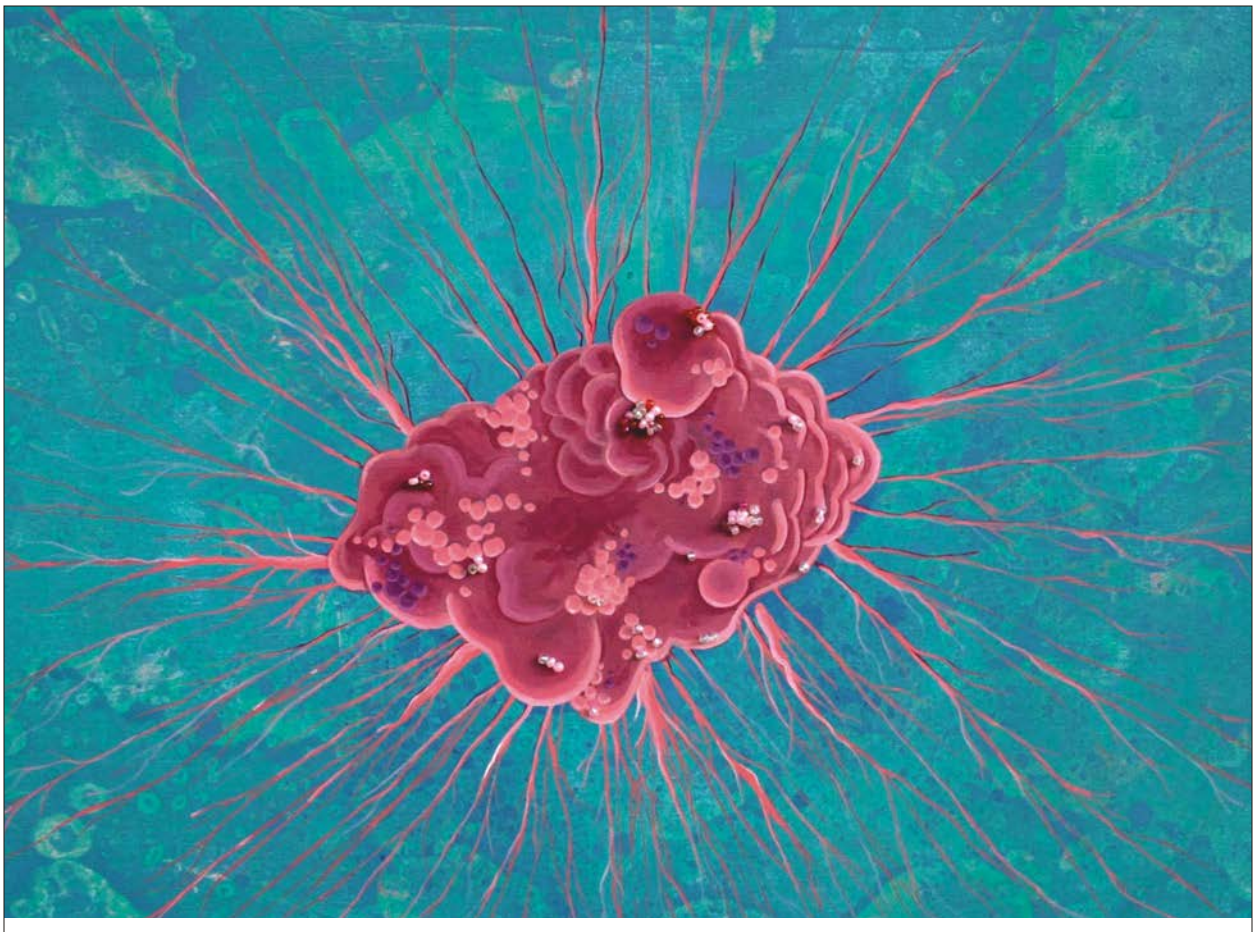

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The Effect of Neem (*Azadirachta indica*) Leaves Extract on Alpha-fetoprotein Serum Concentration, Glutathione S-transferase and Glutathione Peroxidase Activity in Hepatocarcinogenesis Induced Rats

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Abstract: This study evaluates the chemopreventive effect of an aqueous extract of fresh leaves of *Azadarichta indica* against liver cancer. Twenty male rats (Spraque dawley), 150-250 g, were acclimatized for 1 week before use. The rats were divided randomly into 4 groups; cancer control group (C), cancer treated with 5% *Azadarichta indica* extract group (CAI), normal control group (N) and normal treated with 5% *Azadarichta indica* extract group (NAI) and each group contains 5 rats. Rats in group C and CAI were induced cancer by intraperitoneal injection of 200 mg kg^{-1} diethyl nitrosamine (DEN) as hepatocarcinogenesis initiator and then followed by 2-acetylaminofluorene as promoter of hepatocarcinogenesis (0.02% in food) for 2 weeks. The rats were then left for 2 weeks. The rats in group N and NAI were injected once intraperitoneally with corn oil and act as control. The plant was fed orally to CAI and NAI groups. Serum concentration of alpha-fetoprotein (AFP) as liver tumor marker was measured. Glutathione S-transferase (GST) and glutathione peroxidase (GPx) were measured in the serum and liver cytosol. The results of this study revealed that there is a significant difference ($p \leq 0.05$) between C and CAI groups in serum alpha-fetoprotein and, in serum and liver cytosol levels of glutathione S-transferase and glutathione peroxidase. While there was no significant difference ($p \geq 0.05$) between NAI and N group in GST and GPx level. As a conclusion from this study that the consumption of 5% of NEEM leaves aqueous extract resulted in complete inhibition of chemically induced hepatocarcinogenesis in *Spraque dawley* rats.

Key words: *Azadarichta indica*, liver cancer, GST, GPx and AFP

INTRODUCTION

The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins, but the development of new screening technologies led to the revival of collections of plants and other organisms in 1986, with a focus on the tropical and sub-tropical regions of the world (Gordon and David, 2005). *Azadirachta indica*, Meliaceae, is widely distributed in Asia, Africa and other tropical parts of the world. Neem has been used as a folk medicine since ancient times. A considerable number of experimental studies also deal with the pharmacological activities of neem. Neem products are also used for the treatment of cardiovascular disorders, diabetes and cancer. In addition, it is reported to possess spermicidal, anti-implantation, anti-inflammatory, anti-pyretic,

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hypoglycemic, abortifacient activities (Brahmachari, 2004), antimicrobial effects (Sai Ram *et al*., 2000), storage behavior (Sacande *et al*., 2000), reduction of paracetamol induced liver damage (Bhanwara *et al*., 2000), insecticidal activity (Et-Shazley and Et-sharnoub, 2000) antibacterial agent (Das *et al*., 1999) and anticancer effect (Subapriya and Nagini, 2005). Numerous scientific reports validate the traditional use of NEEM in both the maintenance of general health and skin care. Practically every part of NEEM (Leaves, bark, fruits, flowers, oil and gum) has been reported to be associated with various remedial properties. Hepatocellular Carcinoma (HCC) is one of the most common malignant tumors in some areas of the world (Hubert, 2003). It's responsible for around 250 000 deaths every year (Kew, 2000). Serological markers for HCC are important for early diagnosis, as well as monitoring of tumor aggressiveness, treatment responsiveness, recurrence and survival. The most common marker is Alpha-fetoprotein (AFP). The AFP gene, a classic oncofetal antigen, is normally expressed in the fetal liver and only at very low levels in the normal adult liver. However, AFP expression is induced in regenerating liver and liver tumors. But it was the first described as marker for HCC by Abelev in the 1960s and now it is used in the medical laboratories in the diagnosis of adult liver caner (Man-Fung and Chin-Lung, 2005). GST and GPx are assumed to be significant biomarkers of chemoprevention owing antioxidant and detoxification properties (Balasenthil *et al*., 1999). The aqueous extract of NEEM (*Azdarichta indica*) was tested for its anticancer properties in induced liver cancer in rats treated with DEN and AAF. Alpha-fetoprotein, glutathione S-transferase and glutathione peroxidase was measured to evaluate the anticancer effects of the plant extract. This present study was proposed, due to the difficulties associated with recent liver cancer treatment (Sala *et al*., 2004; Majno *et al*., 2005), the promising properties of plants as source for anticancer agents (Gordon and David, 2005) and the rich literature of *Azadarichta indica* (Sai Ram *et al*., 2000; Parida *et al*., 2002).

MATERIALS AND METHODS

Treatment of Animals

Twenty *Sprague dawley* male rats (150-250 g) were divided randomly into 4 groups each group contain 5 rats respectively. Rats in group N and NAI were injected once intraperitoneally with corn oil and acted as control groups. The rats in group C and CAI were induced cancer by intraperitoneally injection 200 mg kg^{-1} Diethyl Nitrosamine (DEN) dissolve in corn oil. DEN intraperitoneal injection at the beginning of experiment was followed by a recovery period of two weeks on food which was mixed with acetyl aminofluorene (0.02% AAF). The 5% NEEM leaves extract was given as a substitute to water in rats in NAI and CAI groups. Animals were sacrificed by decapitation under ether anesthesia at 10 weeks. This study was conducted at the animal house and the Research Laboratories of the Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Kuala Lumpur, Malaysia.

Liver Cytosol

The cytosolic preparation was carried out essentially following the method of Hesham *et al*. (2002).

5% NEEM Leaves Aqueous Extract

The leaves of *Azadirachta indica* were collected from the Herbs Garden at Faculty of Medicine and Health Sciences, University Putra Malaysia. Aqueous extract of NEEM was prepared from the modification method of green tea extraction according to Conney *et al*. (1992). In this experiment, 5.0% w/v of leaves extract was used.

Assay of Glutathione Peroxidase Activities

Glutathione peroxidase (GPx) in the liver cytosol and were assayed according to the method of Lawrence and Burk (1976) and protein determination was carried out according to the method of Bradford (1976).

Assay of Glutathione S-transferase Activies

The assays for GST activity in liver cytosol serum were conducted according to the method of Habig *et al.* (1974).

Assay of Alpha-fetoprotein

Serum was collected from experimental animals' group to detect the level of alpha fetoprotein AFP and the method was done according to the method described by Premalatha and Sachdanandam (1999).

RESULTS

The Assay of Serum Alpha-fetoprotien

Figure 1 showed the level of AFP in the serum of the experimental rats. The C group showed the highest level of AFP (2.410±1.013) IU mL^{-1}. The other groups (N, NAI and CAI) were significantly lowered ($p \leq 0.05$) compared to the C group. Among cancer treated with NEEM (CAI group), normal control (N group) and normal treated with NEEM (NAI group), there was no significant different ($p \geq 0.05$). This suggested that NEEM could reduce back the level of AFP in the blood of the cancerous rats near to the normal value.

Gluatathione Peroxidase (GPx) Assay

Figure 2 shows the Gluatathione Peroxidase (GPx) specific activity in the liver and serum. Generally, cancer control group (C) showed a significant ($p \leq 0.05$) higher GPx specific activity compared to the other groups normal control (N), normal control rats treated with 5%NEEM (NAI) and cancer control group treated with 5% NEEM extract ($p \leq 0.05$). There was an insignificant

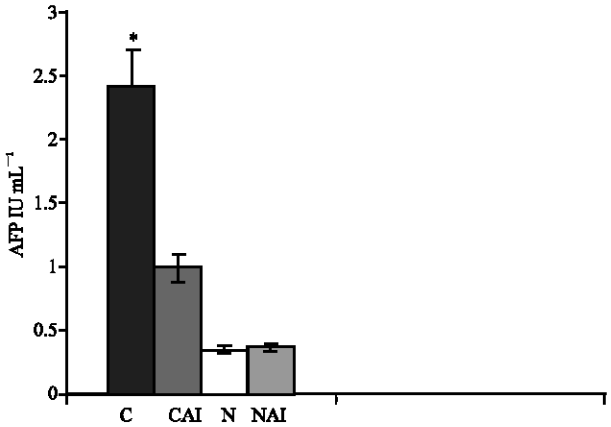

Fig. 1: Blood AFP concentration in the different group of rats. Results are showed as mean±SD (n = 10). ANOVA test showed a significant difference between different groups and by using Duncan post-hoc test, the significant difference was noticed in animals of cancer group *($p \leq 0.05$). N = Normal control; NAI = Normal with NEEM treated; C = Cancer control; CAI = Cancer with NEEM treated

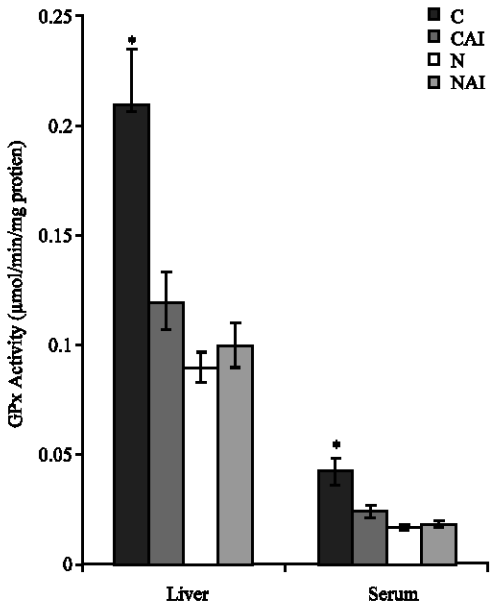

Fig. 2: The effect of 5% NEEM on GPx activity in the liver and serum of DEN and AAF-induced cancer rats. Results are showed as mean±SD (n = 10). ANOVA test showed a significant different between different groups and by using Duncan post-hoc test, the significant difference was noticed in animals in cancer group *($p \leq 0.05$). N = Normal control; NAI = Normal with NEEM treated; C = Cancer control; CAI = Cancer with NEEM treated

Table 1: The effects of 5% NEEM extract on GST activity in the liver and serum of DEN and AAF-induced cancer rats

	GST activity (umol/min/mg protien)	
Animal groups	Liver	Serum
Cancer group	1.32±0.21*	0.48±0.12*
Cancer + *A. indica*	0.61±0.29	0.18±0.09
Normal group	0.52±0.18	0.13±0.06
Neem + Normal	0.54±0.31	0.14±0.07

*Results showed as mean±SD (n = 10). ANOVA test showed a significant difference between different groups and by using Duncan post-hoc test, the significant difference was noticed in animals of cancer group *($p \leq 0.05$)

difference of GPx activities in the normal control group treated with 5% NEEM (NAI) compared to the normal control group (N) and cancer group treated with 5% NEEM ($p \geq 0.05$). The decreased values of GPx specific activity in cancer groups treated with 5% NEEM is a suggestion of the anti-cancer effects of the aqueous extracts of *Azadirachta indica*.

Gluatathione-S-Transferase (GST) Assay

Gluatathione S-Transferase (GST) specific activities in serum and liver samples were summarized in Table 1. The animals in DEN-AAF control group (C) showed the highest level of GST in all samples compared to the different group, NAI, N and CAI ($p \leq 0.05$). The supplementation of 5% NEEM to cancer group (CAI) decreased significantly the activity of GST ($p \leq 0.05$) compared to cancer group (C). There was no significant difference between normal control group (N) and normal control fed with NEEM (NAI) ($p \geq 0.05$) (Table 1). It is noticed that the treatment with 5% NEEM in animals with experimental liver a cancer using DEN-AAF model reduced the specific activity of GST in liver cytosol and serum and this will be an indication to the potential of this plant to be used as anti-cancer agent.

DISCUSSION

Tumor markers are defined as molecular products metabolized and secreted by neoplastic tissue and characterized biochemically in cells or body fluids. They are indicators of tumor stage and grade as well as useful for monitoring responses to treatment and predicting recurrence. Many chemical groups are represented including hormones, antigens, amino and nucleic acids, enzymes, polyamines and specific cell membrane proteins and lipids. Tumors markers are helpful in diagnosis but most are elevated in a broad spectrum of malignancies and some are highly tissue specific (Hall *et al*., 1998). In the study there are 2 enzyme markers evaluated i.e., cytosolic and serum GST and GPx. Form this study, results from the enzyme markers showed the significant chemopreventive effect ($p \leq 0.05$) of *A. indica* on rat hepatocarcinogenesis. As well as AFP in the serum was seen to be higher ($p \leq 0.05$) in group C compared to CAI, N and NAI.

The results obtained in the present study showed highest GST and GPx values in cancer control livers and serum. None of the other groups showed any significant difference ($p \geq 0.05$) among them. The increase in these enzymes level is due to the DEN/AAF treatment is expected since its levels have been shown to increase in the presence of foreign compounds including carcinogens (Ahluwalia and Farber, 1984).

Glutathione peroxidase is an important part of the antioxidant defense system. Today five isoforms are known; therefore it is called more like an enzyme family than a single enzyme. They are present in almost every cell of animals, but the tissue distribution of the isoforms shows high variation (Yu *et al*., 2006). There are several factors affecting the activity of the enzyme. Some of these are internal, individual factors, resulting in significant variation in the enzyme activity of different organs, age groups and sex. Endocrine regulation can also control enzyme activity. However, environmental factors have also definite effect on enzyme action. Nutrition is one of the most essential factors as fat content and fatty acid composition of feed, or trace element intake as well as vitamin status of the animal play crucial role in normal enzyme activity. A seasonal change has also some effect on GPx activity and such changes have been reported in the literature (Erdélyi *et al*., 1999). The present work, clearly showed that GPx activity of CAI group was decreased significantly ($p \leq 0.05$) compared with cancer group (C). Therefore, this study indicated that *A. indica* had effect in reducing liver cancer severity and protecting the structure of hepatic tissue. These results also indicated that *A. indica* did not cause any side effects to the normal cells due to no significant difference was observed between normal cells and normal + *A. indica*. Supporting to the obtained results of this study in antioxidant enzymes, a work done by Pongtip *et al*. (2005), on the antioxidant activity of this plant, NEEM, revealed that all the plant parts when assessed for antioxidant activity *in vitro* using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging assay, total antioxidant activity and inhibition of lipid peroxidation in Chago K1 cancer cell culture by the thiobarbituric acid reactive substances (TBARS) method. Their results showed that leaf aqueous extract, flower and stem bark extracts exhibited higher free radical scavenging effect on the DPPH assay.

The Glutathione S-transferase (GST) is a group of dimeric isoenzymes which have been divided, on the basis of isoelectric points, into three groups: basic, near neutral and acidic, respectively, known as α, μ and π GST. They have a wide distribution within the body and many different functions. In general they conjugate with hydrophobic molecules to allow further metabolism including the detoxification of foreign and endogenous toxins and intracellular transport (Habig and Jakoby, 1981).

In the liver, GST is the most important and most abundant of all the enzymes. It was suggested that GST functions in a detoxification capacity by catalyzing conjugation reaction between GSH and a variety of potentially toxic electrophiles (Chasseaud, 1979).

The results obtained in the present study showed liver cytosolic GST activity increased significantly ($p \leq 0.05$) in the hepatocarcinogens induced group (C) compared with the normal control

group (N). The oral administration of DEN followed by promotion of AAF even without the stimulating factor, partial hepatectomy still can elevate GST activity and this clearly indicated that cancer cells overcome the growth of normal cells.

Usually, an increase of GST activity by a substance actually enhances the protection mechanism against the noxious effects of xenobiotics (Lam *et al*., 1994). This suggests that neoplastic cells of those rats are protected by producing higher amount of GST enzymes in the cells. Whereas, cancer cells which where already protected and suppressed the supplementation of anti-cancer agents such as NEEM don't have to produce high amount of GST to increase cells protection against the xenobiotics. In the present work, clearly showed GST activities of cancer + NEEM (CAI) group were decreased significantly ($p \leq 0.05$) compared with cancer group (C). Therefore, this study indicated that *A. indica* had effect in reducing liver cancer severity. These results also indicated that *A. indica* did not cause any side effects to the normal cells due to no significant difference was observed between normal cells and normal + *A. indica*.

The use of AFP as biomarker is intensively used in the detection of adult liver cancer in human and animals, (Lysandro *et al*. 2005; Masi *et al*., 2005). Tartarinov (1964) found AFP in the serum of the patient with primary liver cancer. Thirty years later, (Marrs, 1996) stated that high level of plasma AFP is believed to be strongly suggestive of HCC. The presence of AFP in serum rapidly returns to normal after complete resection of hepatocellular carcinoma indicating that malignant hepatocytes are responsible for the production of AFP. According to the results obtained from this study, the level of the AFP in cancer groups is higher than non-cancer groups. The level of AFP concentration in cancer control group was the highest and was significantly different ($p \leq 0.05$) compared with the group fed with NEEM. Among cancerous groups, only cancer control group was significantly different ($p \leq 0.05$) compared with normal control group. This indicates that the rats in cancer control group might be completely suffered from hepatocarcinogenesis but the histological examination was still needed to confirm it. There were no significant changes between normal control (N) and normal treated with NEEM group (NAI).

CONCLUSIONS

The 5% aqueous extract of NEEM (*Azadarichta indica*) decreased the level of alpha-fetoprotein in animals treated with DEN and AAF comparing to cancer animals ($p \leq 0.05$). The increased activities of antioxidant enzymes, such as glutathione S-transferase (GST) and glutathione peroxidase (GPx) shows that GST and GPx play a major role in repairing the damage caused by carcinogenesis. The results of the present study opened a new window for more investigations in the future to reveal the multiple mechanism of action possessed by *Azadarichta indica* as chemopreventive agent.

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