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PREFACE

Faculty of Pharmacy UKM was established with a vision to be recognised globally as the pacesetter in the pharmacy education, research and services to the profession and society since June 2008. The faculty is committed to produce graduates who are well versed in pharmaceutical sciences, competent, competitive, confident and able to work effectively in various areas of pharmacy practice.

This research activity is being conducted yearly as part of the faculty programme to engage the interaction between academicians and postgraduate students from various pharmacy disciplines to strengthen their research fields through scientific deliberation, exchange of ideas and experiences. This year, distinguished speakers and participants from the other faculties and outside UKM were invited to join in this event.

This proceeding contains selected conference presentations in the area of pharmaceutical chemistry, pharmaceutics, pharmacology, pharmacy practice and clinical pharmacy.

It is hope that ‘Proceeding of Pharmaceutical Sciences Research Day 2015’ will be a useful document in enhancing research and practice activities.

Endang Kumolosasi
Chief Editor
EXTENDED ABSTRACTS
The Effect of δ-tocotrienol on Insulin Secretion-Associated Genes Expression in Basal and Stimulant Glucose Condition in Rat Pancreatic Islets Culture

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes that develops in individuals with poor glycemic control and high glucose level in the blood [1]. It is a chronic metabolic disorder characterized by impairment of insulin sensitivity, relative deficiency of insulin secretion [2] and loss of insulin responsiveness in peripheral tissues [3]. The chronic complications of T2DM lead to development of hyperglycemia with long-term clinical problems and onset of chronic complications [4].

Palm oil tocotrienol isomers, the naturally occurring vitamin E derivatives have shown therapeutic potential in secondary complications such as hyperlipidemia induced by diabetes [5]. However, there is no current evidence in the effect of γ-tocotrienol with insulin synthesis level. The aim of this study was to evaluate the effect of δ-tocotrienol on the gene expression of rat pancreatic islets culture in basal (2.8 mM) and stimulant (16.7 mM) glucose induction medium.

EXPERIMENTAL METHODS

Male Wistar rats weighing 300-500g were used as pancreatic islet donors. The pancreatic islets were freshly isolated and cultured overnight in supplemented RPMI1640 (Gibco, Invitrogen, USA) medium (10% v/v FBS, 1% v/v AA) pH-7.2 [6, 7]. A group of 20 islets were picked from the culture and divided into negative control group [basal (2.8mM) glucose buffer], positive control group [1 μM glyburide in basal (2.8 mM) or stimulant (16.7 mM) glucose buffer] and tocotrienol treated group (0.25 mg/ml or 0.125 mg/ml of δ-tocotrienol, added with basal or stimulant glucose buffer respectively). All groups were incubated for 1 hour at 37°C [8].

The cells were collected for the total RNA extraction and reverse-transcribed (SuperScript® III Reverse Transcriptase, Lifetechnologies). Quantitative gene expression (qPCR) (SYBR® Select master mix) was performed on peroxisome proliferator-activated receptor (PPAR) and insulin secretion-associated genes [peroxisome proliferator-activated receptors δ (PPARδ), PPARγ, insulin 1 (INS1), and neurogenic differentiation 1 (BETA2)].

RESULTS AND DISCUSSION

The insulin secretion-associated genes mRNA expression levels of δ-tocotrienol treated groups were compared to negative control and glyburide treated positive control group. All δ-tocotrienol treated groups showed higher gene expression compared to the control groups. δ-tocotrienol treated groups in stimulant glucose buffer showed higher mRNA expression level compared to that of basal buffer group. Higher tocotrienol concentration (0.25 mg/ml) further increased the gene expression level compared to 0.125 mg/ml. All the genes from 0.25 mg/ml stimulant group showed significantly greater mRNA gene expressions compared to the negative control group.

PPARδ mRNA gene expressions in 0.125 and 0.25 mg/ml basal group were 28% and 68.9% greater than that of positive control group. In stimulant group, the gene expressions in 0.125 and 0.25 mg/ml were 55.2% and 74% greater than positive control group respectively. PPARγ showed 10.6% and 119.9% greater than its positive control groups in 0.125 and 0.25 mg/ml in mRNA expression respectively. The gene expression further increased to 22.6% and 44.5% greater than positive control group in 0.125 mg/ml and 0.25 mg/ml stimulant group. INS1 gene expressions in 0.125 and 0.25 mg/ml basal group were 10.6% and 170.3% respectively, significantly increasing to 81.8% and 248.9% in stimulant group compared to the positive control group. BETA2 gene expressions in 0.125 and 0.25 mg/ml basal group were 36% and 62.8% respectively, significantly increasing to 55.5% and 128.1% in stimulant group compared to the positive control group.

δ-tocotrienol increased mRNA expression of PPARδ and γ, through direct binding to the ligand-gated receptor site, induced an increase of their transcriptional activities in glucose homeostasis [9]. Metabolic regulation of INS1 gene expression by δ-tocotrienol enables the maintenance of sufficient intracellular insulin in β-cell to sustain the secretory demand especially in high glucose condition [10]. The activation of BETA2 by δ-tocotrienol mediates the binding of insulin promoter elements to corresponding sites in glucose-induced insulin gene transcription especially by stimulatory glucose treatment group, promoting preproinsulin mRNA synthesis which is crucial for insulin synthesis [11].
Figure 1. Comparison of relative gene expression of (A) PPAR δ, (B) PPAR γ, (C) INS1 and (D) BETA2 in pancreatic islets for negative control: basal (2.8 mM) glucose buffer, positive control: 1 μM glyburide, basal: 2.8 mM glucose buffer, and stimulant: 16.7 mM glucose buffer

* P<0.05 significance difference when compared to negative control
@ P<0.05 significance difference when comparing basal and stimulant within same concentration of δ-tocotrienol treatment group
# P<0.05 significance difference when comparing 0.125 mg/ml and positive control in stimulant group
## P<0.05 significance difference when comparing 0.125 mg/ml and 0.25 mg/ml stimulant group
### P<0.05 significance difference when comparing 0.25 mg/ml and positive control in stimulant group

P<0.05 significance difference when comparing 0.125 mg/ml and 0.25 mg/ml basal group

CONCLUSION

These results showed that δ-tocotrienol could increase insulin secretion-associated genes expression in high glucose-contained medium, with the concentration of 0.25 mg/ml δ-tocotrienol showing higher insulin secretion-associated genes expression than 0.125 mg/ml. From the pharmaceutical point of view, the role of δ-tocotrienol has significant clinical implications in the management of T2DM.

REFERENCES


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Novel Synthesis of 2-(3, 4-Dimethoxyphenyl)-3-(4-fluorophenyl)-6-methoxy-4H-chromen-4-one as a Selective COX-2 Inhibitor

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are useful therapeutics for the treatment of pain and inflammation, particularly arthritis. The main drug target of NSAIDs is cyclooxygenase (COX), which bio-catalyzes the first committed step in arachidonic-acid metabolism [1]. Most NSAIDs not only selectively inhibit the production of prostaglandins (PGs) at the sites of inflammation but also other PGs which serve important functions in other parts of the body, a factor which accounts for some of the toxicity of these agents. The most frequent complication associated with NSAIDs usage can be found in the gastrointestinal tract (GIT). GI bleeding, ulceration, perforation, and obstruction are a significant cause of morbidity and mortality in patients who are treated with these agents [2, 3]. To counter these limitations, novel scaffolds with selective COX-2 or mPGES-1 inhibition need to be designed and evaluated [4].

Since COX-2 enzyme, also known as PGI synthase was cloned in 1992 [5], DuPont company had developed a compound namely DuP-697, that was potent in many anti-inflammatory assays and did not have the ulcerogenic effects like any conventional NSAIDs in rats at a single dose up to 400 mg/kg [6]. DuP-697 eventually became the major building-block for the synthesis of many other new COX-2 inhibitors. Celecoxib and rofecoxib, the first two COX-2 inhibitors that reached the market, were generally based on DuP-697. Celecoxib (Celebrex®) is also the first and only COX-2 inhibitor available in the United States. However, rofecoxib (Vioxx®) and valdecoxib (Bextra®) were known to increase the risk of heart attacks and strokes with long term use, therefore, both drugs were withdrawn from the market in September 2004 and April 2005 [7-11]. This eventually led to the development of new selective COX-2 inhibitors that could act effectively without exerting major side effects associated with the traditional NSAIDs [12]. The solving of the 3D structures of an unliganded murine COX-2 and SC-558-protein complex by Kurumbail et al. (1996) finally shed some light on the structural basis for the selective inhibition of COX-2 which also demonstrates some of the conformational changes associated with time-dependent inhibition [13]. The development of new generation of selective COX-2 inhibitors typically relied on the structural modifications of coxib drugs, for example DFU, etoricoxib, parecoxib, and MPO-0029 [14-16].

Recent results published by Gilad et. al showed that coxibs actually interfere with the action of aspirin by binding tightly to one monomer of cyclooxygenase-1 [17]. The administration of celecoxib to dogs interfered with the ability of a low dose of aspirin to inhibit AA-induced ex vivo platelet aggregation. Because coxibs exhibit cardiovascular side effects, they are often prescribed in combination with low-dose aspirin to prevent trombosis. Their studies have shown that the cardioprotective effect of low dose aspirin on COX-1 may be blunted when taken with coxibs. This interesting finding has encouraged our group in finding and developing potential novel selective COX-2 inhibitors from the lead structure of 2-(3, 4-dimethoxyphenyl)-3-(4-fluorophenyl)-6-methoxy-4H-chromen-4-one.

EXPERIMENTAL METHODS

Chemical synthesis and characterization

All reagents were purchased from Aldrich, Acros and Merck and were used without further purification. All the solvents used in the syntheses were analysis and synthesis grade. The solvents used in spectroscopic measurements were spectroscopic grade. Melting points were determined on a STUART SMP10 melting point apparatus. 1H, 13C NMR, DEPT, HMOC and HMB spectra were recorded on a Bruker 500 MHz spectrometer. ESI-HRMS spectra were recorded on a Bruker microTOF Mass Spectrometer.

Biology evaluation

Determination of PGE2. The cell culture supernatants were collected and analyzed for PGE2 secretion PGE2 EIA kits (Cayman Chemical, Ann Arbor, MI, USA). The protocols provided by the manufacturers were followed to the detail. The data was acquired using a SpextraMax Plus microplate reader (Molecular Device, Sunnyvale, CA, USA). The concentration of PGE2 for each sample was calculated from their respective standard curves. Cyclooxygenase Assay. The effect of synthetic compound on COX-1 and COX-2 was determined by using a COX Inhibitor Screening Kit (Catalog No. 560131) from Cayman Chemicals, Ann Arbor, Michigan, U.S.A. The reaction mixture was prepared with 1μM heme and COX-1 (ovine) or COX-2 (human recombinant) enzymes in 100mM Tris-HCl buffer, and then pre-incubated for 10 min in a water...
bath (37 °C) at pH 8.0. The reaction was started by adding 10 μL arachidonic acid (final concentration in reaction mixture 100 μM). After 2 min, the reaction was terminated by adding 1M HCl and lastly PGE₂ was quantified by ELISA. The compounds were dissolved in DMSO and diluted to desired concentration with potassium phosphate buffer. After transferring the compounds to 96 well plate coated with a mouse anti-rabbit IgG, tracer prostaglandin acetylcholine esterase and primary antibody (mouse anti PGE₂) were added and the plates were kept for overnight. Thereafter, the reaction mixtures were removed and the wells were washed with 10 mM potassium phosphate buffer containing 0.05% Tween 20. Ellman’s reagent (200 μL) was then added to all the wells and were incubated at room temperature for 60 min, until the control wells gave an OD = 0.3-0.8 at 412 nm. A standard curve with PGE₂ was generated from the same plate, which was used to calculate the PGE₂ levels produced in the presence of test compounds. The results were expressed as percentage relative to control (solvent treated samples). All the experiments were performed in triplicate and values normally agreed within 10%.

Molecular docking
Docking studies were performed with the LibDock protocol under the receptor-ligand interaction section in Discovery Studio® 3.1 (Accelrys, Inc., San Diego, CA, USA). All of the 3D structures of the compounds were built with ChemBioOffice® 2008 (Perkin Elmer, Inc., Waltham, MA, USA). The protein crystal structure of the inhibitor-bound COX-1/COX-2 was retrieved from the Brookhaven Protein Data Bank (PDB codes: 6COX and 3KK6). Both proteins were pre-treated before the docking. Hydrogen atoms were added to the protein structure, and all ionisable residues were set at their default protonation of pH 7.4 while the ligands were prepared and minimized. For compound 22, the crystal structure obtained from X-ray crystallographic analysis was used as the starting conformation for the docking study. During the docking process, the receptor was held rigid while the ligands were allowed to flex during the refinement. Number of polar or apolar receptor hotspots for conformer matching starting were set at 500 with the docking tolerance 0.25 Å. The conformations generate of the ligands was set 500 within this relative energy threshold 20 kcal/mol.

RESULTS AND DISCUSSION

![Figure 1](attachment:figure1.png)

Figure 1. Replacement of the central core structures of known COX-2 inhibitors with a chromone ring

![Figure 2](attachment:figure2.png)

Figure 2. Retrosynthetic analysis for the synthesis 2,3-diaryl benzopyran

![Scheme 1](attachment:scheme1.png)

Scheme 1. Reagents and condition of synthesis: (a) KOH 40%, EtOH, (i) sonicate 1 h at 40ºC (ii) stir at room temperature for overnight; (b) I₂, DMSO, reflux 1 h; (c) Bu₄NBr/Phl(OAc)₂, CH₂Cl₂, rt 8 h; (d) 4-Fluorophenylboronic acid, Pd(OAc)₂, PEG 400, K₂CO₃, 60 ºC 20 h.
Synthesis of (E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxy-4-methoxy phenyl) prop-2-en-1-one (3)

Compound 3 as yellow solid, yield 95%, mp. 157-159 °C; 1H NMR (500 MHz, CDCl3) δ 13.56 (s, 1H), 7.87 (t, 2H), 7.46 (d, J = 15.4 Hz, 1H), 7.27 (dd, J = 8.3, 1.8 Hz, 1H), 7.18 (d, J = 1.9 Hz, 1H), 6.93 (d, J = 8.3 Hz, 1H), 6.51 (dd, J = 11.5, 2.5 Hz, 2H), 3.99 (s, 3H), 3.96 (s, 3H), 3.88 (s, 3H). 13C NMR (126 MHz, CDCl3) δ 191.95, 166.81, 166.24, 151.79, 149.50, 144.72, 131.26, 127.99, 123.44, 114.24, 111.38, 110.51, 107.78, 101.23, 77.16, 56.17, 55.71. ESI-HRMS: (C18H18O5) calc. [M+Na+] 337.1052, found 337.1078.

Synthesis of 2-(3,4-dimethoxyphenyl)-7-methoxy-4H-chromen-4-one (4)

Compound 4 as pale yellow crystal, yield 52%, mp. 184-185 °C; 1H NMR (500 MHz, CDCl3) δ 8.14 (d, J = 8.8 Hz, 1H), 7.55 (dd, J = 8.5, 2.1 Hz, 1H), 7.38 (d, J = 2.1 Hz, 1H), 6.99 (dt, J = 6.9, 2.6 Hz, 3H), 6.72 (s, 1H), 4.00 (s, 3H), 3.98 (s, 3H), 3.95 (s, 3H). 13C NMR (126 MHz, CDCl3) δ 177.76, 164.14, 163.11, 157.93, 152.01, 149.32, 127.03, 124.36, 119.87, 117.74, 114.25, 111.20, 108.88, 106.36, 100.47, 56.11, 56.08, 55.85. ESI-HRMS: (C18H18O5) calc. [M+Na+] 335.0896, found 335.0950.

Synthesis of 3-bromo-2-(3,4-dimethoxyphenyl)-7-methoxy-4H-chromen-4-one (5)

Compound 5 as white solid, yield 98%, mp. 165-166 °C; 1H NMR (500 MHz, CDCl3) δ 8.20 (d, J = 8.9 Hz, 1H), 7.52 (dd, J = 8.4, 2.1 Hz, 1H), 7.43 (d, J = 2.1 Hz, 1H), 7.05-7.00 (m, 2H), 6.91 (d, J = 2.3 Hz, 1H), 4.00 (s, 3H), 3.98 (s, 3H), 3.94 (s, 3H). 13C NMR (126 MHz, CDCl3) δ 172.47, 164.42, 161.24, 157.31, 151.35, 148.53, 127.95, 125.23, 123.06, 115.67, 115.08, 112.47, 110.57, 108.73, 99.92, 56.19, 56.06, 55.90. ESI-HRMS: (C18H16O5) calc. [M+Na+] 415.0001, found 415.0003.

Synthesis of 2-(3,4-dimethoxyphenyl)-3-(4-fluorophenyl)-7-methoxy-4H-chromen-4-one (6)

Compound 6 as white crystals, yield 64%, mp. 197-198 °C. Good quality crystals were obtained by recrystallization from absolute ethanol. 1H NMR (500 MHz, CDCl3) δ 8.20 (d, J = 8.9 Hz, 1H), 7.25 (dd, J = 8.4, 2.1 Hz, 1H), 7.43 (d, J = 2.1 Hz, 1H), 7.05-7.00 (m, 2H), 6.77 (d, J = 8.5 Hz, 1H), 3.96 (s, 3H), 3.91 (s, 3H), 3.61 (s, 3H). 13C NMR (126 MHz, CDCl3) δ 176.68, 164.21, 163.19, 160.93, 157.66, 150.61, 148.53, 127.95, 125.23, 123.06, 115.67, 115.08, 112.47, 110.57, 108.73, 99.92, 56.19, 56.06, 55.90. ESI-HRMS: (C24H19FO5) calc. [M+Na+] 429.1115, found 429.1120.

Figure 3. Effects of 6 on PGE2 production in LPS-stimulated RAW 264.7 cells. The cells were co-incubated with LPS (5 µg/mL) and different concentrations of 6 ranging from 0.78 to 50 µM. The supernatants were then collected for the measurement of PGE2 using EIA kit. Compound 6 significantly inhibited PGE2 levels in LPS-stimulated macrophages. The values are expressed as the means ± S.D of three individual samples. ***P<0.001 as compared with the LPS-treated macrophages; significant differences between groups were determined using one-way ANOVA test followed by a Dunnett’s multiple comparison test. *P<0.05 solvent control compared with the LPS-treated cells; significant difference was determined using unpaired student’s t-test (n.s is non-significant).

Table 1. In vitro COX-1 and COX-2 inhibitory activities of 6 and Indomethacin

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (µM)</th>
<th>SI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>&gt;100</td>
<td>38.6</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.21</td>
<td>3.24</td>
</tr>
</tbody>
</table>

*Indomethacin a non-selective COX inhibitor was used as the positive control.
SI* Selectivity index (IC50 COX-1/IC50 COX-2 COX-1).
ΩDeviation from the mean is <10% of the mean value.
CONCLUSION

A hypothetical model of binding interactions between compound 6 bearing a chromone scaffold structure and human recombinant COX-2 protein is proposed in this study. With the successful structural elucidation of compound 6 using simple spectroscopic tools followed by the retrieved atomic coordinates from X-ray diffraction analysis and biological results, it can be concluded that the compound inhibited the PGE₂ synthesis by selectively binds to COX-2 over COX-1 protein. The major factor that contributes to the selectivity mainly due to the presence of chromone ring and bulky dimethoxyl groups as predicted by the molecular modeling programs. The important information obtained from this study will be utilized in the next design of more potent and selective COX-2 inhibitors.

REFERENCES


ACKNOWLEDGEMENTS

This work was financially supported by Science Fund (02-01-02-SF00665) and Fundamental Research Grant Scheme (FRGS/2/2014/ST01/UKM/02/3), Ministry of Science, Technology & Innovation, Malaysia. Authors also special thanks to UKM for the Zamalah scholarship.
Inhibitory Effects of Novel Chalcone Derivatives and Curcumin Analogues on Oxidized Palmitoyl Arachidonoyl Phosphorylcholine (OxPAPC)-induced Proinflammatory Activities in U-937 Cell Line

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INTRODUCTION
Atherosclerosis and related cardiovascular diseases represent one of the greatest threats to human health worldwide. Despite important progress in prevention and treatment, these conditions still account for one third of all death cases reported annually. Variety of research has been carried out to show that the major underlying risk factor of atherosclerosis is related to chronic inflammation [1]. In recent decades, it is well accepted that the accumulation of OxPAPC plays an important role in the development of vascular inflammation in atherosclerosis by inducing the production proinflammatory chemokines [2]. However, the underlying mechanisms involved are not yet fully elucidated. Increasing of evidence has been reported regarding the roles of chalcones and curcumin as potential therapeutic agents in the inhibition of various proinflammatory chemokines induced by lipopolysaccharide [3, 4], but less is known about their effects on the inflammatory activities induced by OxPAPC. Hence, this study was aimed to investigate the inhibitory potentials of newly synthesized chalcone derivatives and curcumin analogues induced by OxPAPC tested on the proinflammatory activities in phorbol myristate acetate (PMA)-differentiated U-937 macrophages.

EXPERIMENTAL METHODS
Autoxidation of PAPC
OxPAPC was yielded from autoxidation of PAPC with methods as previously described [5]. Solvent evaporation was carried under nitrogen stream and PAPC was allowed to autoxidize at room temperature (28ºC) for 7 days. Analysis of positive ion-electrospray ionization-mass spectrometry (ESI-MS) was carried out to monitor the purity of PAPC and autoxidation status of the yielded OxPAPC.

Cell Culture and cellular differentiation
U-937 cell line (ATCC® CRL-1593.2 TM) was purchased from ATCC, USA. The cells were cultured in RPMI medium supplemented with 10% foetal bovine serum (FBS) and 5% penicillin-streptomycin. The culture was maintained in humidified condition of 37ºC, 5% CO₂. Prior to the addition of tested compounds, cells were differentiated into macrophages phenotypes by incubation of PMA at 200 nM/mL for 24 hours. Recovery phase of the cells was carried out by incubation with serum-free media overnight.

Preparation of chalcone derivatives and curcumin analogues
Chalcone derivatives and curcumin analogues used were synthesized by Drug and Herbal Research Centre, Faculty of Pharmacy, UKM. Stock for every compound (50 µM) was prepared by dissolved in dimethyl sulfoxide (DMSO) solution. Compounds were diluted to desirable concentrations with serum-free media before use. In the present study, 6 chalcone derivatives (compound 1.1-1.6) and 6 curcumin analogues (compounds 2.1-2.6) were used.

Determination of cell viability
The cytotoxic effects of tested compounds on PMA-differentiated U-937 macrophages were determined using MTT assay adapted from protocols as previously described [6]. Cells were first treated with tested compounds with range of concentrations (6.25, 12.5, 25, 50, 100 µM) and incubated for 24 hours. Subsequently, MTT salt solution was added and incubated for another 4 hours. DMSO solution was used to dissolve the formazan crystal formed and analyzed at 565 nm using microplate reader (Tecan, Switzerland). Viability of cells was determined based on the optical density of formazan crystals. Concentrations of tested compounds with cell viability more than 90% were used for subsequent assays.

Analysis of chemokines released
PMA-differentiated U-937 macrophages were pre-treated with tested compounds (0, 6.25, 12.5, 25, 50 µM) for 1 hour and followed by incubation of OxPAPC (30 µg/mL) for 6 and 9 hours for interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) quantifications, respectively. Supernatants were collected and ELISA assays (eBioscience, USA) were carried out in accordance to manufacturer’s instructions. Cells with media and DMSO only were served as vehicle control while cells treated with simvastatin (20 µM) was served as a positive control.
Quantification of protein expression
PMA-differentiated U-937 macrophages were treated with tested compound, harvested, washed and lysed as prior study [7]. Lysates were subject to SDS-PAGE electrophoresis for separation of proteins according to molecular weights and transferred onto a polyvinylidene difluoride (PVDF) membrane. Western immunoblot was carried out to determine the target proteins; XBPI protein using anti-XBP1 antibody [ab37152] and beta-actin protein using anti-beta actin antibody [ab75186]. Bands of protein expression were determined by exposed to chemiluminescent substrate (ECL) and images were captured using FUSION FX-7 (Vilber Lourmat, Germany).

Statistical analysis
Data were obtained from three individual experiments and statistical analysis was carried out using GraphPad Prism5 software. Data with p-values ≤ 0.05 were regarded as statistical significant.

RESULTS AND DISCUSSION

Positive-ion ESI-MS Spectra

MTT assay
At 24 hours of incubation at 50µM of tested compounds, compounds 1.2, 1.4 and 1.5 of chalcone derivatives showed cell viability of 91.76 ± 0.83%, 92.03 ± 1.89% and 96.29 ± 0.32%, respectively. For curcumin analogues, compounds 2.1, 2.2, 2.3, 2.4 and 2.6 at 50 µM showed viability of cell of 93.81 ± 0.97%, 101.42 ± 1.14%, 106.99 ± 1.47% and 97.28 ± 0.92%, respectively.

ELISA assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀(µM) for IL-8</th>
<th>IC₅₀(µM) for MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>45.46 ± 1.78</td>
<td>ND</td>
</tr>
<tr>
<td>1.4</td>
<td>27.32 ± 4.84</td>
<td>23.45 ± 0.47</td>
</tr>
<tr>
<td>1.5</td>
<td>18.33 ± 1.59</td>
<td>13.05 ± 1.37</td>
</tr>
<tr>
<td>2.1</td>
<td>11.56 ± 4.68</td>
<td>35.65 ± 1.48</td>
</tr>
<tr>
<td>2.2</td>
<td>21.42 ± 1.65</td>
<td>15.31 ± 2.73</td>
</tr>
<tr>
<td>2.3</td>
<td>7.33 ± 2.81</td>
<td>20.66 ± 2.68</td>
</tr>
<tr>
<td>2.6</td>
<td>32.02 ± 3.19</td>
<td>12.98 ± 1.97</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>5.97 ± 0.93</td>
<td>8.42 ± 1.05</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM; n=3. ND represents IC₅₀ value not determined.

Table 1. Inhibitory activities (IC₅₀) of test compounds
Western immunoblot

XBP-1

Comp. 1.2
Comp. 1.4
Comp. 1.5

Beta actin (loading control)

CONCLUSION

The present study demonstrated that chalcone derivatives and curcumin analogues possessed potential inhibitory effects against OxPAPC-induced inflammation. Thus, further molecular investigation to provide useful evidence in developing more specific anti-inflammatory agents.

REFERENCES


ACKNOWLEDGEMENTS

This research was supported by a grant GGPM-2013-059 from Universiti Kebangsaan Malaysia (UKM). The authors would like to extend appreciation to the Molecular Structure Characterization Laboratory, Centre for Research and Instrumentation Management (CRIM), UKM for providing the ESI-MS services. Special thanks also to UKM for the Zamalah scholarship.
Inhibitory Effects of *Labisia Pumila* on Monosodium Urate Crystals-Stimulated Cytokine Production: *In Vitro* and *In Vivo* Studies

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*Corresponding author: eldizapujirahmi89@gmail.com

**INTRODUCTION**

Inflammation in joint, known as gouty inflammation is characterized by recurrent attack of acute inflammation in response of accumulation of monosodium urate (MSU) crystals in joint, tendon and tissue. During inflammatory response, a variety of soluble factors are involved in leukocyte recruitment such as inflammatory lipid metabolites (platelet activating factor, prostaglandins, leukotrienes and lipoxins) and a group of cell-derived polypeptides, known as cytokines, which play a key role in the inflammatory response [1].

*Labisia pumila* (Primulaceae) is a Malaysian traditional plant and popularly known as “Kacip Fatimah”. It has been used by many generations of the Malay women as traditional medicine to reproductive function, in terms of its ability to induce and facilitate childbirth and also for ‘sickness in the bones’ [2]. Botanically, there are three varieties of *L. pumila*, that are, var. *alata*, var. *pumila* and var. *lanceolata*, and these can be differentiated by the characteristics of the petioles and leaf-shape [3]. Previous scientific studies revealed that extracts of *L. pumila* had various activities such as anti-oxidant [4], xanthine oxidase inhibitory activity [5] and anti-inflammatory [6]. However, there has been no report of effects of *L. pumila* extracts on MSU-induced inflammation. Thus, this study was an attempt to explore the possibility of effect of *L. pumila* extracts on gouty inflammatory response by *in vitro* and *in vivo* assays.

**EXPERIMENTAL METHODS**

**Plant materials**

The leaves and roots of three varieties of *Labisia pumila* were collected from Hutan Gunung Bujang Melaka, Kampar, Perak, Malaysia. The species and varieties were differentiated and authenticated, and the voucher specimens were deposited at the Herbarium of Faculty of Science and Technology, UKM (var. *pumila* UKMB 30006(SM s.n), var. *alata* UKMB 30007 (SM s.n), and var. *lanceolata* UKMB 30008(SM s.n)).

**Preparation of plant extract**

Dried powder of each plant part was successively extracted with 80% ethanol by maceration for 3 days. The organic filtrate was collected and concentrated under reduced vacuum pressure to obtain the crude extracts. This procedure was repeated ten times, the extracts were combined and then freeze-dried.

**Synthesis of monosodium urate (MSU) crystals**

MSU crystals were synthesized by carrying out the modified procedure described by Sabina *et al.* [7]. Briefly, about 4 g of uric acid was dissolved and heated in 800 mL H$_2$O with NaOH (9 mL/0.5 N); adjusted to pH 8.9 at 60°C by adding HCl; cooled for 3 days in cold room; washed and dried. Then, needle-shaped crystals were suspended in sterile saline (100 mg/mL).

**Effect of cytokine production in monosodium urate (MSU) crystals-stimulated peripheral blood mononuclear cells (PBMCs)**

a. **Cell preparation**

Cell preparation procedure was according to modified method of Boyum [8]. Briefly, venous blood from healthy volunteer was diluted in a ratio of 1:1 with RPMI-1640 medium containing L-glutamine. PBMCs were separated from the blood by Lymphoprep gradient centrifugation at 600 x g for 20 minutes at 25°C. The cells were washed twice with medium and suspended in a complete medium containing RPMI-1640 medium with L-glutamine buffered with 20 mM HEPES to pH 7.4 and supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal bovine serum, and adjusted up to 5x10$^7$/mL. Cell viability was determined by MTT assay using 3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide; thiazolyl blue (MTT) as described by Mosmann [9] with slight modification.

b. **Cytokine Assay**

Cells were pre-incubated with control or test extracts at 37°C containing 5% CO$_2$ for 3 hours prior to stimulation with MSU crystals (20 mg/dL). After 24 hours of stimulation, samples were collected and cell-free supernatants were obtained by centrifugation at 300 x g and 4°C for 10 min and stored at -80°C until analysed by ELISA for interleukin (IL)-1α, IL-1β, IL-6 and tumor necrosis factor (TNF)-α [10]. Furthermore, extract that gave the highest inhibitory activity was used in the *in vivo* study.
Effect of monosodium urate (MSU) crystals-induced inflammation in rats

An experimental model of gouty inflammation using MSU crystals as inducer was performed according to Zhang et al. [11] with slight modification. Thirty-six male Sprague-Dawley rats (6-8 weeks old, 300-350 g) were divided into six groups (n=6). Groups 1 and 2, normal control and gouty inflammation control, respectively, received only vehicle. Group 3, positive control, animals were treated with indomethacin (3 mg/kg body weight). Groups 4, 5 and 6 were treated with L. pumila var. pumila root extract (50, 100, 200 mg/kg BW). Treatments were administered once a day by oral gavage, for 14 days. At 11th day, the inflammation was induced by intra-articular injection of 50 μL of MSU crystals suspension inside the knee joint of rat’s right limb. In order to obtain synovial fluid, rats were anaesthetized with ketamine and xylazine (100 and 20 mg/kg, respectively). After skin shaving, a 27 needle was inserted into the synovial cavity to inject 100 μL sterile saline, while the second needle was inserted next to the first needle to collect synovial fluid. The inflammation was quantified by measuring IL-1α, IL-1β, IL-6 and TNF-α in the synovial fluid by using a multi-cytokine bead array detection system (Luminex assay) according to manufacturer’s instruction.

Statistical Analysis

All the data were analysed using Statistical Package for Social Sciences (SPSS) version 21.0. The IC50 values were calculated using GraphPad Prism 5 software. The values were obtained from at least three determinations (n=3) and the data presented as mean±standard error of mean (SEM). Data were analysed using a one-way analysis of variance (ANOVA) for multiple comparisons and p<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of cytokine production in monosodium urate (MSU) crystals-stimulated peripheral blood mononuclear cells (PBMCs)

Viability of PBMCs was assessed by the MTT assay, which was based on the conversion of MTT to purple coloured formazan by mitochondrial dehydrogenases from viable cells. It can be solubilised and read visually or quantified by spectrophotometric measurement at 570 nm and was proportional to the number of living cells present [9]. This step was crucial to show that the inhibition of cytokines production was not due to cell death. At the concentration of 50 μg/mL of all of L. pumila extracts and 5 μg/mL of dexamethasone, percentage of cell viability was more than 90% (data was not shown). It indicated that 50 μg/mL of extract and 5 μg/mL of dexamethasone had no significant effect on cell viability after 24 hours of incubation.

Preliminary screening of six extracts of L. pumila varieties (50 μg/mL) on PBMCs was carried out (Table 1). IC50 values were then determined for the active extracts (inhibitory activity > 50%) in a concentration range of 3.125 to 50 μg/mL (Table 2). The results revealed that L. pumila var. pumila root extract possessed the highest inhibition on cytokines production for IL-1α, IL-1β and TNF-α in a dose-dependent manner with IC50 values of 35.71, 25.06, and 17.68 μg/mL, respectively.

Effect of monosodium urate (MSU) crystals-induced inflammation in rats

The inflammation was quantified by examining the changes in inflammatory markers attenuated by the treatment. Table 3 represents the activity of L. pumila var. pumila root extract on inflammatory mediators in the synovial fluid of experimental animals. The higher levels of IL-1α, IL-1β, IL-6, and TNF–α were found in synovial fluid of gouty inflammation control rats when compared with normal control animals. However, both extract and indomethacin significantly reduced levels of these inflammatory mediators to control animal values in dose-dependent manner.

Table 1. Inhibition of Labisia pumila extracts (50 μg/mL) on cytokines production in MSU-stimulated human PBMCs

<table>
<thead>
<tr>
<th>Samples</th>
<th>Percentage Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1α</td>
</tr>
<tr>
<td>Lanceolata</td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>52.53 ± 3.13</td>
</tr>
<tr>
<td>Leaves</td>
<td>47.22 ± 3.49</td>
</tr>
<tr>
<td>Pumila</td>
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<tr>
<td>Roots</td>
<td>65.28 ± 2.29</td>
</tr>
<tr>
<td>Leaves</td>
<td>38.54 ± 4.06</td>
</tr>
<tr>
<td>Alata</td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>26.51 ± 3.96</td>
</tr>
<tr>
<td>Leaves</td>
<td>22.63 ± 0.70</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>87.45 ± 0.83</td>
</tr>
</tbody>
</table>

Percentage inhibition values are presented as mean ± SEM (n = 3). ND = not detected.
Percentage inhibition > 8% was significant at p<0.05 when compared with negative control.
*p<0.05 was considered not significant compared with dexamethasone (positive control).
Gout is caused by increased levels of uric acid in blood (hyperuricemia) due to metabolism disorder of purine, leading to crystallisation of MSU and are deposited in the joint, tendon and tissue, and could be characterized by recurrent attack of acute inflammation [12]. These microcrystals interact with all of the major synovial cell types to produce a variety of inflammatory mediators. MSU crystals promote inflammation response by stimulating cells via toll-like receptor signaling and culminating in secretion of inflammatory mediators such as cytokines with a significant influx of neutrophils into the joint [13]. Pro-inflammatory cytokines undoubtedly have a critical role in the inflammatory response to MSU crystals. Recent attention has focused particularly on the role of IL-1. Besides IL-1, IL-6, IL-8 and TNF-α are also upregulated when MSU crystals interact with all of the major synovial cell types to produce a variety of inflammatory mediators. MSU crystals promote inflammation response by stimulating cells via toll-like receptor signaling and culminating in secretion of inflammatory mediators such as cytokines with a significant influx of neutrophils into the joint [13]. Pro-inflammatory cytokines undoubtedly have a critical role in the inflammatory response to MSU crystals. Recent attention has focused particularly on the role of IL-1. Besides IL-1, IL-6, IL-8 and TNF-α are also upregulated when monocytes are in contact with MSU in vitro and with gouty tissues in vivo.

Previous scientific studies have been conducted to identify the phytochemicals from L. pumila. Karimi et al. [14] reported the presence of phenolics and flavonoids in L. pumila. The presence of these compounds might contribute to the inhibitory effect of L. pumila on MSU-induced inflammation. Flavonoids have been reported to present significant anti-inflammatory effects. Huang et al. [15] reported that quercetin has a significant inhibitory effect on MSU crystal-induced inflammation in rat in dose-dependent manner by reducing levels of inflammatory mediators such as IL-1β, TNF-α, NO, COX-2 and PGE2. Another study by Yoon et al. [16] revealed that gallic acid reduced the expression of pro-inflammatory gene in rheumatoid arthritis fibroblast-like synoviocytes.

Since quercetin and gallic acid have been observed in L. pumila var. pumila root, the cytokine production on MSU-induced inflammation might be suppressed by these compounds. However, the inhibitory activity of the extracts was found to be uncorrelated with the amount of these compounds. Karimi et al. [14] reported that L. pumila var. pumila leaves had the highest concentration of quercetin (106.7 μg/mg dry sample) compared to the others, while L. pumila var. alata leaves had the highest concentration of gallic acid (449.5 μg/mg). These results indicated that there may be other compounds that gave a potent inhibition of MSU-stimulated cytokine production.

CONCLUSIONS
This study has demonstrated that L. pumila var. pumila root extract has high potential inhibitory effect on MSU-stimulated pro-inflammatory cytokines production in vitro and in vivo in dose-dependent manner that may be useful as an adjuvant for the treatment of gouty inflammation. However, further work is required to determine the bioactive constituent(s) responsible for the effect.

REFERENCES

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Table 2. IC50 values (μg/mL) of cytokine production inhibitory activities of Labisia pumila extracts on cytokine production in MSU-stimulated human PBMCs

<table>
<thead>
<tr>
<th>Samples</th>
<th>IL-1α IC50 (μg/mL)</th>
<th>IL-1β IC50 (μg/mL)</th>
<th>IL-6 IC50 (μg/mL)</th>
<th>TNF-α IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanceolata</td>
<td>45.96 ± 5.23</td>
<td>44.72 ± 5.53</td>
<td>-</td>
<td>15.38 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>35.69 ± 1.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pumila</td>
<td>35.71 ± 2.08</td>
<td>25.06 ± 1.60</td>
<td>-</td>
<td>17.68 ± 1.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alata</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.02 ± 0.002</td>
<td>0.71 ± 0.24</td>
<td>0.46 ± 0.03</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>

Data are mean ± SEM (n = 3).

Table 3. Effect of Labisia pumila var. pumila root extract on inflammatory mediator production in monosodium urate crystal-induced inflammation on rat knee joint

<table>
<thead>
<tr>
<th>Treatments</th>
<th>IL-1α Concentration (pg/mL)</th>
<th>IL-1β Concentration (pg/mL)</th>
<th>IL-6 Concentration (pg/mL)</th>
<th>TNF-α Concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>576.83 ± 83.25*</td>
<td>1002.2 ± 73.99*</td>
<td>796.8 ± 92.78*</td>
<td>1373.67 ± 93.29*</td>
</tr>
<tr>
<td>MSU Control</td>
<td>1056.11 ± 60.4</td>
<td>1448.73 ± 75.21</td>
<td>1170 ± 48.11</td>
<td>1825.33 ± 138.26</td>
</tr>
<tr>
<td>Indomethacin (3 mg/kg)</td>
<td>503.49 ± 40.73*</td>
<td>1055.13 ± 107.95*</td>
<td>720.27 ± 88.22*</td>
<td>1361.67 ± 104.26*</td>
</tr>
<tr>
<td>Extract (50 mg/kg)</td>
<td>887.61 ± 46.87</td>
<td>1398.4 ± 66.6</td>
<td>997.87 ± 61.89</td>
<td>1765 ± 121.26</td>
</tr>
<tr>
<td>Extract (100 mg/kg)</td>
<td>643.33 ± 33.06*</td>
<td>1238.2 ± 85.25</td>
<td>901.33 ± 45.53</td>
<td>1510.67 ± 63.09</td>
</tr>
<tr>
<td>Extract (200 mg/kg)</td>
<td>647.45 ± 66.63*</td>
<td>1126.8 ± 33.22*</td>
<td>828.8 ± 36.61*</td>
<td>1372 ± 106.44*</td>
</tr>
</tbody>
</table>

Data represent mean±SEM of 6 animals.
For statistical significance, Student’s t-test was used between control and drug groups. *< 0.05, versus MSU control group.


ACKNOWLEDGEMENTS
The authors greatly appreciate financial support from The Ministry of Agriculture and Agro-Based Industry Malaysia, grant number NH0711D002.
Designing and Improving Linker Stability of Demethoxycurcumin Analogues as Pro-inflammatory Prostaglandin E₂ Modulators and Nitric Oxide Attenuators against Human and Murine Macrophages

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INTRODUCTION

Macrophages are the main pro-inflammatory cells responsible for occupying pathogen by releasing many pro-inflammatory molecules such as nitric oxide (NO) and prostaglandin E₂ (PGE₂). High levels of NO and PGE₂ in a chronic inflammation state can result in various pathological conditions. PGE₂, a product of the cyclooxygenase (COX) pathway is well identified as the lipid mediator that contributes to inflammatory diseases such as cancer, rheumatoid arthritis, atherosclerosis, and pain. Inhibition of both COX isoforms (COX-1 and COX-2) may lead to undesirable side effects such as life threatening gastrointestinal perforation, ulcer and bleeding (PUB) as well as renal toxicity. Accordingly, control of the production of NO and PGE₂ in macrophages are current research topics for the development of new anti-inflammatory agents. The main objective of this study is to synthesize and optimize a series of novel COX-2 inhibitors based on the structure of demethoxycurcumin, a natural unsymmetrical curcumin derivative and to determine the biological effects of the compounds on COX-1 and COX-2 activities as well as PGE₂ and NO attenuation in vitro (human and murine macrophages), respectively.

RESULTS AND DISCUSSION

Despite the fact that cyclohexanone analogues of curcumin and bisdemethoxycurcumin have been well studied in recent years, the anti-inflammatory effects of cyclohexanone analogues of demethoxycurcumin have never been disclosed to date. To initiate this study, first the β-diketone fragment and methoxy group were replaced with a rigid cyclohexanone and different substituents, respectively (See Fig. 1). In order not to limit the structure-activity relationship scope, different linker with different substituted benzene were proposed (See Fig. 2). Therefore, ring A and ring B was altered by varying substituents on aromatic ring to test the bulkiness and electronic influences to the binding pocket. Linker was also varying to search for others possible binding region. All spectroscopy methods consistent with the assigned structure of the synthesized compounds. We hypothesized that the designed compounds could shows promising anti-inflammatory activity via inhibition of PGE₂ production through COX-2 pathway without exerting cardiovascular side effects. Since demethoxycurcumin have been reported to be more active than curcumin and bisdemethoxycurcumin in attenuation of NO and PGE₂ production, we predict that the cyclohexanone analogues of demethoxycurcumin could possibly exhibits better NO and PGE₂ attenuation via COX pathway than cyclohexanone analogues of curcumin (BHMC) and bisdemethoxycurcumin, respectively.

Figure 1. Design strategy of cyclohexanone analogues of demethoxycurcumin synthesis
CONCLUSION
This project is expected to lead to the discovery of novel demethoxycurcumin analogues as potential COX-2 inhibitor candidates, which eventually results in highly reputable journals. We believe that the output from this research project is highly significant challenges for the future to give a lead molecule based on plant traditional medicine use in Malaysia with a strong scientific base and develop research and clinical capability to consistently produce new drugs based on advances in modern biological sciences and the development of innovative therapeutic strategies to address local issues regarding arthritis and pain.

REFERENCES


ACKNOWLEDGEMENT
This work was financially supported by the Science Fund (02-01-02-SF00665), Research University Grant (GUP) (UKM-GUP2011-014), and Fundamental Research Grant Scheme (FRGS).
Correlation Between the Content of 80% Ethanol Extracts of *Phyllanthus amarus* and Their *in vitro* Immunomodulatory Activity

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INTRODUCTION

The immune system refers to organization of immune cells (neutrophils, macrophages and T-lymphocytes) and specialized immune molecules that have evolved to mediate resistance to infections. The coordinated interaction of these molecules with several immune cells evoke an appropriate immune response [1]. This immune responses of the human body against any non-self have been divided into two interconnected subsystems, i.e. the innate immunity and the adaptive immunity [2]. The major cellular components of innate immune system involve neutrophils and macrophages [3]. In contrast to the innate immune response, the adaptive immune response specifically recognize distinct substances produced by microbes and capable of providing a long lasting protection [4].

Despite the complex immunological mechanisms, the effectiveness and efficiency of this system depends on dynamic interplay between antigen and a network of immunologically competent cells as there are many mechanisms by which the normal immune restoration process can go awry. The failure to maintain an adequate redox balance of immune cells may leads to impaired immune response which may pose serious threats to health [5]. Modulation of immune response by medicinal plants and their active components provide alternative potential to conventional chemotherapy for a variety of immunologic diseases. In sight of this, *Phyllanthus amarus* (Family: Euphorbiaceae), is of immense interest due to its wide spectrum of biological activities such as anti-inflammatory, antimicrobial, antiviral activities against Hepatitis B, anti-mutagenic, anti-carcinogenic, hepatoprotective, nephroprotective, cardioprotective and hypoglycemic agent [6]. In this study we have demonstrated that standardized extracts of *P. amarus* and their major constituents, corilagin, geraniin, phyllanthin and hypophyllanthin potentially inhibit in vitro chemotactic migration as well phagocytic ability of phagocytes, suppress the release of reactive oxygen species by granulocytes.

EXPERIMENTAL METHODS

Samples

Plant Collection

The whole plants of *P. amarus* were collected from Marang, Kuala Terengganu, Malaysia. The voucher specimens (*P. amarus* UKMB 30078 and *P. urinaria* UKMB 30077) were identified by Dr Abdul Latif Mohamad of Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), and deposited at the Herbarium of UKM, Bangi, Malaysia.

Preparation of 80% ethanol extracts of *Phyllanthus amarus*

The whole plants of *P. amarus* were obtained from Marang, Kuala Terengganu, Malaysia. The plants were authenticated by Dr Abdul Latif Mohamad of Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), and voucher specimens (P. amarus UKMB 30078) were deposited at the Herbarium of UKM, Bangi, Malaysia. The whole plant of *P. amarus* (1kg) were ground and extracted with 80% EtOH (3 x 3 L) at room temperature for 72 h and filtered through Whatman No.1 filter paper (Whatman, England). The filtrate was collected and excess solvent was evaporated under reduced pressure using rotary evaporator at temperature between 55- 60°C.

Quantitative determination of the major components of plant extracts by HPLC

Briefly, the HPLC analysis was performed using the following conditions: Column: Reverse Phase, C-18 column (250 mm ×4.6 mm i.d., 5 m, Xbridge, Waters, Ireland), and detector: PDA (Waters 2998) of wavelength ranging from 205 to 270 nm. Identification and quantification of components of extracts and standard compounds including gallic acid, ellagic acid, corilagin, geraniin, phyllanthin and hypophyllanthin was performed using two different chromatographic conditions, method1 and method 2 as described in our previous study. For the analysis of the extracts and standard solutions of phenolic compounds (gallic acid, ellagic acid, corilagin, geraniin) corilagin had been performed using method 1 with corilagin as an external standard. Whereas the identification of each compound was carried out by comparing the retention times and UV-Vis spectra of the peaks with that of the standard compounds. Whereas Identification and quantification of the lignans (phyllanthin and hypophyllanthin) were carried out based on the chromatographic condition described in method 2. The validation of HPLC for the standardization of extract was carried out by determination of linearity, precision, limits of quantification (LOQ) and detection (LOD).
Isolation of human PMNs and MNCs

Ten mL of fresh venous blood was obtained in heparin-containing tubes from healthy human volunteers. The whole blood was aliquoted into falcon tubes and equal amount of dextran and PBS were added and the mixture was left for sedimentation and supernatant was layered on Ficoll before centrifugation. Whereas for the isolation of mononuclear (MNC) was performed by using modified method of Gmelig et al., 1980 [14], 10 mL of venous blood from healthy individual was diluted 1:1 with PBS. Diluted blood was then carefully layered on lympho prep and centrifuged. The cells were adjusted to final concentration of 1 × 10^6 cells/mL. The use of human blood was approved by Human Ethical Committee of UKM (approval number FF/2012/Ibrahim/23-May/432-May 2012–August 2013).

Cell viability

Cytotoxicity of P. amarus and P. urinaria extracts and standard compounds on PMNs and MNCs was determined by the standard trypan blue exclusion method. The standard trypan blue exclusion assay was carried out by incubating cells with samples in triplicate at 37°C for 2 h.

Chemotaxis assay

The assay was performed using a modified 48-well Boyden chamber with formyl-methionylleucyl-phenylalanine (fMLP) a chemoattractant, as previously described by Jantan et al.[8]. Briefly, aliquots of 25 µL of fMLP (10−8 M) were added to the wells in lower chamber. Migrated cells which had adhered to the distal part of the filters were fixed and stained by haematoxylin and xylene. The cell migration distance was measured by using a light microscope.

Phagocytic assay

The assay was carried out according to the protocol given by the manufacturer (ORPEGEN Pharma). Briefly, 100 µL heparinized peripheral whole blood was incubated with 20 µL pre-cooled FITC-labelled opsonized E. coli and 20 µL of test samples (extracts: 6.25 and 100 µg/mL; pure compounds: 3.125 and 50 µg/mL) in a closed shake water bath at 37°C for 10 min. The phagocytic ability of neutrophils and monocytes was determined by flow cytometry using the blue-green excitation light (488 nm argon-ion laser). Live populations were gated by the software program in the scatter diagram (FCS versus SSC).

CD18 integrin expression of leukocytes

The CD18 integrin expression assay was performed using modified method of Mazzone et al. [7]. Peripheral blood was obtained from heparinized venous blood of normal voluntary donors. Aliquots (100 µL) of whole blood were then incubated in the presence or absence of samples with different concentrations at 37°C for 30 min. Thereafter, cells were stimulated with LPS (0.25 µg/mL) for 90 min at 37°C in a CO2 incubator. Subsequently 10 µL of CD18-FITC or Immunoglobulin G-FITC (negative control) was added into the mixture. Expression of adhesion molecules was determined by flow cytometry and compared with adhesion molecule expression of untreated cells. Neutrophils and monocytes, were discriminated in terms of forward and side scatter.

Chemiluminescence assay

Luminol-augmented chemiluminescence assays were carried out as described by Jantan et al.[8] Briefly, 25 µL diluted 25 µL PMN or MNCs (adjusted to 1 × 10^6 ) suspended in HBSS++ were incubated with 25 µL of sample in 96 well flat bottom microplate. The cells were induced with 25 µL of polymyristate acetate (PMA) or zymosan. This is followed by the addition of 25 µL of luminol and the final volume of each well was adjusted to 200µL. The microplate was incubated for at 37°C for 50 min. The luminometer results were monitored as chemiluminescence RLU (reading per luminometer unit).

Statistical analysis

All the data are presented as means ± standard error median (SEM) from triplicate experiments and were analysed using statistical package for social sciences (SPSS) software version 20.0. The IC50 values were calculated using Graph PAD Prism 6 Analysis software. Data were analysed using a one-way analysis of variance (ANOVA) for multiple comparisons. P<0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Quantitative determination of the major components of the 80% ethanol extracts of Phyllanthus amarus and P. urinaria.

The chromatograms of the reversed-phase HPLC column of the methanol extracts of P. amarus showed four major peaks of gallic acid, geraniin, corilagin, ellagic acid, phyllanthin and hypophyllanthin corresponding to retention times at 8.172, 23.694, 26.436, 27.251 and 28.079 min, respectively (Fig. 1). Concentration range of 62.5–500 µg/mL showed a correlation coefficient (r2) of 0.9958. The reproducibility of the results was confirmed by results of relative standard
deviation (RSD %) of mean area under peak 1.81% and mean of retention time values 0.93% for interday assay, whereas the % RSD values for intra-assay precision of peak area and retention time were 3.68 and 1.09%. Limit of detection (LOD) and limit of quantification (LOQ) of corilagin were found to be 1.86 and 5.65 ng/mL, respectively. Quantitative determination of the major compounds by HPLC indicated that P. amarus contained the amounts of phyllanthin (103.5 µg/mL), gallic acid (163.3 µg/mL) and ellagic acid (601.29 µg/mL). Corilagin (413.42 µg/mL) was found in highest concentration. The variations in the amounts of the major compounds in the plants of similar species collected from different geographical locations were partly due to the different environmental factors related to altitude and genetic adaptation of the populations growing at different altitudes to specific environment [26].

Chemotaxis Assay

The cell viability test was carried out using trypan blue and MTT assays for PMNs and monocytes, respectively, to evaluate the cytotoxicity of extracts of Phyllanthus amarus and P. urinaria as well as the standard compounds. The extracts and the standard compounds at concentrations ranging from 6.25 to 100 µg/mL showed more than 95% of cell viability except for phyllanthin and hypophyllanthin which possessed cell viability > 95% at concentrations ranging from 0.3125 to 5 µg/mL. The inhibitory activities of the extracts and standard compounds at the serial dilutions of 10 to 0.625 µg/mL, on the migration of PMNs towards the chemoattractant (fMLP), were determined and their percentage inhibitions (%) were shown in Figure 2. P. amarus extract depicted the most potent inhibitory activity on PMNs and monocytes migrations with IC50 values of 1.22 µg/mL and 1.44 µg/mL, respectively (Table 1). Amongst the major compounds identified in P. amarus and P. urinaria, geraniin, corilagin and ellagic acid demonstrated lower IC50 values than that of ibuprofen, with geraniin being the strongest inhibitor with IC50 values of 1.09 and 1.69 µM against PMNs and monocytes, respectively.

Phagocytic assay

The ability of PMNs and monocytes to phagocytize opsonized E. coli was evaluated by phagotest kit and analyzed by flow cytometry. The E coli was opsonized with immunoglobulin and complement of pooled sera. The plant extracts of Phyllanthus amarus and P. urinaria at 100 and 6.25 µg/mL exhibited moderate inhibition of E. coli uptake by monocytes and neutrophils. At 6.25 µg/mL, all plant extracts showed moderate engulfment inhibitory activity with percentage of phagocytizing cells ranging from 53.08 to 59.7% for monocytes. The engulfment inhibitory activity at normal condition at 37°C was used as a positive control and normal condition at 0°C as negative control (Table 1).

The effects of the P. amarus extracts on β2 integrins (CD18) expression on PMNs and monocytes are shown in Table 2. The inhibition of CD18 expression by plant extracts and compounds was tested with the aid of flow cytometry. Plant extracts possessed moderate inhibition of Cd18 expression on human leukocytes. Phyllanthin and hypophyllanthin depicted significant inhibition at 50 µg/mL with percentage of CD18 expression of 67.17% and 61.56% for PMNs and monocytes, respectively.

Chemilluminescence assay

Preliminary screening of the plant extracts on whole blood showed that P. amarus exhibited high inhibitory activity for luminol-enhanced chemiluminescence with IC50 values of 0.95 and 1.52 µg/mL, respectively, for zymosan induced and 1.24 and 1.98 µg/mL, respectively, for PMA induced (Table 3).

Figure 1. Representative HPLC chromatograms of (a) P.amarus for identification of phyllanthin (1) and hypophyllanthin (2) at RT 27.279 and 28.051 respectively. (b) P. amarus for identification and quantification of gallic acid (1) at RT 7.891 minutes, geraniin (2) at RT 23.764minutes, corilagin (3) at RT 26.416 minutes, ellagic acid (4) at RT 33.421, unidentified peak at RT (23.860 minutes).
Table 1. Percentage of phagocytic activity (%) of neutrophils and monocytes at various concentrations of Phyllanthus extracts and their major compounds. (Mean ± SEM, n = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition (%)</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMNs MNCs PMNs MNCs PMNs MNCs PMNs MNCs PMNs MNCs PMNs MNCs</td>
<td></td>
</tr>
<tr>
<td>PA (Mal)</td>
<td>79.40 ± 1.23</td>
<td>69.70 ± 0.15</td>
</tr>
<tr>
<td>GA</td>
<td>95.90 ± 1.52</td>
<td>77.80 ± 2.18</td>
</tr>
<tr>
<td>EA</td>
<td>88.18 ± 1.19</td>
<td>75.50 ± 1.97</td>
</tr>
<tr>
<td>Ger</td>
<td>99.10 ± 3.05</td>
<td>82.28 ± 2.06</td>
</tr>
<tr>
<td>Cor</td>
<td>97.60 ± 2.82</td>
<td>80.70 ± 1.51</td>
</tr>
<tr>
<td>Phyll</td>
<td>14.20 ± 1.08</td>
<td>27.10 ± 0.55</td>
</tr>
<tr>
<td>Hypophyll</td>
<td>49.11 ± 1.04</td>
<td>64.6 ± 0.51</td>
</tr>
<tr>
<td>Positive control</td>
<td>87.80 ± 1.25</td>
<td>84.6 ± 4.84</td>
</tr>
</tbody>
</table>

Table 2. Percentage of CD18 expression (%) on neutrophils, monocytes and lymphocytes at various concentrations of Phyllanthus extracts and their major compounds. (Mean ± SEM, n = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition (%)</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMNs MNCs PMNs MNCs PMNs MNCs PMNs MNCs PMNs MNCs</td>
<td></td>
</tr>
<tr>
<td>PA (Mal)</td>
<td>93.61 ± 1.70</td>
<td>90.39 ± 2.13</td>
</tr>
<tr>
<td>GA</td>
<td>90.77 ± 1.80</td>
<td>90.70 ± 1.20</td>
</tr>
<tr>
<td>EA</td>
<td>93.13 ± 2.31</td>
<td>91.39 ± 0.57</td>
</tr>
<tr>
<td>Ger</td>
<td>96.97 ± 1.71</td>
<td>84.49 ± 1.71</td>
</tr>
<tr>
<td>Cor</td>
<td>98.80 ± 0.24</td>
<td>88.76 ± 1.10</td>
</tr>
<tr>
<td>Phyll</td>
<td>67.17 ± 1.34</td>
<td>61.56 ± 0.98</td>
</tr>
<tr>
<td>Hypophyll</td>
<td>74.70 ± 0.09</td>
<td>74.42 ± 0.77</td>
</tr>
<tr>
<td>Positive control</td>
<td>86.77 ± 0.54</td>
<td>84.79 ± 0.55</td>
</tr>
</tbody>
</table>

Table 3. IC50 values (µg/mL) of ROS inhibitory and chemotactic activities of Phyllanthus sp. and their major compounds on phagocytes (Mean±SEM, n = 3). IC50 values in µM are in parentheses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PMNs</th>
<th>MNCs</th>
<th>Whole Blood</th>
<th>PMNs</th>
<th>MNCs</th>
<th>Whole Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA (Mal)</td>
<td>1.22 ± 0.10</td>
<td>1.44 ± 0.80</td>
<td>0.95 ± 0.24</td>
<td>0.58 ± 0.10</td>
<td>0.10 ± 0.17</td>
<td>1.24 ± 0.98</td>
</tr>
<tr>
<td>GA</td>
<td>6.40 ± 0.02</td>
<td>2.00 ± 0.03</td>
<td>1.70 ± 0.03</td>
<td>0.45 ± 0.15</td>
<td>1.29 ± 0.05</td>
<td>0.87 ± 0.05</td>
</tr>
<tr>
<td>EA</td>
<td>1.53 ± 0.44</td>
<td>1.81 ± 0.19</td>
<td>1.36 ± 0.73</td>
<td>0.56 ± 0.57</td>
<td>0.45 ± 0.01</td>
<td>0.83 ± 1.07</td>
</tr>
<tr>
<td>Ger</td>
<td>1.04 ± 0.08</td>
<td>1.61 ± 0.51</td>
<td>0.54 ± 0.04</td>
<td>0.18 ± 0.01</td>
<td>0.37 ± 0.05</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>Cor</td>
<td>1.49 ± 0.72</td>
<td>1.99 ± 0.19</td>
<td>0.37 ± 0.02</td>
<td>0.36 ± 0.25</td>
<td>0.44 ± 0.01</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.40 ± 0.56</td>
<td>1.98 ± 0.18</td>
<td>0.50 ± 2.74</td>
<td>0.50 ± 0.44</td>
<td>0.50 ± 0.10</td>
<td>0.50 ± 0.05</td>
</tr>
</tbody>
</table>

IC50 Values (µg/mL)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chemotaxis</th>
<th>Zymosan</th>
<th>Chemiluminescence</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>2.16 ± 0.06</td>
<td>0.17 ± 0.07</td>
<td>0.14 ± 0.01</td>
<td>0.58 ± 0.25</td>
</tr>
<tr>
<td>Phyll</td>
<td>5.20 ± 0.15</td>
<td>5.61 ± 0.15</td>
<td>5.80 ± 1.35</td>
<td>1.40 ± 0.14</td>
</tr>
<tr>
<td>Hypophyll</td>
<td>4.40 ± 0.17</td>
<td>4.90 ± 0.60</td>
<td>5.90 ± 1.03</td>
<td>1.87 ± 0.03</td>
</tr>
</tbody>
</table>

CONCLUSION

The HPLC analysis methods proposed in this study enable identification and quantification of all major compounds found in the 80% ethanol extracts of P. amarus. The HPLC quantification analysis indicated the presence of high amount of phenolic compounds in the plant extracts obtained from both Malaysia and Indonesia. The in vitro studies revealed that, the plant extracts, especially Malaysian P. amarus exhibited potent inhibitory activity of ROS generation and chemotactic activity of phagocytes stimulated by different stimuli. Potential inhibitory action on both phagocytic and CD18 expression of...
phagocytes. Thus, the in vitro study suggest that the presence of high levels of the major compounds of P.amarus and P.urinaria reported in this study could be the major contributors to the strong immunomodulatory effect of the plants.

REFERENCES
Transcriptional Response of Methicillin Resistant *Staphylococcus aureus* to Secondary Metabolites Compounds Isolated from Endophytic *Streptomyces* sp., SUK 25.

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INTRODUCTION

The set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA transcribed in one cell or a population of cells is known as transcriptome. GeneChip DNA microarray can be used for identification of genes in multiple regions within a bacterial genome, or it can be applied to samples consisting of different genomes [1]. Transcriptional profiles generated by GeneChip analysis of bacteria have been used to investigate differential gene expression in response to antimicrobial agents [2, 3]. Methicillin Resistant *Staphylococcus aureus* (MRSA) is one of the most important bacterial pathogens that infect human and animal [4]. Its ability of resistance to all currently available antibiotics over the last two decades lead to search for new and save potential natural antimicrobial activity against MRSA [5]. Natural chloramphenicol (CAP) and one type of diketopiperazine known as cyclo (L-Pro-L-Val) were isolated from endophytic *Streptomyces* sp., SUK 25 which exhibits a high degree of potency against MRSA. In this study we determined the impact of exposure of MRSA to subinhibitory concentration (4 µg/mL) of CAP and cyclo (L-Pro-L-Val) on the gene transcriptional profile of MRSA ATCC 43300 at one time point (45 mins) using three biological replication set of Affymetrix *S. aureus* GeneChip™.

EXPERIMENTAL METHODS

MRSA Treatment with CAP and cyclo (L-Pro-L-Val). MRSA strain ATCC 43300 was grown overnight at 200 rpm in a rotary shaker at 37°C in 10ml of Mueller Hinton Broth (MHB). Two 250-ml Erlenmeyer flasks, each of which contain 100 ml of MHB, were inoculated with 1 ml of an overnight culture to an initial reading of OD at A_625 of 0.05. The bacteria were grown at 37°C at 200 rpm until OD A_625 of 0.7. Subsequently, 80 µl of a 5000 µg/ml of CAP and cyclo (L-Pro-L-Val) stock solution, prepared in ethyl acetate, was added to one of the cultures experimental flask culture that giving a final concentration of 1/2×MIC (4 µg/ml). The other one culture containing 10 % (v/v) solvent ethyl acetate only lacking CAP or cyclo (L-Pro-L-Val) was used as the positive control. All bacterial suspensions both experimental and control suspensions were further incubated for 45 min at 37°C. RNA isolation was then performed at this time. Three independent bacterial cultures for each CAP and cyclo (L-Pro-L-Val) treatment or control condition were prepared as biological replicates for RNA isolation on different days. cDNA was synthesized with starting material of 10 µg of total high quality RNA, fragmentation and terminal labelling and hybridization were carried out according to manufacturer’s protocol (Affymetrix, USA). The expression level of each probe set was analyzed by the GeneSpring version 12. To identify differentially expressed genes, pairwise comparisons were conducted using the heat map, gene ontology biological process terms. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were generated using criteria of a false discovery rate (FDR) of< 5 % and a minimum of a 1.5 -fold change between examined groups.

RESULTS AND DISCUSSION

Exposure of MRSA to CAP and cyclo (L-Pro-L-Val) revealed significant modulation of gene expression with up regulation of about 60 genes and down regulation of 120 genes. The genes which showed some changed involved genes encoding proteins essential to translation for ribosomal pathway, transcription, ATP metabolites, immunoglobulin G binding protein A, lipoprotein precursor and cell division protein.

This study revealed that the mode of action of CAP through interaction with the ribosomal protein synthesis. The transcriptome analysis of CAP revealed up regulation in ribosomal assembly pathway, preprotein translocase and adenylate kinase. This is may be because CAP is activating as a translation inhibitor, which induces the cell to synthesize more ribosomes, in order to reduce the turnover and degradation of existing ribosomes. Whereas, the mode of action of cyclo (L-Pro-L-Val) may be affected in translation, ATP binding and some of other metabolic process such as down regulation of all ribosome genes, alpha-glucosidase and maltose operon repressor. The amino acid transport, energy metabolism, virulence and some Signal transduction were also down regulated.
CONCLUSIONS
This study showed that Streptomyces sp., SUK 25 represent an important source of natural product with potential activity against MRSA. The mode of action of CAP is an inhibitor of translation process. Whereas, the mode of action of cyclo (L-Pro-L-Val) may be affected for many biological and metabolic process.

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ACKNOWLEDGEMENTS
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Molecular Modeling and Synthesis of Nucleoside Analogues as Potential Dengue Virus RNA-Dependent RNA-Polymerase (DENV NS5 RdRp) Inhibitors

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INTRODUCTION

Dengue fever is a neglected emerging disease for which no effective antiviral agent and no clinically approved vaccine exist at present. It is caused by dengue virus (DENV), a member of Flavivirus genus [1]. DENV spreads rapidly and contributes to increasing threats to public health and economic worldwide [2]. Therefore, the studies of novel target sites are critical for the development and discovery of antiviral drugs [3]. Among the strategies is to target DENV NS5 RNA-dependent RNA-polymerase (RdRp). Molecular docking study using CHARMM-based DOCKER (CDOCKER) was implemented in this research to examine the orientation, conformation and bonding interaction of 39 nucleoside analogues on DENV NS5 RdRp. Prediction for suitable nucleosides will be made and those nucleosides will be synthesized. Two series of acyclic thionucleoside analogues that resemble the structures of ligand that showed a good docking interaction among the tested nucleoside analogues will be synthesized using the Michael-type Addition reaction. Suitable protective groups and deprotection methods will be investigated. Crude products obtained will be purified by column chromatography or crystallization. The structure of the isolated chemical components will be determined by the nuclear magnetic resonance (NMR), mass spectrometry (MS), microanalysis and infrared spectroscopy (IR).

EXPERIMENTAL METHOD

Docking

Protein (DENV NS5 RdRp) were taken from RCSB Protein Data Bank while 19 comercial nucleoside analogues were taken from PubChem & DrugBank and 20 non-comercial nucleoside from previous literature [4, 5, 6]. Molecular docking studies were conducted on selected nucleoside analogues & DENV NS5 RdRp through CDOCKER Program to identify bonding interaction. CHARMM-based Docker (CDOCKER) algorithm was implemented for the docking studies in this research.

Synthesis

Nucleoside analogues that show a better docking interaction will be chosen and two series of acyclic thionucleoside analogues that resemble the structures analogues will be synthesized using the Michael-type Addition reaction.

Extraction and Purification

Crude products were purified by using column chromatography or crystallization process. Crude product were stored in refrigerated for 2 hours and then recrystallization by using methanol or ethyl acetate.

Analysis

Product will be analyzed by using nuclear magnetic resonance (NMR), infrared spectroscopy (IR), mass spectrometry (GCMS) and microanalysis.

RESULT & DISCUSSION

Docking

4'-ethyl-thioarabinofuranosyluracil (Structure 13) shows the highest value of –CDOCKER energy and had the highest number of interaction with DENV NS5 RdRp compared to others selected non-commercial nucleoside analogues. From docking result, structure 13 form a two hydrogen bond and one cation π-aromatic interaction with crucial amino acid which is Lys-401 and Asn-405. The value of –CDOCKER energy is 10.1273 kcal/mol while the value of –CDOCKER interaction energy is 25.9081 kcal/mol. For commercial nucleoside analogues, 5-(2-chloroethyl)-1-(2-deoxy-2-fluoro-4-thio-D-arabino-pentofuranosyl) uracil (structure 5'), 5-methyl-1-(4-thio-D-arabinofuranosyl) uracil (structure 8') and 4'-thio-araT (structure 12') shows the highest number of interaction with three hydrogen bond and two charge interaction between crucial amino acid. However, structure 5' shows the best docking interaction among this structure 8' and 12' because the value of –CDOCKER energy are much higher.
REFERENCES


ACKNOWLEDGEMENTS

Special thanks to Dr. Malina, lecturers and friends that have contributed their help in this studies.
Immunostimulatory effects of the standardized extract of *Tinospora crispa* on neutrophile functions in Wistar Kyoto rats

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INTRODUCTION

The immune system is a sophisticated and intricate network comprising organs, tissues, special cells and proteins that function collectively to protect the body against foreign invasions such as toxins, parasites and germs [1]. The state of good health is maintained by the regulation of several cellular and humoral factors functioning in the immuno-regulatory mechanism. The immune system is involved in the pathophysiology and etiology of several diseases. The dysfunction of immune system causes a number of diseases such as cancer, infectious diseases, parasitic diseases, allergy, asthma, ulcerative colitis and arthritis [2]. The adjustment of immune responses to alleviate such diseases has been of interest for several years. The immunostimulation by natural substances is believed to be a promising way to prevent and cure diseases [3]. Herbal drugs possess immunomodulatory characteristics and normally act by suppressing or stimulating both specific and non-specific immunity.4 Tinospora crispa (Wild.) Hook. f. &Thomson (TC) is a tree which belongs to the family Menispermaceae. This specie is widely used in Indonesia, Malaysia and Thailand as a bitter tonic for the treatment of intermittent fever, urinary disorders, rheumatism and jaundic. Thus, the present study was aimed to investigate the immunomodulatory effects of TC using neutrophils in an *in vivo* animal model.

EXPERIMENTAL METHODS

Samples

The whole plants of *Tinospora crispa* were collected from Marang, Kuala Terengganu, Malaysia. Voucher specimens were deposited at the UKM Herbarium. The stems of *T. crispa* were ground and extracted with 80% EtOH (3 x 3 L) at room temperature for 72 h and then filtered through Whatman No.1 filter paper (Whatman, England).

Standardization of the *Tinospora Crispa* extract (TCE)

HPLC was performed on a Waters (USA) instrument equipped with a 2998 photodiode array detector. The extract was analyzed on a RP, Xbridge® C-18 column (5 µm, 250 mm ×4.6 mm i.d, Waters, Ireland). The mobile phase consisted of acetonitrile and water + 0.02% of trifluoroacetic acid.

Animals and experimental design

Male Wistar Kyoto rats of inbred strains (6-8 week old) were randomly divided into six groups. Each experimental group consisted of six animals.

Group I: Control group rats received only the regular diet and tap water.
Group II: The vehicle control group received 0.01% tween 20 solution.
Group III: Positive control group received levamisole, orally at a dose of 10mg/kg body weight.
Groups IV: Received 80% ethanolic *T. crispa* extract at dose of 50 mg/kg body.
Groups IV: Animals were given oral dose of 100 mg/kg of 80% ethanolic *T. crispa*.
Groups IV: Animals were given oral dose of 200 mg/kg of 80% ethanolic *T. crispa*.

Isolation of neutrophil from rat whole blood

The neutrophils were isolated from the whole blood of treated rats which was drawn before immunization. It was carried out using modified Histopaque gradient technique by using Lymphprep®.

Neutrophil migration assay

The effect on the migration of neutrophils was quantitatively assessed using CytoSelect 24-well Cell Migration Assay kits (Cell Biolabs, Inc.). The number of cells migrating in response to chemoattractant (FBS) determined by fluorescence reader.

Determination of phagocytosis

The effect on the phagocytic activity of the neutrophils was determined by a commercially available kit by using FITC-labelled *E. coli*. The phagocytic activity was determined as the percentage of phagocytizing neutrophils using flow cytometer.
RESULTS AND DISCUSSION

Standardization of *Tinospora Crispa* extract

The *Tinospora crispa* extract was standardized on the basis of two marker compounds, syringin and magnoflorine. The reversed phase-HPLC chromatogram of 80% ethanol extract exhibited peaks of syringin and magnoflorine corresponding to retention times at 6.360 ± 0.072 and 20.967 ± 0.208 min, respectively (Figure 1). The quantitative determination of marker compounds by HPLC indicated that extract contained syringin and magnoflorine at 466.92 ± 2.23 and 281.21 ± 2.078 µg/mL, respectively.

![HPLC chromatogram of Tinospora crispa 80% ethanol extract.](image1.png)

**Figure 1.** (a) HPLC chromatogram of *Tinospora crispa* 80% ethanol extract. (b) HPLC chromatogram of syringin and magnoflorine. Detection was at 254nm.

Effect of *Tinospora Crispa* extract neutrophile migration

It was seen that the migration increased in neutrophils obtained from extract-treated rats in contrast to controls. A dose-dependent increase in the number of migrated cells was observed, i.e increasing the dose lead to increase in the number of migrated cells.

![Effect of the ethanolic extract of Tinospora Crispa (100-400mg/kg) on neutrophil migration.](image2.png)

**Figure 2.** Effect of the ethanolic extract of *Tinospora Crispa* (100-400mg/kg) on neutrophil migration.
Effect of TCE on neutrophil phagocytic activity
All animals treated with the doses of 100, 200 and 400 mg/kg exhibited an increase in the phagocytosis of FITC-labelled E. coli. The phagocytic activity is expressed by percentage of phagocytosis as shown in Figure 3. At the doses of 200 and 400 mg/kg the phagocytic activity of neutrophils was found to be significant as compared to control group.

Figure 3. Flowcytometric evaluation of the effect of the ethanolic extract of *Tinospora Crispa* (100-400mg/kg) on phagocytosis of FITC- conjugated E.coli by neutrophils.

CONCLUSIONS
The results demonstrate that the extract of TC was able to activate the cell mediated immunity by enhanced chemotactic and phagocytic activity of neutrophiles. The present findings suggest that the ethanolic extract of TC possesses potent immunostimulatory properties. It can be a potential immune-therapeutic agent possibly beneficial in enhancing the immune response in immune-compromised diseases.

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ACKNOWLEDGEMENTS
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Effects of Curcumin on Gene Expression of Inflammation and Immune Response in Epileptic Rats

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INTRODUCTION
Temporal lobe epilepsy (TLE) is the most common and difficult-to-treat type of partial epilepsy [1, 2] There are up to 40% of epileptic patients that are resistant to current antiepileptic drugs and remain in uncontrolled state [3]. Current antiepileptic drugs only provide symptomatic relief by suppressing seizures, with none of them exerting significant effect in preventing or inhibiting progression of epilepsy [4, 5]. Therefore, in recent years, epilepsy research has changed the focus from targeting control of seizures to strategies for prevention and cure of epilepsy [4, 6].

To discover the potential agents with antiepileptogenic properties, animal models play a crucial role. The kainate-induced epilepsy model is a well-established model of TLE [7, 8]. On the other hand, this model has been recommended as a useful tool for studying mechanism of epileptogenesis and discovering antiepileptogenic treatment [9, 10]. Epileptic insults cause numerous neurobiological changes that lead to epileptogenesis and these changes might be attributed to alteration in gene expressions [11]. Therefore, this study employ the microarray technique to understand the cellular and molecular mechanisms that involve the epileptogenesis process in kainate-induced epileptic rat model.

EXPERIMENTAL METHODS
Animals
Female Wistar rats (aged 3 months old), weighing 200 – 220 g were purchased from Laboratory Animal Resource Unit, Faculty of Medicine, UKM. They were kept under controlled environment conditions with free access to food and water. The rats were housed three per cage and allowed to acclimatize about one week before the experiment. All animal care and handling were conducted in accordance with the procedures approved by UKM Animal Ethical Committee (UKMAEC), Faculty of Medicine.

Seizure induction and drug administration procedure
For seizure induction, the rats were intraperitoneally injected with a single dose of kainic acid (KA) at a dose of 10 mg/kg and monitored behaviourally for 6 hours. In the control rats, vehicle (saline) injections were given intraperitoneally at the same volume as KA-induced group. Seizure severity was scored according to the Racine scale [12]. Only rats achieving stage 4 and 5 limbic seizures were used and randomly distributed to group 2, 3 and 4. However, only rats that survived and complete treatment were included in the study. Each group consisted of a minimum of six animals as shown below:
- Group 1: DMSO 50%-treated/without seizure induction.
- Group 2: DMSO 50%-treated/with seizure induction.
- Group 3: levetiracetam treated/with seizure induction
- Group 4: curcumin treated/with seizure induction

On the day after seizure induction, treatment with levetiracetam (100mg/kg/day) and curcumin (100mg/kg/day) was started in group-3 and -4, respectively. Animals of group-1 and -2 received vehicle (DMSO 50%) injections. Vehicle or drug was administered intraperitonially, once daily, 7 days per week.

Tissue sampling and RNA extraction
After the treatment, six rats were selected from each group for decapitation and both hippocampi were dissected. Approximately 75 mg of the tissue sample from each rat was used for RNA purification using RNeasy® Microarray Tissue Mini Kit. The concentration and purity of RNA was determined by standard methods.

Microarray analysis
A volume of 200 ng of total RNA from each sample was used as template to generate amplified cDNA using Applause® WT-Amp Plus ST System. The GeneChip® Rat Gene 2.0 ST Array was used in this study. As per Affymetrix’s protocol, hybridization mixes were prepared and put onto chips which were then washed, stained and scanned using Affymetrix GeneChip Fluidics Workstation.

Statistical analysis of gene expression data
Data transformations (log2 conversion), selection and statistical analyses were performed with Affymetrix® Transcriptome Analysis Console (TAC) software. A set of differential expression changes was determined by dual fold change in either
direction and at least \( p < 0.05 \) using one way ANOVA (unpaired) for a significant difference.

**Gene annotation and pathway analysis**
The dataset of the significantly altered genes was imported to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (available at http://david.abcc.ncifcrf.gov/) for bioinformatics analysis (Huang et al. 2009).

**RESULTS AND DISCUSSION**

**Seizure induction**
Thirty-four rats were given KA, of which 28 (82.35\%) rats developed stage 4 (\( n = 8 \)) or stage 5 (\( n = 20 \)) of status epilepticus, which lasted for 169.30 ± 53.36 minutes, but only 19 (67.86\%) survived until completion of treatment. The distribution of the seizure severity according to Racine scale is shown in Table 1.

**Table 1.** Severity of seizure induction

<table>
<thead>
<tr>
<th>Seizure induction</th>
<th>( n )</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
<th>Stage 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kainic acid</td>
<td>34</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

**Gene expression profile**
Data filtering identified 36,685 out of 220,231 probe sets (17\%) reached informative signal intensity values on the array. A list of 370 genes was differentially expressed based on fold change more than two in either direction and \( p \) value less than 0.05 by ANOVA analysis. Among the genes, the total number of genes up-regulated and down-regulated by respective treatment was showed in Table 2. Figure 1 shows the hierarchical clustering of differentially expressed genes for respective treatment.

**Table 2.** The total number of up-regulated and down-regulated genes by respective treatment

<table>
<thead>
<tr>
<th>Number of genes</th>
<th>Up-regulated(^a)</th>
<th>Down-regulated(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kainic acid</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Levetiracetam</td>
<td>146</td>
<td>17</td>
</tr>
<tr>
<td>Curcumin</td>
<td>61</td>
<td>25</td>
</tr>
</tbody>
</table>

The effect of kainic acid refered to genes expression that altered by kainic acid, in comparison to the control group (KA-DMSO versus saline-DMSO group). The effect for levetiracetam or curcumin was determined by genes that altered expression in levetiracetam-treated or curcumin-treated epileptic rats, in comparison to the kainic acid treated group (i.e. KA-LEV versus KA-DMSO group or KA-DMSO versus KA-CUR group). \(^a\)Based on fold change < -2 or > 2 and \( p < 0.05 \).

**Figure 1.** The hierarchical clustering of differentially expressed genes for respective treatment.
Gene annotation and pathway analysis

Inflammation and immune response was the most prominent process involved. A list of 59 genes related to inflammation and immune response was differentially expressed based on fold change more than two in either direction and p value less than 0.05 by ANOVA analysis.

Kainic acid up-regulated pro-inflammatory cytokines included \( \text{Il}18 \) and \( \text{Ifngr1} \), and cell surface receptor, \( \text{Cd74} \). Despite treatment given, pro-inflammatory cytokines remained up-regulated in the kainate-induced status epilepticus rodents (levetiracetam: \( \text{Il}18 \), \( \text{Ifngr1} \), \( \text{Ccl2} \), \( \text{Ccl3} \), \( \text{Ccr5} \), \( \text{Cx3cr1} \); curcumin: \( \text{Cx3cr1} \), \( \text{Cx3cl1} \)). However, anti-inflammatory cytokines were also overactivated in addition to pro-inflammatory cytokines by both levetiracetam (\( \text{Il}10\text{rb} \), \( \text{Tgfb}1 \), \( \text{Cxcl16} \)) and curcumin (\( \text{Il}10\text{rb} \), \( \text{Cxcl16} \), \( \text{Cxcl17} \), but not by kainic acid treatment. This suggested that both levetiracetam and curcumin may be involved in activation of these anti-inflammatory factors, which was likely to contribute in counteracting neuroinflammation induced by kainic acid.

For molecular pathway analysis, KEGG pathways were identified from DAVID. Due to the small amount of genes identified in kainic acid treatment, there was no relevant pathways identified. The levetiracetam treatment in kainate-induced epileptic rats suggested it associated mainly with immune response-related pathway. On the other hand, curcumin treatment groups showed less association with the immune response-related pathway. This suggests that curcumin may possibly exert a more potent anti-inflammatory effect than levetiracetam and beneficial in ameliorating the inflammation process induced by kainic acid.

CONCLUSION

The microarray analysis yielded several number of genes involved in the epileptogenesis and those gene altered by levetiracetam and curcumin. Both levetiracetam and curcumin are likely to be involved in modulating neuroinflammation by activating anti-inflammatory factors.

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ACKNOWLEDGEMENTS

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In vivo Efficacious study of Alginate Bilayer Film containing Hidrox® in Freud’s Adjuvant induced-Arthritic Rat Model

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INTRODUCTION
Hidrox is an aqueous olive extract, freeze-dried powder which is rich in HT content (minimum 2.5%). HT has been studied and proven to have antioxidant and anti-inflammatory activity [1]. Some studies also indicate that the use of Hidrox can relieve the symptoms of a disease, such as psoriasis, eczema and RA [2]. The olive extract is proved to be nontoxic and is considered safe at a dose of 1200mg / day in humans [3]. Therefore, topical Hidrox bilayer film formulation can be potential to be used as an alternative treatment for RA that is usually treated with corticosteroids and NSAIDs which cause toxicity if prolonged use.

EXPERIMENTAL METHODS
The 5% and 10% Hidrox alginate bilayer film formulations are shown in Table 1. To investigate in vitro permeation, Franz cells studies using cellulose acetate and rat skin were carried out. Next, the acute dermal irritation studies of the formulations were done on Wistar rats according to the OECD guidelines [4]. To study the efficacy of the bilayer films on treatment of arthritis, Freund’s adjuvant induced rats were treated with the films, and Salonpas was used as positive control treatment. Then, the arthritic scores were observed over 2 weeks and ELISA on serum IL-6 was also measured and investigated at the end of study. All animal study protocols were approved by Animal Ethics Committee Universiti Kebangsaan Malaysia (UKMAEC) with the approval number of FF/2014/FERN/21-MAY-2014-JAN.-2015

Table 1. 5% and 10% Hidrox bilayer film formulations

<table>
<thead>
<tr>
<th></th>
<th>Alginate (%w/v)</th>
<th>Gelatin (%w/v)</th>
<th>Glycerol (%v/v)</th>
<th>PG (%v/v)</th>
<th>D.W (%v/v)</th>
<th>Hidrox (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Hidrox bilayer film</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>upper layer</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>96</td>
<td>5</td>
</tr>
<tr>
<td>lower layer</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>10% Hidrox bilayer film</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>upper layer</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>lower layer</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
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</tbody>
</table>

PG - Propylene glycol

RESULTS AND DISCUSSION
From Figure 1, HT permeation through rat skin gave the lower drug flux as compared to cellulose acetate membrane. This was due to the fact that the skin is a multilayered, heterogenous and very complex barrier system to drug transport. On the other hand, the synthetic membrane is just a simple homogenous monolayer system which gives the less resistance to drug transport. For 5% Hidrox bilayer film, the difference in flux rate was not significant (p>0.05) and the difference in HT cumulative amount was significant (p<0.05). For 10% Hidrox bilayer film, the difference in both flux rate and HT cumulative amount was significant (p<0.05).
Table 2 shows the erythema scores and edema scores for the negative control and treatment group with 10% Hidrox bilayer films over 3 days (Score 0 being no edema while Score 4 being the most severe edema). Throughout the study, both erythema scores and edema scores did not show any significant difference between negative control and treatment group (p>0.05). Besides, there was no significant difference of scores between the time before application of film and any specific hours in the study (p>0.05). The highest average erythema score and edema score recorded for treatment site were only 0.056±0.136 and this reading was approaching to 0. It was indicated that 10% Hidrox bilayer films caused “no edema and no erythema” to the rat skin, hence it may reveal that the films did not cause acute dermal irritation.

Table 2. Erythema and Edema Scores

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Erythema Scores</th>
<th>Edema Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative control</td>
<td>Treatment</td>
</tr>
<tr>
<td>0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>1</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>24</td>
<td>0.056±0.136</td>
<td>0±0</td>
</tr>
<tr>
<td>48</td>
<td>0±0</td>
<td>0.056±0.136</td>
</tr>
<tr>
<td>72</td>
<td>0±0</td>
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</tbody>
</table>

For arthritic scores in Figure 2, non-treatment arthritic group (NT) showed no significant difference from group treated with 5% Hidrox bilayer (H5), 10% Hidrox bilayer (H10) and Salonpas (P) groups until Day 9. However, at Day 12 and Day 15, the differences between NT and other groups (H5, H10 and P) were significant (p<0.05). At Day 15, H10 achieved the lower arthritic score (1.89±0.27) compared to H5 (2.33±0.21) and the difference was significant (p<0.05). This could prove that H10 which contained double amount of Hidrox than H5 gave the better treatment to arthritis. However, there was no significant difference between H5 and H10 with P (p>0.05). This means that the bilayer films had the same efficacy as the commercial patch. When comparing arthritic scores within each group at Day 6 and Day 15, NT had increased arthritic score but H5, H10 and P groups had lower readings. Among them, only H10 achieved the significant difference of arthritic scores between Day 6 (2.67±0.56) and Day 15 (1.89±0.27) (p<0.05). Hence, the assessment of arthritic scores indicates that H10 gave the best anti-inflammatory effect.
Figure 2. Arthritic scores of for different rat treatment groups (n=6). Score 0 being no edema while Score 4 being the most severe edema. * indicates statistically significant to NT p<0.05.

Figure 3 revealed the rat serum IL-6 levels of NT, H5, H10 and P treated groups. H5, H10 and P showed the lower serum IL-6 level than NT. H10 (22.08±3.43 pg/ml) recorded the significant different serum IL-6 level from NT (28.61±2.65 pg/ml) (p<0.05). However, serum IL-6 in H5 showed no significant difference from NT although it achieved a lower level (25.90±2.35 pg/ml) compared to NT (p>0.05). The result of ELISA might indicate that 10% Hidrox bilayer films are effective in arthritis treatment and has same efficacy as marketed patch.

CONCLUSIONS

In vitro Franz diffusion study showed that HT can be released steadily from the formulations. From the clinical observations, the films produced did not cause acute dermal irritation. The arthritic score and suppression of inflammatory cytokine IL-6 in Freund’s adjuvant-induced arthritic rat model showed that Hidrox bilayer films is effective in relieving the inflammation and synovitis in RA. Therefore, the bilayer film containing HT can potentially be exploited as a new approach for topical RA treatment.

REFERENCES


ACKNOWLEDGMENTS

The authors would like to thank Faculty of Pharmacy, University Kebangsaan Malaysia for funding (Grant no. GUP-2013-013) and research facility support.
Probing the Effectiveness of Fish Oil on Imiquimod Induced Inflammation

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INTRODUCTION
Skin cancer is the most common cancer in humans that derives from non-keratinizing cells that originate in the basal and squamous layers of the epidermis. Basal cell carcinoma originates from the basal layer of the epidermis and accounts for the 75% of the skin cancer all over the world and Squamous cell carcinoma, the second most common type of skin cancer, develops in the upper layers of the epidermis and accounts for about 20% of the non-melanoma skin cancers, but due to their more obvious nature and growth rates, they represent 90% of all head and neck cancers [1-3]. Imiquimod, an immune response modifier, is a chemotherapeutic agent for skin cancer, but it has also been associated with inflammatory side effects [4]. The aim of this study was to prevent the inflammatory effect of commercial imiquimod (Aldara ®) by controlling the release of imiquimod through a hydrogel/oleogel colloidal mixture (CA bigel) containing fish oil as an anti-inflammatory agent.

EXPERIMENTAL METHODS
Skin Permeation
The permeability of imiquimod from Aldara ® cream and bigel through mice skin was evaluated, and the drug content residing in the stratum corneum, epidermis, and dermis of the skin via the tape stripping technique was quantified. The fish oil fatty acid content inside the skin layers and the increase in the lipophilic environment of the skin was also determined.

Inflammation study on animal models
An inflammation study was conducted using animal models, and Aldara ® cream was found to potentially cause psoriasis-like inflammation, which could be owing to prolonged application and excessive drug permeation. CA bigel released the drug in a controlled manner, and it is composed of fish oil, which may have caused reduced imiquimod inflammation.

NMR study on fish oil-imiquimod mixtures
NMR studies were conducted to observe whether the fish oil and imiquimod formed a complex that was responsible for improving imiquimod transport and reducing its side effects. NMR spectra showed dose-dependent chemical shifts between the EPA/DHA triglycerides from fish oil and imiquimod, which could help reduce imiquimod inflammation.

Molecular Modeling of fatty acid-imiquimod complexes
Molecular models of EPA- and DHA-imiquimod and found a π-σ interaction between EPA and imiquimod, indicating a possible push and pull phenomenon that is usually associated with skin permeation, but in this scenario, it may also help reduce inflammation caused by imiquimod.

CONCLUSION
CA bigel demonstrated the controlled release of imiquimod which showed equal release to Aldara ® cream till 8 hrs of permeation and also composed of fish oil which is known for its anti-inflammatory activity. Furthermore, inflammation study on animal models exhibited psoriasis kind of inflammation when applied with Aldara ® cream and histopathological study of skin confirms the presence of inflammation. CA bigel does not cause any severe inflammation which may be due to controlled release of drug and increase of lipophilic environment of the stratum corneum, epidermis and dermis layers with the help of anti-inflammatory fish oil fatty acids (EPA and DHA). We also studied for the possibility of any interaction between fish oil and imiquimod through proton NMR and it revealed a clear pattern of changes in chemical shift of aromatic protons in imiquimod, the magnitude of which depends on the concentration of fish oil. Through computational molecular modeling, a chance of complex formation between imiquimod and EPA of fish oil through π-σ interaction between the aromatic ring of drug and long chain fatty acid was confirmed. Such π-σ systems interaction may also be beneficial for synergetic effects as EPA has a known anti-inflammatory activity and along with permeation it may also help in reducing drug related inflammatory side effects.
REFERENCES


Lineage-related and Particle-Size Dependent Cytotoxicity of Chitosan Nanoparticles on Mouse Bone Marrow-derived Hematopoietic Stem and Progenitor Cells

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INTRODUCTION
Chitosan, a polycationic polymer has been used as a carrier for the delivery of drugs, genes and vaccines [1]. Being a natural polymer, chitosan has been widely regarded as biocompatible [2]. Nanoparticles have high potential in stem cell research including intracellular delivery of DNA, RNAi, and drugs for stem cell differentiation [3]. It is important to assess the effectiveness of uptake into the target cells. However, biological adverse effects of chitosan nanoparticles (CSNPs) need to be evaluated. Information regarding nanotoxicology is insufficient and requires further investigations. The objectives of the study are to determine and correlate the cellular uptake and cytotoxicity of CSNPs. CSNPs of low molecular weight were prepared via ionotropic gelation method with tripolyphosphate (TPP) ions and characterized for size, polydispersity index (PDI), and zeta potential. For quantification and visualization of the cell-associated chitosan, chitosan was conjugated with fluorescein-5-isothiocyanate (FITC). Uptake and cytotoxicity studies were performed ex vivo on cells of mouse ICR strain origin (MBMCs). MTT assay, immunophenotyping and ROS assays were used to measure the cytotoxicity of CSNPs.

EXPERIMENTAL METHODS
Preparation and Characterization of CSNPs
CSNPs were prepared by ionotropic gelation of chitosan with TPP at high speed agitation [4]. Nanoparticles formed spontaneously when 1.2 ml of TPP (0.10% in water) solution was added gradually to 10 ml of chitosan (0.20% in 0.1 M CH3COOH) with stirring at 700 rpm (Corning Stirrer/Hot Plate) at ambient temperature. Nanoparticles were measured for size, PDI, and zeta potential using Malvern Zetasizer (Nano ZS®, Malvern Instruments, UK). All formulations were characterized immediately upon preparation.

Viability Assessment of MBMCs
The effects of CSNPs on viability of MBMCs were assessed by MTT assay following protocol as previously described [5]. Briefly, MBMCs were seeded at 1×10^4 cells/well in a 96-well plate and cultured in the presence of CSNPs with concentrations ranging from 31.25–1000 µg/mL for 24, 48 and 72 h. The cells were incubated at 37°C in 5% CO2 incubator. At the pre-determined exposure times, 100 µL of MTT solution (1 mg/mL) was added to the culture and the cells were further incubated for another 4 h at 37°C. Following aspiration of the MTT solution, formazan crystals produced were solubilized with 150 µL of DMSO for 10 min at 37°C and quantified by measuring absorbance at 570 nm using a microplate reader (BioRad, USA) and the defined optical density (OD) correlates with the viability of the cells in culture.

Immunophenotypic analysis of HSPCs
Expression of surface antigens was analyzed using flow cytometer BD FACS Canto II (BD Biosciences, USA). Phenotypic analysis was performed following 72 h exposure of hematopoietic stem and progenitor cells (HSPCs) to CSNPs at a concentration range of 62.5–1000 µg/mL. Immunophenotyping technique was performed according to a previously established method [6] with the involvement of fluorescent phycoerythrin (PE)-conjugated monoclonal antibodies rat anti-mouse CD45 and CD3e; as well as fluorescent FITC-conjugated monoclonal antibodies rat anti-mouse Sca-1+, CD11b and Gr-1 (Miltenyi Biotec, Germany). Cells stained with isotype matched antibody were used as a control for the gating of mouse CD45 and CD3e; as well fluorescent FITC-conjugated monoclonal antibodies rat anti-mouse Sca-1+, CD11b and Gr-1. The level of ROS was determined using a redox- or oxidant-sensitive probe, hydroethidine (DHE) into the cells [8]. Hydrogen peroxide (H2O2) was used as the positive control for ROS generation. Briefly, 1×10^6 cells/mL of MBMCs was exposed to different sizes of CSNPs at various concentrations (62.5-1000 µg/mL). Cell suspension without CSNPs exposure acted as a control. After 72 h of incubation, MBMCs were harvested and centrifuged at 2500 rpm for 5 min at 15°C. Supernatant was discarded and the pellet is re-suspended in 1 mL pre-warmed...
(37°C) DMEM. In the dark, 1 μL of HE (10 mM) was loaded to the sample and further incubated in 5% CO2 at 37°C for 15 min. Subsequently, the cells were washed twice in chilled PBS by centrifugation at 2500 rpm for 5 min at 4°C. Lastly, the pellet was re-suspended in 500 μL of chilled PBS. ROS production was quantified by measuring the intensity of HE fluorescence using flow cytometry.

Cellular Uptake Study on MBMCs
The uptake of FITC-CSNPs into MBMCs was evaluated using a fluorescent microscope (Leitz Laborlux S, Leica, Germany) following described protocol [9] with some modifications. The cells were seeded at 1×10^5 cells/mL in a 12-well plate and were treated with 0.2% w/v FITC-CSNPs at concentration of 1000 μg/mL for 24 h. Subsequently, 150 μL of cell suspension from each well was harvested and loaded onto the slide. The cells were directly fixed in 200 μL of 4% paraformaldehyde for 30 min at room temperature. The fixed cells were washed thrice in PBS and permeabilized in 200 μL of methanol at room temperature for 15 min. Finally, slides were stained with 8 µL of propidium iodide (PI) and stored in the dark at 4°C prior to analysis.

RESULTS AND DISCUSSION

SNPs characterization
CSNPs had a mean particle size that ranged from 200 to 700 nm and zeta potential values ranged from +57 to +75 mV, depending on the concentration of chitosan used. Smaller particle size was obtained with lower chitosan concentration and vice versa. Particles obtained were categorized into small (164.0±6.3 nm), medium (384.6±4.6 – 475.2±5.3 nm) and large (684.8±4.5 nm) CSNPs. Different sizes of particles were obtained by varying the chitosan concentration from 0.2% to 0.6% w/v (Table 1).

<table>
<thead>
<tr>
<th>Chitosan % (w/v)</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
<th>0.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (nm)</td>
<td>164.0 ± 6.3</td>
<td>384.6 ± 4.6</td>
<td>458.9 ± 3.2</td>
<td>475.2 ± 5.3</td>
<td>684.8 ± 4.5</td>
</tr>
<tr>
<td>PDI</td>
<td>0.393</td>
<td>0.488</td>
<td>0.518</td>
<td>0.862</td>
<td>0.884</td>
</tr>
<tr>
<td>Zeta-potential (mV)</td>
<td>+63.2 ± 5.4</td>
<td>+62.1 ± 5.1</td>
<td>+72.1 ± 4.6</td>
<td>+71.3 ± 5.3</td>
<td>+74.4 ± 4.6</td>
</tr>
</tbody>
</table>

MTT assay
At 24 h, no significant difference in MBMCs viability was noted between the treated groups and control group for all CSNPs concentrations tested. At 48 h, a significant reduction in MBMCs viability was observed at CSNPs concentration of 750 (76.8±8.0%) and 1000 μg/mL (26.3±0.9%) for small CSNPs. Following 72 h incubation, significant reductions in MBMCs viability were observed for groups treated with small CSNPs at 750 (76.8±8.0%) and 1000 µg/mL (27.6±4.2%).

Immunophenotyping assay
Exposure to small and medium particle sizes at CSNPs concentrations lower than 750 µg/mL showed reduced viability on Sca-1+ cells with noted significant reduction at 62.5 µg/mL (16.4±2.8%) for medium particle size (0.3% w/v chitosan only). Exposure to CSNPs for 72 h mediated particle size-dependent cytotoxicity in myeloid-committed progenitors (CD11b+ and Gr-1+ cells). CSNPs had no significant cytotoxicity effect on lymphoid-committed progenitors (CD45+ and CD3e+ cells).

ROS assay
Level of ROS production in MBMCs was not significantly different as compared to the ROS level in control group following CSNPs exposure for 72 h.

CONCLUSIONS
The data obtained from this study suggest that CSNPs mediate lineage-related and particle size-dependent cytotoxicity on mouse bone marrow-derived HSPCs ex vivo. Exposure to small and medium-sized CSNPs at concentrations less than 750 µg/mL showed cytotoxicity in Sca-1+ cells. In contrast, greater cytotoxicity was observed in myeloid-committed
progenitors exposed to medium-sized and large CSNPs. Interestingly, the percentage of lymphoid-committed progenitors remained unaffected by CSNPs exposure. Conversely, no evidence of oxidative stress was seen in the ROS analysis performed. This finding could greatly improve the degree of confidence regarding the safety of nanomaterials for diagnostic and/or therapeutic applications in humans. The evaluation of adverse effects of CSNPs targeting HSPCs deserves further investigation.

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ACKNOWLEDGEMENT
This research was funded by UKM grant: FRGS/1/2012/SKK02/UKM/02/1.
INTRODUCTION
Hydrogel dressings have generated the greatest interest as dressings for burns because they produce an ideal hydration environment for healing [1]. Hydrogels also have the ability to absorb exudates, and their transparency facilitates observation of the healing process [2]. Additionally, hydrogel have been widely investigated because of their biocompatibility with the human skin [3]. Recent studies have shown that bacterial cellulose (BC), synthesized by Acetobacter xylinum sp., has potential for use in wound dressings and artificial skin [4]. BC combined with AA (acrylic acid) at several ratios were used to fabricate hydrogels by exposure to accelerated electron beam (EB) irradiation at different doses. Fourier transform infrared spectroscopy (FTIR) results revealed that AA had been successfully grafted onto the cellulose fibers. Morphological analysis showed that hydrogels prepared by the mixture of BC and AA had a highly macroporous sponge-like structure. Pore size in the hydrogels decreased as AA content and irradiation doses increased [5]. These hydrogels exhibited many promising features for an effective wound dressing. The highly macroporous structure and water absorption capability of bacterial cellulose acrylic acid (BC/AA) hydrogels maybe beneficial for exudate absorption and preservation of moisture in the wound area. Furthermore, the degree of crosslinking of these hydrogels can be fine-tuned by varying the irradiation dose. This can be helpful in controlling the mechanical strength and water absorption capacity of the hydrogels. The objectives of this study were to develop and characterize BC/AA hydrogels synthesized by EB irradiation specifically used for wound dressing material and investigate its wound healing potential in an animal model.

EXPERIMENTAL METHODS
BC/AA hydrogel preparation
Purified BC was ground to a powder with particle sizes ranging from 20 to 200 μm. AA was added to a 1% (w/v) dispersion of BC in distilled water to produce a 40:60 AA:BC mixture, which was stirred using a mechanical homogenizer at room temperature (26°C) for 30 min and poured into petri dishes (10 mL each) for exposure to electron-beam radiation of 35 and 50 kGy in air at the accelerator facility (NHV-Nissin High Voltage, EPS 3000, Japan) at the Malaysian Nuclear Agency.

Animal studies
The wound healing characteristics of the H35 hydrogel preparation was evaluated in a rat model. All experiments were performed with the approval of the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). Female Sprague-Dawley rats (N = 62) weighing 150–200 g were obtained from the laboratory animal resource unit of Universiti Kebangsaan Malaysia (UKM). The rats had access to a standard diet and water ad libitum. The rats were maintained in a controlled environment with a 12-h light/12-h dark cycle at 24°C ± 2°C and 60 ± 5% humidity.

Establishment of skin burns
The rats were anesthetized by isoflurane inhalation. Partial-thickness burns were produced on the shaved area by a 10s application of a 1 cm diameter stainless steel template that was heated in boiling water for 5 min [6].

Treatment of burns wounds
The rats were divided into three groups (n = 6/group). Treat-ments were applied to each burn shortly after it was produced and rinsed with 70% alcohol. Group 1 animals were treated with H35, group 2 was treated with IntracSite Conformable®hydrogel (positive control), and group 3 received no treatment (negative control). The dressings were applied to the wounds daily for 14 days after cleaning with alcohol [6]. After positioning the dressings, bandaging tape and surgical tape were used to secure them, and wounds were allowed to heal. All rats were housed in individual cages.

Visual observation of wound closure
Standardized digital photographs of the wounds were taken after 1, 3, 7, and 14 days during replacement of the dressings. Each photograph was taken at the same position with a ruler placed at the bottom (proximal end) of the wound. The burn wound size for each animal was measured at specific times to calculate the percentage of wound closure.
Histological analysis
Animals were euthanized on days 1, 7, and 14. Excised burn sites were fixed with formaldehyde (10%), and embedded in paraffin. The samples were cut into sections (4 μm) with a cryomicrotome. After tissue sections were dewaxed and rehydrated, sections were stained with hematoxylin and eosin (H&E) and Masson’s trichrome stain. The stained samples were examined using an optical microscope under 20× and 40× magnification.

RESULTS AND DISCUSSION

Wound closure
Macroscopic observations of wound closure, as shown in Fig.1, revealed clear differences between the H35-treated and control groups. Each burn was observed for at 1, 3, 7, and 14 days post-injury. At days 1 and 3, inflammation (redness and swelling) was observed at the burn site. At day 7 post-injury, the untreated control groups showed hemorrhagic, scabbed wounds. However, no scabbing was observed at the burn sites in the H35-treated group.

Figure 1. Photographs on days 1, 3, 7, and 14 of wounds treated with H35, the positive control treatment, or untreated. H, hemorrhage; R, redness; S, scab

The rate of wound closure was evaluated by calculation of the open wound area as a function of time, as shown in Fig. 2. On day 1, there were no significant differences between the groups. However, on days 3, 7, and 14, H35-treated burns showed a greater percent-age of wound closure compared with the untreated burns.

Figure 2. Rate of wound healing of wounds treated with H35, the positive control treatment, or not treated on days 1, 3, 7, and 14.

Histological observation
The healing patterns of partial-thickness burns were studied by histological examination of the test and control samples at days 1, 7, and 14 post-burn by H&E staining and Masson’s trichrome staining. In comparison to the positive control, treatment with H35 markedly increased the formation of keratin and hair follicles, and the proliferation of blood vessels. The H35 and the positive control treatment groups showed organized superficial epithelium and were nearly healed.
CONCLUSION
Animal studies revealed that H35 accelerated healing by promoting neovascularization, reepithelialization, and proliferation of fibroblasts. Thus, BC hydrogels with BC:AA at a 60:40 ratio synthesized with 35 kGy have the potential to be used for burn dressings.

REFERENCES

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Effects of Genetic Polymorphism on Drug Metabolizing Enzymes in Primaquine Safety and Efficacy

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INTRODUCTION
As malaria global transmission is concerned, an estimated 3.4 billion people are at risk (WHO 2013). In 2012, WHO estimates that 207 million cases of malaria occurred globally with 627,000 deaths mostly occurring in Africa. Unfortunately most deaths were in children under the age of 5 years (WHO 2013). In Malaysia, there has been a reduction of malaria cases over the years and the incidence has declined from 16.1 per 100,000 populations in 2012 to 12.9 per 100,000 populations in 2013 (Vector Section, Disease Control Division, Ministry of Health, Malaysia, 2014). The pharmacoepidemiology of malaria infection in malaria has demonstrated to differ between years. This is especially true recently with the emergence of \( P. \) knowlesi. Apart from identifying the different types of infection, a study on pharmacoepidemiology can be conducted to assess the efficacy and safety profile of antimalarials in a large number of people in certain regions. Treatment of malaria is dependent on a handful of drugs, mainly the newer artemisinins and the much older quinolines. Despite the high use of primaquine in malaria, recent work has shown that there is potential influence of host genetic factors that may affect treatment outcomes (Roederer et al., 2011). This is due to the involvement of the human liver cytochrome P450 (CYP 450) enzymes. Polymorphisms in CYP450 may lead to primaquine levels being inadequate or otherwise. The effect of this is treatment failure or at times may lead to the presence of adverse effects. Host genetic factors thus, may play an important role in patient outcome of malaria patients, of which has been recently highlighted (Roederer et al., 2011). This study aims to identify effects of drug metabolizing enzymes systems, Cytochrome P450 and monoamine oxidase genetic polymorphism on efficacy and safety of primaquine.

EXPERIMENTAL METHODS
Study Design
The study comprises two sections:

i. Retrospective observation study
   Study of efficacy and safety profile of primaquine in human malarial infection obtained from national database.

ii. Prospective observation study
   Study on effect of genetic polymorphism on drug metabolizing enzymes in primaquine efficacy and safety from clinical progress of patients, genotyping and primaquine assay

Study Population
300 patients will be studied in retrospective study and 150 will be recruited in prospective study.

Inclusion Criteria
i. All adult patients with primaquine age 18 years old and over
ii. Uncomplicated malaria infection

Exclusion Criteria
i. Incomplete records
ii. Defaulted primaquine therapy
iii. Pregnancy and lactation

Data Collection
In retrospective study, data on efficacy of primaquine therapy will be identified based on parasite clearance from Blood Film Malaria Parasite and fever parameter as documented in the National Malaria Case registry, E-Vekpro and epidemiology weekly updates on malaria cases from various health state offices. Data on safety will be identified based on ADR reported through MADRAC and also from health state offices.

In prospective study, identified genetic polymorphisms of CYP2D6, CYP3A4, CYP1A2, CYP2C19 and Mao-A through PCR-RFLP will be compared with primaquine metabolism and clinical progress of patients mainly efficacy and
safety as documented in patient clinical notes and confirmed ADR reports. Elimination rate constant (kel) of parent drug primaquine and its main metabolite carboxyprimaquine is obtained simultaneously to measure the metabolism activity.

**Data Analysis**

Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 21.0. Demographic data will be analyzed using descriptive analysis. The type of statistical tests used will be based on the objectives as shown below:

i) Variables for response of therapy: (number of days to parasite clearance and temperature) will be presented as mean and median SD.

ii) Chi-square is used to measure the exposure of difference cumulative doses with presence of adverse drug events.

iii) Logistic regression is used to estimate the crude odds ratio (OR) for the associations between variables for response of therapy with primaquine cumulative doses.

iv) ANOVA statistical analysis is used to determined the differences of mean k and clinical outcome parameters in three groups identified from RFLP.

Chi square statistical analysis is used to determined the proportions of presence / severity of ADR in three groups identified from RFLP.

**REFERENCES**


**ACKNOWLEDGEMENTS**

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Study of Anxiety-related Behavior in Kainate-induced Epileptic Mice

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INTRODUCTION
Common comorbidities in epilepsy patients include major depression, anxiety disorders, psychosis and cognitive dysfunction [1]. Animal models of epilepsy, such as the kainic acid model of acquired epilepsy, are useful to study the relationship between epilepsy and behavioral dysfunctions. Such studies may also help to enhance our understanding of the causal mechanisms between epilepsy and behavioral abnormalities [2 -4] (Post, 2004, Majak and Pitkanen, 2004 and Heinrichs and Seyfried, 2006). Curcumin ([1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which is a yellow pigment isolated from the medicinal plant Curcuma longa [5]. It has been shown to inhibit acute seizures and neuronal death in kainic acid animal model through radical scavenging and SOD-like activities [6,7]. The purpose of the present study is to identify the effect of curcumin (CUR) on anxiety related behavior in ICR mice.

EXPERIMENTAL METHODS
Adult male ICR mice weighing 20-35 g were used in this study. Behavioral dysfunction and cognitive alterations were compared in three different groups. Group 1 was the control group (DMSO-saline); Group 2 was kainic acid (KA) without Cur (DMSO-KA) and Group 3 was KA with Cur (CUR-KA). Behavioral studies done were light/dark box test (LDT) and open field test (OFT). The test were done before curcumin (CUR), after CUR and after KA administration. LDT consisted of a plastic enclosure divided in two compartments. One compartment was dark (painted black and covered with a black lid) and the other compartment was non-covered, white and illuminated. The total time of mice spent in the light and dark compartments, respectively, was recorded. Rears, grooming duration, dark zone duration, light-dark transitions, light zone duration, defection, and urination were recorded manually [1].

OFT consisted of a one compartment plastic box. The arena is divided into 16 squares (4 x 4 cm). The following behavioral items are observed and recorded: sniffing, locomotion, stretch-attend, leaning, rearing, grooming, face-washing, digging, jumping, gnawing, freezing, pausing, central and total transitions [1].

RESULTS AND DISCUSSION
Increase time spent in dark zone in LDT indicate higher level of anxiety. Rearing and number of line crosses correlate with locomotor and exploratory activity. Increase in latency showed decrease anxiolysis [8] (refer Table 1).

Table 1. Behavioral measures of mice in LDT (N=18)

<table>
<thead>
<tr>
<th>Behavioural measures</th>
<th>DMSO-Saline N=6</th>
<th>DMSO-KA N=6</th>
<th>CUR-KA N=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency</td>
<td>Pre-CUR 20.0±24.6, 29.2±36.8, 21.5±52.0</td>
<td>Post-CUR 15.0±20.22, 3.0±3.16, 1.7±1.6</td>
<td>Post-KA 3.5±3.2, 7.7±8.6, 22.8±40.6</td>
</tr>
<tr>
<td>Rearing</td>
<td>Pre-CUR 0, 0</td>
<td>Post-CUR 2.0±2.53, 1.0±1.1</td>
<td>0.33±0.82, 2.5±4.0</td>
</tr>
<tr>
<td>Grooming</td>
<td>Pre-CUR 0</td>
<td>Post-CUR 12.3±17.7, 34.3±41.6</td>
<td>10.6±10.03, 16.2±19.0</td>
</tr>
<tr>
<td>Duration in dark zone</td>
<td>Pre-CUR 151.7±19.4, 156.3±47.8, 273.0±64.6</td>
<td>Post-CUR 138.7±49.2, 211.5±70.3, 291.7±54.8</td>
<td>Post-KA 138.7±58.2, 136.8±95.5, 190.7±99.7</td>
</tr>
<tr>
<td>Duration in light zone</td>
<td>Pre-CUR 133.7±38.9, 113.8±108.9, 75.2±39.3</td>
<td>Post-CUR 131.3±68.3, 206.3±132.8, 209.7±53.4</td>
<td>Post-KA 131.3±68.3, 207.3±132.8, 209.7±53.4</td>
</tr>
<tr>
<td>Defecation</td>
<td>Pre-CUR 1.83±1.94, 0.5±1.22, 3.2±1.83</td>
<td>Post-CUR 1.17±1.13, 1.5±1.76, 1.2±2.4</td>
<td>Post-KA 1.0±1.6, 2.6±2.9, 1.2±1.8</td>
</tr>
<tr>
<td>Urination</td>
<td>Pre-CUR 0, 0</td>
<td>Post-CUR 0, 0.17±0.4</td>
<td>0.17±0.4</td>
</tr>
</tbody>
</table>
For OFT, increase frequency of rearing and line crosses showed decrease in anxiety level. Number of central square entries and duration of time spent in central square measure exploratory and anxiety level (Bailey KR and Crawley JN, 2009).

### Table 2. Behavioral measures of mice in OF (N=18)

<table>
<thead>
<tr>
<th>Behavioural measures</th>
<th>DMSO-Saline N=6</th>
<th>DMSO-KA N=6</th>
<th>CUR-KA N=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sniffing Pre-CUR</td>
<td>1.33± 0.52</td>
<td>1.00± 0.00</td>
<td>1.00± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.83± 0.41</td>
<td>1.83± 0.41</td>
<td>1.83± 0.41</td>
</tr>
<tr>
<td>Locomotion Pre-CUR</td>
<td>2.00± 0.00</td>
<td>2.00± 0.00</td>
<td>2.00± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.67± 0.52</td>
<td>1.83± 0.41</td>
<td>1.83± 0.41</td>
</tr>
<tr>
<td>Stretching Pre-CUR</td>
<td>1.17± 0.41</td>
<td>1.33± 0.52</td>
<td>1.33± 0.52</td>
</tr>
<tr>
<td></td>
<td>1.17± 0.41</td>
<td>1.00± 0.00</td>
<td>1.17± 0.41</td>
</tr>
<tr>
<td>Rearing Pre-CUR</td>
<td>1.67± 0.52</td>
<td>1.50± 0.55</td>
<td>1.50± 0.55</td>
</tr>
<tr>
<td></td>
<td>1.17± 0.41</td>
<td>1.33± 0.52</td>
<td>1.33± 0.52</td>
</tr>
<tr>
<td>Defecation Pre-CUR</td>
<td>1.67± 0.52</td>
<td>1.17± 0.41</td>
<td>1.50± 0.55</td>
</tr>
<tr>
<td></td>
<td>1.5± 0.55</td>
<td>1.67± 0.52</td>
<td>1.33± 0.52</td>
</tr>
<tr>
<td>Urination Pre-CUR</td>
<td>1.00± 0.00</td>
<td>1.00± 0.00</td>
<td>1.00± 0.00</td>
</tr>
<tr>
<td></td>
<td>1.17± 0.41</td>
<td>1.33± 0.52</td>
<td>1.33± 0.52</td>
</tr>
<tr>
<td>Line crossing Pre-CUR</td>
<td>132.50± 34.3</td>
<td>102.67± 22.18</td>
<td>13.03± 32.29</td>
</tr>
<tr>
<td></td>
<td>61.17± 17.53</td>
<td>130.33± 32.29</td>
<td>79.67± 4.27</td>
</tr>
<tr>
<td>Centre square entries Pre-CUR</td>
<td>13.50± 5.13</td>
<td>7.5± 5.32</td>
<td>13.67± 4.27</td>
</tr>
<tr>
<td></td>
<td>2.83± 3.60</td>
<td>13.67± 4.27</td>
<td>5.50± 3.62</td>
</tr>
<tr>
<td>Centre square duration Pre-CUR</td>
<td>70.00± 85.95</td>
<td>10.67± 7.20</td>
<td>16.00± 5.44</td>
</tr>
<tr>
<td></td>
<td>3.5± 3.89</td>
<td>16.0± 5.44</td>
<td>5.83± 3.87</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Curcumin has been reported to possess neuroprotective effects and act as an anxiolytics (Cole GM et al, 2007). Current study showed epileptic mice treated with curcumin were less anxious compared to epileptic mice without treatment.

**REFERENCES**


**ACKNOWLEDGEMENTS**

This research was funded by UKM-GUP Khas 2011. Special thanks to UKM for the Zamalah scholarship.
Identification and Applicability of Themes in Group Behavioural Therapy Module Developed for Workplace Smoking Cessation Program in Malaysia: A Qualitative Approach

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*Corresponding author: faizal_maarof85@yahoo.com

INTRODUCTION
Smoking related diseases have been the primary cause of mortality for the past three decades in many developing countries including Malaysia. [1] Even though, the Ministry of Health (MOH), Malaysia provides smoking cessation services through 294 quit smoking clinics (QSCs), [2] the prevalence of smoking has increased by 1.6% between 2006 and 2011.[3,4] The potential cause of the rising number of smokers are failure in training adequate number of workers [2] and lack of counseling skills among the health care providers [5] in QSCs, which may potentially contribute to the poor service delivery. Non–pharmacological approach such as behavioural support is important since smoking is associated with nicotine addiction, physical and behavioural involvement. [6]

There are several modules of smoking cessation programme (SCP) that has been used for the purpose of health promotion activities or research in Malaysia. The training module for healthy lifestyle campaign at workplace has been introduced by MOH in 2004 which involved various parties such as smoker and non-smoker staffs, and employers as the target groups. [7] Meanwhile, the Malaysian Pharmaceutical Society has launched the Corporate Smoking Cessation program in 2004 which aimed to help workers to drop their strong smoking habit. [8] To date, the data on the success and continuation of these programs at workplace is limited.

There is a lack of study in Malaysia that evaluates the acceptability of the components in the smoking cessation module. Therefore, this study aimed to identify and propose the components in the group behavioural therapy module developed for WSCP.

EXPERIMENTAL METHODS
Setting and Participants
This study was conducted from May to August 2014 in the Universiti Kebangsaan Malaysia, KL Campus. Purposive sampling method was conducted. Staffs who were regular smokers and at least in the contemplator stage of quitting smoking and those who were interested to participate were invited to enroll in this study. The discussions were conducted at the Health Psychology Clinic, Faculty of Health Sciences, UKM.

Study Tools
Behavioural module was developed based on extensive literature review, SCP guidelines search and discussions among expert panelists. The focus groups were conducted in a close session for 60 to 90 minutes in two groups for four sessions. It covers the elements of psycho-educative, behavioural and physical change technique, cognitive therapy and also prevention of weight gain as recommended by the smoking cessation guideline.

Data Collection
Identification of themes in the module for group behaviour therapy

Analysis
All discussions were audio recorded and transcribed verbatim by MFM with the participants’ permission. The accuracy of the transcripts was verified by the independent rater who listened to the audio-taped, and later returned to the participants for comment(s) and/or correction(s). The interview data were analysed using the thematic analysis [9] with ATLAS.ti v7. Each transcript was repeatedly read to identify the common themes and significant points raised by the participants. The
discussions were continued until theoretical saturation was reached.[10] The analysis primarily drew on deductive thematic coding, established by the discussion guides, with secondary inductive coding that helped disclose any emergent themes from the discussion transcripts.[11] All authors discussed the themes to refine the analysis.[9] The themes were then discussed with independent researchers to ensure their reliability and trustworthiness.[12] This lead to the establishment of the final inclusive thematic map (Table 1), which addressed the common themes across all participants.

Table 1. Themes identified by the smokers

<table>
<thead>
<tr>
<th>Themes, subthemes, and components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preparation to quit</strong></td>
</tr>
<tr>
<td>Reasons of continuing smoking</td>
</tr>
<tr>
<td>The advantages of smoking</td>
</tr>
<tr>
<td>The disadvantages of cessation</td>
</tr>
<tr>
<td>Love about cigarette</td>
</tr>
<tr>
<td>Identifying reasons for quitting</td>
</tr>
<tr>
<td>The expectations in study involvement</td>
</tr>
<tr>
<td>The disadvantages of smoking</td>
</tr>
<tr>
<td>The advantages of cessation</td>
</tr>
<tr>
<td>Comprehending smoking characteristics</td>
</tr>
<tr>
<td>Smoking habit</td>
</tr>
<tr>
<td>Smoking pattern</td>
</tr>
<tr>
<td>Smoking environment</td>
</tr>
<tr>
<td>Nicotine dependence</td>
</tr>
<tr>
<td>Quit attempts experiences</td>
</tr>
<tr>
<td>Presence of withdrawal symptoms</td>
</tr>
<tr>
<td>Smoking triggers</td>
</tr>
<tr>
<td>Quit challenges</td>
</tr>
<tr>
<td><strong>Quitting</strong></td>
</tr>
<tr>
<td>Support and encouragement</td>
</tr>
<tr>
<td>Intra-treatment social support</td>
</tr>
<tr>
<td>Extra-treatment social support</td>
</tr>
<tr>
<td>Learn new skills and behaviour</td>
</tr>
<tr>
<td>Coping with smoking triggers</td>
</tr>
<tr>
<td>Coping with withdrawal symptoms</td>
</tr>
<tr>
<td>Reward for not smoking</td>
</tr>
<tr>
<td><strong>Staying quit</strong></td>
</tr>
<tr>
<td>Be prepared for lapse/relapse or difficult situations</td>
</tr>
<tr>
<td>Reasons of previous relapse</td>
</tr>
</tbody>
</table>

**RESULTS**

<table>
<thead>
<tr>
<th>Preparation to quit</th>
<th>Quotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reasons of continuing smoking</td>
<td>I feel the sense of pleasure and enjoyment during smoking. I feel the masculinity of it. (Smoker 02, 32 yrs)</td>
</tr>
<tr>
<td>Identifying reasons for quitting</td>
<td>Smoking is <em>haram</em> in Islam. A lot of my friends have quit smoking successfully and they asked me to quit too. Personally, I feel bad about the smoking habit. (Smoker 03, 30 yrs)</td>
</tr>
<tr>
<td>Comprehending smoking characteristics</td>
<td>...now, I just realised about my smoking pattern...I smoked without thinking of the smoking pattern in the office. If I can focus (not to smoke) during office hour, I should do the same thing when I’m outside especially with my friend(s). (Smoker 01, 28 yrs)</td>
</tr>
<tr>
<td>Quit attempt experiences</td>
<td>I also had experienced restlessness, unstable emotion and getting angry easily. (Smoker 06, 30 yrs)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quitting</th>
<th>Quotes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Support and encouragement</td>
<td>I need my colleagues to provide me with support during the quitting process. But, there are a lot of them (smokers) at my workplace. My work station is just next to the smoking port. They will tease me to</td>
</tr>
</tbody>
</table>
DISCUSSION

The aim of this study was to identify and propose the components in intervention program which mainly focused on behavioural approach for Malaysian smokers. The focus groups provide an opportunity for participants to explain in their own words their problems in quitting. The themes have been identified to be applied in group behaviour therapy. The themes were linked with the available interventions which have been discussed in the literature and guidelines as a reference. The participants felt that those items in the module would help them to quit successfully.

This finding has shown that the themes in the module would help smokers to gain knowledge and increase their awareness and confidence in smoking cessation. This is essential to ensure the modules are delivered effectively and successfully.

There are several limitations to our present study. The study was conducted among supporting staffs and predominantly Malay male. The findings of this study may not be generalised to the other occupational level, gender and ethnicity.

CONCLUSION

This qualitative study provides a preliminary data on the experiences and issues of the smokers for smoking cessation. These results will lead to an effective intervention program emphasising important behavioural approach and knowledge acquisition. The findings suggested that all behavioural components developed could be applied in delivering GBT.

REFERENCES


ACKNOWLEDGEMENT

The research was supported by National University of Malaysia Research Grant (UKM-Komuniti-2013-018). We thank to the participants and senior management of the faculty for permission to conduct this study among their staff and independent researchers for their help in transcript analysis.
Improving Medication Safety: The Preparation and Administration of Parenteral Medications among Nurses in General Intensive Care Unit (GICU) in a Tertiary Teaching Hospital Before and After Education Intervention

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2Department of Anaesthesiology & Intensive Care, Universiti Kebangsaan Malaysia Medical Centre, Malaysia
*Corresponding author: tansuetyin@gmail.com

INTRODUCTION
Up to 90% of inpatients receive some form of intravenous (IV) therapy during their admission [1]. However, IV therapy is associated with a higher error rate and risk compared to other routes of administration [2, 3]. Studies had shown drug infusions prepared manually often fall out of acceptable pharmaceutical standard [4]. Intensive care units (ICUs) and medical wards were identified as the highest chance of the error among all hospital wards [5]. Critical care settings are prone to errors due to the complexity of care, frequent need for high-risk interventions and inability of patient to communicate errors [6]. Several studies identified lack of education as the main cause of IV drug error [7]. To the best of our knowledge, there is a lack of interventional study conducted on parenteral medication administration errors. Hence, this study aims to determine the error rate in the preparation and administration of parenteral medications among nurses in GICU before and after education intervention using observation method. In addition, the accuracy of selected drug infusion concentration before and after intervention will also be measured.

EXPERIMENTAL METHODS
Observation
Convenience sample of nurses who prepare and administer parenteral medications in the GICU were observed pre and post intervention. Nurses were told that their common practice of parenteral medication administration will be observed by a researcher. Parenteral medications include all intravenous (bolus and infusion), intramuscular, and subcutaneous medications. The observer followed nurses and recorded details of each parenteral medication preparation and administration using a modified checklist based on the Manual of Clinical Nursing and World Health Organization. Any deviation in the administration of parenteral medications from doctor’s prescription, the hospital protocols, or from the manufacturer’s instructions was considered an error. Errors classified according to the American Society of Health-System Pharmacists. Intervention was an education package developed based on the pre intervention findings. Education tools included a 8 minutes education video of standard operating procedure for parenteral medication preparation and administration, memory aid and power point presentation. Teaching sessions were conducted weekly for GICU nurses using the education tools developed.

Sample collection
The concentration of selected reconstituted and diluted drug infusions were measured using isocratic HPLC pump (Waters 1515, Massachusetts, US). IV noradrenaline infusion was chosen based on its high usage in the ward and it requires preparation prior to use. The infusion was considered an error when the actual concentration prepared deviated by more than 10% above or below the expected concentration.

Ethical Issue
The study was approved by the Hospital Research Ethics Committee (NF-046-2013)

Statistical Analysis
The outcome measure of error rate was calculated as number of observations with one or more errors divided by the total number of observations plus dose omissions. All data were entered into and analysed using the Statistical Package for Social Sciences, version 22. Student t test and chi square test were used when comparison were made between pre and post intervention. Data were tested for normality using the Kolmogorov-Smirnoff test; then the Mann-Whitney non-parametric test was used to compare drug infusion concentrations before and after the intervention.

RESULTS & DISCUSSION
Demographic Data
The nurses and medication characteristic are shown in Table 1. There was no significant difference in nurses’ characteristic in terms of age and clinical experience before and after the intervention. Types of administration and class of medication were also not significantly different pre and post intervention.
Table 1. Nurse and medication characteristic pre and post intervention

<table>
<thead>
<tr>
<th>Nurse characteristic</th>
<th>Pre</th>
<th>Post</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year ± SD)</td>
<td>30±4</td>
<td>30±4</td>
<td>0.99</td>
</tr>
<tr>
<td>Clinical experience (year ± SD)</td>
<td>7±4</td>
<td>8±4</td>
<td>0.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medication characteristics</th>
<th>(n=122)</th>
<th>(n=105)</th>
<th>0.14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Types of administration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bolus</td>
<td>34</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Infusion</td>
<td>87</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Class of medication</td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>Analgesia/sedation</td>
<td>10</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Vasopressor/catecholamine</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial</td>
<td>44</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Diuretic</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Electrolytes</td>
<td>33</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Gastro-intestinal</td>
<td>21</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Error rate (pre and post)
Preparation and administration of 122 parenteral medication doses were observed during the pre-intervention period while 105 doses during post intervention. The incidence of errors decreased from 79% to 51% following education intervention (P<0.001). Pre-intervention, 40 (33%) doses had two or more errors while 7 (6%) doses had 3 errors. Post-intervention, 17 (16%) had 2 or more errors while none had 3 errors. Table 2 summarised the types of error performed during preparation and administration of parenteral medication.

Table 2. Incidence of medication errors observed before and after education intervention expressed in number (%)

<table>
<thead>
<tr>
<th>No (%) of error</th>
<th>Pre (N=122)</th>
<th>Post (N=105)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error rate</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>One or more error per dose</td>
<td>96 (79)</td>
<td>47 (45)</td>
<td></td>
</tr>
<tr>
<td>Two or more error per dose</td>
<td>40 (33)</td>
<td>17 (16)</td>
<td></td>
</tr>
<tr>
<td>Three error per dose</td>
<td>7 (6)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Types of medication error</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wrong preparation</td>
<td>69 (57)</td>
<td>32 (30)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Incompatible medication</td>
<td>13 (11)</td>
<td>3 (3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Incorrect dose administration</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>0.92</td>
</tr>
<tr>
<td>Incorrect rate administration</td>
<td>43 (35)</td>
<td>28 (27)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Incorrect time administration</td>
<td>7 (6)</td>
<td>0 (0)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Concentration error
A total of 40 samples of IV noradrenaline infusion were collected pre intervention and 15 samples post intervention. Concentration errors were found in 19 samples (48%) pre-intervention and 53% post intervention. Pre-intervention, 7 samples (18%) deviated by more than 30% above or below the expected concentration, while 3 samples (8%) deviated by more than 50%. Post intervention, 1 sample (6%) deviated by more than 30% and none of the sample deviated by more than 50%. However the effect is not significant (P=0.212).
CONCLUSION

Education intervention was effective in reducing the parenteral medication preparation and administration error. However, the concentration error was not reduced after intervention. The pharmacy department could supply pre-prepared parenteral medications as a measure to reduce error rate. However, further study is recommended to strengthen the evidence base for this practice.

REFERENCES

Evidence of Estrogenic Property of *Mimosa pudica* L. In silico and in vitro Evaluation

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**INTRODUCTION**

*Mimosa pudica* L. belongs to the Fabaceae family is native to Brazil and abundantly found in tropical areas [1]. Traditionally, the plant is consumed to treat gynaecological problems and as a sex stimulant by Garo tribes of Bangladesh, as a birth control agent in Assam, India, and for the treatment of anxiety, depression, neurasthenia, insomnia, traumatic injury and pulmonary tuberculosis in China [2-5]. *M. pudica* has also been reported to cure blood diseases, congestion with phlegm and biliousness [6]. Phytochemical constituents previously isolated from this plant were flavonoid glycosides, flavonoids, phenolics, alkaloids, triterpenoids, steroids and others. Studies on this plant suggest a number of pharmacological uses such as antimicrobial, antimalarial, anthelminthic, antioxidant, analgesic, anti-inflammatory, antiestrogenic, antifertility, wound healing, antiulcer, anticonvulsant, anti-hepatotoxic, hypoglycemic, antivenom and antidepressant [1-2,7-9].

**EXPERIMENTAL METHODS**

**Preparation of plant fractions**

Whole plants of *Mimosa pudica* were collected from Kuala Lumpur, Malaysia and identified by Mr. Sani Miran from the Herbarium of Universiti Kebangsaan Malaysia. A voucher specimen (UKM b 30,000) was deposited at the herbarium. The specimens were air-dried and ground to powder. The powder was sequentially macerated at room temperature with n-hexane followed by dichloromethane and methanol for at least 48 hours each time. The resultant n-hexane, dichloromethane and methanol fractions were evaporated to dryness in vacuo using a rotary evaporator. The aqueous fraction was prepared by reflux extraction for 2 hours and the filtrate was freeze-dried. The dried fractions were re-dissolved in DMSO to give the desired test concentrations by serial dilution in assay media.

**Data sets for docking study and Molecular docking**

To predict the estrogenic activity of *M. pudica*, a list of 16 experimental ligands were obtained from different published literatures related to phytochemical isolation or characterisation of *M. pudica*. 17β-estradiol (E2) was also docked. The chemical structures were drawn using ChemDraw Ultra (version 8.0) and then converted to PDB format. Docking studies were performed with the CDOCKER protocol under the receptor-ligand interaction section in Discovery Studio® 3.1 (Accelrys, Inc., San Diego, CA, USA). All of the 3D structures of the compounds were built with ChemBioOffice® 2008 (PerkinElmer, Inc., Waltham, MA, USA). The crystal structure of human ERα receptor protein (1ERE: 3.1 Å) was retrieved from Brookhaven Protein Data Bank and the 16 ligands were retrieved from published literature.

**MCF-7 Cell Proliferation Assay**

One week prior to treatment cells were cultured in phenol red free RPMI 1640 media with 5% charcoal stripped FBS. 2000 cells/well were seeded in 96 well plate and allowed to attach overnight. The following day, treatment was given with appropriate concentration of extracts and incubated for 7 day. After MTT treatment for 4 hours the absorbance were taken at 550 nm.

**Statistical analysis**

Results are presented as the mean ± standard error of mean (SEM) of three separate experiments (n=3). One-way analysis of variance (ANOVA) with Dunnett's Multiple Comparison post test was determined using GraphPadInStat Software where p<0.05 value was considered statistically significant.

**RESULTS AND DISCUSSION**

Docking of compounds reported in previous literatures

Compounds isolated from *Mimosa pudica* retrieved from previously published literature were docked into an estrogen active site of ERα receptor and were compared with the interaction of established endogenous estrogen, E2.
Compounds | Amino acid bindings in ERα with bond distance (Å)
---|---
17β-estradiol | His 524, Thr 347, Glu 353, Arg 394, Leu 387, Gly 521, Met 421
Myricetin | 2.13, 1.95, 1.94
5,7,3’,4’-tetrahydroxy-8-C-β-apinose-(1→4)β-D-glycopyranosyl flavone | 2.4, 1.97, 2.16
Potassium-5-O-β-D-glucopyranosyl gentisate | 2.11, 2.43, 2.16, 2.45
Isoorientin | 2.19, 2.02, 1.89, 1.86, 2.15, 2.23, 1.86
Orientin | 2.00, 2.00, 2.06, 2.04, 2.16
Quercetin-7-rhamnoside | 2.12, 1.96, 2.07, 2.32, 1.83, 2.23

MCF-7 cell proliferation assay

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RPE values (%) at different concentrations (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-5}</td>
</tr>
<tr>
<td>Hexane</td>
<td>55.91</td>
</tr>
<tr>
<td>Dichloro methane</td>
<td>107.9</td>
</tr>
<tr>
<td>Methanol</td>
<td>77.24</td>
</tr>
<tr>
<td>Aqueous</td>
<td>68.0</td>
</tr>
</tbody>
</table>
CONCLUSION

In conclusion, our in silico and in vitro studies clearly show the potential estrogenic value of *M. pudica*. Some of the compounds found in this plant showed ERα receptor binding characteristics when docked. The dichloromethane fraction also demonstrated significant estrogenicity in the cell proliferation assay. The results provide preliminary indication of the traditional use of this plant in gynecological and post-menopausal problems that could possibly be applied as a supplement for hormone replacement.

REFERENCES


Tumor Targeted Delivery of Doxorubicin by Folate Functionalized Chitosan-Pluronic F127 Micelles

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INTRODUCTION

Discovery of arsphenamine and concept of “magic bullet” by Paul Ehrlich were the cornerstones of chemotherapy and targeted drug delivery systems. Doxorubicin, an anthracycline anticancer chemotherapeutic agent, is widely used for the treatment of solid tumors. However cytotoxicity in normal tissues and multidrug resistance (MDR) limit use of doxorubicin so a vehicle specifically targeting cancer cells is required for the delivery of doxorubicin and other anti-cancer drugs [1]. Pluronic F127, a block copolymers of polyethylene oxide (shell) and polypropylene oxide (core), is an FDA approved excipient and is used for micelle preparation owing to increased circulation times for incorporated drugs. Chitosan has been used as a vehicle for delivery of genes and drugs owing to positive charge which also promotes endocytosis of the resulting nano carrier [2]. Chitosan also has the ability to rupture endosome which is required for the release of drug into cytosol. Cancer cells over express the receptors for folic acid (the folate receptors) on their surface [3] so folic acid functionalized drug delivery vehicles are highly tumor specific and can enter the cells by endocytosis. Based on aforementioned characteristics, a folic acid functionalized chitosan was synthesized. Pluronic F127-Chitosan micelles were prepared encapsulating doxorubicin. Micelles were characterized for size, zeta potential, morphology, CMC, drug release, cytotoxicity and drug uptake. Furthermore, the tumor inhibition of micelles was evaluated using 4T1 breast cancer models in BALB/c mice.

EXPERIMENTAL METHODS

Synthesis of Folic acid conjugates

For synthesis of folic acid modified chitosan (Scheme 1) the folic acid was reacted first with N-hydroxysuccinimide (NHS) to activate the carboxylic groups. For activation, folic acid and NHS were dissolved in DMSO with aid of heating (50 °C) for 1 hour. Then N,N’-Dicyclohexylcarbodiimide (DCC) was added to the above solution and stirring was continued for another 3-5 hours. After this the above reaction mixture was added to 1% chitosan. Reaction was allowed to continue for 24 hours under continuous stirring. After this, the pH was raised up to 9 using 0.1N sodium hydroxide. It was then dialyzed against PBS pH 10 until no folic acid was detected in the dialysis medium. Absorbance measurements were taken at 360 nm. Then it was dialyzed against distilled water for another 3 days. Folic acid conjugated chitosan was recovered by freeze drying the product.

FTIR

Folic acid modified chitosan was then confirmed by FTIR spectroscopy. The FTIR spectra of the conjugate were recorded against background using a universal ATR sampling assembly in range of 4000 to 550 cm⁻¹.

Preparation and characterization of micelles

Micelles were prepared by direct dissolution where a 10 % solution of Pluronic F127 in 10 mM acetate buffer was mixed with varying concentrations of chitosan solution. It was then followed by incubation at 40 °C for 30 minutes. Then this micelle suspension was filtered through 0.45 micron filter to achieve uniform sized micelles. Micelles were then characterized for size, zeta potential, CMC and morphology using a zetasizer (Zetasizer Nano ZS, Malvern instruments, UK) and transmission electron microscopy (TEM) respectively.
Cytotoxicity
Cytotoxicity studies were carried out to evaluate the in vitro efficiency of polymeric micelles in human colon carcinoma cell line (DLD-1) and doxorubicin resistant DLD-1 cell line (DLD-1-DOX) in order to observe and compare this effect with doxorubicin as single regimen.

RESULTS AND DISCUSSION
FTIR spectra of chitosan, folic acid and folic acid conjugated chitosan are shown in Figure 1. The FTIR spectrum of chitosan shows a broad band at ~ 3450 cm\(^{-1}\) which corresponds to the stretching N – H vibration, the small peaks at 2850 cm\(^{-1}\) are typical C – H stretch vibrations while the small peak at nearly 900 cm\(^{-1}\) shows the wagging of saccharide structure of chitosan. FTIR spectra for folic acid shows a number of characteristic bands occurring at ~ 3550, 3400, 1700, 1650 and 1500 cm\(^{-1}\) corresponding to O – H stretching (between 3500 – 3000), C=O stretching of –CONH\(_2\) at 1650 and the characteristic absorption band of phenyl ring at 1500 cm\(^{-1}\).

The conjugation of folic acid to chitosan could be confirmed by the presence of characteristic peaks for both chitosan and folic acid [2].

Size of micelles was determined by dynamic light scattering method using zetasizer. Effect of chitosan concentration of size was observed. This increase in size could be attributed to presence of hydrophilic chitosan in the micelle suspension which lead to reduced hydrophilic-hydrophobic interactions resulting in loosening of micelles structure and thus increased size. Similarly the zeta potential of micelles also increased and decreased corresponding to increase or decrease in concentration of chitosan. When the chitosan concentration was increased from 0 to 5 mg/ml the zeta potential varied from 0 to 25 mV. This was the result of the cationic nature of chitosan as concentration of pluronic was fixed in all the formulations. The morphology and size of the micelles was observed using FESEM (Figure 2).
CMC of the micelles was found not to be affected with the change in chitosan concentration. The presence of chitosan had pronounced affect on the light intensity changes as depicted by the sudden increase in intensity of scattered light. A stronger signal was observed for the formulations with more chitosan.

Cytotoxicity studies in DLD-1 and DLD-1\textsubscript{DOX} indicated that free doxorubicin had little or no effect on DLD-1\textsubscript{DOX} cells, whereas doxorubicin loaded micelles were 4 fold more effective than free doxorubicin (Figure 3).

Tumors were developed by inoculation of 4T1 cells in the 4th inguinal mammary fat pads of 8-12 week old female Balb/c mice. Mice were divided into 4 groups (6 mice per group) receiving saline, free doxorubicin, doxorubicin loaded Chitosan-Pluronic F127 micelles and folate functionalized doxorubicin loaded chitosan-Pluronic F127 micelles. When the tumor size reached 50-100 mm\textsuperscript{3}, treatment with doxorubicin or micelles was started. Tumor size, body weight and general health was measured every 3 days and mice showing any signs of illness were euthanized. Doxorubicin loaded folate functionalized micelles show the maximum tumor growth inhibition.

CONCLUSIONS
Folic acid modified conjugates of chitosan were successfully prepared and characterized. Micelles prepared from chitosan and pluronic had positive zeta potential values which makes them a potential candidate for gene delivery. Further, doxorubicin loaded micelles were more effective in DLD-1-DOX cells compared to free doxorubicin.

REFERENCES

ACKNOWLEDGEMENTS
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Induction of Annexin A1 by Selected Phytoestrogens to Inhibit Various Human Leukaemic Cells

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INTRODUCTION
Chemotherapy, radiotherapy, operative management, monoclonal antibodies [1] are a few approaches for the management of various types of cancer and leukemia. Among these, chemotherapy is the major and most successful therapeutic approach for the treatment of localized and metastasized cancer [2]. However, its use is limited due to lack of selectivity, severe toxicity and continuous development of resistance to the chemotherapeutic agents [3]. Hence, there is a need to discover novel anticancer agents by using alternative approach to outclass the limitations of current therapeutic regime. Annexin A1 (ANXA1), an endogenous anti-inflammatory protein, has roles in many diverse cellular functions, such as membrane aggregation, inflammation, phagocytosis, proliferation and apoptosis. The role for ANXA1 in apoptosis and cancer has also been recently proposed [4,5]. ANXA1 is a glucocorticoid-inducible protein. It is postulated that compounds like phytoestrogens which have structural and functional resemblance with the estrogens may have possible role in the induction and secretion of ANXA1. Studied by Ahmad et al. 2014, showed that phytoestrogens can induced ANXA1 production in normal peripheral blood mononuclear cells. Hence, this study aims to explore the role of phytoestrogens in inducing ANXA1 production in leukemic cells and effect on the ANXA1 induction in various leukemic cells.

EXPERIMENTAL METHODS
Cell Cultures
Human Leukemic Monocyte lymphoma U937 cells, Human Chronic Myelogenous Leukaemia K562 cells and human T-cell Leukaemia Jurkat cells were used as model cells in this study. U937 cells and Jurkat cells were maintained in RPMI 1640 medium supplemented with 2mM L-glutamine, 10% heat-inactivated foetal bovine serum (FBS), 1% streptomycin at 37°C in 5% CO₂, 95% air humidified atmosphere. K562 cells were maintained in Iscove’s Modified Dulbecco’s medium (IMDM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1% streptomycin at 37°C in 5% CO₂, 95% air humidified atmosphere.

Methyl Thiazolyl Tetrazolium (MTT) assay for cell viability
Cell viability was assessed by MTT assay. Cells were seeded at an initial density of 2×10⁵ cells/mL in a 96-well plate for 24 h. Cells then incubated with fresh medium containing various concentrations of phytoestrogens and pure ANXA1. After incubation, MTT (5mg/mL) were added into each well. The insoluble formazan will be collected and dissolved in dimethylsulfoxide (DMSO 0.5%) and after complete dissolution, absorbance were detected at 570 nm by a microtiter plate reader. Cell viability (%) were calculated using the following equation: survival ratio (%) = (Atreatment / Acontrol) x 100%.

Methyl Thiazolyl Tetrazolium (MTT) assay for cell growth assay:
Cells were plated at a density of 3000 per well in 96-well plates in 200µL medium, and incubated for 24 h and 72 h at 37°C. MTT (5 mg/mL) were added into each. The insoluble formazan will be collected and dissolved in dimethylsulfoxide (DMSO 0.5%) and after complete dissolution, absorbance were detected at 570 nm by a microtiter plate reader. Cell growth (%) werecalculated using the following equation: survival ratio (%) = (Atreatment / Acontrol) x 100%.

Analysis of Annexin A1:
Expression of ANXA 1 were quantified by using sandwich Enzyme linked immunosorbat Assay (ELISA). Protocol was carried on according to protocol provided by the manufacturer.

Analysis of apoptosis:
The cells were seeded at 1×10⁶ cell per well in 6-well plate, and exposed to compound at selected concentration. Cells were washed with cold phosphate-buffered saline (PBS) and resuspended in Annexin V Binding buffer. Cells were stained with Annexin V FITC and Propidium Iodide (PI) prior to analysis by flow cytometry.

Analysis of cell cycle by Flow cytometer:
The cells were seeded at 1×10⁶ cell per well in 6-well plate, and exposed to compound at different concentration. After pre-determined time intervals, cells were harvested and fixed in cold 70% ethanol at 20°C. Cell cycle profiles were
evaluated by DNA staining with propidium iodide in phosphate-buffered saline (PBS) supplemented with 100 U/mL ribonucleases A, for 30 min at room temperature. Samples were analysed with a flow cytometer.

**Real-Time quantitative PCR analysis:**
Quantitative PCR was used to analyse ANXA 1 mRNA level in controlled cells and in cells cultures for 4, 8 and 24 h in the presence of compounds to verify the increase of ANXA 1 expression at the transcriptional level. Real-time PCR amplifications and analyses were performed on a Light-cycler system (Roche) using the Light Cycler-Fast Start DNA Master plus SYBR Green I kit and procedure were carried on according to the manufacturer's protocol.

**Analysis of phagocytosis:**
Human acute monocytic leukemia (THP-1) cell line (0.5×10^6 cells /35 mm dish) were first differentiated by stimulation with PMA (160 nM) for 72 h to obtained a macrophage-like phenotype that closely resemble. Leukemia cells were cultured at a density of 0.2×10^6 cells/mL in the presence and absence of compounds and positive control for 24 h, after which medium were changed. PMA-treated THP-1 cells were washed with PBS, and leukemia cells added. Cells then incubated for 3 h at 37°C. Non-ingested cells were removed by three washes with cold PBS. The engulfment by macrophages was visualized by the immunofluorescence detection of macrophages with PE-conjugated anti-CD14 mouse monoclonal antibody. Cells will be analysed under a Zeiss LSM 510 laser confocal microscope.

**Statistical analysis:**
All results were analysed statistically. Experiment was performed in duplicate and results were presented as ± SEM of three experiments performed in triplicate. Statistical comparison between groups were made using One-way ANOVA followed by Bonferroni post hoc test and Student t-test (GraphPad Prism) were used to compare differences between treated cells with untreated cells. Differences was considered significat if p< 0.05.

**RESULTS AND DISCUSSION**

**Cell Viability Assay**

(a) K562

(b) Jurkat

(c) U937

Cell viability percentage of cell (a) K-562; (b) Jurkat and (c) U937 following incubation with each compound with respective concentration.
Increased concentration above 20µg/mL for the tested compound showed a decrease in percentage of viability below 80% for the cell following incubation with some of the compounds. Thus, concentration of 20µg/mL was selected for the next assay in the project.

REFERENCES


ACKNOWLEDGEMENTS

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Inhibitory Effects of *Zingiber officinale* Extract, Essential Oil and Compounds on Human Low-Density Lipoprotein Oxidation

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INTRODUCTION
Cardiovascular diseases (CVDs) are the leading cause of death globally. CVDs were accounted for more than 17 millions mortality worldwide in with coronary heart disease being the main contributor [1]. The modification of low-density lipoproteins (LDL) by oxidation has been considered as a vital factor in the progression of atherosclerosis and the development of cardiovascular diseases [2]. At the early stage of the atherosclerosis, LDL is exposed to oxidative modifications by arterial wall cells including macrophage under oxidative stress that leads to the formation of atherosclerotic plaques [3]. These plaques will subsequently enlarge as cells and lipids accumulate in them and the plaque, if it becomes unstable or ruptured, may result in coronary thrombosis, leading to acute coronary syndrome. Bioactive components from natural resources were found to have effectively inhibited LDL oxidation and prevent the progression atherosclerosis by reducing LDL oxidation [4].

Ginger (*Zingiber officinale* Roscoe) is one of the world’s well-known spices, and it has also been universally used throughout history for its health benefits. The principal components comprises of volatile essential oil and non-volatile polyphenolic compounds such as 6-gingerol, 6-shogaol, 8-gingerol among others have been widely investigated for its many pharmacological activity such as anti-inflammatory, analgesic, antipyretic, chemopreventive, and antioxidant properties [5].

EXPERIMENTAL METHODS

**Essential oil and extract preparation**
Rhizomes of ginger were collected, identified and deposited at the Herbarium of Universiti Kebangsaan Malaysia, Bangi. Rhizomes were washed, dried, ground and macerated in 80% ethanol. The ethanol extract was then filtered and ethanol was removed under reduced pressure. For essential oil, 200 g of each sample was hydrodistilled in Clevenger apparatus for 8 hours. The essential oil obtained was refrigerated prior to analysis. Sample and compounds (6-gingerol and 6-shogaol) intended for LDL oxidation were prepared in dimethyl sulfoxide (DMSO).

**Analysis of essential oil**
The ginger oil composition was analyzed using Shimadzu GC 14A using a FID detector and DB-5 stationary phase column (30m / 0.25mm, 0.25mm film thickness) programmed from 75°C for 10 min, then 3°C/min to 230°C and held for 10 min). Nitrogen was used as carrier gas at 50 cm/sec, injector and detector temperatures were maintained at 250°C. The areas and retention times from chromatogram were used to measure linear retention indices of the compounds, which were relative to *n*-alkanes (Adam, 2001).

The gas chromatograph mass spectrometer (GC/MS) analysis was performed on an Agilent 7890A gas chromatograph (GC) directly coupled to the mass spectrometer system (MS) of an Agilent 5975C inert MSD with triple-axis detector. The MSD Chemstation was used to find all the peaks in the raw GC chromatogram. A library search was carried out for all the peaks using the NIST/EPA/NIH version 2.0, and the results were combined in a single peak table.

**Human LDL isolation**
LDL was isolated from human plasma using density gradient ultracentrifugation with slight modification using Optiprep™ as the density gradient medium. LDL was then characterized by the Bradford protein assay and diluted to 200µg protein/mL using PBS prior to oxidation.

**LDL oxidation**
Oxidation of LDL was done by exposing it to CuSO₄ at 37°C for 5 hours and terminated by rapid freezing. The extract, compounds and probucol (positive control) were added to LDL directly before incubation.

**TBARS Analysis**
Cu-catalysed LDL oxidation inhibition by the extracts and compounds were determined using TBARS (thiobarbituric acid reactive substances) assay. After 5 hours incubation, sodium dodecyl sulphate (SDS) and thiobarbituric acid (TBA) were added to the mixture followed by 1 hour incubation at 95°C to increase the peroxidation. TBARS are expressed in terms of malondialdehyde (MDA) equivalents and the results are expressed as nmoles of MDA/mg LDL protein. The percentage of LDL oxidation inhibition was calculated.
RESULTS AND DISCUSSION

Essential oil analysis
Hydrodistillation of the rhizome oils of *Zingiber officinale* yields 1.2% calculated on a dry weight basis. Figure 1 shows the GCMS spectrum of ginger oil. There are 14 compounds identified as monoterpenes (10.89%) and 19 compounds as oxygenated monoterpenes (15.46%) in the ginger oil using GC. Total monoterpenes compounds identified are 33 comprising 26.35% of total oil yield. For sesquiterpenes (19.97%), there are 19 compounds identified and 18 more compounds are group as oxygenated sesquiterpenes (13.52%). Total sesquiterpenes in the oil is 33.49%. The major compounds in sesquiterpenes are α-zingiberene followed by β-terpinene β-sesquiphellandrene camphene, β-bisabolene and ar-curcumene. The composition matches well to the previous studies [5,6] that indicate that these are the major components of the oil although the amounts were varied.

![Figure 1. GCMS spectrum of ginger oil.](image)

In vitro LDL peroxidation assay
Extract and compounds gave a strong inhibition towards LDL oxidation (Figure 2). In comparison between gingerol and shogaol, the latter exhibits higher antioxidant activity against LDL oxidation. This maybe due to the presence of α, β-unsaturated ketone moiety in shogaol, which was showed to have influenced the antioxidant properties [7]. Other than that, 6-shogaol is more stable than gingerols as gingerols were easily converted to shogaols [8]. On the contrary, the antioxidant activity of the essential oil showed relatively weak activity. The result of our study was in accordance to the previous study, which indicated that the major components in *Z. officinale* essential oil; zingiberene and ar-curcumene, were not active in the inhibition of LDL peroxidation. Structure-activity analysis indicated that non-oxygenated bisabolene-type sesquiterpenes exhibited weak antioxidant activity [9].

![Figure 2. Percentage inhibition of *Zingiber officinale* extract, essential oil and compounds on LDL antioxidant activity. Results are expressed as mean ± SD (n=3).](image)
CONCLUSIONS
Findings in the present study reveals that the extracts of ginger and its compounds exert significant antioxidant activity thus making it a potential natural source for the treatment of cardiovascular related diseases.

REFERENCES

ACKNOWLEDGEMENTS
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Biochemical Effects of *Pasteurella multocida* Vaccine Using Palm Oil and Coconut Oil as Adjuvant in Ducks

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**INTRODUCTION**

Fowl cholera or *Pasteurella multocida* infection is one of the major threats to all duck-growing farms because of its high mortality. Vaccination is the only way of protection from such types of infections [1,2]. Various adjuvants have been used in order to enhance the immune response of vaccines against specific antigens. These are aluminium salts, saponin, muramyl di- and tripeptides, bacterial toxoides, living vectors, mineral oil emulsions and biodegradable oil emulsions. Emulsions adjuvants developed from various vegetable origins include peanut oil and soybean oil [3,4]. Palm oil and coconut oil are biodegradable vegetable oils and the important advantage of these oils is that they have minimal adverse side-effects. Biochemical effects of vaccines are important for safety of body organs as well as to obtain the optimal immunisation response[5,6,7]. The aim of our study was to the compare biochemical effects of a new formulation of vaccine using a mixture of palm oil and coconut oil as adjuvant.

**EXPERIMENTAL METHODS**

Vaccine was prepared by using palm oil/coconut oil mixture as adjuvant mixed killed *P. multocida* (the antigen). A nanoemulsion was produced by using a suitable emulsifying agent and high pressure homogeniser. Two-week old ducklings (N=120) was divided into three groups (40 ducklings in each group). Group A was injected with vaccine containing alum, group B was injected with vaccine containing palm oil/ coconut oil as adjuvant and group C was the control. Blood samples were taken before vaccination and on day 1, 2 and 7 after vaccination. Biochemical analysis of blood serum for liver enzymes (ALT, AST), triglycerides, urea, glucose, creatinine and cholesterol was performed [8]. Analysis was performed on Reflotron Plus using specific analysis kit for each parameter. Data was analysed using SPSS software.

**RESULTS AND DISCUSSION**

Our analysis showed that liver enzymes (ALT, AST) increased significantly in group A as compared to control while group B did not show any significant increase. As for triglyceride level, group B showed a higher level as compared to group A which is not significant. Glucose level was slightly high in group B as compared to group A. Blood urea level was slightly high in group A and creatinine level was low in group B. There was no significant difference in cholesterol levels between the three groups.

<table>
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<tr>
<th></th>
<th>ALT u/L</th>
<th>AST u/L</th>
<th>Tg mg/L</th>
<th>Urea mg/dl</th>
<th>Gluc mg/dl</th>
<th>Creat mg/dl</th>
<th>Chol mg/dL</th>
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<tbody>
<tr>
<td>Gp A</td>
<td>36.91</td>
<td>31.85</td>
<td>155.9</td>
<td>32.69</td>
<td>247.1</td>
<td>1.81</td>
<td>179.8</td>
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<td></td>
<td>± 2.10</td>
<td>± 3.19</td>
<td>± 2.37</td>
<td>± 1.01</td>
<td>± 3.09</td>
<td>± 0.09</td>
<td>± 2.91</td>
</tr>
<tr>
<td>Gp B</td>
<td>26.15</td>
<td>22.74</td>
<td>173.6</td>
<td>27.49</td>
<td>278.4</td>
<td>1.41</td>
<td>175.1</td>
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<tr>
<td></td>
<td>± 1.59</td>
<td>± 1.98</td>
<td>± 2.86</td>
<td>± 0.51</td>
<td>± 2.78</td>
<td>± 0.07</td>
<td>± 2.71</td>
</tr>
<tr>
<td>Gp C</td>
<td>24.12</td>
<td>21.63</td>
<td>143.7</td>
<td>25.31</td>
<td>224.9</td>
<td>1.39</td>
<td>163.5</td>
</tr>
<tr>
<td></td>
<td>± 0.79</td>
<td>± 1.07</td>
<td>± 1.81</td>
<td>± 0.41</td>
<td>± 9.21</td>
<td>± 0.04</td>
<td>± 2.10</td>
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</tbody>
</table>

**CONCLUSION**

Our results suggest that nanovaccine formulated by using a mixture of palm oil and coconut oil as adjuvant is biochemically safe and protective for organs such as liver and kidneys.

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Vaccine **23**:2665–2675.


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**In Vitro Antioxidant Activity of Syzygium polyanthum (Wight) Walpers**

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**INTRODUCTION**

Syzygium polyanthum (Wight) Walpers (Synonym: Eugenia polyantha var. sessilis M.R. Hend, Eugenia lucidula Miq., Eugenia balsamea Ridley, and Myrcia inaequiloba (DC.) D. Legrand) is a tree native to Indonesia, Malaysia, Thailand, Vietnam, and Philippines [1,2]. The vernacular names of this species are salam (Indonesia), daun salam (Suriname), kelat samak (Malaysia), daengkluai (Thailand) and Indonesian bay leaf (Europe). The plant has edible flowers and fruits, and its leaves are used as an important flavoring condiment in Indonesian cuisine [1,3] and several South East Asia countries [4]. The aromatic fragrance of the leaves is unique and difficult to recommend a substitute for this plant even among Syzygium species [5]. The ripe fruits are edible although slightly astringent [2]. The stem bark of the plant is used as a dye for wattle and bamboo handcraft in Indonesia [3]. Meanwhile, the timber is used for house building and furniture [2].

In addition to significant role of *S. polyanthum* in Indonesian cuisine, natives have used the plant traditionally as a medicine to cure several diseases such as diarrhea, hypercholesterolemia, hypertension, diabetes mellitus, gastritis, scabies, itches, and swelling as well as to relieve alcohol consumption [2,6,7,8]. However, despite of its interesting medicinal uses, the chemistry and pharmacology aspects of this species have not been widely investigated [9] and thus requiring more research.

In this study, authors investigated preliminary *in vitro* antioxidant activity of stem and root barks of the plant. We also estimated the content of phenolics and flavonoids in the crude methanol extracts and fractions, which are known to be responsible for the antioxidant action of the plant.

**EXPERIMENTAL METHOD**

**Samples**

*Syzygium polyanthum* was collected from the Bende district, a region of Kendari city, Sulawesi Tenggara, Indonesia. The plant specimen was authenticated and deposited in the Herbarium Bogoriense, Bogor, Indonesia with voucher number BO-1639378.

**Extraction and fractionation**

The dried powder of stem barks (2 x 2.5 kg) and root barks (1.0 kg) of *S. polyanthum* were separately macerated with methanol (8 and 3 L, respectively) for 24 hr and shaken periodically. The extracts were then filtered and the solvent was evaporated under reduced pressure. The steps were executed three times to yield a total of 600 g crude methanol extract (12%) of stem barks (CSB-SP) and 300 g crude methanol extract (13%) of root barks (CRB-SP).

Precise amount of 10 g of each crude methanol extract was suspended in methanol and partitioned successively with petroleum ether, ethyl acetate and methanol. Six fractions were evaporated under vacuum to yield residues of petroleum ether, ethyl acetate, and remaining methanol.

**Estimation of phenolics**

Phenolics content in crude methanol extracts and fractions of *S. polyanthum* was determined according to the Folin-Ciocalteu method [10]. Triplicate twenty-μL of samples (100 μg/mL), standard solutions of gallic acid (10 – 100 μg/mL) and DMSO (100% v/v) as a blank were added with 100 μL Folin-Ciocalteu (10%) and incubated for 5 min at room temperature. After incubation, the mixtures were added with 80 μL Na2CO3 (75%) and incubated for 30 min at room temperature. The absorbance was detected at 735 nm using a Thermo Multiskan Go. The total phenolics was expressed as...
mg of gallic acid equivalent per g of crude methanol extracts and fractions, using an equation obtained from gallic acid calibration curve:

\[
\text{Absorbance} = 4.0418 \times \text{gallic acid (mg)} + 0.0003 \quad (r^2 = 0.999)
\]

**Estimation of flavonoids**

Flavonoids content in crude methanol extracts and fractions of *S. polyanthum* was determined using aluminum method [11,13]. Triplicate one hundred-μL of samples (100 μg/mL), standard solutions of quercetin (10 – 100 μg/mL) and DMSO (100% v/v) were added with 100 μL AlCl₃ (2%) and incubated for 15 min at room temperature. After incubation, the absorbance was detected at 435 nm. The total flavonoids was expressed as mg of quercetin equivalent per g of crude methanol extracts and fractions, using an equation obtained from quercetin calibration curve:

\[
\text{Absorbance} = 6.888 \times \text{quercetin (mg)} + 0.0159 \quad (r^2 = 0.999)
\]

**Free radical scavenging activity (DPPH)**

One hundred-μL of standards (ascorbic acid, trolox, gallic acid, quercetin; ranged from 0.781 – 100 μg/mL), samples (100 μg/mL) and DMSO (100% v/v) as a control were added with 100 μL DPPH radical solution (10% v/v) and incubated for 15 min in dark at room temperature. The absorbance was detected at 540 nm [11,13]. The ability to scavenge DPPH radical known as radical scavenging activity (RSA) was calculated using an equation below. The IC50 values were calculated using GraphPad Prism 5.

\[
\% \text{ Inhibition (DPPH)} = \left( \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \right) \times 100\%
\]

**Reducing antioxidant power activity (FRAP)**

The reducing power activity of crude methanol extracts and fractions of *S. polyanthum* was determined according Benzie and Strain [12] converted into a micromethod [13]. Twenty-μL of standards (0.078125 – 10 μg/mL) and samples (100 μg/mL) was added with 180 μL of FRAP reagent, shaken for 10 sec and incubated for 4 min at 37°C. The absorbance was detected at 593 nm. The equivalent trolox amount of samples was obtained by the interpolation of absorbance using a trolox calibration curve:

\[
\text{Absorbance} = 0.0782 \times \text{trolox (μg)} + 0.0045 \quad (r^2 = 0.999)
\]

**RESULTS AND DISCUSSION**

**Estimation of phenolics and flavonoids**

Crude MeOH extracts of *S. polyanthum* root (CR-SPR) and stem bark (CR-SPS) showed high amount of phenolics and flavonoids similar with the MeOH fractions (MF-SPR and MF-SPS) as displayed in Figure 2. These compounds are well-known representative for polar compounds in nature. The application of methanol as a solvent for extraction and fractionation may increase extractive yield of these type of compounds, resulted in the good distribution of both compounds in the crude MeOH extracts and MeOH fractions.

![Figure 2. Phenolics and flavonoids content in crude methanol extracts and fractions of root and stem barks of *S. polyanthum* (*mean ± SD, n = 3)*](image-url)
Interestingly, non-polar petroleum ether fraction of the stem bark (PEF-SPS) showed high amount of phenolics, indicated the presence of non-polar type of phenolics such as eugenol that prevalent occurred in the *Syzygium* species [1-5,9]. On the other hand, petroleum ether and ethyl acetate fractions of the root bark (PEF-SPR) and stem bark (EAF-SPS), respectively, contained low amount of both phenolics and flavonoids, and indicated their less distribution in these fractions.

**Antioxidant activity**

Crude MeOH extracts and fractions of the root and stem bark of *S. polyanthum*, except petroleum ether fraction of the root bark showed promising radical scavenging activity ranging from 61.99 – 82.07% at a concentration of 100 μg/mL as compared with ascorbic acid, quercetin, trolox and gallic acid (Figure 3).

The crude MeOH extracts and fractions also exhibited dose-dependent inhibition of DPPH radical, and out of these, crude MeOH extract of the root bark (CR-SPR) was the most active with IC$_{50}$ value of 2.82 μg/mL followed by MeOH fraction of the stem bark (MF-SPS) with IC$_{50}$ value of 3.4 μg/mL (Figure 4). These evidences were highly correlated with the presence of phenolics than flavonoids, and the present of flavonoids was evidently decreased the potential of fractions to scavenge DPPH radical.

As we compared further regarding the content of both type of compounds in MF-SPR (IC$_{50}$ 4.42 μg/mL) and MF-SPS fractions (IC$_{50}$ 3.40 μg/mL), we found that there is no significant differentiation of phenolics content in both fractions (50.3 and 49.7% of phenolics, respectively). In contrast, the flavonoids content in the MF-SPS was 8.0% higher than MF-SPR. This evidence suggested that flavonoids may reduce the radical scavenging activity of methanol fractions of *S. polyanthum*. We also noticed that both CR-SPR and CR-SPS contained almost same content of phenolics (57.2%) and flavonoids (42.8%), but gave very different IC$_{50}$ values (2.82 and 4.44 μg/mL, respectively). Finally, the ratio of 3:2 of total both compounds in the CR-SPR and CR-SPS, respectively, confirmed the fact that IC$_{50}$ value of CR-SPR is lower than CP-SPS.

![Figure 3](image3.png)

**Figure 3.** Percentage inhibition (%) of crude MeOH extracts and fractions of root and stem barks of *S. polyanthum* at 100 μg/mL (*mean ± SD, n = 3)

![Figure 4](image4.png)

**Figure 4.** IC$_{50}$ values of crude MeOH extracts and fractions of root and stem barks of *S. polyanthum* (*mean ± SD, n = 3)

The FRAP values expressed as Trolox equivalent were found to vary from 1.00 – 7.02 μg. As displayed in Figure 5, crude MeOH extract of the root bark (CR-SPR) was the strongest antioxidant consistent with DPPH radical assay, and this was similar to MeOH fraction of the stem bark (MF-SPS). On the other hand, the lack antioxidant action was observed for petroleum ether fraction of the root bark (PEF-SPR) followed by ethyl acetate fraction of the stem bark (EAF-SPS), which
was also consistent with their lack DPPH radical scavenging activity. One possibility suggested is because of the low amount of phenolics and flavonoids in these fractions. Meanwhile, antioxidant activity of other fractions was highly correlated with their phenolics and flavonoids, as both types of compounds are believed to play significant role as antioxidant agent.

**CONCLUSION**

In this study, we found that crude MeOH extracts and fractions of root and stem barks of *S. polyanthum* possessed radical-scavenging and reducing antioxidant power activity. These activities are suggested due to the presence of phenolics and flavonoids.

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**ACKNOWLEDGEMENTS**

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Quantitative Determination of Phyllantin, Hypophyllantin and Corilagin in Different *Phyllanthus niruri* Extracts by High-Performance Liquid Chromatography (HPLC)

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INTRODUCTION

Herbal medicine also known as botanical medicine or phytomedicine refers to the use of a plants seeds, berries, roots, leaves, bark and flowers for medicinal purposes. Plants have been used for medicinal purposes long before recorded history. WHO estimated that 80% of people worldwide rely on herbal medicines for some part of their primary health care. Herbal medication is said to be more safe and non-toxic. Global demand for herbal medication is increasing day by day [2]. Natural products, such as plants extract, either as pure compounds or as standardised extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. Extraction is the crucial first step in the analysis of plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterisations. *Phyllanthus niruri* L., known locally as “dukong anak”, is found in most tropical and subtropical regions, commonly in fields, grasslands and forests. It is a small herb that grows up to 60 cm in height. *P. niruri* has been the subject of much phytochemical studies since the mid 1960s. Different classes of organic compounds with various medical interest have been reported, the major being the lignans, tannins, polyphenols, alkaloids, flavonoids, terpenoids and steroids[4]. *P. niruri* is highly valued in the treatment of liver and kidney stones and has been shown to possess anti-hepatitis B virus surface antigen activity. It has also been used to treat jaundice, gonorrhea, dysentery and diabetes. Topically it has been used for skin ulcer and itchiness [1].

EXPERIMENTAL METHODS

Air-dried leaves of *P. niruri* were separately extracted with either hexane, ethyl acetate or ethanol. In each case, the extraction was carried out with 200 ml solvent in cold procedure at room temperature (25 ± 5°C) and in hot procedure soxhlet extraction each for three times and three days and the solvent was removed from the combined extract under reduced pressure to yield the respective crude residue [7]. The contents of the species in sample was determined by High Performance Liquid Phase Chromatography (HPLC) method which is suitable for standardisation of *Phyllanthus niruri* based on their marker compound which are phyllantin, hypophyllantin and corilagin in order to ensure safety and efficacy of these species to consumer [3,5,6].

EXPECTED OUTCOME

This research will be helpful to assess the actual method of extraction with appropriate solvent for quantitative determination of chemical markers in different *Phyllanthus niruri* extract using HPLC.

REFERENCES

[3] Eike Reich, Anne Schibli,High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants
**Determination of Fluoride in Toothpaste by Ion Chromatography**

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**INTRODUCTION**

Fluoride may be an essential element for animals and humans. For humans, however, the essentiality has not been demonstrated unequivocally, and no data indicating the minimum nutritional requirement are available. Quantitative measurement of Fluoride enables the Fluoride content in children’s toothpaste of the Malaysian market monitored by governing bodies. Several methods have been used previously to determine Fluoride in dental products including the gas chromatography, ion selective electrodes, and colorimetry. These methods are subject to interferences, poor reproducibility and laborious. In this application note, we describe the use of Shim-pack IC-A3, a polymer based column with an anion exchange group for ion chromatography which is optimized for non-suppressed ion chromatography with excellent selectivity enabling separation of many organic and inorganic anions.

**EXPERIMENTAL METHODS**

**Materials and Equipment**

Shim-pack IC-A3, sonicator, weighing balance, syringe with filter, glass funnel with 0.25um filter, 20ml volumetric flasks, 50ml volumetric flasks, 2L mobile phase bottles, 10ml pipet, measuring cylinder, sample container, electric stove with magnetic stirrer, Sodium Fluoride Standard, p-hydroxybenzoic acid, Bis(2hyrosyethyl)iminotris(hydroxylmethyl)methane, Boric Acid (Reagent grade), deionized water, HPLC grade.

**Apparatus**

The main instrument being used in this experiment is the non-suppressed ion chromatography with a polymer gel based column with an anion exchange group together with conductivity detector. The mobile phase used containing 8 mM p-hydroxy benzoic acid, 3.2 mM Bis-Tris and 50 mM boric acid will be degassed using the sonicator before introducing into the column. The flow rate is set at 1.0ml/min and the injection volume is 20uL.

**Standards Preparation**

Stock solutions of F\(^-\) will be prepared from Sodium Fluoride. 100ppm of F was produced by dissolving 11.139mg Sodium Fluoride standard in 50ml of deionized water. Then it was further diluted and Fluoride standards were prepared in ppm: 1.25, 2.5, 5, 10, 20, 30, and 40.

**Sample Preparation**

As many as possible children’s toothpaste brands will be obtained from the market. One gram of the toothpaste sample will be dissolved in 100ml of deionized water.

**RESULTS**

Data will be collected to validate the method based on the parameters in ICH guidelines. As an outcome, accountable results can be obtained from the analysis of toothpaste samples allowing the proposed method to be adapted by concerning bodies.

**REFERENCES**


[3] Validation of analytical procedures: text and methodology, Q2 (R1), ICH guideline
INTRODUCTION
Adherence to medication refers to whether patients take their medications as prescribed and continue taking the prescribed medications [1]. Poor adherence to medication carries a huge economic burden to healthcare system. The prevalence of poor adherence to medications is reported to be high worldwide. It is estimated that in developed countries, 50% of patients with chronic diseases do not have good adherence to their medication treatment plan [3]. Poor adherence to medication often caused by many factors. Previous studies using Health Belief Model (HBM) of medication adherence reported that cost play an important role in influencing patients’ medication adherence [8, 9]. Approximately one-quarter of older adults in USA were reported forgoing prescription medications because of the cost [5]. In contra, although the medical and medication are highly subsidized in Malaysia, patients pay a minimum cost of RM 1 to get both medical care and medication in the government healthcare facilities, the returned unused subsidized medication were still high. The study by Hassali et al. estimated that the direct cost of returned unused medication across public hospitals in Malaysia would exceed a few million dollars in a year. To our knowledge, no study has been done to evaluate the effect of lowering the medication costs on patients’ medication adherence [2]. In addition, no study has been done to evaluate the medication adherence pattern and patients’ HBM between patients who received subsidized medications and patients who are self-paying for their medications. Since MOH is planning to introduce a national health financial insurance scheme to patients who seek treatment in public hospitals or clinics (part of healthcare reform initiative to reduce the dependency on subsidized medical care and medications [16]), it will be timely to investigate how such system would affect patients’ HBM on adherence and also the medication adherence behavior.

EXPERIMENTAL METHODS
Study Design
An observational study will be conducted in the dispensary pharmacies of selected government hospitals/clinics and community pharmacies.

Study Population
Patients identified at the sampling sites will be conveniently sampled and categorized into: (1) patients who receive subsidized medications from a government hospital or clinic (2) patients who are self-paying their own medications. The inclusion and exclusion criteria are:

Inclusion Criteria
i. Adults age 18 years and above.
ii. Patients with chronic disease prescribed at least three medications for more than 6 months
iii. Patients received medications that were subsidized or self-paying for their own medications

Exclusion Criteria
i. Diagnosed with terminal ill disease
ii. Stay in assisted living facilities
iii. Patients with mental illness
iv. Patients using pill box
v. Patients that had physical difficulty in opening bottle cap

Data Collection
During data collection, patients will be explained about the study. Upon agreeing to participate, patients will be asked to provide their consent and their demographic data and treatment information will be collected. During the first meeting, patients will also be asked to score their self-perceived medication adherence (using the Morisky 8-item Medication Adherence Scale). Patients’ medications will be reviewed and only one medication (which is perceived as the most important medications for patients’ health conditions) will be chosen to be re-packed into a new bottle that is attached with the Medication Event Monitoring Systems (MEMS) cap. Patients will be explained on the MEM cap and will be asked to return the cap upon completion of the study (during the final follow-up).
Patients who agree to participate will be given an option whether to conduct the interview through phone call or receive a home visit/face to face by the researcher. The interview date, time and place will be set at a convenient time for patients. Patients will be asked on their perceptions of perceived susceptibility, severity, benefits, barriers, cues to action, self-efficacy to medication adherence (proposed in the Health Belief Model on medication adherence) and also their perceptions of the proposed government health financial insurance. After 6 weeks, patient will be follow up and the MEMS cap will be collected.

**Data Analysis**

Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 21.0. Demographic data will be analyzed using descriptive analysis. The type of statistical tests used as shown below:

i) T test will be used to compare the medication adherence score between the two groups.

ii) Multiple linear regression will be used to determine factors that may influence patients’ adherence score such as patients’ demographic and perceived medication cost.

Qualitative interview will be analyzed with constant comparison and grounded theory using NVivo 9 software.

**REFERENCES**


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Comparative in-vitro Evaluation of Four Brands of Diclofenac Sodium Delayed Release Tablets Commercially Available in Malaysia

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INTRODUCTION
Diclofenac 2((2, 6 – dichlorophenyl) amino) benzene acetic acid is one of the most important Non-steroidal anti-inflammatory drugs. It is available in different dosage forms as injection, film coated tablets, enteric coated tablets and prolonged release tablets. Diclofenac formulations are ranked 18th in term of use in Malaysia and number 4th in term of expenditure (medical statistics in Malaysia, ministry of health). It is very potent analgesic (40 times more aspirin). Also it possess anti-inflammatory activities (assumed due to inhibition of lipo-oxygenase enzyme).

Delayed release tablets are typically produced by coating the tablet with enteric coating polymers. These polymers provide the resistance of drug release in acidic environment of stomach and allow the drug to be released in alkaline environment of the intestine. A large number of enteric polymers are available which provide excellent protection to drug release in acidic environment. However, the variations between different polymers and variations in manufacturing processes may result in difference in ability to protect the tablet from dissolution in gastric fluids.

Discovery and formulation of a novel drug cost the company a huge amount of effort and money. That is why the drug company is rewarded through achieving its drug patent for a certain period of time. After the expiration of this period, we face a flood of new brands in the markets. An efficient monitoring of these different brands is very important to ensure an appropriate quality of the pharmaceutical products. And to avoid the presence of substandard products (substandard pharmaceutical product is a product manufactured by an authorized manufacturer but does not meet the fixed quality standards by the authority).

A great effort is done by the ministry of health to monitor the quality of the pharmaceutical products. Although the health professionals have to share the responsibilities of improving the health care system. The researches should contribute assessing and improving the quality of pharmaceutical products. The in-vitro quality control is very important as it can be a valuable predictor of the in-vivo parameters. And contribute efficiently in monitoring the drug efficacy. Example of that, if an oral tablet does not disintegrate completely, this will affect the dissolution and consequently the dose absorbed and thereby the efficacy. The substandard and-or counterfeit drugs may cost the patient his life. So that a great attention must be paid to monitor the quality of different pharmaceutical products. This study will be conducted to assess the in-vitro quality control parameters Diclofenac Sodium tablet brands marketed in Malaysia through the evaluation of the content uniformity, weight variation, hardness, friability, disintegration time and dissolution profile between the commercially available of Diclofenac sodium tablets in Malaysia.

EXPERIMENTAL METHODS
A comparative in-vitro quality control parameters between different brands Diclofenac (enteric coated) tablets will be conducted through evaluation of content uniformity, weight variation, friability, hardness, disintegration time and dissolution performance. The study will be conducted to test these quality control parameters using acceptance criteria cited in the USP for delayed release tablets.
Sample selection and collection
A four different generic brands of Diclofenac Sodium 50 mg tablets delayed release (enteric coated) were purchased from a retail pharmacy in Kuala Lumpur. The selection was based on the availability on the market, where, these brands were the only generic brands of delayed release Diclofenac Sodium tablets available in randomly selected ten pharmacies in Kuala Lumpur. Three brands have only enteric coat and one brand has both film coat and enteric coat. The four brands are within their shelf life.

Materials and equipment
The test samples were purchased from KL retail pharmacy. All the chemicals used were of analytical grade and were provided by faculty of pharmacy, UKM. Hydrochloric acid, sodium hydroxide, tri-sodium phosphate, and Diclofenac reference standards. All the equipment are periodically monitored and calibrated and in a good-working condition.

Visual inspection
The uniformity and homogeneity of the tablets affect patient compliance and adherence. Furthermore it may affect the results through affecting the patient satisfaction. The visual inspection was carried out by testing the shape, size and colour uniformity of the tablets within the same brand.

Friability
Friability describe the ability of the tablet to withstand abrasion and chipping during packaging, transportation and handling. Chipping and abrasion affect the dose of the formulation, furthermore they affect both appearance and consumer acceptance. The test was carried out on 20 tablets using the Electrolab friabilitor, 25 rpm for 4 min. followed by determining the loss of weight.

Hardness
This test is provided to determine, under defined conditions, the resistance to crushing of tablets measured by the force needed to disrupt them by crushing. Hardness can be defined as the strength of the tablet to withstand the applied pressure. It describes the ability of the tablet to withstand the mechanical shocks during handling in manufacturing, packaging and shipping. Hardness is affected by a lot of factors as the compression process, type and amount of binder as well as the efficient granulation. The too hard tablet will not disintegrate efficiently, consequently less disintegration, dissolution and bioavailability. The test carried out for 10 tablets by using pharm test hardness tester. The direction of the tablets were considered in each measurement.

Uniformity of content
The test for content uniformity is based on the assay of the individual content of drug substance(s) in a number of individual dosage units to determine whether the individual content in the limit set or not. The test was done using HPLC. The content of each tablet will be determined as a percentage of the labelled amount.

Disintegration test
Disintegration is defines as that a state in which any residue of the unit except fragments of insoluble coating or capsule shell remaining on the screen of the test apparatus or adhering to the lower surface of the disc, if used, is a soft mass having no palpably firm core (USP). Place one tablet in each of the six tubes of the basket. Carry out the test using 0.1 M hydrochloric acid as the liquid. Operate the apparatus using simulated gastric fluid TS maintained at 37 ± 2 Celsius as the immersion fluid for 2 hours without the discs and examine the state of the tablets. The raising and lowering capacity of the basket in the immersion fluid at a frequency rate between 29 and 32 cycle per minute. After 2 hour of the operation lift the basket from the fluid and observe the tablets. The tablets show no evidence of disintegration (apart from fragment of the coating) or cracks that would allow the escape of the content. Replace the acid by phosphate buffer pH 6.8 R and add a disc to each tube. Operate the apparatus for 60 minutes and examine the state of the tablets. If the tablets fail to comply because of the adherence to the discs, the results are invalid and repeat the test on further 6 tablets omitting the disc.

Dissolution
Dissolution test is provided to determine the compliance with the dissolution requirements for solid dosage forms administered orally (BP2012). Disintegration does not imply complete dissolution of the unit or even its active constituents (USP). Dissolution is very important because it is a determining factor in the absorption. Using apparatus 1, method B USP pharmacopeia. Tablets were tested in the acid stage using 0.1 N HCL 900ml. after two hours, addition of 20 ml 5 N NaOH and stirring for 5 min and analysis of an aliquot using UV-spectrophotometer Shimaduz apparatus at 276 nm (USP). Directly after the completion of 2 hours the test tablets were transferred from the acid medium to a phosphate buffer 6.8 equilibrated to 37 C and the test proceeded for 45 min and an aliquot taken from each jar and diluted with buffer medium 1:1 and examined using UV-spectrophotometer, Shimaduz.
REFERENCES


Carbapenem Stewardship: Impact on Prescribing Pattern, Consumption and Clinical Outcomes at Kajang Hospital

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INTRODUCTION

The emergence of antibiotic resistant organisms secondary to excessive use of antibiotics is now a linked global issue that contributes to increased rate of morbidity and mortality, longer hospital stay and increased cost of healthcare treatment [1]. Carbapenems are broad-spectrum beta-lactam antibiotics that are resistant to most beta-lactamases such as chromosomal cephalosporinases and extended-spectrum beta-lactamases (ESBL) of Enterobacteriaceae [2]. Uncontrolled and excessive use of carbapenem has resulted in causing positive selective pressure for the emergence of carbapenem resistant organisms [3]. An increased consumption of antipseudomonas carbapenems (meropenem and imipenem) was associated with a higher prevalence of carbapenem resistant Pseudomonas and Acinetobacter isolates that contributes to poor clinical outcome such as a higher rate of mortality and longer hospital stay compared with infections by susceptible isolates [4,5]. To address the issue of carbapenem resistance and injudicious use of antibiotic, strategies such as Antibiotic Stewardship Program (ASP) can be implemented. Different strategies are recommended by IDSA to reduce the consumption and improve appropriate prescribing of carbapenems [6]. The aim of this study is to evaluate whether Antibiotic Stewardship (ASP) will be able to improve appropriate prescribing and consumption of carbapenems and improve clinical outcomes in the local setting.

EXPERIMENTAL METHODS

This is a retrospective observational study that compares the use of carbapenem pre and post-intervention. The study comprises of retrospective data collection in the pre-intervention period from June 2014 to Nov 2014 (6 months) and data collection in the post intervention period from Dec 2014 to May 2015 (6 months). The Antibiotic Management Team (AMT) from Dec 2014 will perform the intervention period. The recruitment of patient is based on the prescribing of carbapenem as well as fulfillment of the listed criteria. Complete and detailed clinical data on patient’s demographic, infection, as well as the treatment and its outcomes, will be retrieved from patients’ medical record. The number and type of intervention by AMT and carbapenem consumption (Defined Daily Dose) will be identified. Statistical analysis involves the application of SPPS version 21 with a p-value of <0.05 for statistical significance.

POTENTIAL APPLICATIONS

This study will allow healthcare professions to assess the need for antibiotic stewardship in clinical practice. Close monitoring of antibiotic dispensing in admitted patients should be performed by pharmacists to ensure the appropriateness of antibiotic prescribed. This is to ensure improvement of clinical outcomes as well as reduction in antibiotic resistance and antibiotic consumption.

REFERENCES


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Special thanks to Dr. Farida, lecturers and friends that have contributed their help in this study.
Antibiotic Knowledge, Attitude and Shared Decision-making Preferences among Adolescents in Malaysia

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INTRODUCTION
Mismanagement of antibiotics has become the primary cause of rising levels of antibiotic resistance, which is a major threat worldwide [1]. Non-adherence to antibiotic in adolescents has been shown to occur due to the adolescent’s unwillingness to take medication [2]. Traditionally, adolescents have had little involvement during their medical care visits or in decisions regarding their health care [3]. Lack of knowledge of antibiotic among adolescents could further contribute to their passive involvement in decision making and poor adherence [4]. Thus, giving sufficient knowledge and encouraging active involvement in decision making will improve adolescent adherence to medication [5]. There is still lack of data looking into the level of knowledge of antibiotic in adolescents, their perceived benefits and involvement towards antibiotic in the local population. Therefore, this work is performed as a baseline study to assess adolescent’s knowledge, attitude and experience on antibiotic use and their preference to engage in the shared decision-making process.

EXPERIMENTAL METHODS
Study Design
A prospective, cross-sectional survey was conducted using a validated questionnaire in secondary schools in Malaysia from May to November 2014.

Study Population
1105 students were recruited in this study through stratified random sampling.

Inclusion criteria
i. Students aged 13 to 17 years old.
ii. Able to read Malay or English language.

Exclusion criteria
i. Incomplete questionnaire

Data Collection
Questionnaires were adapted and developed based on previous work [6,7,8,9]. The five-part questionnaire collected responses on demographic characteristics, knowledge, attitude and experience statements, and their preference in shared decision-making process. Control Preference Scale (CPS) was used as a scale to measure respondents’ preference in decision making process. The Cronbach’s alpha for knowledge of antibiotic use, attitude towards antibiotic and experience with antibiotic were 0.72, 0.87 and 0.82 respectively.

Data Analyses
All analyses were performed using the statistical packages SPSS version 22.0. Demographic characteristics, knowledge, attitude and experience level were summarized using descriptive statistics. Pearson correlation and chi-squared tests were used to evaluate the association of knowledge, attitude, experience and preference to engage in shared decision-making. In all statistical analyses, a p-value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION
Of the 1105 students, majority had a poor level of antibiotic knowledge (n= 786, 71.13%), 26.79% (n=296) had moderate knowledge and 2.08% (n=23) had high level of knowledge. The mean attitude score towards antibiotic use was 3.30±0.05 (range: 0-8 points). The experience towards medication use in general was on average fairly positive with a mean score of 2.90 ± 0.029 (range 0-4 points). A positive correlation was observed between knowledge and attitude scores with better knowledge level associated with a more positive attitude towards antibiotic (r=0.257, p<0.001). Interestingly, a higher knowledge score was associated with a more negative experience with medication (r=-0.83, p=0.006). When assessing preference in shared decision-making, more adolescents preferred an active role (n=408, 37%) compared to collaborative (n=360, 32.6%) and passive (n=337, 30.5%) (p=0.028). No association was observed between decision-making preferences and antibiotic knowledge, attitude and experience.
CONCLUSION
The current work demonstrates the need to improve antibiotic knowledge among adolescents. Furthermore, this work also identified a large majority of adolescents were willing to play a more collaborative and active approach than initially expected. Education packages therefore should be introduced to improve adolescents’ antibiotic knowledge and medication practice as well as to encourage their participation in decision making.

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Patient’s Perception and Practice of Complementary and Alternative Medicine (CAM) for the Management of Epilepsy in Universiti Kebangsaan Malaysia Medical Center

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INTRODUCTION
Statistics from World Health Organization showed that approximately 50 million people suffer from epilepsy [1], and it was estimated to account for nearly 0.5% of total global disease burden [2]. In Asia per se, the prevalence of epilepsy ranges between 1.5 and 14 per 1000 people [3], and it is estimated that approximately 1% of Malaysia’s population were diagnosed with epilepsy [4]. Although the use of anti-epileptic drugs (AEDs) remain as the mainstay of epilepsy treatment, there are patients who opt for other non-drug therapies such as complementary and alternative medicine (CAM). Common CAM used among patients with epilepsy included mind-body approaches e.g. prayer [5, 6, 7], and herbal medicines [8]. A study showed that approximately 40% of Western countries population use CAM for various health conditions [9], including epilepsy; while local data showed that the prevalence of ever-used traditional and complementary medicine in their lifetime was 69.4% [10]. Although earlier studies conducted among patients with epilepsy revealed CAM users thought that CAM was beneficial [6, 7], safe and harmless [11], and offered more patient empowerment [11], data on local population is scarce. Hence, it is the aim of this study to assess factors influencing patient’s perception and practice of CAM for the management of epilepsy, as well as their beliefs about anti-epileptic drugs.

EXPERIMENTAL METHODS

Study Design
A cross-sectional survey will be conducted in the Neurology clinic, UKMMC from February to July 2014.

Study Population
285 subjects will be recruited by convenience sampling. The inclusion and exclusion criteria will be as below:

Inclusion Criteria
i. Diagnosis of epilepsy
ii. Patient ≥18 years old
iii. Patients who can read and understand English or Malay language

Exclusion Criteria
i. Patients who cannot and/or unwilling to complete the research questionnaire
ii. Patients with documented psychosis and schizophrenia

Data Collection
Questionnaires will be adapted and developed based on previous studies and literature review [6, 7, 8, 11, 12, 13, 14, 15]. Socio-demographic data, patients’ perception on CAM use, practice of CAM, and beliefs about anti-epileptic drugs will be included in the questionnaires. Prior to the study, a pre-test and a questionnaire validation will be conducted among patients with epilepsy in Neurology clinic, UKMMC.

The validated questionnaires will be distributed to participating subjects at the Neurology Clinic of UKMMC. They will be assisted by the researcher to answer the questionnaire. Subject’s registration number will be recorded in order to obtain medication regimen from the Pharmacy computer system at the Pharmacy department.

Data Analysis
The results of this study will be presented as both descriptive and analytical data. Data analysis will be done by using IBM® Statistical Package for Social Sciences (SPSS) Desktop version 22. Descriptive data will be computed by using the same software. In inferential analysis, confidence interval of 95% will be utilized. Hence, results obtained will be considered statistically significant when P-value <0.05 (two-tailed). The type of statistical tests used will be based on the objectives as shown below:

i) Chi-squared analysis will be used to compare perception of CAM use with categorical socio-demographic data.
ii) Independent t-test will be used to compare perception of CAM use with continuous socio-demographic data.
iii) Wilcoxon rank sum test will be used to associate between perception and practice of CAM, as well as between BMQ-Epilepsy Specific and practice of CAM.

iv) Spearman rank correlation will be used to analyse association between perception of CAM and BMQ-Epilepsy Specific

**POTENTIAL FINDINGS**

Patients with epilepsy will be expected to have positive perception on use of CAM for the management of epilepsy and have low beliefs about anti-epileptic drugs, which will lead to their greater use of CAM.

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Special thanks to Assoc. Prof. Dr. Mohd Makmor Bakry, Dr. Adyani Md Redzuan, lecturers, friends and my family members that have contributed their help in this study
Expression Pattern of P2Y Purinergic Receptor in Oral Squamous Cell Carcinoma (OSCC) and Its Interaction in 5-fluorouracil (5-FU) Mediated Apoptosis in OSCC Cell Line

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a subset of head and neck squamous cell carcinoma (HNSCC). Of all head and neck cancers, OSCC is the most common malignant epithelial neoplasm of oral cavity [1]. It is also ranked as the third most common cancer in Malaysia and most of the cases are being diagnosed at the late stage [2, 3]. The etiology of OSCC appears to be multifactorial and strongly related to the lifestyle. Despite the readily accessible of oral cavity for direct examination, these malignancies are not detected until the later stages [3]. Metabotropic P2Y receptor belongs to the superfamily of G-protein coupled receptor and widely expressed in variety of tissues and organs [4]. Recent studies have found that P2 purinergic receptor subtypes (P2Y) are involved in the growth inhibitory response in different type of malignant cells as being challenged with ATP or other nucleotides and high concentration of ATP, in hundred micromolar range can be observed at tumor sites [5, 6]. Increase in extracellular ATP has been found to involve in anti-tumor immunity [7]. To date, there are three P2Y receptors namely P2Y1, P2Y2 and P2Y11 have been primarily implicated in the growth inhibition of cancer cells [8]. MAPK signaling pathway involve in modulation of gene expression, cell proliferation, and apoptosis. MAPK consist of JNK, ERK and p38. Activation of the MAPK members under stress environment can lead to apoptosis [9, 10, 11]. 5-fluorouracil (5-FU) is an antimetabolite drug which inhibits on thymidylate synthase and lead to depletion on deoxythymidine triphosphate [12]. Study has found that 5-FU would able to induce phosphorylation of MAPK [13]. However, it is still unclear that whether activation of MAPK pathway by 5-FU has linked to apoptosis and whether activation of P2Y receptor will effect on these intearaction. In this study, we aim to investigate the inhibitory effect of P2Y receptor activation in OSCC cell line treated with 5-fluorouracil (5-FU) and its relation with MAPK signaling pathway.

EXPERIMENTAL METHODS

Characterisation of P2Y receptor subtypes in OSCC cell lines

Prior to the in vitro studies using OSCC cell lines, the expression profile of P2Y receptor subtypes in the OSCC cell lines will be determined by western blot method.

Characterisation of the apoptotic effects of 5-FU, P2Y activation and its combination in OSCC cell lines

For the in vitro cell line study, two established OSCC cell lines derived from the patients, one for the early stage and one for the late stage of OSCC, which show high expression of P2Y receptors, are chosen to compare the effects of P2Y activation at different severity of OSCC. These cells will be treated with 5-FU, ATP alone and drug together with ATP to identify the time course and concentration of each drug to induce apoptosis. Specific P2Y receptor agonist and antagonist will also be used to verify the apoptosis results for ATP by flow cytometry analysis.

Characterisation of the cell cycle arrest effects of 5-FU, P2Y activation and its combination in OSCC cell lines

Same as apoptosis, early stage and late stage OSCC cell lines with high expression of P2Y receptors are chosen to compare the effect of P2Y activation on cell cycle arrest. These cells will be treated with 5-FU alone, ATP alone and combination to induce cell cycle arrest. Specific P2Y receptor agonist and antagonist will also be used to verify the apoptosis results for ATP by flow cytometry analysis.

Quantification of the expression level of p-JNK, p-ERK and p-p38

Early stage and late stage OSCC cell lines will high expression of P2Y receptors are chosen and will be treated with 5-FU alone, ATP alone and combination. Western blot will be used to quantify the expression level of p-JNK, p-ERK and p-p38 using the treated cell sample. The expression level will be normalised to total JNK, total ERK and total p38.

Determination of the relation between 5-FU, MAPK pathway and P2Y

Early stage and late stage OSCC cell lines will high expression of P2Y receptors are chosen and will be treated with 5-FU alone, ATP alone and combination. Cell also will be treated with specific MAPK antagonist to study the inter-relation between the action of 5-FU and P2Y on MAPK signalling pathway and apoptosis.
REFERENCES

ACKNOWLEDGEMENTS
Special thanks to Dr Ng Pei Yuen, lecturers and friends who’s providing guidance and assistance in this study.
Expression Pattern of P2Y Purinergic Receptor in Oral Squamous Cell Carcinoma (OSCC) and Its Interaction in Cisplatin-mediated Apoptosis in OSCC Cell Lines

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INTRODUCTION
Oral squamous cell carcinoma (OSCC) is the third most common cancer in Malaysia, with most cases diagnosed at late stages [1]. However, the treatment of OSCC is limited by the lack of efficacy and resistance to the current therapeutic agents, such as cisplatin [2]. Thus, the development of new adjuvant therapy is required to increase the efficacy of cisplatin and to overcome the cisplatin-resistance issue. A promising target of adjuvant therapy is the purinergic P2Y receptor family as its agonist, adenosine triphosphate (ATP), is an extracellular ubiquitous messenger found abundantly in the tumour microenvironment [3]. The role of P2Y receptor activation in combination with standard treatment regime for various cancers has been studied [4, 5]. However, the results are contradicting with one another, possibly due to tissue specificity and activation of specific receptor subtype. In this study, we aim to investigate the inhibitory effect of P2Y receptor activation in a model of cisplatin treated OSCC cell line. We hypothesised that high P2Y expression in OSCC patients is correlated with disease staging and activation of P2Y receptors inhibits cisplatin-mediated apoptosis in OSCC cell lines. Overall, this study attempts to explore P2Y receptor as a potential therapeutic approach to improve the efficacy of cisplatin in the treatment of OSCC.

EXPERIMENTAL METHODS
Immunohistochemistry
Paraffin slides of tumour tissue from OSCC patients (n=60) will be obtained from the Oral Cancer Research and Coordinating Centre (OCRCC), University of Malaya. The samples will be incubated with P2Y receptor antibodies of various subtypes (i.e. P2Y1 and P2Y2) and stained with horseradish peroxidase tagged secondary antibody. Receptor expression and staining intensity will be correlated with disease stage acquired from clinical data.

Immunofluorescence Study
H series of OSCC cell lines obtained will be incubated with different P2Y receptor antibody subtypes and tagged with fluorophore for analysis to determine the expression and location of respective receptor subtypes. Data obtained will be correlated with information of the H series cell lines to determine relationship between receptor expression and cancer stage.

Western Blot
Expression of the phosphorylated form of mitogen-activated protein kinases (JNK, ERK and p38), indicating the activation of cell cycle arrest pathway, will be determined by Western blotting. Treatment of cell lines included stimulation with ATP, cisplatin, and coadministration of ATP and cisplatin. Data obtained will be analysed to determine the effect of ATP on cisplatin mediated apoptosis pathway.

Flow Cytometry
H series cell lines will be treated with ATP, cisplatin, and in combination to investigate cell viability of each treatment.

Statistical Analysis
Data collected will be analysed using GraphPad Prism version 5.00. Figures will be represented as mean ± standard error of mean. Type of statistical test will be performed as shown below:
1. Correlation test will be used to find out the degree of relationship between two parameters. Results from immunohistochemistry and immunofluorescence studies will be analysed using this statistical test.
2. One-way ANOVA will be used to compare between multiple means. Data obtained from Western Blotting and flow cytometry will be analysed by this statistical method.
REFERENCES


ACKNOWLEDGEMENTS

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The Healthcare Professionals’ Perception and Expectation of Pharmacists’ Competencies

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INTRODUCTION
A competent person is defined as an individual who possesses the required knowledge, skills and attributes sufficient to successfully and consistently perform a specific function or task to a desired standard [1]. Perception is the process of attaining awareness or understanding of sensory information [2]. Expectations are “pre-trial beliefs” about a product or service and is an important concept because they provide the frame of reference for evaluation [3]. A competency framework is a collection of competencies that are thought to be central to effective performance [4]. The framework of competency of pharmacist has been widely used by many countries such as United Kingdom, Australia, Singapore, New Zealand and Canada. Pharmacists are responsible for the promotion of the rational use of drugs for treatment and prevention of illnesses by the community, patient, doctors, nurses and other healthcare professionals. Hence, they are required to perform their task competently. Development of a guideline to assess their competency in pharmacy is needed. Currently, the competency standard for pharmacist in the Ministry of Health (MOH) and Ministry of Education (MOE) in Malaysia is yet to be available. Hence, a study is needed to measure the pharmacists and other health care professional perception and expectation of pharmacists’ competencies.

EXPERIMENTAL METHODS
Study Design
A cross-sectional survey will be conducted in UKMMC from March to August 2015.

Study Population
315 subjects will be recruited by convenience sampling. The inclusion and exclusion criteria will be as below.

Inclusion Criteria
iv. All pharmacists, specialist, medical officer, houseman officer, matrons, head of nurses and nurses
v. Working experience for more than 1 years

Exclusion Criteria
vi. Contract staff
vii. Staff who is on maternity leave
viii. Staff who is on study leave
ix. Staff who is on leave due to medical illness

Data Collection
Two questionnaires will be adapted from the Singapore competency framework and research of competencies of pharmacy graduates by Paraidathathu et al [5,6]. One for the perception and expectation by other healthcare professionals. Demographic data will also be included.
Questionnaires will be distributed to all pharmacists and other healthcare professionals (specialists, medical officers, houseman officers, matrons, head of nurses and nurses) in UKMMC from different discipline (medical, surgical, orthopaedic, paediatric, ICU, psychiatric, obstetrics and gynaecology, neurology, emergency department, cardiology) and will be collected upon completion.

Data Analysis
Collected data will be analysed using Statistical Program for Social Sciences (SPSS) version 22.0. Descriptive analysis will be presented by frequency and percentages. Spearman correlation will be used to compare the differences between working experience on their perception and expectation on pharmacist competencies. Chi square test will be use to compare the differences between the educational level on their perception and expectation on pharmacist competencies.

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Special thanks to Dr. Adliah, Prof. Madya Dr. Mohd Bin Makmor Bakry, Puan Lau Chee Lan, lecturers and friends that have contributed their help in this study.
Effect of Chalcone Derivatives and Curcumin Analogues on Lipopolysaccharide-induced Chemokine Production

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INTRODUCTION
Cardiovascular diseases (CVD) have long imposed great burden on global healthcare systems. It comprises of various vascular and heart diseases. Atherosclerosis is well known as an underlying factor contributing to many CVD such as ischemic heart disease, cerebrovascular disease, hypertension and peripheral vascular disease [1]. Inflammation plays an important role in atherogenesis and it could be triggered by various conditions such as dyslipidemia, hypertension, diabetes, bacterial infection and obesity [2]. Lipopolysaccharide (LPS) from the cell walls of Gram negative bacteria has been demonstrated to cause intimal hyperplasia by enhancing the secretion of inflammatory cytokines such as interleukin (IL)-6, monocyte chemotactic protein (MCP)-1 and tumor necrosis factor (TNF)-α in primary human adventitial fibroblasts via nuclear factor kappa B (NF-κB) signalling pathway [3]. Activation of mitogen-activated protein kinase (MAPK) and NF-κB signalling cascades is associated with atherosclerotic lesion formation [4,5]. Inhibition of NF-κB activation and MAPK signaling pathway has been found to dampen inflammation by reducing production of TNF-α, IL-1β and IL-6 in RAW 264.7 macrophages [6]. Previous studies have reported the anti-inflammatory property of chalcone derivatives and curcumin analogues in RAW264.7 macrophages stimulated by LPS [7,8]. Thus, this study is aimed at investigating the effects of synthesized chalcone derivatives and curcumin analogues on LPS-induced chemokine secretion in phorbol myristate acetate (PMA)-differentiated U937 cells and human aortic smooth muscle cells (HASMCs).

EXPERIMENTAL METHODS
Preparation of chalcone derivatives and curcumin analogues
Chalcone derivatives (compounds 1.1-1.6) and curcumin analogues (compounds 2.1-2.6) which will be used in this study were synthesized by Drug and Herbal Research Centre, Faculty of Pharmacy, UKM. Stocks at concentrations of 50μM will be prepared by dissolving the compounds in dimethyl sulfoxide (DMSO) solution. These solutions will then be diluted to desirable concentrations with serum-free media for use in subsequent cell treatment.

Cell culture and differentiation of U937 cells
U937 monocytic cell line (ATCC® CRL-1593.2) and human aortic smooth muscle cells (ATCC® PCS-100-012) will be purchased from ATCC, USA. U937 cell line will be cultured in ATCC-formulated Roswell Park Memorial Institute 1640 medium enriched with 10% fetal bovine serum and 5% penicillin-streptomycin. HASMCs will be cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS and 5% penicillin-streptomycin. The cells will be incubated in humidified atmosphere of 5% CO₂ at 37°C. U937 cells will be differentiated into macrophages by incubation with PMA at 10nM/mL for 24 hours. The cells will then undergo recovery phase by incubation with serum-free media overnight.

Determination of cell viability
The cytotoxic effects of each compound on PMA-differentiated U937 macrophages and HASMCs will be determined using (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay as described earlier [9]. Cells seeded in 96-well plates will be treated with each compound at several concentrations (6.25, 12.5, 25.0, 50.0 and 100.0μM). After 24 hours incubation, MTT salt solution will be added, followed by 4 hours incubation at 37°C. DMSO solution will then be added to dissolve the formazan crystal and absorbance will be measured at 565nm using a microplate reader (Tecan, Switzerland). Concentrations at which 90% of cells remain viable will be used for subsequent cell treatment.

Determination of chemokine secretion
Cells will be seeded on 24-well plates and supplemented with tested compounds (12.5, 25.0 and 50.0μM) for 1 hour, followed by incubation with LPS (10ng/mL) for 24 hours at 37°C. Supernatants will be collected and the secretion of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) will be determined by enzyme-linked immunosorbent assay (eBioscience, USA) in accordance with manufacturer’s instructions. Cells with media and DMSO only will serve as vehicle control while cells treated with dexamethasone (100nM) will be used as a positive control.

Quantification of protein expression
Cells will be seeded on 24-well plates and supplemented with tested compounds (12.5, 25.0 and 50.0μM) for 1 hour, followed by incubation with LPS (10ng/mL) for 24 hours at 37°C. Supernatants will be collected and the secretion of interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) will be determined by enzyme-linked immunosorbent assay (eBioscience, USA) in accordance with manufacturer’s instructions. Cells with media and DMSO only will serve as vehicle control while cells treated with dexamethasone (100nM) will be used as a positive control.

Quantification of protein expression
Cells will be seeded on 12-well plates and treated with each compound at various concentrations (12.5, 25.0 and 50.0μM). The cells will be harvested and whole cell lysate will be prepared based on previously described protocol [10]. The concentrations of protein from whole-cell lysate will be determined by Bradford assay. Lysates containing equal amounts of protein will be separated by SDS-PAGE electrophoresis and transferred onto a polyvinylidene difluoride membrane.
The membrane will then be blocked with 5% (w/v) bovine serum albumin for 2 hours. Thereafter, the membrane will be successfully probed with one of the following target primary antibodies: IkBα antibody [ab32518], NFκB p65 antibody [ab28856] and p38 [ab4822], and horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody. The membrane will be incubated in enhanced chemiluminescence reagent before exposure to chemiluminescent light. Detection of bands will be performed using Fusion FX7 gel documentation system. (Vilber Lourmat, Germany).

**Statistical Analysis**

Statistical analysis will be performed using the GraphPad Prism version 5.0. Data will be expressed as mean ± standard error of mean. One-way ANOVA will be used to compare variables between groups. Two-tailed p values of <0.05 will be considered statistically significant.

**EXPECTED OUTCOME**

Chalcone derivatives and curcumin analogues are expected to maintain 90% of cell viability when added at concentrations ranging from 3.125μM to 50μM. These compounds should inhibit LPS-induced MCP-1 and IL-8 secretion in PMA-differentiated U937 cells and HASMCs. The inhibitory activities of the compounds are most likely due to suppression of the activation of NF-κB signaling pathway as well as the phosphorylation of p38 MAPK.

**REFERENCES**


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A Study on Patients’ Perception on Cognitive Side Effect of Antiepileptic Drug Treatment in UKM Medical Centre

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INTRODUCTION
Cognitive function is a higher order behaviour which involves the ability to adapt, solve problems, memorise data and focus attention [1]. Antiepileptic drugs (AEDs) are known to cause cognitive side effects (CSEs). Cognitive side effects were more prevalent in patients receiving polytherapy, higher dose and blood level as well as rapid up-titration of AEDs [2,3]. The cognitive impairment affects the patient’s life more than the actual seizure itself [1]. Studies done in America and Europe have shown that AED has affect on the cognition and quality of life of epileptic patients [4,5]. To date, no cross cultural studies of patients’ perception on cognitive side effect of antiepileptic drug have been performed in Malaysia. We wish to conduct a study on perception of cognitive side effect of antiepileptic drug in Universiti Kebangsaan Malaysia Medical Centre (UKMMC).

EXPERIMENTAL METHODS
Study Design
A cross-sectional study involving neuromedical patients of UKMMC from February 2015 to June 2015 will be conducted.

Study Population
Approximately 281 patients are required in the study using convenient sampling will be conducted. The inclusion and exclusion criteria will be as below.

Inclusion Criteria
i. 18 years old and above who are diagnosed with epilepsy.
ii. Stable on current AEDs for epilepsy for at least 6 months.
iii. Understand English or Malay.

Exclusion Criteria
i. Comorbid with psychiatric problems
ii. On chronic drugs that affects cognition for example antihistamine
iii. Mentally retard before the start of antiepileptics
iv. Alcoholics
v. History of abuse
vi. Serious illness that can affect cognition for the past 3 months

Data Collection
Questionnaires will be adapted and developed based on literature review [6,7]. Researcher assisted survey will be conducted where participants will be interviewed.

Data Analysis
SPSS version 22 will be applied for statistical analysis with a p-value of <0.05 denotes statistical significance. Demographic data will be analysed using descriptive analysis. The type of statistical tests used will be based on the objectives as shown below:

i) Chi-square test will be used to compare the rate of cognitive side effect between antiepileptics, relationship between demographic variables with the frequency of cognitive side effects and relationship between the cognitive side effects with the quality of life.
ii) Tables and figures will be used to show the demographic data of the patients.
POTENTIAL APPLICATIONS

The observational study will allow the practitioners of UKMMC to determine future treatment of choice for the patients locally.

REFERENCES


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Special thanks to staffs of neuromedical department and friends that have contributed their help in this study.
Neuroprotective Effects of Phenolic-Rich Fractions of *Ocimum basilicum* Linn. Leaves on Kainate-Induced Excitotoxicity in SK-N-SH Neuroblastoma Cells

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INTRODUCTION

Excitotoxicity event due to overstimulation of glutamate receptors eventually leads to neuronal death [1]. Meanwhile, death of neurons has been associated with neurodegenerative diseases such as Alzheimer’s, Parkinson’s as well as Huntington’s disease. These diseases affect the central nervous system (CNS) which is linked to the production of too much reactive oxygen species (ROS) [2]. Until recently, it has been reported that secondary metabolites of plants such as phenolic compounds showed promising results to be developed as neuroprotective agents and these compounds can be found ubiquitously in plants. For instance, resveratrol which is one of polyphenol compounds reduced NADPH oxidase which in turn inhibited ROS generation [3]. *Ocimum basilicum* Linn. or also known as sweet basil has been categorised as herb plants which have several medicinal values. Traditionally, people used sweet basil to treat headaches, cough, diarrhea, and kidney disorders [4]. According to the previous study, rosmarinic acid is one of the phenolic compounds which have been found in *O. basilicum* Linn. which exhibited antiviral, antibacterial, anti-inflammatory, and antioxidant properties [5]. The latter has been contributed by the conformational structure of phenolic compounds in which their ability to terminate free radical chains by donating a hydrogen or an electron and also stabilising unpaired electron via electron delocalisation within their aromatic structure [6]. In this study, *O. basilicum* Linn. leaf extracts will be assessed for their phenolic contents before being subjected for *in vitro* studies in order to investigate their neuroprotective effects on kainate-induced excitotoxicity in neuroblastoma cells.

EXPERIMENTAL METHODS

Plant Extraction

*O. basilicum* Linn. leaves will be separated from other parts of the plant and air-dried. The dried leaves will be grinded into powder and subjected for 80% ethanolic extraction using cold solvent extraction method. The extract will be filtered and freeze-dried. Fractionation of the ethanolic extract will be conducted sequentially using different organic solvents such as hexane and ethyl acetate.

Total Phenolic Content (TPC) Determination

Folin-Ciocalteu (F-C) assay will be conducted to determine the total phenolic content of the extract by using Gallic acid as a standard. Total phenolic content will be expressed as mg of gallic acid equivalents per g (mg GAE/g). Fractions with high TPC will be subjected for *in vitro* studies using SK-N-SH neuroblastoma cell.

Neuroprotective Effects of *O. basilicum* Linn. in kainate-induced excitotoxicity in SK-N-SH cells

SK-N-SH neuroblastoma cell line will be purchased from ATCC and cultured in conditioned media for 24 hours. Then, cells will be exposed to selected fractions of *O. basilicum* extract for 24 hours at up to IC10 concentrations. After 24 hours, the cells will be induced by kainic acid at IC50 concentration. Both of the IC10 of the extract and IC50 of kainic acid will be determined by MTT assay. At the end of the experiment, reduction in markers of cell toxicity will be determined using the methods outlined below.

i) Mode of cell death assessment

The mode of cell death will be assessed based on the externalisation of phosphatidylserine (PS) and membrane integrity. In this assay, cells will be stained by Annexin V-FITC which bind specifically to PS and propidium iodide which differentiate the late apoptotic and necrotic population. After staining, the cells will be analysed using a flow cytometer. Negative staining for both markers represents viable population, while positive staining for Annexin V-FITC only represents early apoptosis. Meanwhile, positive staining for propidium iodide only represents necrosis whereas positive staining for both markers represents late apoptosis.

ii) Determination of free thiols content
Reduced GSH or free thiols will be detected using monochlorobimane (MCB). MCB is nonfluorescence. However, the interaction of MCB with free thiols will form fluorescence complex which can be detected by flow cytometer. Thus, this assay will be used to detect the level of free thiols in the SK-N-SH cells.

iii) Reactive oxygen species (ROS) assessment

The involvement of ROS such as superoxide anion and hydrogen peroxide will be assessed using Mitosox and DCFH-DA respectively. These chemicals will be oxidized by ROS to form fluorescence product in cells which can then be detected using flow cytometer.

iv) Intracellular cytoplasmic calcium

Intracellular calcium influx will be determined by Fura-2 AM assay which is a ratiometric and UV light—excitable technique.

EXPECTED RESULTS

• Ethanol fraction of *Ocimum basilicum* Linn. leaves shows the highest total phenolic content followed by ethyl acetate and hexane fractions.
• SK-N-SH cells pre-treated with *Ocimum basilicum* Linn. leaves phenolic-rich fractions are able to reduce apoptosis rate, oxidative stress level, as well as intracellular cytoplasmic calcium level in kainate-induced neuroblastoma cells.

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Development and Characterisation of Simvastatin Loaded Alginate-Based Film for Accelerated Wound Healing

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INTRODUCTION
Wound dressing has a vital function in wound management and promoting wound healing. Film dressings are transparent, semi-permeable to water vapor and oxygen, and considered as a simple and efficient dressing in creating moist environment that can be a useful candidates for application on various areas of body due to their pliable nature [1-3]. The current wound dressings for diabetic foot ulcer are not ideal as they have their own disadvantages. Alginate based dressing is suggested to be suitable for exudative wound due to its unique characteristics i.e. it can absorb fluids on wound site and release active ingredients at the same time. Simvastatin (SIM) has recently shown successful its potential to promoting wound healing in diabetic wound by augmenting angiogenesis and lymphangiogenesis [4]. In this study, alginate based dressing loaded with simvastatin is proposed to be beneficial for acceleration of diabetic ulcer healing as well as the avoidance of systemic side effects. Therefore the aim of the present work was to develop several films of either sodium alginate (SA) alone or blend of SA and Pectin (PC) or SA and Gelatin (GL) containing SIM and assess its successful development. Here, we described formulation, characterization, and physicochemical properties of alginate-based film dressing for its potential as slow-release wound dressing.

EXPERIMENTAL METHODS
Preformulation
The composition of 5% (w/w) hydrogel is listed in Table 1. Films of only SA and blends of SA-PC, SA-GL of equal mass fractions (50:50) were prepared by solvent casting method.

<table>
<thead>
<tr>
<th>Starting Material (g)</th>
<th>SA</th>
<th>SA-PC</th>
<th>SA-GL</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA</td>
<td>5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>PC</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>GL</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>GLY</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>SIM</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*GLY - glycerol

Film thickness and scanning electron microscopy (SEM)
The thickness of films was measured by using a digital caliper at five designated positions (one at center, four around the edges). Surface morphology of films was determined by using SEM (LEO 1450 VPSEM, Zeiss, Germany). Films were trimmed to small pieces and sputter coated with a layer of gold and mounted onto the aluminum stub under an argon atmosphere and images were recorded at 1000× magnification.

Films expansion and water vapor transmission rate (WVTR)
A gelatin (4%) model was used to determine expansion profile. The circular film sample of known size was placed at the center of the gelatin surface, change in diameter of the films was recorded and percentage ratio was obtained. For WVTR, films were cut into disc-like shape. Calcium chloride as desiccant was added to a dry glass vial and films (exposed diameter = 1.1 cm) were sealed to the cap using a rubber ring then mounted on the brim of vial. The vials were weighed and put at constant relative humidity (84%) and temperature of (25 °C). Gain in the weight of vial was recorded at every 1 h and WVTR was calculated.

Rheology and mechanical properties
The film samples were rehydrated into gel by introducing the amount of solvent lost after drying the gel in oven. Flow properties assessed using a cone and plate measuring system with diameter of 20 mm and angle of 2° at a constant shear
rate of 500 s⁻¹. Tensile strength (TS) and percentage elongation at break (%E) of the film formulations were determined by Instron Universal Testing Machine (Model 5567, USA) according to ASTM D 882-02 standard [5].

**FTIR and X-ray diffraction**

Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectrophotometer (PerkinElmer Spectrum, USA) was used to analyse the samples. Small pieces of films were placed on an ATR crystal and maximum force was applied by using an ATR pressure clamp to allow optimum contact with samples. X-ray diffraction patterns were obtained using D8 Advance X-ray diffractometer (Bruker AXS, Mannheim, Germany). The pure powder samples of drug and polymers were filled to the cavity of the sample tray and the film specimens of about 0.5 mm thickness were cut to fit the square tiles of the sample holder before running the test.

**In vitro drug release study**

Franz diffusion cell (Permegear. Inc., USA) was used to generate the in vitro drug release profile of simvastatin from the film formulations using a cellulose acetate membrane. The concentration of the simvastatin in the samples was determined spectrophotometrically at 238 nm. The cumulative release of SIM from the films was calculated and results were plotted over time.

**RESULTS AND DISCUSSION**

**Preformulation**

SA-PC films showed to be more transparent. Transparency is an important feature in wound dressing as it allows wound inspection without removing the dressing [6]. Besides, SA-PC was odorless as compare the other two with seaweed and gelatin odor.

**Film thickness and SEM**

The thickness of films was slightly increased by loading the drug as well as presence of PC in the film formulation. SEM revealed absence of any pores or cracks. SA-PC fiber-like structure was changed in the presence of drug and a rod-shaped network appeared which may be due to crosslinking of drug with polymers and formation of microaggregates that can be a reason to a better mechanical properties.

**Expansion and WVT rate**

The films hydrated slowly on gelatin surface and expanded in all directions and transformed into a gel. The slowest hydration rate was observed in the blended film containing PC (43.94 ± 9.46), followed by plain SA (48.48 ± 2.64) and GL (90.91 ± 0.0) films. Additionally, film of SA-PC retained its disc-like shape for a longer duration. The WVTR rate for SA, SA-PC, SA-GL were approximately 0.14 ± 0.004, 0.139 ± 0.139 ± 0.0020, 0.139 ± 0.0023 (g/cm²/24h) respectively. Results indicated that all the films were permeable to water vapor. Maintaining high humidity is necessary in healing of wound which accelerates cell migration and helps in epithelialization on wound site [7].

**Rheological studies and Mechanical properties**

The rehydrated films exhibited pseudoplastic flow behavior. This is an indication that the formulations were able to maintain the flow properties during application. Moreover, the apparent viscosity of SA-PC found to be highest. A good wound dressing on wound site is capable of maintaining sufficient viscosity in order to prevent flow for a prolonged time [8]. It is reported that the values of tensile strength and elongation at break for skin is in the range of 2.5-16 MPa and 70 % respectively [9]. By comparing the mechanical profile of films with these values it was revealed that the film prepared by blend alginate and pectin presented a better mechanical properties (3.32 ± 0.58 MPa and 61.49 ± 2.06 %) for skin applications.

**FTIR and X-ray diffraction**

ATR analysis reveals no characteristic peak of drug observed in the drug-loaded films that could be a good indication of uniform inclusion or dispersion of drug within film matrix or lack of obvious interaction between the drug and the matrix [3, 10]. In XRD patterns simvastatin peaks were appeared in the drug loaded films with much lower intensities compared to powder form, indicating reduction in crystallinity of drug after formulation into in the film. Overall, the diffraction patterns of films suggested that there is a good compatibility between matrix film and simvastatin that can be attributed to a good hydrogen and ionic interaction between them. Moreover, the crystallinity index between blank and drug loaded films was not significantly different [10].

**In vitro drug release**

The SA-PC formulation revealed slower release of drug that may be due to its swelling properties or thickness, which can be considered as a rate-limiting step. Moreover, it could also be because of formation of microaggregates or good crosslinking between drug and blend of SA-PC.
CONCLUSION
In the present study three different media were compared to find out the potential media for incorporation of simvastatin for wound dressing. Blend SA-PC showed promising evidence to be a better candidate in terms of transparency, mechanical properties and drug release. FTIR and XRD revealed a good homogeneity and absence of any interaction between polymers and drug.

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Scanning Electron Microscope (SEM) Analysis of Polymer Polyvinylpyrrolidone Wrapped Single Wall Carbon Nanotubes and Its Superior Antimicrobial Activity

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INTRODUCTION

Single wall carbon nanotubes (SWNTs) are a form of carbon nanotubes with a layer of graphitic rolled up sheet. They are proposed to have outstanding properties in material science, drug delivery, biomedical devices and cell biology [1]. The use of carbon nanotubes in medicine and health area has gained great attention from researchers. SWNTs are known for its toxicity. That behaviour has contributed to their antimicrobial and antiseptic properties [2]. The first evidence of SWNTs having strong antimicrobial activity towards E. coli as a model organism was reported by Kang et al [2]. However, SWNTs because of their high surface energy tend to aggregate and form bundles [3]. Liu et al. reported that individually dispersed SWCNTs are more toxic to bacteria than aggregated SWNTs, as they act as moving “nano darts” in solution and constantly attack bacterial cells, causing degradation of bacterial cell integrity, resulting cell death [4], [5], [6]. Through various studies, researchers investigated the antimicrobial activity of SWNTs with covalent surface modification that showed improved antimicrobial activity [7]. However, surface modification has inappropriately alters the sidewall and changes the intrinsic property of nanotubes [8]. A more preferable method of solubilization is non-covalent wrapping with polymers [9], [10]. In this study we intend to validate i) the morphology of polyvinylpyrrolidone (PVP) wrapped SWNTs and ii) its antimicrobial activity against E. coli as compared to that of naked SWNTs.

EXPERIMENTAL METHODS

Materials

The SWNTs used were purchased from Guangzhou Jiechuang Trading Co. Ltd. China and was purified as mentioned elsewhere [10]. The PVP-SWNTs were synthesized in the Laboratory of Fundamental Pharmaceutics, Faculty of Pharmacy, Universiti Teknologi MARA (UITM), Malaysia. The bacteria strain Escherichia coli (E. coli) (ATC 25922) was freshly subcultured in the laboratory and standardized (1.5 x 10^8 CFU) using the MacFarland standard.

Methods

Preparation of samples A to C:

Samples A to C were prepared with method provided elsewhere [11] with modification. All test tubes A (E. coli, 0.5 ml and distilled water, 1.5 ml), B [(E. coli and SWNTs (0.2 mg/ml)] and C [(E. coli and PVP-SWNTs (0.2 mg/ml)] were incubated for 2 days (48 h) at 37 °C with shaking. After incubation the suspensions were subjected to (SEM).

SEM

The solid samples of PVP, PVP-SWNTs and SWNTs were coated with a Pd-Au film by an Emitech Magnetron Sputter Coater before imaging to avoid electric charge build-up. The imaging of bacteria morphology was carried out following previous literature [12] with modification. It was carried out using JEOL JSM-6701F SEM. Further morphology PVP-SWNTs were dispersed and observed for their physical state.

RESULT AND DISCUSSION

SEM of solid state

Fig. 1 shows image of PVP, PVP-SWNTs and typical image of purified SWNTs with no wrapping. SWNTs in this state are still in bundles and appeared in the form of bundled network of ropes and severely being aggregated together. The SEM image observed is also supported by previous literature [13]. Whereas, image of PVP-SWNTs shows no traces of single rope of SWNTs rather a flaky stack of solid molecules, those are comparatively smaller than that of the original PVP.
Both PVP-SWNTs and SWNTs with concentration of 0.2 mg/ml were solubilized in water and incubated with *E. coli* samples for two days. Results are shown in Fig. 2. It is seen that in case of PVP-SWNTs addition (as in C); there have been a massive aggregation of bacterial structure indicating the higher extent of damage to the cell wall as compared to A and B, representing the control and that of the addition of SWNTs (elongated, shortened and mild aggregation).

Vecitis et al. reported that incorporation of SWNTs with bacteria can cause damages to the cell membrane and release of cellular enzymes [14]. Obviously, from the SEM photos, we revealed that at low concentration 0.2 mg/ml PVP wrapped SWNTs are able to damage the bacterial morphology.

PVP-SWNTs were dispersed in ethanol (they are soluble in polar and non-polar solvents) and observed for their physical state. The solubilized PVP-SWNTs are found as many single ropes of SWNTs in the liquid media (Result no shown). These single ropes might act as the moving “nano darts” that continuously attacked the *E. coli*.

**Fig. 2:** Electron micrograph of A: *E. coli*-distilled water, B: *E. coli*-SWNTs, C: *E. coli*-PVP-SWNTs.

**CONCLUSIONS**

On the basis of the morphological verification of *E. coli* it was concluded that the surface contact of the solubilized PVP-SWNTs with *E. coli* resulted superior antimicrobial activity even in lower concentration. From the present study, we conclude that wrapping of SWNTs with water soluble polymer PVP display better antimicrobial potential than that of the virgin SWNTs.

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Preclinical Safety Evaluation of Hydrocortisone-hydroxytyrosol Co-loaded Chitosan Nanoparticles Cream in Albino Wistar Rats

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INTRODUCTION
Synergistic effects of biologically active naturally occurring and synthetic compounds expand the new ways for treatment of diseases. Hydroxytyrosol (HT) or phenyl ethyl alcohol, 2-(3, 4-dihydroxyphenyl) ethanol (3, 4-DHPEA) is one of the most abundant phenolic component present naturally in olive oil (1). HT possesses anti-oxidant, (2) anti-inflammatory, (3) cardio-protective, (4) and anti-microbial activities especially against Staphylococcus aureus (S. aureus), (5). The skin of 90% of patients is colonized by S. aureus. (7). Topical glucocorticoids (TGs) are the mainstay of drug therapy for AD due to their broad immunosuppressant and anti-inflammatory effects (8). However, TGs have been associated with unwanted local and systemic side effects, such as dermal atrophy, acneiform eruption, striae, telangiectasia, hypothalamic-pituitary-adrenal axis suppression and glaucoma development due to increased intraocular pressure (9). These findings prompt the development of a drug delivery system that is able to co-deliver HC and HT into the skin layers of AD patients with minimal systemic absorption. Therefore, our present study evaluated the effects of CSNPs as the carrier for HT and HC on skin irritation. Acute and repeated dose dermal toxicity on healthy albino Wistar rat skins were also undertaken in an attempt to gain better understanding of toxicity/adverse effect caused by local delivery of HC-HT incorporated into CSNPs.

EXPERIMENTAL METHODS
1. Preparation of nanoparticles
HC-HT CS NPs were prepared by ionic cross-linking of CS with TPP ions (ionic gelation). Mixture of two active ingredients (HC and HT, 1 mg/mL each in 30:70 ratios of ethanol and water) was added to CS solution (0.2%, w/v in 1%, v/v acetic acid) and left to achieve equilibrium for 30 min. The pH of mixture was adjusted to 5.0 by adding 1 M sodium hydroxide (NaOH) solution. Co-loaded CSNPs were formed spontaneously by adding TPP solution drop wise to CS solution containing both HC and HT at CS to TPP mass ratio of 5:1 under a constant magnetic stirring at 700 rpm. Co-loaded CSNPs were collected by ultracentrifugation.

2. Physicochemical characterization
2.1 Size and zeta potential measurement
Laser Doppler anemometry technique (Nano-zetasizer, Malvern, UK) was used to measure particle size, polydispersity index (PDI) and zeta potential. The data were presented as the mean ± standard deviation (SD).

2.2 Drug encapsulation efficiency
Encapsulation efficiency (%EE) measured with respect to the total amount of drug used. HC and HT were analyzed at 248 nm and 280 nm on UV-visible spectrophotometry analysis (UV-1601, Shimadzu, Japan), respectively.

3. In-vivo study
3.1 Acute dermal toxicity
The experiment was carried out according to the Organization for Economic Co-operation and Development Guidelines, OECD-402 on albino wistar rats. A dose of 100-times higher than the normal human dose which is 125 mg/BSA of active substance was used. Body weight, food consumption and abnormal clinical sign were recorded.

3.2 Acute dermal irritation
Primary irritation study was conducted according to the guidelines of OECD-404. Animals were treated with aqueous (AQ) cream containing HC-HT CSNPs (G1), AQ cream containing HC-HT (G2), AQ cream only (Vehicle) (G3), and 0.8% v/v aqueous solution of formaldehyde (G4) as a standard irritant. Untreated animals were used as a negative control (G5).

(a) Biometrics and Clinical assessment
Animals’ skin barrier integrity was assessed by measuring transepidermal water loss (TEWL) (g/m2/h) and erythema intensity, indicators of skin irritation using Tewameter⁸ TM 300 and Mexameter⁸ MX-18 (Courage +Khazaka, Kohn, Germany), respectively at 0, 4, 24, 48, 72, 96, 120 and 134 h in triplicate. Erythema intensity was visually scored using the standard Draize skin irritation scoring system.
4. 28-days Repeated dose dermal toxicity
OECD guidelines followed. Albino Wistar rats (6/sex/dose; housed in the animal house, UKM) were treated with HC-HT CSNPs to the nominal doses of 250 mg, 500 mg, 1000 mg/BSA/day for 28-days.

(a) Clinical observation, body weight and food consumption
Body weight and food consumption of each animal were recorded every two days up to 28 days. The terminal body weight was calculated prior to necropsy.

(b) Hematology, clinical biochemistry and urinalysis
Animals were fasted overnight (with free access to water) prior to necropsy and blood collection. Complete blood count was determined by using XE-5000™ Hematology Analyzer (Sysmex, Japan). Complete clinical biochemistry was determined by using ADVIA 2400 chemistry system (Siemens Diagnostics, New York, USA).

Animals were fasted overnight and housed individually in metabolic cages after final administration of doses for the collection of urine to perform complete urinalysis using Clinitek500 (Siemens Diagnostics, Saint-Denis, France).

(c) Gross Necropsy, macroscopic examination and organ weight
All animals were necropsied after collecting their blood on the day at which the final dose was administered to the animals. Organs that were weighed included the skin, stomach, heart, lung (including bronchus), liver, kidneys, spleen, testes and ovaries.

(d) Histopathology
Organ samples were fixed and preserved in 10% neutral formalin. All fixed organs were embedded in paraffin prior to sectioning, and stained with haematoxylin and eosin (H & E).

RESULTS AND DISCUSSION
1. Particle size, zeta potential and encapsulation efficiency
The mean particle size of HC-HT loaded CSNPs was 235 ± 3 nm with a narrow distribution (PI=0.021±0.025). The mean zeta potential of the nanoparticles was +39.2 ± 1.6 mV. Drug encapsulation efficiency was 70% and 65% of HC and HT, respectively.

2. Acute dermal toxicity
The acute dermal LD50 of HC-HT CSNPs to rat skin in both genders was more than 125 mg/BSA.

3. Acute dermal irritation
TEWL of animal skins treated with cream containing HC-HT CSNPs was not significantly different from the normal baseline (untreated) group, suggesting that the formulation did not cause any skin irritation.

4. Repeated dose dermal toxicity
a) Clinical observation, body weight and food consumption
Both sexes did not show any significant difference in food consumption and body weight as compared to untreated group.

b) Blood hematology
Application of creams containing CSNPs with three different doses did not cause any significant hematological changes in both male and females.

c) Blood biochemistry
The individual parameter values for both sexes were generally comparable to their control means.

d) Urine Analysis
No treatment-related change was detected for the urine parameters in any experimental group at the end of treatment period.

e) Organ weight and histopathology
There was no sign of toxicity observed in the organs weight of animals treated with HC-HT CSNPs and normal architecture was observed.

CONCLUSION
This work demonstrated that skin irritation in AD patients could be reduced by incorporating HC and HT into CSNPs by improving local accumulation of drugs in the skin and minimizing direct contact of HC with the skin. Long term use of HC-HT in the form of nanoparticles is expected to prevent severe local and systemic side effects associated with HC. The
study also further confirms that HC-HT CSNPs is non-toxic, safe and well tolerated therapy for AD patients. However, human clinical study is needed to provide further insight into the tolerance of HC-HT CSNPs as the alternative treatment of AD.

REFERENCES


Indole Alkaloid Inhibitory Effect of *Kopsia Larutensis* King & Gamble on Histamine and Beta-Hexosaminidase Release in RBL-2H3 Cell

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**INTRODUCTION**

*Kopsia larutensis* is one of the plants in the Apocynaceae family, found in southeastern Asia and Malaysia. It has been used in traditional medicine for the treatment of ulcerated noses in tertiary syphilis, skin infections and wounds [1]. *Kopsia* species are known to be a rich source of indole alkaloid such as harmana and buchtienina [2], plumeran group from *K. profunda* [3] and aspidofractinine group in bark of *K. teoi* [4]. Extracts and pure isolates of *Kopsia* species has been reported to exhibit various biological activities like antimicrobial, anticancer, anti-inflammatory, cardiovascular and antitussive effects [5][6][7].

The uses of herbal medicines are now becoming the promising approach for the alternative treatment of allergies as many drugs available as antihistamine and anti-allergic agents have undesirable side effects and adverse reaction, such as drowsiness, headache, gastrointestinal tract disturbance, fatigue and dry mouth [7]. In recent years, phenylflavonoids [8], xanthones [9] and acridone alkaloids [10], which have been isolated from various plants, showed potent anti-allergic activities. Therefore, indole alkaloid isolated from *K. larutensis* possessed high potential as a new anti-allergic agent. So, the aim of this study is to investigate the effect of indole alkaloid from *K. larutensis* as an inhibitor of histamine and beta-hexosaminidase release in RBL-2H3 cells.

**EXPERIMENTAL METHODS**

**Samples**
Ten kilogram of *K. larutensis* was collected by FRIM and Mayer’s test was used for alkaloid screening. The samples were identified by the botanist of Universiti Kebangsaan Malaysia and the voucher specimens were deposited at UKM Herbarium (UKMB5971).

**Extraction of Alkaloid**
Ten kilogram of dried *K. larutensis* will be ground and macerated with methanol at room temperature, three times. Acid-base extraction will be carried out to get crude alkaloid.

**Isolation of Indole Alkaloid**
Pure compounds will be isolated using chromatographic techniques (vacuum liquid chromatography, column chromatography, preparative high performance liquid chromatography and chromatotron). The structure of bioactive compounds will be elucidated using spectroscopic techniques (nuclear magnetic resonance, ultraviolet-spectroscopy, infrared-spectroscopy and time of flight-mass spectrometry).

**Histamine Release Assay**
RBL-2H3 cells will be inoculated into a 24-well plate (5x10^5 cells/mL) and cultured for 1 hour. After incubation, monoclonal mouse IgE anti DNP will be added to the cultured medium at a concentration of 0.45 μg/mL and incubated for 24 hour. Treated cells will be washed twice by Siraganian buffer (NaCl 119 mM, KCl 5 mM, MgCl₂ 0.4 mM, PIPES 25 mM, NaOH 40 mM, pH 7.2) and 160 μL of Siraganian buffer will be added including 5.6 mM glucose, 1 mM CaCl₂, and 0.1% bovine serum albumin (BSA).

After incubation at 37 °C for 10 min, the IgE-sensitized cells will be treated with 20 μL of extracts or compounds (20 μL) at 37 °C for 30 min. As the next operation, 20 μL of dinitrophenyl-labeled bovine serum albumin (DNP-BSA) will be added to cultured medium at a concentration of 10 μg/mL and will be incubated for 10 min correctly. To terminate the reaction, the treated cells will be stayed on ice for 10 min. Then, the reacted buffer will be collected in a 1.5 mL tube and centrifuged at 300 g at 4°C for 10 min.

To measure histamine release, 75 mg of NaCl and 50 μL of 1 N NaOH, 500 μL of a 3:2 (v/v) mixture of n-butanol and chloroform will be added to 200 μL of Ag-stimulated Siraganian buffer and mixed for 5 min. The organic layer will be recovered and mixed with 15 μL of 1 N NaOH and 10 μL of 0.2% o-phthalaldehyde, and stayed for 5 min at room temperature. This reaction will be terminated by adding 15 μL of 0.5 N H₂SO₄, and then the absorbance will be measured with microplate reader at 450 nm. The percentage inhibition of histamine release will be calculated as follows: inhibition of histamine release (%) = [1 - (test - negative control)/ (positive control - negative control)] x 100 [9][11].

**Inhibitory Effects on the Release of Beta-hexosaminidase from RBL-2H3 cells Assay**
Briefly, RBL-2H3 cells will be dispersed in a 24-well plates at a concentration of 2x 10^5 cells/well using minimum essential
eagle (MEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 U/mL), and anti DNP IgE (0.45 mg/mL), then will be incubated overnight at 37°C in 5% CO₂ for sensitization of the cells.

The cells will be washed twice with 500 mL of Siraganian buffer that contain 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 1mM CaCl₂, 25 mM piperazine–N, N'-bis (2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA), and 40 mM NaOH, pH 7.2. After that it will be incubated in 160 mL of another Siraganian buffer for an additional 10 min at 37 °C before 20 mL of test sample solution will be added to each well and will be incubated for 10 min, followed by addition of 20 mL of antigen (DNP-BSA, final concentration was 10 mg/mL at 37 °C for 20 min to stimulate the cells degranulation.

The supernatant will be transferred into a 96-well plate and will be incubated with 50 mL of substrate (1 mM p-nitrophenyl-N-acetyl-b-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1h. The reaction will be stopped by adding 200 mL of stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance will be measured with a microplate reader at 405 nm [11].

Beta-hexosaminidase Inhibitory Assay
The cell suspension (5 x 10⁶ cells) in 10 mL of phosphate-buffered saline (PBS) will be sonicated. The solution then will be centrifuged, and the supernatant will be diluted with Siraganian buffer. The enzyme solution (45 mL) and test sample solution (5 mL) will be transferred into a 96-well microplate and will be incubated with 50 mL of the substrate solution at 37 °C for 1 hour. The reaction will be stopped by adding 200 mL of stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance will be measured with a microplate reader at 405 nm [11].

Structure-Activity Relationship Study
Structure-activity analysis will be performed to identify the lead structures which can be further developed into potent anti-allergic agents by using computer software (Computer-aided drug design (CADD), quantum chemistry models, molecular graphics and conformational analysis).

Statistical Analysis
The results will be expressed as a mean ± S.E.M of three determinations at each concentration for each sample. The IC₅₀ values will be calculated using the Microsoft Excel program. Statistical significance will be test by using one-way ANOVA, followed by Dunnett’s test.

EXPECTED RESULTS
There is a significant high anti-allergic activity of indole alkaloid compound isolated from K. larutensis as compared to positive control, ketotifen fumarate.

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ACKNOWLEDGEMENT
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Development and Characterization of Novel Polymeric Micelles Based on Cholic acid-Polyethylenimine-Folic Acid Loaded with Doxorubicin and siRNA for the Targeted Delivery to Cancer Cells

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INTRODUCTION

Several therapeutic anticancer drugs, though pharmacologically effectual in cancer treatment, are restricted in their clinical applications owing to their severe toxicities [1]. To overcome these complications, researchers have emphasized on developing nanoscale anticancer drug carriers for improving therapeutic efficacy and lessening unwanted side effects [2]. Polymeric micelles self- assembled from amphiphilic copolymers have generated great attention for targeted anticancer drug delivery since they own a number of physico- and bio-chemical advantages over other types of nanocarriers.

Bile acids (Cholic acid used here) are naturally synthesized steroid amphiphilic molecules, which organize into micelles above the critical micelle concentration, vary the permeability of membrane [3] and alter drug bioavailability [4]. PEI is a cationic synthetic vector which has shown promising transfection efficacy both in vitro and in vivo [5].

Based on the aforementioned information, CA-PEI-FA copolymer micelles were prepared, characterised and evaluated for the targeted delivery and cytotoxicity to the Nu/Nu nude mice bearing xenografts of human colorectal adenocarcinoma (DLD-1) to investigate the in vivo co-delivery efficiency and tumor targeting of doxorubicin and siRNA.

EXPERIMENTAL METHODS

Cholic acid-polyethyleneimine copolymer decorated with folic acid was synthesized by carbodiimide-mediated coupling. The characterization of the functional groups was done by HNMR spectroscopy. Critical micelle concentration of the copolymer was investigated by dynamic light scattering method. TEM micrographs of the micelles were obtained.

In vivo anti-tumor effect assay was carried out as following, mice bearing visible DLD-1 tumor were randomly divided into following groups: saline, CA-PEI, doxorubicin, D-CA-PEI, D-CA-PEI-S, D-CA-PEI-FA and D-CA-PEI-FA-S, and administrated intravenously on day 0 and day 7 at the dose of 8 mg/kg. Body weight and tumor volumes were monitored and recorded twice a week over a period of 20 days. Then the mice were sacrificed and tumors were excised.

RESULTS AND DISCUSSIONS

The FTIR spectra confirmed the presence of amide linkage between CA and PEI (Figure 1).

The CMC of CA-PEI molar feed ratio 3:1 was least of all (Figure 2). Molar ratio combinations having CA either more or less than 3:1 had higher values of CMC.
From the TEM micrographs the CA-PEI-FA micelles appeared as spherical moieties with diameter less than 200 nm (Figure 3). Occasionally, aggregates were seen of the CA-PEI micelles.

The *in vitro* release profiles of doxorubicin loaded CA-PEI micelles exhibited two phases of drug release (Figure 4). The first phase was the burst phase of 12 hours in which rapid release of drug took place. Later, the drug released in a sustained pattern.

The *in vivo* antitumor study on Nu/Nu nude mice revealed that the doxorubicin loaded CA-PEI-FA micelles exhibited better cytotoxic activity than blank doxorubicin (Figure 5).
CONCLUSION
The synthesis of doxorubicin incorporated novel CA-PEI-FA micelles has been reported for the first time. The conjugates readily form micelles and exhibit uniform spherical morphology as observed by TEM. Increasing quantity of incorporated doxorubicin decreases the release rate of the drug. Doxorubicin incorporated CA-PEI micelles have better in vivo antitumor activity compared to doxorubicin itself. On the contrary, when blank micelles were exposed to normal (V79) cells, they did not exhibit considerable toxicity. The collective results indicate the promise of doxorubicin incorporated CA-PEI-FA micelles as vehicles for antitumor drug targeting.

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Design and Evaluation of pH-Sensitive Bacterial Cellulose-g-P(Acrylic Acid) Hydrogel Microparticles for Oral Insulin Delivery

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INTRODUCTION
Insulin is administered parenterally; however, the pain, trauma, and discomfort associated with frequent injections often leads to low patient compliance. Patient compliance may best be obtained with safe and effective oral administration of the insulin but oral administration is challenged by chemical, enzymatic, and absorbance barriers in the gastrointestinal tract (GIT) [1]. Numerous strategies have been adapted to circumvent these challenges, such as the use of absorption enhancers, enzyme inhibitors, particulate drug delivery systems, mucoadhesive biopolymers, and formulations to protect drugs from the harsh environment of the GIT. Most of these strategies offer long-term possibilities for oral insulin delivery, but none of them has been proven to be successful for clinical application [1-2]. Hence, drug delivery scientists are still presented with the challenge of developing safe and effective oral delivery technique. In this prospect, stimuli-responsive mucoadhesive polymeric networks offer many features to overcome these challenges [1]. Therefore, this study aimed to develop and characterize bacterial cellulose-g-p(acrylic acid) hydrogels for oral insulin delivery.

EXPERIMENTAL METHODS
Hydrogels were synthesized by electron beam irradiation (at 35kGy) of three different ratios (20:80, 30:70 & 40:60) of acrylic acid (AA) and bacterial cellulose (BC). The resultant hydrogels were crushed and pulverized to produce BC-g-P(AA) hydrogel microparticles (MPs) in size range of 50-100µm. The hydrogel microparticles were characterized by nuclear magnetic resonance (NMR) spectroscopy, X-ray diffraction (XRD) and scanning electron microscope (SEM). The swelling of hydrogels MPs was determined at stomach and intestinal pH using simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 6.8).

Insulin was loaded into hydrogel MPs by swelling equilibrium method and entrapment efficiency was determined. In-vitro insulin release was determined in SGF and SIF. The enzyme inhibition activity of hydrogels was determined using Trypsin ELISA kit. Then, in-vitro and ex-vivo insulin degradation studies were performed in the presence and absence of hydrogel MPs. The ex-vivo mucoadhesion of hydrogels MPs was determined using everted gut model. For this purpose, stomach, intestine and colon section of Wistar rats were used.

The cell viability of hydrogel MPs was determined in Chinese hamster lung fibroblast (V-79), human colorectal adenocarcinoma (Caco-2) and methotrexate treated colorectal adenocarcinoma (HT29-MTX) cell lines using alamarBlue® assays. The ability of MPs to improve the paracellular transport was assessed by measuring the reduction in transepithelial electrical resistance (TEER) in Caco-2 and Caco-2/HT29-MTX cells Transwell® monolayers. Similarly, the insulin transport studies across Caco-2/HT29-MTX Transwell monolayers were performed and apparent permeability coefficient ($P_{app}$) of insulin was calculated in presence and absence of MPs.

Acute oral toxicity studies of BC-g-P(AA) hydrogel MPs were performed in male Wistar rats according to OECD guidelines and histological examination of organs were performed to observe any pathological changes. The hypoglycemic effect of the insulin loaded BC-g-P(AA) hydrogel MPs was determined in streptozotocin (STZ) induced diabetic rats. The insulin loaded MPs were administered orally (50IU/kg body weight) to overnight fasted rats (6 rats per group) and blood glucose levels were determined hourly using glucometer. Oral insulin solution (50IU/Kg) was administered to control negative group while subcutaneous (SC) insulin (5IU/kg) was injected to control positive group. The insulin concentration in plasma was determined in using insulin ELISA kit and pharmacokinetic parameters were calculated.

RESULTS AND DISCUSSION
The results suggested that MPs were successfully prepared. The NMR analysis of hydrogels MPs exhibited characteristic peak of BC and PAA confirming that the chemical structure of the hydrogels was not affected by mechanical processing during preparation of MPs. SEM analyses of the MPs showed highly porous, irregular shaped MPs were formed (Figure 1). The irregular shape of the MPs can enhance the mucoadhesion of MPs with intestinal cell wall. The MPs exhibited pH responsive swelling behaviour with lower swelling in SGF as compared to SIF. The swelling of hydrogels in SIF was higher as compared to disks form of same hydrogels, as reported earlier. The pH responsive swelling behaviour of hydrogel MPs can evade the pH-barriers faced by the insulin drugs in the GIT.
Insulin was efficiently loaded into MPs (upto 84% EE). The hydrogel MPs demonstrated pH responsive insulin release with limited release in SGF (<10%) and rapid higher release insulin in SIF (Figure 2). The BC-g-P(AA) hydrogels MPs demonstrated the ability to inhibit the enzymatic activity of Ca\(^{2+}\) dependent intestinal proteases (Trypsin and α-chymotrypsin) indicating potential of MPs to overcome enzymatic barriers. Consequently insulin underwent lower degradation, in enzymes solutions, in the presence of MPs as compared insulin without MPs. Hydrogel MPs exhibited excellent mucoadhesion to intestinal tissues suggesting that MPs can maintain close contact with intestinal mucosa and facilitates to reduce the permeability barrier of oral protein delivery.

Table 1: Apparent permeability coefficient (\(P_{app}\)) and absorption enhancement ratio for insulin across Caco-2/HT29-MTX co-culture (mean ± SD, n = 3, *\(p < 0.05\)).
Acute oral toxicity studies of BC-g-P(AA) hydrogel MPs in Wistar rats demonstrated that BC-g-P(AA) hydrogel MPs were biocompatible as no signs of toxicity were observed after oral administration of MPs (1g/kg/day). Moreover, no histopathological changes were observed in H&E stained tissue sections (Figure 3).

Finally, orally administered insulin-loaded MPs exhibited significant decrease in initial blood glucose level (upto 50%) as compared to orally administered insulin solution and blank MPs, as shown in Figure 4. Similarly, pharmacokinetic parameters suggested that relative bioavailability (BA_R%) of insulin from insulin loaded 208025 MPs (7.45%) and insulin loaded 307035 (6.98%) were much higher than oral insulin solution (0.64%). This higher bioavailability as compared to oral insulin solution suggested that BC-g-P(AA) hydrogel MPs assisted in enhancing bioavailability of orally administered insulin.

CONCLUSIONS
These findings allowed us to conclude that BC-g-P(AA) MPs hold potential to enhance oral bioavailability of the insulin due to their features to overcome GI barriers of protein delivery. However, the oral bioavailability of the insulin can be further improved by incorporating absorption enhancers in hydrogels.

REFERENCES
Synthesis of Cholic Acid-Polyethyleneimine-Polyarginine Triblock Polymeric Micelles for Targeted Delivery of Anticancer Drugs

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INTRODUCTION
Polymeric micelles (PM) are promising candidates to be hired for drug delivery because of their self-assembled small size under aqueous conditions and targeting properties based on the enhanced permeability and retention (EPR) effect. Micelle is a spherical nanoparticle with core and shell structure, in which the hydrophobic anticancer drugs can be encapsulated in the inner core hydrophobic region. The biomedical applications of micelles in drug delivery have been widely investigated in recent decades owing to its special characteristics in delivering insoluble drugs to specific target sites, however many problems yet to be solved such as instability issue upon administered into blood circulation, premature drug release and toxicity due to non-specific targeting [1]. Much attention had been given in modifying the structure and function of micelles to make them more desirable for drug delivery. Cholic acid (CA), is one of the bile acid in body that will naturally organizes into micelles above the critical micelle concentration (CMC). However, CA itself is insufficient to form an effective delivery system due to the poor transfection ability [2]. Polyethyleneimine (PEI) is a cationic polymer often employed in gene delivery because it has good transfection ability and shows strong potential for therapeutic gene delivery. Previous research also showed that the toxicity effect of PEI can be reduced by conjugating with a biocompatible material to form nanoparticles [3]. Polyarginine (pArg), a type of cell-penetrating peptide which is able to translocate through cell membranes and facilitate the uptake of molecules [4]. The combination of CA, PEI and pArg will compensate the weakness of each component by forming triblock copolymers which are able to form micellar delivery system to deliver anticancer drugs effectively into the tumor cells. In the present study, CA-PEI-pArg triblock copolymers were formulated and synthesized. The aim of this study is to demonstrate the formation of micelle based on the triblock copolymer synthesized for the future use of anticancer drugs delivery.

EXPERIMENTAL METHODS
Materials
CA, PEI (average MW approximately 1,300), PArg (average MW approximately 5,000), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), triethylamine and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). The Spectra/Por™ dialysis membrane (MW cutoff (MWCO) = 1,000 g/mol) was purchased from Spectrum Labs (Rancho Dominguez, CA, USA).

Synthesis of the CA-PEI-pArg copolymer
CA-PEI-pArg was prepared by a two-step reaction (Scheme 1). First, CA (100 mg) in methanol (10 ml) was activated with EDC (70 mg) and NHS (42 mg) at room temperature for 24 h. PEI (212 µl) was added drop-wise into the activated CA solution and stirred at room temperature overnight to form CA-PEI. The methanol was evaporated to dryness by using rotary evaporator. The formation was dialysed in 1kDa dialysis tubing against methanol and water (1:1, vol:vol) mixing solvent, dialysis against deionised water and dried by lyophilisation.

Second, 5, 10 and 15 mg of pArg (Set A, Set B and Set C) in 5 ml methanol were activated with EDC (5, 10, 15 mg) at room temperature for 24 h. CA-PEI in methanol (5 ml) was added drop-wise into pArg solution and the mixture was stirred at room temperature overnight. The methanol was evaporated by using rotary evaporator. The formation was dialysed against deionised water in 14 kDa dialysis bag for 24 hours and dried by lyophilisation.
Characterization of copolymer
CA-PEI-pArg copolymers were characterised by using a Fourier transform infrared (FTIR) spectrophotometer. The spectra of the copolymer was determined by KBr disc method and recorded over 4000 to 500 cm⁻¹.

Preparation and characterization of micelles
Micelles were prepared by direct dissolution where CA-PEI-pArg triblock polymer was mixed in 0.01M phosphate buffer and followed by filtration through 0.45 micron filter to obtain uniform sized micelles. Micelles were then characterised for size, zeta potential and morphology using zetasizer (Zetasizer Nano ZS, Malvern Instruments, UK) and transmission electron microscopy (TEM) respectively.

RESULTS AND DISCUSSION
Characterization of copolymer
In the FTIR spectra (Figure 1), peaks for the N-H stretch, C-H stretch, C=O stretch and N-H bending were appeared at absorbance of 3400 cm⁻¹, 2850 to 2900 cm⁻¹, 1646 cm⁻¹ and 1564 cm⁻¹, respectively. This indicating the formation of amide linkage between CA, PEI and pArg [5]. FTIR spectra from different sets of CA-PEI-pArg were showing consistent results indicating the amide linkage had formed in all the formulations.

Physicochemical Characteristics of CA-PEI-pArg Polymeric Micelles
CMC and zeta potential of the micelles were determined by using dynamic light scattering (DLS) method. CMC is the concentration at which the surfactants will self-assembled into micelles. It can be determined by recording the changes in light intensity across a series of diluted samples. Figure 2 shows that there is an abrupt increase in the light intensity at 1.49 x 10⁻⁷ M indicating the formation of micelles. PM should have CMC around 10⁻⁶ – 10⁻⁷ M, which is 1000 times lower than...
the CMC of other low-molecular-weight surfactants [6]. The low CMC of the PM may provide good stability in vivo because the micellar system will not be degraded in diluted condition [7]. Results from DLS also showed that all sets of micelles exhibited positive surface charges. The surface charge of Set A, Set B and Set C micelles are consistent, which are 64.3±0.4 mV, 66.0±1.3 mV and 57.9±0.7 mV respectively.

Figure 2 CMC of the CA-PEI-pArg polymeric micelles

Morphology of the CA-PEI-pArg micelles were determined by using TEM (Figure 3). TEM micrographs showed that the micelles showed spherical shape with consistent size range from 120 to 210 nm. Size was one of the most significant factors in the efficiency of PM. A PM should have size below 200nm. The small PM size, combined with the pathophysiological characteristics of the solid tumor tissues (hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors and absence of effective lymphatic drainage) would allowed the drug-loaded PM to be delivered passively into tumor interstitium due to EPR effect [7].

Figure 3 TEM micrographs of CA-PEI-pArg micelles. Scales are 100nm (a) and 200nm (b).

CONCLUSIONS
The CA-PEI-pArg triblock copolymer had successfully been designed and synthesized. The formation of the copolymer were confirmed by using FTIR. The copolymer readily formed micelles at an ideal molar concentration and showing a spherical morphology when observed with TEM. The size of the micelles were in the ideal range in order to achieve the EPR effect.

REFERENCES

ACKNOWLEDGMENT
Authors would like to thank Universiti Kebangsaan Malaysia (UKM) and Ministry of Higher Education for the financially supporting this project through research grants (ERGS/1/2013/SKK02/UKM/02/4).
Identification of Zingiber cassumunar Rhizomes using HPTLC and ATR-FTIR Combined with Chemometrics

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INTRODUCTION
For many years, the rhizome of Zingiber cassumunar (cassumunar ginger) has been used in traditional preparations to treat digestive problems like stomach ache, flatulence, diarrhoea and inflammatory conditions like ulcers and asthma. More recently, studies have reported that it also shows promising anti-microbial, anti-cancer and anti-oxidant properties. Recognizing its various potential usefulness, there is a need to ensure that the correct herb is identified before being processed or manufactured into a medicinal product. This is particularly important in cases where the raw material is sold in dried powder form, making visual identification difficult. Such quality control measures are in line with the national Economic Transformation Program (ETP), in which the production of high value herbal products have been designated as a project under the agricultural sector. The aim of this project is to improve the quality of herbal supplements and tap the global demand for high-value herbal remedies. Therefore, methods for identifying Z. cassumunar rhizomes using HPTLC and ATR-FTIR combined with chemometrics were developed to enable rapid and accurate authentication of raw herb material.

EXPERIMENTAL METHODS
Materials
Six Zingiber cassumunar samples comprising rhizomes were collected from six different locations (Table 1). The rhizomes were washed, dried and grounded into fine powder (Ethno Resources Sdn. Bhd., Sg. Buloh). For HPTLC analysis, three standard substances (Figure 1): curcumin (Sigma-Aldrich, USA), (3,4-dimethoxyphenyl)butadiene or DMPBD (isolated from previous research) and (3,4-dimethoxyphenyl)but-3-enyl acetate or DMPBA (isolated from previous research) were used as chemical markers while silica gel 60 F254 TLC plates 20mm x 10mm (Merck, Germany) were used as the stationary phase. All reagents were of analytical grade.

Table 1: Z. cassumunar rhizomes collected from various locations

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample Code</th>
<th>Sample Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARDI Kelantan</td>
<td>1</td>
<td>Reference*</td>
</tr>
<tr>
<td>Pahang</td>
<td>2</td>
<td>Commercial</td>
</tr>
<tr>
<td>Melaka</td>
<td>3</td>
<td>Commercial</td>
</tr>
<tr>
<td>Kuala Selangor</td>
<td>4</td>
<td>Commercial</td>
</tr>
<tr>
<td>Negeri Sembilan</td>
<td>5</td>
<td>Commercial</td>
</tr>
<tr>
<td>Indonesia</td>
<td>6</td>
<td>Commercial</td>
</tr>
</tbody>
</table>

*Sample 1 is an authentic sample from the Malaysian Agricultural Research & Development Institute (MARDI)

Figure 1: Chemical structures of curcumin, DMPBD and DMPBA
Extraction of marker compounds

Five grams of rhizome powder samples were extracted with 50ml absolute ethanol by reflux for 30 minutes. The extracts were filtered (Whatman No. 2 filter paper) and concentrated under vacuum at 50°C using a rotary evaporator (Büchi, Switzerland). The resulting extracts were dried and kept in glass vials at -20°C until further use.

HPTLC analysis

HPTLC analysis was carried out using a semi-automated TLC system (Camag, Switzerland) equipped with Linomat 5 autosampler (attached to a nitrogen tank), glass flat bottom twin trough chamber, Visualizer and Scanner 4, and controlled by WinCATS software. Dried extracts were dissolved in methanol to produce sample solutions of 1000 μg/ml. Standard solutions were prepared by dissolving curcumin, DMPBD and DMPBA in methanol to a final concentration of 1000 μg/ml.

Standards were applied in 3 different concentrations (2.5, 10 and 40 μg) for additional quantitative analysis to be carried out. Samples were applied as 10 μl, 8 mm bands. Plates were developed with chloroform:dichloromethane (32.5:67.5 v/v) in a chamber saturated with the mobile phase. After developing over a path of 80 mm, plates were dried in an oven at 60°C for 5 minutes. Evaluation and documentation of plates were conducted under UV 254 nm light.

ATR-FTIR analysis

ATR-FTIR analysis was carried out using a Perkin Elmer (USA) Spectrum 100 spectrometer equipped with universal ATR Diamond/ZnSe sampling accessory and controlled by Spectrum software. Crude powder samples were dried in an oven at 80°C and spectra were recorded from 4000 cm⁻¹ to 400 cm⁻¹ with 4 cm⁻¹ resolution and 16 scans. The background spectrum was recorded on air prior to sample analysis.

Multivariate analysis

Multivariate analysis was performed on ATR-FTIR spectral data using the Unscrambler X Version 10.0.1 software (CAMO, Norway). After pretreatment by SNV, samples were examined using principle component analysis (PCA) and hierarchical cluster analysis (HCA) across the fingerprint region of 2000-600 cm⁻¹.

RESULTS AND DISCUSSION

HPTLC analysis

HPTLC chromatogram of the standards and six samples are shown in Figure 2. Plates were visualized under UV 254 nm as it gave the best detection compared to UV 366 nm and visible light. The mobile phase used was in accordance with previously published literature that had optimized its ratio. Curcumin, one of the known compounds responsible for the yellow colour of the herb appeared at Rf 0.23. The presence of multiple bands indicated that its metabolites had likely affected the purity of the standard. DMPBD, a major constituent of the rhizome appeared near the solvent front at Rf 0.93 while DMPBA came up at Rf 0.18. Secondary bands could be seen at the highest concentration of DMPBD, again indicating lack of purity in the standard. All spots of interest were well separated from each other indicating that the method was selective and robust.

Figure 2: HPTLC chromatogram of 3 concentrations of standards (curcumin, DMPBD & DMPBA) and Z. cassumunar samples obtained from 6 different locations

ATR-FTIR analysis

ATR-FTIR spectra of the six crude samples are shown in Figure 3. All six samples gave similar spectral patterns but with varying levels of transmittance. In the fingerprint region of 2000-600 cm⁻¹, three prominent bands could be seen: Band 1 and Band 2 (~1620 cm⁻¹ and ~1510 cm⁻¹) are attributed to the stretching vibration of C=C in aromatic rings while Band 3 (~1020 cm⁻¹) is due to stretching vibration of C-O in esters. These findings are consistent with the HPTLC results which showed DMPBD and DMPBA as major components in the samples.
Multivariate analysis

Principal Component Analysis (PCA)
PCA is used as an exploratory tool to detect trends and pattern in the data. It is especially useful for spectroscopic data since it reduces the dimensionality of very complex data by extracting important information and eliminating unwanted noise. From the PCA scores plot (Figure 4), the samples were likely differentiated from one another based on their geographical location of growth. The first two principal components accounted for 90% variation in the data, indicative of a good model.

Hierarchical Cluster Analysis (HCA)
The main objective of HCA is to group data in natural clusters. Hierarchical clustering techniques are based on the creation of branched structures called dendrograms, which permit visualisation of correlations amongst samples. From the HCA dendrogram plot of crude Z. cassumunar rhizomes (Figure 5), there were three groups: the first comprised Samples 1, 2 and 4, the second Samples 3 and 5 while Sample 6 was in its own cluster possibly due to its origin from another country.
CONCLUSION

In this work, two different techniques of analysis were employed in the identification of *Z. cassumunar* rhizomes. Using HPTLC, both DMPBD and DMPBA were found to be reliable markers for identification of this herb. ATR-FTIR spectroscopy coupled with chemometric analysis was helpful in authenticating commercial samples by comparison with a reference sample. Both these methods could be used as effective tools for quality control of *Z. cassumunar* rhizomes.

REFERENCES


Stress Degradation Studies of a Prenylated Chalcone and Development of Validated Stability-Indicating RP-HPLC method

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INTRODUCTION
Excessive production of prostaglandin E₂ (PGE₂), a product of the cyclooxygenase (COX) pathway contributes to inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and pain as it triggers a vast array of biological signals and physiological events [1]. Therefore, discovery of potent inhibitors of PGE₂ synthesis is very crucial for the pharmaceutical industry. Dihydrochalcones, prenylated chalcones, prenylated flavones, aurones, and prenylated aurones are categorised into the group of minor flavonoids where these compounds possess many interesting biological activities [2]. For example, xanthohumol, a prenylated chalcone, was found to be active in a wide range of anti-inflammatory activities [3]. Similarly, (E)-3-(3,4-dimethoxyphenyl)-1-(2-hydroxy-4-methoxy-5-(3-methylbut-2-enyl)phenyl)prop-2-en-1-one, a novel prenylated chalcone has shown 90% inhibition on PGE₂ synthesis when tested using mouse macrophage (RAW 264.7) cell line, stimulated by lipopolysaccharide (LPS). Suppression upon PGE₂ secretion was not due to cell death as the compound did not reduce the cell viability in close proximity to the PGE₂ inhibition concentration. The compound physically appears as needle-yellow crystals with empirical formula C₂₃H₂₆O₅. Docking study showed that the compound engage in a number of crucial binding interactions in the active site of murine COX-2 which promote COX-2 inhibitory activity. The ¹H-NMR, ¹³C-NMR and ESI-HRMS spectra of the compound has been studied and was found to be consistent with the assigned structure. The compound has also been characterized using single crystal X-ray structural analysis [4]. Since the compound is a potential PGE₂ synthesis inhibitor and it has great prospect to be developed into an anti-inflammatory drug, evaluating the stability of the compound is highly crucial. Stress testing of the compound can aid in establishing the degradation pathway and the intrinsic stability of the molecule, thus validating the stability-indicating power of the analytical procedure used [5]. Therefore, this study will focus on proposing a validated selective and stability-indicating RP-HPLC method for this prenylated chalcone compound.

EXPERIMENTAL METHODS
Development of RP-HPLC method
RP-HPLC method will be developed by trying different mobile phases, buffer compositions and flow rates in order to obtain best separation of the compound.

Validation of RP-HPLC method according to ICH guideline
i) Linearity
Calibration curve will be generated using 5-concentration points where each concentration will be injected in triplicates.

ii) Precision
Intra-day and inter-day precision will be tested using three consecutive injections at three varying concentrations.

iii) Robustness
Effect of changing oven temperature, flow rate and buffer composition on the developed RP-HPLC method will be studied.

Stress degradation studies
The compound will be subjected to acid and base hydrolysis, oxidation and thermal stress. The parameters to be used for each degradation study will be adopted from literature review [5, 6, 7, 8] and applied on this compound. The parameters will be modifying according to response of the compound on forming degradation products. General parameters for each stress degradation studies are as below:

i) Acid and base hydrolysis
Compound will be subjected to HCl and NaOH concentration of 0.1N to 1.5N at room temperature for 2 to 48 hours.

ii) Oxidation
Compound will be subjected to 1% to 30% hydrogen peroxide at room temperature for 2 hours.

iii) Thermal stress
Compound will be subjected to oven temperature of 50°C to 100°C for 2 to 24 hours.
EXPECTED OUTCOMES

- RP-HPLC method which produces good separation of the compound at a retention time more than 5 minutes by using mobile phase ratio with percentage of water not exceeding 10%.
- $R^2$ value of more than 0.99 for linearity, %RSD of less than 1.5% for precision and changes in retention time and peak area less than 2% for robustness [8].
- Intrinsic stability of the compound when exposed to forced degradation studies and the formation of degradation products.

REFERENCES


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Special thanks to Dr. Lam Kok Wai, lecturers, lab assistants and friends who have contributed to this study.
Influence of Proton Pump Inhibitor on Helicobacter pylori Adherence to the Gastrointestinal Cell Line

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INTRODUCTION
The prevalence of Helicobacter pylori infection globally is more than 50% and it is closely tied to socioeconomic development [1]. It usually associated with asymptomatic and will further develop into peptic ulcer, gastric or duodenal ulcer. Therefore, it is a well known carcinogenic bacteria [2].

Proton pump inhibitor (PPI) with antibiotic is the standard regimen to eradicate H. pylori infection [3]. Recently this standard regimen showed decrease in recovery rate up to 70%. Besides, failure of eradication therapy was also due to antibiotic resistant to bacterial strain [4]. Another condition that may lead to failure in eradication therapy is due to reduced permeability of cell membrane to certain drugs, reduce binding of drug to intracellular target [5] and role of p-glycoprotein [6]. Since p-glycoprotein is well known to be multidrug resistance protein (MDRI), patients have a big problem to cure from illness and this may lead to poor H. pylori eradication therapy [7].

Understanding the effect of proton pump inhibitor as substrate, inhibitor or inducer to p-glycoprotein is very important to prevent further occurrence into gastric cancer. Therefore, this study will highlight the influence of proton pump inhibitor on H. pylori adherence to the human gastrointestinal cell line, Caco-2 (with p-glycoprotein) and LS174T (low level of p-glycoprotein) with and without rifampicin (P-glycoprotein inducer). In clinical study, the p-gp level will be possibly affected due to previous administered drug that are p-gp inhibitor, substrate or inducer. Similar implication will be applies in this study using animal model prior to giving a standard regimen therapy. This may raise the objective on pharmacokinetic effect of valsopodar and rifampicin as p-glycoprotein level in gerbil’s stomach and subsequently may increase understanding on standard regimen effect toward p-glycoprotein activity that has been disturbed by H. pylori.

EXPERIMENTAL METHODS

In vitro study
Two type of cell line derived from colon adenocacinoma (Caco-2 and LS174T) has been selected to determine p-glycoprotein expression before and after treatment using western blot analysis. LS174T cell will be growth further for 6 days to induced p-glycoprotein level.

The bacterial preparation will be done by scrapping the bacterial colonies into cold PBS prior to adding Bac Light green solution. The bacteria solution will be added with Hank Balanced Salt Solution (HBSS, Hepes and glucose) and Fetal Calf serum (FCS). This preparation also added with and without PSC-833 (Valsopodar). PSC-833 (Valsopodar) is potent p-glycoprotein inhibitor. Different timing will be applies on the plate for 4 hours (30, 60, 120, 150, 180, 210 and 240 minutes). The result will be analysed using Immunofluorescent, TECAN plate reader at 485nm excitation and 520nm emission [8].

Omeprazole (Prilosec) and Esomeprazole (Nexium) will be tested for viability of cell using MTT assay. Different concentration of drugs (0µM-20µM) will be used to determine the exact concentration that can allow 85% to 90% cell survival. Omeprazole and Esomeprazole was dissolved using solvent and 0.9% NaCl respectively. Caco-2 and LS174T cell line were growth overnight prior to giving a proton pump inhibitor treatment. DMSO will be added after 4 hours incubation. Shake the plate for 3 minutes to dissolve the formazan color before read the absorbance at 570nm [9].

Each cell line will be tested for p-glycoprotein expression before and after treatment using western blot analysis.

In vivo study
Moriones unguiculatus is the only rodent types that can induce peptic ulcer and cancer due to H. pylori infection. They will be maintained in 12 hour light and 12 hour night at room temperature. Then, each of gerbil will be dividing into 3 groups with low, normal and higher p-glycoprotein. Low and increase p-glycoprotein will be induced with PSC-833 (valsopodar) and rifampicin respectively. After 5 to 10 days, 10^8 colony forming unit of H. pylori will be induced in gerbils. Approximately 7 weeks of inoculation, ulcer will develop in gerbil stomach. Changes on the behavior support the result. H. pylori antigen testing through ELISA will be tested using stool collection. Positive detection of H. pylori antigen test will be injected with 4 types of treatment, which are 2 types of antibiotic, proton pump inhibitor alone and combination of antibiotic and proton pump inhibitor. One group will be excluded from any of treatment. After 14 days of treatment, each group will be sacrificed and stomach biopsy will be collected for western blot analysis, histological section and H. pylori culture for confirmation.
**Statistical analysis**

SPSS version 18 (SPSS Inc., Chicago, IL, USA) have been chosen using Independent t-test and ANOVA. P-value should be less than 0.05 to get the significant result.

**EXPECTED OUTCOMES**

1. Proton pump inhibitor is inhibitor and substrate to p-gp or it will gives direct inhibition on *H. pylori*
2. Longer incubation time may give severe *H. pylori* colonization on the cell line
3. P-glycoprotein inhibitor and inducer may lead to drug interaction in gerbil’s rat
4. 1 - 2 ulcers will develop due to *H. pylori* colonization in stomach
5. Proton pump inhibitor may increase the effectiveness of antibiotic treatment *in vivo*

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**ACKNOWLEDGEMENT**

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Extraction and Quantification of Shogoal and Gingerol from *Zingiber officinale* Rhizomes

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**INTRODUCTION**

*Zingiber officinale* or also known as the common household ginger are best known for their pungent smell and often used as an aromatic spice. Besides being used as flavorings, ginger also has many medicinal uses [1]. The fresh or dried rhizome (root) can be used in oral or topical preparations to treat a variety of ailments while the essential oil can be applied topically as an analgesic [2]. The extraction of *Zingiber officinale* will be carried out using a few method of extraction to determine which method can maximize the extraction of marker compounds. The compounds of interest that will be extracted and quantified are gingerol and shogaol. 6-gingerol is found abundant in ginger and has high bioactivity [3]. 6-shogaol is the dehydrated form of 6-gingerol and it is also known to have many bioactivity as well [4]. Since the method of extraction will influence the extract quality and quantity, three types of extraction will be done to determine the most optimum method [5]. Solvents with different polarity will be utilized for each extraction because the nature of extraction solvent affects strongly the extraction yields [6]. High performance liquid chromatography (HPLC) coupled with an ultraviolet detector has been developed as a method to quantify and identify gingerol and shogaol [7]. Therefore, the extracted compounds will be quantified using HPLC [8]. The retention time of the extract will be compared with the standards to determine the identity of the marker compounds. Validation will be done to ensure the method used is suitable for its intended purpose.

**EXPERIMENTAL METHODS**

(1) **Extraction**

Three type of extraction will be done on dry ginger at two different condition. Three type of solvent ranging from non-polar to polar will be used for each extraction.

- **Type of Extraction**
  - Cold extraction (room temperature)
    - i. Single extraction
    - ii. Sequential extraction
    - iii. Fractionation extraction
  - Hot extraction (60°C)
    - i. Single extraction
    - ii. Sequential extraction
    - iii. Fractionation extraction

- **Solvents**
  - Non-polar: n-hexane
  - Semi polar: ethyl acetate
  - Polar: ethanol

(2) **Data Analysis**

Data analysis will be done using high performance liquid chromatography.

**Method Validation**

Validation characteristics that will be considered according to the ICH Harmonised Tripartite Guideline [9] are:

- Linearity (R² ≥ 0.999)
- Accuracy (Recovery ≥ 80%)
- Precision (RSD ≤ 2.00)
- Robustness
- Detection Limit
- Quantitation Limit

**Quantification**

Extract will be analysed using HPLC and the marker compounds will be quantified using calibration curve prepared using standards of marker compounds. The quantity obtained will be used to determine which type of extraction has the capacity to optimize the extract produced.
REFERENCES


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Comparative Pharmacognostical Studies of the Leaves of Labisia pumila Varieties

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INTRODUCTION

Labisia pumila (Primulaceae) is locally known as ‘Kacip Fatimah’ and traditionally used among women in Malaysia to induce and facilitate childbirth, to help contract the birth channel and as a partum medicine [1]. Other uses are for dysentery, rheumatism, gonorrhea, elimination of excessive gas from the body, and ‘sickness in the bone’ [2]. The plant has been reported to possess antioxidant [3], antimicrobial [4], anti-aging [5], anti-inflammatory [6] and phytoestrogenic [7] properties. There are eight varieties of Labisia pumila [8], three of which are well known in Malaysia including L. pumila var. alata, L. pumila var. pumila and L. pumila var. lanceolata. The first two varieties have been commonly used in traditional preparations and reported in research articles, probably because these are more readily available than the lanceolata variety [9]. However, identification of the two varieties is often difficult due to only slight difference in the leaf and petiole characters. Thus, an efficient method of identification need to be established [10]. Based on our knowledge, there is a very little work on the pharmacognostical studies of these varieties except for L. pumila var. alata. Hence, the present work was carried out to investigate the three varieties of L. pumila based on the detailed pharmacognostical studies of the leaves, particularly morphological and anatomy microscopical characteristics, phytochemical screening, proximate analysis and determination of total phenolics and flavonoids content.

EXPERIMENTAL METHODS

Preparation of plant material

Three varieties of L. pumila were collected from the Bujang Melaka Forest Reserve, Kampar in August 2012. The voucher specimens of L. pumila var. alata (30006), L. pumila var. pumila (30007) and L. pumila var. lanceolata (30008) were deposited in the Herbarium of Universiti Kebangsaan Malaysia. The leaves were washed, air-dried under shade and then coarsely powdered by using a rotary grinder.

Morphological and anatomy microscopical characteristics

Fresh leaf materials (including petiole part) were fixed in AA (1:3) of acetic acid (30%) and ethanol (70%). Fixation, embedding, sectioning, epidermal mechanical scrapping and stained were done according to the procedure by [11] and [12], with suitable modifications.

Phytochemical analysis

The dried powdered leaves of L. pumila var. alata (200 g), L. pumila var. pumila (800 g), and L. pumila var. lanceolata (50 g) were successively macerated with DCM and MeOH respectively, then followed by reflux with water in ratio 1:10. All solvents used were analytical grade purchased from Merck (Germany).

The DCM, MeOH and water extracts were screened for presence of phytochemical content by using standard chemical tests for flavonoids, tannins, alkaloids, terpenoids, steroids and saponins [13,14]

Proximate analysis

Determination of percentage loss on drying and extractive values of water (100 mL) and ethanol (100 mL) extracts were determined for each 4 g of the dried powdered leaves of L. pumila by following the WHO guidelines [15].

Determination of total phenolics and flavonoids content

Total phenolics content (TPC) and total flavonoids content (TFC) of DCM and MeOH extracts of three varieties of L. pumila leaves were estimated by micromethod of Folin-Ciocalteu’s [16] and aluminum chloride (AlCl₃) colorimetry [17] with slight modification.

RESULTS AND DISCUSSION

Morphological and anatomy microscopical characteristic

The main characters that differentiated the three varieties were the leaf size, shape and petiole. The broad wing petiolate distinguished L. pumila var. alata from L. pumila var. pumila with marginate wing. Terete petiole (absence of wing) was observed in L. pumila var. lanceolata. The common characteristics in anatomical microscopic examination showed the presence of anisocytic stomata, scale, and capititate glandular trichomes in all varieties. However, L. pumila varieties also gave significant anatomical characters in terms of structure outline of midrib, petiole and lamina; types of stomata and trichome; organization of vascular system and pattern of anticlinal walls (Figures 1-2, Tables 1-2).
Based on previous studies, most of authors identified these characteristics to differentiate plant species. The type of trichome was used to characterize *Myrsine* species [18] According to [19], the shape and arrangement of the vascular system could be used in the differentiation of species in which these were proved to be useful for taxonomy [20]. Hence, the analysis of anatomy characteristic in this study might be potentially used to identify and differentiate these varieties.

**Figure 1** - Whole plant of *Labisia pumila*: (a) var. *alata*, (b) var. *pumila* and (c) var. *lanceolata*. Macroscopic characteristic of leaves of *L. pumila*: (d) var. *alata*, (e) var. *pumila* and (f) var. *lanceolata*.

**Table 1**: Anatomical characteristic of leaf lamina and margin of *Labisia pumila* varieties

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Main vascular bundle in the leaf lamina Location</th>
<th>Marginal outline</th>
<th>Marginal direction</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pumila</em> var. <em>alata</em></td>
<td>Close to the adaxial epidermis</td>
<td>Tapering</td>
<td>30-45° upwards</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>pumila</em></td>
<td>Equidistant to abaxial and adaxial epidermis</td>
<td>Rounded</td>
<td>10-30° downwards</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>lanceolata</em></td>
<td>Equidistant to abaxial and adaxial epidermis</td>
<td>Rounded</td>
<td>10-30° downwards</td>
</tr>
</tbody>
</table>

**Table 2**: Anatomical characteristic of leaf epidermis of *Labisia pumila* varieties

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Type of trichome</th>
<th>Type of stomata</th>
<th>Pattern of anticlinal walls</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pumila</em> var. <em>alata</em></td>
<td>Absent</td>
<td>Anisocytic, Staurocytic</td>
<td>Straight to curved</td>
</tr>
<tr>
<td></td>
<td>Scale, Capitate glandular</td>
<td></td>
<td>Straight to wavy</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>pumila</em></td>
<td>Simple, 2-armed, Scale</td>
<td>Anisocytic, Diacytic</td>
<td>Straight to curved</td>
</tr>
<tr>
<td></td>
<td>Scale</td>
<td></td>
<td>Straight to wavy</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>lanceolata</em></td>
<td>Absent</td>
<td>Anisocytic</td>
<td>Straight to wavy</td>
</tr>
<tr>
<td></td>
<td>Scale</td>
<td></td>
<td>Straight to wavy</td>
</tr>
</tbody>
</table>
Figure 2- Transverse section of midrib of *Labisia pumila*: (a) var. *alata*, (b) var. *pumila* and (c) var. *lanceolata*.

Figure 3- Transverse section of petiole of *Labisia pumila*: (a) var. *alata*, (b) var. *pumila* and (c) var. *lanceolata*.

**Proximate analysis**

Generally, the water-soluble extractive values in all varieties were higher than that of the ethanol soluble value; suggesting the varieties contained mainly polar components. The result showed no significant different among varieties. The recent monographic specification of *L. pumila* var. *alata* roots reported that the hot water and ethanol extractive values should not be less than 7% [21]. Lower extractive value than the specified limit could indicate presence of immature plant, exhausted material, adulteration, or incorrect processing during drying or storage [22].

*L. pumila* var. *pumila* leaves had the highest loss on drying compared to others two varieties (Table 3). The high value of loss on drying could be due to high content of volatile materials in plants [23], environmental factors, poor storage conditions, and inefficient drying method [24]. However, low moisture content is always desirable for higher stability of drugs [25], in order to preserve the plant material from possible deterioration as a result of either phytochemical degradation due to plant enzyme activity or microbial attack or infestation.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Ethanol-soluble extractive value</th>
<th>Water-soluble extractive value</th>
<th>Loss on drying</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pumila</em> var. <em>alata</em></td>
<td>4.49±0.71</td>
<td>8.50±0.71</td>
<td>9.50±0.04</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>pumila</em></td>
<td>4.98±0.02</td>
<td>8.50±0.71</td>
<td>10.45±0.04</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>lanceolata</em></td>
<td>3.99±0.01</td>
<td>9.00±2.83</td>
<td>8.46±0.06</td>
</tr>
</tbody>
</table>

**Phytochemical analysis**

Phytochemical screening (Table 4) revealed that DCM extracts of all *L. pumila* varieties contained steroids, while the MeOH and H$_2$O extracts showed the presence of flavonoids and tannins. We noticed two differences of phytochemical contents in the three varieties that is the presence or absence of terpenoids and saponins in MeOH and H$_2$O extracts. Terpenoids were absence only in *L. pumila* var. *lanceolata*. Meanwhile, the presence of saponin in H$_2$O fractions was only detected in *L. pumila* var. *alata*. Alkaloids were not detected in all extracts of the three varieties. According to [26], alkaloid was rarely found in family Myrsinaceae.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Extract</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>alata</em></td>
<td>DCM</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DCM</td>
<td>-</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>pumila</em></td>
<td>MeOH</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>+</td>
</tr>
<tr>
<td><em>L. pumila</em> var. <em>lanceolata</em></td>
<td>DCM</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>+</td>
</tr>
</tbody>
</table>

-absent  + present;

Flavonoid (1), Tannin (2), Alkaloid (3), Terpenoid (4), Steroid (5), Saponin (6)
Determination of total phenolics and flavonoid content

Generally, the results showed that MeOH extracts had high TPC while the DCM extracts had high TFC (Table 5). The TPC varied in the following order of L. pumila: var. lanceolata > var. pumila > var. alata; whereas the TFC had different order of L. pumila: var. pumila > var. alata > var. lanceolata. These results are consistent with previous studies [3,27].

Table 5: Total phenolics content (TPC) and total flavonoids content (TFC) of Labisia pumila varieties

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Fraction</th>
<th>TPC (mg GAE/g)*</th>
<th>TFC (mg QE/g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. pumila var. alata</td>
<td>MeOH</td>
<td>83.94±1.15</td>
<td>5.48±0.10</td>
</tr>
<tr>
<td>L. pumila var. pumila</td>
<td>DCM</td>
<td>79.50±3.54</td>
<td>115.04±1.28</td>
</tr>
<tr>
<td>L. pumila var. lanceolata</td>
<td>MeOH</td>
<td>121.17±2.99</td>
<td>9.19±0.04</td>
</tr>
<tr>
<td>L. pumila var. lanceolata</td>
<td>MeOH</td>
<td>57.38±1.59</td>
<td>96.98±1.67</td>
</tr>
</tbody>
</table>

*Results are presented as mean ± standard deviation (n = 3)

CONCLUSION

The comparative studies on the leaves of three varieties of L. pumila showed differences in morphological and anatomical microscopic characteristics, proximate analysis, as well as the contents of total phenolics and flavonoids, which could be used for proper identification and authentication of L. pumila varieties.

REFERENCES


ACKNOWLEDGMENTS
The authors are grateful to Ministry of Agriculture and Agro-Based Industry NRGS research grant (NH0711D002) and UKM Zamalah (2011/2012) for financial support.
Antibacterial Activity of Orthosiphon stamineus Benth & Ficus deltoidea Jack Against Selected Pathogenic Oral Bacteria

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INTRODUCTION

Oral health problems, particularly periodontal disease, dental caries and endodontic infections, are the most significant destructive processes in the oral cavity and costly burden to the public globally. Despite some improvements on the treatment modality, a high prevalence are still recorded due to the worldwide emergence of antibiotics resistance. There are also some controversial issues regarding the proper management procedure for these diseases which continuously be a significant challenge for society in general and healthcare professionals in particular due to various complications such as i) treatment cost, ii) dental care awareness among public and iii) difficulty in recognizing the relationship between pathological conditions, appropriate treatment for those conditions, and oral health status. Furthermore, with the treatment coverage that is still limited, any outbreak phenomena of changes in pathogenicity and virulence may result in serious burden in the near future [1][8]. It is of interest to explore and gain better understanding of mechanisms responsible for the development and progression of these diseases with a hope to develop effective future therapeutic and preventive strategies. Besides, integrated study of cellular and molecular level of both pathogens and its management is of fundamental importance to understand the basis in managing oral diseases. Noteworthy many studies are presently being conducted to know herbal plants (phytomedicine) of dental world and it may reflect the attempt to create a better dental treatment modality. This is due in part to the acknowledgement of the specialty of herbal plants and their identification that have been shown to have significant healing power, either in their natural state or as the source of new pharmaceuticals [7]. Orthosiphon stamineus, a valuable herbaceous woody plant that can be found in regions with tropics climate. It is a plant with a wide range of beneficial phytochemicals that have shown to improve various functions of the human body [5][6]. Meanwhile, Ficus deltoidea Jack or mistletoe fig is commonly known as Mas cotek is a type of herbal plant from the family Moraceae that also can be found in the South East Asia region. It has been proven to possess phytomedicinal abilities which significantly improve human body condition [3]. Scientific studies have found that both plants exhibit a dynamic ability as pharmaco-medicinal agents. However, there is still no information regarding their effect on the health and well-being of the oral cavity. Besides, answering these questions will also allow us to gain better insight of the significant contribution of Orthosiphon stamineus Benth and Ficus deltoidea Jack towards combating the selected oral pathogens. This later may pave the way to pharmaceutical applications in near sight and consequently resulting in the development of a novel preventive strategy to prevent the infections caused by these pathogens.

EXPERIMENTAL METHODS

Study Design

An in vitro study consists of viability tests which are the disc diffusion assay, the determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) and also the checkerboard and time kill assay to assess the viability of the bacterial cells upon treatment with the herbal extracts (essential oil and 80% ethanol). Besides, the physiological changes of the bacterial cells after exposure to the herbal extracts also will be observe through the scanning and transmission electron microscope (SEM and TEM respectively) and the anti-biofilm and anti-adhesion assays.

Data Collection

The OD readings from the MIC, anti-biofilm and anti-adhesion assays will be collected and the percentage of inhibition will be calculated by using specific formula. The photomicrograph from the SEM and TEM analysis also will be collected and analyzed visually.

Data Analysis

Collected data will be analyzed quantitatively and also qualitatively.

REFERENCES


ACKNOWLEDGEMENTS
Special thanks to NRGS research grant (NH0513D019) from the Ministry of Education and also to Prof Ibrahim, Dr Shahida, Dr Zamirah lecturers, DrugBio Research Group, staffs and friends that have contributed their help in this study.
Isolation of Major Phenolic Compounds, 6-gingerol and 6-shogaol from Zingiber officinale Roscoe by using Recycling Preparative HPLC

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INTRODUCTION

Ginger (Zingiber officinale Roscoe, Zingiberaceae) is widely used worldwide as a food, spice and herb. Ginger has found to possess many therapeutic benefits, it has been suggested particularly for the treatment of symptoms such as inflammation, sprains, muscular aches, cramps, constipation, hypertension, fever, infectious diseases and helminthiasis, as well as rheumatic and gastrointestinal final symptoms [1]. The fresh rhizome contains a rich source of biologically active constituents including the main pungent principles, gingerols, with 6-gingerol be the most abundant. Gingerols comprise a series of homologue substances differentiated by the length of their alkyl chains with [6]-, [8]- and [10]-gingerol having 10, 12 and 14 carbons in their unbranched alkyl chains, respectively [2]. [6]-Gingerol has been found to possess various beneficial pharmacological effects including anti-inflammatory, analgesic, antiplatelet, chemopreventive, angiogenesis, and antioxidant application [3, 4, 5]. 6-shogaol has many biological effects such as antibacterial, antifouling and anti-oxidative properties in vitro and in vivo [3, 6, 7]. This study presents new method for isolation and purification of 6-gingerol and 6-shogaol by using Recycling Preparative HPLC. It is simple, highly efficient, environmentally friendly, and has been demonstrated to be effective for preparation and purification of 6-gingerol and 6-shogaol from Zingiber officinale Roscoe.

EXPERIMENTAL METHODS

General experimental procedures

The following instruments were used: 1H-NMR and 13C-NMR spectra were measured with a Bruker Advance Model (300 MHz), JAI LC-9130G NEXT Recycling Preparative HPLC was used for purification step. The following adsorbents were used for purification: vacuum liquid chromatography with Merck Si-gel 60 (5-40μm, cat. no. 1.07747), column chromatography with Merck Si-gel 60 (40-63μm, cat. no. 1.09385), and TLC analysis with Merck Kieselgel 60 F254 0.25 mm (cat. no. 1.05554). All HPLC solvents and other solvents used were purchased from Merck by high purity. The reference standards of 6-gingerol and 6-shogaol were purchased from Chromadex.

Plant material

The rhizome of Z. officinale was collected from Kuala Krau, Temerloh, Pahang, Malaysia.

Extraction and Isolation.

The powdered dried rhizomes of Zingiber officinale Roscoe were extracted with ethanol. The ethanolic extract was further fractionated by different organic solvents: hexane, ethyl acetate and methanol. TLC screening and HPLC profiling of the fractions revealed the presence of 6-gingerol and 6-shogaol in hexane fraction and some 6-gingerol in ethyl acetate fraction. Hexane fraction (25g) was subjected to Vacuum-Liquid Chromatography (Silica gel, 135g; Mobile Phase, system solvents of n-hexane/EtOAc 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 25:75, 100% EtOAc, 50% MeOH and 100% MeOH) to give 48 fractions. Fractions containing 6-gingerol (FG) and fractions containing 6-shogaol (FS) were combined respectively. The Shogaol mixture and Gingerol mixture was fractionated by using Silica gel Column Chromatography with mobile phase of Hexane:EtOAc 9:1 and Hexane:EtOAc 75:25 respectively. The eluents containing the target compounds were then subjected to Reverse Phase C18 Column Chromatography with isocratic solvent system of MeOH:H2O 7:3 for 6-gingerol purification and MeOH:H2O 9:1 for 6-shogaol purification. The final purification step of the target compounds was done by using Recycling Preparative HPLC. For 6-shogaol purification, 100% MeOH with flow rate of 7.0ml/min. was used as mobile phase, whereas for 6-gingerol purification, 100% Acetonitrile with flow rate of 6.0ml/min. was used. Sample concentration and volume of 20 mg/ml and 3.0 ml was applied to the Recycling Preparative HPLC for the isolation of both compounds.

RESULTS AND DISCUSSIONS

This study presents isolation of 6-gingerol and 6-shogaol by using Reversed Phase C18 Column Chromatography as one of the purification step. Based on our study, it was found that Shogaol homologue substance, 6, 8 and 10-shogaol and Gingerol homologues, 6, 8, and 10-gingerol are best separated by using Reversed Phase Column Chromatography. This is due to they are only differs by the alkyl chain length.

The fractions obtained from Reversed Phase C18 Silica gel CC was further purified by using Recycling Preparative HPLC. 6-shogaol can be purified by using 100% Methanol as mobile phase, and flow rate of 7.0ml/min. By using this system, the retention time of 6-shogaol is about 22min. 6-shogaol can be purified up to 4 cycles, by draining of other compounds in the first cycle. After 2 cycles, compound which co-elute at nearly the same retention time as 6-shogaol will
be separated completely. By draining of the separated co-eluted compound, 6-shogaol can be collected. By applying this system, it was found that separation and purification of 6-shogaol by using Recycling Preparative HPLC takes about 100 minutes.

Isolation of 6-gingerol by using Recycling Preparative HPLC can be purified by using 100% Acetonitrile as mobile phase, and flow rate of 6.0 ml/min. The retention time of 6-gingerol is about 22min. The separation step may be done by first draining the minor neighboring compounds. The compound which co-elute at nearly the same retention time as 6-gingerol was allowed to separate during the first cycle and can be collected at the second cycle. Pure 6-gingerol can be obtained and collected at 5th cycle.

1H and 13C NMR Spectral data of both isolated compounds isolated are corresponds with literature [8].

6-gingerol (1) Yellow oil. C17H26O4. ESI-MS m/z: 293 [M-H]−. 1H NMR (600MHz, CDCl3): 0.89 (3H, t, J= 7.0 Hz, H-10), 1.24 – 1.51 (8H, m, H-6, H-7, H-8, H-9), 2.49 (1H, dd, J=17.5, 8.6 Hz, H-4), 2.57 (1H, dd, J=17.4, 3.3Hz, H-5), 2.74 (2H, m, H-2), 2.84 (2H,m, H-1), 3.87 (3H, s, -OCH3), 4.03 (1H, m, H-5), 6.61 (1H, dd, J=8.0, 2.0 Hz, H-6), 6.68 (1H, d, J=2.0Hz, H-2'), 6.83 (1H, d, J=8.0 Hz, H-5'). 13C NMR (150 MHz, CDCl3): 211.6, 146.4, 144.0, 132.7, 120.7, 114.40, 111.0, 67.7, 55.9, 49.4, 45.5, 36.4, 31.7, 29.3, 25.2, 22.6, 14.1.

6-Shogaol (2) Yellow oil. C17H24O3. ESI-MS m/z: 299 [M+Na]++. 1H NMR (600 MHz, CDCl3): 0.87 (3H, t, J=7.0Hz, H-10), 1.23-1.45 (6H,m, H-7, H-8, H-9), 2.15-2.19 (2H, m, H-6), 2.79-2.86 (4H, m, H-1, H-2), 3.80 (3H, s, -OCH3), 6.07 (1H, dt, J=16.1, 1.4 Hz, H-4), 6.66 (1H, dd, J=8, 2 Hz, H-6), 6.69 (1H, d, J=1.7Hz, H-2'), 6.80 (1H, d, J=5.9Hz, H-5'), 6.95 (1H, dt, J=15.8, 7.0Hz, H-5). 13C NMR (150 MHz, CDCl3): 200.0, 148.1, 146.6, 144.1, 133.43, 130.5, 121.0, 114.5, 111.3, 56.1, 42.2, 32.6, 31.5, 29.9, 28.0, 22.6, 14.1.

**Fig. 1 Structure of 6-gingerol and 6-Shogaol**

**Fig. 2 Recycling Preparative HPLC Chromatogram of 6-shogaol purification**
CONCLUSIONS
This study presents new Recycling HPLC method for purification of 6-gingerol and 6-shogaol from *Zingiber officinale* Roscoe. Isolation and purification of 6-gingerol and 6-shogaol from *Zingiber officinale* Roscoe can be achieved by repeated column chromatographic techniques including Reversed Phase C18 Column Chromatography and final purification by using Recycling Preparative HPLC.

REFERENCES

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Evaluation of Self Management Behavior of Chronic Kidney Disease Patients at UKMMC

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INTRODUCTION
Chronic Kidney Disease (CKD) has affected more than 20 million people in US. [1] In Malaysia, prevalence of End Stage Renal Failure (ESRF) patients on dialysis has increased from 325 per million population (pmp) in 2001 to 762 pmp in 2009. [2] CKD and ESRF are associated with many complications and co-morbidities which in turn have led to complicated management. [3] There is no cure for chronic kidney disease; instead management over time is essential. Self management strategies have been recommended to be important key components of a multifaceted treatment plan in managing patients with chronic kidney disease. [4] A qualitative research suggested that ESRF patients’ self management behavior may be related to their overall functioning and well being [5,6] A cross sectional study measuring self management of 372 patients on hemodialysis has suggested that the patients studied were low self managers. The study also showed that self care during hemodialysis was positively associated with physical functioning. [7] A prospective randomized controlled trial involving 54 patients with CKD stage 3-5 has shown that patients with self management interventions had higher absolute estimated glomerular filtration rate (eGFR) and lower hospitalization events. Higher absolute eGFR at the end of the study suggested a slowdown in the progression of CKD. [8] For effective treatment, patients are encouraged to involve actively in the management of their disease and good self management has been associated with positive outcomes. However, there is a lack of study on evaluation of self management behavior status of patients with CKD in Malaysia. Therefore, in this study, self management behavior of patients with different stages of CKD will be assessed and this background information can be useful for development of targeted self management interventions in the future.

EXPERIMENTAL METHODS

Study Design
A cross-sectional survey will be conducted in the nephrology outpatient clinic, UKMMC from March to May 2015.

Study Population
300 subjects will be recruited by convenience sampling.

Inclusion Criteria
I. Chronic kidney disease stage I to V
II. Diagnosed with for at least 12 months
III. Aged ≥ 18 years old

Exclusion Criteria
I. Mental illness/mental incapability
II. Not consented

Data Collection
Demographic characteristics such as age, gender, race, education level, marital status, employment, duration of illness and number of co-morbidities will be collected. Study outcome will be measured as self management behavior score. Partners in Health (PIH) Scale will be used to assess general self management behavior of CKD patients . [9]

Data Analysis
Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 22.0. Demographic data of patients will be presented as descriptive statistics of n (%) for categorical variables. Difference in categorical variables between CKD stage I to V will be analyzed using chi-square test. Continuous variables of demographic data will be tested for normality. If it is normally distributed, data will be presented as mean ± SD and difference in continuous variables between CKD stage I to V will be analyzed by using a one way ANOVA test. If it is not normally distributed, data will be presented as median (IQR) and Kruskal Wallis test will be used instead. The association of self management behavior and different CKD stages will be tested with a one way ANOVA if normally distributed. Otherwise, Kruskal Wallis test will be used. Pearson Correlation test will be used to analyze the correlation of self management behavior with duration of illness and number of co-morbidities if normally distributed. Otherwise, Spearman Correlation test will be used.

REFERENCES


Evaluation of Serum Biomarkers and Antioxidants of Isoproterenol-induced Myocardial Infarction Rats Pre-treated with Aqueous Extract of Labisia pumila

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INTRODUCTION
Myocardial infarction (MI) occurs as a result of imbalance between supply and demand of the blood to the heart. It may cause necrosis or death of tissue of myocardium due to lack of oxygen supply. Reperfusion of the blood flow to an ischemic region may further damage the cell that is far worse than ischemic alone [1,2]. Isoproterenol-induced MI rat is a common model used in pre-clinical stage since it is able to produce an infarct-like myocardial lesion in a high dose which is resemble to changes observed in human MI [3,4].

Several natural products have been listed to give cardioprotectant towards isoproterenol-induced MI. The effects are probably due to its antioxidant potentials and its ability to reduce the damage of the myocardiac muscle cell [5]. Thus, Labisia pumila was evaluated for its protective effects against isoproterenol induced MI in rats. Previous studies have identified several flavonoids and phenolics in L. pumila as well as alkyl resorcinols and saponins [6,7]. These secondary metabolites are believed to be responsible compounds for the antioxidant capacity and enzyme modulation activities [8].

EXPERIMENTAL METHODS

Plant sample and extraction
Dried and ground whole plant of Labisia pumila was purchased from a local supplier which was origin from Perak. The plant identification has been done by a botanist from Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM) and the voucher specimen has been deposited at Herbarium UKM (UKMB 30010).

Plant sample (1 kg) was refluxed with 10 L of distilled water for 3 hours. The extract was filtered, pooled and subjected to freeze drying to get powdered extract AqELP. This crude extract was kept at 4°C for further use.

Animals and ethics
Male, Wistar rats (150-200 g) were used for this study. The animals were housed at 25±2°C in a well-ventilated animal house under 12/12 h light and dark cycle. Animals were maintained under standard condition (humidity 60±10%), fed with conventional diet and water provided ad libitum. This study was carried out following an approval from the Universiti Kebangsaan Malaysia Animal Ethics Committee (FF/2012/IBRAHIM/23-MAY/433-MAY-2012-SEPTEMBER-2013).

Experimental design and protocols
Rats were divided into nine groups of six rats each.

- **Group I**: normal-control group.
- **Group II**: ISO-control group (received 85 mg/kg of ISO, s.c. on 29th and 30th day).
- **Group III**: rats treated with 10 mg/kg of propranolol, orally, for 28 days and received 85 mg/kg ISO, s.c. on 29th and 30th day.
- **Group IV**: rats treated with 100 mg/kg of AqELP orally, for 28 days and received 85 mg/kg of ISO, s.c. on 29th and 30th day.
- **Group V**: rats treated with 200 mg/kg of AqELP, orally, for 28 days and received 85 mg/kg of ISO, s.c. on 29th and 30th day.
- **Group VI**: rats treated with 400 mg/kg of AqELP, orally, for 28 days and received 85 mg/kg of ISO, s.c. on 29th and 30th day.

Extract AqELP (100 mg/mL) and propranolol (5 mg/mL) were dispersed homogenously in 2% Tween 20. Isoproterenol (10 mg/mL) was dissolved in water for injection and injected subcutaneously to the rats at interval of 24 h for 2 consecutive days. At 31th day, rats were sacrificed under mild diethyl ether anesthesia. Animals were made to fast 12 h before sacrificing. Blood was collected via abdominal aorta and allowed to clot for 1 h at room temperature. Serum was separated by centrifugation at 4000 rpm for 20 min and kept at -80°C for further biochemical analysis. Heart tissue was excised immediately and rinsed with cold-saline solution. The cardiac apex was dissected out and fixed in 10% formalin for histopathological examination. The remaining part was immediately kept at 80°C for further analysis.
Assays of serum biomarkers
Serum MI biomarkers was analyzed by measuring cardiac troponin I (cTnI), creatine kinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), aspartate dehydrogenase (AST) and alanine transaminase (ALT) using commercial standard assay kits.

Assays of antioxidant system
Analysis of serum antioxidants was performed by measuring the glutathione peroxide (GPx), catalase (Cat) and superoxide dismutase (SOD) levels on serum using commercial standard assay kits.

Histopathological examination
The formalin-fixed tissues were dehydrated in a series of graded alcohol and toluene followed by embedding in paraffin wax. The tissue was then cut into 4 μm thickness and stained with hematoxyline and eosin.

Statistical analysis
Results were analyzed statistically by using IBM SPSS Statistics 21. One-way ANOVA and Tukey’s test were carried out to statistically compare the data among the groups. The $p$-values less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION
Figure 1 shows a significant rise ($p<0.05$) of cTnI level on the serum of MI rats (control negative) which was about 12-fold of the normal rats. Pre-treatment with AqELP had successfully reduced ($p<0.05$) the release of cTnI in dose-dependent manner indicating a protection effect against the injury due to isoproterenol. As showing in Fig. 2, CK-MB level in normal group was considering low and more CK-MB released into circulation during the injured cardiac rising its serum level to nearly 6-fold in MI rats ($p<0.05$). Pretreatment with AqELP significantly ($p<0.05$) reduced the released of this enzyme dose-dependently. At dose 400 mg/kg, AqELP gave similar protecting effect to propranolol (10 mg/kg).

Table 1 shows a significant increase in the levels of all other diagnostic marker enzymes (LDH, AST and ALT) in the MI rats ($p<0.05$). Pre-treatment with AqELP prior the challenge showed significant ($p<0.05$) decreasing levels of all those enzymes dose-dependently as compare to ISO-induced group (negative control). At dose 400 mg/kg, AqELP reduced the released of AST to the level that comparable to the propranolol. The activities of enzyme antioxidants GPx, CAT and SOD in the serum of the normal, ISO-induced and experiment rats are shown in Table 2. Administration of isoproterenol causing significant ($p<0.05$) decrease in GPx, CAT and SOD level as compared to normal group. Beta-blocker propranolol
has significantly \((p<0.05)\) reduced oxidative stress in injured cardiac. Pretreatment with AqELP provided significant \((p<0.05)\) defense towards oxidative stress in dose-dependent pattern.

### Table 1. Effect of aqueous extract of *L. pumila* on serum cardiac marker enzymes

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>LDH (U/L)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>127.83 ± 13.52</td>
<td>51.40 ± 2.26</td>
<td>21.10 ± 0.73</td>
</tr>
<tr>
<td>Control negative ISO</td>
<td>215.11 ± 18.11</td>
<td>92.44 ± 4.22</td>
<td>55.91 ± 1.70</td>
</tr>
<tr>
<td>Propranolol 10mg/kg</td>
<td>157.10 ± 5.28</td>
<td>56.48 ± 0.67</td>
<td>18.46 ± 0.96</td>
</tr>
<tr>
<td>AqELP 100mg/kg</td>
<td>190.36 ± 1.08</td>
<td>71.14 ± 0.83</td>
<td>35.98 ± 0.49</td>
</tr>
<tr>
<td>AqELP 200mg/kg</td>
<td>185.44 ± 1.36</td>
<td>64.75 ± 1.52</td>
<td>28.22 ± 0.35</td>
</tr>
<tr>
<td>AqELP 400mg/kg</td>
<td>181.04 ± 1.10</td>
<td>59.01 ± 1.51</td>
<td>26.92 ± 1.26</td>
</tr>
</tbody>
</table>

*Significantly different to isoproterenol-induced (negative control) group at \(p<0.05\)

### Table 2. Effect of aqueous extract of *L. pumila* on antioxidant system

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>GPx (U/L)</th>
<th>CAT (nmol/min/mL)</th>
<th>SOD (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>81.69 ± 11.04</td>
<td>296.45 ± 15.60</td>
<td>290.37 ± 3.68</td>
</tr>
<tr>
<td>Control negative ISO</td>
<td>15.52 ± 3.37</td>
<td>63.63 ± 12.86</td>
<td>217.91 ± 8.91</td>
</tr>
<tr>
<td>Propranolol 10mg/kg</td>
<td>58.05 ± 5.78</td>
<td>240.33 ± 16.89</td>
<td>282.33 ± 1.42</td>
</tr>
<tr>
<td>AqELP 100mg/kg</td>
<td>22.81 ± 2.21</td>
<td>131.38 ± 2.99</td>
<td>238.42 ± 4.50</td>
</tr>
<tr>
<td>AqELP 200mg/kg</td>
<td>27.65 ± 0.40</td>
<td>176.41 ± 5.67</td>
<td>250.86 ± 4.11</td>
</tr>
<tr>
<td>AqELP 400mg/kg</td>
<td>30.22 ± 1.17</td>
<td>201.65 ± 9.10</td>
<td>272.16 ± 1.58</td>
</tr>
</tbody>
</table>

*Significantly different to isoproterenol-induced (negative control) group at \(p<0.05\)

Hispathological observations on cardiac tissues of control normal rats showed clear cell membrane integrity, normal structure of myofibrillar with striations, appearance of branched and continuity with adjacent myofibrils. No evidence of inflammatory cell infiltration, edema or inflammation itself. (Fig. 3A) However, the tissues of ISO-induced rats showed loss or blurring of striations following the necrosis of myofibrillar. Degenerated myofibrillar lost their nuclei due to infiltration of neutrophil granulocytes and macrophage causing an interstitial edema. (Fig. 3B). Pretreatments with AqELP (100, 200 and 400 mg/kg, respectively) have reduced the degree of necrosis and edema with obvious reduced infiltration of inflammatory cells. The improvements were observed to be in line with the given doses. At higher dose, AqELP shows better protections to the myofibrillar as compared to rats given a lower dose (Fig 3D-F). The same evidences were observed in tissues of propranolol group. (Fig. 3C)
Figure 3. Effect of AqELP on cardiac muscle histopathological changes, A: control (normal), B: ISO (negative control), C: propranolol 10mg/kg (positive control), D: AqELP 100mg/kg, E: AqELP 200mg/kg, F: AqELP 400mg/kg. Heart tissues (4 μm thickness) were stained with hematoxylin and eosin and visualized under light microscope at ×10 magnification.

CONCLUSION
In conclusions, pretreatment with *L. pumila* offers dose-dependent cardioprotective effects towards isoproterenol-induced myocardial infarction rats. Our study demonstrates that the effects are possibly derived from its antioxidant capacity which directly reduced the oxidative stress during the MI and augmented the myocardial antioxidant enzyme level while enhancing the permeability and integrity of myocardial cell membrane.

REFERENCES

ACKNOWLEDGEMENT
Authors thank Ministry of Agriculture of Malaysia for the financial support (NH0811S003) and Dr. Shamsul Khamis from IBS, UPM for the plant sample identification.
Venous Thromboembolism (VTE) Risk Assessment and Prophylaxis Modalities in Critically Ill Patients in Universiti Kebangsaan Malaysia Medical Centre

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INTRODUCTION
Venous thromboembolism (VTE) is a disease that includes both deep vein thrombosis (DVT) and pulmonary embolism (PE)[1]. VTE is seen commonly as a complication in critically ill patients, who are admitted to medical and surgical Intensive care unit (ICU), due to their premorbid conditions, trauma, surgical procedures and immobilization etc [2,3]. After myocardial infarction and stroke, VTE is the third most common cardiovascular disease[4]. The overall rate of VTE is 100 per 100,000 population per year, of which 70% are hospital-acquired[5]. It has long been thought that VTE is uncommon in Asia. However, most of the epidemiological studies that are conducted recently seem to refute this perception. The occurrence rate of VTE after surgery, stroke, medically ill and critical care patients is almost similar to the rate of VTE that occurs in western countries[6]. In Malaysia limited studies were published in critically ill patients. Thromboprophylaxis rates of critically ill patients in Malaysia are not well studied as compared to western countries. Knowing the association of VTE risk factors in critically ill patients may provide useful information for the healthcare provider whether to initiate VTE prophylaxis in critically ill patients or not in order to minimize the risk of mortality and hospital stay. The aim is to assess the risk of VTE and prophylaxis modalities in critically ill patients.

EXPERIMENTAL METHODS
Study Design
A cross-sectional study will be conducted in intensive care unit in critically ill patients, UKMMC from March to June 2015.

Study Population
80 subjects will be recruited by convenience sampling. The inclusion and exclusion criteria will be as below.

Inclusion Criteria
i. Patient ≥ 18 years old.
ii. Patient admitted for a minimum of 2 days at ICU.

Exclusion Criteria
i. Patient is admitted solely for treatment of VTE.
ii. Patient is transferred to the other ward.

Data Collection
A structured proforma is designed for VTE risk assessment in critically ill patients using a previously published standard protocol [7] (Caprini’s risk stratification score card) and bleeding risk assessment proforma based on Malaysian guidelines to assess patient’s bleeding risk[1]. This proforma will be marked by the researcher by looking at the patient’s record who is still admitted in ICU.

VTE total risk factor score and risk level calculation will be based on Caprini’s risk assessment score card while protocol evaluation for prophylaxis regimen will be based on UKMMC GICU anticoagulant prescribing guide.

Data Analysis
Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 21.0. Demographic data, determination of common VTE risk factors and risk of bleeding will be analyzed using descriptive analysis. The type of statistical tests used will be based on the objectives as shown below:

i) Chi-square test or independent T test will be used to evaluate comparison between gender and risk level of VTE.
ii) Pearson correlation will be used to determine an association between risk level of VTE and age.
iii) Pearson correlation will be used to determine an association between risk of bleeding and prophylactic regimen.
POTENTIAL APPLICATIONS
As most of the critically ill patients are at high or very high risk of VTE, so identification of patients at risk and the implementation of appropriate preventive protocols can be enhanced. This study will help to reduce the occurrence of VTE in the patients by early recognition of risks and reduce the hospital stay, thus reducing the cost as well. This study will also focus on patients whether they are a good candidates for VTE prophylaxis or not as risk of bleeding in ICU patients is usually very high.

REFERENCES

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Special thanks to Dr. Adyani Md Redzuan, co-supervisors and friends that have contributed their help in this study.
Isolation and Structure Elucidation of Bioactive Compounds from F. aurantiaca

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INTRODUCTION

Ficus (Moraceae) comprise one of the largest genera of angiosperms [3] with more than 800 species of trees, shrubs, hemi-epiphytes and climbers in the tropics and sub tropics worldwide. The genus is an important genetic resource due to its high economic and nutritional value and also an important part of the biodiversity in the rainforest ecosystem. It is also a good source of food for fruit eating animals in tropical area. Chemical investigations of various Ficus species have shown the present of flavonoids, coumarins, alkaloids, steroids, triterpenes, simple phenols and salicylic acids from F. bengalensis, F. carica, F. hirta Vahl, F. hispida L.f., F. microcarpa, F. nymphaefolia Mill, F. ruficulis Merr and F. septic [13], [16]. Some of these plants exhibited a wide range of biological activities such as anti-inflammatory [8], [13], [14], antioxidant, hypolipidemic and hypoglycemic activities [17]. Some Ficus species are well known in Asia as medicinal plants and are widely used in folk medicines for the treatment of flu, malaria, tonsillitis, bronchitis and rheumatism [1].

Ficus aurantiaca Griff is an evergreen tree that can grow up to 9 metres. It is widespread in the lowland forests in Kelantan, Terengganu, Perak, Pahang, Melaka and Johor. In Malaysia, F. aurantiaca is locally known as tengkuk biawak or akar tengkuk biawak hitam. Traditionally, the plant parts have been used for the treatment of headache, wound and toothache [7].

Xanthine oxidase (XO) serves as a significant biological foundation of oxygen-derived free radicals that give to oxidative break to living tissues which are implicated in many pathological processes such as inflammation, atherosclerosis, cancer and aging [3]. This is the enzyme which is responsible for the formation of uric acid from the purines, hypoxanthine and xanthine. It is accountable for the medical condition known as gout due to over-activity of this enzyme. One of the most universal metabolic problem which distressing to human is Gout 5-lipoxygenase enzyme (5-lox), 12-lipoxygenase enzyme (12-lox) and 15-lipoxygenase enzyme (15-lox) are the members of a family of enzymes called Lipoxygenase. The catalyzation of 5-lipoxygenase (5-lox) synthesizes the fatty acid signal molecules leukotrienes from arachidonic acid in the cell. The over production of leukotrienes causes the reductions in the soft muscle’s inside layer which is the source of inflammation like asthma and allergic diseases [20].

EXPERIMENTAL METHODS

Plant Material

Ficus aurantiaca was collected from Banting, Selangor, and a voucher specimen (SM 2109) was deposited at the Herbarium of University Kebangsaan Malaysia (UKM), Bangi.

Extraction and Isolation

Extraction and isolation were done by modification of the method described by Rukachaisirikul [13]. The air-dried stem (196 g) of F. aurantiaca was ground and the powder was sequentially extracted thrice with n-hexane, ethyl acetate and methanol by soaking at least 48 hours at room temperature for each time. The resultant extracts were evaporated to dryness in vacuo using rotary evaporator to yield crude extracts of hexane (7.8 g), ethyl acetate (4.0 g) and methanol (10.0 g) respectively. All the crude extracts were screened for bioactivity. Based on the activity of crudes, F. aurantiaca was further investigated for pure isolates. The separation of hexane, ethyl acetate and methanol extracts were done by using vacuum liquid chromatography, column chromatography and chromatotron over silica gel respectively. Total ten compounds were isolated from three extracts of stem of F. aurantiaca. Compound 4 and 5 were obtained from hexane extract while compound 1, 2, 3, 6 and 7 were isolated from ethyl acetate extract. Compound 8, 9 and 10 were isolated from the methanol extract. The structure elucidation of isolated compounds was performed using UV, IR, ESIMS and NMR spectroscopy data.

Xanthine oxidase inhibition assay

All of the extracts were investigated for in vitro xanthine oxidase inhibitory activity and the assay was run spectrophotometrically in an aerobic environment [13]. Allopurinol which is a recognized inhibitor of XO has been used as a positive control.

Soybean lipoxygenase inhibition assay

Soybean lipoxygenase (SBL) inhibition was evaluated spectrophotometrically with the measurement of change in the absorbance at 234 nm from linoleic acid to hydroperoxylinoleic acid following the published method by Malterud and
Rydland [11]. Phenidone was used as a standard soybean lipoxigenase (SBL) inhibitor.

RESULTS AND DISCUSSION

Compound 1 (28-dihydroxy, 30-hydroxy lupeol)

Compound 1 was isolated as white powder (20.0 mg), melting point 222-224\(^\circ\) C, molecular weight \(M=474.716\) with molecular formula \(C_{30}H_{50}O_4\). Based on 1D and 2D NMR spectral data and compare with lupeol, compound 1 was identified as 28-dihydroxy, 30-hydroxy lupeol. It is isolated first time as trihydroxy lupeol among other hydroxy derivatives of lupeol in natural products.

Compound 2 (taraxerone)

Compound 2 was isolated as white powder (50 mg), m. p. 239-240\(^\circ\) C, molecular weight \(M=424\) with molecular formula \(C_{30}H_{48}O\). Based on 1D and 2D NMR spectral data and compare with published literature [6], compound 2 is identified as taraxerone. Teraxerol significantly inhibited growth of the human lung cancer cell line H157 and exhibited visible antibacterial activity against the bacteria \emph{S. aureus, E. feacalis} and \emph{E. coli} [3].

Compound 3 (taraxerol)

Compound 3 was isolated as white powder (26 mg) with m. p. 279-280\(^\circ\) C, molecular weight \(M=426\) corresponded to molecular formula \(C_{30}H_{50}O\). Based on the above spectral data, and comparison of these data with those of reported data [6], compound 3 is identified as taraxerol. Terraxerol significantly inhibited growth of the human lung cancer cell line H157 and exhibited visible antibacterial activity against the bacteria \emph{S. aureus, E. feacalis} and \emph{E. coli} [3].

Compound 4 (ethyl palmitate)

Compound 4 was isolated as sticky Pale yellow (36 mg) with m. p. 40-43\(^\circ\) C (literature [4], 42\(^\circ\) C). The molecular weight is \(M=284.27\) with molecular formula \(C_{18}H_{36}O_2\). Based on the NMR spectral data of 4 and comparing those with literature [3], compound 4 is identified as ethyl palmitate.

Compound 5 (Herniarin)

Compound 5 was isolated as light pink crystal (120 mg), m. p. 120-122\(^\circ\) C (literature, 120-121\(^\circ\) C, [10]). Molecular formula is \(C_{30}H_{50}O\) with molecular weight \(M=176.04\). Based on the 1H and 13C NMR spectral data of 5, comparing those to published data [10], the structure of 5 is characterized as herniarin.

Compound 6 (3, 26-hydroxy,21-dihydroxy lanostanoic acid)

Compound 6 was isolated as white powder (27.2 mg) with m. p. 150-155\(^\circ\) C. Molecular formula is \(C_{30}H_{50}O\) with the molecular weight \(M=502.32\). Comparing the NMR data of 6, with those of other lanostanoic acid [16], compound 6 is identified as 3, 26-hydroxy, 21-dihydroxy lanostanoic acid. This is first time isolated as trihydroxy lanostanoic acid among other hydroxy derivatives of lanostanoic acids in natural products.

Compound 7 (stigma sterol)

Compound 7 was isolated as white powder (17 mg) with a melting point 168\(^\circ\) C (167-170\(^\circ\) C [19]). The molecular formula is \(C_{29}H_{48}O\) with molecular weight \(M=412.37\). Based on the 13C and 1H and other spectral data and comparing those to published data [5], compound 7 is identified as stigma sterol. The steroid is extensively distributed in many plants. In the genus \emph{Ficus} stigmasterol has been reported previously from \emph{Ficus carica}.

Compound 8 (ursolic acid)

Compound 8 was isolated as white amorphous powder (32.3 mg), with a melting point 282-284\(^\circ\) C (283-285\(^\circ\) C, [19]). The molecular formula is \(C_{30}H_{48}O_3\) with molecular weight \(M=456.36\). Based on the spectral data and comparing the NMR data with those of published data [19], compound 8 is identified as ursolic acid.

Compound 9 (dehydroxy betulinic acid)

Compound 9 was isolated as white powder (17.4 mg) with a melting point 285-290\(^\circ\) C. The molecular weight is \([M+]=440.365\) corresponding to molecular formula \(C_{30}H_{48}O_2\). The 1D and 2D NMR spectral data indicated that compound 9 is a pentacyclic lupeol type triterpenoic acid without a secondary hydroxyl bearing carbinol carbon at (C-3) position. This is the first report of this compound along with other lupeol type triterpenoic acid (for example, betulinic acid) in natural products.
Compound 10
Compound 10 was isolated as white powder with the molecular weight M=498, corresponded to molecular formula C\textsubscript{32}H\textsubscript{50}O\textsubscript{4}. Based on NMR spectral data and compare with literature [19], Compound 10 is identified as acetyl ursolic acid.

Biological activity of isolated compounds

Xanthine oxidase inhibition assay
Xanthine oxidase inhibitory activity (% of inhibition) of active compounds are shown in Figure 1.

Lipoxygenase inhibition assay
5-lipoxygenase inhibitory activity (% of inhibition) of active compounds are shown in Figure 2.
CONCLUSION
Compounds 1, 4, 5, 6, 7, 8 and 9 strongly inhibited both xanthine oxidase and lipoxygenase enzyme compared with compound 2 and 3 while 10 did show any activity.

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ACKNOWLEDGEMENTS

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**In Vitro** Inhibitory Effects of Isolated Compounds from The Extracts of *Artocarpus* sp. on Prostaglandin E\(_2\) and Thromboxane B\(_2\) Production and Platelet Activating Factor Receptor Binding

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**INTRODUCTION**

Thousands of plants in Malaysia have been used for medicinal purposes included *Artocarpus* species. The knowledge from folk about the medicinal uses of *Artocarpus* species could lead to discover new therapeutic drugs [1]. In recent years, several studies have produced evidence of the biological properties from the bark of *Artocarpus* species such as anti-inflammatory [2], anti-malarial [3] and anti-bacterial [4].

Prostaglandin E\(_2\) (PGE\(_2\)) is a metabolite of AA through the cyclooxygenase-2 (COX-2) pathway. PGE\(_2\) has an established role in causing inflammation and also contributes to other functions such as febrile responses, vasodilation and alteration of microvascular permeability [5]. Thromboxane A\(_2\) (TXA\(_2\)) is produced by activated platelets [6]. TXA\(_2\) is unstable and rapidly hydrolyzed into almost inactive, stable and measurable metabolite thromboxane B\(_2\) (TXB\(_2\)) [7]. The determination of serum TXB\(_2\) production by platelets following blood coagulation is a specific method for evaluation of COX-1 activity in human and other species [6, 8]. The radioimmunoassay technique (RIA) is the common method to quantify PGE\(_2\) and TXB\(_2\) and thus will be used in this study since it has the ability to detect and quantify the antigen-antibody interaction.

Platelet activating factor (PAF) is made up of a pair of phospholipids produced by pro-inflammatory cells like endothelia cells, platelets, macrophages and monocytes [9]. PAF involves in several pathophysiological conditions such as inflammation [10], allergy [11], asthma [12] and thrombosis [13]. It may also influence the pharmacological actions, such as the induction of platelet and neutrophil aggregation, bronchoconstriction, hypotension and increased vascular permeability [14].

The present study is aimed at investigating the inhibitory effect of isolated compounds from the bark of *Artocarpus* species, namely *A. lowii*, *A. anisophyllus* and *A. scortechinii* on PGE\(_2\) and TXB\(_2\) production and PAF receptor binding. The bioactive compounds that contribute to the inhibitory effects will be identified. Knowledge of these compounds could did the discovery of new modern therapeutic agents.

**EXPERIMENTAL METHODS**

**Isolated Compounds**

The isolated compound from the extracts of *Artocarpus* species such as *A. lowii*, *A. anisophyllus* and *A. scortechinii* will be provided by researcher from Universiti Teknologi Malaysia (UTM).

**Radioimmunoassay for PGE\(_2\)**

Venous blood will be obtained in polypropylene tube containing 10% (v/v) of 2% EDTA by aseptic vein puncture from healthy human volunteers who fulfilled the following inclusion criteria: non-smoker fasted overnight and did not take any medicine or supplements within the last two weeks. One mL of blood will be incubated at 37 °C for 24 h with 10 µL of LPS and 10 µL of serial dilutions of each compound in DMSO and ethanol (1:1 ratio) (1.25–10 µg/mL) or control. DMSO and ethanol (1:1 ratio) as a negative control and indomethacin, as a positive control. After incubation, the blood will be centrifuged at 2,600 × g for 15 min at 4 °C to separate the plasma. The reaction mixtures consisted of 100 µL of plasma, 100 µL of anti-PGE\(_2\) and 100 µL of [3H]-PGE\(_2\) will be incubated at 4 °C for 18–24 h. After incubation, the mixtures will be added with 200 µL of dextran charcoal and will be incubated again for 10 min. After centrifugation at 3,000 × g for 15 min at 4 °C, 3 mL of liquid scintillation cocktail will be added to 300 µL of supernatant. The radioactivity will be measured by a liquid scintillation counter.

**Radioimmunoassay for TXB\(_2\)**

TXB\(_2\) assay will be carried out with the same method used to quantify PGE\(_2\).

**PAF Receptor Binding Inhibitory Assay**

The assay will be carried out whereby 200 µL washed rabbit platelet suspension will be mixed with 25 µL H-PAF (with or without unlabeled PAF) and followed by 25 µL samples or control. These reaction mixtures will be incubated at room temperature for 1 hour. The free and bound ligands will be separated by using a glass microfiber filter in cell harvester. The radioactivity will be measured by a scintillation counter. The difference between total amounts of H-PAF bound in the
absence and in the presence of excess unlabeled PAF is defined as specific binding H-PAF. The IC\textsubscript{50} values of the samples will be determined (Moharam et al., 2010)

**Structure-activity Relationship Study to Identify Lead Compounds**

Structural features of the most active compounds that are important for anti-inflammatory activity will be determined.

**Data Analysis**

Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) software Version 17. Each sample will be measured in triplicate and the data will be presented as means ± standard deviation (SD). Data will be analyzed using one way ANOVA. A P value of <0.05 will be considered statistically significant.

**EXPECTED OUTCOMES**

Any compounds from the bark of several *Artocarpus* species, namely *A. lowii*, *A. anisophyllus* and *A. scortechinii* that shown the inhibitory effects on PGE\textsubscript{2} and TXB\textsubscript{2} production in human whole blood and displacement of \textsuperscript{3}H-PAF-specific binding in washed rabbit platelets will be observed. Thus, the bioactive compounds that contribute to the inhibitory effects will be determined.

**REFERENCES**


**ACKNOWLEDGEMENTS**

Special thanks to Dr. Juriyati, lecturers and friends that have contributed their help in this study.
Effect of Poloxamer 188 on PLGA Nanoparticle Size: An Acoustic Spectroscopic Analysis

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INTRODUCTION
Despite enormous advances in brain research, brain and central nervous system disorders remain the world's leading cause of disability. The major problem in drug delivery to brain is the presence of the blood-brain barrier (BBB). Overcoming the difficulty of delivering therapeutic agents to specific regions of the brain presents a major challenge to treatment of most brain disorders. Nanoscale drug delivery systems that have been developed include liposomes and nanoparticles. Nanoparticles are solid colloidal particles ranging in size from about 10 nm to 1000 nm. The major goal in designing nanoparticles as a delivery system is to control particle size in order to achieve the site-specific action of drugs at therapeutically optimal concentrations [1,2]. There are many factors contributing to the size of the nanoparticles. One of them is the systematic aggregation of the surfactant and the nanoparticle there by increasing their size. In this pretext acoustic spectroscopy based attenuation measurement is preferable for the characterization of particle size distribution even without the requirement of dilution [3]. The aim of this work was to study the extent of aggregation and the subsequent effect on PLGA nanoparticles size and distribution because of the presence of poloxamer 188 at different concentrations using acoustic spectroscopy.

EXPERIMENTAL METHODS
PLGA nanoparticles were prepared by water/oil/water double emulsion solvent evaporation method [4]. After 72 hours storage in freeze dryer, the lyophilized nanoparticles were dispersed in poloxamer 188 solutions at concentrations ranging from (S1) 0.1% to (S10) 1.0% respectively. The nanoparticles are also dispersed in water (S0) for comparison. The systems were analyzed at attenuation mode being stirred by ElectroAcoustic Spectrometer (DT1200) Dispersion Technology Inc. (USA). The particle size distribution was obtained with weight basis and as well cumulative weight basis plots.

RESULTS AND DISCUSSION
In our study the nanoparticle size is seen (Figure 1&2) to shift from nanometer to micrometer even in the presence of the lowest studied concentration of poloxamer 188 as compared with PLGA nanoparticles (27.92 nm) dispersed in water. The variations in attenuation spectra (Figure 3) is in agreement with the fact that, increase in concentration of poloxamer 188 from 0.1% to 1.0% (w/v), increased the extent of aggregation between the nanoparticles and the former, hence enlarging the overall size. Surfactant plays an important role in the formation of polymeric nanoparticles and to control the size of the nanoparticles. The results obtained in the present study suggest that the concentration of surfactant must be adjusted to an optimum concentration so to retain the particle size distribution in the nanometer range.
CONCLUSION
The results obtained in the present study suggest that Poloxamer 188 can significantly modify the size of the prepared PLGA nanoparticles.

REFERENCES

ACKNOWLEDGEMENTS
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Sonication Aided, Cinnamaldehyde Containing Microemulsion: Attenuation Spectra Based Dispersion Stability Analysis.

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INTRODUCTION
Cinnamaldehyde has been investigated extensively for its utility as an antimicrobial agent [1]. Its lipophilic characteristic has instigated many to study, the microemulsion prospective so as to produce better activity[2]. High speed homogenization is one of the method to produce microemulsion with suitable surfactant system. Sometimes high speed homogenization alone is not sufficient to produce a stable microemulsion. In this pretext sonication with definite power and exposure time ensures better emulsification. In this study we investigated trans-cinnamaldehyde containing microemulsion and its stability[3], on the basis of attenuation spectra based dispersion dynamics using an acoustic spectrometer. Attenuation spectra is used to characterize the colloidal systems using acoustic spectrometer[4]. It has been observed that attenuation spectra is indicative of the inherent instability within the system.

EXPERIMENTAL METHODS
Samples
Trans-Cinnamaldehyde 99%, Tween 80, Tween 20 and sunflower oil were purchased from sigma (Sigma–Aldrich Chemical Co., Steinh-ein,Germany).

Microemulsion preparation
On the basis of previous literature[5], a composition (0.5% Tween 80, 0.5% Tween 20, 10% oil phase and 89% aqueous phase) for 100 ml has been subjected to six different speed of homogenization (1,000, 2,000, 4,000, 6,000, 8,000 and 10,000) for a duration of 5 minutes and are denoted as F1 to F6 respectively. The preparations F4, F5 and F6 were further subjected to stability analysis as the initial preparations are separated after one hour.

On the basis of one month stability analysis, the formulation F6 has been improved with the exposure of sonication duration as the variable (20s, 40s, 60s, 90s, 120s, 180s, 240s, 360s, 480s and 600s respectively) with 120 watt power as a constant and formulations were denoted as F6-1 to F6-10[4]. On the basis of fitting error (not presented here) and the particle size distribution (PSD) value is further F6-8 was selected to be the optimum formulation and 4% of trans-cinnamaldehyde (F6-8C) was incorporated within the oil phase and the final formulation was prepared, which was further subjected for stability study.

RESULTS AND DISCUSSION
Stability analysis
The stability analysis was discussed based on the attenuation spectra and particle size distribution (cumulative, weight basis) of the formulation.

Attenuation spectra in figure 1 illustrates the precision pattern of the attenuation spectra on formulation F6-8C within 60 day. It can be seen from the graph that though the pattern is not exactly superimposable but however very close to each other. Also it is evident that the spectrums at day 30 and 60 are almost superimposable indicating the further sonication sample going stability on standing. Meanwhile as in figure 2 the PSD (cumulative, weight basis) plot for sample F6-8C are almost superimposable further support the contentions of attenuation spectra.
It is seen in table 1, that the size grew from 132.3 to 155.8 μm but with a decreasing trend of fitting error indicating the stabilization of the droplet size. The data is in well agreement with the cumulative weight basis PSD.

Table 6: Stability data for F6-8C

<table>
<thead>
<tr>
<th>Day</th>
<th>Fitting error, %</th>
<th>Mean size, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>9.1</td>
<td>132.3</td>
</tr>
<tr>
<td>Day 15</td>
<td>4.7</td>
<td>135.2</td>
</tr>
<tr>
<td>Day 30</td>
<td>4.4</td>
<td>140.5</td>
</tr>
<tr>
<td>Day 60</td>
<td>3</td>
<td>155.8</td>
</tr>
</tbody>
</table>

CONCLUSIONS
It is concluded that the attenuation spectra can be utilized for the prediction of stability as that is a reflection of the dispersion dynamics.
REFERENCES


ACKNOWLEDGEMENTS

It is highly thankful to University Technology Malaysia (UiTM), and Ministry of Education (MOE) Malaysia, for financial support through Fundamental Research Grant Scheme-600-RMI/FRGS 5/3 (55/2013).
**Study on the Factors Influencing Lipid Profile among Acute Coronary Syndrome Patients**

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**INTRODUCTION**

Acute Coronary Syndrome (ACS) is one of the commonest cardiovascular diseases¹. The incidence of ACS in Malaysia is 141 per 100,000 population per year². Proper risk factors management such as achieving lipid goal attainment is important to improve survival and the quality of life³. There are a few factors that are associated with the lipid goal attainment such as age, gender, race, underlying illness, intensity of statin therapy, patients knowledge and patients belief towards medication⁴⁻⁵. Identification of these factors helps in solving treatment gaps in achieving the lipid goal attainment. Older age had significant predictors for successful LDL-C goal attainment (p-value=0.0001) while younger age patients have significantly lower HDL-C level (p value < 0.0001)⁶. In addition, Chinese significantly had the highest HDL-C (1.26 ± 0.3) compared to Malay and Indian (p value= 0.014)⁶. Men are found to be the significant predictor for effective LDL-C reduction among dyslipidaemic patients (p-value <0.0001)⁴. Diabetes and hypertension are significant predictors of LDL-C outcome (p<0.05)⁴ and study conducted in Malaysia by Al-Khateeb in 2011 found that coronary heart disease (CHD) or CHD risk equivalent obtained the lowest LDL-C attainment compared to other groups of patients (p-value<0.001)⁷. Over the years, the mortality rate has gone up to 7% and from 2008 to 2011, over 8000 percutaneous coronary intervention was performed in Malaysia⁷. Study by Whitley in 2011 found that the targeted lipid measurement among majority of ACS patients are still unachievable. Thus, factors that associate with the failure in attaining the cholesterol level shall be identified. Identification of these factors may play as useful information for the healthcare provider to optimize therapy and educate patients for better achievement of lipid goal.

**EXPERIMENTAL METHODS**

**Study Design**

A cross-sectional survey will be conducted in the cardiology clinics, UKMMC from March to June 2015.

**Study Population**

A target of 165 subjects will be recruited by systematic sampling. The inclusion and exclusion criteria is as following:

**Inclusion Criteria**

i. Aged more than 18 years old
ii. Treated for ACS for at least 2 months
iii. On secondary prevention medication

**Exclusion Criteria**

i. Patient who are not willing to join the survey
ii. Participants who do not understand English or Malay

**Data Collection**

Questionnaires will be adapted and developed based on literature review⁸⁻⁹. Socio-demographic data will also be included. Researcher assisted survey will be conducted where participants will be interviewed.

The medication list will be collected by accessing UKMMC pharmacy system. The latest lipid measurement will be obtained from the UKMMC laboratory system.

**Data Analysis**

Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 22.0. Socio-demographic data, pattern of lipid lowering therapy, knowledge score on risk factors, belief about medicine score and lipid measurement will be analyzed using descriptive analysis. For inferential statistic in determining the association of factors (i.e gender, race, age, patient’s premorbid, lipid lowering therapy, patient’s knowledge and belief about medicine) influencing lipid measurement, multiple linear regression will be used.
EXPECTED FINDINGS
This study opts for an overview on the association of patient’s factor, disease factor, intensity of statin therapy, patient’s knowledge and belief about medicines towards the lipid goal attainment. This may help the healthcare provider to optimised therapy and formulate individualized strategy of patient education and counselling in order to meet the desired outcome.

REFERENCES

ACKNOWLEDGEMENTS
Special thanks to Dr. Marhanis, Dr Adyani, lecturers and friends who have contributed their help in this study.
Development and Validation of a HPLC Method for Standardization of Zingiber cassumunar based on Selected Chemical Markers

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INTRODUCTION

Herbal medicines have been used to treat and relieve the symptoms of diseases since thousands years ago [1]. Zingiber Cassumunar is one of the famous herb used in traditional medicines to treat diseases such as sprains, diarrhoea [2], asthma [3] and skin disease [4]. Several biological activities have been reported for this plant such as anti-inflammatory [5], antimicrobial [6] and anti-oxidative [7]. This herb can be found easily in the Southeast Asia, China and India [8]. However, due to high price, manufacturers of herbal medicines tend to fake this herb with other related species that may have some similar biological activity such as Z. Zerumbet and Z. americans. Therefore, the important of this research is to establish standardization of Z. Cassumunar based on selected chemical markers and to ensure the quality control for efficacy and quality of herbal medicines. It is also important to overcome the impediment in the acceptance of the herbal medicines by improving standard quality control profile.

EXPERIMENTAL METHODS

Z. Cassumunar will be collected from two different locations and will be extracted using ethanol and water. The concentration of marker compounds in each extractions will be measured. In this study, marker compounds that will be used are (E)-4-(3, 4-dimethoxyphenyl)-butadiene [DMPBD] [9], (E)-4-(3, 4-dimethoxyphenyl) but-3-enyl acetate [10] and (1E, 6E)-1, 7-bis(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione [Curcumin] [11]. High Performance Liquid Chromatography (HPLC) method will be developed for simultaneous determination of these chemical markers and the method will be validated according to ICH guideline. Then, Some herbal-based products that are available in market will be tested using the previous developed HPLC method.

Data analysis

RP-HPLC analysis.

EXPECTED OUTCOMES

The method will be developed to obtain the best separation among marker compounds. The developed method is expected to be the best method for analysis of Z. Cassumunar extract and some finished products that are available in market. Method will be validated according to the ICH guideline. The method developed is expected to give best correlation coefficient, percentage recovery, and precision. All the data obtained is expected to be within accepted range.

REFERENCES


ACKNOWLEDGEMENTS
Special thanks to Prof. Madya Dr. Juriyati Jalil, lecturers and friends that have contributed their help in this project.
Safety of Fish Oil Containing Parenteral Lipid Emulsion as Compared to Soybean Lipid Emulsion Administered to Extremely Low Birth Weight Neonates

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INTRODUCTION

Lipid emulsion as an essential component in parenteral nutrition (PN), serving as a source of energy and at the same time provides fatty acid supply which promotes growth, visual and cognitive development [1, 2]. Soybean oil based lipid emulsion (SOLE), which has been used as standard intravenous fat emulsion for years is known to be associated with inflammatory complication such as PN associated liver disease (PNALD) [3]. A new generation lipid emulsion, SMOFlipid\textsuperscript{®} 20% consists of 30% soybean oil, 30% medium chain triglycerides, 25% olive oil and 15% fish oil enriched in vitamin E which is expected to have anti-inflammatory properties has been introduced in University Kebangsaan Malaysia Medical Centre (UKMMC) to replace SOLE [4].

It has been identified that the duration of PN and the birth weight of preterm neonates are the risk factors in developing PNALD [5]. Preterm neonates and small-in-gestational-age neonates are vulnerable to PNALD probably due to hepatic immaturity [6]. Effect of lipid emulsion specifically administered to extremely low birth weight (ELWB) neonates has not been studied. With the growing use of this fish oil containing lipid Emulsion (FOLE), this study aims to evaluate the short term effect of FOLE (SMOFlipid\textsuperscript{®} 20%) compared to SOLE (Intralipid\textsuperscript{®} 20%) in ELBW neonates on liver and lipid safety profile.

EXPERIMENTAL METHODS

Study Design

This is a retrospective observational cohort study on all extremely low birth weight neonates given intravenous lipid in Neonatal Intensive Care Unit (NICU), UKMMC. The studied subjects will be divided according to the type of lipid emulsion they receive, which include SOLE (Intralipid\textsuperscript{®} 20%) and FOLE (SMOFlipid\textsuperscript{®} 20%).

Study Population

All ELBW neonate given intravenous lipid in NICU from 1\textsuperscript{st} January 2012 to 1\textsuperscript{st} January 2015 will be recruited according to the study criteria:-

Inclusion criteria:

i. Birth weight $\leq$ 1000g
ii. Infant that received parenteral nutrition with lipid emulsion
iii. Gestational age $\leq$ 34 weeks
iv. Duration of parenteral nutrition $\geq$ 7 days

Exclusion criteria

i. Underlying liver and haemolytic disease
ii. primary biliary disorders

Data collection

The list of patient from NICU given parenteral nutrition within the study period will be obtained from the pharmacy record. The following data will then be collected from medical record, pharmacy parenteral nutrition record as well as laboratory information system using a standardized data collection form.

- Demographic – gender, gestational age, birth weight, medical and medication history, concomitant disease, neonatal morbidity (APGAR score)
- Nutrition information- type of lipid emulsion given: Intralipid\textsuperscript{®} 20% or SMOFlipid\textsuperscript{®} 20%, maximum fat intake, constituents delivered through parenteral nutrition, duration of parenteral nutrition, lipid emulsion infusion rate.
- Biochemistry test- serum total bilirubin, serum direct bilirubin, $\gamma$-glutamyl transpeptidase (GGT), alanine transaminase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglycerides (TG).
These biochemistry tests data will be collected within the first week of parenteral nutrition initiation, and weekly monitoring of these parameter till parenteral nutrition is stopped. This is in line with the routine monitoring standards in NICU, UKMMC

- Incidence of cholestasis, defined as a serum direct (conjugated) bilirubin >2 mg/dL for ≥2 consecutive weeks

Data analysis
Statistical analysis will be performed using SPSS version 22, using descriptive statistics including 95% confidence interval. Student t-test will be used to compare changes within group and paired t-test to compare changes in TG, liver enzymes and bilirubin pre and post intravenous lipid. A value of P<0.05 were considered statistically significant. Result will be presented as mean± SD.

A stratified analysis for subject weight 500g-1000g and subject weight <500g will be done in order to analyse effect of PN within the two birth weight categories. The difference between the subgroup is examined using student’s t test.

Multiple regression analysis will be constructed to assess the possible independent effect of the type of lipid emulsion on the incidence of cholestasis and after controlling for possible confounders.

POTENTIAL APPLICATIONS
The finding of this study serves as an audit to the changes in type of lipid emulsion used in neonatal intensive care unit since 2013. If FOLE is found to be as safe as SOLE, it can safely continue to replace SOLE in preterm neonates in view of the benefit in preventing inflammation and treating parenteral nutrition associated liver disease.

REFERENCES
“Moving Towards Scientific Excellence”
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T1BE, UKM Kuala Lumpur

Research Utilisation in Clinical Practice among Pharmacists in Malaysia:
Knowledge, Attitudes, Practices and Barriers

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INTRODUCTION
Research is defined as systematic inquiry or investigation of hypotheses that aims to establish facts or reach new conclusions. [1] Research utilisation is defined as the review and critique of scientific research, and then the application of the findings to clinical practice. [2] During evidence-based practice, research evidence is critically appraised to assess the usefulness and to interpret the results into the area of work or on a patient’s condition. When utilising research in evidence-based practice, clinicians need to be able to identify problems from clinical practice, search the related literature for the clinical questions, critically appraise research articles on their validity, importance and applicability, implement the research findings into clinical practice and evaluate the action. [3] The utilisation of research with the best quality in clinical practice will promote excellent and reliable healthcare that can improve patients’ health outcomes and reduce variations in disease management.[4] Although evidence-based practice promotes the utilisation of research findings, this is not always implemented and is known as “research-practice gap”. [5] Previous study of research utilisation among Australian paediatric occupational therapies showed that the participants had positive attitudes and willing to access new information to guide their clinical practices. However, they were less confident about their knowledge and not fully implementing research findings into practice due to insufficient time to read research and implement new ideas into practice. [6] Barriers to research utilisation reported in studies among nurses include insufficient time to read research articles and implement new ideas, unable to evaluate the quality of research articles, inadequacy of facilities for implementation, implication of research utilisation into practice are not made clear and lack of authority to change. [7,8,9,10] Pharmacists play an important role in providing safe, effective and affordable use of medications. With the introduction of pharmaceutical care, pharmacists are responsible to ensure that patients have positive medication-related outcomes and reduced medication related problems. Such role requires pharmacists to utilise important research findings related to medication use. [11] However, up to date, no study has been done to evaluate the research-practice gap among pharmacists in Malaysia. Therefore, in this study, pharmacists' knowledge, attitude, practice and barriers to research utilisation will be identified. The information gained from this study will help in designing the plan to enhance research utilisation activities among pharmacists.

EXPERIMENTAL METHODS
Study Design
A cross-sectional survey will be conducted among pharmacist in Malaysia from February to June 2015.

Study Population
487 subjects will be recruited by convenience sampling after including a 30% of allowance for non-response. The inclusion and exclusion criteria will be as below.

Inclusion Criteria
i. Registered pharmacist and provisional registered pharmacist practicing in Malaysia
ii. Pharmacists who provide healthcare services in healthcare institution or organization. This includes retail pharmacy, hospitals and health clinics

Exclusion Criteria
i. Pharmacists working in pharmaceutical industry or in enforcement office

Data Collection
The questionnaires will be adapted and developed based on the literature review [3,12]. The full survey consists of three parts: 1) the questionnaire on knowledge, attitudes and practices of research utilisation, 2) the barriers in research utilisation and 3) the demographics data of the participants.

Corresponding pharmacists from each facility who are willing to help with survey distribution and re-collection will be identified. According to the number of pharmacists working in the institution, a total number of surveys, information sheet about the study and returned self-addressed stamped envelope will be mailed to corresponding person. Researchers will follow-up with the corresponding pharmacists to ensure they received the survey. Another follow-up will be done after two weeks if the completed surveys were not returned.
Data Analysis
Collected data will be analysed using Statistical Program for Social Sciences (SPSS) version 22.0. Descriptive statistic such as mean scores of research utilisation knowledge, attitudes, practices and barriers will be presented using graphs or chart where appropriate. Inferential statistics to test the correlation between pharmacists’ knowledge- attitudes, knowledge-practices and attitudes-practices will be done using Pearson’s correlation test. Factors that may influence the score of pharmacists’ knowledge, attitudes and practices for example demographic data, involvement in research will be modelled using multiple linear regression.

REFERENCES

ACKNOWLEDGEMENTS
Special thanks to Dr. Ernieda, lecturers and friends that have contributed their help in this study.
**Determination of Steroids in Herbal Products**

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**INTRODUCTION**

Herbal products are gaining popularity among consumers due to the perception of the general public that they are natural substances which are not harmful towards one's health [1, 2]. However, not all herbal products are natural because a portion of them has been found to be adulterated with scheduled poisons to increase its effectiveness [2]. Steroids are among the many scheduled poisons which are commonly used to adulterate herbal products claiming to relieve inflammation and improve general well being [1]. Screening for adulterants in herbal products are done by the National Pharmaceutical Control Bureau (NPCB) using expensive instruments like high performance liquid chromatography and gas chromatography mass spectrometer [3]. The cost for running analysis using these instrument is high and also time consuming. Therefore, a more easier and cheaper screening method should be developed to allow for on site screening.

This research uses chemical tests: Zak test, Liebermann Burchard test and Salkowski test for cholesterol as basis since cholesterol is a modified steroid [4]. Brady's reagent test make use of the reaction of carbonyl group with hydrazine as all selected steroids (cortisone, prednisolone and dexamethasone) contains the carbonyl group [5]. Brady's reagent test, Zak test, Liebermann Burchard test and Salkowski test are simple chemical test that is cheap and can be done fast in field work as a method of preliminary screening test for steroids in herbal products. Cortisone, prednisolone and dexamethasone were chosen as each steroid represents a range of potency from low to high. Quantification of steroids using high performance liquid chromatography will be done as to confirm the identity of steroid and also determine the amount of steroid in test samples showing positive results from the chemical tests.

**EXPERIMENTAL METHODS**

**Samples**

20 registered herbal products with indications of pain relieve, giving external energy and for general well being were randomly purchased from the market.

**Chemical Reaction Test**

Brady's reagent test, Zak test, Liebermann Burchard test and Salkowski test are carried out for the methanol extracts of herbal samples and compared with steroid standards (cortisone, prednisolone and dexamethasone). Determination of the limit of steroid detection is done by carrying out serial dilutions of up to 20 concentrations.

**High Performance Liquid Chromatography (HPLC) analysis** [6]

The herbal samples with positive chemical reaction tests are to be analysed with HPLC. Kinetex XB-C18 HPLC column (50 x 5.60 mm, 2.6 µm) will be used with column temperature set at 40°C. Acetonitrile and ultrapure water will be used as mobile phase with gradient elution and the injection volume and flow rate set at 15 µL and 1.2 mL/minute respectively. Perform UV detection in the range of 210 - 400 nm.

**EXPECTED OUTCOME**

To develop method using simple chemical test that is cheap and can be done fast in field work as a method of preliminary screening test for steroids in herbal products.

**REFERENCES**


Pharmacoeconomic Analysis of Providing Fixed Dose Combination Tenofovir / Emricitabine / Efavirenz Versus Free Dose Combination Tenofovir + Emricitabine + Efavirenz in HIV Patients in Malaysia

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INTRODUCTION

Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome (HIV/AIDS) is a major public-health problem worldwide. About 8 million adults and children are living with HIV/AIDS and more than a million have died.(1) Data 2011 showed that these number of people living with HIV in Malaysia are 81,000 and death due to AIDS was 5800 (2). It also estimated that by the end of 2015, Malaysia will have 81,946 people living with HIV.(3) The majority of HIV/AIDS cases in Malaysia are found to be among drug users, commercial sex workers and transgenders.(1) It is known worldwide that the economic burden of HIV infection involves the use of health care service for treatment and AIDS-associated symptoms or opportunistic infections, and other costs associated with morbidity/premature mortality of adult working patients.(2) The Government of Malaysia has allocated RM 500 million (USD 143 million) for a period of 5 years during the National Strategic Policy 2006-2010 for the provision of antiretroviral (ARV) treatment for almost 10,000 people living with HIV.(3) The current HIV therapeutic options available in Malaysia include more than 20 approved antiretroviral drugs which are categorized into six classes such as nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitor (NNRTI), Protease Inhibitor (PI), fusion inhibitors, CCR5 antagonist and integrase inhibitors (InI). Each of these groups works on HIV in a different way. Highly active antiretroviral therapy (HAART), a regimen of two (NRTI) and a (PI) or a (NNRTI) was, introduced in Malaysia in year 1997. HAART has modified the clinical course of the HIV infection, reducing the rate of disease progression, the incidence of opportunistic infections, and mortality. As consequence, combination antiretroviral therapy has resulted in longer survival and better quality of life for many HIV infected patients.(5) Each drug varies greatly in terms of efficacy, resistance, pill burden, safety, and price. Although HAART regimen is effective, pill burden, drug-related toxicity and adverse effects, and subsequent difficulties with patient adherence lead to treatment failure and development of resistant strains. Reducing number of pills per day and importance of adherence in predicting treatment outcomes of HIV/AIDS patients highlighted in numerous studies. Combining different antiretroviral agents into single tablet while maintaining efficacy, tolerability and safety of the regimen reduces patients’ daily pill burden and enhance patients’ adherence to antiretroviral medication which increased survival chances and increased life expectancy among HIV patients.(14) Therefore an intervention study will be conducted to compare fixed dose combination form (fxd) of Tenofovir / Emricitabine / Efavirenz to free dose combination (frc) form Tenofovir / Emricitabine + Efavirenz in which the latter is currently used in the Medical clinic Hospital Sungai Buloh. Fixed Dose Combination therapy was defined as antiretroviral formulations that have more than one active antiretroviral agent, either from same or different drug class, combined in a single dosage form. Formulations consisting of only 1 antiretroviral agent were termed as free dose combination (FRC). Airolidi et al (7) found that patients who were switched to a one pill once-a-day showed statistical increase in Health Related Quality of Life (HRQoL) scores only in the last month of the 6-month follow-up period. HRQOL is a multidimensional construct of individuals’ perception of the impact of disease and treatment on their physical, psychological, and social well-being, is fast becoming an important health status measurement. It could be influenced by the social, economic, and physical environments, personal health practices, individual capacity and coping skills, human biology, early childhood development, and health service (13). The objective of this study is to perform a cost effectiveness analysis of HIV patients undergoing fxd therapy versus s frc therapy. This study will be conducted at the Malaysia Ministry of Health institution since the majority of funding for HIV treatment comes from government supported programs. Generic Medicines Policy in Malaysian insist generic prescribing should be encouraged, and generic substitution permitted and eventually legislated, in order to improve affordability of medicines. As we know HIV is a prolonged disease, the treatment should also be evaluated in a context of Ministry of Health care resource allocation.(5) Therefore economic analysis on ARV treatment regimens in Malaysia is in line to the goals of the Malaysian National Strategic Plan 2011 – 2015 which stress on reducing the social and economic impact resulting from HIV and AIDS on the individual, family and society.(6)

EXPERIMENTAL METHODS

Study Design

A cross-sectional survey will be conducted at the infectious diseases clinics, Hospital Sungai Buloh from March 2015 to March 2016.

Study Population

100 subjects will be recruited. The inclusion and exclusion criteria will be as below.
Inclusion Criteria
- ages above 18 years
- patients with confirmatory serology results CD4 count and viral load

Exclusion Criteria
- pregnancy
- has co-morbid medical condition unrelated to HIV such as hypertension
- Lost to follow-up (defined as not having been seen in the clinic for more than 6 months after the last visit)

Data Collection
After signing the informed consent, patients will be given EQ-5D questionnaires and Morisky Medication Adherence Scale. Patients will be given rightful privacy to answer the two questionnaires in their own time. The investigator or co-investigator will be available to provide assistance when needed, without influencing patients’ answers. The same investigator obtained the socio-demographic and clinical information from patients’ medical records.

Surrogate markers such as CD4+ count and viral load after 6 months treatment initiation and 12 months after the treatment initiation. This is taken to monitor disease progression and treatment effectiveness between fixed dose form free dose form.

The acquisition costs of the antiretroviral will be obtained from the Sungai Buloh Hospital Pharmacy store for the year 2015/16.

The cost of the treatment will be estimated by identifying the resources used in the process, quantifying the resources, and assigning a standard price in monetary units to each medication. Information concerning cost and consumption will be acquired from the hospital’s accounting department and from inventory files. The following will be accounted: personnel costs (doctors’ and pharmacist service provision cost, other healthcare staff such as nurses, medical assistants and healthcare assistants’ costs) and laboratory costs.

The number of medications and pharmacy refill records will be collected by accessing Hospital Sungai Buloh pharmacy system. Pharmacy refill records in combination with other tools will be used to estimate adherence to antiretroviral (22).

Data Analysis
Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 21.0. Demographic data will be analyzed using descriptive analysis. The type of statistical tests used will be based on the objectives as shown below:

i) Chi-square test will be used to evaluate any significance difference between fixed dose form and free dose form.

ii) Chi-square test will be used to determine any significant difference between fixed dose form’s CD4 count and free dose form’s CD4 count.

iii) Cost-effectiveness analysis is conducted using data from incremental cost of fixed dose form and CD4 count from HIV patients as health effects.

iv) Cost Utility Analysis incremental cost of a fixed dose form compared to the incremental Quality of Life to the fixed dose form.

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ACKNOWLEDGEMENTS

Special thanks to Dr. Farida, lecturers and friends that have contributed their help in this study.
Use of Intravenous Immunoglobulin Therapy among Paediatric Patients at Universiti Kebangsaan Malaysia Medical Centre (UKMMC)

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INTRODUCTION
Intravenous Immunoglobulin (IVIG) has been widely used as ‘replacement dose’ in antibody deficiency diseases and also as ‘immunomodulatory’ agent in immune and inflammatory disorders [1]. Nermeen (2013) reported the use of IVIG in a Paediatric Intensive Care Unit (PICU) of a tertiary referral pediatric hospital based on clinical diagnoses included neurology (35%), neonatology (16%), hematology (11%), autoimmune disorders (11%) immunodeficiency disorders (11%), infections other than neonatal sepsis (9%) and cardiology (6.5 %) [2]. A recent review of 12 United States (US) academic centres reported the use of IVIG for more than 50 different conditions, with 52% of patients receiving IVIG for ‘off-label’ indications. The net result is that patients throughout the US are often treated with IVIG based on case report, case series, and individual clinicians’ experiences [3]. In terms of dosing, Orange et al. (2006) reported that the usual dose of IVIG for antibody replacement is between 0.3 and 0.6 g/kg per month, delivered every 2 to 4 weeks through the intravenous route in most primary and secondary immunodeficiency cases. For other uses, the doses range between 0.4 g/kg per day for 5 days or a more rapid course of 1 or 2 g/kg administered in 1 or 2 days [4]. IVIG was known to be a complex therapy which may lead to adverse effects. A survey by the Immune Deficiency Foundation in 2002 on more than 1000 patients with primary immunodeficiency found that 44% report experiencing adverse reactions such as fever, headache, chills, myalgia, nausea and hypotension that were not related to the rate of infusion. There is a consistent growing range of clinical indications for IVIG treatment among paediatric patients. In Malaysia, there is still a lack of established guideline and protocol on the use of IVIG especially in paediatric patients. To our knowledge, there is no published study done on the use of IVIG in local population so far. The current use of IVIG is based on the positive outcome from clinical trials and case reports. In addition, the usage of IVIG in clinical practice also had been limited by its cost and inadequate funding. Thus, there is a crucial need on a study to show the utilization of IVIG among paediatric patients.

EXPERIMENTAL METHODS
Study Design
An observational, retrospective chart review study will be conducted from March to June 2014.

Study Population
Paediatric patient (Age: birth to 12 years old). The inclusion and exclusion criteria will be as below.

Inclusion/ Exclusion Criteria
i. All patients receiving IVIG treatment during hospital stay from January 2007 to December 2014 will be included in this study.

Data Collection
List of patients given IVIG will be traced from the computerized pharmacy system. Patient’s registration number (RN) will be recorded and submitted to the medical record office to obtain the patient’s medical record. The following relevant information will be collected retrospectively from medical records:

i. Demographic data
ii. Diagnosis
iii. Dose and administration of IVIG
iv. Clinical indication and outcome

Data Analysis
Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 22.0. Demographic data, IVIG indication, dosing regimen and outcome will be analyzed using descriptive analysis. Simple logistic regression will be applied to investigate the factors that influence the clinical outcome of paediatric patients prescribed with IVIG (demographic factors, indication, dosing). P values < 0.05 will be accepted as statistically significant.

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A Novel Coenzyme Q10 Nanoemulsion Formulation Containing Omega -3, -6 and -9 Oils For Dermal Wound Healing

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INTRODUCTION
Nanoemulsions are submicron sized emulsions typically in the range of 20 – 200 nm consisting of two immiscible liquid (water and oil) stabilized by interfacial film of surfactant molecules. Nanoemulsion possesses various advantages such as good thermodynamic stability, increases bioavailability of drug, protects hydrolysis and oxidation of drug and ability to deliver both hydrophilic and lipophilic drugs that makes it a promising technique for drug delivery [1]. Wound healing is an orderly progression of events yet overlapping that re-establish the integrity of damaged tissue which involves several phases including hemostasis, inflammation, proliferation and remodeling [2]. However, neutrophils and cytokines released during the inflammatory phase produce oxidants resulting in delay of the restoration process. Ubiquinol (reduced form of Coenzyme Q10) serves as an endogenously lipid-soluble antioxidant which helps to prevent the damage of the tissue [3]. Fatty acids particularly polyunsaturated fatty acids (PUFA) namely linolenic (n-3) and linoleic (n-6) exert major influences in the immune responses by participating in the biosynthesis of inflammatory mediators together with monounsaturated fatty acid (MUFA) which is an oleic acid (n-9) for the synthesis of membrane phospholipids contributing to the control of signaling mechanisms of cell proliferation in the healing process [4]. The purpose of this project is to determine the effects of each omega -3, -6, and -9-loaded with coenzyme Q10 in nanoemulsion form for the treatment of dermal wound healing.

EXPERIMENTAL METHODS

Samples
Coenzyme Q10 will be purchased from Wisapple Technology Co. Ltd (China). Linseed oil, evening primrose oil and olive oil will be purchased from Fluka (Malaysia).

Screening of surfactants
Various types of surfactants will be screened. In water, 2.5 mL of 15 wt. % surfactant solution will be prepared and 4 µL of respective oils will be added with vigorous vortexing. If a one-phase clear solution is obtained, the addition of oil will be repeated until the solution becomes cloudy [5].

Screening of Co-Surfactants
The surfactant that shows high solubilization capacity with three oils will be selected and combined with co-surfactants. Six types of co-surfactants namely Transcutol P, ethanol, propylene glycol, span 80, span 20 and PEG 400 will be screened. At a fixed Smix ratio of 1:1, the pseudoternary phase diagrams will be constructed. Twelve different combinations in different weight ratios of oil and Smix, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 6:4, 7:3 and 9:1 will be taken so that maximum ratio will be covered to delineate the boundaries of phases precisely formed in the phase diagrams [5].

Pseudoternary phase diagram
Surfactant will be blended with co-surfactant in the weight ratios of 3:1, 2:1, 1:1, 1:0, 1:2 and 1:3. For each phase diagram, the ratio of oil to the Smix will be varied as 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 6:4, 7:3 and 9:1. Aqueous titration method will be used for construction of the pseudoternary phase diagrams, which involves stepwise addition of water to each weight ratio of oil and Smix, then mix the components with the help of vortex mixer at 25°C. The nanoemulsion phase will be identified as the region in the phase diagram where clear, easily flowable and transparent formulations will be obtained based on visual observation. One axis of the pseudo-three-component phase diagram represented the aqueous phase, the other represented the oil phase and the third represented a mixture of surfactant and co-surfactant at a fixed weight ratio (Smix) [5].

Preparation of Coenzyme Q10-loaded in nanoemulsion formulations
Nanoemulsion formulation of CoQ10 will be prepared by dissolving 1% of CoQ10 in heated respective oils at 60°C. Then, required quantity of selected surfactant and co-surfactant will be added to the oil phase with continuously stirring. The aqueous phase composed of distilled water will be heated at the same temperature separately. Then, the distilled water will be added slowly to the oil phase to make 100% w/w of final formulation. This coarse emulsion will be homogenized with a high-speed blender for 2 min followed by five passes at 12K psi through a high pressure homogenizer. The oil and aqueous phases will be kept warm (>50°C) during mixing and homogenization to avoid any chances of crystallization.
Characterization of nanoemulsion
The optimized nanoemulsion formulation will be characterized for various attributes including droplet size and size distribution, viscosity, pH, morphology, drug content, texture analysis, FTIR and skin permeation.

In vitro cell culture analysis
Scratch wound healing assay
Human dermal fibroblast will be cultured. The migration of HDF cells to fill the created scratch area will be captured by digital camera attached to microscope and analysed by NIH image J software at different time interval [6].

Cell proliferation assay
HDF cells seeding in 96-well plate will be incubated with various concentrations of nanoemulsion. After 24 hours, MTT reagent will be added and the absorbance at 570 nm will be measured using microtiter plate reader with reference standard of 630 nm. Graph of absorbance against number of cells will be plotted to determine the HDF cells proliferation as per standard methods [7].

In vivo animal healing studies
Rat will be divided into five groups containing six rats for each group. The wound will be created on rat and 1 cm² area of nanoemulsion samples will be applied once a day until they completely healed. Wound contraction will be measured and histological examination will be performed.

Data Analysis
Collected data will be analyzed using Statistical Program for Social Sciences (SPSS) version 22.0

EXPECTED OUTCOMES
• A stable, novel nanoemulsion of CoQ10 can be developed by using different sources of omega fatty acids oils.
• Any of the three fatty acids has a potential in wound healing.
• Delivery of CoQ10 in fatty acids can be enhanced through skin.
• CoQ10 nanoemulsion in omega fatty acids oils would enhance the wound healing activity.

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Nurses’ Knowledge and Practice in Drug Administration through Enteral Feeding Tubes in UKM Medical Centre

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INTRODUCTION
Medications given in addition to nutrients through enteral feeding tubes especially in patients with swallowing difficulties or critically ill patients is a common practice. Nurses are primarily responsible for administering medications in this manner. Therefore, appropriate techniques of drug delivery via enteral feeding tubes (such as preparing and administrating the medication, flushing the tube before, during and after administration and assessing potential complications) are required [1,2]. Oral drug administration through enteral feeding tubes raises several issues such as obstruction of feeding tubes with inappropriate administration techniques or drug formulations, inadequate crushing of enteric coated drugs leading harmful consequences, risk of cross-contamination from sharing of tablet crushing devices and risks of occupational exposure to drug powders through inappropriate handling [3]. All these issues may result in decreased drug efficacy, increased adverse effects, or drug-formula incompatibilities [4]. Health care providers need to have the knowledge about the characteristics of different drug dosage forms available in the market and the correct handling technique when administer medication through enteral feeding tubes [5]. Studies showed that practices among nurses in enteral medication administration are inconsistent [6]. There is no standard precaution guideline about the drug administration via enteral feeding tubes is available in Malaysia. Therefore, the aim of the study is to explore nurses’ knowledge and their practice in drug administration through enteral feeding tubes in Universiti Kebangsaan Malaysia Medical Centre (UKMMC) for future reference at least at the local setting.

EXPERIMENTAL METHODS

Study Design
A cross sectional, mixed methods observational study with self-administered questionnaire and direct observational method using checklist. Data are being collected from questionnaire and observational checklist that will be conducted in adult neurology surgical ward and neurology medical ward, UKMMC from March to June 2015.

Study Population
Convenience sampling will be used to enrol participants in the study. The minimal recommended sample size is 38.

The inclusion and exclusion criteria will be as listed below.

Inclusion Criteria
i. For Questionnaire part: Staff nurses working in neurology wards
ii. For Observation part: Staff nurses who prepare and administer drugs through enteral feeding tubes during data collection period

Exclusion Criteria
i. For Questionnaire part:
   • Staff nurses who are not available during data collection period (eg. on maternity leave)
   • Incomplete questionnaire
ii. For Observation part:
   • Preparation and administration drugs beyond working hours (8am- 6pm) or during weekend
   • Preparation and administration drugs when the investigator is not available

Data Collection
The questionnaires will be adapted and developed based on literature review [2,3,4,6,7,8]. Nurses will asked to complete a questionnaire which has 14 questions to gauge their knowledge for medication preparation and administration via enteral feeding tubes.

An observational checklist developed from guidelines will be used to observe the nurses’ practice in drug preparation and administration through enteral feeding tubes [2,3,7,8].

Data Analysis
Statistical analysis involves the application of Statistical Program for Social Sciences (SPSS) version 22. Data for subjects’ demographic, knowledge and the practice in enteral medication preparation and administration through enteral feeding
tubes will be analyzed using descriptive statistics (average and percentage). T test will be used to determine any significant difference between nurses’ knowledge and their practice in enteral drug administration through enteral feeding tubes.

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Toxicity Analysis and Liquid Chromatography-Mass Spectrometry Profiling of Methanol Extracts of *Bryopsis pennata*, *Padina australis* and *Sargassum binderi*

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INTRODUCTION
Seaweeds are found extensively along Malaysia’s coastline with high diversity. Malaysian seaweeds have been reported for their antibacterial [1] and antioxidant activities [2, 3]. Recently, the mosquito larvicidal activity of extracts and compounds of thirty seaweed species was described in the review of Yu *et al* [4]. However, there is no report on the usage of seaweeds for mosquito control in Malaysia. Therefore, it is necessary to conduct the study to screen through the mosquitoecidal potential of seaweeds especially in Malaysia where dengue cases are still a serious health problem in both the rural and urban areas.

The study aimed to determine the mosquito larvicidal and adulticidal properties of the methanol extracts of *Bryopsis pennata*, *Padina australis* and *Sargassum binderi* against vectors of dengue fever - mosquitoes *Ae. aegypti* and *Ae. albopictus*. The toxicity of methanol extracts was assessed towards brine shrimp nauplii. Besides, the methanol extracts were also subjected to LC-MS analysis to determine their phytochemical composition.

MATERIALS AND METHODS

Seaweed materials and extract preparation
The seaweed materials were collected from Peninsular Malaysia. Identification of the seaweeds was mainly based on comparison to the published taxonomic literature. The voucher specimens were deposited at the Herbarium of Universiti Kebangsaan Malaysia. The seaweeds collected were cleaned, air dried at 25±2°C with relative humidity of 72±2%. Then, the samples were ground, sieved, and stored in airtight container at -20°C. Methanol extract was prepared for LC-MS analysis and toxicity assay. The dried seaweeds were soaked in methanol (Merck, German) (60 g/L) and concentrated by rotary evaporator at 40°C [3]. The methanol extract was kept in vials at -20 °C for further use.

Mosquito larvicidal assay
Batches of 25 of third instar lab strain of *Ae. aegypti* and *Ae. albopictus* larvae were introduced to the 200 mL filled paper cups with seaweed extracts diluted from 1.0% stock solution in methanol and distilled water [5]. Abate at 1.11 ppm (Cyanamid, American Cynamid Company) was used as positive control. Three replicates were set up for each test samples as well as negative control (which only distilled water and methanol were used) and the experiment was repeated three times. The larval mortality was recorded after 24 h. Mortality of the control, which is not more than 20%, was corrected by Abbott’s formula [6]. LC50 value was calculated by using the Probit Analysis Program [7].

Mosquito adulticidal assay
Lab strains of mosquito *Ae. aegypti* and *Ae. albopictus* were maintained in insectarium of Institute for Medical Research. Adulticidal assay was carried out according to World Health Organization [8] with slight modifications. Stock solutions of 10 000 µg/mL were prepared by dissolving in ethanol solution. Four mL solution of different concentrations were then impregnated on filter papers (140 X 115 mm) making concentrations of 0.248, 0.496, 0.993 and 1.987 mg/cm². Impregnated papers were left to dry at room temperature prior to testing. Malathion, at a diagnostic dosage of 5% was used as a positive control. Negative control was impregnated with 5% ethanol solution. Batches of 15 adult females were exposed to the impregnated paper for 3 h continuously with mortality recorded every 10 min throughout the exposure period. At the end of the 3 h exposure, the mosquitoes were transferred in the holding tube and given 10% sugar solution enriched with vitamin B complex as food. The experiment was repeated 3 times with 3 replicates. Mortality was recorded again after 24 h of monitoring period.

Brine shrimp toxicity assay
Ten newly hatched *Artemia salina* nauplii were introduced to 5 mL of seaweed extract solution with concentration ranged from 300 to 600 µg/mL. The extract solution was prepared by using seaweed extract stock solution (20 mg/mL) and brine medium. Three replicates were set up for each test sample and the experiment was repeated 3 times [9]. Potassium dichromate solution was used as a positive control and mixture of methanol and brine medium was prepared as negative control. The nauplii mortality was recorded after 24 h. LC50 was calculated by using the Probit Analysis Program [7].
Liquid chromatography-mass spectrometry analysis
The sample was prepared in methanol of the concentration of 20 ppm and filtered through a 0.45 μm nylon filter before being loaded into the system. The sample was analyzed by using Agilent 6530 Accurate-Mass Q-TOF liquid chromatography-mass spectrometry (LC-MS) system with Agilent Zorbax Eclipse XDB-C18 column (2.1 x 50 mm, 1.8 micron) (Agilent Technologies Canada Inc., Canada), and eluted with acetonitrile and water using gradient system. The experiments were performed in the positive ion mode. The flow rate of the drying gas was set at 8 L/min at the temperature of 350°C. The nebulizer pressure was set at 35 PSIG with the capillary and injection volume set at 3000 V and 5 µl, respectively.

RESULTS AND DISCUSSION
The mosquito larvicidal and adulticidal effects of the methanol extracts of seaweeds Bryopsis pennata, Padina australis and Sargassum binderi against Ae. aegypti and Ae. albopictus were investigated. Of all the seaweed methanol extracts tested, green seaweed B. pennata exhibited the strongest larvicidal effect against Ae. aegypti (LC_{50} value = 156.97±7.415 µg/mL) and Ae. albopictus (LC_{50} value = 177.50±6.685 µg/mL). The results of adulticidal assay showed that brown seaweed P. australis exhibited the strongest toxic effect towards female adults of Ae. aegypti (LC_{50} value = 31.035±4.521 mg/cm²) and Ae. albopictus (LC_{50} value = 36.949±3.435 mg/cm²). The results of brine shrimp assay showed that all the methanol extracts were having very mild toxic effect against the nauplii, as S. binderi exhibited LC_{50} value of 423.395 µg/mL, B. pennata and Padina australis exhibited LC_{50} values of more than 1000 µg/mL. The results of brine shrimp toxicity assay indicated that the three seaweeds are potential target specific bioinsecticide towards Aedes mosquitoes.

All the main peaks belonging to various compounds in LC-MS profile were tentatively assigned by comparing the data acquired by Mass Hunter Acquisition Data to Dictionary of Marine Natural Products [10]. The LC-MS profiles of seaweed extracts of B. pennata, P. australis and S. binderi showed that each of the seaweed had different composition of compounds.

CONCLUSION
The toxic effect of the seaweed extracts was more profound against larvae of Ae. aegypti and Ae. albopictus as compared to Artemia salina nauplii. The results indicated the potential of seaweed extract as a target specific mosquito larvicidal agent and further investigation of the responsible lead and its mode of action should be carried out.

REFERENCES

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Quality Control Study on Two Brands of Ibuprofen Syrups Available in Malaysian Market

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ABSTRACT
The quality control of pharmaceutical product is important to insure the safety of the patient. Different parameters of quality control can guarantee the quality and bioavailability of optimal therapeutic activity. Therefore, the present study was undertaken with aim of assuring the quality and therapeutic activity of ibuprofen syrup available in Malaysian market.

INTRODUCTION
Ibuprofen or alpha-methyl-[4 - (2-methylpropyl) phenyl] propanoic acid (Fig. 1) is a non-steroidal analgesic and anti-inflammatory drug (NSAID). It is available in different dosage forms as tablet, syrup, gel and suppository. Ibuprofen syrup is used as pain relief for children in case of osteoarthritis, rheumatoid arthritis and can also be used for the short term treatment of fever in children.

EXPERIMENTAL METHODS
1. Visual inspection.
2. Viscosity determination.
3. PH measurement.
4. Light Transmittance.
5. H.P.L.C.

RESULTS AND DISCUSSION
- The PH Measurement: PH measurement was run by (METER TOLEDO-SWISS. MOLDEL FE20-ATC KIT) the results were
  - The first brand’s PH (BRUFEN SYRUP) = 4.02 at 27 °C.
  - The second brand’s PH (NUROFEN SYRUP) = 3.89 at the same temperature.
  - From the above results we found that both are compliance with the requirements of The united states pharmacopeia (U.S.P) which was between 3.6 and 4.6.
- In term of viscosity determination the results were:

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CONCLUSION
The Ibuprofen syrups marketed in Malaysia by pharmaceutical companies are of satisfactory quality and met U.S.P standard.

REFERENCES

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Special thanks to Dr. Sayed Nasir Abbas Bukhari. lecturers and friends that have contributed their help in this study.