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In vivo Anti-tumor Effects of *Azadirachta indica* in Rat Liver Cancer

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Abstract: The aim of the current study is to determine the effects of *A. indica* aqueous extract on Diethyl Nitrosamine (DEN) and 2-Acetylaminofluorene (AAF) induced-hepatocarcinogenesis on Spraque-Dawley rats. The plant, *A. indica*, extract was prepared into 5% w/v in distilled water. Spraque-Dawley male rats were divided into 3 groups of 7 rats each. The groups were: DEN/AAF-induced rats (C), DEN/AAF-induced rats treated with 5% *A. indica* (CAI) and normal control group (N). *In situ* detection of DNA fragmentation, TUNEL assay, was used to investigate the apoptogenic properties of *A. indica*. RT-PCR was used to amplify AFP mRNA. TUNEL assay supported that there was more numbers of apoptotic cells in the liver of (CAI) group compared with (C) group. AFP gene was suppressed by the supplementation of *A. indica* to DEN/AAF rats (CAI). *A. indica* (Neem) has revealed a chemopreventive capability by regressing the hepatocarcinogenesis induced by DEN/AAF carcinogens. This capability can be seen from the modulating effects of the plant in the biological indicators used in this study.

Key words: *Azadirachta indica*, apoptosis, AFP gene, DEN/AAF, rats

INTRODUCTION

Nowadays, cancer is widely recognized as one of the most formidable human afflictions. It exists in >100 forms and has many causes, from genetic factors to infections. Probably, more than any other single disease, cancer provokes fearful images of pain, disfigurement and inevitable death (Nishma *et al*., 2006). Hepatocellular Carcinoma (HCC) is one of the most common malignancies in the world. Due to the global pandemic of hepatitis B and C viral infections, the incidence of HCC is rapidly increasing in Asian and Western countries (Keiichi *et al*., 1995; Seeff and Hoofnagle, 2006) and this trend is expected to continue for the next 50 years because of the long latency between infection and the development of HCC. The prognosis of advanced HCC remains poor and novel treatment and diagnosis strategies are urgently needed (Bruix *et al*., 2006). Despite the existence of recently used liver cancer treatment, which include percutaneous ethanol injection, transarterial chemoembolisation, radiofrequency thermal ablation, liver resection and liver transplantation (Majno *et al*., 2005). This can be countered by graft (liver organs) shortage and the considerable time spent by patients with HCC in the waiting list, facing the risks of not being transplanted because of contraindications developed, while waiting (dropouts) (Majno *et al*., 2005). Neem (*A. indica* Juss), abundantly prevalent in tropical countries of the world and has been reported to be anti-inflammatory, anti-pyretic and hypoglycaemic and also exhibits antimicrobial and anti-cancerous properties (Parida *et al*., 2002). Practically, every part of *A. indica* (leaves, bark, fruit, flowers, oil and gum) have been reported to be associated with various remedial properties such as, antimicrobial effects, storage behavior (Parida *et al*., 2002), reduction of paracetamol-induced liver damage, enhancer of hepatic glutathione and glutathione-dependent enzymes, *in vitro* antiviral activity and antibacterial agent. Numerous scientific reports validate the traditional uses of Neem in both the maintenance of general health and skin care used by Indian people. However, its effect towards some other diseases or some other cancers has never been studied, thereby, suggesting this study as complementary research for its anticancer effects towards

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neoplastic hepatocytes *in vivo*. This research was also proposed, due to the complicatedness linked with current liver cancer management (Sala *et al*., 2004; Majno *et al*., 2005), the promising usage of plants as the resource for new anticancer compounds (Steinmetz and Potter, 1991; Gordon and David, 2005) and the rich literature of *A. indica* (Sai *et al*., 2000; Parida *et al*., 2002).

MATERIALS AND METHODS

Preparation of 5% Neem leaves aqueous extract: The leaves of *A. indica* were collected from the Herbs Garden of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Aqueous extract of Neem was prepared from the modification method of green tea extraction according to Conney *et al*. (1997). Briefly, 25 g of fresh leaves was grinded and steeped in 250 mL (60°C) and later cooled at room temperature to be filtered. Filtrate 1 was kept and the grounded plants were grinded again, steeped in 250 mL (60°C) and filtered. The Two filtrates were mixed to obtain 5% (w v^{-1}) of leaves extract.

Experimental design: Twenty male rats (Spraque-Dawley), 150-250 g, were acclimatized under control condition in the laboratory for one week before use. The body weight was taken and blood was withdrawn from the animals and serum was immediately prepared and kept at -20°C. The rats were divided into 3 groups (C, CAI and N) and each group contained 7 rats. Rats in group C and CAI were induced with cancer by intraperitoneal injection of 200 mg kg^{-1} Diethyl Nitrosamine (DEN) dissolved in corn oil and then followed by a recovery of 2 weeks on food, which was mixed with 2-Acetylaminofluorene (0.02% AAF) as promoter of hepatocarcinogenesis. Then the rats were left for 2 weeks. The rats in group N were not induced with cancer but injected once intraperitoneally with corn oil and act as normal control. The Neem leaves extract 5% was given as a substitute for water to rats in group CAI and N. But rats in group C did not receive any treatment.

Post treatment: At the termination of experiment, the rats were weighed and complete autopsies were performed after the rats had been sacrificed by decapitation under Ether anesthesia. Blood was collected by heart puncture and the serum placed into plain tubes. The samples were centrifuged at 3800 rpm with bench centrifuge for 10 min and the serum was stored at -20°C until the assay was done. Liver were removed and washed in ice-cold 1.15% KCl solution immediately. These samples were processed for TUNEL assay and viewed under confocal microscopy.

Detection of apoptotic cells (TUNEL assay): The DeadEnd flourmetric TUNEL system measures the fragmented DNA of apoptotic cells by catalytically incorporating flourescein 12-dUTP at 3'-OH DNA end using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). The liver tissues of rats in each group were sliced from the largest lobe and they were fixed in 4% paraformaldehyde solution, embedded in paraffin blocks, processed and sectioned. DeadEnd™ Fluorometric TUNEL System was conducted according to the manufacturer's (Promega) instruction. TUNEL-stained slides were immediately analyzed under the fluorescent microscope. All the slides were examined and a systematic scoring method was done at ×200 magnification and 5 spots randomly selected. On each spot, green stained cells were counted as a TUNEL-positive apoptosis cells.

Reverse transcriptase polymerase chain reaction RT-PCR: RNA isolation process was carried out following the manufacturer's instructions (Qiagen, Germany). RT-PCR was carried out using Promega Access Quick™ RT-PCR System with master mix system for setting up one-tube RT-PCR reactions. The following components were pipetted in sterile, nuclease-free tubes: Access Quick Master Mix, AFp primer (Sense-5'-AAC ACA TCC AGG AGA GCC AG-3', Anti-sense-5'-TTC TCC AAG AGG CCA GAG AA-3'), RNA Template and Nuclease free water. Access Quick™ Master Mix was thoroughly mixed before removing aliquots. About 1 μL of AMV Reverse Transcriptase was added as the final component and mix by gentle pipetting. Individual pipettes tips were used for all additions, to avoid cross contamination of the samples. The reaction tubes were incubated at 45°C for 45 min for reverse transcription process and the synthesis of cDNA. RT-PCR product was documented in 1.2% gel electrophoresis.

Statistical analysis: Data are expressed as mean±S.D. By using SPSS version 16.0 (Chicago, USA), ANOVA test was utilized to analyze the difference between different groups and multi-comparison test (Duncan's Test) was used as post hoc test. $p<0.05$ were considered as an indicator for the significant difference between study groups.

RESULTS

The detection of apoptosis using TUNEL system: TUNEL stained liver tissues of DEN/AAF induced rats (C), showed that the fluorescent green stained cells were observed to be fewer in numbers and the orange color showed that the cells were still viable. The TUNEL

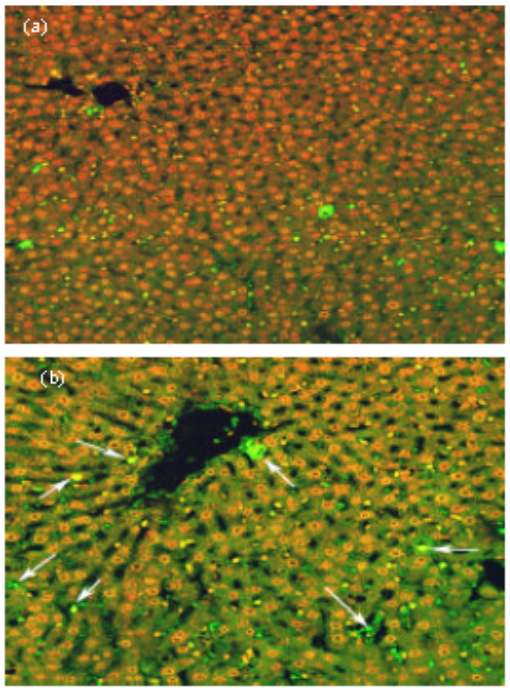

Fig. 1: Confocal micrograph of TUNEL assay for liver sections from DEN/AAF induced hepatocarcinogenesis rats (C). Mixture of PI and FITC labeled cells. The fluorescent green stain absorbed by final stages of apoptotic cell. Red to orange stained cells nuclei showed the viable cells. (A 20X), (B 60 X)

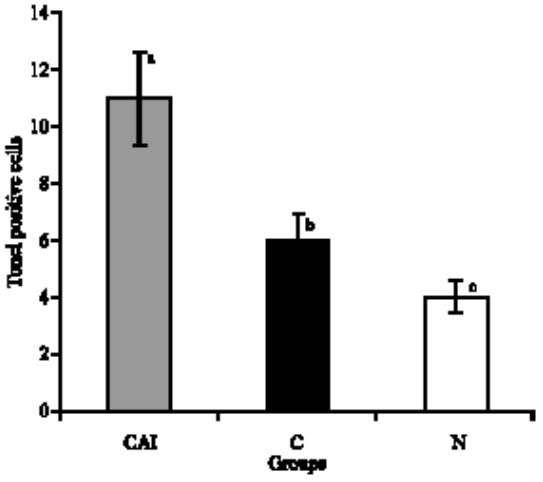

Fig. 2: Graph showing the effect of 5% *A. indica* aqueous extract on DEN/AAF induced cancer in rats. TUNEL positive cells were counted in liver sections and data are expressed as mean±S.D. Apoptosis is significantly induced by *A. indica* treatment in cancerous animals' group (CAI) comparing with others groups ($p<0.05$)

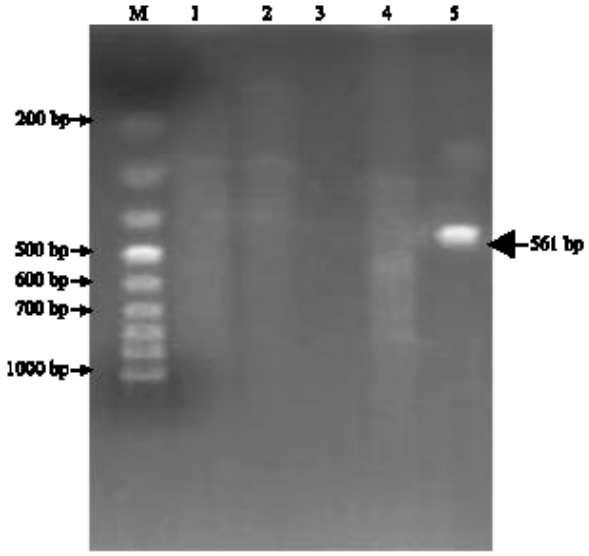

Fig. 3: Effect of *A. indica* aqueous extract on the expression of AFP gene of hepatocarcinogenized rats with DEN/AAF model. PCR products were analyzed on a 2% agarose gel. Lane 5, represents the quality of PCR preparation from cancer control group versus different groups (Lane 1-4), M, DNA Ladder 100 bp, 1, 2 and 3): Normal Control (N) group, 4): Cancer treated with *A. indica* (CAI) group, 5): Cancer control (C) group (positive control)

staining also showed that apoptotic cells with intensively green fluorescence of liver sections obtained from DEN/AAF induced rats treated with 5% aqueous extract of *A. indica* leaves (CAI) (Fig. 1). The common features are condensation of chromatin, fragmentation of DNA and apoptotic bodies. Scoring of TUNEL positive cells (Apoptotic cells) were performed and the results has shown in Fig. 2, which demonstrates the difference was statistically significant ($p<0.05$) between CAI, C and N.

Expression of AFP gene (RT-PCR): Figure 3 clearly demonstrate AFP gene expression in DEN/AAF-induced rats group (C). The oral supplementation of 5% aqueous extract of *A. indica* leaves to cancerous rats clearly inhibited the expression of AFP gene and that was proven by the absence of the expected size band in electrophoresis (CAI). In normal control, there was absence of band in agarose gel and that was expected for healthy rats.

DISCUSSION

Neem (*A. indica* Juss), abundantly prevalent in the tropical countries of the world and has been reported to

be anti-inflammatory, anti-pyretic and hypoglycaemic (Parida *et al*., 2002) and also exhibits antimicrobial and anti-cancerous properties (Sai *et al*., 2000). However, its effect towards some other diseases or some other cancers has never been studied, thereby, suggesting this study as complementary research for its anticancer effects towards neoplastic hepatocytes *in vivo*, could provide some contribution to cancer research. This study on the anticancer effect of *A. indica* during the hepatocarcinogenesis was based on the popular hepatocarcinogenesis model (Solt and Farber, 1976; Motalleb *et al*., 2008).

Apoptosis is the most common and well-defined form of programmed cell death. It is a physiological cell suicide that is essential for the maintenance of homeostasis in embryonic, fetal and adult tissues. Both induction and inhibition of apoptosis can be related to either physiological stimulus, exposure to stress-inducing agents, or pathological conditions. Among pharmacological agents, many cancer chemotherapeutic drugs are known to activate apoptotic mechanisms of tumor cell death, suggesting this may be a mechanism for current cancer treatment (Kerr *et al*., 1994). Moreover, apoptosis has been proposed as a novel target for cancer chemoprevention whose rationale is to remove cells undergoing neoplastic transformation, in situations where other defense mechanisms fail to block the carcinogenesis process upstream (Kun-Young *et al*., 2003). However, TUNEL assay was used intensively in the detection of chemopreventive induced apoptosis in hepatocellular carcinoma in animal models and patients (Luzzi *et al*., 1998; Ding *et al*., 2006; Guha *et al*., 2006; Chowdhury *et al*., 2006).

Detection of DNA fragments *in situ* using the terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick and labeling (TUNEL) assay is increasingly applied to investigate apoptosis and this procedure rely on labeling of breaks in the DNA strands that have been observed to occur in apoptosis (Kerr *et al*., 1994). Apoptosis occurs at low frequency as part of normal cell turnover but is dramatically increased during involution of liver hyperplasia. In pre-stages of liver cancer, apoptosis largely counterbalances cell proliferation (Grasl-Kraupp *et al*., 1994). Quantitative determination of apoptosis in histological specimens revealed that the rat apoptosis tends to increase from normal to (pre) neoplastic to malignant cells (Schulte-Hermann *et al*., 1995). In this study the difference in TUNEL-positive cells were significantly different ($p<0.05$) between cancer control and cancer group treated with Neem (CAI). There was insignificant difference ($p>0.05$) between normal control, (N). From the present result the normal cells were not affected by the plant treatment, however, preneoplastic cells are more susceptible than normal liver cells to stimulation of either cell replications or cell death and in the steady state condition of the organism; cell division must be counterbalanced by cell death (Raff, 1992). There is evidence that hepatocelullar is essential in all three stages of experimental hepatocarcinogenesis from initial genotoxic insult (initiation), through the clonal expansion from the premalignant to malignant lesion (promotion) and finally progression of tumor growth by further clonal expansion (Grasl-Kraupp *et al*., 1994).

In the absence of reliable liver protective drugs in allopathic medical practices, herbs play a role in the management of various liver disorders. A number of plants have shown hepatoprotective properties (Suresh and Mishra, 2008). There is growing interest in identifying new chemopreventive agent from dietary sources (Hae-Jeung *et al*., 2005). In harmony with the previous study by Hae-Jung *et al*. (2005) the present study, showed that the supplementation of Neem has a potential anticancer effect against DEN/AAF induced liver cancer supported by TUNEL-positive apoptotic cells count in liver sections.

AFP expression is induced in regenerating liver and liver tumors. DEN is a genotoxic carcinogen that has been shown to induce liver tumors and liver damage (Jin *et al*., 2005). In this study, AFP gene expression in DEN/AAF control group might be due to DEN/AAF intoxication that caused necrosis of the hepatocytes. Hepatocyte localization within the liver plate or outside it is the defining factor that regulates the activity of AFP synthesis on a cellular level (Lazarevich, 2000), however, the expression of AFP gene was not observed in CAI rats' group. Regulation of the AFP gene expression has been shown by a number of investigators to be extremely complex. Its expression is limited to a small number of tissues during a specific time in development (Jin *et al*., 2005). It has been generally accepted that the liver contains cells with stem-like properties and that these cells can be activated to proliferate and differentiate into mature hepatocytes and cholangiocytes under certain pathophysiologic circumstances (Farber, 1956; Sell, 1994; 2001).

Neem leaf aqueous extract efficiently suppresses oral squamous cell carcinoma provoked by 7, 12-dimethylbenz [a] anthracene, as revealed by reduced incidence of neoplasm. Neem may exert its chemopreventive effect in the oral mucosa by modulation of glutathione and its metabolizing enzymes. That Neem leaf extract exerts its protecting activity in Nmethyl-N¢-nitro-N-nitrosoguanidine (MNNG) (a carcinogenic material)-induced

oxidative stress has also been demonstrated by the reduced formation of lipid peroxides and enhanced level of antioxidants and detoxifying enzymes in the stomach, a primary target organ for MNNG as well as in the liver and in circulation (Kausik *et al*., 2002).

CONCLUSION

It could be said that *A. indica* has revealed a hepato-protective effects on DEN/AAF induced liver carcinogenesis. These results are encouraging for the adoption of this plant in further detailed preclinical studies.

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