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

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

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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. ON SELECTED CANCER CELL LINES**

By

HADIZA ABUBAKAR ANKA

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-dimethylthiozol-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 IC$_{50}$ 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 IC$_{50}$ 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 IC$_{50}$ 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 dikesukak oleh 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

HADIZA ABUBAKAR ANKA

September 2014

Pengerusi : Loh Su Peng, PhD
Fakulti : Perubatan dan Sains Kesihatan

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 merawati 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 milinjau 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-dimethylthiozol-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 jual kepekatan (15.625-1000 µg/mL) dan tempoh pengeraman 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 dan 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 IC_{50} apabila titisan sel kanser dirawat dan dieram selama 72 jam. Walau bagaimanapun, 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 IC_{50} 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 IC_{50} 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 IC_{50} 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* 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, laktion, 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 IC_{50} yang sangat tinggi (100 µg/mL ). Walau bagaimanapun, 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.
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I certify that a Thesis Examination Committee has met on 19 September 2014 to conduct the final examination of Hadiza Abubakar Anka on her thesis entitled "In Vitro Cytotoxicity Properties of Gnetum gnemon L. on Selected Cancer Cell Lines" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>vii</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvii</td>
</tr>
</tbody>
</table>

## CHAPTER

1 INTRODUCTION 1

1.1 Background 1
1.2 Problem Statement and Significance of the Study 1
1.3 Research Question 2
1.4 Objectives of the Study 3

1.4.1 General Objective 3
1.4.2 Specific Objectives 3

2 LITERATURE REVIEW 4

2.1 Cancer 4
2.2 Cancer Prevalence 5
2.3 Cancer as a World Health Problem 5
2.4 Types of Cancer 6
2.4.1 Liver Cancer 6
2.4.2 Breast Cancer 7
2.4.3 Colon or Colorectal Cancer 9
2.5 Cancer Chemoprevention 10
2.6 *Gnetum gnemon* species 11
2.7 Anticancer Assay 13

2.7.1 MTT (3-(4,5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide) Assay 13
2.7.2 Acridine Orange / Propidium Iodide (AO/PI) staining 14
2.8 Apoptosis and Necrosis 14
2.9 Identification of Functional Groups and Bioactive Compounds 15
2.9.1 Fourier Transform Infrared Spectroscopy (FT-IR) 15
2.9.2 Gas chromatography-Mass spectroscopy (GC-MS) 17

3 MATERIALS AND METHODS 18

3.1 Plant Materials 18
3.2 Media, cell lines, chemicals and reagents 18
3.2.1 Preparation and Extraction of Plant Material 18
3.3 Cell Culture 19
3.3.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT Assay) 19

3.4 Morphological studies 20
3.4.1 Inverted Light Microscopy 21
3.4.2 Fluorescent microscopy of Propidium Iodide and Acridine Orange (AO/PI) Stained Cancer Cells 21

3.5 Fourier Transform Infrared Spectroscopy (FT-IR) 22

3.6 Gas Chromatography-Mass spectroscopy (GC-MS) 23

3.7 Statistical analysis 23

4 RESULTS 24
4.1 Cytotoxicity Study 24
4.2 Morphological Study 34
4.3 Quantification of Apoptosis Using Acridine Orange and Propodium Iodide Double Staining 37
4.4 Fourier transform infrared (FT-IR) Analysis of functional groups from the ethanolic extracts of G. gnemon 39
4.5 GC-MS Analysis of bioactive components from the ethanol extract leaves of G. gnemon 40

5 DISCUSSION 47
5.1 Cytotoxicity Study 47
5.1.1 Morphological Studies 48
5.1.2 Fluorescence Microscopy 49
5.1.3 Fourier Transform Infrared Spectroscopy 49
5.1.4 Gas chromatography- Mass Spectroscopy (GC-MS) analysis 49

6 CONCLUSION AND RECOMMENDATIONS 51
6.1 Conclusion 51
6.2 Future Research Recommendations 51
6.3 Limitation of Study 52

REFERENCES 53
APPENDICES 62
BIODATA OF STUDENT 66
PUBLICATION 67
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Differential features and significance of necrosis and apoptosis</td>
</tr>
<tr>
<td>4.1</td>
<td>Concentration that inhibits 50% of cell viability (IC$_{50}$) by young and matured <em>G. gnemon</em> leave extracts against selected cell lines after 72 hr incubation.</td>
</tr>
<tr>
<td>4.2</td>
<td>Summary of Fit</td>
</tr>
<tr>
<td>4.3</td>
<td>Parameter Estimates</td>
</tr>
<tr>
<td>4.4</td>
<td>Apoptotic feature(s) on Figure 4.11</td>
</tr>
<tr>
<td>4.5</td>
<td>Apoptotic feature(s) on Figure 4.12</td>
</tr>
<tr>
<td>4.6</td>
<td>FTIR absorption bands from ethanol extracts of young and mature leaves of <em>G. gnemon</em></td>
</tr>
<tr>
<td>4.7</td>
<td>Phytochemical components Identified in <em>G. gnemon</em> YE extract</td>
</tr>
<tr>
<td>4.8</td>
<td>Phytochemical components Identified in <em>G. gnemon</em> ME extract</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>12</td>
</tr>
<tr>
<td>4.2</td>
<td>28</td>
</tr>
<tr>
<td>4.3</td>
<td>29</td>
</tr>
<tr>
<td>4.4</td>
<td>29</td>
</tr>
<tr>
<td>4.5</td>
<td>30</td>
</tr>
<tr>
<td>4.6</td>
<td>30</td>
</tr>
<tr>
<td>4.7</td>
<td>31</td>
</tr>
<tr>
<td>4.8</td>
<td>31</td>
</tr>
<tr>
<td>4.9</td>
<td>32</td>
</tr>
<tr>
<td>4.10</td>
<td>33</td>
</tr>
</tbody>
</table>
4.11 The non-centrable phase-contrast micrographs of the adherent HepG2 cells. (a) untreated HepG2 cells, (b) treated HepG2 cell lines with IC50 concentration of YE after 24 hrs, (c) treated HepG2 cell lines with IC50 concentration of YE after 48 hrs, (d) treated HepG2 cell lines with IC50 concentration of YE after 72 hrs, (e) treated HepG2 cell lines with IC50 concentration of ME after 24 hrs, (f) treated HepG2 cell lines with IC50 concentration of ME after 48 hrs, (g) treated HepG2 cell lines with IC50 concentration of ME after 72 hrs respectively (magnification 400X). Arrow on (a) shows untreated cells having intact cell structure, 80% confluent and well differentiated, (b) shows the appearance of floating dead cells in the culture media and 50% confluency, arrow on (d) shows decrease cell number and 40% confluency, arrow on (g) shows clear space, 30% confluency and cells losing contact with adjacent cells.

4.12 Fluorescent micrograph of acridine orange and propodium iodide double-staining of HepG2 cancer cell lines showing (a) untreated HepG2 cells, (b) treated HepG2 with IC50 concentrations of YE after 24 hrs, (c) treated HepG2 with IC50 concentrations of YE after 48 hrs, (d) treated HepG2 with IC50 concentrations of YE after 72 hrs, (e) treated HepG2 with IC50 concentrations of ME after 24 hrs, (f) treated HepG2 with IC50 concentrations of ME after 48 hrs and (g) treated HepG2 with IC50 concentrations of ME after 72 hrs respectively (magnification 400X). Arrow on (a) shows a normal intact cells, (b) shows a necrotic cell, arrow on (d) shows a necrotic cell and arrow on (g) shows membrane blebbing.

4.13 FT-IR Spectrum of ethanolic extract of young leaves of G. gnemon (YE).

4.14 FT-IR spectrum of ethanolic extract of matured G. gnemon leave (ME)

4.15 GC-MS chromatogram of ethanolic extract of G. gnemon young leaves

4.16 GC-MS chromatogram of ethanolic extract of G. gnemon mature leaves

A.1 Whole Model Actual by predicted Plot

A.2 Residual by predicted plot

A.3 Extract leverage plot

A.4 Mean IC50 value Against extracts
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.5 Maturity Leverage Plot</td>
<td>64</td>
</tr>
<tr>
<td>A.6 Mean IC&lt;sub&gt;50&lt;/sub&gt; value Against Maturity</td>
<td>64</td>
</tr>
<tr>
<td>A.7 Time Leverage Plot</td>
<td>65</td>
</tr>
<tr>
<td>A.8 Mean IC&lt;sub&gt;50&lt;/sub&gt; value Against Time</td>
<td>65</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO/PI</td>
<td>Acridine orange/Propidium iodide</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuated Total Reflectance</td>
</tr>
<tr>
<td>BCA</td>
<td>1,2-Benzenedicarboxylic acid</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole (fluorescent stain)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric Reducing Ability of Plasma</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GBL</td>
<td>2-hydroxy-gamma-butyrolactone</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatocellular carcinoma tissue</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colon adenocarcinoma tissue</td>
</tr>
<tr>
<td>HCA</td>
<td>Hydrazine carboxylic acid</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration that inhibits 50% cell viability</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal carcinoma</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive lobular carcinoma</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium Bromide</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>MCF-7</td>
<td>Hormone dependent breast adenocarcinoma</td>
</tr>
<tr>
<td>ME</td>
<td>Ethanol extract of matured leaves of G. gnemon</td>
</tr>
<tr>
<td>MH</td>
<td>Hexane extract of matured leaves of G. gnemon</td>
</tr>
<tr>
<td>MHT</td>
<td>Menopausal hormone therapy</td>
</tr>
<tr>
<td>MTTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide assay</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NCR</td>
<td>National Cancer Registry</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NIST</td>
<td>National institute of standards and technology</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>PAL</td>
<td>Propionaldehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Pyrazinecarboxamide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Roswell park memorial institute media</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRIC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>XTT</td>
<td>Sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulphonic acid hydrate</td>
</tr>
<tr>
<td>YE</td>
<td>Ethanol extract of young leaves of <em>G. gnemon</em></td>
</tr>
<tr>
<td>YH</td>
<td>Hexane extract of young leaves of <em>G. gnemon</em></td>
</tr>
<tr>
<td>2,4-DTBP</td>
<td>Phenol, 2,4-bis(1,1-dimethyl)</td>
</tr>
<tr>
<td>3T3</td>
<td>Mouse embryo fibroblast</td>
</tr>
</tbody>
</table>
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 conventional 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 lines namely Hepatocellular carcinoma tissue HepG2, Colon adenocarcinoma tissue HT-29 and Hormone dependent breast adenocarcinoma MCF-7.

This study will further investigate the 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 IC$_{50}$ 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 leaves 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.
REFERENCES


of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis.


