



UNIVERSITI PUTRA MALAYSIA

***In Vitro* CYTOTOXICITY PROPERTIES OF *Gnetum gnemon* L.
ON SELECTED CANCER CELL LINES**

HADIZA ABUBAKAR ANKA

FPSK(M) 2015 80



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By

HADIZA ABUBAKAR ANKA

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

September 2014

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DEDICATIONS

Mum

Dad

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

***In Vitro* CYTOTOXICITY PROPERTIES OF *Gnetum gnemon* L.
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September 2014

Chairman : Loh Su Peng, PhD
Faculty : Medicine and Health Sciences

Most people dreaded the side effects of conventional cancer treatment and have resorted to alternative treatments such as herbal therapy which is often have less adverse side effects and less expensive. This study was conducted to evaluate the potential anticancer properties of *Gnetum gnemon* leave extracts on liver cancer (HepG2), colon adenocarcinoma (HT-29), hormone-dependent breast cancer (MCF-7) cell lines and normal mouse embryo fibroblast (3T3).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Acridine Orange/Propodium Iodide (AO/PI), Fourier Transform Infrared (FT-IR) and Gas chromatography-Mass Spectroscopy was analysed on both young and mature leave extracts of ethanol and hexane (YE, ME, YH and MH). All extracts inhibited proliferation of HepG₂ in a concentration and time-dependent manner with high IC₅₀ values 240.53, 138.53, 249.83 and 257.21 g/mL respectively. The outcome of the model used (fixed effect test) on the comparing parameters (maturity, solvent and time) on HepG₂ cell lines, the young leaves showed a high significant difference ($p < 0.05$) when compared to matured leaves. There was no significant difference between the two extraction solvents. On the other hand, time showed highly significant effect ($p < 0.001$).

All extracts was found to induce apoptosis at the IC₅₀ concentrations in HepG₂ due to presence of clear space, decrement in cell number, floating cells and decrease in attached cell number after 72 hours. The AO/PI double staining of both treated and untreated HepG₂ cells with IC₅₀ concentrations of YE and ME confirmed some apoptotic features such as membrane blebbing, cell size decreases and several necrotic cells after 72 hours. The FT-IR analysis of YE and ME showed several intense, sharp absorption peaks due to the different functional groups present (primary amines, secondary amines, alcohol, phenol, carboxylic acid, aromatics, esters, lactone, aldehyde and ketones). And the

GC-MS analysis revealed important bioactive components such as squalene, α -tocopherol, α and β -sitosterol and Inositol in YE and phytol, inositol, β and γ -sitosterol, α -tocopherol and phenol,2,4-bis(1,1-dimethyl) in ME.

In summary, the morphological studies and AO/PI of treated HepG2 cells with both YE and ME for up to 72 hours incubation period showed some features of apoptosis. Conducting cytotoxicity assay with different extraction solvents is recommended.



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

CIRI-CIRI SITOTOKSISITI *Gnetum gnemon* L. *In Vitro* KE ATAS TITISAN SEL KANSER YANG TERPILIH

Oleh

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Pengetahuan mengenai antioksidan daripada sumber tumbuh-tumbuhan telah meningkat dengan begitu drastik kerana ia terbukti dapat mencegah penyakit kanser serta kardiovaskular. Pelbagai rawatan secara konvensional untuk merawat kanser seperti melalui pembedahan, kemoterapi dan radioterapi walaubagaimanapun tidak memberangsangkan disebabkan pesakit harus menjalani beberapa kesan sampingan yang sering memburukkan lagi keadaan kesihatan pesakit. Ramai yang telah bertukar kepada rawatan alternatif yang mempunyai kurang kesan sampingan dan juga murah seperti rawatan yang berasaskan herba. *Gnetum gnemon* L. biasanya dikenali sebagai melinjau adalah spesies daripada gymnosperma dari keluarga Gnetaceae berasal dari Asia Tenggara. Daunnya biasa digunakan secara meluas dalam masakan Melayu-Indonesia dan getah daun tersebut juga telah digunakan untuk menyembuhkan komplikasi pada mata dalam perubatan tradisional. Kajian terdahulu menunjukkan bahawa kedua-dua daun matang dan muda mengandungi komponen antioksidan dan aktiviti mencegah kerosakan komponen DNA yang tinggi. Kajian ini dijalankan untuk menentukan potensi ciri-ciri anti-kanser bagi ekstrak daun *G. gnemon* ke atas titisan sel kanser hati (HepG₂), titisan adenokarsinoma sel kanser usus (HT-29), titisan sel kanser payudara yang bergantung hormon (MCF-7) dan normal fibroblast embrio tikus (3T3). Ujian MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) telah digunakan untuk mengkaji kesan sitotoksik bagi daun muda dan matang *G. gnemon* di dalam ekstrak etanol dan heksan ke atas titisan sel kanser 3T3, HepG₂, HT-29 dan MCF-7 pada julat kepekatan (15.625-1000 µg/mL) dan tempoh penderaman selama 24, 48 dan 72 jam. Analisis pendarfluor nukleoprotein (Acridine Orange / Propodium Iodida, AO / PI) berganda telah dijalankan untuk mengukur dan menetapkan kekerapan apoptosis. Kumpulan berfungsi serta komponen bioaktif daripada kedua-dua ekstrak etanol daun *G. gnemon* yang muda dan matang (YE dan ME) ditentukan melalui analisis Fourier Transform Infrared (FT-IR) dan Gas

kromatografi-Mass Spektroskopi. Peningkatan dos / kepekatan kedua-dua ekstrak etanol dan heksan bagi daun *G. gnemon* yang muda dan matang tidak menunjukkan kesan sitotoksik ke atas sel kanser HT-29 dan MCF-7 seperti yang ditunjukkan oleh nilai IC₅₀ apabila titisan sel kanser dirawat dan dieram selama 72 jam. Walaubagaimanapun, daun *G. gnemon* yang muda dan matang (YE, ME, YH dan MH) dalam ekstrak etanol dan heksan dapat menghalang proliferasi titisan sel kanser HepG₂ yang bergantung kepada kepekatan dan masa. Selepas tempoh 72 jam pengeraman, daun *G. gnemon* yang muda dan matang dalam ekstrak etanol dan heksan menunjukkan nilai IC₅₀ yang sangat tinggi 240.53, 138.53, 249.83 dan 257.21 mg / mL masing-masing. Berdasarkan keputusan model yang digunakan (ujian kesan tetap) ke atas perbandingan parameter (kematangan, pelarut dan masa) pada titisan sel kanser HepG₂, daun muda menunjukkan perbezaan yang signifikan ($p < 0.001$) berbanding dengan daun matang. Tidak terdapat perbezaan yang signifikan antara kedua-dua ekstrak pelarut yang digunakan (etanol dan heksan). Sebaliknya faktor masa telah menunjukkan kesan yang amat signifikan ($p < 0.001$).

Aruhan apoptosis telah ditunjukkan dengan morfologi kultur hidup pada titisan sel kanser HepG₂. Kedua-dua ekstrak etanol daun muda dan matang dari *G. gnemon* menunjukkan ia dapat mengaruhkan apoptosis pada kepekatan IC₅₀ ke atas sel HepG₂ berbanding dengan sel HepG₂ yang tidak dirawat yang dapat ditunjukkan melalui kehadiran ruang yang jelas, penurunan dalam bilangan sel, sel terapung, dan penurunan jumlah sel yang melekat selepas 72 jam masa pengeraman. Pewarnaan Acridine Orange / Propodium Iodide berganda dari kedua-dua sel HepG₂ yang dirawat dan tidak dirawat dengan kepekatan IC₅₀ daripada kedua-dua ekstrak etanol daun muda dan matang dari *G. gnemon* (YE dan ME) menunjukkan beberapa ciri-ciri apoptosis seperti tonjolan membran, saiz sel berkurangan dan beberapa sel nekrotik dengan peningkatan tempoh pengeraman. Analisis Fourier Transform Infrared (FT-IR) pada daun *G. gnemon* yang muda dan matang bagi ekstrak etanol menunjukkan beberapa puncak serapan yang tajam yang disebabkan oleh hadir kumpulan-kumpulan berfungsi yang berbeza dalam molekul-molekul (amina primer, amina sekunder, alkohol, fenol, asid karboksilik, aromatik, ester, lakton, aldehid dan kumpulan berfungsi keton) pada gelombang yang hampir sama. Akhir sekali, keputusan analisis gas kromatografi-Mass Spektroskopi menunjukkan kehadiran komponen bioaktif penting seperti Squalene, -tokoferol, dan -sitosterol dan Inositol pada daun *G. gnemon* muda (YE) ekstrak etanol dan Fitol, Inositol, dan β dan γ -Sitosterol, α -tokoferol dan Fenol, 2,4-bis (1,1-dimetil) dan antioksidan pada daun *G. gnemon* matang (ME) dalam ekstrak etanol.

Kesimpulannya, kedua-dua daun muda dan matang *G. gnemon* (YE, ME, YH dan MH) bagi ekstrak etanol dan heksan tidak menunjukkan kesan sitotoksik kesan ke atas titisan sel kanser HepG₂ dan 3T3 seperti yang ditunjukkan dengan nilai kepekatan IC₅₀ yang sangat tinggi (100 $\mu\text{g/mL}$). Walaubagaimanapun, berdasarkan kajian morfologi, titisan kanser sel HepG₂ yang dirawat dengan kedua-dua ekstrak etanol daun muda dan matang dari *G.*

gnemon untuk tempoh pengeraman selama 72 jam menunjukkan beberapa ciri-ciri apoptosis, kesan yang sama juga ditunjukkan dengan ujian Acridine Orange / Propodium Iodide pewarnaan berganda ke atas titisan sel kanser HepG₂ yang dirawat. Pengasingan dan pengenalpastian sebatian bioaktif dalam kedua-dua daun muda dan matang *G. gnemon* serta menjalankan ujian sitotoksik dengan menggunakan pelarut pengekstrakan yang berbeza disarankan pada masa akan datang.



ACKNOWLEDGEMENTS

In the Name of Allah, the most beneficent and the most merciful. May the glory of Allah and his mercy be upon Muhammad his household, his dedicated companions and the people that follows his own path till the day of resurrection. Thank be to Almighty God who through his wisdom showed me through this long journey.

I wish to express my appreciation and regards to my humble supervisor Associate Prof. Dr. Loh Su Peng for the time, advice, patience, support to mention a few throughout my study period. I would like to express my gratitude to my co-supervisor Prof. Dr. Asmah bint Rahmah, for her untiring advice, support and considerations. To my entire family, may God almighty reward you abundantly for the prayers, financial and moral support given to me. My special regards to my dear husband Nasiruddeen Umar Matazu, my my loving children Muhammad Almustapha, Hafsat, Fatima and Mukhtar I thank you for being there for me throughout this journey.

I would like to also extend my gratitude to Hassan Usman Katsina Polytechnic, Katsina Nigeria, my able lecturers, non academic staff and students from my great department (Dept. of Nutrition and Dietetics, FPSK, UPM), supporting staff of Nutrition Laboratory 2, 3 and 7 for their support in one way or the other. May Allah reward you all abundantly, amin.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AO/PI	Acridine orange/ Propodium iodide
ATCC	American type culture collection
ATR	Attenuated Total Reflectance
BCA	1,2-Benzenedicarboxylic acid
DCIS	Ductal carcinoma <i>in situ</i>
DAPI	4',6-diamidino-2-phenylindole (fluorescent stain)
DMEM	Dulbecco's modified eagle media
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FRAP	Ferric Reducing Ability of Plasma
FT-IR	Fourier transform infrared spectroscopy
GBL	2-hydroxy-gamma-butyrolactone
GC-MS	Gas chromatography/mass spectrometry
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepG ₂	Hepatocellular carcinoma tissue
HT-29	Colon adenocarcinoma tissue
HCA	Hydrazine carboxylic acid
IC ₅₀	Concentration that inhibits 50% cell viability
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
KBr	Potassium Bromide
LCIS	Lobular carcinoma <i>in situ</i>
MCF-7	Hormone dependent breast adenocarcinoma
ME	Ethanol extract of matured leaves of <i>G. gnemon</i>
MH	Hexane extract of matured leaves of <i>G. gnemon</i>
MHT	Menopausal hormone therapy
MTT	3-(4,5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide assay
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
NCR	National Cancer Registry
NIH	National Institute of Health
NIST	National institute of standards and technology
OD	Optical Density
FBS	Fetal bovine serum
ORAC	Oxygen radical absorbance capacity
PAL	Propionaldehyde
PBS	Phosphate buffered saline
PCA	Pyrazinecarboxamide
ROS	Reactive oxygen species
RPMI 1640	Roswell park memorial institute media

SE	Standard Error
TNF	Tumor necrosis factor
TRIC	Tetramethylrhodamine
XTT	Sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrate
YE	Ethanol extract of young leaves of <i>G. gnemon</i>
YH	Hexane extract of young leaves of <i>G. gnemon</i>
2,4-DTBP	Phenol, 2,4-bis(1,1-dimettylethyl)
3T3	Mouse embryo fibroblast



CHAPTER 1

INTRODUCTION

1.1 Background

An appreciable amount of plants have abundant quantity of bioactive complexes that plays a significant role in growth and development as well as a defence mechanism against predators. Most of these phytochemicals have proven to have high antioxidant activities with a number of actions in humans in health and diseases. This is the reason why intake of edible parts of these plants is encouraged (Di Carlo et al., 1999; Sun, 1990). The phytochemicals found in such plant parts are becoming of great interest in food industries due to their promising potency in preventing free radicals scavenging activities in food during processing and storage (Halliwell, 1999).

G. gnemon is gymnosperm specie from the family Gnetaceae, with common names such as melinjo or belinjo (Indonesia), Melinjau (Malaysia), and its internationally used name is *G. gnemon*, joint fir, or Spanish joint fir (English). This plant is a native from Assam (northeastern India). The nuts/seeds are eaten raw, boiled, fired or roasted. In East Java, “blinjo” chips made from *G. gnemon* seeds are an important home industry (Manner and Elevitch, 2006). The leaves and the inflorescence are eaten raw or boiled.

Previous researches conducted on different parts of *G. gnemon* (twig, bark, young and matured leaves, seeds and roots) has demonstrated the plant has high antioxidant activity such as reducing power activity, high total phenolic content, DNA damaging activity, peroxyl radical-scavenging activity as well as strong inhibitory effect on murine tyrosinase activity, anti-inflammatory effect and few natural compounds of biological importance were isolated and identified in the plant (Wazir and Shukor, 2011; Santoso et al., 2010; Kato et al., 2009; Tangkanakul et al., 2005; Iliya et al., 2003b,a; Ohguchi et al., 2003; Iliya et al., 2002a; Ito et al., 2002; Huang et al., 2001; Kimura et al., 1985; Wallace, 1979).

1.2 Problem Statement and Significance of the Study

The interest on natural antioxidant from plants is dramatically increasing as they have been associated with prevention of cancer and cardiovascular diseases (Tadhani et al., 2007; Virgili et al., 2001). Cancer being one of the non-communicable chronic diseases that are a world health problem, is at an increase about 18% cases of cancer each year (2 million) death from cancer worldwide are projected to rise to over 11 million by 2030, which contributes to a few specific chronic infections (World Health Organization, 2014).

People suffering from cancer and other chronic diseases value herbal therapy and its medicinal potentials. The herbal or natural therapy gives hopefulness, may decrease side effect of biomedical medicine and may lack the toxicity of biomedical medicine (Abebe, 2003). The convectional treatments of cancers including surgery, chemotherapy and radiotherapy have somehow become less promising as patients who have undergone such treatments are subjected to adverse side effects which often aggravate the patients' health condition. Mostly people have dreaded these side effects and have resorted to alternative treatments such as herbal therapy which often associates with less adverse side effects and less expensive.

So far no work has been done on human cancer cell with *G. gnemon* leave extract. Therefore this research is aimed at investigating whether young and matured leaves *G. gnemon* ethanol and hexane extracts has anticancer effects on a normal cell line (3T3 rouse embryo fibroblast) and several human cancer cell linen namely Hepatocellular carcinoma tissue HepG₂, colon adenocarcinoma tissue HT-29 and Hormone dependent breast adenocarcinoma MCF-7.

This study will further investigatethe potential anticancer properties of *G. gnemon* leave in normal and several human cancer cell lines. The information can be useful for cancer therapy to help decrease morbidity and mortality rate of cancer. And the alternative usage for the *G. gnemon* plant especially the leaves increase their value.

1.3 Research Question

Therefore, this study is formulated with the hope that it will be able to provide more information and highlights regarding to the below questions:

- Do *G. gnemon* young and matured leave extracts (ethanol and hexane) have antitumor properties or various cancer cell lines namely Hepatocellular carcinoma tissue HepG₂, Colon adenocarcinoma tissue HT-29 and Hormone dependent breast adenocarcinoma MCF-7?
- How can the IC₅₀ concentration of both YE and ME bring about morphological changes to the various cancer cell lines?
- What type of cell death do the extracts cause (apoptotic or necrotic) via the Fluorescent microscopy of AO/Pr stained cancer cells?
- What are the functional groups and phytochemical compounds of biological importance in both young and matured loaves of *G. gnemon* via FT-IR spectroscopy and GC-MS?

1.4 Objectives of the Study

1.4.1 General Objective

To study the potential anticancer properties of young and matured *G. gnemon* ethanol and hexane leave extracts on normal cell line (3T3 fibroblast cell) and several human cancer cell lines namely Hepatocellular carcinoma tissue HepG₂, Colon adenocarcinoma tissue HT-29 and Hormone dependent breast adenocarcinoma MCF-7.

1.4.2 Specific Objectives

- To determine the *in vitro* cytotoxic effect of young and matured *G. gnemon* leave extracts (ethanol and hexane) on normal cell line (3T3 fibroblast cell) and several human cancer cell lines namely Hepatocellular carcinoma tissue HepG₂, Colon adenocarcinoma tissue HT-29 and Hormone dependent breast adenocarcinoma MCF-7.
- To examine the *in vitro* morphological changes of both treated and untreated cell lines
- To examine the probable mode of cancer cell death induced by the young and matured *G. gnemon* leave ethanol extracts on normal cell line (3T3 fibroblast cell) and several human cancer cell lines namely Hepatocellular carcinoma tissue HepG₂, Colon adenocarcinoma tissue HT-29 and Hormone dependent breast adenocarcinoma MCF-7 by fluorescence microscopy.
- To identify the various functional groups and phytocomponents present in the *G. gnemon* extract by FT-IR Spectroscopy and GC-MS.

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