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Effect of *Strobilanthes crispus* on the Histology and Tumour Marker Enzymes in Rat Liver During Hepatocarcinogenesis

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The administration effect of *Strobilanthes crispus* extract (SC) during hepatocarcinogenesis was studied to investigate the possible cancer suppressive effect of the component existed in the leaves. The rats (84 male Sprague Dawley) were divided into 14 groups N (Normal), C (Cancer), NS 1 (Normal+ SC 1%), NS 2.5 (Normal+SC 2.5%), NS 5 (Normal+SC 5%), NS 7.5 (Normal+SC 7.5%), CS 1 (Cancer+SC 1%), CS 2.5 (Cancer+SC 2.5%), CS 5 (Cancer+SC 5%), CS 7.5 (Cancer+SC 7.5%), CG 1 (Cancer+Glycyrrhizin 1%), CG 2.5 (Cancer+Glycyrrhizin 2.5%), CG 5 (Cancer+Glycyrrhizin 5%), and CG 7.5 (Cancer+Glycyrrhizin 7.5%). 1, 2.5, 5 and 7.5% (w/v) of *S. crispus* extract were used, compared with Glycyrrhizin, the commercial anticancer drug used mainly for liver. Administration effect during liver cancer in rats that have been induced with cancer by diethylnitrosamine (DEN) and 2-acetyl-aminoflourence (AAF) was studied by estimation of glutathione S-transferase (GST) and uranyl diphosphate glucoronyl transferase (UDPGT) in liver. The severity of neoplasia in induced liver cancer was also evaluated by histological examination. Treatment with DEN/AAF caused increase in all enzyme activities measured when compared to control. Significant differences were observed among all the treatment groups for GST and UDPGT activities. Histological evaluations showed the loss of normal morphology and organizations of hepatocytes when carcinogens were introduced into the rats. The severity of liver cell dysplasia was evidently decreased by SC extract treatment group as compared to glycyrrhizin. Furthermore SC did not affect the normal organization in the liver. The findings suggest that supplementation of SC on DEN/AAF rats reduced the severity of hepatocarcinogenesis.

Key words: Tumour marker enzymes, GST, UDPGT, hepatocarcinogenesis, *Strobilanthes crispus*

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INTRODUCTION

Strobilanthes crispus ZII 109 (L) Bremek or *Saricocalyx crispus* ZII 109 (L) Bremek (Acanthaceae) plant is native to countries from Madagascar to Indonesia and was first authored by Anderson, Thomas who classified the plant under Spermatophyta[1]. This plant has many cystoliths of calcium carbonate and an infusion is mildly alkaline[2]. A study in Indonesia indicated that the water extract of *S. crispus* contained compounds, which inhibit the proliferation of retrovirus, an agent in viral disease such as acquired immune deficiency syndrome (AIDS) and adult T-cell leukemia[3]. The leaves of this plant contained high antioxidant activity and cytotoxic properties against colon carcinoma (Caco-2), breast carcinoma (MCF-7) and liver carcinoma (HepG2) cell lines[4,5].

By 2000, liver cancer was the fifth most common cancer worldwide, responsible for approximately 551,000 new liver cancer cases[6]. The highest incidence rates are in West and Central Africa, Eastern and South Eastern Asia and in Melanesia[7]. Various ways of monitoring the carcinogenic process have been reported; these could either be by examination of morphology either histology or/and ultrastructural study[8] and by determination of reported preneoplastic marker enzymes glutathione S-transferase (GST)[9] and uridyl diphosphate glucoronyl transferase (UDPGT)[10]. This study was conducted to determine the effect of administration of *S. crispus* extract on the histology and the tumour marker enzymes, GST and UDPGT, in rat liver induced with hepatocarcinogens DEN and AAF.

MATERIALS AND METHODS

Plant extract: The leaves of *S. crispus* were harvested at Faculty of Medicine and Health Sciences, UPM and authenticated by Mr. Ahmed Zainuddin (AZ-6803), Department of Botany, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Crude extract SC was prepared from the modification of green tea extraction according to Conney *et al.*[11]. Ten gram of *S. crispus* leaves were ground in 100 mL of distilled water (10%) and filtered. The filtrate was diluted with distilled water to obtain the concentration of 1, 2.5, 5 and 7.5% and stored at 4°C.

Diethylnitrosamine (DEN) preparation: Diethylnitrosamine (DEN) as an initiator agent, were prepared by dissolving 1.0 mL DEN in 2.33 mL corn oil (Mazola), which is equivalent to 200 mg DEN/kg/body weight of the rats. About 0.1 mL of the solution was injected to each rat, having weight between 150-200 g.

2-Acetylaminofluorene (AAF) preparation: 1.0 g AAF (a promotion agent) was dissolved in 50 mL acetone 1.5 mL of this solution was dropped and mixed to 150 g rat chow to obtain the final concentration of 0.02% (w/w) AAF in the diet. The acetone was dried in vacuum at 15 mmHg for an hour.

Experiment protocol: A total of 84 male Sprague-Dawley rats (*Rattus norwegicus*), each initially weighing between 150-200 g were purchased from Faculty of Veterinary Medicine, 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 I: Normal (basal diet) (N), Group II-V: SC supplemented-diet (1, 2.5, 5 and 7.5% SC in drinking water (NS 1, NS 2.5, NS 5, NS 7.5), Group VI: Cancer (DEN/AAF) with basal diet (C), Group VII-X: Cancer (DEN/AAF) SC extract supplemented diet (CS 1, CS 2.5, CS 5, CS 7.5), Group XI-XIV: Cancer (DEN/AAF) treated with glycyrrhizin (CG 1, CG 2.5, CG 5, CG 7.5).

Hepatocarcinogenesis was induced according to the method of Solt and Farber[12] but without partial hepatectomy. Animals in the Groups VI until XIV were intra peritoneally given single injection 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 extract (at different concentration) was given as a substitute to distilled water in Groups II-V, VII-X and glycyrrhizin with different concentration in Group XI-XIV. A summary of the protocol is presented in Table 1.

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, 2.5, 5.0 and 7.5%)		Basal diet+water		
C (DEN+AAF)		DEN/AAF+water		
C+SC (1, 2.5, 5.0 and 7.5%)	DEN	AAF	Basal diet+SC	
C+G (1, 2.5, 5.0 and 7.5%)	DEN	AAF	Basal diet+G	
Weeks	0	2	4 Sacrifice	14

N : Normal
SC : *Strobilanthes crispus*
AAF : 2-Acetylaminofluorene
C : Cancer
DEN : Dietylnitrosamine
G : Glycyrrhizin

Termination of experiment: All rats were starved for 24 h before being sacrificed. The rats were sacrificed by cervical dislocation at 14 weeks from DEN injection. The livers from each rat were weight and washed in ice-cold 0.9% NaCl solution as soon as possible. The liver tissues from 6 rats in each group were sliced from the largest lobe (about 1 cm thick) and were fixed in 10% buffered formalin, embedded in paraffin blocks and processed for routine histological observation with the use of hematoxylin and eosin stain. Light microscope was used to determine the severity of liver lesion. Lesion scoring was done on all the liver sections and the mean lesion score was determined. The other part of the liver were stored at -70°C until used for the tumour marker enzyme assays.

Cytosolic and microsomal fractions of the liver were prepared according to the method of Speir and Wattenberg[13]. Cytosolic fraction was used to determine the activity of GST while UDPGT assay was determined in the microsomal fraction.

The activities of GST in the liver cytosol were assayed according to the method of Habig *et al.*[14] using 1-chloro-2, 4-dinitro-benzene (CDNB) as the substrate. Specific activity was defined as μmol/min/mg protein in the cytosol.

UDPGT activity in the liver microsome was assayed by the method of Vessey and Zakim[15] 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 analysis of the data was conducted by using SPSS version 11.0. The results obtained were analyzed by analysis of variance (ANOVA) followed by Fisher's LSD-test. Probability level of $p<0.05$ was chosen to determine statistical significance. The values were reported as mean± SEM.

RESULTS

Mean lesion score of liver induced carcinogenesis treated with different doses of *S. crispus* were shown in Fig. 1. The histological results based on the lesion score were shown in Fig. 2-5. There was significant difference between cancer group compared to the other groups ($p<0.05$). However, there was no significant difference between normal control compared to normal control supplemented with *S. crispus* groups ($p>0.05$). Based on the lesion score result, 5% of *S. crispus* extract has shown better suppressive effect compared to 1, 2.5 and 7.5% of *S. crispus* extract.

The N, NS 1, NS 2.5, NS 5 and NS 7.5 showed a very low mean lesion score. The mean lesion score of C was 2.76.

Table 2: Effect of diethylnitrosamine/2-acetylaminofluorine (DEN/AAF), *S. crispus* extract and glycyrrhizin at different doses on liver glutathione S-transferase (GST) level

Kumpulan	Aktiviti GST (μmol/min/mg protein)
Normal (N)	1.27±0.10b
SC 1% (NS 1%)	1.23±0.08b
SC 2.5% (NS 2.5%)	1.25±0.01b
SC 5% (NS 5%)	1.18±0.07b
SC 7.5% (NS 7.5%)	1.16±0.07b
DEN/AAF (C)	1.69±0.07a-f
DEN/AAF/SC 1% (CS 1%)	1.22±0.05b
DEN/AAF/SC 2.5% (CS 2.5%)	1.10±0.05b
DEN/AAF/SC 5% (CS 5%)	1.08±0.02ab
DEN/AAF/SC 7.5% (CS 7.5%)	1.01±0.04ab
DEN/AAF/G 1% (CG 1%)	1.20±0.09b
DEN/AAF/G 2.5% (CG 2.5%)	1.11±0.03b
DEN/AAF/G 5% (CG 5%)	1.09±0.08b
DEN/AAF/G 7.5% (CG 7.5%)	1.07±0.01b

Values shown are mean±SEM
a= $p<0.05$ compared with normal control
b= $p<0.05$ compared with cancer
c= $p<0.05$ compared with CG 1
d= $p<0.05$ compared with CG 2.5
e= $p<0.05$ compared with CG 5
f= $p<0.05$ compared with CG 7.5
N: Normal
DEN: Diethylnitrosamine
AAF: 2-Acetylaminofluorine
G: Glycyrrhizin
NS: Normal *S. crispus*
C: Cancer
SC: *S. crispus*

Table 3: Effect of diethylnitrosamine/2-acetylaminofluorine (DEN/AAF), *S. crispus* extract and glycyrrhizin at different doses on liver uridyl diphosphoglucuronyl transferase (UDPGT) level

Treatment Group	UDPGT (μmol/min/mg protein)
Normal (N)	2.94±0.30b
SC 1% (NS 1%)	2.59±0.22b
SC 2.5% (NS 2.5%)	2.43±0.27b
SC 5% (NS 5%)	2.16±0.05b
SC 7.5% (NS 7.5%)	2.30±0.29b
DEN/AAF (C)	4.46±0.30a-f
DEN/AAF/SC 1% (CS 1%)	3.04±0.30b-f
DEN/AAF/SC 2.5% (CS 2.5%)	2.82±0.11b
DEN/AAF/SC 5% (CS 5%)	2.30±0.29b
DEN/AAF/SC 7.5% (CS 7.5%)	2.65±0.14b
DEN/AAF/G 1% (CG 1%)	2.73±0.49b
DEN/AAF/G 2.5% (CG 2.5%)	2.48±0.11b
DEN/AAF/G5% (CG 5%)	2.30±0.17b
DEN/AAF/G7.5% (CG 7.5%)	2.23±0.15b

Values shown are mean±SEM
a= $p<0.05$ compared with normal control
b= $p<0.05$ compared with cancer
c= $p<0.05$ compared with CG 1
d= $p<0.05$ compared with CG 2.5
e= $p<0.05$ compared with CG 5
f= $p<0.05$ compared with CG 7.5
N: Normal
DEN: Diethylnitrosamine
AAF: 2-Acetylaminofluorine
G: Glycyrrhizin
NS: Normal *S. crispus*
C: Cancer
SC: *S. crispus*

However, the cancer treated group showed a reduction in the mean lesion score. The lowest mean lesion score was seen in both the 5% SC and G group.

Tumour marker study showed that cytosolic GST and microsomal UDPGT activities in DEN/AAF treated rats increased significantly compared to control rats (Table 2 and 3). SC extract supplementation to the

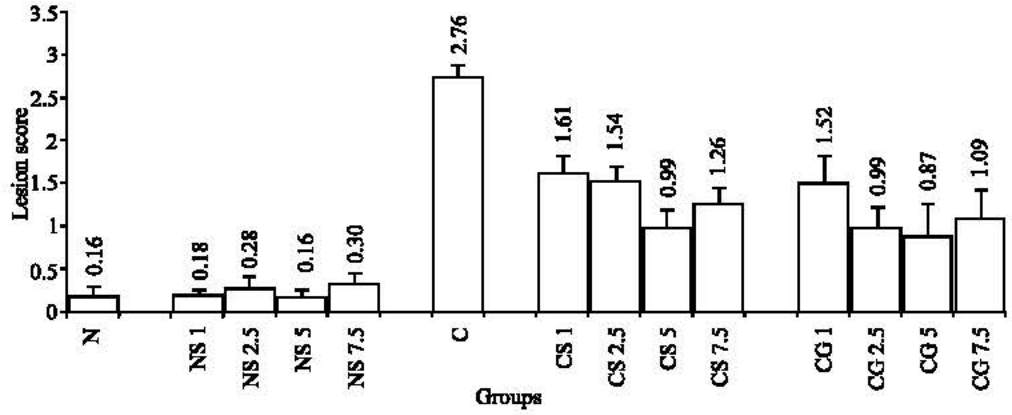

Fig. 1: Mean of the lesion score of liver induce carcinogenesis treated with different doses of *S. crispus*

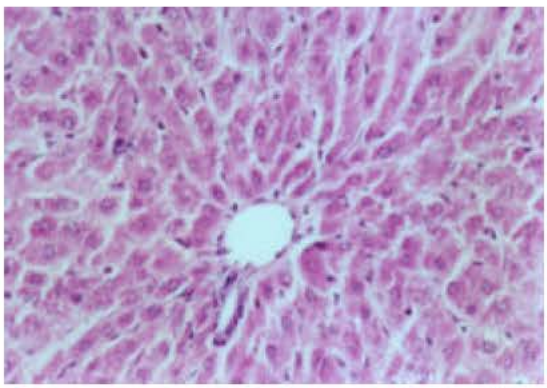

Fig. 2: Photomicrograph of normal liver demonstrating O lesion score (H and E, x 400)

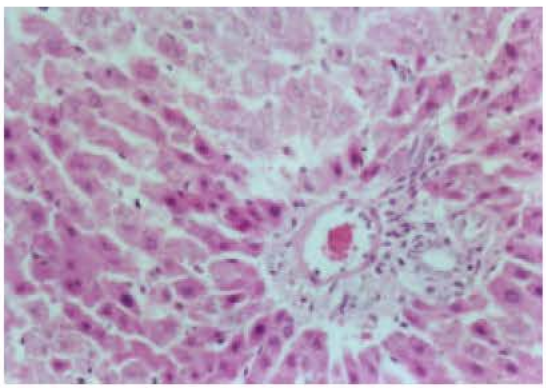

Fig. 4: Photomicrograph of normal liver demonstrating +2 lesion score (H and E, x 400)

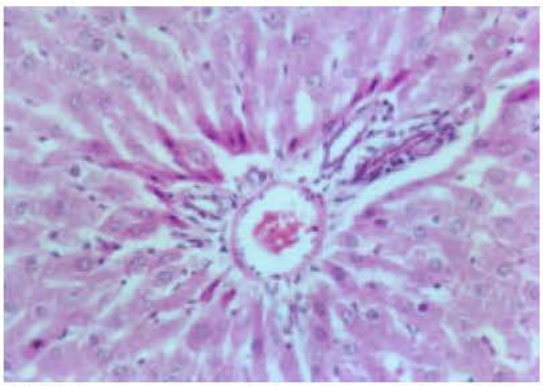

Fig. 3: Photomicrograph of liver demonstrating +1 lesion score (H and E, x 400)

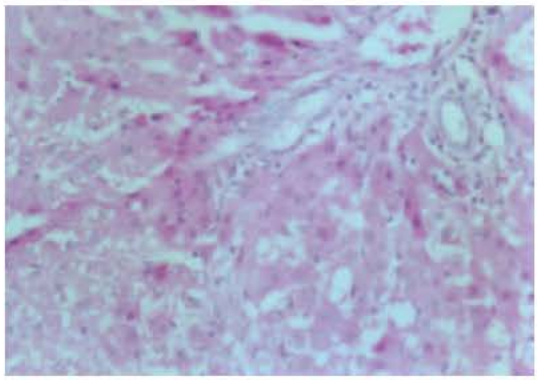

Fig. 5: Photomicrograph of liver demonstrating +3 lesion score (H and E, x 400)

DEN/AAF-treated rats significantly reduced UDPGT and GST activities ($p<0.05$) (Table 2 and 3).

DISCUSSION

The effect of *S. crispus* extract during rat hepatocarcinogenesis was studied by using the determination of tumour marker enzymes and histological pattern. Results showed that the hepatocytes which were found in this study is similar to the features described by Gartner and Hiatt[16]. Hepatocyte are polygonal, approximately 20 to 30 μm in diameter, that are closely packed together to form anatomizing plates of liver cells. They exhibit variations, in their structural, histochemical and biochemical properties, depending on their location within liver lobules. This indicates the importance in

choosing the best and constant part of the lobules during histology preparations. Normally liver cell dysplasia shows cellular enlargement, nuclear pleomorphism with hyperemia and multinucleation of hepatocytes[17]. However, the present study did not show any evidence of multinucleate of the hepatocytes in the cancer group. Binucleation of hepatocytes was occasionally seen in the SC extract treated groups.

Cameron *et al.*[18] also found that the cancerous cells continue to grow in size but not in numbers. These features can be clearly comprehended in cancer control group but uncommon in normal livers. The normal hepatocytes were well organized and arranged with clear cell membranes and similar shape and size of the nuclei, can be seen in normal control cells and normal cells treated with SC extract. However, tumour cells were larger, with pale hepatocytes and lost of arrangement. The nuclei of these cells were pleomorphic and many were hyperchromatic. This histological evidence show that carcinogens combination of DEN/AAF cause cancer in the liver.

Existence of two nuclei in a hepatocyte in normal+SC rat was distributed in the liver cells. This feature showed evidence of normal hepatocytes[16], indicating SC extract did not alter normal distribution of hepatocytes. The severity of liver cell dysplasia decreased in the SC and G supplemented cancer groups, indicating the anticancer effect of G and SC. SC extract evidently showed a better effect towards dysplasia of the hepatocytes arrangement, nucleus shape and cell stain were similar to the normal. Cell membrane and nuclei were clear causing less pleomorphism, hyperemia and giving a normal trabecular pattern in the liver.

Conversely, cellular enlargement and disarrangement, nuclear pleomorphism and hyperemia still existed evidently, though less likely to the cancer control, in the cancer cells treated with G. The possibility of the better anticancer effect of SC might be due to the lower of concentration of G used during the treatment to the cancer cells. Though G and SC supplemented cancer group respond positively.

The optimal concentration of SC used is 5% in treating the liver cancer. This support earlier work by Elizabeth *et al.*[19]. Both SC and G supplemented cancer group at 5% showed promising result, in which the lesion score was lowest in the cancer group. This shows that SC has a great potential to be used as an anti-cancer agent for liver cancer.

The enzymes tumour marker study showed that DEN/AAF-carcinogens caused an increase in all enzyme activities measured when compared to control. Supplementation of SC caused a reduction in the GST. Determination of GST was reported to be able to detect 90% of enzyme altered hepatic foci induced by chemical carcinogen[9]. GST activity has also been used as a tumour marker enzyme in the study of hepatocarcinogenesis in mice[10]. Besides GST, changes of other enzyme during hepatocarcinogenesis have also been reported. UDPGT determination and its application as a marker to detect preneoplastic nodule had been reported by Sato[10]. UDPGT activity also increased in DEN/AAF group compared to control group and SC supplementation decreased the activity significantly.

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