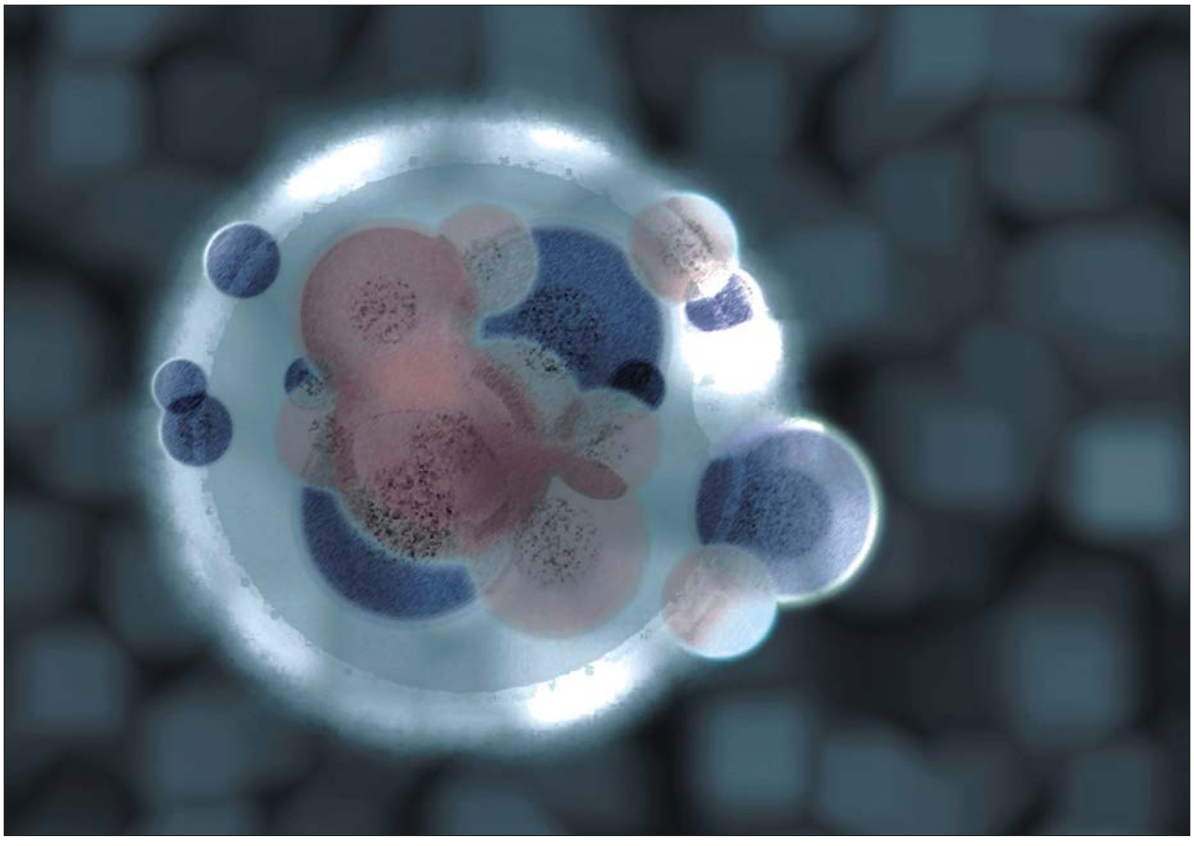

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Histological Study During Hepatocarciniogenesis in Rats Treated With *Strobilanthes crispus* Extract

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Abstract: This study was conducted to investigate the effectiveness of 5% (w/v) *Strobilanthes crispus* extract on rat liver during chemically induced hepatocarcinogenesis. Histological evaluation of liver was conducted to observe the cellular and morphological changes during hepatocarcinogenesis treated with *Strobilanthes crispus*. The histological evaluation revealed that a certain grade of inflammation or necrosis at portal and lobular region and stages of fibrosis during hepatocarcinogenesis was successfully reduced after administration of *Strobilanthes crispus* extract. However, these changes did not fully recovered by supplementation of *Strobilanthes crispus* to normal histological features of liver. This could be due to a short experimental duration and in addition, the supplementation of this extract to normal rat did not show any changes in normal hepatocytes. Thus, *Strobilanthes crispus* can be considered as a potential chemopreventive agent.

Key words: *Strobilanthes crispus*, hepatocarcinogenesis, lesion score, light microscopy

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in some areas of the world[1]. Carcinogenesis is a complex and protracted multistage process. Earlier studies of chemically induced hepatocarcinogenesis indicated that altered hepatic foci appeared in the livers of treated animals at early stage. In 1960s, altered hepatic foci had been noted, with each laboratory using an independent histological protocol for their identification[2]. The cellular events in HCC emergence are still not completely understood. In early models of chemical hepatocarcinogenesis in rats, the initiated hepatocytes started to proliferate[3]. HCC has trabecular, pseudoglandular, compact or mixed architectural patterns with diverse cytological features and found in cirrhotic liver[4].

The *Strobilanthes crispus* ZII 109 (L.) Bremek or *Saricocalyx crispus* ZII 109 (L.) Bremek (Acanthaceae) plant is commonly known as "daun picah beling" in Jakarta or "enyoh kelo," "kecibeling," or "kejibeling" in Java. This plant is native to countries from Madagascar to Indonesia[5]. This plant can be found in shaded terrain, especially in region of Indonesia with a strong East monsoon, in coconut tree gardens, at roadsides and in the woods[6]. A study in Indonesia found that an infusion of the dried leaves of *Strobilanthes crispus* has been used as antidiabetic, diuretic, antilytic and laxative. The recent study found that 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[7]. Besides that, stigmasterol from *Strobilanthes crispus* induced apoptosis on MCF-7 breast cancer cell lines when observed under fluorescence microscope[8].

Many studies have been carried out using edible plants and vegetables to treat hepatocarcinogenesis. Most of these plants such as *Emblica officianalis, Phyllanthus amarus* and *Picrorrhiza kurroa* tend to reduce the severity of hepatocarcinogenesis[9] but this study did not report any histological changes during hepatocarcinogenesis. Moreover, the histological study of the use of *Strobilanthes crispus* during hepatocarcinogenesis was also not well documented. Thus, the objective of this study was to score the histological lesion of rat induced hepatocarcinogenesis and treated with *Strobilanthes crispus.*

MATERIALS AND METHODS

Chemicals: Glutathione, 1-chloro-2,4-dinitrobenzene and all other reagents used were of highest grade

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commercially available (Sigma Chemical Co., St. Louis, Mo, USA).

***In vivo* bioassay:** In this study, male Sprague-Dawley rats (*Rattus norwegicus*) weighing 150-200 g (6-8 weeks) were maintained in the animal house at the Faculty of Medicine and Health Sciences and 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 at 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.

The *in vivo* bioassay study for hepatocarcinogenesis was based on the modified technique of Solt and Farber[10], without partial hepatectomy (selective pressure) stage. In this study, rats were randomly divided into six groups and each group consists of 12 rats. Rats in Group 1, 2 and 3 were injected with 200 mg/kg/body wt. diethylnitrosamine (DEN) intraperitoneally which acts as an initiator to hepatocarcinogenesis and 2 weeks later, the rat chow which was mixed with acetylaminofluorene (AAF) was given to these rats as a promoter of hepatocarcinogenesis. Rats in Group 4, 5 and 6 were not induced with liver cancer and left untreated with neither DEN nor AAF.

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. Three rats from each group were sacrificed at week 12. All rats were starved for 24 h before being sacrificed. Upon termination of the experiment, rats were weighed and livers were removed.

Lesion scoring: The rat liver samples were cut into a numbers of 1 mm^3 slices and processed according to the methods of sample preparation for transmission electron microscope.

The processed samples were cut into 1 μm thick sections using ultramicrotome LEICA ULTRACUT-UCT. These sections were placed onto glass slide and stained with methylene blue. The stained sections then viewed under light microscope Leica DMRA II equipped with Qwin and Qfluoro software under x200 magnification for lesion scoring. The severity was based on the modified method of Stevens[11].

Statistical analysis: The lesion scoring data were analysed using the Mann-Whitney test (non-parametric t-test) to observe the difference between group. Meanwhile, 2-way ANOVA was used to differentiate between weeks.

RESULTS

Lesion scoring of portal area of rat liver: At the highest score at week 10, the cancer induced and untreated rats group showed moderate or severe necrosis of periportal hepatocytes and was significantly different from cancer induced rats treated with *Strobilanthes crispus* and glycyrrhizin group (Fig. 1).

In rats induced cancer treated with *Strobilanthes crispus* extract group, the mild necrotic cells were seen at periportal area at lowest score on week 12 and was not significant with normal rats group, normal rats treated with *Strobilanthes crispus* and glycyrrhizin group (Fig. 1).

The cancer induced rats treated with glycyrrhizin group showed the lowest score at week 10 and this group did not show any significant different compared to normal rats group, normal rats administered with *Strobilanthes crispus* and glycyrrhizin group but significantly different compared to cancer induced and untreated rats group.

There was no inflammation or necrosis seen at portal area in the normal rats group, normal rats treated with *Strobilanthes crispus* and glycyrrhizin group. However, in normal rats treated with glycyrrhizin group, the portal regions were seen infiltrated with inflammatory cells at both week 10 and 12.

Lesion scoring of lobular area of rat liver: The grade of inflammation and necrosis at lobular area in cancer induced and untreated rats group showed significantly higher score compared to normal rats groups at week 2, 4, 10 and 12. At the highest score at week 10, necrotic liver cells were seen bridging the portal tract (Fig. 2).

Cancer induced rats treated with *Strobilanthes crispus* group showed significantly higher score at lobular region compared to normal rats group, normal rats treated with *Strobilanthes crispus* group and normal rats treated with glycyrrhizin group at week 2, 4 and 10. However, at the lowest score at week 12, this group was significantly different compared to normal rats group, normal rats treated with *Strobilanthes crispus* group and untreated cancer induced rats group.

Cancer induced rats treated with glycyrrhizin group showed significant difference compared to normal rats group, normal rats treated with *Strobilanthes crispus* group and normal rats treated with glycyrrhizin group at week 2, 4 and 10 (Fig. 2).

In normal rats group and normal rats treated with *Strobilanthes crispus* group, there was no lesion seen. Both groups were significantly different to cancer induced

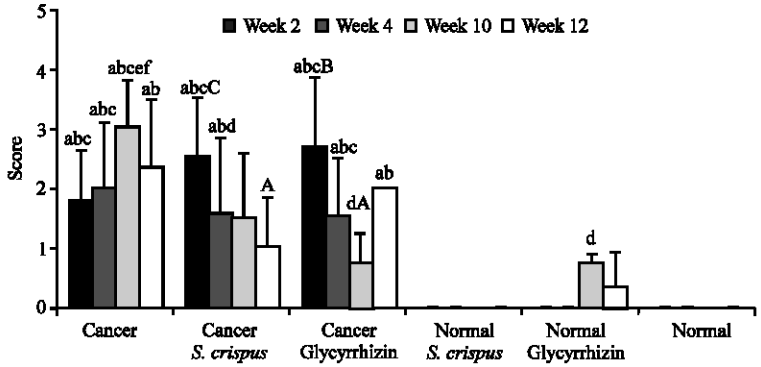

Fig. 1: The lesion score of portal area of the liver during induced hepatocarcino-genesis in rats treated with *Strobilanthes crispus* and glycyrrhizin at 2, 4, 10 and 12 weeks post induction

a: Significant ($p \leq 0.05$) compared to normal
b: Significant ($p \leq 0.05$) compared to normal *Strobilanthes crispus*
c: Significant ($p \leq 0.05$) compared to normal glycyrrhizin
d: Significant ($p \leq 0.05$) compared to cancer
e: Significant ($p \leq 0.05$) compared to cancer *Strobilanthes crispus*
f: Significant ($p \leq 0.05$) compared to cancer glycyrrhizin
A: Significant ($p \leq 0.05$) compared to week 2
B: Significant ($p \leq 0.05$) compared to week 10
C: Significant ($p \leq 0.05$) compared to week 12

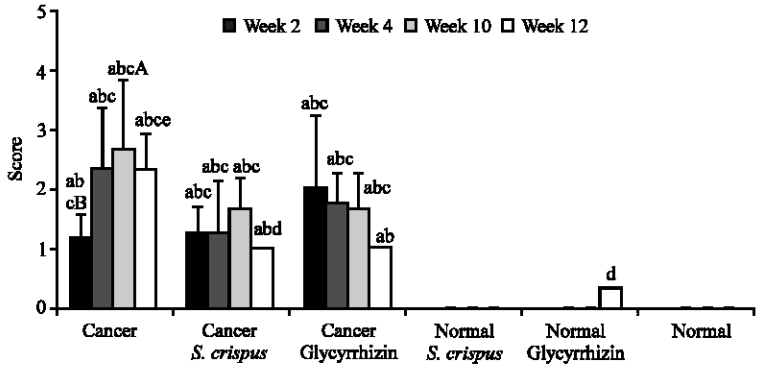

Fig. 2: The lesion score of lobular area of the liver during hepatocarcinogenesis in rats treated with *Strobilanthes crispus* and glycyrrhizin at 2, 4, 10 and 12 weeks post induction

a: Significant ($p \leq 0.05$) compared to normal
b: Significant ($p \leq 0.05$) compared to normal *Strobilanthes crispus*
c: Significant ($p \leq 0.05$) compared to normal glycyrrhizin
d: Significant ($p \leq 0.05$) compared to cancer
e: Significant ($p \leq 0.05$) compared to cancer *Strobilanthes crispus*
f: Significant ($p \leq 0.05$) compared to cancer glycyrrhizin
A: Significant ($p \leq 0.05$) compared to week 2
B: Significant ($p \leq 0.05$) compared to week 10

and untreated rats group, cancer induced rats treated with *Strobilanthes crispus* and cancer induced rats treated with glycyrrhizin group. In normal rats treated with glycyrrhizin group, there was no lesion at week 2, 4 and 10. However, at week 12, the inflammation or necrosis score was 0.33±0 at week 12.

Stages of fibrosis of rat liver: The stages of fibrosis in cancer induced and untreated rats group showed significant higher score compared to normal rats group, normal rats treated with *Strobilanthes crispus* and normal rats treated with glycyrrhizin group at week 10 and 12. At week 12, this group was also significantly different with

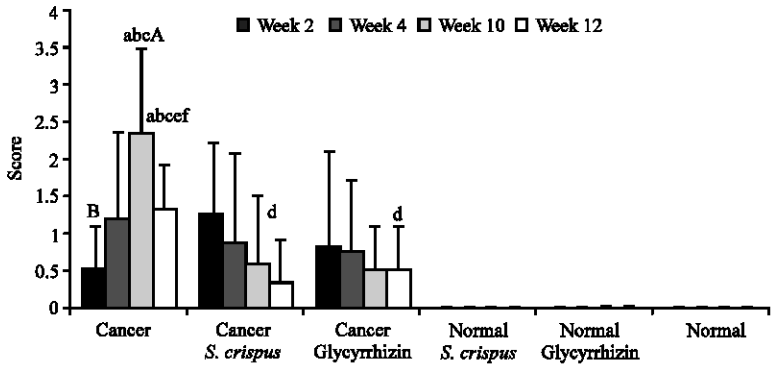

Fig. 3: The stages of fibrosis of liver during hepatocarcinogenesis in rats treated with *S. crispus* and glycyrrhizin at 2, 4, 10 and 12 weeks post induction
a: significant ($p \leq 0.05$) compared to normal
b: significant ($p \leq 0.05$) compared to normal *Strobilanthes crispus*
c: significant ($p \leq 0.05$) compared to normal glycyrrhizin
d: significant ($p \leq 0.05$) compared to cancer
e: significant ($p \leq 0.05$) compared to cancer *Strobilanthes crispus*
f: significant ($p \leq 0.05$) compared to cancer glycyrrhizin
A: significant ($p \leq 0.05$) compared to week 2
B: significant ($p \leq 0.05$) compared to week 10

cancer induced rats treated with *Strobilanthes crispus* and glycyrrhizin group (Fig. 3).

Cancer induced rats treated with *Strobilanthes crispus* and glycyrrhizin group showed significantly lower stages of fibrosis compared to cancer induced and untreated rats group at week 12.

In normal rats group, normal rats treated with *Strobilanthes crispus* and normal rats treated with glycyrrhizin group, there was no fibrosis seen.

DISCUSSION

The results presented in this study clearly indicated that hepatocarcinogenesis which was induced by DEN/AAF was effectively inhibited by 5% (w/v) *Strobilanthes crispus* crude extract. The cancer induced and untreated rats group showed the highest score in inflammation or necrosis at portal, lobular and stages of fibrosis. The severity of cancer at portal, lobular and fibrosis was reduced after rats were supplemented with 5% (w/v) *Strobilanthes crispus* extract for 12 weeks. Glycyrrhizin treatment also found to decrease the severity of cancer but not effective as *Strobilanthes crispus* extract treatment. Moreover, results in previous study[12], clearly demonstrated that *Strobilanthes crispus* 5% (w/v) extract is very effective dose in treating hepatocarcinogenesis in DEN/AAF induced rats. Supplement of 5% (w/v) *Strobilanthes crispus* to normal rats did not cause any side effects towards the normal cells. The result also indicates that *Strobilanthes crispus* treatment 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.

Liver plays an important role in toxicological response and pollutant induced pathological changes. These can be observed by liver histological analysis. Histopathology may be an indication of environmental stress[13]. The damaged hepatocytes, inflammatory cells or cytokines such as tumor necrosis factor-α could contribute to the production of reactive oxygen species (free radical such as superoxide ion [O_2^-] and hydrogen peroxide [H_2O_2]). The lipid peroxides formed increased inflammation by chemotactic for neutrophils, which further drives oxidant-mediated injury in the liver[14].

In this study, *Strobilanthes crispus* might act as antioxidant agent which can inhibit or slow down the severity of cancer. The elements in *Strobilanthes crispus* extract might function as antioxidants, antimitogens and antimutagens. The elements especially antioxidant elements neutralized free radicals and intermediates of metabolism that are highly reactive since they contain a non-paired electron[15]. The antioxidant elements prevent DNA damage which was caused by free radicals[16].These elements also avoiding DNA damage by preventing free radicals from forming, protecting cells from damage by free radicals, binding to free radicals to inactive or kill them and enhancing body's own defense system[17].

A study on antioxidant[14] revealed that the levels of antioxidant in liver were lower in patients with the highest grades of fibrosis. It was clearly indicated that lower levels of antioxidant could only be a reflection of increased fibrosis and the absence of active liver tissue.

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