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Evaluation of Lesion Scoring and Aniline Hydroxylase Activity in Hepatocarcinogenesis Rats Treated with *Strobilanthes crispus*

O. Fauziah, P. Hanachi, S. Yogespiriya and R. Asmah

The effect of 5% w/v of *Strobilanthes crispus* (SC) extract and glycyrrhizin in diethylnitrosamine and acetylaminofluorene induced hepatocellular carcinoma, which is a vital mechanism in cancer treatment, was studied in male Sprague-Dawley rats. The obtained results have shown a significant, increase ($p<0.05$) of liver microsome Aniline Hydroxylase (AH) in cancer group rats after 12 weeks. Treatment with glycyrrhizin caused decrease in liver AH activity compared to control. Meanwhile, treatment with SC caused overall decrease in liver AH activity almost near to control groups. Meanwhile, microscopic observation of the lesion score during hepatocarcinogenesis revealed that cells of cancer group without treatment were severely necrotic at week 12. However, *S. crispus* treatment was reduced the severity in cancer group rats at week 12. The result also indicate that SC only ameliorated the cancer incidence in the liver, however did not fully recover the liver tumor similar to the normal cells. This might be due to short experimental duration.

Key words: *Strobilanthes crispus*, hepatocarcinogenesis, lesion score, aniline hydroxylase

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For further information about this article or if you need reprints, please contact:

Dr. Parichehr Hanachi
Department of Biomedical Sciences
Faculty of Medicine and
Health Science
University Putra Malaysia
43400 UPM
Serdang, Selangor, Malaysia

Tel: 0060389568440
Fax: 0060389436178
E-mail: paricheh@medic.upm.edu.my

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Department of Biomedical Science, Faculty of Medicine and Health Science, Universiti Putra, Malaysia

INTRODUCTION

Recently, there has been renewed interested in hepatocellular carcinoma (HCC) in developing countries, because it accounts for 15% of total cancer mortality burden. Accumulating epidemiological and experimental evidence has revealed the influence of number of naturally occurring and synthetic compounds on drug detoxification and HCC incidence[1]. Hepatocarcinogenesis induced by diethylnitrosamine (DEN) and acetylaminofluorene (AAF) is a cancer model rat as it facilitates the study of mechanism of chemical carcinogenesis and response of HCC to anticancer drug therapy.

Since the increase in the use of synthetic chemicals in cancer therapy has led to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources (medical plants) which are therapeutically effective, culturally acceptable and economically within the reach of even the neediest people.

Over the centuries no fewer than 3000 plant species have been used to treat cancer[2]. Many plants are introduced and studied to increase the discovery of natural product cancer chemotherapeutic agents[3]. *Strobilanthes crispus* (L) Bremek or *Saricocalyx crispus* (L) Bremek (Acanthaceae) (SC) plant is native to countries from Madagascar to Indonesia which is commonly known as' picah beling' in Jakarta or ' kejibeling' in Java. It has been found that an infusion of the dried leaves of this plant has been used as antidiabetic, diuretic, antilytic and laxative[4].

The liver is the principal site of drug-metabolizing activity, the possible significance of all the biochemical patterns, including the biotransformation enzymes, in analysing the diversity of biochemical expression of cancer and mechanism of cancer development, in addition to the understanding of a possible role of physiological importance. The liver is main target and particularly susceptible for chemically induced toxicity. Firstly, it is an organ with the highest complement of cytochrome P450 and secondly, the liver is the first site for the metabolism of xenobiotics absorbed from gastrointestinal tract[5]. AH is one of the isozymes of P450 2E1 subfamily which activates diethylnitrosamine (DEN)[6]. In this study, we have undertaken efforts to ascertain the anticancer potency of *S. crispus* (SC) extract on (DEN) and (AAF) induced HCC with special attention to hepatic drug metabolism and to investigate the effect of (SC) on preneoplastic marker enzyme activity specifically of microsomal AH activity and lesion scoring in control and treated with DEN and AAF.

MATERIALS AND METHODS

Chemicals: Diethylnitrosamine, Acetylaminofluorene, Aniline and all other reagents used were of highest grade commercially available (Sigma Chemical Co., St. Louis, Mo, USA)

Animals: Thirty male 200-250 g (6-8 weeks) Sprague-Dawley rats (*Rattus norwegicus*) were purchased from the animal colony unit, Universiti Putra Malaysia (UPM) . These rats were acclimatised for at least a week before use. They were kept in separate cages in a ventilated room with equal periods of day light and darkness with temperature (32±2°C). Rat chow (Ridley Rat Chow, Australia) and water *ad libitum* were given to these rats daily. Each cage was cleaned every week and bedded with wood chip for urine absorption.

Preparation of *S. crispus* extract for rat bioassay: The leaves of SC were collected from the Herbs Garden of Faculty of Medicine and Health Sciences, UPM. Crude extract of (SC) was prepared from a modified method described in Conney *et al.*[7]. In this experiment, 5.0% (w/v) of SC leave extract was used. In this study, 5% (w/v) of SC was chosen because the previous study by Elizabeth[8] revealed that 5% (w/v) SC extract found to be very effective in treating hepatocarcinogenesis in DEN/AAF induced rats.

Animals treatment: The protocol of inducing rat hepatocellular carcinoma in this study was basically according to Solt and Farber[9] method. The method was modified, as the rats did not go the partial hepatectomy (selective pressure) stage. In this study, rats were divided to groups consisted of 5 rats/group. Rats in Group 1, 2 and 3 were injected 200 mg/kg/bodywt. diethylnitrosamine (DEN) intraperitoneally as an initiator to hepatocarcinogenesis and after 2 weeks, the rat chow which was mixed with acetylaminofluorene (AAF) were given to these rats as promoter of hepatocarcinogenesis. However rats in Group 4, 5 and 6 were not induced liver cancer. At the first week, treatment with 5.0% (w/v) *Strobilanthes crispus* extract was given *adlibitum* to the rats in Group 2 and 4. Rats in Group 3 and 5 received 0.005% glycyrrhizin as treatment. However, rats in Group 1 and 6 were not given any treatment. These treatments were given to the rats for 12 weeks. At week 12, three rats from each group were sacrificed. All rats were starved for 24 h before being sacrificed. Upon termination of the experiment, rats were weighed and livers were removed.

Preparation of microsome: The microsomal preparation was carried out essentially following the method described in Hasham *et al.*[10].

Lesions scoring analysis: The toluidine blue stained sections were used for lesion scoring by using digital light microscope Leica DMRA II equipped with Qwin and Qfluoro software under power x200. The severity was based on inflammation and necrosis grade using method described in Stevens *et al.*[11].

Enzyme assays: Aniline hydroxylase assay was performed according to the method of Imai and Sato[12] with the some modifications according Waxman *et al.*[13]. A unit of activity was defined as 1 μmol p-aminophenol liberated/mg protein/min. Protein determination was carried out according to the method of Bradford[14].

Statistical analysis: The results obtained was analysed by inferential statistic in terms of t-test and Analysis of Variance (ANOVA) in which post-hoc comparisons were made using the Benferonni's test. The level of significance was 0.05 or difference with a $p<0.05$ were considered to be significant.

RESULTS

Lesion scoring analysis: In untreated cancer induced group, the grade of inflammation or necrosis was 2.3 and higher compared to other groups. However, the score is not significantly different when compared with cancer induced rats treated with SC and glycyrrhizin group and normal rat treated with glycyrrhizin group. Hepatocytes with necrosis were seen at portal area in the former group. However, the grade of cancer with *Strobilanthes* treatment group is 1.0 and this group did not showed any significant difference compared to normal rats and normal rats with SC and glycyrrhizin treatment groups. The portal of this group was inflamed but necrotic cells were not found (Fig. 1).

In rats induced hepatocarcinogenesis, the score of inflammation or necrosis of liver lobular was found to be at 2.3 and found to be at significant changes in hepatic lesion when compared with normal groups and cancer with SC treatment group. Moderate and severe focal necrotic cells were seen in this group. However, cancer with (SC) group differs significantly when compared to normal, normal with *Strobilanthes* and cancer group. In this group, inflammatory cells without necrosis were seen and in some area normal cells without inflammation were seen.

Cancer group showed the highest stage of fibrosis and showed significant different between cancer with *Strobilanthes* group and normal (SC) group. In this group, fibrosis at portal area was seen.

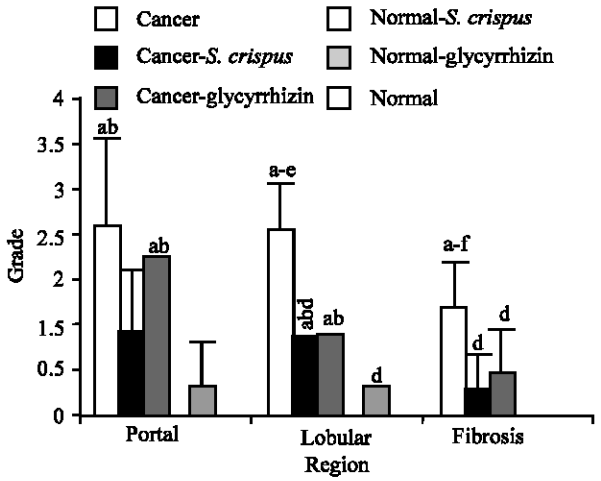

Fig. 1: Lesion scoring of control and SC treated rat liver at week 12. (CC: cancer control, CS: cancer with (SC) treatment, CG: cancer with glycyrrhizin treatment, NS: normal with (SC) treatment, NG: normal with glycyrrhizin treatment and NC: normal without treatment)

a: Significant ($p\leq0.05$) compared to normal
b: Significant ($p\leq0.05$) compared to normal *Strobilanthes crispus*
c: Significant ($p\leq0.05$) compared to normal glycyrrhizin
d: Significant ($p\leq0.05$) compared to cancer
e: Significant ($p\leq0.05$) compared to cancer-*Strobilanthes crispus*
f: Significant ($p\leq0.05$) compared to cancer-glycyrrhizin

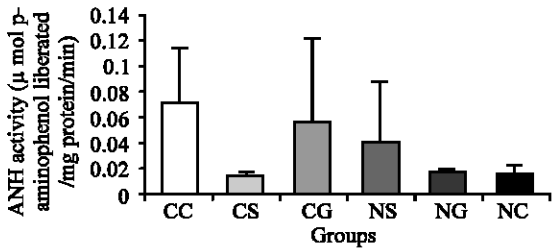

Fig. 2: The activity of aniline hydroxylase enzyme in experiment and control liver rat at week 12 (CC: Cancer Control, CS: Cancer with *Strobilanthes crispus* treatment, CG: Cancer with Glycyrrhizin treatment, NS: Normal with *Strobilanthes crispus* treatment, NG: Normal with Glycyrrhizin treatment and NC: Normal without treatment)

Aniline hydroxylase activity: Oral administration of 5.0% w/v of SC extract is found to be effective in reducing aniline hydroxylase activity. The obtained results have

shown decreased ($p<0.05$) of liver microsome AH in liver cancer animals after 12 weeks (Fig. 2). The SC extract affords anticancer activity by enhancing enzyme activities to near normal levels after 12 weeks.

DISCUSSION

The effect of *S. crispus* extract in DEN/AAF induced hepatocellular carcinoma, was studied in male Sprague-Dawley rats. Histological evaluation of rat liver revealed DEN/AAF induced and untreated rats group showed higher score of inflammation or necrosis at portal, lobular and stages of fibrosis compared to all the other groups. Five percent (w/v) SC extract administration successfully reduced the score of inflammation or necrosis at portal, lobular and stages of fibrosis

Glycyrrhizin also found to be reduced the histopathological changes during hepatocarcinogenesis in rats but not effective as SC treatment. Five percent (w/v) of SC did not cause any side effect towards normal cells. SC did not fully recovered the histopathological changes during hepatocarcinogenesis. This could be due to short experimental duration. In this study, SC might act as antioxidant agent which can inhibit or slow down histopathological changes which induced DEN/AAF.

P450 2E1 is phase I enzyme that responsible in metabolizing catalyst many low molecular weight carcinogens and potentially toxic chemicals including ethanol, nitrosamines, halogenated alkanes and aromatic compounds[10]. At low substrate concentrations, cytochrome P450 2E1 is the major enzyme responsible for the oxidative demethylation of DEN.

The *S. crispus* extract exerts anticancer activity by inhibiting enzyme activity to near normal levels after 12 weeks. The P450 2E1 isoform is responsible for AH that activates diethylnitrosamine (DEN)[6]. DEN activity depends on its conversion to 8-hydroxyguanine by oxidative stress and formation of alkyl-DNA adduct. Thus the chemopreventive action may be due to the scavenging of the reactive oxygen radicals from the system, as well as inhibition of the enzymes responsible for the activation of DEN[15]. In this study, SC extract may act as a chemopreventive agents which exerts its protective effects by inhibition of enzymes involved in metabolic activation of carcinogen (phase I enzyme i.e ANH)[6].

Distinct evidence from this study contribute that oral administration of 5% *Strobilanthes crispus* extract demonstrated anticancer activity by reducing the severity of cancer in treated group. Moreover, there were no evidence suggestion side effects of SC towards normal cells indicating SC as a potent preventive agent for cancer.

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