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# CYTOTOXICITY OF *Eurycoma longifolia*, *Nigella sativa* AND *Hibiscus sabdariffa* ON CERVICAL AND OVARIAN CANCER CELL LINES

BY

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# ABSTRACT

Cervical and ovarian cancers are the lethal gynaecological malignancies that affect the local health scene among women. *Eurycoma longifolia, Nigella sativa* and *Hibiscus sabdariffa* are widely used as herbal remedies and natural supplements in Malaysia. The current study is designed to investigate the cytotoxic effects of standardised quassinoid *E. longifolia* (TAF 273), *N. sativa* (NSE) and *H. sabdariffa* (HSE) extracts on cervical cancer (HeLa) and ovarian cancer (Caov-3) cells and the mechanism of cell death. The cytotoxicity of TAF 273, NSE, and HSE were evaluated using MTT assay and the mode of cell death was detected by Hoechst 33258 nuclear staining. The analysis of apoptosis and cell cycle arrest were assessed via flow cytometry. The gene expression of *Bax, Bcl-2* and *caspase-3* were investigated through real-time PCR. The findings showed that TAF 273 suppressed the cell growth of Caov-3 with the lowest $IC_{50}$ value (5.3µg/mL), comparable to the cisplatin ($IC_{50}$ value of 6.07 ± 1.87 µM; ~6.1 µM). The $IC_{50}$ values of TAF 273 on HeLa as well as NSE and HSE on both Caov-3 and HeLa were however not identified. TAF 273 and cisplatin showed no toxicity on normal human ovarian surface epithelial cells (nHOSEpiC). The characteristics of apoptosis such as cell shrinkage, chromatin condensation and nuclei fragmentation were found following TAF 273 and cisplatin treatment. The flow cytometry analyses showed that TAF 273 and cisplatin induced the apoptosis on Caov-3 in dose- and time-dependent manner and arrested the cell cycle at $G_2$ phase. The real-time PCR results showed that TAF 273 down-regulated the *Bax, Bcl-2,* and *caspase-3* expression. Meanwhile, cisplatin up-regulated the *Bax* and *caspase-3* and down-regulated the *Bcl-2* expression. Results from this study suggests that TAF 273 was cytotoxic on Caov-3 cells through the induction of the apoptosis via the mitochondrial pathway, initiated by proteins other than *Bax* and it was executed through other group of executioner caspase or via caspase-independent pathway. The output of this study may indicate the potential of the standardised quassinoid of *Eurycoma longifolia* extract in the successful treatment of ovarian cancer as part of cancer therapy.

# خلاصة البحث


سرطان عنق الرحم وسرطان المبيض هي أورام خبيثة قاتلة تؤثر على الصحة المحلية بين النساء. يتم استخدام اليوركوما لونغيفوليا، والنايجيلا ساتيفا، والهيبيسكوس سابادريفيا على نطاق واسع كعلاجات عشبية ومكملات غذائية طبيعية في ماليزيا. تم تصميم الدراسة الحالية للتحقيق في السمية الخلوية لليوركوما لونغيفوليا (TAF 273) والنايجيلا ساتيفا (NSE)، والهيبيسكوس سابادريفيا (HSE) ضد سرطان عنق الرحم (HeLa) وسرطان المبيض (Caov-3) وكيفية آلية موت الخلايا التي تؤثر عليها. تم تقييم السمية الخلوية لـ TAF 273، وNSE، وHSE باستخدام فحص MTT، وتم الكشف عن آلية موت الخلية بواسطة التلوين النووي Hoechst 33258. تم تقييم تحليل موت الخلايا المبرمج ودورة الخلية عن طريق اختبار التدفق الخلوي. تم التحقيق في التعبير الجيني لـ *Bax*، و*Bcl-2*، و *caspase-3* باستخدام PCR الوقت الحالي. أظهرت النتائج أن TAF 273 قامت بتثبيط نمو خلايا Caov-3 بأقل معدل لل $IC_{50}$ (5.3 مكروغرام/مل) مقارنة مع السيسبلاتين (قيمة $IC_{50}$ حوالي6.1 مكرومولار). لم يتم تحديد قيم الـ$IC_{50}$ للـTAF 273 على HeLa و NSE و HSE على كل من Caov-3 و HeLa. لم تظهر TAF 273 والسيسبلاتين أي سمية على الخلايا الطلائية للمبيض البشري الطبيعية (nHOSEpiC). تمت ملاحظة صفات موت الخلايا المبرمج مثل الانكماش الخلوي وتكثف الكروماتين وتجزئة النواة بعد علاج الخلايا بالسيسبلاتين والـTAF 273. أظهرت تحاليل التدفق الخلوي أن TAF 273 والسيسبلاتين سببا موت الخلايا المبرمج على Caov-3 بطريقة تعتمد على الجرعة والوقت وأوقفت دورة الخلية في المرحلة G2. أظهرت نتائج PCR الوقت الحالي أن TAF 273 قد قام بالتنظيم التخفيضي لبروتينات *Bax*، و*Bcl-2*، و caspase-3، في حين قام السيسبلاتين بالتنظيم الرفعي لـ *Bax*، و *caspase-3*، و *Bcl-2*. أشارت نتائج هذه الدراسة إلى أن TAF 273 كان ساما خلويا على Caov-3 عن طريق تنشيط موت الخلايا المبرمج عبر مسار الميتوكوندريا والتي تم بدؤها من قبل جينات أخرى غير *Bax* وتم تنفيذها من خلال المسار المستقل عن الـcaspase. تشير نتائج هذه الدراسة إلى إمكانية مركب الكوسينويد المعياري لمستخلص اليوريكوما لونغيفوليا في معالجة سرطان المبيض بنجاح وذلك كجزء من علاج السرطان.

# APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

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# DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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# TABLE OF CONTENTS

Abstract ..... ii
Abstract in Arabic… ..... iii
Approval page ..... iv
Declaration ..... vi
Copyright page ..... vii
Acknowledgements ..... viii
Table of contents ..... ix
List of figures ..... xiii
List of tables ..... xvii
List of equations ..... xviii
List of abbreviations ..... xix
List if symbols… ..... xxi

**CHAPTER ONE: INTRODUCTION ..... 1**
1.1 Background of the Study ..... 1
1.2 Problem Statement ..... 3
1.3 Significance of the Study ..... 4
1.4 Research Questions ..... 6
1.5 Research Objectives ..... 6
1.5.1 General Objective ..... 6
1.5.2 Specific Objectives ..... 6
1.6 Flowchart of Work ..... 7
1.7 Research Hypothesis ..... 9

**CHAPTER TWO: LITERATURE REVIEW ..... 13**
2.1 Cancer ..... 10
2.1.1 Cervical Cancer ..... 11
2.1.2 Ovarian Cancer ..... 15
2.2 Natural products ..... 19
2.2.1 *Eurycoma longifolia* ..... 19
2.2.1.1 General information ..... 19
2.2.1.2 Active constituents ..... 19
2.2.1.3 Physiological effects ..... 22
2.2.1.4 Anticancer effects ..... 22
2.2.2 *Nigella sativa* ..... 23
2.2.2.1 General information ..... 23
2.2.2.2 Active Constituents ..... 26
2.2.2.3 Physiological effects ..... 26
2.2.2.4 Anticancer effects ..... 27
2.2.3 *Hibiscus sabdariffa* ..... 28
2.2.3.1 General information ..... 28
2.2.3.2 Active constituents ..... 28
2.2.3.3 Physiological effects ..... 30
2.2.3.4 Anticancer effects ..... 30
2.3 Treatment for Cancer ..... 31

2.3.1 Cisplatin ..... 32
2.3.1.1 Medical uses ..... 32
2.3.1.2 Side effects ..... 34
2.3.1.3 Mechanisms of action ..... 35
2.4 Mechanisms of Programmed Cell death ..... 39
2.4.1 Apoptosis ..... 37
2.4.1.1 Extrinsic pathway ..... 38
2.4.1.2 Intrinsic pathway ..... 38
2.4.1.3 Caspase ..... 39

**CHAPTER THREE: METHODOLOGY ..... 42**
3.1 Materials ..... 45
3.1.1 Chemicals and Reagents ..... 45
3.1.2 Consumables ..... 45
3.1.3 Laboratory Equipment and Apparatus ..... 45
3.2 Plant Materials ..... 49
3.2.1 *Eurycoma longifolia* ..... 49
3.2.2 *Nigella sativa* ..... 49
3.2.3 *Hibiscus sabdariffa* ..... 47
3.2.4 Preparation of stock and working solutions for TAF 273, NSE, HSE, and Cisplatin. ..... 47
3.3 Cell Culture ..... 48
3.3.1 Cell and Medium Preparation ..... 48
3.3.2 Thawing, Growing and Maintaining Cells ..... 53
3.3.3 Passaging Cells ..... 54
3.3.4 Cell Counting ..... 55
3.3.5 Plating Cell ..... 56
3.4 Cell Viability Assay ..... 57
3.5 Hoechst 33258 Staining ..... 58
3.6 Apoptosis Analysis ..... 59
3.7 Cell Cycle Arrest Analysis ..... 57
3.8 Real-time Polymerase Chain Reaction ..... 58
3.8.1 RNA Extraction ..... 58
3.8.1.1 Quantification of RNA ..... 59
3.8.1.2 Purity and Integrity of RNA ..... 59
3.8.2 Primers and Housekeeping Genes ..... 63
3.8.3 Reverse Transcription (cDNA Synthesis) ..... 65
3.8.4 Optimisation of Real-time PCR ..... 69
3.8.4.1 Optimum Annealing Temperature (Ta) ..... 69
3.8.4.2 Melting Curve Analysis ..... 68
3.8.4.3 Standard Curve ..... 68
3.8.4.4 Control ..... 68
3.8.5 Quantitative Real-time PCR ..... 69
3.8.5.1 Relative Quantification and Normalisation ..... 75
3.9 Statistical Analysis ..... 76

**CHAPTER FOUR: RESULTS AND FINDINGS ..... 77**
4.1 Cell Viability assay ..... 77
4.1.1 Cell Viability of Cervical Cancer Cell Line (HeLa) ..... 77

4.1.1.1 Cell Viability of HeLa treated with *E. longifolia* Extract (TAF 273) ..... 77
4.1.1.2 Cell Viability of HeLa treated with *N. sativa* Extract (NSE) ..... 77
4.1.1.3 Cell Viability of HeLa treated with *H. sabdariffa* Extract (HSE)..... 77
4.1.1.4 Cell Viability of HeLa treated with Cisplatin..... 77
4.1.2 Cell Viability of Ovarian Cancer Cell Line (Caov-3)..... 83
4.1.2.1 Cell Viability of Caov-3 treated with *E. longifolia* Extract (TAF 273)..... 83
4.1.2.2 Cell Viability of Caov-3 treated with *N. sativa* Extract (NSE) 83
4.1.2.3 Cell Viability of Caov-3 treated with *H. sabdariffa* Extract (HSE)..... 83
4.1.2.4 Cell Viability of Caov-3 treated with Cisplatin..... 83
4.1.3 Cell Viability of Normal Human Ovarian Surface Epithelial Cell Line (nHOSEpiC)..... 89
*4.1.3.1* Cell Viability of nHOSEpiC treated with *E. longifolia* Extract (TAF 273) ..... 89
4.1.3.2 Cell Viability of nHOSEpiC treated with Cisplatin ..... 89
4.1.4 Summary Table of $IC_{50}$ Values..... 89
4.2 Cell Presentation..... 94
4.3 Hoechst 33258 staining..... 96
4.4 Assessment of Apoptosis..... 98
4.4.1 Apoptosis Analysis of Caov-3 after 48 h of Treatment..... 98
4.4.1.1 Untreated Caov-3 after 48 h ..... 98
4.4.1.2 Caov-3 treated with TAF 273 after 48 h..... 98
4.4.1.3 Caov-3 treated with Cisplatin after 48 h..... 98
4.4.2 Apoptosis Analysis of Caov-3 after 72 h of Treatment..... 103
4.4.2.1 Untreated Caov-3 after 72 h ..... 103
4.4.2.2 Caov-3 treated with TAF 273 after 72 h..... 103
4.4.2.3 Caov-3 treated with Cisplatin after 72 h..... 103
4.4.3 Apoptosis Analysis ..... 108
4.5 Assessment of Cell Cycle Arrest ..... 110
4.5.1 Cell Cycle Distribution of Caov-3 after 72 h of Treatments ..... 110
4.5.1.1 Untreated Caov-3 after 72 h ..... 110
4.5.1.2 Caov-3 treated with TAF 273 after 72 h..... 110
4.5.1.3 Caov-3 treated with Cisplatin after 72 h..... 110
4.5.2 Cell Cycle Analysis ..... 115
4.6 Real-Time PCR..... 117
4.6.1 RNA Integrity ..... 117
4.6.2 Standard Curve, Melting Curve and Amplification Efficiency... 119
4.6.2.1 *GAPDH*..... 119
4.6.2.2 *B-Actin*..... 119
4.6.2.3 *Bax*..... 119
4.6.2.4 *Bcl-2* ..... 119
4.6.2.5 *Caspase-3* ..... 119
4.6.3 Quantification of Apoptosis Regulatory Proteins..... 125

**CHAPTER FIVE: DISCUSSION** ..... **127**
5.1 Cell Viability ..... 127
5.1.1 Cell viability of normal human ovarian surface epithelial cell line (nHOSEpiC) ..... 127
5.1.2 Criteria of selection the best $IC_{50}$ value ..... 128
5.1.3 Cervical (HeLa) and ovarian (Caov-3) cancer cell viability ..... 129
5.1.4 Effects of *E. longifolia* root extract (TAF 273) ..... 129
5.1.5 Effects of *H. sabdariffa* extract (HSE) ..... 131
5.1.6 Effects of *N. sativa* extract (NSE) ..... 132
5.1.7 Cell viability of cells after treatment with cisplatin ..... 133
5.1.8 Comparison between TAF 273, HSE and NSE ..... 134
5.2 Cell presentation ..... 134
5.3 Characteristic features of apoptosis ..... 135
5.4 Flow Cytometry analysis ..... 136
5.4.1 Apoptosis analysis ..... 136
5.4.2 Cell cycle analysis ..... 137
5.5 Expressions of *Bax, Bcl-2* AND *Caspase-3* ..... 138
5.5.1 RNA integrity and selection of SYBR Green ..... 138
5.5.2 Expression of *Bax* and *Bcl-2* ..... 139
5.5.3 Expression of *caspase-3* ..... 140

**CHAPTER SIX: CONCLUSION** ..... **144**
6.1 Conclusions ..... 144
6.1.1 Limitations and Future Recommendations ..... 146

**REFERENCES** ..... **148**

**APPENDIX A** ..... **164**
**APPENDIX B** ..... **165**
**APPENDIX C** ..... **166**
**APPENDIX D** ..... **167**
**APPENDIX E** ..... **168**
**APPENDIX F** ..... **169**
**APPENDIX G** ..... **170**
**APPENDIX H** ..... **171**

# LIST OF FIGURES


| Figure No. | | Page No. |
|---|---|---|
| **1.1** | The flowchart of work in this study. | 8 |
| **2.1** | The structure of cervical cancer cell line (HeLa) sourced from ATCC. The ATCC number of this cell is CCL-2. Figure A show the structure of the cells in low density while Figure B show the cells in high density. (Scale bar = 100 µm). | 14 |
| **2.2** | The structure of ovarian cancer cell line (Caov-3) sourced from ATCC. The ATCC number of this cell is HTB-75. Figure A show the structure of the cells in low density while Figure B show the cells in high density. (Scale bar = 100 µm). | 18 |
| **2.3** | *E. longifolia* root (A) adapted from website Nutrient Journal, 2017 and its active components (B) (self-drawed). | 21 |
| **2.4** | *N. sativa* seeds (A) (self-photographed) and the structure of thymoquinone (B) (self-drawed). | 25 |
| **2.5** | Roselle (*H. sabdariffa*) (A) and the structure of anthocyanin (B) (reproduced with permission from (Abou-Arab, Abu-Salem, & Abou-Arab, 2011)). | 29 |
| **2.6** | Chemical structure of cisplatin (self-drawed). | 33 |
| **2.7** | Extrinsic and intrinsic pathways of apoptosis (Illustration is reproduced with permission of Wali, Masters, & Thomas, 2013). The intrinsic pathway is activated by cellular stress and lead to the activation of the BH3-only members of the Bcl-2 family (such as *Bim* and *Puma*). This initiates the apoptosis signaling by binding to Bcl-2 and Bcl-xL and release of *Bax* and/or *Bak*. This promotes the loss of mitochondrial membrane potential and triggers the release of *cytochrome c* and activates *caspase-9*, *caspase-3* and eventually lead to apoptosis. The extrinsic pathway is initiated by the activation of *Fas*. This promotes a death-inducing signaling complex in which *caspase-8* is activated by its adaptor Fas-associated death domain (*FADD*). Eventually, this activates the caspase cascade and apoptosis. | 41 |

| | | |
|---|---|---|
| **4.1** | HeLa at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value 838.47 ± 27.7 µg/mL), and 72 h ($IC_{50}$ value NIL) following treatment with TAF 273. Values are expressed as mean ± SD (n = 3). | 75 |
| **4.2** | HeLa at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value NIL), and 72 h ($IC_{50}$ value NIL) following treatment with NSE. Values are expressed as mean ± SD (n = 3). | 76 |
| **4.3** | HeLa at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value 625.54 ± 156.31 µg/mL), and 72 h ($IC_{50}$ value 586.1 ± 137.34 µg/mL) following treatment with HSE. Values are expressed as mean ± SD (n = 3). | 77 |
| **4.4** | HeLa at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value 5.41 ± 2.2 µM), and 72 h ($IC_{50}$ value 4.92 ± 1.6 µM) following treatment with cisplatin. Values are expressed as mean ± SD (n = 3). | 78 |
| **4.5** | Caov-3 at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value 56.1 ± 2.6 µg/mL), and 72 h ($IC_{50}$ value 5.3 ± 0.6 µg/mL) following treatment with TAF 273. Values are expressed as mean ± SD (n = 3). | 81 |
| **4.6** | Caov-3 at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value NIL), and 72 h ($IC_{50}$ value NIL) following treatment with NSE. Values are expressed as mean ± SD (n = 3). | 82 |
| **4.7** | Caov-3 at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value 866.66 ± 102.63 µg/mL), and 72 h ($IC_{50}$ value 666.41 ± 106.02 µg/mL) following treatment with HSE. Values are expressed as mean ± SD (n = 3). | 83 |
| **4.8** | Caov-3 at 24 h ($IC_{50}$ value NIL), 48 h ($IC_{50}$ value NIL), and 72 h ($IC_{50}$ value 6.07 ± 1.87 µM) following treatment with cisplatin. Values are expressed as mean ± SD (n = 3). | 84 |
| **4.9** | nHOSEpiC at 24, 48 and 72 h following treatment with TAF 273 (No $IC_{50}$ values but cell viability of 83.9 ± 2.7%, 81.02 ± 6.4% and 73.73 ± 9.7% at 100 µg/ml respectively). Values are expressed as mean ± SD (n = 3). | 87 |
| **4.10** | nHOSEpiC at 24, 48 and 72 h following treatment with cisplatin (No $IC_{50}$ values but cell viability of 77.85 ± 6.3%, 86.82 ± 5.2% and 80.51 ± 2.9% at 10 µM respectively). Values are expressed as mean ± SD (n = 3). | 88 |

| | | |
|---|---|---|
| **4.11** | The adherence of Caov-3, HeLa and nHOSEpiC following exposure to TAF 273 (5.3 µg/mL) and cisplatin (6.1 µM) for 72 h. The red arrows show the space between cells grow bigger than the untreated groups while the yellow arrows show the shrinkage of the cells (4×, scale bar = 200 µm, Evos Life Technologies). | 92 |
| **4.12** | Apoptotic nuclear changes observed in (A) untreated Caov-3, (B) Caov-3 treated with TAF 273 (5.3 µg/mL) and (C) Caov-3 treated with cisplatin (6.1 µM) for 72 h. Caov-3 cells stained with Hoechst 33258 (10 µg/mL) were observed with fluorescence imager under magnification of 20×. | 93 |
| **4.13** | Flow cytometry measurements of apoptosis of untreated Caov-3 after 48 h of incubation. | 96 |
| **4.14** | Flow cytometry measurements of apoptosis of Caov-3 treated with TAF 273 (5.3 µg/mL) for 48 h. | 97 |
| **4.15** | Flow cytometry measurements of apoptosis of Caov-3 treated with cisplatin (6.1 µM) for 48 h. | 98 |
| **4.16** | Flow cytometry measurements of apoptosis of untreated Caov-3 after 72 h of incubation. | 101 |
| **4.17** | Flow cytometry measurements of Caov-3 treated with TAF 273 (5.3 µg/mL) for 72 h. | 102 |
| **4.18** | Flow cytometry measurements of apoptosis of Caov-3 treated with cisplatin (6.1 µM) for 72 h. | 103 |
| **4.19** | Percentage of apoptosis by Annexin V-PE /7-AAD assay of Caov-3 treated with 5.3 µg/mL TAF 273 and 6.1 µM cisplatin. Statistical analysis was performed using one-way ANOVA. Values are expressed as mean ± SD. *$p$ < 0.05 significantly different compared to untreated cells. | 106 |
| **4.20** | The cell cycle distribution of untreated Caov-3 after 72 h of incubation. | 108 |
| **4.21** | The cell cycle distribution of of Caov-3 treated with TAF 273 (5.3 µg/mL) for 72 h. | 109 |
| **4.22** | The cell cycle distribution of Caov-3 treated with cisplatin (6.1 µM) for 72 h. | 110 |
| **4.23** | Cell cycle analysis by Propidium iodide staining of Caov-3 treated with TAF 273 (5.3 µg/mL) and cisplatin (6.1 | 113 |

| | | |
|---|---|---|
| | µM) for 72 h. Statistical analysis was performed using one-way ANOVA. Values are expressed as mean ± SD. *$p$ < 0.05 significantly different compared to untreated group. | |
| **4.24** | The RNA integrity test of untreated Caov-3 and Caov-3 treated with TAF 273 (5.3 µg/mL) and cisplatin (6.1 µM) for 72 h using agarose gel in 1× TAE at 100V for 120 min. | 115 |
| **4.25** | The optimisation of *GAPDH*. | 117 |
| **4.26** | The optimisation of *β-actin*. | 118 |
| **4.27** | The optimisation of *Bax*. | 119 |
| **4.28** | The optimisation of *Bcl-2*. | 120 |
| **4.29** | The optimisation of *caspase-3*. | 121 |
| **4.30** | Expression level of pro-apoptotic protein; *Bax*, anti-apoptotic protein; *Bcl-2* and executioner caspase; *caspase-3* in Caov-3 cell line treated with TAF 273 (5.3 µg/mL) and cisplatin (6.1 µM) for 72 h which were normalized with untreated Caov-3 cells. Statistical analysis was performed using one-way ANOVA. Values are expressed as mean ± SD. *$p$ < 0.05 significantly different compared to control group. | 123 |
| **5.1** | Suggested illustration of pathway how TAF 273 induced apoptosis in Caov-3. "Sensitizers" from BH3-only pro-apoptotic proteins bind directly to *Bcl-2* anti-apoptotic protein (solid line). The binding triggered the inhibition of *Bcl-2* proteins without the involvement of *Bax* (dashed line). The decrease of Bcl-2 level may play a positive role in increasing the susceptibility of the cells to undergo apoptosis without the involvement of caspase through caspase-independent pathway. | 139 |

# LIST OF TABLES


| Table No. | | Page No. |
|---|---|---|
| **3.1** | List of general chemicals and reagents. | 43 |
| **3.2** | List of consumables items. | 44 |
| **3.3** | List of equipment and apparatus. | 45 |
| **3.4** | List of cell lines used. | 49 |
| **3.5** | The list of primers. | 61 |
| **3.6** | The genomic DNA removal reaction. | 63 |
| **3.7** | The Reverse Transcription reaction components. | 64 |
| **3.8** | The steps of Reverse Transcription reaction. | 65 |
| **3.9** | The components for optimising annealing temperature. | 67 |
| **3.10** | The reaction mix of quantitative real-time PCR. | 70 |
| **3.11** | The conditions of quantitative real-time PCR. | 71 |
| **4.1** | The summary of $IC_{50}$ values. | 90 |

# LIST OF EQUATIONS


| Equation No. | | Page No. |
|---|---|---|
| **3.1** | The formula was used to calculate the desired samples concentration. | 48 |
| **3.2** | The formula for cell density calculation. | 52 |
| **3.3** | The formula to calculate the percentage of cell viability. | 55 |
| **3.4** | The formula to calculate the average threshold cycle ($C_T$). | 72 |
| **3.5** | The formula to calculate the difference between the $\Delta C_T$ values of treated samples and untreated samples. | 72 |
| **3.6** | The formula to calculate the fold change. | 72 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| TAF 273 | standardized quassinoid-rich *Eurycoma longifolia* extract |
| NSE | *Nigella sativa* extract |
| HSE | *Hibiscus sabdariffa* extract |
| Caov-3 | ovarian cancer cell line |
| SKOV-3 | ovarian cancer cell line |
| HeLa | cervical cancer cell line |
| nHOSEpiC | normal Human Ovarian Surface Epithelial Cell Line |
| DNA | deoxyribonucleic acid |
| HIV | human immunodeficiency virus |
| DMEM | Dulbecco's Modified Eagle Medium |
| RPMI-1640 | Roswell Park Memorial Institute-1640 Medium |
| FBS | Fetal Bovine Serum |
| PenStrep | Penicillin-Streptomycin |
| PBS | Phosphate Buffered Saline |
| DMSO | dimethyl sulfoxide |
| MTT assay | 3-[4,5-dimethylthiazol-2-yl]-2,diphenyltetrazolium bromide assay |
| $IC_{50}$ | inhibitory concentration by half |
| CV | cell viability |
| PE | phycoerythrin |
| 7-AAD | 7-Aminoactinomycin D |
| PCR | polymerase chain reaction |
| RNA | ribonucleic acid |

| | |
|---|---|
| NIL | none |
| w/w | concentration |

# LIST OF SYMBOLS

| | |
|---|---|
| & | And |
| ˚C | Degree Celcius |
| < | Less than |
| µg | Microgram |
| µL | Microliter |
| µM | Micromolar |
| mL | Milliliter |
| µg/mL | Microgram/microliter |
| et al., | (et alia): and others |
| H | Hour |

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND OF THE STUDY

Cancer is a group of disease characterised by the unbalanced cell growth and death of cell together with the spread of abnormal cell. It is the major cause of fatality worldwide. The incidence and mortality reports due to cancer have been increasing despite the advancement in understanding and treating other diseases. It is estimated that in 2018, there will be 18.1 million new cancer cases and 9.6 million cancer deaths worldwide (Bray et al., 2018). According to the newest version of International Agency for Research on Cancer (IARC) under World Health Organization (WHO), in the report Globocan (2012), 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people were diagnosed with cancer in 2012 globally. Rate is the number of new cases or deaths per 100,000 persons per year. Meanwhile, the age-standardised rate (ASR) is defined as the rate that a population had as a standard age structure. The overall age-standardised cancer incidence rate is reported to be 25 % higher among men as compared women. In Malaysia, almost 40,000 new cases were detected in 2012 of whereby 18,000 new cases were reported from male cancer patients and 19,300 new cases were reported from female cancer patients. Around 22,000 cancer deaths were reported for both sexes in 2012 in Malaysia. From that figure, 11,300 deaths were reported from male cancer patients and 10,400 deaths were reported from female cancer patients. The ASR in males were 144.9/100,000 and 143.4/100,000 in females. Based on the ASR reported by IARC in 2012, the top 10 leading cancer for Malaysia population were the breast, colorectal, lung, cervix uteri, prostate, ovary, stomach,

nasopharynx, liver and corpus uteri. The top 5 most frequent cancer among Malaysian females reported by IARC were breast, colorectal, cervix uteri, lung and ovary cancers (IARC, 2012).

Gynaecological malignancies contribute to the high cases of fatality among women in Malaysia. Globally, in 2012 cervical cancer is ranked as the fourth most common cancer among women and the seventh for overall cancer with almost 530,000 new cases and 265,672 death reported. At the same time, the ovarian cancer is ranked as the seventh most common cancer among women and the ninth for overall cancer with 238,719 number of incidence and 151,917 death reported (IARC, 2012). In Malaysia, cervical cancer is the third leading cancer with 2,145 number of incidence and 621 mortality cases reported while ovarian cancer is the fifth leading cancer with 1,098 incidence and 645 mortality cases reported in 2012 (Globocan, 2012). Globally, in 2018, it is estimated that there will be almost 600,000 new cases and 311,000 number of deaths due to cervical cancer. While almost 300,000 new cases and 185,000 number of deaths will be reported due to ovarian cancer (Bray et al., 2018). Of all gynaecological malignancies, epithelial ovarian cancer is the most lethal type of cancer in women with the worst prognosis (Manimaran & Rajneesh, 2009; Johari, Sidek, Othman, & Anuar, 2013). With the high incidence cases reported, the cervical and ovarian cancers remain in the top five of common cancer among women in Malaysia despite the advance treatment given to the patients. Hence, the treatment of these two types of cancers are worth investigating in order to identify new anticancer agents that can potentially reduce the number of cases.

In Malaysia, the natural resources have long been applied or consumed as the remedies for multiple diseases and improvement of health-related conditions. The usage of plants as healing medium or supplements has been based on the perception that plant-

based remedies are safer than conventional drugs due to the natural origin of former. Plant-derived natural products have held a great promise in providing invaluable lead compounds for the development of new potential drugs (Ramasamy et al., 2011). Nowadays, most of cancer research works on the treatment which derived from natural sources. Numerous natural products and their analogues have been identified as potent anticancer agents. Based on various previous researches, a broad range of diseases such as lymphomas, skin carcinomas, cancer of breast, testicular, ovarian, head and neck have applied natural products as the treating agent (Demain & Vaishnav, 2011). Hence, it could be perceived that anticancer agent based from natural sources may widen the choices in searching for the new alternatives in decreasing the occurrence of cancer.

## 1.2 PROBLEM STATEMENT

The current treatment of cancer includes the surgical removal and radiation treatment followed by systemic chemotherapy to maintain the efficacy. The main challenges of chemotherapy are the recurrence of cancer associated with resistance to drugs and severe side effects that can limit the administration of anticancer agents, thus affecting the quality of life for cancer patients. Despite these disadvantages, chemotherapy is still widely used as a the treatment for all cancers and at every stage of cancer progression (Rayan, Raiyn, & Falah, 2017).

Cisplatin is a well-established chemotherapeutic drug that has been used for the treatment of several cancers including the ovarian and cervical cancers (Dasari & Tchounwou, 2014). The mode of action for cisplatin is related with the cross-linking of DNA such as interference of DNA repair mechanism, initation of DNA damage and eventually induction of the apoptosis in cancer cells (Marsh et al., 2007; Dasari &