



Ain Shams University
Faculty of Science
Botany Department

Evaluation of some coumarin derivatives as potential anticancer drugs

Thesis

Submitted for the Degree of Doctor of Philosophy
of Science in Botany
(Cytology and Genetics)

By

Dina Salim Abdel Monem Salim

(M. Sc. Faculty of Science Ain Shams University 2013)

Supervisors

Prof. Dr. Hoda Sobhi Barakat

Professor of Cytogenetics, Botany
Department, Faculty of Science
Ain Shams University

Dr. Noha Sayed Farag Khalifa

Assistant Professor of Molecular
Genetics, Botany Department,
Faculty of Science, Ain
Shams University

Prof. Dr. Nahla Ahmed Farag

Professor of Pharmaceutical
Medicinal Chemistry, Faculty
of Pharmacy, Misr international
University

Dr. Ahmed Esmat Abdelrazek

Lecturer of Pharmacology and
Toxicology, Faculty of Pharmacy
Ain Shams, University

2018



Ain Shams University
Faculty of Science
Botany Department

Student's name: Dina Salim Abdel Monem Salim

Title of Thesis: Evaluation of some coumarin derivatives as
potential anticancer drugs

Degree of Thesis: Doctor Philosophy of Science in Botany
(Cytology and Genetics)

Advisory committee

Prof. Dr. Hoda Sobhi Barakat

Professor of Cytogenetics, Botany
Department, Faculty of Science
Ain Shams University

Dr. Noha Sayed Farag Khalifa

Assistant Professor of Molecular
Genetics, Botany Department,
Faculty of Science, Ain
Shams University

Prof. Dr. Nahla Ahmed Farag

Professor of Pharmaceutical
Medicinal Chemistry, Faculty
of Pharmacy, Misr international
University

Dr. Ahmed Esmat Abdelrazek

Lecturer of Pharmacology and
Toxicology, Faculty of Pharmacy
Ain Shams, University

2018

**This thesis has not been previously
submitted for any degree at this or any
other University**

Dina Salim Abdel Monem Salim

Abstract

Thousands of coumarin compounds have been isolated and identified in different plant species. These compounds play an important role in anticancer drug development due to their wide range of biological activities and their effect against different types of cancer cells. Therefore, this study was carried out to gain information about the mechanism of these compounds and searching for novel cytotoxic agents. Seven plants containing coumarin derivatives and ten new furocoumarin analogues were used in this work. HepG-2 cells (liver cancer cell line) were treated with concentrations ranged from 0.1 -1000 µg/ml for 72 hours. Compound number 9 showed highest reduction in cell viability percentage, and exhibited the highest inhibitory activity with IC₅₀ (inhibitory concentration for 50% of cells) equal 11.97 µg/ml, and fall within the American National Cancer Institute criteria; while the other compounds recorded medium or weak effect as compared with the standard drug Doxorubicin (IC₅₀=0.87 µg/ml). The plant extracts also reduced the viability of cancer cells in a dose dependent manner. Their cytotoxic effect was more observed at the higher concentrations. IC₅₀ of the seven plant extracts was ranged from 121.4 µg/ml for *Verbascum thapsus* to 511.8 µg/ml for *Ammi majus*.

Root tip cells of *Pisum sativum* plant were treated with concentrations ranged from 0.05–1.0 mg/ml of compounds number 9 and 8 and with 2.19 – 35 mg/ml for the natural plant extracts of *Achillea millefolium* and *Verbascum thapsus*. Results showed significant decrease in mitotic index that increased gradually by increasing the concentration and the time of treatments; in addition to different types of mitotic abnormalities were recorded such as stickiness and chromosome breakage. The expression level of two cell cycle regulatory genes; cyclin B₁ and cyclin D₁ were examined using RT-PCR techniques. The results indicated that cyclin B₁ was down regulated in response to treatment of *Pisum sativum* roots with the concentrations 0.5 mg/ml of compound 9 and 1.0 mg/ml of compound 8, and with 17.5, 35 mg/ml of the extracts of *Verbascum thapsus* and *Achillea millefolium* as compared with the reference gene β-tubulin-3. Moreover, only compound number 9 caused decrease in cyclin D₁ in response to treatment

with 0.5 mg/ml, as compared with compound number 8; extracts of *Verbascum thapsus* and *Achillea millefolium* which showed no effect, indicating the effect of compound number 9 on the cell cycle arrest at the transition point G₁/S. The genomic stability percentage in *Pisum sativum* plant cells treated with the previous materials using ISSR-DNA fingerprints showed change in band number and decrease in genomic template stability (GTS) values in all treatments. Compound number 9 recorded the lowest GTS (46%) while compound number 8 recorded 64% in comparison to the untreated control, indicating that compound 9 is more effective on genome stability. The GTS value of plant extracts decreased with increasing the concentrations. The lowest GTS (76.12%) recorded with the highest concentration (35 mg/ml) of *Verbascum thapsus*; while recorded 88.06% in samples treated with the same concentration of *Achillea millefolium*. Thus, 0.5 mg/ml of compound 9 have greater damage on cell's genetic material indicative of the genotoxicity at this concentration. On the other hand, the molecular docking technique showed that this compound has a good ability to inhibit topoisomerase I which in turn will lead to cancer cell inhibition through the inhibition of DNA replication.

Contents

1-Introduction	1
2-Literature review	7
2.1. Natural and synthetic coumarins	7
2.1.1. Antitumor activity of coumarins	16
2.2. Cytological studies	25
2.3. Plant cyclins and cell cycle progression	31
2.4. Genome instability and molecular markers	39
3-Materials and methods	44
3.1. Materials	44
3.2. Anticancer activity of natural and synthetic coumarin derivatives using sulforhodamine B (SRB) assay	48
3.3. Cytological studies	53
3.4. Expression level of plant cyclin B1 and cyclin D1 genes using RT-PCR	55
3.4.1. Isolation of total RNA	56
3.4.2. Primer selection and determination of T_m	57
3.4.3. Reverse transcription and RT-PCR	58
3.4.4. Gel electrophoresis	60
3.5. Genomic template stability using ISSR assay	60
3.5.1. Genomic DNA extraction	60
3.5.2. ISSR assay	61
3.6. Molecular modelling studies	64
4-Results	65
4.1. Anticancer activity of natural and synthetic coumarin derivatives using SRB assay	65
4.1.1. Cytotoxic activity of ten synthetic coumarin derivatives	65
4.1.2. Cytotoxic activity of seven plant extracts containing coumarin derivatives	69

Contents

4.2. The cytological studies	73
4.2.1. Cytological effect of two synthetic coumarin compounds	73
4.2.2. Cytological effect of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> extracts	87
4.3-Expression level of cyclin B1 and cyclin D1 genes using (RT-PCR)	100
4.3.1.Effect of two synthetic coumarin compounds on expression level of cyclin B1 and cyclin D1 genes	100
4.3.2.Effect of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> on the expression level of cyclin B1 and cyclin D1 genes	102
4.4. Genomic template stability using ISSR-assay	104
4.4.1-Effect of two synthetic coumarin derivatives on GTS	104
4.4.2.Effect of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> extracts on GTS	114
4.5-Molecular docking analysis for compound 9	124
5-Discussion	126
6-Summary	160
7-References	171
8-Arabic summary	

List of tables

List of Tables

Table No.		Page
(3.1)	Scientific names, family, locality and arabic name of the seven plants containing coumarin used in this study	44
(3.2)	Names and the chemical structure of the ten synthetic coumarin derivatives used in this study	46
(3.3)	Genes ID, Tm and primers sequence for cyclin B1, D1 and <i>B</i> - Tubulin-3 used for RT-PCR technique	58
(3.4)	List of ISSR primers used with ISSR assay	62
(4.1)	<i>In vitro</i> cell viability percentage of ten synthetic coumarin compounds tested against HepG-2 cancer cell line after 72 hours treatment	67
(4.2)	IC50 values of ten synthetic coumarin derivatives tested against HepG-2 cell line.	67
(4.3)	<i>In vitro</i> cell viability percentage of seven plants containing coumarin derivatives tested against HepG-2 cancer cell line after 72 hours treatment	71
(4.4)	IC50 values of the seven plant extracts tested against HepG-2 cell line	71
(4.5)	Number of total examined cells, total mitosis, percentage of mitotic phases and the mean of mitotic index after treating <i>Pisum sativum</i> root tips with different concentrations of compound number 9 for 4 and 24 hours	76
(4.6)	Number of total examined cells, total mitosis, percentage of mitotic phases and the mean of mitotic index after treating <i>Pisum sativum</i> root tips with different concentrations of compound number 8 for 4 and 24 hours	77
(4.7)	Frequency of abnormal mitotic phases after treating <i>Pisum sativum</i> root tips with compound number 9 for 4 and 24 hours	84
(4.8)	Frequency of abnormal cells at different mitotic phases after treating <i>Pisum sativum</i> root tips with compound number 8 for 4 and 24 hours	85
(4.9)	Number of total examined cells, total mitosis, percentage of mitotic phases and the mean of mitotic index after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> extract for 4 and 24 hours	89

List of tables

(4.10)	Number of total examined cells, total mitosis, percentage of mitotic phases and the mean of mitotic index after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Achillea millefolium</i> extract for 4 and 24 hours	90
(4.11)	Frequency of abnormal cells at different mitotic phases after treating <i>Pisum sativum</i> root tips with <i>Verbascum thapsus</i> extract for 4 and 24 hours	96
(4.12)	Frequency of abnormal cells at different mitotic phases after treating <i>Pisum sativum</i> root tips with <i>Achillea millefolium</i> extract for 4 and 24 hours	97
(4.13)	Number of new bands appeared (a), bands disappeared (b) as related to control and Genome Template Stability (GTS) percentage in <i>Pisum sativum</i> treated with synthetic compound number 8 and 9 using six ISSR primers	113
(4.14)	Band Sharing Index (BSI) in <i>Pisum sativum</i> treated with the synthetic compound number 8 and 9 using six ISSR primers	113
(4.15)	Number of new bands appeared (a), bands disappeared (b) and Genome Template Stability (GTS) percentage in <i>Pisum sativum</i> treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> extracts using six ISSR primers	122
(4.16)	Band Sharing Index (BSI) in <i>Pisum sativum</i> treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using six ISSR primers	123

List of figures

List of figures

Fig. NO.		Page
(4.1)	Viability percentage of HepG-2 cancer cell line treated with different concentrations of compounds number 5, 8, 9, 10 and the positive control doxorubicin	68
(4.2)	IC ₅₀ values of the synthetic coumarin compounds tested against HepG-2 (liver cancer cell line).	68
(4.3)	Viability percentage of HepG-2 (liver cancer cell line) treated with different concentrations of <i>Verbascum thapsus</i> , <i>Achillea millefolium</i> and the positive control doxorubicin	72
(4.4)	IC ₅₀ value of plant extracts containing coumarin derivatives tested against HepG-2 (liver cancer cell line)	72
(4.5)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of compound number 9 for 4 hours	78
(4.6)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of compound 9 for 24 hours	78
(4.7)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of compound 8 for 4 hours	79
(4.8)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of compound 8 for 24 hours	79
(4.9)	Mitotic indices after treatment <i>Pisum sativum</i> root tips with different concentrations of compound 8 and 9 for 4 hours	80
(4.10)	Mitotic indices after treatment <i>Pisum sativum</i> root tips with different concentrations of compound 8 and 9 for 24 hours.	80
(4.11)	Mean percentage of total abnormal cells after treating <i>Pisum sativum</i> root tips with different concentrations of compound number 8 and 9 for 4 hours	86
(4.12)	Mean percentage of total abnormal cells after treating <i>Pisum sativum</i> root tips with different concentrations of compound number 8 and 9 for 24 hours	86
(4.13)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> extract for 4 hours	91
(4.14)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> extract for 24 hours	91

List of figures

(4.15)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Achillea millefolium</i> extract for 4 hours	92
(4.16)	Frequency of mitotic phases after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Achillea millefolium</i> extract for 24 hours	92
(4.17)	Mitotic indices after treatment <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> extracts for 4 hours	93
(4.18)	Mitotic indices after treatment <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> extracts for 24 hours	93
(4.19)	Mean percentage of total abnormal cells after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> for 4 hours	98
(4.20)	Mean percentage of total abnormal cells after treating <i>Pisum sativum</i> root tips with different concentrations of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> for 24 hours	98
(4.21)	Types of mitotic abnormalities induced by different concentrations of compound number 8 and 9 and the extracts of <i>Achillea millefolium</i> and <i>Verbascum thapsus</i>	99
(4.22)	Effect of compound 8 and compound 9 on the expression of cyclin B1 and cyclin D1 in <i>Pisum sativum</i> roots using RT-PCR	101
(4.23)	Effect of compound 8 and compound 9 on band intensity of <i>Pisum sativum</i> roots	101
(4.24)	Effect methanol extract of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> extracts on the expression of cyclin B ₁ and cyclin D ₁ in <i>Pisum sativum</i> using RT-PCR	103
(4.25)	Effect of <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> on band intensity of <i>Pisum sativum</i> roots	103
(4.26)	ISSR profile of <i>Pisum sativum</i> DNA, treated with compound 8 and 9 using primer ISSR7	105
(4.27)	ISSR profile of <i>Pisum sativum</i> DNA, treated with compound 8 and 9 using primer ISSR8.	106
(4.28)	ISSR profile of <i>Pisum sativum</i> DNA, treated with compound 8 and 9 using primer ISSR11	107
(4.29)	ISSR profile of <i>Pisum sativum</i> DNA, treated with compound 8 and 9 using primer ISSR12	108
(4.30)	ISSR profile of <i>Pisum sativum</i> DNA, treated with	109

List of figures

	compound 8 and 9 using primer ISSR13.	
(4.31)	ISSR profile of <i>Pisum sativum</i> DNA, treated with compound 8 and 9 using primer ISSR18	110
(4.32)	ISSR profile of <i>Pisum sativum</i> DNA, treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using primer ISSR7	115
(4.33)	ISSR profile of <i>Pisum sativum</i> DNA, treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using primer ISSR8	116
(4.34)	ISSR profile of <i>Pisum sativum</i> DNA, treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using primer ISSR11	117
(4.35)	ISSR profile of <i>Pisum sativum</i> DNA, treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using primer ISSR12	118
(4.36)	ISSR profile of <i>Pisum sativum</i> DNA, treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using primer ISSR13	119
(4.37)	ISSR profile of <i>Pisum sativum</i> DNA, treated with <i>Verbascum thapsus</i> and <i>Achillea millefolium</i> using primer ISSR14	120
(4.38)	Binding mode of camptothecin in the binding site of Topoisomerase I	125
(4.39)	Binding mode of compound 9 in the binding site of Topoisomerase I	125

Acknowledgment

In the name of Allah, I would like to thank Allah for giving me the strength, knowledge, ability and opportunity to complete this thesis.

I would like to express my sincere gratitude to my advisor Prof. Dr. Hoda Mohamed Sobhi Barakat Professor of Cytogenetics, Botany Department, Faculty of Science, Ain Shams University, for the supervision of my Ph.D study, revising the manuscript her patience, insightful comments, suggesting the research point and fruitful discussions throughout the thesis work.

I would like to thank Dr. Noha Sayed Farag Khalifa, Assistant professor of Molecular Genetics, Botany Department, Faculty of Science, Ain Shams University for keen supervision, suggesting the research point, her guidance, encouragement and advice she has provided throughout my thesis.

Special appreciation represented to Prof. Dr. Nahla Ahmed Hassan Professor of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, Misr international University for supervision of thesis, supplying me with compounds she had synthesized and valuable advice.

I wish to express my sincere thanks to Dr. Ahmed Esmat Abdelrazek Lecturer of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams, University for keen supervision and guidance.

I am also greatly indebted to Prof. Dr. Magda El-Araby. Head of Botany Department, Faculty of Science, Ain Shams University.

I wish to express my sincere appreciation to Prof. Dr. Maher Shehata, the former Head of Botany Department, Faculty of Science, Ain Shams University.

Special thanks to all staff and all members of Cytology and Genetics Unit, Botany department, faculty of Science. Ain Shams University.

Last but not the least; I would like to thank my family: my mother Mrs Soad Ahmed and sisters Dr/Wesam Salem and Mrs Reham Salem for supporting me spiritually throughout writing this thesis and my life in general.

1. Introduction

The plant kingdom is gaining global recognition as unique and renewable resource for the discovery of phytochemicals that may represent pharmacological active compounds (**Brower, 2008 and Newman and Cragg, 2012**). Plants are important sources of medicinal compounds worldwide. Plant materials have a long history of being successfully used in the treatment of cancer, both as chemotherapeutic agents and as complementary treatments (**Newman *et al.*, 2003**). According to **Newman and Cragg (2012)**, 40% of all anticancer drugs are developed from natural products while 20% are synthetic derived from natural ones. Continuous efforts have been made for discovering bioactive compounds or designing new drugs with more potent effect against cancer.

Coumarins are class of natural compounds widely distributed in plants, having multiple biological activities and anti-oxidant properties. About 1300 coumarin compounds have been identified, principally as secondary metabolites in green plants (**Hoult and Paya, 1996**). They found free or as hetero-sides in many