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Effect of cola nut (*Cola nitida*) on tumour marker enzymes in rat liver during hepatocarcinogenesisSuherman Jaksa¹, Susi Endrini², Michiko Umeda¹, Wan Nor Izzah Wan Mohd. Zen³, Fauziah Othman^{3*} and Asmah Rahmat³¹⁶¹Faculty of Medicine and Health Sciences, University of Muhammadiyah Jakarta, Indonesia²⁴²School of Medicine, YARSI University, Indonesia³Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia.

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The administration effect of Cola nut extract (Cn) during hepatocarcinogenesis was studied to investigate the possible cancer suppressive effect of the component that existed in the leaves. The rats (66 Male Sprague Dawley) were divided into 11 groups: N (Normal), C (Cancer), NCn1 (Normal + Cn 1%), NCn2.5 (Normal + Cn 2.5%), NCn5 (Normal + Cn 5%), CCn1 (Cancer + Cn 1%), CCn2.5 (Cancer + Cn 2.5%), CCn5 (Cancer + Cn 5%), CG1 (Cancer + Glycyrrhizin 1%), CG2.5 (Cancer + Glycyrrhizin 2.5%) and CG5 (Cancer + Glycyrrhizin 5%). 1, 2.5, and 5% (w/v) of cola nut extract were used, compared with Glycyrrhizin, the commercial anticancer drug used mainly for liver. Rats were induced with cancer by using diethylnitrosamine (DEN) and 2-acetyl-aminofluorene (AAF), the administration effect was studied by estimation of glutathione S-transferase (GST) and gamma glutamyl transpeptidase (GGT) in liver. The supplementation effect of cola nut extract and DEN/AAF into the body and liver weight of rats were also studied. Treatment with DEN/AAF caused increase in rats liver weight and all enzyme activities measured when compared with the control. However, DEN/AAF caused decrease of rats body weights. Significant differences were observed among all the treatment groups for GST and GGT activities.

Key words: Tumour marker enzymes, gamma glutamyl transpeptidase (GGT), glutathione S-transferase (GST), cola nut, hepatocarcinogenesis.

INTRODUCTION

Cola nut tree is native to West Africa. It has been naturalized to South America, Central America, the West Indies, Sri Lanka, Malaysia, and Indonesia. Related to cocoa, cola nut is the source of a stimulant, and contains the methylxanthine alkaloids that occur also in coffee, cocoa, and tea. Of the 40 known species, *Cola acuminata* and *Cola nitida* bear the nuts most readily available in the United States and Europe; other species frequently used in commerce include *Cola verticillata* and *Cola anomala*. West Africans have been chewing cola nuts for thousands of years. Its stimulant effects are its predominant application in the United States and Europe.

In Africa, however, cola nuts have been used as an appetite and thirst suppressant, enabling soldiers who chewed them to travel long distances without much food. Cola twigs, with an extremely bitter taste, are used to clean the teeth and gums (Mitchell, 2008).

Today, cola nut is exported worldwide. It is used in the manufacture of methylxanthine-based pharmaceuticals. Cola nut is also used in non-pharmaceutical preparations, including (at least formerly) cola-based beverages such as Coca Cola. It is on the generally recognized as safe (GRAS) list for food additives in the United States (Mitchell, 2008). Our previous study suggested the

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possibilities that cola nut extract have high antioxidant activities and cytotoxic properties against liver carcinoma (HepG2) cell lines (unpublished data). However, there were no reports on the administration effect of cola nut extract during hepatocarcinogenesis.

At present, hepatocellular carcinoma (HCC) is still a worldwide health issue for which the medical oncology community is largely unprepared. Each year, 550,000 new patients are diagnosed with HCC worldwide (Motola-Kuba et al., 2006; Coleman, 2003). Primary liver cancer remains the fifth most frequent neoplasm and, because of its poor prognosis, the third leading cause of cancer deaths (Parkin, 2001). Various ways of monitoring the carcinogenic process have been reported; these could either be by examination of morphology and by determination of reported preneoplastic marker enzymes glutathione S-transferase (GST) (Hendrich and Pitot, 1987) and gamma-glutamyl transpeptidase (GGT). This study was conducted to determine the effect of administration of cola nut extract on the tumour marker enzymes, GGT and GST, in rat liver induced with hepatocarcinogens diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF).

MATERIALS AND METHODS

Plant extract

Cola nuts were harvested at herbs garden Nasuha Ent, Pagoh, Johor, Malaysia. Crude extract of cola nut was prepared according to Suherman et al. (2005). Cola nuts (10 g) were grounded in 100 cm³ of distilled water (10%) and were filtered. The filtrate was diluted with distilled water to obtain the concentration of 1, 2.5, and 5% and was stored in the refrigerator at 4°C.

DEN preparation

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 to 200 g.

AAF preparation

One gram AAF (a promotion agent) was dissolved in 50 ml acetone, 1.5 ml of this solution was mixed with 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 66 male Sprague Dawley rats (*Rattus norvegicus*), each initially weighed between 150 to 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 eleven groups, that is, Group I: normal (basal diet) (N), Group II to IV: Cn supplemented diet (1, 2.5, and 5% Cn in drinking water (NCn1, NCn2, and NCn5)), Group V: cancer (DEN/AAF) with basal diet (C), Group VI to VIII:

cancer (DEN/AAF) Cn extract supplemented diet (CCn1, CCn2.5, and CCn5), Group IX to XI: cancer (DEN/AAF) treated with glycyrrhizin (CG1, CG2.5, and CG5).

Hepatocarcinogenesis was induced according to the method of Solt and Farber (1976), but without partial hepatectomy. Animals groups V to VI were intraperitoneally given single injection of 200 mg DEN/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 cola nut extract (at different concentration) was given as a substitute to distilled water in Groups II to IV, VI to VIII and glycyrrhizin with different concentrations in Group IX to XI. A summary of the protocol is as shown in Figure 1.

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 weighed and washed in ice-cold 0.9% NaCl solution weighed and washed immediately. The liver tissues from 6 rats in each group were stored at -70°C until it is used for tumour marker enzyme assays.

Cytosolic and microsomal fractions preparation

Cytosolic and microsomal fractions of the livers were prepared by the method of Speir and Wattenberg (1975). Briefly, rat livers were rinsed in 1.15% w/v potassium chloride (KCl). Tissues were cut into small pieces in 1.15% KCl at a volume of 3 ml of KCl per gram liver and were homogenized for 5 min in an Ultra Turrax homogenizer (Janker and Kunkel, FRG). The homogenate was centrifuged at 9000 g at 4°C for 20 min in a Sorvall RC-5B superspeed centrifuge. The supernatant was pipetted into clean centrifuge tubes and centrifuged further at 104,000 g (35,000 rpm) at 4°C in a Beckman L-60 centrifuge. The pellet obtained represents the microsomal fraction and was used for GGT assay. The cytosol was used for GST activities.

Enzyme assays

GGT

GGT was assayed by the method of Jacobs (1971), with some modifications. Gammaglutamyl carboxynitride was used as substrate. The reaction mixture comprised 0.05 M Tris-HCl buffer pH 8.2 containing 2.9 mM substrate, 22 mM glycylglycine, and 11 mM MgCl₂ in a total volume of 1 ml. Plasma (0.1 ml) was added and allowed to incubate for 45 min. The reaction was stopped by adding 5 ml 7.5 mM NaOH. The absorbance of the final mixture was measured at 405 nm. Microsomal GGT was also assayed in a similar way except that the microsomal pellet was resuspended in 5 volumes of 0.1 M Tris-HCl buffer, pH 8.2, containing 1 mM MgCl₂. Microsomal GGT was expressed as IU/g protein.

GST

The activities of GST in the liver cytosol were assayed according to the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) or 2,4 dichloro-1-nitrobenzene (DCNB) as the second substrate. The reaction mixture consisted of 0.1 M phosphate buffer pH 7.5 (pH 7.5), 1 mM GSH, 1 mM CDNB (or DCNB) and cytosol in a final volume of 1.0 ml. The reaction was

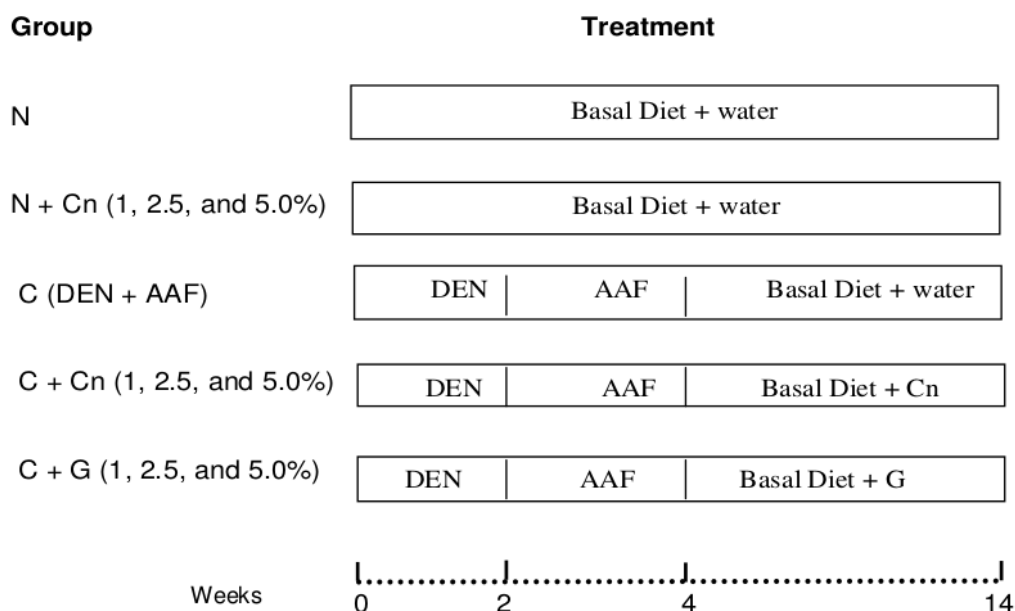


Figure 1. Protocol of the experimental design. Different groups of rat's cancer induced and non-cancer induced rats treated with different regimens of drinking water either/neither with Cn or G. N: Normal; C: DEN/AAF induced hepatocarcinogenesis; Cn: *Cola nitida*; DEN: Diethylnitrosamine; AAF: 2-Acetylaminofluorene; G: Glycyrrhizin.

Table 1. Effect of DEN/AAF, cola nut extract and glycyrrhizin on microsomal GGT after 14 weeks. Values are means \pm SEM.

No	Group (%)	GGT homogenate ($\mu\text{m}/\text{min}$)
1	N	1.63 \pm 0.54 ^a
2	NCn1	1.85 \pm 0.41 ^{ab}
3	NCn2.5	1.58 \pm 0.35 ^a
4	NCn5	1.49 \pm 0.60 ^a
5	C	3.77 \pm 0.57 ^f
6	CCn1	3.63 \pm 0.30 ^f
7	CCn2.5	2.57 \pm 0.35 ^{cd}
8	CCn5	1.98 \pm 0.29 ^{abc}
9	CG1	3.21 \pm 0.31 ^{ef}
10	CG2.5	2.79 \pm 0.30 ^{de}
11	CG5	2.42 \pm 0.32 ^{bcd}

^aP < 0.05 compared with normal control; ^bP < 0.05 compared with cancer; ^cP < 0.05 compared with CG1; ^dP < 0.05 compared with CG2.5; ^eP < 0.05 compared with CG5; ^fP < 0.05 compared with CCn5; N= Normal; AAF= 2-acetylaminofluorene; NCn = normal + cola nut; Cn = cola nut; DEN = diethylnitrosamine; G = glycyrrhizin; C=Cancer.

followed in a Shimadzu 2101 PC spectrophotometer at 340 nm. One unit of GST activity is expressed as the amount of enzyme required to conjugate 1 μM of the second substrate with GSH per minute at 29 $^{\circ}\text{C}$. Specific activity was defined as the units of enzyme per mg protein in the cytosol. Protein was assayed by the method of Bradford (1976).

Statistical analysis

Statistical analysis of the data was conducted by using Statistical Package for Social Sciences (SPSS) version 12.0. The results obtained were analyzed by analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Probability level of P < 0.05 was chosen to determine statistical significance. The values were reported as mean \pm SEM.

RESULTS

Cola nut extract had no effect on the plasma and liver microsomal GGT (Tables 1 and 2). DEN/AAF increased plasma and liver GGT activity as compared to that of controls (P < 0.05). However, when cola nut extract was supplemented in the diet of DEN/AAF treated rats, GGT activity was significantly less than that in rats treated with DEN/AAF only.

Rats treated with the carcinogens DEN/AAF showed increased cytosolic GST (Table 3). Rats supplemented with cola nut extract (Cn) and treated with carcinogens showed increases in this enzyme, but the increases were less than those receiving carcinogens only (P < 0.05).

DISCUSSION

Methanolic extract of cola nut showed free radical scavenging activities significantly and dose dependently

Table 2. Effect of DEN/AAF, cola nut extract and glycyrrhizin on plasma GGT after 14 weeks. Values are means \pm SEM.

No	Group (%)	GGT plasma ($\mu\text{m}/\text{min}$)
1	N	1.18 \pm 0.21 ^{ab}
2	NCn1	1.06 \pm 0.33 ^a
3	NCn2.5	1.22 \pm 0.15 ^{ab}
4	NCn5	1.56 \pm 0.26 ^b
5	C	2.15 \pm 0.61 ^c
6	CCn1	1.39 \pm 0.49 ^{ab}
7	CCn2.5	1.15 \pm 0.19 ^{ab}
8	CCn5	1.18 \pm 0.16 ^{ab}
9	CG1	1.38 \pm 0.12 ^{ab}
10	CG2.5	1.27 \pm 0.08 ^{ab}
11	CG5	1.33 \pm 0.19 ^{ab}

^aP < 0.05 compared with normal control; ^bP < 0.05 compared with cancer; ^cP < 0.05 compared with CG1; N= Normal; AAF= 2-acetylaminofluorene; NCn = normal + cola nut; Cn = cola nut; DEN = diethylnitrosamine; G = glycyrrhizin; C=Cancer.

(unpublished data). Results of the present investigations indicate that the aqueous extract of *C. nitida* nut is an effective chemopreventive agent against the DEN/AAF induced hepatocellular carcinoma. This observation is supported by various biological properties of the extract. Treatment of the extract prior to the DEN/AAF administration significantly reduced the tumour incidence compared to the control group of the animals. Increase in microsomal GGT activity with DEN/AAF treatment was observed in the liver. The plasma GGT activity was significantly elevated in the DEN/AAF alone treated group of animals indicating the induction of hepatocellular carcinoma (Ajith and Janardhanan, 2006). This is in agreement with elevated hepatic GST activity in DEN/AAF treated animal. Supplementation with cola nut extract resulted in less of an increase in GGT activity in both the plasma and liver in rats treated with cola nut extract in addition to DEN/AAF. In humans, plasma GGT activity has been reported to be useful as a marker of neoplasia and to correlate well with the extent of cancer diseases (Sahm et al., 1983). In animal studies, the degree of severity of cancer process is directly proportional to the enzyme activities (Ura et al., 1987). However, treatment of the extract prior to DEN/AAF showed a significant reduction of the tumour marker in a dose dependent manner.

Changes in molecular forms of hepatic cytosolic GST during rat chemical hepatocarcinogenesis were investigated by Kitahara et al. (1984). GST activities toward substrates CDNB and DCNB increased with the increased area of GGT-positive foci and hyperplastic nodules induced by DEN followed by AAF plus hepatectomy. GST types A and P were markedly increased and induced in livers bearing foci and nodules. These enzymes are the preneoplastic enzymes for chemical

Table 3. Effect of DEN/AAF, cola nut extract and glycyrrhizin on liver cytosolic glutathione S-transferase after 14 weeks. Values are means \pm SEM.

No	Group	GST ($\mu\text{m}/\text{min}/\text{mg}$)
1	N	0.77 \pm 0.11 ^{ab}
2	NCn1	0.93 \pm 0.08 ^{abc}
3	NCn2.5	0.86 \pm 0.14 ^{abc}
4	NCn5	0.74 \pm 0.11 ^{abc}
5	C	1.49 \pm 0.41 ^d
6	CCn1	1.19 \pm 0.34 ^{bcd}
7	CCn2.5	0.89 \pm 0.13 ^{abc}
8	CCn5	0.84 \pm 0.14 ^{abc}
9	CG1	1.22 \pm 0.22 ^{cd}
10	CG2.5	0.97 \pm 0.16 ^{abc}
11	CG5	0.91 \pm 0.58 ^{abc}

^aP < 0.05 compared with normal control ; ^bP < 0.05 compared with cancer; ^cP < 0.05 compared with CG1; ^dP < 0.05 compared with CG2.5; ^eP < 0.05 compared with CG5; N= Normal; AAF= 2-acetylaminofluorene; NCn = normal + cola nut; Cn = cola nut; DEN = diethylnitrosamine; G = glycyrrhizin; C=Cancer.

hepatocarcinogenesis (Kitahara et al., 1984; Lewis et al., 1989). This study also showed increases in GST activities, whereas cola nut extract supplementation caused a decrease in the activities, suggesting a protective effect of cola nut. Similar results were also observed in similar researches (Ajith and Janardhanan, 2006; Suherman et al., 2005).

Conclusion

Conclusively, the glutathione-dependent enzymes GST were increased with DEN/AAF treatment. Supplementation with cola nut extract brought these enzymes to values in between control and DEN/AAF treatment, suggesting a protective role of cola nut. This observation was further supported by a similar pattern of increases in GGT activities.

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