UNIVERSITI PUTRA MALAYSIA

EFFECT OF AZADIRACWTA INDICA EXTRACT ONHEPATOCARCINOGENESIS-INDUCED RATSMANAL MOHAMED

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EFFECT OF *AZADIRACHTA INDICA* EXTRACT ON HEPATOCARCINOGENESIS-INDUCED RATS

By

MANAL MOHAMED ELHASSAN TAHA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science.

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DEDICATION

Specially dedicated to,

My beloved parents, husband, sisters Manahil and Sarah, brother Ahmed, daughter Roa, supervisors

For their invaluable support, love, patience and intellectual stimulation.....

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

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Chairman: **Associate Professor Fauziah Othman, PhD**

Faculty: **Medicine and Health Sciences**

The effects of 5% *A. indica* aqueous extract (AI), or more commonly as Neem, on hepatocarcinogenesis induced *Spraque-Dawley* male rats were investigated. Hepatocarcinogenesis was induced in rats by employing a two carcinogen system: an intraperitoneal injection of 200 mg/kg diethyl nitrosamine (DEN) as initiator; followed by 0.02% of 2-acetylaminofluorene (AAF) in rat chow for two weeks to promote carcinogenesis. The rats were then left for two weeks to allow hepatic preneoplastic lesions to occur. The plant extract was prepared in 5% w/v in distilled water. Fresh leaves were collected, blended and mixed with distilled water. Twenty male rats *Spraque-Dawley* weighing 150-250g, were acclimatized for 1 week before use. The rats were divided into four groups of five rats each.

The groups were: DEN/AAF-iduced rats (C), DEN/AAF-iduced rats treated with 5% *A. indica* (CAI), normal control rats (N) and normal rats treated with 5% *A. indica* extract group (NAI). The rats in group N and NAI were not induced with cancer however were injected once intraperitoneally with corn oil and act as normal control. The plant extract was fed to CAI and NAI groups to study its effects on both cancer and normal groups, respectively.

In this study several parameters were evaluated as means of determining the effects of AI on DEN/AAF-induced hepatocarcinogenesis in rats. Body and liver weight profiles, foremost, hepatic lesions were scored in rats induced with DEN/AAF cacinogens especially in the portal and lobular regions of the liver sections examined for histology. Loss of normal cell organization was also observed once the hepatocarcinogenesis was induced. In addition to histological observations, the TUNEL Assay, liver antioxidant enzyme Glutathione S-transferase (GS-T), Glutathione Peroxidase (GPx) in the serum and liver, tumor marker alpha-fetoprotein (AFP) in serum and molecular detection of AFP and albumin genes expressions were conducted. The observation of the lesion scoring have shown significant difference ($p<0.05$) between DEN/AAF and normal control groups (N, NAI). Histologically there were significant changes in the lesion scoring of the liver in portal and lobular region in DEN/AAF induced

group (C) compared to the DEN/AAF treated with *A.indica* (CAI). TUNEL assay supported that there was more numbers of apoptotic cells in the liver of (CAI) group compared to(C) group. The liver enzymes (GST & GPx) activity was measured and the result for both glutathione S-transferase and glutathione peroxidase were significantly ($p<0.05$) higher in the (C) compared to the other groups (CAI, N, NAI). This result revealed that *A. indica* extract could reduce the activity of liver and serum GPx and GST enzymes of rats during hepatocarcinogenesis. However, the results of body and liver weight profiles showed that the CAI group was not significantly different ($p>0.05$) from N, C and NAI groups.

Alpha fetoprotein (AFP), a notable liver tumor marker, level was measured. The DEN/AAF induced group (C) showed the highest increase in AFP levels while in CAI group illustrated significant ($p<0.05$) decrease in AFP level. There was no significant ($p>0.05$) difference between N, NAI and CAI group.

Molecular detection of gene expression was done by RT-PCR for α-fetoprotein and albumin specific genes. However, the expression of the AFP gene was observed only in DEN/AAF induced group (C). Albumin gene expression was observed in all the study groups C, N, NAI and CAI proving the hepatic nature

of the studied tissue and used as a housekeeping control gene in the RT-PCR experiments.

As a conclusion, *A. indica* (Neem) has revealed a chemopreventive capability by regressing the hepatacarcinogenesis 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 which can encourage the researchers to consider the *A. indica* (Neem) for further on mechanism and toxicology study.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

KESAN EKSTRAK *AZADIRACHTA INDICA* TERHADAP TIKUS ARUHAN HEPATOKARSINOGENESIS

Oleh

MANAL MOHAMED ELHASSAN TAHA

Februari 2007

Pengerusi: **Profesor Madya Fauziah Othman, PhD**

Fakulti: **Perubatan dan Sains Kesihatan**

Kesan 5% larutan ekstrak akuas *A. indica* (AI) atau lebih di kenali sebagai Neem pda hepatokarsinogenesis terhadap tikus jantan *Sprague-Dawley* telah dikaji. Hepatokarsinogenesis telah diaruh pada tikus menggunakan dua sistem karcinogen, melalui suntikan 200 mg/kg diethylnitrosamine (DEN) secara intraperitoneal sebagai pencetus hepatocarcinogenesis dan diikuti dengan memberikan makanan yang dicampurkan dengan 2-acetylaminofluorene (0.02% AAF) sebagai 'promoter' hepatokarsinogenesis selama 2 minggu. Ekstrak tumbuhan disediakan di dalam air suling pada kepekatan 5% w/v. Daun tumbuhan tersebut dikumpul, dikisar dan dicampurkan bersama air. 20 tikus jantan Spraque Dawley, 150g–250g diaklimatisasikan selama satu minggu sebelum diujikaji. Tikus tersebut dibahagikan kepada empat kumpulan dengan

lima ekor tifus setiap kumpulan. Kumpulan tersbut ahila: tikus yang diaruh dengan DEN/AAF (C), tikus yang diaruh dengan DEN/AAF dirawat dengan 5% *A. indica* (AI), tikus kewalan normal (N) dan tikus normal yang dirawat dengan 5% ekstark *A. indica* (NAI). Tikus kumpulan N dan NAI tidak diaruhkan dengan kanser tetapi disuntik secara intraperitonial dengan minyak jagung dan dijadikan sebagai kawalan. Ekstrak tumbuhan tersebut diberikan kepada kumpulan CAI dan NAI untuk dikaji kesannya terhadap kumpulan kanser dan normal.

Dalam kajian ini, beberapa parameter telah ditentukan sebagai purata untuk menentukan kesan bagi AI terhadap DEN/AAF yang memyebabkan hepatokarsinogenesis pada tikus. Profil berat dan hati, selain itu, kesan hati telah dikira dalam tifus disuntik dengan bahan karsinogen DEN/AAF terutama dalamm kawasan portal dan lobular hati telah dikaji dalam pemeriksaan histologi. Kehilangan organisasi pada sel normal juga telah ditihat apabila karsinogenesis diaruh. Pemeriksaan histologi juga telah dijalankan menggunakan asai TUNEL, enzim antioksida hati Glutathione S-transferase (GST), Glutathione peroxidase (GPx) di dalam serum dan hati, penanda tumor alpha-fetoprotein (AFP) di dalam serum dan pengesananan molekular AFP dan ekspresi gen albumin juga telah dijalankan. Pemerhatian terhadap ujian skor

kesan menunjukkan perbezaan yang signifikan ($p \leq 0.05$) di antara DEN/AAF dan kumpulan kawalan normal (N, NAI). Pemeriksaan histologi menunjukkan perubahan pada ujian skor kesan hati dalam kawasan portal pada kumpulan aruhan DEN/AAF (C) berbanding dengan DEN/AAF yang dirawat dengan *A. indica* (CAI). Asai TUNEL mengukuhkan bahawa terdapat beberapa sel apoptotic di dalam hati kumpulan CAI berbanding kumpulan C. Aktiviti enzim hati (GST & GPx) diukur dan keputusan bagi kedua glutathione S-transferase and glutathione peroxidase adalah berbeza secara signifikan pada aras keertian ($p<0.05$) di antara kumpulan kanser (C) dan kumpulan rawatan (CAI, N, NAI). Keputusan ini menunjukkan A.indica mampu mengurangkan aktiviti enzim hati dan serum GST dan GPx pada tikus semasa hepatokarsinogenesis. Walau bagaimanapun keputusan profil berat jisim tubuh dan hati menunjukkan kumpulan CAI tidak memberikan perbezaan yang signifikan pada aras keertian ($p > 0.05$) daripada kumpulan N, C dan NAI.

Aras alpha fetoprotein (AFP) telah diukur sebagai penanda pertumbuhan sel kanser hati. Kumpulan DEN/AAF (C) menunjukkan peningkatan aras AFP yang tertinggi, manakala kumpulan CAI menunjukkan penurunan yang signifikan pada aras keertian ($p < 0.05$) pada aras AFP. Namun tiada perbezaan yang signifikan ($p > 0.05$) di antara kumpulan N, NAI dan CAI.

Pengesahan secara molecular ke atas ekspresi gen telah dilakukan menggunakan RT-PCR ke atas gen spesifik α–fetoprotein dan albumin. Tetapi , ekspresi gen AFP hanya dapat dilihat dalam kumpulan yang diaruh dengan DEN/AAF (C). Ekspresi gen albumin diperhatikan dalam kumpulan kajian C, N, NAI dan CAI yang membuktikan sifat semulajadi hepatic pada organ yang dikaji dan pembersih gene kawalan dalam ujikaji RT-PCR.

Kesimpulannya, *A. indica* (Neem) telah dinyatakan sebagai kemopreventif untuk penyakit hepatokarsinogenesis yang diaruhkan oleh bahan karsinogen DEN/AAF. Keupayaan ini boleh dilihat melalui kesan tumbuhan dalam penunujuk biologi yang digunakan dalam kajian ini dimana akan menggalakkan penyelidik *A. indica* (Neem) untuk mempertimbangkan mekanisma dan toksikologi yang akan datang.

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LIST OF ABBREVIATIONS

AFP	Alpha-fetoprotein
A.indica	*A. indica*
Abs	Absorbance
°C	Centigrade
cDNA	Complementary DNA
cm	Centimeter
CDNB	1-chloro-2,4-dinitrobenzene
DEN	Diethylnitrosamine
DNA	Deoxyribonuecleic acid
EDTA	Disodium Ethylene Diaminetetracetate
FITC	Fluorescein isothiocynate
g	Gram
GSH	Glutathione
GST	Glutathione S-transferase
GPx	Glutathione Peroxidase
GSSG reductase	Glutathione reductase
H_2O_2	Hydrogen peroxide
$_4H_3PO$	Phosphoric acid

H&E	Hematoxylin and eosin
HCL	Hydrochloric acid
KH_2PO_4	Potassium dihydrogen orthophosphate
$K H_2PO_4$	Potassium dihydrogen orthophosphate
Kg	Kilogram
KCL	Botassium chloride
Ml	Mililitre
Mn	Minute
µl	Microlitre
mg	Miligram
NaOH	Sodium hydroxide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMDA	N-dimethylnitrosamine
Neem	*A. indica*
NaN_3	Sodium nitrate
PBS	Phosphate buffer saline
Bp	Basepair
pH	Hydrogen ion concentration
PI	Propidium iodide
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

RNA	Ribonucleic acid
RLUs	Relative light units
S.D	Standard deviation
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotydil transferase-mediated dUTP nick end-labeling
TBE	Tris base EDTA
UPM	Universiti Putra Malaysia
UV	Ultraviolet