Sprague-Dawley* rats were divided into 14 groups viz control (N), DEN/AAF (C), SC1% (NS1), SC2.5% (NS2.5), SC5% (NS5), SC7.5% (NS7.5), DEN/AAF/SC1% (CS1), DEN/AAF/SC2.5% (CS2.5), DEN/AAF/SC5% (CS5), DEN/AAF/SC7.5% (CS7.5), DEN/AAF/G1% (CG1), DEN/AAF/G2.5% (CG2.5), DEN/AAF/G5% (CG5) and DEN/AAF/G7.5% (CG7.5). The effect of SC was investigated by identifying activities of liver and plasma γ-glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) and glutathione (GSH) concentrations. Treatment with DEN/AAF caused increase in all enzymes activities when compared to control. The administration of SC (1, 2.5, 5 and 7.5%) to the induced cancer rats decreased the microsomal GGT. T-test showed a significant difference ($p<0.05$) when the GGT level of all the treatment groups were compared to the control (N) and DEN/AAF (C). The findings suggest that supplementation of SC on DEN/AAF rats reduced the severity of hepatocarcinogenesis by reducing liver GGT and ALP activities and also the levels of GSH.

Key words: *Strobilanthes crispus*, GGT, ALP, GSH, hepatocarcinogenesis

INTRODUCTION

Hepatocellular carcinoma (HCC), the predominant form of liver cancer, is the fourth most common cause of cancer related mortality in the world[1]. The highest incidence rates are in West and Central Africa, Eastern and South Eastern Asia and in Melanesia[2]. Due to the high tolerance of liver, it is seldom detected at the early stage and once detected treatment has a poor prognosis in most cases[3]. An increased risk for developing liver cancer is correlated with either virus infection[4] or consumption of alcoholic beverages[5].

Over the centuries, no fewer than 3000 plant species have been used to treat cancer[6]. Many plants are introduced and studied to increase the discovery of natural product cancer chemotherapeutic agents[7]. *Strobilanthes crispus* (L.) Bremek or *Saricocalyx crispus* (L.) Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia which is commonly known as 'picah beling' in Jakarta or 'kejibeling' in Java[8]. This bush-like plant can be found on riverbanks or abandoned fields. Top surfaces of the leaves are darker green in colour and less rough compared to the below surface. The leaves are very scabrid on both surfaces and covered with short hairs, whereas the flowers are short, dense and are panicled spikes[9]. It has been found that an infusion of the dried leaves of this plant has been used as antidiabetic, diuretic, antilitic and laxative[8]. There are very few researches done on the therapeutic activity of *Strobilanthes crispus*. Its potential of curing diseases only has been determined traditionally.

A recent research reported that the water extract of this plant inhibit the proliferation of retroviruses; an agent in viral disease such as acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia[10]. However, there were no research on hepato-carcinogenesis has been reported. Therefore, the aim of this study is to determine the effect of the water extract of *S. crispus* (SC) supplementation on GGT and ALP activities and also GSH level in hepatocarcinogenesis induced by Diethylnitrosamine (DEN) and 2-Acetylaminofluorene (AAF) in rats.

MATERIALS AND METHODS

Plant: *Strobilanthes crispus* (L.) Bremek or *Saricocalyx crispus* (L.) Bremek (Acanthaceae) was harvested from herbal garden of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor and authenticated by Mr. Ahmed Zainuddin (AZ-6803), Department of Botany, Universiti Kebangsaan Malaysia.

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Table 1: Protocol of the experimental design. Different groups of rat's cancer induced and non-cancer induced rats treated with different regimens of drinking water either/neither with SC or G

Groups		Treatments	
N		Basal diet + water	
N + SC 1.0		Basal diet + SC 1.0	
N + SC 2.5		Basal diet + SC 2.5	
N + SC 5.0		Basal diet + SC 5.0	
N + SC 7.5		Basal diet + SC 7.5	
C	Basal diet + water	AAF + water	Basal diet + water
C + SC 1.0	Basal diet + SC1.0	AAF + SC1.0	Basal diet + SC 1.0
C + SC 2.5	Basal diet + SC2.5	AAF + SC2.5	Basal diet + SC 2.5
C + SC 5.0	Basal diet + SC5.0	AAF + SC 5.0	Basal diet + SC 5.0
C + SC 7.5	Basal diet + SC7.5	AAF + SC 7.5	Basal diet + SC 7.5
C + G 1.0	Basal diet + G1.0	AAF + G1.0	Basal diet + G 1.0
C + G 2.5	Basal diet + G2.5	AAF + G2.5	Basal diet + G 2.5
C + G 5.0	Basal diet + G5.0	AAF + G 5.0	Basal diet + G 5.0
C + G 7.5	Basal diet + G7.5	AAF + G 7.5	Basal diet + G 7.5
	DEN		
Weeks	0	2	4	14

N: Normal AAF: 2-Acetylaminofluorine DEN: Diethylnitrosamine G: Glycyrrhizin C: Cancer SC: *Scripus*

Chemicals: A basal diet of rat chow was purchased from Ridley Agriculture, Australia. γ-glutamyl carboxynitroanilide, 2-Acetylaminofluorene (AAF), glycylglycine, p-nitrophenol phosphate, diethanolamine, 5-5'-dithiobis (2-nitrobenzoic acid) and all other reagents used were the highest grade commercially available (Sigma Chemical Co, St-Louis, MO).

Treatment of animals: A total of 84 male Sprague-Dawley rats (*Rattus norwegicus*), each initially weighing between 150-200 g were purchased from Faculty of Veterinary, UPM, Serdang, Selangor. The rats were housed individually at 27°C and were maintained on normal or treated rat chow. The rats were divided into fourteen groups i.e. group 1 : control (basal diet) (N), group 2-5 : SC supplemented-diet (1%,2.5%, 5 and 7.5% SC in drinking water) (NS1, NS2.5, NS5, NS7.5), group 6 : cancer (DEN/AAF) with basal diet (C), group 7-10 : cancer (DEN/AAF)-SC supplemented diet (CS1,CS2.5, CS5, CS7.5), group 11-14: cancer (DEN/AAF)-treated with Glycyrrhizin (CG1, CG2.5, CG5, CG7.5). The crude extract of *S. crispus* was prepared from the modification of previous method[11]. A 10 g of *S. crispus* leaves were grounds in 100 ml of distilled water (10%) and filtered. The filtered were diluted with distilled water to obtain the concentration that will be used (1, 2.5, 5 and 7.5%). The extract was stored at -20°C until further used.

Hepatocarcinogenesis was induced according to the method of Solt and Farber[12], but without partial hepatectomy. Animals in the groups 2, 7 until 14 were intraperitoneally given single injections of DEN (200 mg kg^{-1} 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 SC (at different concentration) was given as a substitute to distilled water in groups 7-10 and glycyrrhizin with different concentration in group 11-14. A summary of the protocol is presented in Table 1.

Determination of glutathione, γ-glutamyl transpeptidase 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 liver 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[13]. 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 were assayed following the method of Jacobs [14] and the activity 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 $MgCl_2$. Alkaline phosphatase activity was assayed by the method of Jahan and Butterworth[15]. One unit of activity is expressed as the amount of enzyme required to catalyse the release of 1 μmol p-nitrophenol/min under the condition stated. Plasma and liver ALP were expressed as units per liter and units per milligram protein, respectively. The level of GSH in the homogenate was determined by using the method of Ellman[16] and GSH concentration was expressed as μM/g liver. Protein concentration was determined by using the method of Bradford[17].

Statistical analysis: The results obtained were analysed by ANOVA student's t-test. Probability level of $p<0.05$ was chosen as the criterion of statistical significance. The values were reported as mean ± SEM.

RESULTS

Treatment with *S. crispus* extract had no effect on either plasma or liver microsomal GGT activities (Table 2).

Table 2: Effect of Diethylnitrosamine/2-Acetylaminofluorine (DEN/AAF), *Strobilanthes crispus* extract and Glycyrrhizin at different doses on γ-Glutamyl Transpeptidase (GGT) activities in plasma and liver

Groups	Liver GGT (IU/g protein)	Plasma GGT (IU/L)
Normal Control (N)	1.79±0.08b	2.87±0.03be
DEN/AAF (C)	2.56±0.16a-f	11.43±0.34a-f
NS1	1.90±0.27b	3.40±0.16be
NS2.5	1.79±0.18b	3.63±0.16be
NS5	1.66±0.08b	4.26±0.85be
NS7.5	1.42±0.07b	4.11±0.80be
DEN/AAF/SC1% (CS1)	1.88±0.18bf	9.86±0.74a-f
DEN/AAF/SC2.5% (CS2.5)	1.63±0.11b	5.02±1.83a-e
DEN/AAF/SC5% (CS5)	1.54±0.13b	3.22±0.49be
DEN/AAF/SC7.5% (CS7.5)	1.52±0.08b	5.18±0.84a-e
DEN/AAF/G1% (CG1)	1.82±0.14b	3.97±0.08be
DEN/AAF/G2.5% (CG2.5)	1.74±0.19b	2.81±0.24b
DEN/AAF/G5% (CG5)	1.60±0.07b	1.32±0.31a-f
DEN/AAF/G7.5% (CG7.5)	1.46±0.09b	3.54±0.14be

Table 3: Effect of Diethylnitrosamine/2-Acetylaminofluorine (DEN/AAF), *Strobilanthes crispus* extract and Glycyrrhizin at different doses on alkaline phosphatase (ALP) activities in plasma and liver

Groups	Liver ALP (IU/g protein)	Plasma ALP (IU/L)
Normal Control (N)	4.88±0.31b-f	186.26±7.28b
DEN/AAF (C)	7.02±0.17a-f	233.12±6.68a-f
NS1	5.01±0.55b-f	187.94±11.77b
NS2.5	4.69±0.61b-f	175.63±8.33b
NS5	4.87±0.45b-f	180.33±19.03b
NS7.5	4.87±0.95b-f	183.34±23.04b
DEN/AAF/SC1% (CS1)	4.03±0.23b-f	180.93±19.45b
DEN/AAF/SC2.5% (CS2.5)	3.76±0.16b-f	173.02±8.61b
DEN/AAF/SC5% (CS5)	3.56±0.33a-f	183.88±2.04b
DEN/AAF/SC7.5% (CS7.5)	3.47±0.50a-e	172.78±10.12b
DEN/AAF/G1% (CG1)	2.44±0.31ab	174.38±4.26b
DEN/AAF/G2.5% (CG2.5)	2.11±0.15ab	171.90±14.47b
DEN/AAF/G5% (CG5)	2.11±0.23ab	164.90±18.80b
DEN/AAF/G7.5% (CG7.5)	2.19±0.07ab	163.85±5.76b

Values shown are mean ± SEM N : Normal C : Cancer SC : *S.cripus* G: Glycyrrhizin AAF : 2- Acetylaminofluorine
DEN : Diethylnitrosamine NS : Normal *S.cripus*
a = p< 0.05 compared with normal control b = p< 0.05 compared with DEN/AAF c = p< 0.05 compared with CG1
d = p< 0.05 compared with CG2.5 e = p< 0.05 compared with CG5 f = p< 0.05 compared with CG7.5

Table 4: Effect of Diethylnitrosamine/2-Acetylaminofluorine (DEN/AAF), *Strobilanthes crispus* extract and Glycyrrhizin at different doses on liver glutathione (GSH) level

Groups	GSH (μM/g liver)
Normal Control (N)	0.121±0.009b
DEN/AAF (C)	0.344±0.016a-f
NS1	0.125±0.012b
NS2.5	0.111±0.013bc
NS5	0.104±0.004bc
NS7.5	0.127±0.026b
DEN/AAF/SC1% (CS1)	0.097±0.004a-f
DEN/AAF/SC2.5% (CS2.5)	0.111±0.014bc
DEN/AAF/SC5% (CS5)	0.086±0.006a-f
DEN/AAF/SC7.5% (CS7.5)	0.103±0.017bce
DEN/AAF/G1% (CG1)	0.137±0.005bd
DEN/AAF/G2.5% (CG2.5)	0.111±0.009bc
DEN/AAF/G5% (CG5)	0.126±0.003b
DEN/AAF/G7.5% (CG7.5)	0.122±0.014b

Values shown are mean ± SEM
N : Normal C : Cancer SC : *S.cripus* G: Glycyrrhizin
AAF : 2- Acetylaminofluorine DEN : Diethylnitrosamine
NS : Normal *S.cripus*
a = p< 0.05 compared with normal control
b = p< 0.05 compared with DEN/AAF
c = p< 0.05 compared with CG1
d = p< 0.05 compared with CG2.5
e = p< 0.05 compared with CG5
f = p< 0.05 compared with CG7.5

On the other hand, treatment with DEN/AAF caused a significant increase in plasma and liver GGT activity compared to that of control ($p<0.05$). However, when SC (all doses) were supplemented to DEN/AAF treated rats, GGT activities were significantly less than in rats treated with DEN/AAF only ($p<0.05$).

DEN/AAF also increases ALP activities when compared to control and SC supplemented rats (Table 3). While the supplementation of DEN/AAF treated rats, with SC or G caused lowering of ALP activities. Rats treated with carcinogens DEN/AAF also caused a significant increase in GSH levels (Table 4).

Supplementation of SC and G to DEN/AAF decrease GSH values ($p<0.05$) significantly for all doses and the lowest values was observed on dose 5% of SC.

DISCUSSION

The present study demonstrates the effect of *S.crispus* extract (SC) during hepatocarcinogenesis in the rat treated with DEN/AAF on enzyme tumour markers, GGT and ALP. The determination of ALP and GGT had been used extensively as a marker of neoplasia especially for liver cancer in humans. The overall results showed that SC supplementation in the diet of the carcinogen-treated rats lowered the GGT and ALP activities and GSH levels.

In humans, plasma GGT determination have been reported correlate closely with the clinical status i.e, significantly elevated GGT levels corresponded to disease progression and death. Patients with low enzyme levels were found to be free from disease [18,19]. In the other report, GGT isoenzyme was determined as a potential specific marker for primary or metastatic liver neoplasia[20]. The results of the present study showed a similar pattern of enzyme activities in both the plasma and liver microsomes. The result obtained showed that plasma and microsomal GGT activities increased in DEN/AAF rats and complied with previous report [21] which stated that plasma and liver microsomal GGT activities increased in carcinogen-treated rats. Increases in plasma GGT activities could be due to an overflow from the neoplastic cells in the liver and could be considered to a more accurate measure of the extent of the carcinogenic process.

Supplementation of SC to the cancer induced rats caused a reduction in the GGT activity. The reduction in plasma GGT activity maybe due to the regression of a proportion of the altered hepatic foci while the remainder progress towards the neoplastic state[22].

The determination of ALP as a marker of neoplasia has not been used as extensively as GGT. However, there have been reports of the diagnostic use of ALP and its isoenzymes for liver cancer in humans[23]. Elevation in ALP enzyme activities has also been reported in lung cancer and ovarian cancer[24]. In this study, plasma and liver ALP also showed higher activities in carcinogen-treated rats compared to control.

The different doses of SC used in this study showed the great variation in terms of their effect on the severity as assessed by measurement of the ALP and GGT levels. It seemed that the optimum dose for supplementation of SC was 5% (5 gram *S. crispus* in 100 ml water) extract. The lowest dose used in the present study (SC 1%) was insufficient for maximum protection while higher doses (SC 7.5%) did not show further attenuation of the plasma GGT activities.

The detoxification of carcinogens, which are in neture strong electrophiles, depends on the rate of reaction and concentration of GSH. It has been suggested that GSH may be important as a free radical trap in antimutagenesis and anticarcinogenesis, since GSH readily donates a hydrogen atom to free radicals, notably hydroxy and carbon radicals (Ketterer, 1988). The results obtained in this present study showed higher GSH level in carcinogen-treated rats compared to control. The increase in GSH level due to DEN/AAF treatment is expected since GSH levels have been shown to increase in the presence of foreign compound including carcinogen[25]. Again, SC supplementation reduced the levels significantly.

In conclusion, SC supplementation attenuated the impact of carcinogens and showed a protective effect in the early stages of hepatocarcinogenes. The optimum dose appears to be 5% extract of *S.crispus*.

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