UNIVERSITI PUTRA MALAYSIA

GLYCOSIDATION OF BETULINIC ACID USING NOVOZYME 435

HAMISU ABDU

FS 2016 37

GLYCOSIDATION OF BETULINIC ACID USING NOVOZYME 435

By

HAMISU ABDU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, In Fulfilment of the Requirementsfor the Degree of Masters of Science

March 2016

COPYRIGHT

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

GLYCOSIDATION OF BETULINIC ACID USING NOVOZYME 435

By

HAMISU ABDU

March 2016

Chairman : Associate Professor Intan Safinar Ismail, PhD
Faculty : Science

In this study, 3-*O*-*β*-D-glucopyranoside-betulinic acid was successfully synthesized *via* the reaction between betulinic acid and glucose catalyzed by immobilized lipase from *Candida antarctica* (Novozyme 435) in *t*-butanol. The structure of the product obtained were elucidated using spectroscopic data. Effects of individual reaction parameters such as reaction time, reaction temperature, amount of enzyme and substrate molar ratio, were investigated and optimized. The optimum conditions for the reaction between betulinic acid and glucose were obtained at the reaction time of 48.50 h, temperature of 26^{o}C, 175 mg of enzyme, and substrate molar ratio of 1:1.2; giving 85.83 % of yield.

The Response Surface Methodology (RSM) based on five-level, three variables Central Composite Rotatable Design (CCRD) was employed using Design Expert software to evaluate the effect of synthesis parameters and its mutual interactions. It was observed that the maximum conversion yield of 3-*O*-*β*-D-glucopyranoside-betulinic acid 88.69% was obtained using 30.67 h, 54.30^{o}C and 180 mg of enzyme using betulinic acid (0.05 mmol) and glucose (0.1 mmol) respectively. The experimental value obtained was 88.69%, closer to the results obtained using single parameter.

Finally, the anticancer activity of the synthesized compound was evaluated against cultured mouse embryonic fibroblast normal cell line (3T3), human cervical carcinoma cancer (HeLa), human breast cancer (MCF-7), human T-promyelocytic leukaemia (HL-60), and cell lines. From the results, BA showed high activity against cultured human T-promyeloctic leukaemia (HL-60), human breast cancer (MCF-7), and human cervical carcinoma cancer (HeLa) cell lines with IC_{50} values of MCF-7 0.8 µg/ml, HL-60 4.4 µg/ml and HeLa 4.8 µg/ml, respectively. On the other hand, 3-*O*-*β*-D-glucopyranoside-betulinic acid also showed strong activity against cultured, HL-60, MCF-7and 3T3 with IC_{50} values of 8.4 µg/ml, 8.5 µg/ml and 2.75 µg/ml respectively. However, it was found to have moderate activity against HeLa cell line with IC_{50} value of 12.0 µg/ml.

In conclusion, an enzymatic synthesis of 3-*O*-*β*-D-glucopyranoside-betulinic acid was successfully carried out by the reaction between betulinic acid and D-glucose in an organic solvent using Novozyme 435. The activity of 3-*O*-*β*-D-glucopyranoside-betulinic acid against cancer cell lines was found to be better than betulinic acid.

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

PENGLIKOSIDAAN ASID BETULINIK MENGGUNAKAN NOVOZYME 435

Oleh

HAMISU ABDU

Mac 2016

Pengerusi : Profesor Madya Intan Safinar Ismail, PhD
Fakulti : Sains

Di dalam kajian ini, 3-*O*-*β*-D-glucopyranoside- asid betulinik telah berjaya disintesis melalui tindak balas antara asid betulinik dan glukosa dimangkinkan oleh lipase pegun dari *Candida antarctica* (Novozyme 435) dalam *t*-butanol. Struktur produk yang diperolehi telah dijelaskan menggunakan data spektroskopi. Kesan parameter tindak balas individu seperti masa reaksi, suhu tindak balas, jumlah enzim dan substrat nisbah molar, telah dikaji dan dioptimumkan. Keadaan optimum bagi tindak balas antara asid betulinik dan glukosa yang diperoleh adalah pada masa reaksi 48.50 jam, suhu pada 26^{o}C, 175 mg enzim dan substrat nisbah molar daripada 1:1.2; memberikan hasil 85.83%.

Kaedah Permukaan Sambutan (RSM) berdasarkan kepada lima tahap, dengan tiga pembolehubah Reka Bentuk Komposit Berputar (CCRD) digunakan dalam perisian Pakar Rekabentuk untuk menilai kesan parameter sintesis dan interaksi bersama. Pemerhatian menunjukkan bahawa hasil penukaran maksimum asid 3-*O*-β-D-glucopyranoside-betulinik (88.69%) telah diperolehi pada 30.67 jam, 54.30 ^{o}C dan 180 mg enzim yang menggunakan asid betulinik (0.05 mmol) dan glukosa (0. 1 mmol) masing-masing. Hasil ujikaji yang diperolehi ialah 88.69%, lebih dekat kepada keputusan yang diperolehi dengan menggunakan parameter tunggal.

Akhirnya, aktiviti antikanser sintesis kompaun telah diuji terhadap barisan sel kanser tikus embrio fibroblast sel garisan normal (3T3), kanser karsinoma serviks manusia (HeLa), kanser payudara manusia (MCF-7), dan leukemia T-promyelocytic manusia (HL-60). Keputusan menunjukkan Asid betulinik (BA) mencatatkan aktiviti yang tinggi terhadap kultur sel kanser payudara manusia (MCF-7), leukemia T-promyeloctic manusia (HL-60), dan kanser karsinoma serviks manusia (HeLa) dengan nilai IC_{50}, 0.8 μg/ml, HL-60 4.4 μg/ml dan HeLa 4.8 μg/ml, masing-masing . Pada masa yang sama, asid 3-O-β-D-glucopyranoside-betulinik menunjukkan aktiviti yang kuat terhadap kultur HL-60, MCF-7 dan 3T3 dengan nilai IC_{50} 8.4 μg/ml, 8.5

μg/ml, dan 2.75μg/ml masing-masing. Walau bagaimanapun, ia didapati mempunyai aktiviti sederhana terhadap barisan sel HeLa dengan IC_{50} nilai sebanyak 12.0 μg/ml. Sebagai kesimpulan, sintesis enzim 3-O-*β*-D-glucopyranoside-betulinic asid telah berjaya dijalankan oleh tindak balas antara asid betulinic dan D-glukosa dalam pelarut organik menggunakan Novozyme 435. Aktiviti asid 3-*O*-*β*-D-glucopyranoside-betulinik terhadap barisan sel kanser didapati lebih baik berbanding asid betulinik.

ACKNOWLEDGEMENTS

All praises be to Almighty Allah, my creator and my sustainer for sparing my life and granted me the opportunity to undertake this research work. My most profound gratitude goes to Prof. Dr. Faujan Hj. Ahmad who tirelessly gave his meaningful suggestions and correction of the drafts; his rightful guidance was responsible for the completion of this work.

It is my great pleasure to acknowledge my indebtedness to my supervisor Assoc. Prof. Intan Safinar Ismail for her relentless efforts and useful suggestions rendered to me, I am most grateful. My acknowledgment also goes to my supervisory committee, Prof. Dr. Mahiran Basri and Prof. Dr. Mohd. Basyaruddin Abdul Rahman for their efforts towards the completion of this program.

I am seizing this opportunity to thank the Management of the Hussaini Adamu Federal Polytechnic, Kazaure, for giving me this opportunity to undertake this course.

This acknowledgement will never be complete without registering my most profound gratitude to my parents for their ceaseless, financial and moral supports they had been rendering throughout the period of my study and to my wife Nabiha Tukur Tafida, my son Ahmad Hamisu and my daughter Khadija Hamisu for accompanying me to stay at Malaysia during the program on schedule.

My appreciation also goes to my colleagues Sabo Wada Dutse, Abbas Ja'afaru Badakaya, Sani Jibrin, Bashir Ahmed Danzomo, Mukhtar Hussaini, Mohd Maijama'a, Abba Lawali Geidam, and Mohd Gidado Liman and also to my lab mates especially Siti Aminah Shah, Leila, Nadiya and many others.

It grieves me so much that I cannot mention all the name of those who has helped me in one way or the other during my study, but your good intentions are permanently registered in my mind, I am most grateful.

Lastly, my sincere and heartily acknowledgement goes to Saleh isyaku whose his selfless humble supports, advise and patient lead to the success and completion of my program here in UPM.

I certify that a Thesis Examination Committee has met on 24 March 2016 to conduct the final examination of Hamisu Abdu on his thesis entitled "Glycosidation of Betulinic Acid using Novozyme 435" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Gwendoline Ee Cheng Lian, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Siti Mariam binti Mohd Nor, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Faridah binti Abas, PhD
Associate Professor
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Internal Examiner)

Zurina Shaameri, PhD
Associate Professor
Universiti Teknologi MARA
Malaysia
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 28 September 2016

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

Intan Safinar Ismail, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Mahiran Basri, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Mohd. Basyaruddin Abdul Rahman,PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software

Signature: _____ Date: _____

Name and Matric No.: Hamisu Abdu, GS27134

Declaration by Members of Supervisory Committee

This is to confirm that:

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

Signature: _____
Name of Chairman of Supervisory Committee: Associate Professor Dr. Intan Safinar Ismail

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Mahiran Basri

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Mohd. Basyaruddin Abdul Rahman

TABLE OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
AKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xii
LIST OF FIGURES		xiv
LIST OF SCHEMES		xvi
LIST OF ABBREVIATIONS		xvii
CHAPTER		
1	**INTRODUCTION**	1
	Enzymes	2
	Nature of enzymes	3
	How enzyme work	3
	Immobilized enzymes	5
2	**LITERATURE REVIEW**	6
	Bioactivity of betulinic acid and its derivatives	6
	Anti-cancer activity	6
	Anti-inflammatory activity	8
	Advantages of betulinic acid glycoside	9
	Example of isolated betulinic acid glycosides from nature	10
	Synthesis of betulinic acid glycosides by chemical reaction	11
	Biosynthesis of betulinic acid glycoside	15
	The use of Novozyme 435 in the synthesis of betulinic acid derivatives	15
	Response surface methodology (RSM)	16
	Application of RSM in enzymatic synthesis	16
3	**MATERIALS AND METHODS**	18
	Chemicals and materials used	18
	Methodology	18
	Enzymatic reaction between of betulinic acid and D-glucose	18
	Purification of the product	18
	Fourier Transform Infrared spectroscopy (FTIR)	19
	Nuclear Magnetic Resonance (NMR)	19
	Effect of single parameters on the glycosidation of betulinic acid using enzyme	19
	Effect of varying the substrate molar ratio	19
	Effect of varying the amount of enzyme	19
	Effect of varying the reaction temperature	20
	Effect of varying the reaction time	20
	Effect of reused enzyme	20

Response surface methodology (RSM) 20
Data analysis 21
Cytotoxic activity 22

4 RESULTS AND DISCUSSION 24
Properties of betulinic acid 24
Enzymatic reaction of betulinic acid (1) and D-glucose 27
Analysis of the product 30
Study of the individual reaction parameters on the reaction of betulinic acid (1) and D-glucose 38
Effect of varying the substrate molar ratio 38
Effect of varying the amount of enzyme 38
Effect of varying the reaction temperature 40
Effect of varying the reaction time 41
Effect of re-used enzyme 41
Model fitting and analysis of variance (ANOVA) 42
Model validation 45
Response surface plots and the effect of parameters 46
The effects of amount of enzyme and reaction temperature 46
The effects of reaction time and reaction temperature 47
The effects of amount of enzyme and reaction time 48
Optimization of the reaction 49
Bioactivity studies 49

5 CONCLUSION AND RECOMMENDATIONS 52
Conclusion 52
Recommendations for further studies 53

REFERENCES 54
APPENDICES 61
BIODATA OF STUDENT 67
LIST OF PUBLICATIONS 68

LIST OF TABLES

Table		Page
1	In vitro cytotoxic activity of compounds 3-4 against cancer cell lines	7
2	Effect of compound 5-7 on VEGF-induced cell proliferation	8
3	Anti-inflammatory activity of compounds 8-10	9
4	The effect of compound IFN-γ release and IL-2 release on compound 16	11
5	Cytotoxicity of compounds 17-19	12
6	Cytotoxicity of compound 20 against some cancer cell lines	13
7	The cytotoxicity and haemolytic activities of compound 21 against cell lines	14
8	The cytotoxicity of compound 22 against some cancer cell lines	14
9	Coded and actual level variables used for the experimental design.	20
10	Experimental design for five-level-three variable central composite rotatable design	21
11	Optimum conditions for the glycosidation of betulinic acid using enzyme obtained using RSM software	22
12	^{13}C NMR chemical shifts of betulinic acid	27
13	Comparison of the percentage yields of the product obtained using different solvents	28
14	^{1}H NMR, ^{13}C NMR, HMBC and ^{1}H-^{1}H COSY Data of the 3-*O*-*β*-D-glucopyranoside-betulinic acid	37
15	ANOVA for the enzymatic glycosidation of betulinic acid (**1**) of the quadratic model	43
16	Statistical regression analysis for the enzymatic glycosidation of betulinic acid (1)	43
17	Enzymatic glycosidation of betulinic acid (**1**) values and significance of regression coefficients	44
18	Coded and actual level variables used for the experimental design.	44
19	Experimental design for five-level-three variable central composite	45

rotatable quadratic polynomial model, experimental and predicted values

20 Actual and predicted model values for data validation 46

21 Optimum conditions obtained by response surface methodology 49

22 Cytotoxicity activity of betulinic acid (**1**) and 3-*O*-β-D-glucopyranoside betulinic acid (BAG) against HeLa, 3T3, MCF-7 and HL-60cell lines 50

LIST OF FIGURES

Figure		Page
1	Lock-and-key model of enzyme-substrate binding	4
2	Induced-fit model of enzyme-substrate binding	5
3	Infrared spectra of betulinic acid	24
4	Mass spectrum of betulinic acid (**1**)	25
5	^{1}H-NMR of betulinic acid	25
6	^{13}C-NMR of betulinic acid	26
7	Infrared spectrum of 3-*O*-β-D-glucopyranoside betulinic acid	30
8	^{1}H-NMR 3-*O*-β-D-glucopyranoside betulinic acid	31
9	Expanded ^{1}H-NMR 3-*O*-β-D-glucopyranoside betulinic acid	31
10	Expanded ^{1}H-NMR 3-*O*-β-D-glucopyranoside betulinic acid	32
11	Expanded ^{1}H-NMR 3-*O*-β-D-glucopyranoside betulinic acid	32
12	^{13}C-NMR of 3-*O*-β-D-glucopyranoside betulinic acid	33
13	Expanded ^{13}C-NMR of 3-*O*-β-D-glucopyranoside betulinic acid	33
14	Expanded ^{13}C-NMR of 3-*O*-β-D-glucopyranoside betulinic acid	34
15	Expanded ^{13}C-NMR of 3-*O*-β-D-glucopyranoside betulinic acid	34
16	Expanded ^{13}C-NMR of 3-*O*-β-D-glucopyranoside betulinic acid	35
17	Expanded ^{13}C-NMR of 3-*O*-β-D-glucopyranoside betulinic acid	35
18	HMBC spectrum 3-*O*-β-D-glucopyranoside betulinic acid	36
19	HSQC of 3-*O*-β-D-glucopyranoside betulinic acid	36
20	Effect on varying substrate molar ratio	38
21	Effect of varying the amount of enzyme	39
22	Effect of varying reaction temperature	40
23	Effect of varying reaction time	41

24	Effect of reused enzyme	42
25	Response surface plot of the effect of amount of enzyme and reaction temperature	47
26	Response surface plot of the effect of reaction time and reaction temperature	48
27	Response surface plot of the effect of amount of enzyme and reaction time	48
28	Effect of **1** and BAG against HeLa cell line	50
29	Effect of **1** and BAG against 3T3 cell line	51
30	Effect of **1** and BAG against MCF-7 cell line	51
31	Effect of **1** and BAG against HL-60 cell line	51

LIST OF SCHEMES

Scheme		Page
1	Enzymatic reaction of betulinic acid and 2, 2-dimethylsuccinic anhydride in chloroform using Novozyme 435 as the catalysts	16
2	Enzymatic glycosidation of betulinic acid using enzyme	28
3	Mechanisms for the enzymatic glycosidation of betulinic acid	29

LIST OF ABBREVIATIONS

%	Percentage
α	Alpha
β	Beta
V max	Wave number
δ	Delta
^{o}C	Degree centigrade
µg/ml	Microgram per millilitre
µM	Micromolar
^{1}H	Proton NMR
^{13}C	Carbon NMR
3T3	Mouse embryonic fibroblast cell line
A253	Head tumor cell line
A549	Lung tumor cell line
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of Variance
ATTC	American Type Culture Collection
BA	Betulinic acid
B16-F1	Mouse melanoma
br	Broad
CCRD	Central composite rotatable design
CEM	T-lymphoblastic leukaemia
COSY	Correlation spectroscopy
d	Doublet
dd	Doublet of doublet
DLD-1	Human colorectal adenocarcinoma
DMSO	Dimethylsulphoxide
D10	Chloroquine-susceptible strain
EC_{50}	Effective concentration that kill 50 %
F-test	Fisher distribution test
F32	*Plasmodium falciparum*
h	Hour

HeLa	Human cervical carcinoma cell line
HIV-1	Human immunodeficiency virus-1
HL-60	Human T-promyelotic leukemia
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
IC_{50}	Inhibitory concentration that kill 50%
IFN-γ	Interferon gamma
IL-2	Interleukn-2
IR	Infrared
KB	Human epidemoid carcinoma of mouth
KS	Kasposis sarcoma cell
K562	Leukemia cell line
LA	*Leishmania amazonensis*
M^+	Molecular ion
m	Multiplet
MCF-7	Human breast cancer
MEL-2	Cultured human melanoma
MHz	Megahertz
ml	Milliliter
MTT	Microculture tetrazolium salt
M2903	*Leishmania brazilliensis*
MIA PaCa	Pancrease tumor cell
NMR	Nuclear magnetic resonance
ND	Not detected
NO	Nitric oxide
PA-1	Ovary tumor cell line
PC-3	Human prostate adenocarcinoma
PMT	Prodrug monotherapy
R^2	Coefficient of determination
RSM	Response surface methodology
s	Singlet
SD	Standard deviation
SAR	Structure activity relationship

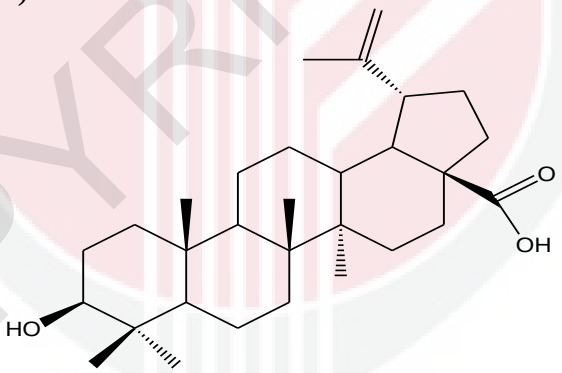
SW620	Colon tumor cell line
t	Triplet
TLC	Thin layer chromatography
TULA	Trypanasoma cruzi tulahuen
U937	Human macrophage cell line
UV	Ultraviolet
WSI	Healthy cell line

CHAPTER 1

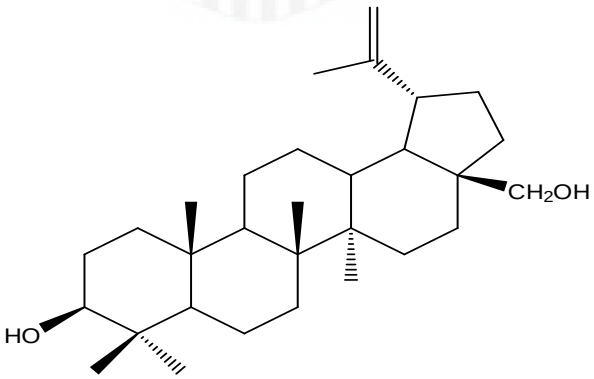
INTRODUCTION

Betulinic acid (**1**), [3β-hydroxy-lup-20(29)-en-28-oic acid] (BA) is a pentacyclic triterpene, with a molecular formula of $C_{30}H_{48}O_3$, a molecular weight of 456.70 g/mol and is a white to off-white powder found to be soluble in dimethylsulphoxide (DMSO). Betulinic acid isolated from the bark of many plants. It is also found abundantly in the outer bark birch trees, *Betula alba* (Pisha *et al*., 1995). White birch bark, *Betula alba* has been used by Americans as a folk remedy. Betulinic acid once enters cancerous cells it provokes apoptosis or "a programmed cell death" within the tumour growth. It was reported that Scientists at the University of North Carolina reported that the chemicals found in the white birch bark slowed down the growth of human immunodeficiency virus (Fujioka *et al*., 1994).

Betulinic acid is a natural agent isolated from birch trees which was first described to induce apoptosis in neuroectodermal tumour cells (Pisha *et al*., 1995). Its botanical sources were reviewed recently, where BA exhibits cytotoxicity in many cancer cell-lines and is capable of inducing apoptosis (Pawel *et al*., 2009). The reduced congener of betulinic acid, betulin (3β-lup-20(29)-en-3, 28-diol) (**2**), is one of the first natural product identified and isolated from plants in 1788 (Alakurti *et al*., 2006)



1



2

Glycosides are important biological molecules that are involved in a vast number of biological processes. Many glycosides have therapeutic uses such as anti-cancer therapies, anti-inflammatories, enzyme inhibitors and antibiotics (Henessian *et al.*, 2000). Glycosidation reactions are generally challenging and laborious, it requires several protection and deprotection steps, these steps often result in time-consuming reaction sequences with low overall yields. To avoid these challenges, glycosidation of betulinic acid using Novozyme 435 was carried out in this work.

Fischer-type glycosidation remain the simplest and most effective method in performing simple glycosidation, it involves the addition of an alcohol to an unprotected sugar in the presence of an acid catalyst. A chemical glycosidation reaction involves the coupling of a glycosylic donor to a glycosylic acceptor forming a glycoside. The reaction requires activation with a suitable activating reagent. The reactions often result in a mixture of products due to the creation of a new stereogenic centre at the anomeric position of the glycosylic donor. The linkage formed may either be axial or equatorial (α or β with respect to glucose).

Generally, glycosides are more water soluble than their corresponding aglycones (Smith *et al*., 1999). Therefore it is hope that glycosidation of triterpenes (e.g. betulinic acid) will increase its hydrosolubility as well as its biological activities.

Enzymes

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. Nonspecific reactions may result in poor product yields. High temperatures and/or high pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures, acidity, or alkalinity need high capital investment, and specially designed equipment and control systems. In a number of cases, some or all of these drawbacks can be virtually eliminated by using enzymes. As we explain in the next section, enzyme reactions may often be carried out under mild conditions, they are highly specific, and involve high reaction rates. Industrial enzymes originate from biological systems; they contribute to sustainable development through being isolated from microorganisms that are fermented using primarily renewable resources.

In addition, as only small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale, both solid and liquid enzyme preparations take up very little storage space. Mild operating conditions enable uncomplicated and widely available equipment to be used, and enzyme reactions are generally easily controlled. Enzymes also reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water and energy, and the subsequent generation of waste.

Among the biocatalysts in organic synthesis, lipases are the most frequently used (Gotor, 1999). In particular, this class of enzyme is able to perform enantioselective hydrolytic reactions. Lipases have been extensively utilized in the synthesis of many biologically active compounds and natural products (Itoh *et al.*, 1993).

Developments in genetic and protein engineering have led to improvements in the stability, economy, specificity, and overall application potential of industrial enzymes. When all the benefits of using enzymes are taken into consideration, it's not surprising that the number of commercial applications of enzymes is increasing every year.

Enzymes are classified according to the guidelines of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) into six major groups, based on the type of chemical reaction catalyzed (Krishna, 2002), as listed below.

1. *Oxidoreductases*: enzymes catalyzing oxidation/reduction reactions that involve the transfer of electrons, hydrogen or oxygen atoms.
2. *Transferases*: enzymes catalyzing the transfer of a functional group from a donor to a suitable acceptor.
3. *Hydrolases:* enzymes catalyzing reactions of hydrolysis, this is, the cleavage of a chemical bond by the action of water.
4. *Lyases*: enzymes catalyzing reactions of non-hydrolytic and non-oxidative cleavage of chemical bonds
5. *Isomerases*: enzymes catalyzing reactions of conversion of a substrate into an isomer.
6. *Ligases*: enzymes catalyzing reactions of covalent linkage of two molecules.

Nature of Enzymes

Enzymes are biological catalysts in the form of proteins that catalyze chemical reactions in the cells of living organisms. In general, these metabolic requirements of enzymes can be defined as:

1. Chemical reactions must take place under the conditions of the habitat of the organisms
2. Specific action by each enzyme
3. Very high reaction rates

How Enzymes Work

At least 21 different hypotheses for how enzymes catalyzed reactions have been proposed (Silverman, 2004). A common link between all these proposals, however, is that an enzyme-catalyzed reaction is always initiated by the formation of an

enzyme-substrate (or E.S) complex, from which the catalysis takes place.The concept of an enzyme-substrate complex was originally proposed independently by Brown in 1902.

Lock-and-key hypothesis

This is the simplest model to represent how an enzyme works. An enzyme is the lock into which the substrate (the key) fits (Figure 1). In this model, the active site of the unbound enzyme is complementary in shape to the substrate (Berg *et al*., 2002). This interaction of the enzyme and substrate will account for a high degree of specificity of enzymes, but the lock-and-key hypothesis does not rationalize certain observed phenomena. For example, compounds whose structures are related to that of the substrate, but with less bulky substituents, often fail to be substrates, even though they could fit into the enzyme. Some compounds with bulkier substituents are observed to bind more tightly to the enzyme than does the substrate. If the lock-and-key hypothesis was correct, one would think that a bulkier compound would not fit into the lock. Some enzymes that catalyze reactions between two substrates do not bind one substrate until the other one is already bound to the enzyme (Silverman, 2004).

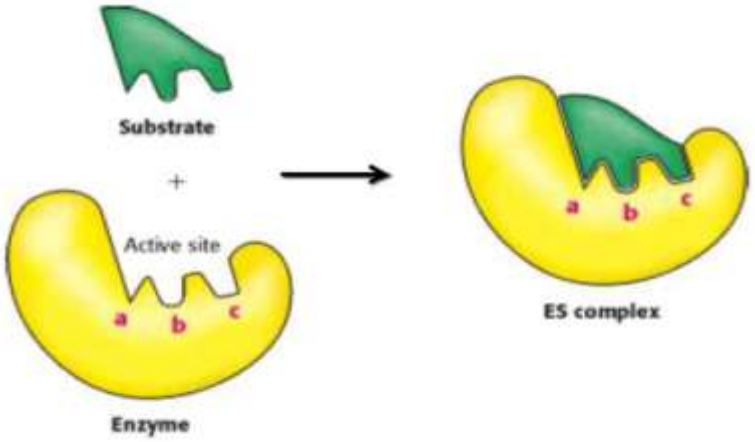


Figure 1: Lock-and-key model of enzyme-substrate binding

Induced-fit hypothesis

In this model, the enzyme molecule changes shape as the substrate molecules gets closer. When a substrate begins to bind to an enzyme, the interactions of various groups on the substrate, with particular enzyme functional groups are initiated, and these mutual interactions induce a conformational change in the enzyme (Figure 2). The active site forms a shape complementary to the substrate only after the substrate has been bound (Berg *et al*., 2002).

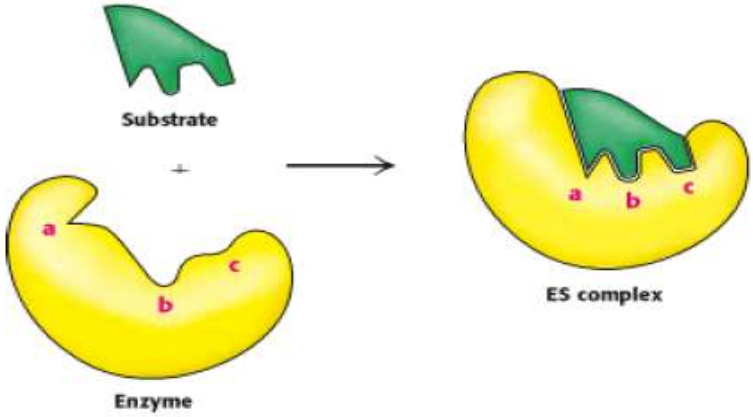


Figure 2: Induced-fit model of enzyme-substrate binding

Immobilized Enzymes

The excellent functional properties (activity, selectivity, and specificity) of enzymes have given them a great potential as industrial catalysts in chemical industry such as fine chemistry, food chemistry, analysis and so on (Koeller and Wong, 2001). For technical and economic reasons, most chemical processes which are catalyzed by enzymes require re-use or continuous use of the biocatalyst for a very long time (Katchalski-Katzir and Kraemer, 2000; Bickerstaff, 1997; Chibata *et al*., 1986). Meanwhile, immobilization of enzymes seems to be the most relevant approach for stabilization and recovery of enzymes. In particular, enzyme immobilization has profound influence on the nature of the catalytic process, which is now heterogeneous in nature, since catalysis occurs in the surface or within a solid structure where the enzyme is located, while substrates and products of reaction are in the liquid reaction medium where its course is being monitored (Illanse *et al*., 2008).

The only major problem of betulinic acid for its future clinical development is its low water solubility (Gauthier *et al*., 2006). However, its vast pharmacological activity aroused the interest of my research especially on cytotoxic activity. Thus the objectives of this research are:

1. To synthesise of betulinic acid glycoside using lipase
2. To characterize the product obtained using spectroscopic techniques
3. To optimize the reaction parameters used in the synthesis using response surface methodology
4. To evaluate the cytotoxic activity of the synthesized compound against cancer cells

REFERENCES

Adnya, I. K., Yasuhiru, T., Suresh, A., Arjun, H. B., Kim, Q. T., Shigetoshi, K. (2000). Quadranosides VII-XI, Six new Triterpenes Glucosides from the seeds of *Combretum quadrangulare*. *Chem. Pharm. Bull*., 48(8): 1 114-1120.

Ahmad, F. B. H.., Ghaffari, M. M., Basri, M., Abdul Rahman, M. B. (2010). Anticancer activity of 3-*O*-Acylated Betulinic acid Derivatives Obtained by Enzymatic Synthesis. *Biosci. Biotechnol. Biochem*., 74 (5): 1025-1 029.

Ahmad, F. B. H.., Ghaffari, M. M., Basri, M., Abdul Rahman, M. B. (2010). Enzymatic synthesis of betulinic acid ester as an anticancer activity agent. Optimization study. *Biocatalysis and Biotransformation*, 28(3): 192-200.

Alakurti, S., Makela, T., Koskimies, S., Y-Li, K. (2006). Pharmacological properties of the ubiquitous natural product betulin. *Eur. J. Pharm. Sci.*, 29: 1-13.

Aminah, Shah, S. M. (2010). *Synthesis and Optimization Study of Lipase Catalyzed Synthesis of 3-O-(3', 3'-Dimethylsuccinyl)-Betulinic acid*, M.Sc. Thesis, Universiti Putra Malaysia, Malaysia.

Aracil, J., Garcia, T. and Martinez, M. (1993). Enzymatic synthesis of an analogue of jojoba oil: Optimisation by statistical analysis. *Enzyme and Microbial Technology*, 15(7): 607-611.

Azim, H., Dekhterma, A., Jiang, Z., Gross, R. A. (2006). *Candida antartica* lipase- catalyzed synthesis of poly (butylen succinate): Shorter chain building blocks also work. *Biomacromolecules* 7: 3093-3097.

Berge, J. M., Tymoczko, J. L., Stryer, L. (2002). *Biochemistry*. New York: W. H. Freeman and Company, 317.

Bickerstaff, G. F. (1997). *Immobilization of enzymes and cells: Methods in biotechnology 1*. Totowa: Humana Press, 367.

Bloomer, S., Adlercreutz, P., Mattiasson, B. (1992). Facile synthesis of fatty acid esters in high yields. *Enzyme and Microbial Technology* 14(7): 546-552.

Carolina, P. R., Marvin, J. N., Ignacio, A. J., Jerome, B., Maria, J. A. and Isabel, L. B. (2006). Activity of lupane-triterpenoids from *Maytenus* species as inhibitors of nitric oxide and prostaglandin E2. *Bioorganic and Medicinal Chemistry* 14: 1573-1579.

Chiang, W. D., Cheng, S. W., Shiah, C. J. (2003). Studies on the optimized biosynthesis of cis-3-hexen-1-yl acetate in n-hexane. *Process Biochem*. 38: 1103-1197.

Chibata, I., Tosa, T. and Sato, T. (1986). Biocatalysis: immobilized cells and enzymes. *Journal of Molecular Catalysis* 37: 1-24.

Copeland, R. A. (2000). Enzymes: A practical introduction to structure, mechanism and data analysis, 2nd Ed. Wiley-VCH, Inc. New York.

Diana, M. C., Gloria, I. G., Carlos, E. O. (2011). Application of response surface design to solvent, temperature and lipase selection for optimal monoglyceride production. *Journal of molecular catalysis*, 72: 13-19.

Dominguez-Carmona, D. B., Escalante-Erosa, K., Garcia-Sosa, G., Ruiz-Pinell, D., Gutierrez-Yapu, Chan-Bacab, M. J., Gimenez-Turba, A. and Peria, L. M. (2010). *Phytomedicine* 17: 379-382.

Erbeldinger, M., Ni, X., Halling, P. J. (1998). Enzymatic synthesis with mainly undissolved substrates at very high concentrations. *Enzyme & Microbial Technology*, 23(1-2): 141-148.

Facioli, N. L. and Barrera-Arellano, D. (2001). Optimisation of enzymatic esterification of soybean oil deoderiser distillate. *Journal of the Science of Food and Agriculture* 81(12): 1193-1198.

Fomuso, L. and Akoh, C. C. (1998). Structured Lipids: Lipase-Catalyzed interesterification of tricaproin and trilinolein. *Journal of American Oil Chemists Society* 75: 405- 410.

Foresti, M.L., Errazu, A., Ferreira, M.L. (2005). Effect of several reaction parameters in the solvent-free ethyl oleate synthesis using *Candida rugosa* lipase immobilised on polypropylene. *Biochemical Engineering Journal*, 25: 69-77.

Fujioka, T., Kashiwada, Y., Kilkuske, R., Cosentino, L. M., Ballas, L.M., Jiang, J.B., Janzen, W. P., Chen, T. S., and Lee, k. H. (1994). Anti-Aids agents, 11. Betulinic acid and platonic acid as an anti-HIV principles from *syzigium clariflorum*, and the anti-HIV activity structure related triterpenoids. *J. Natural Products*, 57: 243- 247.

Fulda, S., Jerimias, I., Debatin, K. M. (2004). Cooperation of betulinic acid and TRAIL to induce apoptosis in tumor cells. *Oncogene* 23**:** 7611-7620.

Fumio, H., Kashiwada, Y., Mark, C. L., Chin-Ho, C., Patricia, E. G., Kuo-Hsiung, L. (1997). Anti-AIDS Agents-XXVII. Synthesis and Anti-HIV Activity of Betulinic Acid and Dihydrobetulinic Acid Derivatives. *Bioorganic & Medicinal Chemistry* 5: 2133-2143.

Gauthier, C. Legault, J., Lebrun, M., Dufour, P., Pichette, A. (2006). Glycosidation of lupane-type triterpenoids as potent in vitro cytotoxic agents. *Biorganic med. Chem.* 14: 6713-6725.

Gauthier, C., Legault, J., Rondeau, S. and Pichette, A. (2009a). Synthesis of betulinic acid acyl glucuronide for application in anticancer prodrug monotherapy. *Tetrahedron Letters*, 50: 988-991.

Gauthier, C., Jean, L., Marianne, P., Serg, L., Samuel, T., Andre-, P. (2009b). Synthesis, cytotoxicity and haemolytic activity of chacotriside lupine-type neosaponins and their germicane-type rearrangement products. *Biorganic and Med. chem. lett*. 19: 2310-2314.

Ghaffari, M. G., Ahmad, F. B. A., Basri, M. And Abdul Rahman, M. B. (2010). Lipsae catalyzed esterification of betulinic acid using phthalic anhydride in organic solvent media: Study of reaction parameters. *Journal of Applied Sciences*, 10, 337-342.

Ghaffari, M. M. (2009). *Response surface methodology (RSM) and Artificial Neural Network (ANN) Analysis for Prediction in the Enzymatic Acylation of Betulinic Acid Using Anhydrides*, PhD Thesis, Universiti Putra Malaysia.

Ghanem, A. (2007). Trends in lipase-catalysed asymmetric access to enantiometrically pure/enriched compounds. *Tetrahedron*, 63, 1721-1754.

Gunawan, E.R. and Dedy, S. (2008). Synthesis of wax esters from kernel oil catalyzed by lipase. *International Journal of Chemistry*, 8(1): 76-83.

Gunawan, E. R., Basri, M., Mohd, B. A. R., Abu, B. S., Raja, N. Z. A. R. (2005). Study on surface methodology (RSM) of lipase-catalyzed synthesis of palm-based wax ester. *Enzyme and Microbial Technology* 37: 739-744.

Haizhen, Z., Yu, Z., Fengxia, L., Xiaomei, B., Zhaoxin, L., Hongmei, N. (2011). Optimized enzymatic synthesis of ascorbyl esters from lard using Novozyme 435 inco-solvent mixtures. *Journal of molecular catalysis B: Enzymatic* 69: 107-111.

Hari Krisna. S., Sattur, A. P., Karant. N. G. (2001). Lipase-catalyzed synthesis of isoamyl isobutyrate optimization using central composite rotatable design. *Process Biochem*. 37: 9-16.

Hamsaveni, D. R., Prapulla, S. G., Divakar, S. (2000). Response surface methodological approach for the synthesis of isobutyl isobutyrate. *Process Biochem*. 36: 1103-1109.

Hasmah, B., Basri, M., Salina, M. R., Arbakariya, A., Raja, N. Z. A. R., Abu, B. S. (2009). Optimization of lipase-catalyzed synthesis of palm amino acid surfactant using response surface methodology (RSM). *Industrial crops and products* 30: 206-211.

Hsiao-Ching, C., Hen-Yi, J., Yao-Kuo, T., Jiann-Hwa, C., Chieh-Ming, J. C., Yuang-Chuan, L., Cheng, C. and Chwen-Jen, S. (2010). Optimized enzymatic synthesis of caffeic acid phenethyl ester by RSM. *New Biotechnology* 27(1): 89-93.

Harish, K., Goran, N. K., Martin, B., Jutta, K., Petra, F., Simone, F., Walter, M., Reinhard, P. (2010). In vitro anti-cancer studies of α- and β-D-glucopyranose betulin anomers. *Chemico-Biological interactions* 185: 128-136.

Hata, K., Hori, k., Ogasawara, H. and Takahashi, S. (2003). Anti- leukaemia action of lup- 28-al-20(29)-en-3-one, a lupine triterpene. *Toxicology letters* 143: 1-7.

Issak, A. (2004). *Preparation of Betulinic Acid using Lipase in Organic Solvents*, Master's Thesis. Universiti Putra Malaysia, Malaysia.

Katchalski-Katzir, E. and Kraemer, D. M. (2000). Eupergit® C, a carrier for immobilization of enzymes of industrial potential. *Journal of Molecular Catalysis: Enzymatic* 10:157-176.

Kim, D. S. H. L., Pezzuto, J. M., Pisha, E. (1998). *Biorg. Med. Chem. Lett*., 8: 1707-1712.

Koeller, K. M., Wong, C. H. (2001). Enzymes for chemical synthesis. *Nature* 409: 232-240.

Linko, Y. Y., Lamsa, M., Huhtala, A., Rantanen, O. (1995). Lipase biocatalysis in the production of esters. *Journal of Americal Oil Chemists Society*, 72(11): 407-411.

Lozano, P., De-Diego, T., Carrie, D., Vaultier, M. and Iborra, J. (2003). Enzymatic ester synthesis in ionic liquids. *Journal of Molecular Catalysis B: Enzymatic*, 21(1-2): 9-13.

Liu, J., Fu, M.L. and Chen, Q.H. (2011). Biotransformation optimization of betulin into betulinic acid production catalyzed by cultured *Armillaria luteo-virens sacc* ZJUQH 100-6 cells. *Journal of Applied Microbiology*, 110: 90-97.

Liu, K. J. (2009). Lipase-catalyzed synthesis and antibacterial activity of N-vanllylnonamide. *Journal of Molecules and Catalysis B: Enzymatic*, 58: 181-186.

Li, H., Phong, H., Kuo-Hsiung, L. and Chin, Ho. (2006). Synthesis and anti-HIV activity of bifunctional betulinic acid derivatives. *Bioorganic and Medicinal Chemistry*, 14: 2279-2289.

Liu, X., Chang, S. and Yook, C. (2006). Lupane-triterpenoids from the leaves of *Acanthopanax gracilistylus*. *Journal of Lanzhou University* (Natural sciences), 42(4): 86-91.

Madan, L. V., Wamik, A. and Shamsher, S. K. (2011). Enzymatic synthesis of isopropyl acetate by immobilized *Bacillus cereus* lipase in organic medium. *Enzyme research*, 2011: 1-7.

Naz, C., Mahiran, B., Mohd, B. A., Atena, A., Abu Bakar, S. (2012). Lipase-catalysed synthesis of ergosterol ester. *Biocatalysis and Agricultural Biotechnology* 1: 51-56.

Newmann, D. J., Cragg, G. M., Snader, K. M. (2003). Natural products as sources of new drugs over the periods 1981-2001. *J. Nat. prod*. 66**:** 1022-1037.

Nguyen, X., Phan,V. K., Chau, V. M., Bhui, H. T., Nguyen, H. T., DO, T. H., Kwang, S. S., Jun, H. K., Ji -Young, A., Young-Mi, L., Young, H. K. (2010). Structure-activity relationship of lupane triterpene glycosides from *Acanthopanax koreanum* on spleen lymphocyte IL-2 and IFN-γ. *Biooganic and medicinal chemistry letters,* 20: 4927-493.

Nguyen, X., Nguyen, H.T., Phan, V. K., Chau, V. M., Yan Ding, Jae-Hyun, Hee-Kuyong., Young, H. K. (2009). Lupane triterpene glycosides from *Acanthopanax koreanum* and their cytotoxicity activity. *Chemical Pharmaceutical Bulletin*, 57(9): 986-989.

Noriyuki, D., Hiroyasu, O.(2010).Organic solvent-tolerant enzymes. *Biochemical Engineering* 48: 270-282.

Paul, A. C. and Cass-Imir, C. A. (1994). Effect of reaction parameters on SP435 lipase- catalyzed synthesis of citronellyl acetate in organic solvent. *Enzyme microb. Technol*., 16: 835.

Pawel, S., Marci, N, D., Verena, M., Manfred, D. and Hermann, L. (2009). Betulinic acid exhibits stronger cytotoxic activity on the normal melanocyte NHEM-neo cell than on the drug-resistant MeWo melanoma cell lines. *Molecular medicine reports* 2: 543-548.

Piotr, C., Zbigniew, P., Jana, S., Miroslav, S. (2008). Synthesis of lupane-type saponins bearing mannosyl and 3, 6-branched trimannosyl residues and their evaluation as anticancer agents. *Carbohydrates Research* 343: 995-1003.

Pisha, E., Chai, H., Lee, I. S., Chagwedera, T. E., Farnsworth, N.R., Cordell., G. A., Beecher, C. W, Fong, H. H., Kinghorn, A. D. and Brown, A. 1995). Discovery of betulinic acid as a selective inhibitor of human melanoma that function by induction of apoptosis. *Nat. Med*. 1: 1046-1051.

Raymond H. M., Douglas C. M., Christine, M. A. (2009). Response Surface Methodology: Process and Product Optimization Using Designed Experiments, Third Edition. John Wiley & Sons, Inc.1.

Rita, C. S., Jorge, A. R. Silvia, M., Marta, C. M., Jiao, N. M. and Teresa, C. P. D. (2010). Synthesis and structure-activity relationship study of novel cytotoxic carbamate and N- acylheterocyclic bearing derivatives of betulin and betulinic acid. *Bioorganic and medicinal chemistry* 18: 4385-4396.

Robert, H. C. and Samir, A. K. (2004). Chemistry, Biological Activity and Chemotherapeutic Potential of Betulinic Acid for the Prevention and Treatment of Cancer and HIV Infection. *Medicinal Research Review* 24(1): 90-114.

Robertson, D. E. and Bornscheuer, U. T. (2005). Biocatalysis and biotransformation: New technologies, enzymes and challenges. *Current Opinion in Chemical Biology* 9: 164-165.

Seong-Cheol, B. Kim, Y. Lee J. H., Ahn, B. Z. (2005). Triterpenoid Saponins from the Roots of *Pulsatilla koreana*. *J. Nat. Prod.* 68**:** 268-272.

Shu-Wei, C., Jei-Fu, S. and Chwen-Jen, S. (2003). Optimization of Enzymatically Prepared Hexyl Butyrate by Lipozyme IM-77. *Food Technol*. *Biotec hnol*. 41(3): 237-243.

Silverman, R.B. (2004). *The organic chemistry of drug design and drug action*. USA: Academic Press. pp. 173-188.

Smith, T.A. (1999). Facilitative glucose transporter expression in human cancer tissue. *British Journal of Biomedical Science* 5: 285–292.

Soo, E. L., Salleh, A. B., Basri, M., Rahman, A., Kamaluddin, K. (2004). Response surface methodological study on lipase-catalyzed synthesis of amino acids surfactants. Process Biochemistry 39: 1511-1518.

Stefania, M., Maria, L. B., Fracesca, F., Charles, E., Antonio, E. G. S., Cosimo, P. (2010). Effects of triterpenes derivatives from *Maytenus rigida* on VEGF induced kaposis sarcoma cell proliferation. *Chemico-Biological interactions* 183: 450- 454.

Syamsul, K.M.W., Salina, M.R., Siti, S.O. and Hanina, M.N. (2010). Green synthesis of Laurel Palmitate *via* Lipase-Catalyzed reaction. *World Applied Science Journal* 11(4): 401- 407.

Thakur, C., Srivastava, V. C. and Mall, I. D. (2008). Electrochemical treatment of a distillery waste water: Parametric and residue disposal study. *Chemical Engineering Journal* 148: 496-505.

Tolstikov, G. A., Flekhter, O. B., Shultz, E.E., Baltina, L. A., Tolstikov, A.G. (2005). Betulin and its derivatives. Chemistry and Biological activity. *Chemistry for sustainable development* 13**:** 1-29.

Vivek, K., Nidhi, R., Pawan, A., Vinod, K. S., Anu, T. S., Manu, J., Narenda, J., Pramod, K. S., Raghuveer, I. and Anand, C. B. (2008). *Bioorganic and Medicinal Chemistry letters* 18: 5058-5062.

Wen-Yong, L., Min-Hua, Z., Yu-Ying, L., Jurfang, W. (2006). Efficient enentioselective hydrolysis of D-L-phehylglycine ester catalyzed by immobilized *Candida antartica* lipase B in ionic liquid containing systems. *Journal of Biotechnology*, 125(1), 64-74.

Wick, W., Grimmel, C., Wagenknecht, B., Dichgans, J., Weller, M. (1999). Betulinic acid induced apoptosis in glioma cells. A sequential requirement for new protein synthesis, format of reactive oxygen species, and caspase processing. *Journal of pharmacology and Experimental Therapeutics* 289: 1306-1312.

Yasin, Y., Basri, M., Ahmad, F., Salleh, A. B. (2008). *J. Chem. Technol. Biotechnol*., 83**:** 694-698.

Yasin, Y. (2004). *Optimization of Lipase Catalyzed Synthesis of 3-O-Benzoylbetulinic acid*, PhD Thesis, Universiti Putra Malaysia, Malaysia.

Ying, W., Chao-Mei, M., De-Yuan, C., Masao, H. (2008). Anti- HIV-1 protease triterpenoids from *Stauntonia obovatifoliola Hayata subs. intermedia*, *Phytochemistry* 69: 1875-1879.

Yuan, X., Liu, J., Zeng, G., Shi, J., Tong, J., Huang, G. (2008). Optimization of conversion of waste rapeseed oil with high FFA to biodiesel using response surface methodology. *Renewable Energy* 33: 1678-1684.