Quality Control of Olive Leaf Extract and Discrimination between Different Cultivars Cultivated in Egypt Using Multivariate Data Analysis

A thesis submitted to

Faculty of Pharmacy, Ain Shams University

In Partial Fulfilment of the Requirements for the Degree of

Master in Pharmaceutical Sciences (Pharmacognosy)

By

Eman Mahmoud Ibrahim Kabbash
Bachelor Degree of Pharmaceutical Science (2012)
Faculty of Pharmacy, Ain Shams University

Under the supervision of

Sherweit Hamed El Ahmady, Ph.D.
Professor of Pharmacognosy
Faculty of Pharmacy, Ain Shams University

Zeinab Talat Abd El Shakour, Ph.D.
Associate Professor of Pharmacognosy
National Organization for Drug Control and Research

Iriny Mohsen Mansour Ayoub, Ph.D.
Lecturer of Pharmacognosy
Faculty of Pharmacy, Ain Shams University

Department of Pharmacognosy
Faculty of Pharmacy, Ain Shams University
Cairo, Egypt
(2019)

Acknowledgments

In the beginning, I would like to thank Allah; without His virtue, I would have never been able to accomplish this work. Thanks Allah for giving me knowledge that I pray I could get a chance to pass on to others.

I would like to express my gratitude to my supervisors: **Prof. Sherweit El-Ahmady,** Professor of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, for the encouragement, the help and being the reason to love Phytochemistry, since I was a college student. **Assoc. Prof. Dr. Zeinab Talat**, Associate Professor of Pharmacognosy, National Organization for Drug Control and Research, for her continuous encouragement, unlimited help and scientific support throughout this work especially in the LC/MS data analysis part. **Dr. Iriny Ayoub**, Lecturer of Pharmacognosy, Faculty of Pharmacy, Ain Shams University, for her great effort and patience for supervising this work. No words can express how thankful I am to all of you.

Members, colleagues and professors of the Department of Pharmacognosy, Ain Shams University. **Dr. Haidy Gad** for her help with the chemometrics work and support. My deep thanks to **Dr. Abdul-Rahman Roshan**; who never held back any information, especially in the multivariate data analysis part.

Members, colleagues and professors at the National Organization for Drug Control and Research. My indefinite thanks to **Dr. Amal Koura**; Former Head of Phytochemistry Laboratory at (NODCAR), for her continuous encouragement and great support.

Greatest thankful are also due to **Prof. Michael Wink**, Professor of Biology, Head of Biology Department, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany, for hosting LC/MS analysis in his laboratory.

. My family and my greatest support. I would like to thank my mother for the endless support, love, care and nourishment she has always been a true helping hand throughout my life may god bless her. I'd like to thank my husband for being my backbone, understanding and extremely supportive. I'd like to thank my brother for being my true guidance, he is not only my brother he has been the father figure in my life.

Finally, I would like to dedicate this thesis to my late Father, may his soul rest in peace.

Eman Mahmoud Kabbash, 2011

Table of Contents

List of Tables iii

List of Figures iv

List of Abbreviations viii

Introduction 1

Aim of work 4

Review of Literature 5

A. Reported phytochemical constituents in *O. europaea* L. 5

1. Secoiridoids reported in *Olea europaea* L. 5

2. Phenolic compounds reported in *Olea europaea* L. 7

3. Lignans reported in *Olea europaea* L. 8

4. Triterpenes reported in *Olea europaea* L. 9

5. Miscellaneous compounds 10

B. Biological activities reported for *O. europaea* L 19

Taxonomy 24

Materials and Methods 30

A. Materials 30

1. Plant Material 30

2. Reagents, Solvents and Apparatus 30

2.1. Extracts preparation 30

2.3. Polyphenols assay 31

2.4. Flavonoids assay 31

2.5. HPLC assay 31

2.6. HPLC-PDA-ESI/MS/MS analysis 31

2.7. *In-vitro* assay of antioxidant activity 31

3. Authentic Reference Standards 32

4. Software 32

B. Methods 32

1. Extracts preparation 32

2. Phytochemical analysis 32

2.1. Ultraviolet Spectroscopic Analysis for *O. europaea* L. Cultivars Leaf Extracts 32

2.2. Quantitative Determination of Total Polyphenol Content of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 33

2.3. Quantitative Determination of Total Flavonoid Content of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 34

2.4. HPLC Assay of Oleuropein Content of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 34

2.5. HPLC-PDA-ESI/MS/MS Based Metabolomics of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 35

3. *In vitro* Assay of Antioxidant Activities of *O. europaea* L. Cultivars Leaf Extracts by DPPH Radical Scavenging Method 36

Results and Discussion .....

Part 1: Phytochemical Analysis 38

Chapter 1: Ultraviolet Spectroscopic Analysis for *O. europaea* L. Cultivars Leaf Extracts 38

Chapter 2: Quantitative Determination of Total Polyphenol Content of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 43

Chapter 3: Quantitative Determination of Total Flavonoid Content of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 48

Chapter 4: HPLC Assay of Oleuropein Content of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 54

Chapter 5: HPLC-PDA-ESI/MS/MS Based Metabolomics of *O. europaea* L. Cultivars Leaf Extracts in Autumn and Spring 59

Part 2: *In vitro* Assay of Antioxidant Activities of *O. europaea* L. Cultivars Leaf Extracts by DPPH Radical Scavenging Method 89

Conclusions 95

Recommendations 97

Summary 98

References 103

Appendix .....

Arabic summary .....

List of Tables

Table 1: Traditional uses of *O. europaea* L. different parts 1
Table 2: Iridoids, secoiridoids and secoiridoid glycosides reported in *O. europaea* L. various parts 6
Table 3: Main phenolic compounds in *Olea europaea* L. different parts 7
Table 4: Main lignans identified in *Olea europaea* L. various parts 9
Table 5: Triterpenes identified in *O. europaea* L. various parts 9
Table 6: Miscellaneous compounds isolated from *O. europaea* L. 10
Table 7: Antimicrobial activities of *O. europaea* L. leaf extracts 21
Table 8: *O. europaea* L. different cultivar included in this study 25
Table 9: Name, code and origin of *O. europaea* L. different cultivars 30
Table 10: Absorbance of gallic acid standard solutions different concentrations at 760 nm 44
Table 11: Total polyphenol concentration (mg/mL) of the olive leaf extracts collected in autumn and spring 45
Table 12: Total polyphenol contents of each extract in mg/g extract in autumn and spring 46
Table 13: The absorbance of rutin standard solutions of different concentration at 415 nm 49
Table 14: Total flavonoid concentration (mg/mL) of the olive leaf extracts collected in autumn and spring 49
Table 15: The total flavonoid contents of each extract in mg/g extract 50
Table 16: Oleuropein standard solutions different concentrations and their measured peak areas at 280 nm 55
Table 17: Oleuropein content in mg/mL of each extract collected in autumn and spring 55
Table 18: Oleuropein content in mg/100 g dried extract in autumn and spring 56
Table 19: Metabolites identified by HPLC-PDA-ESI/MS/MS analysis of *O. europaea* L. leaf extracts in negative and positive ionization modes 60
Table 20: The distribution of the identified compounds in the twelve olive leaf cultivars collected in autumn and spring 75
Table 21: The % inhibition of the DPPH free radical of *Olea europaea* L. different cultivars in autumn and spring 89

List of Figures

Figure 1: Iridoid and secoiridoid compounds in *O.europaea* L. various parts 11
Figure 2: phenolic acids, coumarins and simple phenolic compounds identified in *O. europaea* L. various parts 13
Figure 3: Main flavonoids identified in *O. europaea* L. various parts 14
Figure 4: Lignans identified in *O. europaea* L. various parts 15
Figure 5: Triterpenes identified in *O. europaea* L. various parts 16
Figure 6: Miscellaneous compounds identified in *O. europaea* L. various parts 18
Figure 7: (A) Photograph of *O. europaea* L. *tree* (0.02X) 28
Figure 8: *O. europaea* L. leaf of different cultivars included in this study 29
Figure 9: UV absorbance spectra over the range 250-400 nm for the twelve different olive leaf extracts collected in autumn 39
Figure 10: UV absorbance spectra over the range 250-400 nm for the twelve different olive leaf extracts collected in spring 39
Figure 11**:** Score plot of PC_1 and PC_2 for the twelve olive leaf extracts collected in autumn based on their UV scan. Egyptian cultivars in red; Spanish cultivars in green; Greek cultivars in blue and Italian cultivar in brown 41
Figure 12**:** Score plot of PC_1 and PC_2 for the twelve olive leaf extracts collected in spring based on their UV scan. Egyptian cultivars in red; Spanish cultivars in green; Greek cultivars in blue and Italian cultivar in brown 41
Figure 13: Score plot of PC_1 and PC_2 for all olive leaf extracts collected in autumn and spring based on their UV scan. 42
Figure 14: Calibration curve of gallic acid standard used for the assay of polyphenols in olive leaf extracts 44
Figure 15: Total polyphenol contents for olive leaf different cultivars in autumn and spring 46
Figure 16: Standard calibration curve of rutin used for the assay of total flavonoids in olive leaf extracts 49
Figure 17: Total flavonoid contents for olive leaf different cultivars in both seasons 51
Figure 18: The score plot (A) and loading plot (B) of olive leaf autumn extracts using UV scan, TF and PPh content as variables. Egyptian cultivars in red; Spanish cultivars in green; Greek cultivars in blue and Italian cultivar in brown 52

Figure 19: The score plot (A) and loading (B) plot of olive leaf spring extracts using UV scan, TF and Pph content as variables. Egyptian cultivars in red; Spanish cultivars in green; Greek cultivars in blue and Italian cultivar in brown 53
Figure 20: The standard calibration curve of oleuropein 55
Figure 21: Oleuropein contents for olive leaf different cultivars in autumn and spring..... 57
Figure 22: Proposed fragmentation pathways of oleuropein aglycone 66
Figure 23: ESI-MS/MS spectrum of oleuropein-*O*-deoxyhexoside (23) in the negative ion mode 67
Figure 24: Proposed fragmentation pathways of luteolin as a representative to flavones 70
Figure 25: Proposed fragmentation pathways of kaempferol as representative to flavonols .. 71
Figure 26**:** ESI-MS/MS spectrum of compound (**24**), unknown-*C*-glycoside in the negative ion mode 72
Figure 27**:** Proposed fragmentation pattern of pentacyclic triterpenes 74
Figure 28**:** Representative LC/MS base peak chromatograms of olive leaf extracts analysed in negative ionization mode 79
Figure 29: Total ion chromatogram of the twelve olive leaves cultivars collected in autumn (A) and spring (B), in the negative ionization mode 84
Figure 30**:** Principal Component Analysis of different olive cultivars leaves collected in autumn derived from the negative ionization mode HPLC/ESI/MS-MS data (*m/z* 100–1000); showing the score plot (A) and the loading plot (B) for the Egyptian, Spanish, Greek and Italian cultivars**.** 86
Figure 31: Principal Component Analysis of different olive cultivars leaves collected in autumn after removing MAN cultivar; showing the score plot (A) and the loading plot (B) for the Egyptian, Spanish, Greek and Italian cultivars. 87
Figure 32: Principal Component Analysis of different olive cultivars leaves collected in spring derived from the negative ionization mode HPLC/ESI/MS-MS data (*m/z* 100–1000); showing the score plot (A) and the loading plot (B) for the Egyptian, Spanish, Greek and Italian cultivars 88
Figure 33: The % inhibition of the DPPH free radical for autumn and spring olive leaf extracts different cultivars 90
Figure 34**:** HCA (A), PCA score plot (B) and loading plot (C) based on UV spectroscopy, TF, PPh, oleuropein content and DPPH assays for the twelve olive cultivars collected in autumn. Egyptian cultivars in red; Spanish cultivars in green; Greek cultivars in blue and Italian cultivar in brown 92

Figure 35: HCA (A), PCA score plot (B) and loading plot (C) based on UV spectroscopy, TF, PPh, oleuropein content and DPPH assays for the twelve olive cultivars collected in spring. Egyptian cultivars in red; Spanish cultivars in green; Greek cultivars in blue and Italian cultivar in brown 93
Figure 36: PCA score plot (A) and loading plot (B), showing discrimination of all the extracts into two groups one for autumn (blue colour) and the other for spring (orange colour).....94

List of Abbreviations

Abbreviation	
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AE	Autumn extracts
ANN	Artificial Neural Networks
BBCE	Bovin brain capillary endothelial cells
BHT	Butylated hydroxy toluene
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDA	Exploratory Data Analysis
EIC	Extracted ion chromatogram
ESI	Electospray ionization
EVOO	Extra virgin olive oil
FA	Factor analysis
GC/MS	Gas chromatography coupled to mass spectrometry
HCA	Hierarchical Cluster Analysis
HPLC	High performance liquid chromatography
HPLC/ESI-MS/MS	High-performance liquid chromatography-electrospray ionisation tandem mass spectrometry
HPLC/UV-vis	High performance liquid chromatography with ultraviolet visible detector
HRI	Horticulture Research Institute
KNN	*k*-nearest neighbours
LC/MS	Liquid chromatography/Mass spectrometry
LDA	Linear Discriminate Analysis
MVA	Multivariate analysis
NMR	Nuclear magnetic resonance
OLE	Olive leaf extract
PCA	Principal Component Analysis
PCR	Principal Component Regression
PDO	Protected designation of origin
PGI	Protected geographical origin
PLS	Partial Least Square
PLS-DA	Partial Least Squares Discriminate Analysis
PPh	Polyphenol content
RDA	Retro-Diels-Alder reaction
SE	Spring extracts
SIMCA	Soft Independent Modelling of Class Analogy
TF	Total flavonoids
TIC	Total Ion Chromatogram
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC-QTOF-MS	Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Introduction