UNIVERSITI PUTRA MALAYSIA

NUTRIENT ENRICHMENT OF FERMENTED SOYBEAN TEMPEH VIA ANAEROBIC FERMENTATION WITH VARIOUS BIOLOGICAL ACTIVITIES

HAMIDAH MOHD YUSOF

FBSB 2013 46
NUTRIENT ENRICHMENT OF FERMENTED SOYBEAN TEMPEH VIA ANAEROBIC FERMENTATION WITH VARIOUS BIOLOGICAL ACTIVITIES

By

HAMIDAH MOHD YUSOF

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

December 2013
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

NUTRIENT ENRICHMENT OF FERMENTED SOYBEAN TEMPEH VIA ANAEROBIC FERMENTATION WITH VARIOUS BIOLOGICAL ACTIVITIES

By

HAMIDAH MOHD YUSOF

December 2013

Chairman  :  Assoc. Prof. Noorjahan Banu Alitheen, PhD
Faculty  :  Biotechnology and Biomolecular Sciences

Fermented soybean is found to contain increased level of bioactive contents such as proteins, polyphenolics, vitamins and minerals as a result of its microbial activity. Tempeh is an example of fermented soybean that has been recognized to have various health benefits contributed by the increased level of amino acids and antioxidants. Further enhancement of the bioactive properties of tempeh can be achieved via combination of anaerobic fermentation with selected strain of *Rhizopus* sp. as an inoculant. The purpose of this study is to determine and compare the cytotoxic, immunomodulatory, anti-inflammatory, liver ameliorative activities as well as acute toxicity of aqueous extract of nutrient enriched of soybean tempeh (NESTE) with non-fermented soybean (SBE). The product ion of NESTE involved by normal aerobic fermentation of tempeh followed by anaerobic fermentation using *Rhizopus oligosporus* (*R. oligosporus*) 5351 strain that was obtained from the culture collection of Malaysian Agricultural Research and Development Institute (MARDI). NESTE was produced by soaking soybeans for 18 h, steaming for 40 min, mixing with *R. oligosporus* 5351 strain starter culture prior to packaging with perforated plastic, incubating in aerobic condition for 30 h at 30 °C and continue incubating in anaerobic condition for 20 h at room temperature. Results have demonstrated that anaerobic fermentation on soybean had successfully produced NESTE, which contains 3210 ± 0.01 mg/ 100 g DW (dried weight) total free amino acids, 1100 ± 0.01 mg/ 100 g DW (dried weight) total essential amino acids and 338 ± 0.025 mg/ 100 g DW (dried weight) gamma-aminobutyric acid (GABA). Besides, NESTE also contains 42.64 ± 1.59 µg/ g extract of soluble phenolic acids and 22.56 ± 0.31 mg GAE/ g extract of total phenolic acids. These results indicated that the level of amino acids, GABA and antioxidants had significantly increased (p < 0.05). In addition, NESTE also inhibited the growth of MCF 7 cells in MTT assay with IC\(_{50}\) 3.6 ± 0.22 mg/ mL after 72 h incubation while no cytotoxicity was detected in MCF 10A normal breast cell line. Cell cycle with flow cytometry analysis illustrated that NESTE arrested MCF 7 cells at G\(_0\)/G\(_1\) phase. Furthermore, increment of the cell population in sub G\(_0\)/G\(_1\) has shown that IC\(_{50}\) of NESTE at 72 h was able to...
induce the best apoptotic effect towards MCF 7 cells. Annexin V-FITC/PI assay has further confirmed the apoptotic effect induced by NESTE on MCF 7 cells where substantial amount of early apoptotic cells were detected. On the other hand, the immunomodulatory study of NESTE has shown that NESTE stimulated splenic cells proliferation in time and dosage dependent manner which can be observed through MTT and BrdU assays. Additionally, NESTE was also able to stimulate and enhance cytokine secretion (IL-2 and IFN-gamma) in time and dosage dependent manner. Anti-inflammatory study has shown that NESTE exhibited no sign of cytotoxicity towards RAW 264.7 cells (macrophage cells) and was able to suppress the level of nitric oxide, which is an inflammatory indicator. In vivo tests on mice ear edema and analgesic demonstrated that the best effect was achieved when treated with 1000 mg/ kg of NESTE suggesting that NESTE was able to suppress the edematous effect of mice ear and produce better and lasting analgesic effect. The evaluation of in vivo liver ameliorative activity of NESTE indicated that NESTE could revert the effect of steatosis in hepatocytes to normal condition, increase the antioxidant level and reduce the inflammation of the ethanol treated mice. Moreover, NESTE exhibited no sign of toxicity towards mice up to 5000 mg/ kg. Overall, anaerobic fermentation of soybean using R. oligosporus sp. 5351 strain has successfully produced NESTE with higher bioactive contents such as GABA, amino acid and antioxidants. This finding suggested that NESTE could be formulated as a healthy food supplements that possessed the anticancer, immunomodulatory, anti-inflammatory and liver ameliorative effect.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENINGKATAN NUTRIEN DALAM TEMPEH KACANG SOYA MELALUI KAEDAH PENAPAIAN SECARA ANAEROBIK SERTA KEBELAGAIAN AKTIVITI BIOLOGI

Oleh

HAMIDAH MOHD YUSOF

Disember 2013

Pengerusi : Prof. Madya Noorjahan Banu Alitheen, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Penapaian kacang soya didapati mengandungi peningkatan terhadap tahap kandungan bioaktif seperti protein, polifenolik, vitamin dan mineral akibat daripada aktiviti mikroanya. Tempeh adalah contoh penapaian soya yang telah diketahui mempunyai pelbagai manfaat kesihatan yang disumbangkan oleh tahap peningkatan asid amino dan antioksidan. Peningkatan kandungan bioaktif tempeh boleh dilakukan melalui gabungan penapaian secara anaerobik dengan menggunakan jenis *Rhizopus sp.* yang terpilih sebagai inokulan. Tujuan kajian ini dilakukan adalah untuk menentukan dan membandingkan tahap sitotoksik, imunomodulatori, anti-inflamasi, aktiviti memperbaiki hati serta pengesanan toksik melalui kajian ketoksikan akut terhadap ekstrak akues mentah tempeh kacang soya yang diperkaya dengan nutrisi (NESTE) dan ekstrak akues mentah kacang soya tanpa penapaian (SBE). Penghasilan NESTE adalah melibatkan penapaian secara aerobik biasa diikuti dengan penapaian secara anaerobik dengan menggunakan *Rhizopus oligosporus* (*R. oligosporus*) jenis 5351 yang diperolehi daripada koleksi kultur Institut Penyelidikan dan Kemajuan Pertanian Malaysia (MARDI). NESTE dihasilkan melalui merendam kacang soya selama 18 jam, mengukus selama 40 minit, mencampurkan dengan kultur pemula *R. oligosporus* jenis 5351 sebelum pembungkusan dengan menggunakan plastik yang berlubang, diinkubasi secara aerobik selama 30 jam pada 30 °C dan menyambung proses inkubasi secara anaerobik selama 20 jam pada suhu bilik. Keputusan telah menunjukkan bahawa penapaian anaerobik pada kacang soya telah berjaya menghasilkan NESTE yang mengandungi 3210 ± 0.01 mg/100 g DW (berat kering) jumlah keseluruhan asid amino bebas, 1100 ± 0.01 mg/100 g DW (berat kering) jumlah asid amino perlu dan 338 ± 0.025 mg/100 g DW (berat kering) asid gamma-aminobutyric (GABA). Selain itu, NESTE juga menghasilkan asid fenolik larut yang berjumlah 42.64 ± 1.59 μg/ g ekstrak dan asid fenolik total berjumlah 22.56 ± 0.31 mg GAE/ g ekstrak. Kesemua keputusan data nutrient NESTE menunjukkan bahawa tahap asid amino, GABA dan antioksidan telah meningkat secara signifikan (p < 0.05). Di samping itu, NESTE juga menunjukkan
rencan pertumbuhan sel-sel MCF 7 pada esei MTT dengan menghasilkan IC\textsubscript{50} 3.6 ± 0.22 mg/ mL ekstrak selepas inkubasi selama 72 jam. Di samping itu, tiada sitotoksik yang dikesan pada sel payudara normal, MCF 10A. Ujian kitaran sel dengan menggunakan analisis sitometri aliran menunjukkan bahawa NESTE telah menghalang kitaran sel MCF 7 pada fasa G\textsubscript{0}/G\textsubscript{1}. Tambahan pula, peningkatan populasi sel pada sub G\textsubscript{0}/G\textsubscript{1} telah menunjukkan bahawa IC\textsubscript{50} daripada NESTE pada 72 jam dapat memberi kesan apoptosis terbaik terhadap sel-sel MCF 7. Ujian Annexin V-FITC/PI telah menambahkan lagi bukti bahawa kesan apoptosis pada sel MCF 7 adalah disebabkan oleh NESTE di mana sel-sel apoptotik fasa awal dikesan pada jumlah yang tinggi. Sebaliknya, kajian imunomodulatori pada NESTE melalui ujian MTT dan ujian BrdU telah menunjukkan bahawa NESTE mampu menambah baik pertumbuhan sel-sel limpa mengikut penambahan masa dan dos ekstrak. Selain itu, NESTE juga mampu untuk merangsang dan meningkatkan rembesan sitokin (IL-2 dan IFN-gamma) mengikut penambahan masa dan dos ekstrak. Kajian antiinflamasi pula telah menunjukkan bahawa NESTE tidak menunjukkan sebarang kesan sitotoksik terhadap sel-sel RAW 264.7 (sel makrofaj) dan ia juga menunjukkan kemampuan untuk menyebabkan peningkatan nitrik oksida yang diketahui sebagai penunjuk kesan inflamasi. Ujian in vivo terhadap kesan edema telinga tikus dan analgesik telah menunjukkan kesan pemulihan terbaik apabila dirawat dengan 1000 mg NESTE/ kg berat tikus dan ini menunjukkan bahawa NESTE mampu menyebabkan kesan edema pada sel-sel tikus dan menghasilkan kesan analgesik yang lebih baik dan berkekal. Penilaian aktiviti memperbaiki hati secara in vivo terhadap NESTE menunjukkan bahawa kesan steatosis pada sel hati telah kembali kepada keadaan normal, peningkatan terhadap tahap antioksidan dan pengurangan keradangan etanol pada tikus. Selain itu, NESTE tidak mempamerkan sebarang tanda-tanda keracunan terhadap tikus sehingga pada tahap 5000 mg/ kg. Secara keseluruhan, penapaian secara anaerobik terhadap kacang soya dengan menggunakan R. oligosporus sp. jenis 5351 telah berjaya menghasilkan NESTE dengan menghasilkan kandungan bioaktif seperti GABA, asid amino dan antioksidan yang lebih tinggi. Penemuan ini menunjukkan bahawa NESTE berpotensi untuk dirumuskan sebagai makanan tambahan kesihatan yang mempunyai kesan antikanser, imunomodulatori, anti-inflamasi dan memperbaiki hati.
ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my supervisor, Assoc. Prof. Dr. Noorjahan Banu Mohamed Alitheen who has given me the opportunity to do this project in the first place. Her guidance, mentorship, encouragement, patience and support at all levels have made my thesis completion possible. I also would like to thank my co-supervisory committee members Prof Dr. Suraini Abd. Aziz and Dr. Kamariah Long (MARDI) for their valuable advice, technical guidance and support for the accomplishment of this project and thesis.

Lots of thanks also to all MARDI staff for supplying plant materials, *Rhizopus sp* strain 5351, tempeh production course and all equipment used for the whole study. Deepest appreciation is also due to Dr. Swee Keong Yeap and Dr. Soo Peng Koh who were extremely helpful in offering invaluable assistance and guidance. Without their knowledge and support, this project would not have been successful. Special thanks to all my colleagues for their continuous help and advises starting from Miss Norlaily, Mr. Beh, Miss Ho, Miss Nadia, Miss Elyani, Miss Teoh, Mr. Aimi, Mrs. Roszaimah and Mr. Firdaus. An honorable mention goes to my beloved families, friends and everyone who had contributed directly or indirectly to this project for their understandings and supports. Last but not least, I would like to thank the Ministry of Agriculture (MOA) for providing this project with grant no. RB2198SF10 and Universiti Putra Malaysia for RUGS 6 grant no. 9300373 as well as Graduate Research Fellowship (GRF) funding throughout my study.
I certify that a Thesis Examination Committee has met on 17 December 2013 to conduct the final examination of Hamidah binti Mohd. Yusof on her thesis entitled “Nutrient Enrichment of Fermented Soybean Tempeh via Anaerobic Fermentation with Various Biological Activities” 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.

Members of the Thesis Examination Committee were as follows:

**Ho Chai Ling, PhD**  
Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Chairman)

**Janna Ong binti Abdullah, PhD**  
Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Cheah Yoke Kqueen, PhD**  
Faculty of Medicine and Health Science  
Universiti Putra Malaysia  
(Internal Examiner)

**Salehhuddin Hamdan, PhD**  
Associate Professor  
Universiti Teknologi Malaysia  
(External Examiner)

---

**NORITAH OMAR, PhD**  
Associate Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia  

Date: 21 April 2014
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

**Noorjahan Banu Alitheen, PhD**  
Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Chairman)

**Suraini Abd. Aziz, PhD**  
Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Member)

**Kamariah Long, PhD**  
Department of Bioprocess Biotechnology  
Malaysian Agriculture Research Development Institute  
(Member)

**BUJANG BIN KIM HUAT, PHD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia  
Date:
DECLARATION

Declaration by graduate student

I hereby confirm that:
• this thesis is my original work;
• quotations, illustrations and citations have been duly referenced;
• this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
• intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012 and MARDI;
• written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published in book form;
• there is no plagiarism or data falsification/ fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _______________________ Date: ________________

Name and Matric No. : Hamidah Mohd Yusof (GS 25860)
Declaration by Members of Supervisory Committee

This is to confirm that:
- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____________________     Signature: ______________________
Name of Chairman of Chairman of
Supervisory Supervisory
Committee: _____________________ Committee: ______________________

Signature: _____________________     Signature: ______________________
Name of Chairman of Chairman of
Supervisory Supervisory
Committee: _____________________ Committee: ______________________
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>iv</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>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xix</td>
</tr>
</tbody>
</table>

### CHAPTER

1. **INTRODUCTION**

2. **LITERATURE REVIEW**

   2.1. Soybean
       2.1.1 Types of soybean based foods
       2.1.2 Chemical composition of soybean seeds
       2.1.3 Bioactive compound of soybeans
   2.2. Soybean tempeh
       2.2.1 The production of tempeh
       2.2.2 Biochemical changes in tempeh
       2.2.3 Current studies on soybeans and fermented soybeans
           2.2.3.1 Antioxidant
           2.2.3.2 Amino acid
           2.2.3.3 Gamma aminobutyric acid (GABA)
   2.3. Pigmentation, antipigmentation and tyrosinase activity
   2.4. Cancer
   2.5. Immune system and immunomodulators
   2.6. Inflammation and anti-inflammatory
   2.7. Alcoholic liver disease and protection
   2.8. Risk assessment and fermented food

3. **ANALYSES OF BIOACTIVE COMPOSITION OF NUTRIENT-ENRICHED OF SOYBEAN TEMPEH (NESTE)**

   3.1. Introduction
   3.2. Materials and Methods
       3.2.1. Chemicals and reagents
       3.2.2. Plant material and microorganism
       3.2.3. Preparation of nutrient enriched soybean tempeh (NESTE)
       3.2.4. Preparation of sample extraction
       3.2.5. Analysis of amino acids and GABA
3.2.6. Total phenolic content assay of NESTE and SBE
3.2.7. 2,2'-diphenyl-1- picrylhydrazyl (DPPH) assay of NESTE and SBE
3.2.8. Ferric reduce antioxidant power (FRAP) assay of NESTE and SBE
3.2.9. HPLC quantification of soluble phenolic acid of NESTE and SBE
3.2.10. Antipigmentation assay (Tyrosinase inhibition assay)
3.2.11. Statistical analysis

3.3. Results
3.3.1. Amino acid content
3.3.2. Antioxidant content
3.3.3. Antipigmentation effect

3.4. Discussion
3.5. Conclusion

4. ANTIPROLIFERATIVE AND CYTOTOXIC EFFECTS OF NESTE AGAINST HUMAN BREAST CANCER CELL (MCF 7)
4.1. Introduction
4.2. Materials and Methods
4.2.1. Chemicals, reagents and cultureware
4.2.2. Cell lines
4.2.3. MTT cell viability assay
4.2.4. Trypan blue exclusion assay
4.2.5. Cell cycle progression by flow cytometer
4.2.6. Annexin V-FITC/propidium iodide analysis by flow cytometer
4.2.7. Acridine orange/propidium iodide (AO/PI) staining
4.2.8. Statistical analysis
4.3. Results
4.3.1. MTT assay
4.3.2. Trypan blue exclusion assay
4.3.3. Cell cycle progression effect
4.3.4. AO/PI staining of MCF 7 cells
4.3.5. Annexin V-FITC/PI staining assay
4.4. Discussion
4.5. Conclusion

5. IN VITRO IMMUNOMODULATORY EFFECT OF NESTE ON IMMUNE CELLS
5.1. Introduction
5.2. Materials and Methods
5.2.1. Chemicals and reagents
5.2.2. Animal
5.2.3. Mice splenocytes cell suspension preparation
5.2.4. Splenocyte viability assay (MTT assay)
5.2.5. Splenocyte proliferation assay (BrdU assay) 44
5.2.6. Determination of mice splenocyte cytokine secretion 44
   (IL2 and IFN-γ)
5.2.7. Cell cycle analysis on mice splenocyte 44
5.2.8. Statistical analysis 45

5.3. Results 46
5.3.1. MTT assay 46
5.3.2. BrdU assay 47
5.3.3. Determination of cytokine secretions 48
5.3.4. Cell cycle distribution of splenocyte 49

5.4. Discussion 50
5.5. Conclusion 51

6. THE EVALUATION OF IN VIVO AND IN VITRO ANTI-INFLAMMATORY EFFECT OF NESTE 52
6.1. Introduction 53
6.2. Materials and Methods 53
   6.2.1. Chemicals and reagents 53
   6.2.2. Cell culture 53
   6.2.3. Cell viability assay (MTT assay) 53
   6.2.4. Nitric oxide (NO) inhibitory assay 53
   6.2.5. Animals preparation for in vivo anti-inflammatory assessment 54
   6.2.6. Mice ear inflammation test 54
   6.2.7. Mice paw inflammation test 54
   6.2.8. Statistical analysis 55
6.3. Results 56
   6.3.1. Viability of RAW 264.7 cells 56
   6.3.2. LPS induced NO production in RAW 264.7 cells 57
   6.3.3. Effect of NESTE and SBE on arachidonic acidinduced ear edema in mice 58
   6.3.4. Analgesic effect of NESTE and SBE on mice by hot plate test 59
6.4. Discussion 60
6.5. Conclusion 60

7. THE EVALUATION OF IN VIVO ACUTE TOXICITY OF NESTE ON NORMAL MICE AND AMELIORATIVE EFFECTS OF NESTE ON ALCOHOL-MEDIATED LIVER DAMAGE IN MICE 62
7.1. Introduction 63
7.2. Materials and Methods 63
   7.2.1. Chemicals and reagents 63
   7.2.2. Animals preparation for acute toxicity assessment 63
   7.2.3. Experimental design for acute toxicity assessment 63
   7.2.4. In vivo liver ameliorative study 64
   7.2.5. Serum biochemical analysis 64
7.2.6. Histopathological analysis 64
7.2.7. FRAP assay of mice liver homogenate 65
7.2.8. Determination of malondialdehyde (MDA) level on mice liver homogenate 66
7.2.9. Determination of superoxide dismutase (SOD) level on mice liver homogenate 66
7.2.10. Determination of nitric oxide (NO) level on mice liver homogenate 66
7.2.11. Statistical analysis 67

7.3. Results 68
7.3.1. Limit dose test (LD<sub>50</sub>) 68
7.3.2. Evaluation of mice body weight 69
7.3.3. Evaluation of mice organ weight 69
7.3.4. Evaluation of mice serum biochemistry for acute toxicity test 70
7.3.5. Histopathological of mice organ for acute toxicity test 71
7.3.6. Evaluation of serum biochemistry in mice with alcohol induced liver damage 74
7.3.7. Evaluation of SOD, FRAP, MDA and NO level on liver homogenate of mice with alcohol induced liver damage 76
7.3.8. Liver histopathological evaluation for mice with alcohol induced liver damage 78

7.4. Discussion 81
7.5. Conclusion 82

8. GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 83

REFERENCES 84
APPENDICES 113
BIODATA OF STUDENT 129
LIST OF PUBLICATION 130
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Nutritional composition of soybean based foods</td>
<td>3</td>
</tr>
<tr>
<td>2.2</td>
<td>Components of nutrient in dried soybean</td>
<td>4</td>
</tr>
<tr>
<td>2.3</td>
<td>Bioactive compounds of soybean and its health benefits</td>
<td>5</td>
</tr>
<tr>
<td>2.4</td>
<td>Types of GABA enriched food, inoculant and plant</td>
<td>11</td>
</tr>
<tr>
<td>3.1</td>
<td>Amino acid content of SBE and NESTE (mg/100 g dry weight)</td>
<td>25</td>
</tr>
<tr>
<td>3.2</td>
<td>Antioxidant profiles of SBE and NESTE</td>
<td>26</td>
</tr>
<tr>
<td>3.3</td>
<td>Soluble phenolic acid content in SBE and NESTE</td>
<td>26</td>
</tr>
<tr>
<td>3.4</td>
<td>IC$_{50}$ value of inhibition effect from different extracts against mushroom tyrosinase enzyme activity with L-tyrosine substrate</td>
<td>27</td>
</tr>
<tr>
<td>4.1</td>
<td>MTT assay on cytotoxicity activity of SBE and NESTE (IC$_{50}$ mg/mL ± S.E.M) against cancer cell lines at different incubation time</td>
<td>34</td>
</tr>
<tr>
<td>5.1</td>
<td>Effect of extract treatments toward cell viability (%) of spleen by MTT assay at 24, 48 and 72 h incubation.</td>
<td>46</td>
</tr>
<tr>
<td>6.1</td>
<td>Inhibitory effect of NESTE and SBE extracts towards arachidonic acid-induced ear edema in mice.</td>
<td>58</td>
</tr>
<tr>
<td>6.2</td>
<td>Analgesic activity of NESTE and SBE by hot plate method on mice</td>
<td>59</td>
</tr>
<tr>
<td>7.1</td>
<td>Limit dose (LD$_{50}$) test of different experimental groups of treated ICR mice</td>
<td>68</td>
</tr>
<tr>
<td>7.2</td>
<td>Effect of weight gain of mice on different experimental groups of treated ICR mice.</td>
<td>69</td>
</tr>
<tr>
<td>7.3</td>
<td>Effect of spleen index, kidney index and liver index on different experimental groups of treated ICR mice.</td>
<td>69</td>
</tr>
</tbody>
</table>
7.4 Serum biochemical profile of treated ICR mice from different experimental groups.

7.5 Serum biochemical parameter of liver protective effect from different experimental groups.

7.6 Liver protective effect on liver homogenate parameters of different experimental groups.

7.7 Histopathological scoring of alcohol induced liver injury with different experimental groups.
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Images of soybean tempeh</td>
<td>6</td>
</tr>
<tr>
<td>3.1</td>
<td>Process of making nutrient enriched soybean tempeh (NESTE)</td>
<td>20</td>
</tr>
<tr>
<td>4.1</td>
<td>Percentage viability of MCF 7 cell line treated with different concentration of NESTE by trypan blue exclusion assay</td>
<td>35</td>
</tr>
<tr>
<td>4.2</td>
<td>Cell cycle progression effect of MCF 7 cell lines treated with different concentration of NESTE</td>
<td>36</td>
</tr>
<tr>
<td>4.3</td>
<td>AO/PI staining of treated and untreated MCF 7 viewed under fluorescence microscope with a magnification of 100 times</td>
<td>38</td>
</tr>
<tr>
<td>4.4</td>
<td>Annexin-V staining of MCF 7 cells treated with different concentration of NESTE after 24 h incubation</td>
<td>39</td>
</tr>
<tr>
<td>5.1</td>
<td>Effect of the extract treatment on percentage of spleen cells proliferation by BrdU assay</td>
<td>47</td>
</tr>
<tr>
<td>5.2</td>
<td>Effect of extract treatments on cytokine secretion of splenocytes IFN-γ and IL-2 at 24, 48 and 72 h</td>
<td>48</td>
</tr>
<tr>
<td>5.3</td>
<td>Effect of different experimental treatments on cell cycle distribution of mice splenocytes after 24 h incubation</td>
<td>49</td>
</tr>
<tr>
<td>6.1</td>
<td>The viability effect of RAW 264.7 cells by MTT assay with various concentration of NESTE and SBE after 24 h treatments</td>
<td>56</td>
</tr>
<tr>
<td>6.2</td>
<td>The effect of different concentration of extract treatments towards the percentage of nitric oxide inhibition on LPS-induced NO production in RAW 264.7 cells</td>
<td>57</td>
</tr>
<tr>
<td>7.1</td>
<td>Histological microsection of liver tissues from mice treated with single oral acute dose of 5000 mg/ kg extract and obtained under a light microscope with 10 X 10 magnifications.</td>
<td>71</td>
</tr>
</tbody>
</table>
7.2 Histological microsection of kidney tissues from mice treated with single oral acute dose of 5000 mg/ kg extract and obtained under a light microscope with 10 X 10 magnifications.

7.3 Histological microsection of spleen tissues from mice treated with single oral acute dose of 5000 mg/ kg extract and obtained under a light microscope with 4 X 10 magnifications

7.4 Histopathological microsection of liver after alcohol intoxication and treatment with different experimental group of extracts.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>m</td>
<td>Mili</td>
</tr>
<tr>
<td>AAE</td>
<td>Ascorbic acid equivalent</td>
</tr>
<tr>
<td>ALD</td>
<td>Alcoholic liver disease</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>ASA</td>
<td>Acetyl Salicylic Acid</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Balb/c</td>
<td>Albino, laboratory-bred strain mice</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylatedhydroxytoulene</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromo-deoxyuridine</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethysulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPH</td>
<td>1,1-diphenyl-2-picryl-hydrazil</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Link Immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescein Activated Cell Sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocynate</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
</tbody>
</table>
G  Gap
GABA  $\gamma$-amino butyric acid
GAE  Gallic acid equivalents
h  hour
HBBS  Hank’s Balance Salt Solution
HCL  Hydrochloric acid
HDL  High-density lipoprotein
IC$_{50}$  Inhibition concentration that reduces 50% of cells viability
IFN  Interferon
IL  Interleukin
LD$_{50}$  Lethal dose that cause 50% of death in animal
LDH  Lactate dehydrogenase
LDL  Low-density lipoprotein
LPS  Lipopolisaccharide
MARDI  Malaysian Agriculture Research Development Institute
MCF 10A  Human mammary epithelial cells
MCF-7  Human mammary gland adenocarcinoma cells
MDA  Malondialdehyde
min  Minutes
mL  Mililiter
mm  Millimeter
mM  Milimolar
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na$_2$HPO$_4$  Disodium hydrogen phosphate anhydrous
NaCl  Sodium chloride
NBT  Nitro blue tetrazolium
NESTE  Nutrient enriched soybean tempeh
nm  Nanometer
NO  Nitric oxide
NSAIDs  Non-steroidal anti-inflammatory drugs
PBS  Phosphate buffer saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>RAW264.7</td>
<td>Murine macrophage cell line</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SBE</td>
<td>Non-fermented soybean extract</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TMB</td>
<td>Peroxidase substrate 3,3',5,5''-tetramethylbenzidine</td>
</tr>
<tr>
<td>TPC</td>
<td>Total phenolic content</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2, 4, 6-tripyridyl-s-triazine</td>
</tr>
<tr>
<td>TypLE</td>
<td>Express-Trypsin replacement enzyme for cells dissociation</td>
</tr>
<tr>
<td>UPM</td>
<td>Universiti Putra Malaysia</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>x g</td>
<td>times gravity</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Fermented foods have contributed to one third of food intake all around the world and are one of the oldest forms of biotechnology product that converts complex food substrate into simpler and digestible food with the help of enzymes that are produced from microbial activity (Blandino et al., 2002). According to Steinkraus (1995), food fermentation process could serve five main purposes which are to develop various aromas, flavours and textures; produce proteins, essential amino acids and vitamins; save time and fuel; preserve the food through lactic acid, alcohol, acetic acid and alkaline fermentation and remove the antinutrient substance. Furthermore, production of the end-products of food fermentation such as acids, alcohol and carbon dioxide has the ability to control the growth of spoilage microbe (Paul Ross et al., 2002). Due to these reasons, many researchers are attracted to investigate the process and health benefits of fermented food products for the development of functional foods (Stanton et al., 2005).

For many years, functional food is one of the important food ingredients for people in Asia as they believe that food and medicine are derived from the same source, hence exhibit similar effect (Verschuren, 2002; Siro et al., 2008). Great emphasis has been put on functional foods as they can act as medicine with less side effects (Shah, 2007). Fermentation has been used to produce functional foods since enhanced nutrient content is always observed in fermented foods (Verschuren, 2002). Several fermented foods have been developed into functional foods such as tempeh, fermented rice and prebiotic product such as fermented milk (Aoki et al., 2003a; Su et al., 2003; Park and Oh, 2007; Granato et al., 2010).

Numerous legumes have been consumed in daily dietary either as a staple or nutrient added foods. Among vegetarians, legumes are their protein substitute and through the consumption of legumes, it could help to reduce the risks of multiple diseases (Dunham and Kollar, 2006). Glycine max or generally known as soybean has been extensively consumed all over the world (Cherng et al., 2007; Hartman et al., 2011). Soybean contains several phytochemicals and phytonutrients which are stipulated to possess many potential health benefits (Omoni and Aluko, 2005; Mateos-Aparicio, 2008). Numerous soybean products are available and the most widely consumed products in Asian region are in fermented form. Several studies have revealed that fermented soybeans could potentially enhance the immune system and alleviate several diseases including cancer, cardiovascular, osteoporosis and obesity (Messina, 2003; Villares et al., 2011).

Tempeh is one of fermented soybean that is widely known among vegetarians and also a traditional food for many people in Indonesia and Malaysia. Tempeh has been consumed extensively by people around Europe, United States and Japan due to its high level of nutritional contents (Nout and Kiers, 2005). As one of nutritious
soybean fermented food, tempeh has been claimed to possess many health benefits including lowering the risks of heart disease, stroke, osteoporosis, cancer, digestive disorder and to reduce weight and symptoms of menopause (Astuti et al., 2000; Babu et al., 2009). The elevated nutritious substances in tempeh such as amino acid and antioxidant have attracted many investigators to study the properties of tempeh. Study towards its nutrient quality had brought Aoki et al. (2003a) to develop a method to increase its nutrient content including GABA, amino acids and antioxidants of tempeh via anaerobic fermentation (Aoki et al., 2003a; Watanabe et al., 2007). R. oligosporus 5351 strain from MARDI was chosen in this study as a starter culture of tempeh fermentation since it was observed to generate the highest content of amino acids and GABA during aerobically fermentation of tempeh as compared to other R. oligosporus strain (Koh et al., 2012). In this study, Nutrient Enriched Soybean Tempeh (NESTE) was produced by combination process of aerobic and anaerobic fermentation as well as using R. oligosporus 5351 strain as a starter culture. In addition, bioactivities including cytotoxicity, immunomodulatory, anti-inflammatory and hepatoprotective activities of its nutritional contents were also evaluated.

Therefore, the objectives of this study were:

1. to establish an improved technique in order to enrich the nutrient component in tempeh via anaerobic incubation.

2. to determine the amino acid, GABA and antioxidant content in the fermented tempeh produced via anaerobic incubation.

3. to evaluate the crude sample of anaerobic tempeh for its cytotoxic effect towards breast cancer cell line, immunomodulator, anti-inflammation, alcoholic liver damage ameliorator as well as to determine the acute toxicity level.
REFERENCES


Ahmad, A., Ramasamy, K., Jaafar, S. M., Majeed, A. B. A. and Mani, V. (2014) Total isoflavones from soybean and tempeh reversed scopolamine-induced amnesia, improved cholinergic activities and reduced neuroinflammation in brain, Food and Chemical Toxicology, 65, 120-128.

Ani, V. and Naidu, Kamatham A (2011) Antioxidant potential of bitter cumin (Centratherum anthemumicum (L.) Kuntze) seeds in in vitro models, BMC Complementary and Alternative Medicine, 11, 40, 1-8


max (L.) Merill) and sprouts grown under different conditions, *European Food and Research Technology.*, 222, 201-208.


