CRANFIELD UNIVERSITY

Applied Mycology Group, Cranfield Health

Faculty of Medicine and BioSciences

TITOS NIKOLAOU MAVRAKIS

EXPLOITATION OF BIOACTIVE CONSTITUENTS OF OLIVE LEAVES, GRAPE POMACE, OLIVE MILL WASTE WATER AND THEIR APPLICATIONS IN PHYTOPROTECTION

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ABSTRACT

Pure plants extracts or/and essential oils show antifungal and antibacterial activity against a wide range of fungi and bacteria. Recently there has been increasing interest in the effects of natural compounds against important plant pathogens (fungi and bacteria). Although the antimicrobial activity of oleuropein, grape pomace (GPE) and olive mills waste water (OMWW) extracts have been studied widely, little research has been done on the *in vitro* and *in vivo* evaluation of these extracts efficiency against important plant diseases and postharvest treatments.

The overall objectives of this study were firstly to extract and isolate from olive leaves (oleuropein), olive mills waste water and from grape pomace (winery by-products) natural compounds in order to be used for *in vitro* and *in vivo* experiments.

In particular, all three natural extracts were found to restrain *in vitro* growth of a series of important bacterial and fungal pathogens, such as: *Botrytis cinerea,* the cause of grey mold disease, *Alternaria alternata*, causing leaf spots and moulds on several plant species, *Fusarium oxysporum fsp melonis,* causing Fusarium wilt of melon, a *Rhizopus* species (a genus causing fruit and vegetable decays), the crucifer pathogen *Colletotrichum higginsianum,* causing anthracnose leaf spot disease on several *Brassica* and *Raphanus* species and *Phytophthora parasitica* var. *nicotianae,* the causal agent of the black shank disease of tobacco. Bacterial strains including *Clavibacter michiganensis spp. michiganensis,* the cause of bacterial canker of tomato, *Ralstonia solanacearum* causing bacterial wilt in solanaceous plants, *Pseudomonas syringae* pv. *tomato,* the cause of bacterial speck on tomato and Arabidopsis and *Xanthomonas campestris* pv. *vesicatoria* causing the bacterial spot disease of pepper and tomato were tested. Oleuropein (semipure and pure) showed remarkable antibacterial activity. The minimal inhibitory concentration (MIC), at least of oleuropein was lower than 0.1%. MICs values for GPE and OMWW extract ranged from 0.1% to 0.2%. Moreover, these natural extracts were shown to inhibit and/or restrain spore germination of fungi in solid media, however grape pomace extract, was sufficiently effective to inhibit spore germination and germ tube of *C. higginsianum* and *B. cinerea.*

Furthermore *in vivo* antibacterial activity of pure oleuropein and OMWW extract was assessed in greenhouse experiments, on *Xanthomonas campestris* pv. *vesicatoria*, the cause of bacterial spot of pepper plants and *Pseudomonas syringae* pv. *tomato*, the cause of bacterial speck of tomato plants, respectively. It seems that 0.1% pure oleuropein (98%) and 0.1% OMWW extract had a protective effect against bacterial spot and bacterial speck respectively, which is more obvious when oleuropein application started before infection. Besides endophytic growth of *Phytophthora parasitica* var. *nicotianae,* causal agent of the black shank disease of tobacco, on tobacco leaves by measuring its radial growth inside the infected tissue, was assessed. 0.1% GPE showed the most significant inhibition in all tobacco leaves treatments.

The potential of postharvest treatment of table grapes with the grape pomace extract (GPE) to restrain grey mould (*Botrytis cinerea*) disease incidence and development was estimated. 5% GPE treatments produced the strongest inhibitory effect against *B. cinerea* incidence on grape berries and the time after treatment influenced the numbers of decayed berries.

Finally the use of pure oleuropein as antibacterial in vase solution for cut flowers (carnation) was evaluated. Oleuropein showed strong antibacterial activity as vase solution and carnations vase life was more than doubled by using oleuropein 200ppm and 400ppm, compared with control (water).

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LIST OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS...iii

LIST OF CONTENTS iv

LIST OF FIGURES vii

LIST OF TABLES xiii

NOTATION xv

1.0	**CHAPTER ONE: INTRODUCTION**………………………………	1
1.1	Project background…………………………………………………	1
1.2	Aim and objectives…………………………………………………	1
	1.2.1 Aim……………………………………………………………	1
	1.2.2 Objectives………………………………………………………..	1
1.3	Thesis structure……………………………………………………...	2
2.0	**CHAPTER TWO: LITERATURE REVIEW**……….………….	6
2.1	The Olive……………………………………………………………...	6
2.1.2	Olive oil………………………………………………………………	7
2.1.2.1	Production of Olive Oil on Crete……………………………………..	7
2.1.2.2	Olive Oil Phenols……………………………………………………..	8
2.1.3	Chemical Compounds in Olive Leaves and OMWW………………...	10
2.1.4	Biological Properties of Olive Leaves and Olive oil………………...	13
	a Olive leaves……………………………………………………...	13
	b Olive Oil……………………………………………………………	15
2.2	Grapes and Wine…………………………………………………...	17
2.2.1	History of wine making in Crete……………………………………...	18
2.2.2	Phytochemical Approach of *Vitis vinifera*…………………………...	19
2.2.3	Biological Properties of Grapes and Wine……………………………	21
2.2.4	Resveratrol……………………………………………………………	23
2.2.5	Winery Byproducts…………………………………………………...	24
	2.2.5.1 Polyphenolic compounds of winery byproducts……...……...	26
2.3	Effects of vase solutions microorganisms on cut carnation vase life...	27
3.0	**CHAPTER THREE: EXTRACTION AND ISOLATION OF NATURAL COMPOUNDS FROM OLIVE LEAVES, OLIVE MILS WASTE WATER AND WINERY BY-PRODUCTS**...........	29
3.1	Introduction and objectives………………………………………...	29

3.2	Materials and Methods………………………………………………..	30
3.2.1	Olive leaf extract…………………………………………………...	30
	3.2.1.1 Plant material……..…………………………………………	30
	3.2.1.2 Extraction and isolation……..………………………………	30
3.2.2	Olive mills waste water (OMWW) extract……………………………	33
	3.2.2.1 Amberlite XAD polymeric adsorbents………..……………..	33
	3.2.2.2 Olive mills waste waters...…………………………………...	34
	3.2.2.3 Extraction and isolation...……………………………………	34
	a Filtration......…………………………………………………	34
	b Treatment of OMWW with resin………..…………………..	36
	c Solvent Recovery………..…………………………………...	37
	d HPLC analysis………..……………………………………...	38
3.2.3	Grape Pomace Extract……………………………………………...	39
	3.2.3.1 Plant material…………………………………………………	39
	3.2.3.2 Extraction and isolation procedure…………………………...	39
	3.2.3.3 HPLC analysis………………………………………………..	42
3.3	Results and discussion………………………………………………..	43
3.3.1	Olive leaves…………………………………………………………...	43
3.3.2	Olive Mills Waste Water (OMWW)…………………………………	44
3.3.3	Grape Pomace Extract (GPE)…………………………………………	46
4.0	**CHAPTER FOUR: *IN VITRO* EVALUATION OF EXTRACTED NATURAL COMPOUNDS……………………..**	47
4.1	Introduction and objectives…………………………………………	49
4.2	Materials and Methods………...………………………………...	50
4.2.1	Test microorganisms………………………………………………….	51
4.2.2	Fungi Radial Growth………………………………………………….	51
4.2.3	Spore germination of *Botrytis cinerea* and *Colletotrichum higginsianum*…..	52
4.2.4	Bacteria………………………………………………………………..	53
4.3	Results………………………………………………………………...	54
4.3.1	Fungal Radial Growth natural extracts………………………………..	54
	a *Botrytis cinerea*……………………………………………………	54
	Effects of pure resveratrol on radial growth of *Botrytis cinerea*..….	57
	b *Rhizopus spp*..……………………………………………………..	58
	c *Alernaria alternata*…………………………………………………	61
	d *Phytophthora parasitica*……………………………………………	64
	e *Colletotrichum higginsianum*………………………………………	67
	f *Fusarium oxysporum*……………………………………………….	69
4.3.2	Inhibition of spore germination by natural extracts…………………..	72

	a *Colletotrichum higginsianum*….…………………………………...	72
	b *Botrytis cinerea*….…………………………………..................	74
4.3.3	Bacterial strains….…………………………………....................	77
	a *Clavibacter michiganensis* (4040*)* ….…………………………....	77
	b *Xanthomonas* campestris pv. Vesicatoria (5075) ….………………	78
	c *Erwinia atroseptica* (3217) …..……………………………………	79
	d *Erwinia amylovora* (15) …..………………………………….….....	80
	e *Ralstonia solanacearum (* 819-6) …..………………………………	81
	f *Pseudomonas syringae* pv. *Apii (* 255a) …..………………………..	82
	g *Pseudomonas corrugate* (1157) …..………………………………..	83
	h *Pseudomonas viridiflava* (Acant.2) …..……………………………	84
	i *Pseudomonas syringae* pv. *Tomato* (132) …..……………………..	85
	j *Pseudomonas savastanoi* (1266) …..………………………………..	86
4.4	DISCUSSION….…………………………………......................	87
5.0	**CHAPTER FIVE: *IN VIVO* EVALUATION OF EXTRACTED NATURAL COMPOUNDS………………………………………**	90
5.1	Introduction and objectives…………………………………………	91
5.2	Materials and Methods………...………………………………....	92
5.2.1	Treatments on pepper plants………………………………………	92
	Toxicity test of Oleuropein in Peppers……………………………...	92
	Suppressive treatments of oleuropein against bacterial spot (*Xantomonas campestris* pv. *vesicatoria* 5075) of pepper seedlings-plants. ……………………………………………………………...	93
5.2.2	Suppressive treatments of OMWW extract against bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato plants………………..	96
5.2.3	Tobacco leaf inoculation assay……………………………………...	99
5.2.4	Post harvest treatments of table grapes to control *B. cinerea*………...	100
5.2.5	Potential use of pure oleuropein as antibacterial in vase solution…….	102
5.3	Results….…………………………………………………………...	104
5.3.1	Toxicity test with oleuropein on peppers……………………………..	104
5.3.2	Suppressive treatments of oleuropein against *Xanthomonas campestris* pv. *vesicatoria* of pepper seedlings-plants………………..	104
5.3.3	Suppressive treatments of OMWW extract against *Pseudomonas syningae* of tomato seedlings-plants…………………………………	108
5.3.4	Effects of natural compounds on tobacco leaf infection by *P. parasitica*……………………………………………………………..	111
5.3.5	Efficiency of Grape Pomace Extract on post harvest gray mold control……………………………………………………………...	112
5.3.6	Effect of use of oleuropein as antibacterial in vase solution…………	118
5.4	Discussion……………….…………………………………………	122

6.0	**CHAPTER SIX: SUMMARY OF CONCLUSIONS OF THE THESIS AND FUTURE CONSIDERATION……………………..**	127
6.1	SUMMARY OF THE OUTCOMES OF THIS PROJECT…………	128
	(a) *In vitro* experiments………………………………………………	129
	(b) *In vivo* experiments……………………………………………….	130
	FUTURE CONSIDERATIONS………………………………………	131
7	**CHAPTER SEVEN: REFERENCES……………………………...**	133

LIST OF FIGURES

CHAPTER TWO

Figure 2.1 The major constituents of olive oil. 9

Figure 2.2 Structures of the main antioxidants isolated from OMWW.: **1**. Hydroxytyrosol (HT), **2.** Tyrosol and **3**. (5-ethylidene-2-oxo-tetrahydropyran-4-yl)-acetic acid. 12

Figure 2.3 Chemical structure of grape and wine phenolics that have been isolated and can be divided into two groups: nonflavonoids and flavonoids. 20

Figure 2.4 *cis*- and *trans*- , isomers of resveratrol**.** 23

CHAPTER THREE

Figure 3.1 Chemical structure of polymeric adsorbents XAD-4 XAD-16 XAD-7HP

Figure 3.2 Schematic presentation of the three major steps of the polyphenol extraction from grape pomace 41

Figure 3.3 HPLC chromatogram of pure Oleuropein (98%) 43

Figure 3.4. HPLC chromatograph (UV 254, UV 280) of unteated OMWW. 44

Figure 3.5. HPLC chromatograph (UV 254, UV 280) of OMWW after treatment with XAD-16 resin. 45

Figure 3.6. HPLC chromatogram of Phenolic compounds from enriched red grape pomace extact: 1 gallic acid, 2 caftaric acid, 3 catechin, 4 Caffeic acid, 5 syringic acid, 6 epicatechin, 7 p-coumaric acid, 8 ferulic acid, 9 quercetin glucosides, 10 trans-resveratrol, 11 quercetin, 12 viniferin. 48

CHAPTER 4

Figure 4.1. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Botrytis cinerea* after 4 days. 54

Figure 4.2. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Botrytis cinerea* after 7 days. 55

Figure 4.3 Effect of pure resveratrol on *Botrytis cinerea* radial growth after 4 and 7 days. 57

Figure 4.4 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Rhizopus* after 4 days. 58

Figure 4.5. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Rhizopus* after 7 days. 59

Figure 4.6 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Alternaria alternata* after 4 days. 61

Figure 4.7 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Alternaria alternata* after 7 days. 62

Figure 4.8 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Phytophthora parasitica* after 4 days. 64

Figure 4.9 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Phytophthora parasitica* after 7 days. 65

Figure 4.10 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Colletotrichum higginsianum* after 7 days. 67

Figure 4.11. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Fusarium oxysporum* after 4 days. 69

Figure 4.12 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Fusarium oxysporum* after 7 days. 70

Figure 4.13 Effect of natural compounds on spore germination of *Colletotrichum higginsianum* after 48hr. incubation. 72

Figure 4.14 Effect of natural compounds on spore germination of *B. cinerea* after 36 hr. incubation. 74

Figure 4.15 Effect of extracts on colony forming ability of *Clavibacter michiganensis* subsp. michiganensis (4040) after 3 days incubation. 77

Figure 4.16 Effect of extracts on colony forming ability on colony forming ability of *Xanthomonas campestris* pv. vesicatoria (5075) after 2 days incubation. 78

Figure 4.17 Effect of extracts on colony forming ability of *Erwinia atroseptica* (3217) after 2 days incubation. 79

Figure 4.18 Effect of extracts on colony forming ability of *Erwinia amylovora* (15) after 2 days incubation. 80

Figure 4.19 Effect of extracts on colony forming ability of *Ralstonia solanacearum* (819-6) after 3 days incubation. 81

Figure 4.20 Effect of extracts on colony forming ability of *Pseudomonas syringae* pv. Apii (255a) after 2 days incubation. 82

Figure 4.21 Effect of extracts on colony forming ability of *Pseudomonas corrugate* (1157) after 2 days incubation. 83

Figure 4.22 Effect of extracts on colony forming ability of *Pseudomonas viridiflava* (Acant.2) after 2 days incubation. 84

Figure 4.23. Effect of extracts on colony forming ability of *Pseudomonas syringae* pv. tomato (132) after 2 days incubation. 85

Figure 4.24 Effect of extracts on colony forming ability of *Pseudomonas savastanoi* (1266) after 2 days incubation. 86

CHAPTER 5

Figure 5.1 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 2h before the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean. 104

Figure 5.2 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 2h before and 24h and 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean. 106

Figure 5.3 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 24h, 48h and 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean. 106

Figure 5.4 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean. 107

Figure 5.5 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 72h, 48h, 24, 2h before and 24h, 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean. 107

Figure 5.6 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 2h before the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean. 109

Figure 5.7 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 2h before, 24h and 72h after the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean. 110

Figure 5.8 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 24h, 48h and 72h after the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean. 110

Figure 5.9 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 72h, 48h 2h before and 48h and 72h after the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean. 111

Figure 5.10 Effects of 0.1% pure oleuropein, 0.1% OMWW extract and 0.1% GPE i) 2 days and 2 h before ii) 2h before iii) 2 h and 2 days after iv) 2 and 4 days after inoculation on the adaxial surface by placing discs of *P. parasitica* mycelium cut from the edge of an actively growing colony. The values are reported in endophytic radial growth (lesion formation) % of the control. 112

Figure 5.11 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **2h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. 113

Figure 5.12 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **24h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. The values are the means of three replicates. Bars indicate standard errors of the mean. 114

Figure 5.13 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **48h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. The values are the means of three replicates. Bars indicate standard errors of the mean. 115

Figure 5.14 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **2h and 24h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. The values are the means of three replicates. Bars indicate standard errors of the mean. 116

Figure 5.15 Effect of water (control), 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral treatments on the decay of un-inoculated table grapes clusters during storage at 0-1°C (RH>90%). The values are the means of three replicates. Bars indicate standard errors of the mean. 117

Figure 5.16 Effect of 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral treatments on the decay of inoculated table grapes clusters during storage at 0-1°C (RH>90%). Approximately 10^5spores/ml were used to infect table grapes clusters. The values are the means of three replicates. Bars indicate standard errors of the mean. 118

Figure 5.17 Change in relative fresh weight (%) of initial weight, of cut carnations treated with water (control), DICA 25ppm, DICA 50ppm, DICA 100ppm as vase solutions, after 4, 7 and 10 days. 119

Figure 5.18 Number of colonies (x10^4) in cut carnations vase solutions: a) water (control), b) DICA 25ppm, c) DICA 50ppm, d) DICA 100ppm, e) Oleur. 50ppm, f) Oleur. 100ppm, g) Oleur. 200ppm and h) Oleur. 400ppm, after 10 days. The values are the means of three replicates. Bars indicate standard errors of the mean. 120

Figure 5.19 Longevity effects of water (control), DICA 25ppm, DICA 50ppm, DICA 100ppm, Oleur. 50ppm, Oleur. 100ppm, Oleur. 200ppm and Oleur. 400ppm on carnation vase life longevity. The values are the means of seven replicates. Bars indicate standard errors of the mean. 121

LIST OF TABLES

CHAPTER TWO

Table 2.1 Main references of chemical compounds that have been isolated from olive leaves and OMWW. 10

Table 2.2 Main references of chemical compounds that have been isolated from *Vitis vinifera* plant. 19

CHAPTER THREE

Table 3.1 MPLC solvent system of increasing polarity (CH_2Cl_2:MeOH / 100:0→0:100). 31

Table 3.2 Gradient elution program applied to the HPLC Analysis of OMWW, flow 1 ml/min. 38

Table 3.3 Gradient elution program applied to the HPLC Analysis of grape pomace extract, flow 1 ml/min. 42

CHAPTER FOUR

Table 4.1 Effect of Oleuropein 55% and 98% purity on radial growth of *Botrytis cinerea* after 4 and 7 days. 56

Table 4.2 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Botrytis cinerea*. 56

Table 4.3 Effect of Oleuropein 55% and 98% purity on radial growth of *Rhizopus sp* after 4 and 7 days. 60

Table 4.4 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Rhizopus sp* after 4 and 7 days. *60*

Table 4.5 Effect of Oleuropein 55% and 98% purity on radial growth of *Alternaria alternata* after 4 and 7 days. 63

Table 4.6 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Alternaria alternata* after 4 and 7 days. 63

Table 4.7 Effect of Oleuropein 55% and 98% purity on radial growth of *Phytophthora parasitica* after 4 and 7 days. 66

Table 4.8 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Phytophthora parasitica* after 4 and 7 days. 66

Table 4.9 Effect of Oleuropein 55% and 98% purity on radial growth of *Colletotrichum higginsianum* after 4 and 7 days. 68

Table 4.10 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Colletotrichum higginsianum* after 4 and 7 days. 68

Table 4.11 Effect of Oleuropein 55% and 98% purity on radial growth of *Fusarium oxysporum* after 4 and 7 days. 71

Table 4.12 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Fusarium oxysporum* after 4 and 7 days. 71

Table 4.13 ED_{50} values (mg/ml) after 4 days incubation for all fungi we used. 87

Table 4.14 ED_{50} values (mg/ml) after 7 days incubation for all fungi we used. 88

Table 4.15 Minimum Inhibitory concentrations (MICs) of Oleuropein against bacterial strains used in this study. 89

CHAPTER FIVE

Table 5.1: Treatments for toxicity test with oleuropein on pepper plants 93

Table 5.2 Suppressive treatments of oleuropein against *Xantomonas campestris* pv. *vesicatoria* of pepper seedlings-plants. 95

Table 5.3 Suppressive treatments of OMWW extract against *Pseudomonas syringae* pv. *tomato* of tomato plants. 98

Table 5.4 Vase solutions was used to evaluate the use of oleuropein as antibacterial compound in vase solution. 103

NOTATION

→	to
OMWW	Olive mills waste water
GPE	grape pomace extract
BC	Before Christ
AD	Anno Domini
%	per cent
°C	degree Celsius
e.g.	for example
ca.	approximately
Ca	calcium
UV	Ultraviolet
Ha	hectare
in vitro	outside a living organism
in vivo	inside a living organism
kg	kilogram
μg	μilligram
g	gram
mg	microgram
ppm	part per milion
l	litre
M	molarity
m	metre
m^3	cubic metre
mg	milligram
h	hour
min	minute
ml	millilitre
mm	millimetre
nm	nanometer
mM	millimolar
MW	molecular weight
N	normality
NaCl	sodium chloride

MgCl	Magnesium chloride
DICA	Dichloroisocyanuric acid
CH_2Cl_2	Dichloromethane
MeOH	Methanol
CH_3CN	acetonitrile
EtOH	Ethanol
AcOH	acetic acid
TLC	thin layer chromatography
US	United States
UK	United Kingdom
EU	European Union
viz.	namely
pv.	pathovar
var	variety
et al.	et alii (and others)
v/v	volume by volume
w/w	weight by weight
w/v	weight by volume
ED_{50}	Effective dose
MIC	Minimum Inhibitory Concentration
MPLC	Medium Pressure liquid chromatography
HPLC	High Performance liquid chromatography
LDL	low-density lipoproteins
RT	retention time
PDA	Potato Dextrose Agar
O.D.	optical density
n.d.	not defined
RFW	relative fresh weight

CHAPTER ONE

Introduction

1.1 Project background

There is currently an increasing interest in the isolation, examination and exploitation of agricultural wastes or inexpensive plant sources, rich in polyphenols. Phenolic compounds in *Olea europaea* tissues and olive mills waste waters (OMWW), have pharmacological, antimicrobial properties and are considered natural antioxidants. In a similar manner grape or wine polyphenols have beneficial effects for human health, and have been linked to several functions in plants, such as defence against invading pathogens. The recovery of high added-value products from waste plant material such as olive leaves, OMWW and winery by-products, are therefore a significant issue in non chemical disease control.

This project is funded by a research grant PEP-Crete (KR-19, co-financed by the E.U.-European Regional Development Fund, the Regional Authority of Crete and the Greek Ministry of Development-General Secretariat Research & Technology).

1.2 Aim and objectives

1.2.1 Aim

The aim of this PhD project was to isolate and purify natural antioxidants from olive leaves, olive mills waste waters and grape pomace (winery byproducts) and to exploit these compounds by using them as phytoprotective agents against important pathogens in Greece.

1.2.1 Objectives

The specific project objectives were:

- Extraction and purification of natural antioxidants (oleuropein, hydroxytyrosol, tyrosol) from olive leaves and olive mil waste waters using Amberlite XAD - adsorbent, Medium Pressure Liquid Chromatography (MPLC).

- Extraction and recovery of phenolics (resveratrol, quercetin) from wine industry by-products (grape pomace) using Amberlite XAD adsorbent.
- *In vitro* evaluation of antimicrobial activity of these compounds against various known economically important pathogens in Greece.
- Determination of Minimum Inhibitory Concentration (MIC) of those compounds against Gram-positive or Gram-negative bacteria such as *Clavibacter michiganensis spp. michiganensis, Ralstonia solanacearum, Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria.*
- Effect of these compounds on radial growth, spore germination and determination of ED_{50} of some plant-pathogenic fungi such as *Botrytis cinerea, Alternaria alternata*, *Fusarium oxysporum fsp melonis*, a *Rhizopus* species, *Colletotrichum higginsianum,* and *Phytophthora parasitica* var. *nicotianae.*
- *In vivo* evaluation of antimicrobial activity of these substances. Treatments were conducted on plants and fruits in a greenhouse. Suppressive treatments of oleuropein against bacterial spot (*Xantomonas campestris* pv. *vesicatoria*) of pepper seedlings-plants and of OMWW extract against bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomatoes plants were conducted.
- Effects of natural compounds on tobacco leaf infection by *Phytophthora parasitica*.
- Post-harvest treatments of table grapes with grape pomace extract to control *Botrytis cinerea*.
- Evaluation of potential use of pure oleuropein as antibacterial in vase solution for cut flowers (carnation).

1.3 Thesis structure

The thesis is arranged into seven chapters (Fifure 1.1). Chapter 2 is a review of existing literature. First it describes olive and olive oil generally (historical, production in Crete) and then reports the olive oil phenols, the chemical compounds of olives and olive mills waste water (OMWW) and the biological properties of olives leaves and OMWW. At the end Chapter 2 describes grapes and wine in general and then reports a phytochemical approach of *Vitis vinifera*, biological properties of

grapes and wine, resveratrol properties and winery by-products and their polyphenolic compounds.

Chapter 3 details the extraction and isolation of natural compounds from olive leaves , olive mills waste water and grape pomace (winery by-products). Chapter 4 describes experiments aimed at the in vitro evaluation of isolated natural compounds plant pathogens (fungi and bacteria) such as *Botrytis cinerea, Alternaria alternata* as well as other fungi such as *Fusarium oxysporum fsp melonis* and bacteria such as *Clavibacter michiganensis*, *Pseudomonas syringae* and *Xanthomonas campestris* etc. Although the antimicrobial activity of oleuropein, grape pomace extract (GPE) and OMWW extract have been studied widely, little research has been done on screening these extracts efficiency against phytopathogenic microoraganisms. Results from this work have been presented in:

- 13rd National Conference of Phytopathology, as poster titled "Isolation of natural antioxidants from olive tissues and their use in plant protection". Athens, Greece, October 2006.

Chapter 5 reports greenhouse and postharvest experiments to evaluate *in-vivo* of potential antimicrobial activity of oleuropein, OMWW extract and GPE against commercial varieties of plants and fruits. In particular *in vivo* antibacterial activity of pure oleuropein and OMWW extract was assessed in greenhouse experiments, on *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato*, on pepper and tomato plants, respectively. Besides endophytic growth of *Phytophthora parasitica* var. *nicotianae,* on tobacco leaves by measuring its radial growth inside the infected tissue, was assessed. Furthermore the potential of postharvest treatment of table grapes with the grape pomace extract (GPE) to restrain grey mould (*Botrytis cinerea*) disease incidence and development was estimated and finally the use of pure oleuropein as antibacterial in vase solution for cut flowers (carnation) was evaluated. Results from this work have been presented in:

- 7th Joint Meeting of AFERP, ASP, GA, PSE & SIF , Natural products with pharmaceutical, nutraceutical, cosmetic and agrochemical interest on August 2008 as poster with title: "Exploring the potential of natural substances from olive and grape pomace in phytoprotection"

- 2nd International conference on quality and marketing of agricultural products held in Hersonissos, Crete on September 2008 with a poster titled: "The natural substances of the olive and the vine and use in organic crop protection."
- 14rd National Conference of Phytopathology, held in Nafplion, Greece on October 2008 with an oral presentation titled "Application of bioactive plant substances from olive tissues and grapes in non-chemical disease control. "

Chapter 6 is a summary of conclusions which integrates the results from previous chapters and proposes recommendations for future research in non-chemical disease control.

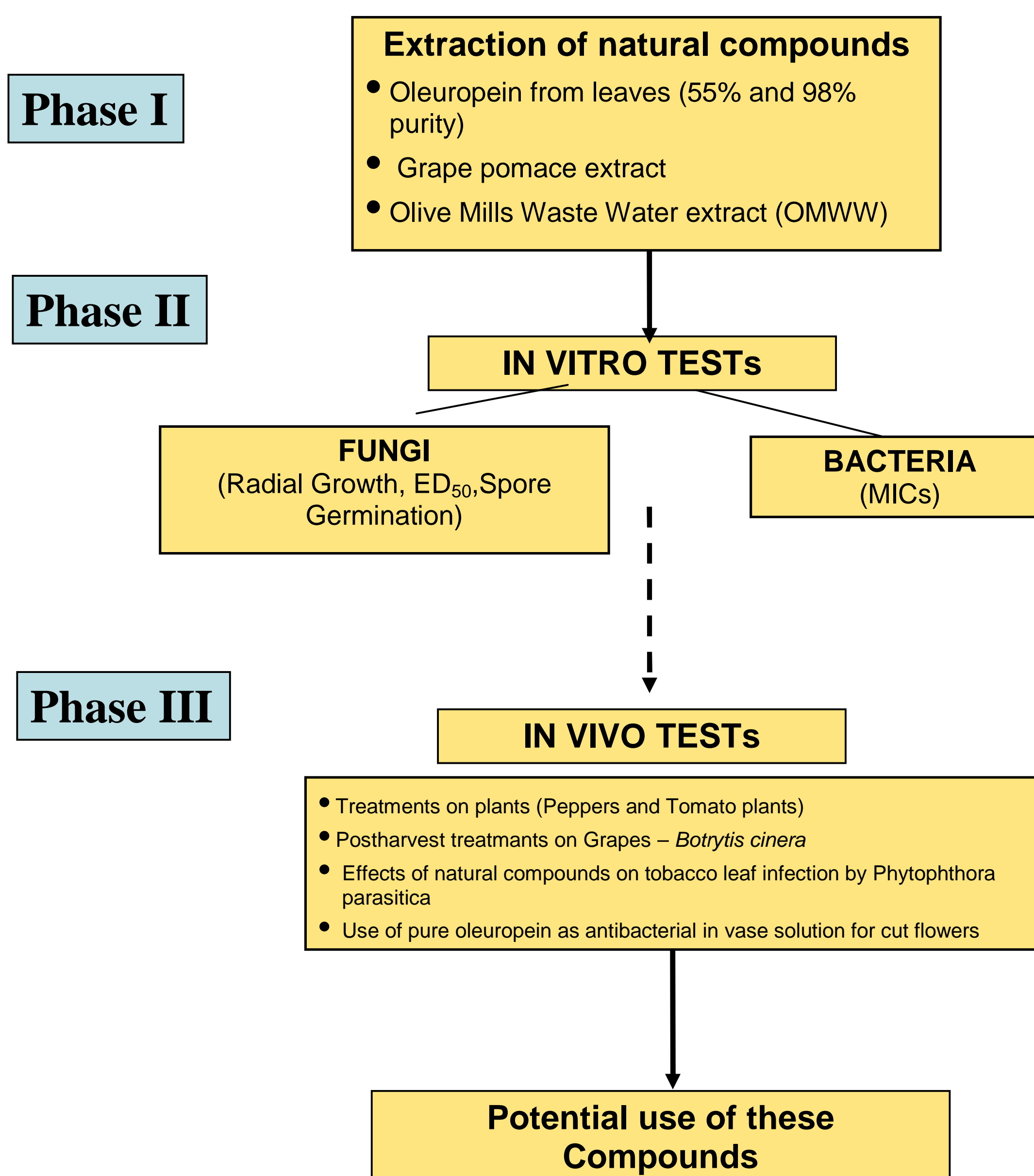


Figure 1.1 PhD plan

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

Literature Review

2.1 The Olive

According to archaeological researches, olive tree cultivation began in the south-eastern area of Mediterrannean at 5th century BC. Olive trees may have been cultivated independently in two places, Crete and Syria. In Crete an olive tree which age has determined to be over two thousands years old has been found (Rackham *et al.,* 2002).

It is believed that olive trees from north Syria olives spread to Crete and Greek islands then to mainland of Greece, then to Italy and other parts of the Mediterranean area (Standish 1960). Olives are also grown commercially in Australia, California, and South Africa.

Olive tree (*Olea europaea*) belongs to Oleaceae family. Tha family comprised from 22 genus and about 500 species, most of them belongs to the subfamily Oleoideae. Genus *Olea* include about 40 species and subspecies and it is best grown between the is native to the Mediterranean region, parts of Africa and Asia (Tutin *et al.,* 1972; Strid, 1997).

Olive prefers the temperate climate of Mediterranean area with no extremities to temperature and dampness. It is an evergreen tree (Plate 2.1), that can reach 8-15 m in heigh. It loves moisture but can survive in conditions of great dryness and grows best in fertile soils but even in the most barren soils it can take root and bear fruit.

On Crete it seems that olive trees have found the most ideal conditions for their development. Crete olive plantations have spread over the years, covering today about 65% of island's agricultural land (~23,000 hectares) and the total population of olive plantations include at least 35 million trees. The main variety of olive trees (90% of trees) is Coroneiki or Psilolia. Fruits of Coroneiki are small, ovoid, ripens early and have high and constant yields almost every year. Olive oil of this variety is green

with fruit flavour. Other varieties of olive trees which are cropped in Crete but to a lesser extend are Hondrolia in Heraklion , Tsounati in Chania and Throumbolia in Rethimnon.

2.1.2 Olive Oil

Olive oil is obtained from the fruits of *Olea europaea* trees. Mediterranean countries supply more than 95% of the world olive oil production, 75% of which comes from the European Union (mostly Spain, Italy, and Greece). About 4% of total vegetable oil production contributes olive oil. Olive oil classified in different grades depending on oil chemical properties and the degree of acidity. Extra virgin olive oil is the most valuable kind of oil, which contains no more than 0.8% acidity, and has a superior taste.

Plate 2.1 Olive tree in Crete

2.1.2.1 Production of Olive Oil on Crete

Oil production on Crete is increasing at an average rate of 3% per year. Between 1994 -1998 production was around 140,000 tons per year while today is greater than 150,000 tons per year. From year to year on Crete the fluctuation in production, was significant up until 10 years ago. Last years however production has reduced noticeably and has stabilized significantly. Well tended olives trees usually give between 8-10 kg of oil per year but it depends on tree size and the year. The fruit yield in terms of oil depends on the variety, the amount of fruit which the trees have,

and the ripeness of the fruit when it is harvested. Thus 1kg of oil can be produced from 3-7 kilos of olives.

2.1.2.2 Olive Oil Phenols

Fruits and vegetables, including olives and grapes, are continuously exposed to environmental stress, including relatively high temperatures and UV radiation, and therefore need a variety of compounds, for example antioxidants, to preserve their integrity (Soleas *et al* 1997, Toguri *et al* 1993). It is well known that olive oil is obtained from whole fruit with physical pressure and without the use of chemicals in contrast with most vegetable oils which are extracted from seeds by solvents. Therefore lipophylic components of olives fruits are transferred to the oil, which in turn retains the organoleptic properties of olives. Among the several minor constituents of virgin olive oil, there are vitamins such as a- and g-tocopherols and b-carotene (which, together with chlorophylls is responsible for the oil color), flavonoids such as luteolin and quercetin and phenolic compounds, usually termed polyphenols , phytosterols, pigments and terpenic acids.

The amount of phenolic compounds in olive oil depends on several factors, including cultivar, degree of maturation, possible infestation by the olive fly *Dacus Olea*, and climate (Boskou *et al* 2000). It usually decreases with over-maturation of olives, although there are some exceptions to this rule: for instance, olives grown in warmer climates, in spite of a more rapid maturation, yield oils that are richer in phenols. On the other hand, intact olives that are hand-picked at the right moment (when the skin color changes from pale green to dark brown), that are immediately brought to the mill, and processed right away in a clean plant, and that are crushed and pressed at temperatures lower than 25–30°C, yield a high-quality oil that is also rich in phenolic constituents.

Elaboration process influence on the amount of phenolic compounds in olive oil is yet to be fully elucidated. It seems that that oils that have been obtained by centrifugation have lower phenols content (Di Giovacchino *et al* 1994) probably because this process used large quantities of warm water, with which the olive paste is continuously hosed during the milling. This is known as olive mill waste water (OMWW) and is produced in extremely large quantities. Olive oil contains only 2%

(50-1000 μg/g) of the total polyphenols that are contained in the olive drupe, while the rest 98% is transferred in the waste waters. A series of experiments performed by Visioli (Visioli *et al* 1999, Visioli *et al* 1995), demonstrated that OMWW extracts have powerful (in the ppm range) antioxidant activity and might therefore be recovered and employed in preservative chemistry as a cheap, source of natural antioxidants.

The major phenolic compounds in olive oil are shown in Figure 2.1. The three phenolic compounds in highest concentration in olive oil are the glycoside oleuropein, hydroxytyrosol (3,4-dihydroxyphenyl ethanol) and tyrosol. These three compounds are related structurally. Hydroxytyrosol and tyrosol are structurally identical except that hydroxytyrosol posses an extra hydroxy group in the *meta* position. Oleuropein is an ester which consists of hydroxytyrosol and elenolic acid. Oleuropein is the major phenolic compound in olive fruit, which can be as much as 14% in dried fruit, hydroxytyrosol is the major phenolic component in olive oil (Amiot *et al* 1996). As the olive fruit matures the concentration of oleuropein decreases and hydroxytyrosol, a hydrolysis product of oleuropein increases (Cimato *et al* 1990, Ryan *et al* 1999).

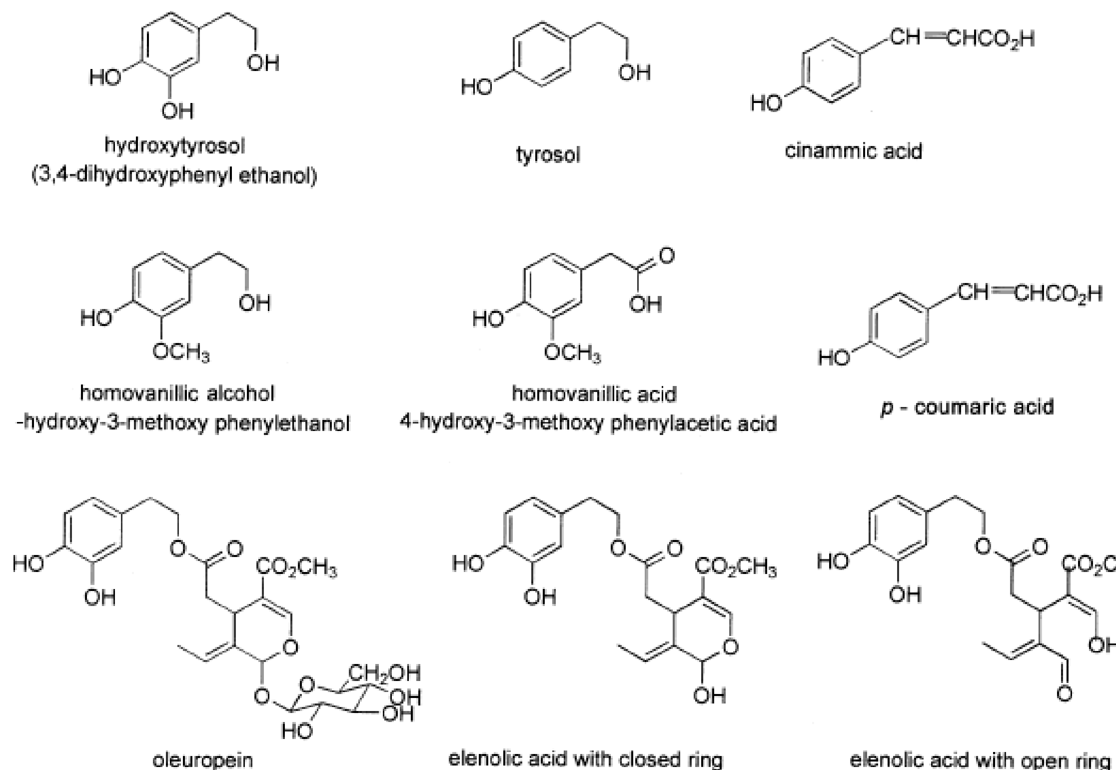


Figure 2.1. The major constituents of olive oil (Tuck and Hayball, 2002).

2.1.3 Chemical Compounds in Olive Leaves and OMWW

Olea europaea comprises one of the richest sources of secondary metabolites. Table 2.1 contains the main references of chemical compounds that have been isolated from olive leaves and OMWW.

Table 2.1 Main references of chemical compounds that have been isolated from olive leaves and OMWW.

Chemical category	Reference
Olive leaves	
Iridoids (Oleuropein)	**Gariboldi *et al.,* 1986; Kuwajima *et al.,*1988; Paiva-Martins and Gordon, 2001)**
Flavonoids	**(Le Tutour and Guedon, 1992; Meirinhos *et al.,* 2005)**
Triterpenes (Oleanolic acid)	**(Caputo *et al.,* 1974; Somova *et al.,* 2003)**
Olive mills waste water	
Polyphenols	**(DellaGreca *et al.,* 2004; Obied *et al.,* 2005; Bianco *et al.,* 2006)**
Flavonoids	**(Obied *et al.,* 2005)**
Iridoids	**(DellaGreca *et al.,* 2000; Mulinacci *et al.,* 2001)**

Phenolic compounds in *Olea europaea* leaves are important factors to consider in order evaluating virgin olive oil quality because they are partly responsible for its autoxidation stability (Vazqueez 1975, Perrin 1992) and organoleptic characteristics (Vazqueez 1978). Moreover these molecules have pharmacological properties (Maestro- Duran 1994), are natural antioxidants (Chimi 1991, Le Tutor 1992) and inhibit the Gram positive microorganisms involved in olive fruit fermentation (Brenes 1992, Brenes 1995).

These properties of olive leaves is due to existing iridoids and especially oleuropein which is responsible for the bitter taste of unripened olives and hydroxytyrosol (Le Tutour and Guedon, 1992, Ghisalberti 1998). Oleuropein, was discovered in 1908 by Bourquelot and Vintilesco and it a 3,4-dihydroxy-phenylethanol (hydroxytyrosol) ester with a β-glucosylated elenolic acid (Esti 1998, Panizzi 1990). The aglycon, which is obtainable from oleuropein hydrolysis, is well-known as a pharmacologically active molecule for its potential application as an antimicrobial agent in some fairly common olive tree diseases (Lo Scalzo *et al* 1992). At any rate olive leave extract show higher antioxidant activity than these substances solely (Le Tutour and Guedon, 1992).

OMWW is an aqua extract which contains polysaccharides, sugars, polyphenols, polyalcohols, proteins, organic acids, and oil (Zouari 1998). For OMWW processing, about 20 million tones of water expanded per year and derived 30 million tones wastes water. In Greece 2500-3000 olive oil mills are operating the great majority of which are three-phase centrifugal mills with average daily olive oil production per mill of 15-20 tones (Manios 2004).

Although OMWW is becoming a serious environmental problem due to its high concentration of organic matter (BOD ranging between $15x10^3$ - $50x10^3$ mg/l and COD reaching about 220 g/l), they are, on the other hand a very rich source of phenolics compounds with antioxidants and antimicrobial activities (Visioli et al 1995b) that are useful for the pharmaceutical and cosmetic industry. OMWW concentration of phenolics compounds is 0.5-1.8 % and contains large amounts of hydroxytyrosol, oleuropein, tyrosol and lactone (Figure 2.2). It should be noted that the concentration of the polyphenolic compounds in olive oil ranges from 50 to 1000 μg/g of oil depending on the olive variety and the extraction system and that this amount of antioxidants in the olive oil is only 1-2% of the available pool of antioxidants in the olive fruit (Rodis *et al* 2002). The rest is lost either in the wastewater (approximately 53%) or in the pomace (approximately 45%).

Hydroxytyrosol is a natural compound with pharmaceutical, antioxidants (Visioli *et al* 2001) antimicrobial and phytotoxic properties (Visioli *et al* 1999). From a financial point of view, it should be noted that the cost of 1 gr. of pure Hydroxytyrosol is about

1400 euro's (Extrasynthese, France) for scientific purposes. One litre of OMWW contains about 130 mg hydroxytyrosol and if we consider that olive mills can produce 30 million tones of OMWW, we understand that this is a hugely and unexploited source of natural antioxidants.

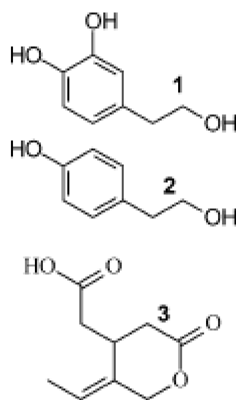


Figure 2.2 Structures of the main antioxidants isolated from OMWW.: **1**. Hydroxytyrosol (HT), **2.** Tyrosol and **3**. (5-ethylidene-2-oxo-tetrahydropyran-4-yl)-acetic acid.

2.1.4 Biological Properties of Olive Leaves and Olive oil

a. Olive leaves

The first official report on the use of olive leaves in medicine dates back to 1854, when Hanbury reported a simple recipe for the use of aqueous extract of olive leaves as a febrifuge in review in Pharmaceutical Journal. Since the early 20th century there are many reports in the literature for the activity of olive leaves. Paticularly it was reported the antioxidant activity (Le Tutour and Guedon, 1992; Ghisalberti,1998; Owen *et al.,* 2000), antimicrobial activity (Walter et al., 1973; Ghisalberti, 1998) the antihypertensive activity (Visioli and Galli, 1994; Ziyyat *et al.,* 1997; Ghisalberti, 1998; Visioli and Galli, 1998), the vasodilatory activity (Pieroni *et al.,* 1996) and the hypoglycaemic activity (Gonzalez *etal.,* 1992).

Currents studies have shown that olive leaf extract had the capacity to lower blood pressure (Samuelsson 1951), vasodilator activity, increase blood flow in the coronary arteries (Zarzuelo 1991), and relieve arrhythmia and prevent intestinal muscle spasms while new possible activities studied.

Olive leaves extract and especially oleuropein has been found to have strong antimicrobial activity against fungi, bacteria viruses and other parasites (Walter et al., 1973; Ghisalberti, 1998, Aziz *et al* 1998, Juven *et al* 1972, Koutsoumanis *et al* 1998, Tassou *et al* 1995). In particular, olive leaves extract was found to be effective in vitro against many microorganisms, such as *Staphylococcus aureus* (at low concentrations reduces the growth rate, at higher concentrations inhibit growth, and inhibits the production of enterotoxin B, regardless of concentration), *Vibrio cholerae, Vibrio parahaemolyticus, Haemophylus influenza, Salmonella* spp., *Bacillus cereus* (inhibits spore germination) etc.

Oleuropein has also recently been shown to inhibit or delay the rate of growth of bacteria and fungi (Aziz *et al* 1998, Tassou *et al* 1995, Fleming 1969, Juven *et al* 1972, Ruiz-Barba *et al* 1990, Nychas *et al* 1990, Capasso *et al* 1995, Capasso *et al* 1995, Bisignano *et al* 1999, Tranter *et al* 1993), so that it might be useful as an alternative food additive (Tassou *et al* 1995, Nychas *et al* 1990, Tranter *et al* 1993).

Studies of six major phenolic compounds contained in ethyl acetate extracts of green olives have shown that these have antimicrobial properties (Fleming 1969). Moreover, *Lactobacillus plantarum, Staphylococcus carnosus, Enterococcus faecalis, Salmonella enteridis, Pseudomonas fragi,* and some fungi were inhibited by the oleuropein and its aglycone (Aziz *et al* 1998, Tassou *et al* 1995, Fleming 1969, Ruiz-Barba *et al* 1990, Tassou 1991, Bisignano *et al* 1999).

The antibacterial efficiency of oleuropein, possibly is due to phenolic substances properties to prevent the formation of cell wall with subsequent leakage of cell components. However, it has not been adequately demonstrated *in vivo* efficacy of olive leaves extract and oleuropein.

Already in the U.S. market but also on the Internet various companies sell olive leaf extracts as diet supplements in tablet form (Plate 2.2). Their recommended use is as antimicrobial in fungal and viral infections such as influenza and herpes, reinforcing the immunogenic system and finally recommended for chronic fatigue.

Plate 2.2 Commercial nutrition supplements of olive leaf extract

b. Olive oil

One of the geographic areas in which dietary habits have attracted the interest of scientists because of protective role of food to substances that cause oxidation, is the Mediterranean, particularly Crete. There are many elements to believe that the Mediterranean diet plays an important role against many diseases such as coronary heart disease (Buzina *et al.,* 1991). The Mediterranean diet is rich in mono-and poly-unsaturated fatty acids, fiber and substances with antioxidant properties, but low in saturated or hydrogenated fats. Major fat component of Mediterranean diet is olive oil (Nestle, 1995; Willett *et al.,* 1995). Mediterranean populations such as Greeks and Italians have great life expectancy and low cancer rates and chronic diseases related with diet (Harman, 1956), although they have high rates of smoking (Menotti, 1991). The study of seven countries after 25 years of observation, the Cretan group of volunteers had lower mortality even showed 5 times lower mortality rates from coronary heart disease in a 25-year base and lower mortality from cancer at 25 years base again. (Hertog *et al.,* 1995; Kafatos *et al.,* 1997; Dontas *et al.,* 1998). Total mortality was 35.3% for the Cretan team of volunteers (Kromhout, 1999). In a recent study by the American Society of Cardiology, 605 volunteers who had suffered myocardial infarction within 27 months the group which put on Cretan diet showed 75% lower mortality (Renaud *et al.,*1995), while after 4 and 5 years rates were 56% (Lorgeril *et al.,* 1998) and 70% (Lorgeril *et al.,* 1994) respectively compared with the control group.The lower death rates from cardiovascular disease and cancer and longer life expectancy observed in these cases were mainly attributed to the high consumption of olive oil, vegetables, fruits and in fiber intake, vitamins, flavonoids and polyphenolic substances characterizing the Cretan diet. Also found that many of the substances consumed in the traditional Cretan diet, have antioxidant properties and are able to prevent lipoprotein oxidation (Ross 1993, Visioli 1998), which is considered to be a key factor in the pathogenesis of atherosclerosis (Quinn *et al* 1987, Steinberg, 1997).

Olive oil comprise a complex mixture of various substances. Great importance and intense pharmacological interest are the polyphenolic constituents of olive oil. Oleuropein and its derivatives and hydroxytyrosol are the main polyphenolics of olive oil (Panizzi *et al.,* 1960) and have a variety of biochemical roles (Manna et al 1999,

Visioli et al 2002), including anti-inflammatory and antithrombotic activities (Carluccio et al 2003). These polyphenols are able to prevent low-density lipoprotein oxidation (Wiseman et al 1996, Visioli et al 1994) and platelet aggregation and to inhibit lipoxygenases and eicosanoid production (Visioli *et al* 1994, Petroni *et al* 1995). Furthermore by using cell-free systems it was demonstrated that oleuropein and hydroxytyrosol are powerful scavengers of peroxyl radicals, HCIO, superoxide radical, hydrogen peroxide and synthetic radicals(Visioli *et al* 1998, Aruoma *et al* 1998, Pellegrini *et al* 2001).

Other substances contained in olive oil are hydrocarbons (saturated and unsaturated) esters (sterols, alcohols and others), aldehyde, alcohols, phenols (tocopherols, epoxyphenols) acids and chlorophyll. The stability of olive oil in oxidation is due to high concentration of oleic acid contained and lesser in other phenols.

2.2 Grapes and Wine

The cultivation of vine goes back to prehistoric times, which shows that both the viticulture and the winery one known to man almost from the beginning of history. Since ancient times (the inhabitants of ancient Greece used grapes in their nutrition from 4000 BC) the vine cultivation and the dietary value of viticultural products were known. Greece location, environment and climate ensure the production of quality grape and wine production.

The size of Greek vineyards (1.317 ha, 1993) is small compared with other countries (Spain 12.238 ha, Italy 10.774 ha, France 10.340 ha, 1987) where characterized by small tracts of land. From 162.296 vineyards of Greece, approximately 77% have an area exceeding 1ha, 14% ranged from 1-2 ha and only 9% have an area over 2 ha, while the average size of vineyards is 0.55 ha (1987) in contrast with 0.9ha of Italy, 1.91 ha of Germany, 3.6 of Spain and 3.76 ha of France.

Plate 2.3 Vineyards on Crete

2.2.1 History of Wine Making in Crete

Crete has the most traditional vineyards of Europe and has a 4000 years of wine tradition, since the Minoan era. This is confirmed from the findings in Zakro area (eastern Crete) where the oldest cultivated grapevine was found and in Vathipetro area (Heraklion) where the oldest stone foot press in the world, dated to 1600 BC was found (Plate 2.4). There were periods when Cretan wine production enjoyed great commercial prosperity. Major stations were, the period between 1st and 2nd century AD in great Crete wine history. During that time Cretan wines were sealed in elaborated jars, transporting throughout the Mediterranean area and the period of Venetian rule over the island (12th -16th century), since the Cretan wine reached the zenith of its reputation with a sweet type of wine known as 'Malvazia wine'

Plate 2.4 Minoan wine press which was found in Vathipetro archaeological site outside the village of Archanes (Heraklion, Crete).

Crete was famous for centuries for its red wines from old native varieties. From these same varieties produced now wines, coming to market place with their place names (Archanes, Dafnes, Peza, Sitia are the main wine producing areas). The main red grapes varieties in Crete are Kotsifali, Mandilari, Liatiko, Romeiko, while the main white grapes varieties are Vilana and Athiri. Of the white varieties, were less important in the Cretan vineyard, 'Vilana' currently holds a significant position in the wine production of Heraklion region.

2.2.2 Phytochemical Approach of *Vitis vinifera*.

Vitis vinifera has been the subject of extended phytochemical study. A lot of research on different varieties of grapes gluts continuously scientific journals, enriching the list of secondary metabolites that have been isolated. Table 2.2 presents only a few publications on the *Vitis vinifera* plant, indicative of chemical category of compounds that have been isolated.

Table 2.2 Main references of chemical compounds that have been isolated from *Vitis vinifera* plant.

Chemical category	Reference
Phenolics	**Guendez R. *et al.,* 2005**
Flavonoids	**Yilmaz Y. &Toledo R.T., 2004**
Terpenes	**Lucker J. *et al.,* 2004**
Tannins	**Vivas N. *et al.,* 2004**
Stilbenes	**Ito J. *et al.,* 2000**
Anthocyanins	**Renault J.H *et al.,* 1997**

As shown in Table 2.2, the molecules that have been isolated from grape belongs to the wider class of polyphenolic molecules. It is therefore obvious that the diverse and intense presence of phenolic compounds in *Vitis vinifera* is the reason of great phytochemical interest of this plant. Figure 2.3 shows the chemical structure of molecules that have been isolated.

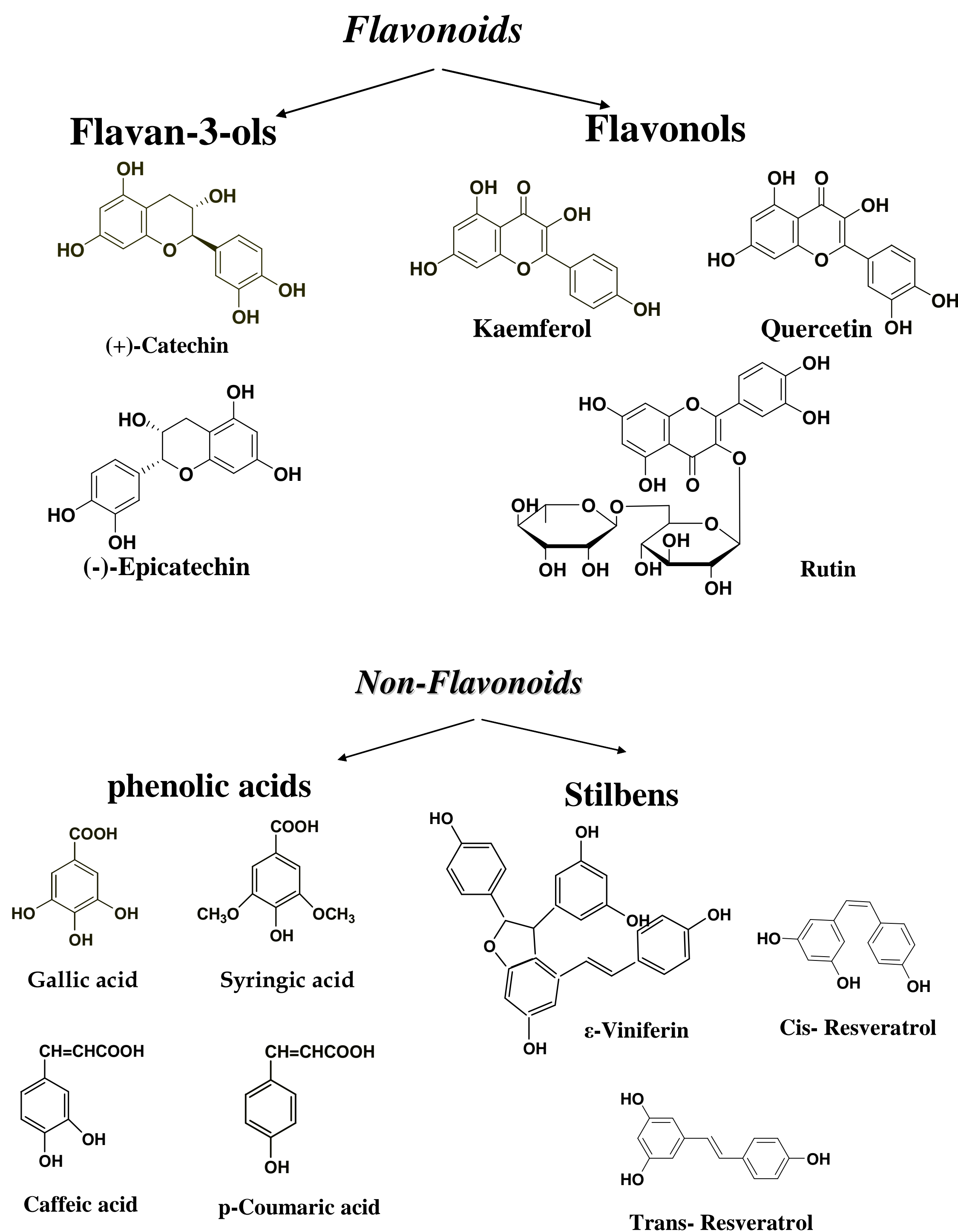


Figure 2.3 Chemical structure of grape and wine phenolics that have been isolated and can be divided into two groups: nonflavonoids and flavonoids.

2.2.3 Biological Properties of Grapes and Wine

Although, phytochemistry of *Vitis vinifera* is very attractive to scientists, there is also a great interest for the beneficial effects of that plant and its metabolites (Renaud 1992, Torres et al 2002, Murthy et al 2002).

Antioxidant activity: It is known that grapes are rich in antioxidants substances such as flavonoids, stilbenes, anthocyanins, etc. Extracts mainly from red grapes have demonstrated significant antioxidant activity which is growing in proportion to the containing percentage of polyphenolic molecules (Negro *et al.* 2003). In addition extracts from seeds, bark and from whole grapes have great obstructive activity against free radicals (Caillet *et al.* 2006). Another category of substances, found in grapes, are the stilbenes. In particular, resveratrol, the main representative of stilbenes, has strong antioxidant activity and protects body from oxidative stress (Li Y. *et al* 2006). The same activity against free radicals is also shown by anthocyanins which are responsible for the coloration of grapes (Stintzing F.C. & Carle R., 2004). Anthocyanins, in synergy with other antioxidants of grape extracts, by preventing the oxidation of cells in pancreas, which produces insulin, becomes a protective agent against occurrence of diabetes (Rahimi *et al.* 2005).

Protective effect of cardiovascular diseases: Beneficial effect of wine is a phenomenon that appeared first in the international literature in 1992 (Renaud S. & De Lorgeril M., 1992) and called "French Paradox". The principle for the formulation of this phenomenon was the conduct of worldwide clinical research on the relationship between a diet rich in fat and many calories with mortality from coronary heart disease. This relationship was found to be similar. However, in some regions of France, the mortality was markedly lower (about 1/3) than in the U.S. and Great Britain and was closer to statistics in China and Japan. This initially seemed paradoxical, since the daily intake of fat from the French was the same as in the other countries. Therefore, scientists have sought to distinguish the difference in the diets of these countries. After more extensive studies it was found that the French have lower rate of heart disease, because of the larger amount of red wine consumption at meals. It has been reported the protective role of grape extract in situations of ischemic attack. (Gross G.J. 2005). Both in vivo experiments and clinical trials have demonstrated the ability to scavenge free radicals, the protection of myocardial cells,

the reduction of athirogenesis possibility and generally better heart function (Bagchi *et al.* 2003). Polyphenols that have been isolated from grapes, have a beneficial effect on hypertension, coronary heart disease and other cardiovascular diseases. It was found an inverse relationship between intake of polyphenols and the occurrence of cardiovascular diseases (Dell' Agli et al. 2004, Stoclet et al., 2004). In addition flavonoids reduce platelet aggregation and prevents oxidation of low-density lipoproteins (LDL), resulting in the reduction of blood cholesterol levels and reducing the risk of cardiovascular diseases (Frankel et al 1995, Teissedre et al 1996). Such activity shows also and Resveratrol (Ethernon *et al*. 2002, Mayer *et al* 1997).

Anticancer activity: *Vitis vinifera* chemoprotective activity has been tested in *in vivo* experiments on skin cancer of mice in which tumours were created on skin surface. It proved that ethanol grape extract prolong the latency of tumours and reduces their number (Alam et al., 2002). Besides, many food components which are beneficial against cancer, one found in grapes (resveratrol, quercetin etc.) (Chen & Kong 2005). Several *in vivo* and *in vitro* studies have shown that grape extracts showed cytotoxicity towards cultured human cancer cells (e.g. breast and lung cancer cells) as well as inhibited human prostate tumor xenograft growth in mice (Agarwal *et al* 2000, Singh et al 2004). Flavonoids of grapes can also affect the metabolism of foods cancer-causing agents and inactivate them (Stavric 1994). Resveratrol also shows significant anticancer activity and is considered one of the most effective antineoplastic molecules of plant origin. It can be applied in regimens with traditional cancer drugs, in order to increase the sensitivity of tumour to them and reduce their side effects (D' Incalci 2005).

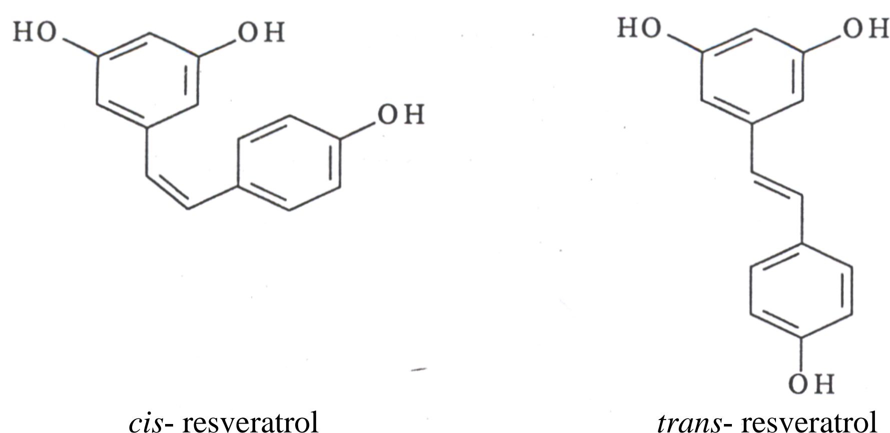
Antimicrobial activity: Grape extracts have shown antibacterial action in several *in vitro* experiments, which is attributed to containing polyphenolic metabolites. In particular, red grape extracts were tested against 15 strains of microorganism such as *Bacillus Brevis, Escherichia coli, Pseudomonas aeruginosa* etc. Results were very encouraging since it was demonstrated that the extracts were active at concentrations of 4% and 20%. This action is particularly important as the grape extracts may be used as antibacterial agents to protect food from spoilage caused by bacteria. Grape seeds and pomace extract have also been studied for their antimicrobial activity (Jayaprakasha *et al.* 2003, Baydar *et al,* 2004). Recently Anastasiadi et al (2009)

reported the antilisterial acivity of grape berries extracts rich in polyphenols and vification byproducts, obtained from Greek islands *Vitis vinifera* varietes.

2.2.4 Resveratrol

Resveratrol as mentioned before, belongs to the stilbenes. Stilbenes are polyphenolic compounds produced by various plants in response to situations of stress (Schultz *et al*., 1990). Thus, when a plant is facing an external risk factor (eg, fungal infection such as *Botrytis cinerea*, bad weather), this initiate mechanisms of synthesis of these substances. The stilbenes and other molecules with defense capabilities are known as phytoalexins and their production is related from how bad the external conditions are.

Resveratrol is a 3,5,4´- trihydroxystilbene. It exists as two isomers *cis*- and *trans*- (Figure 2.4). Trans- resveratrol is responsible for significant beneficial properties on human health.



cis- resveratrol *trans*- resveratrol

Figure 2.4 *cis*- and *trans*- , isomers of resveratrol

The best known natural source of *trans*-resveratrol are grapes. The quanity of resveratol depending on the crop, the time of collection and the magnitude of adverse circumstances affecting the grape varieties studied (Burns J. *et al*, 2002). The presence of resveratrol in vine tissues and induction by fungal infection was first reported by Langcake (Langcake *et al* 1979, Langcake 1976, Langcake 1981).

Langcake also had demonstrated that UV-irradiation, but not natural sunlight, could stimulate resveratrol synthesis (Langcake *et al* 1977).

Wine also contain resveratrol. Specifically the presence of *trans*-resveratrol has been reported in greater quantities in red wine than white (Bavaresco *et al*, 1999). The average content of *trans*-resveratrol in red Greek wines are a hundred times more than the corresponding levels of white Greek wines (Dourtoglou *et al*, 1999). Other sources, rich in resveratrol, are weed *Polygonum cuspidatum* (Nonomura *et al* 1963, Kubo *et al* 1981), peanuts, tea and the wood of *Eucalyptus* and *Finns*.

Resveratrol has rich pharmacological activity such as antioxidant, anticancer, antimicrobial activity, protective effect against cardiovascular diseases and many other activities.

2.2.5 Winery By-products

A large proportion of agricultural by-products are composed of plant tissues rich in phytochemicals, which may possess valuable chemical and biological properties. The recovery of high added-value products from waste plant material has therefore been a significant issue with economic importance for the pharmaceutical and food industries. There is currently an increasing interest in the isolation, examination and exploitation of agricultural wastes or inexpensive plant sources, rich in polyphenols. Only a small amount of this wastes are recycled or exploited for composts or animal feed

Grape pomace is an agricultural waste produced in huge quantities during the vinification. It is composed by solid residues (skin and seed) of grapes that remain often removal of their juice, consisting mainly of sugars, which certainly reduces the rate of polyphenolic constituents and difficult the process of isolation.

Grape pomace as a by-product is approximately 20% of the harvested grapes (Laufenberg *et al* 2003). In Greece the total volume of grape production is 100,000 tons. A large fraction of this amount is processed by 400 wineries located in Greek mainland to produce 400,000 tons of wine. The large volume and content of these

wastes constitutes a serious environmental pollution factor, which is increasing constantly.

On the other hand, most wineries are small to medium size enterprises that cannot afford sufficient management of their waste and strict regulations limit the amount of ethanol produced from the waste by distillation. Thus, they adopt environmentally non-acceptable practices to manage their wastes. Their most usual action refers to the transfer of solid in open fields, where they are transforming slowly to fertilizer by aerobic biodegradation. The sludgy wastewater is also discarded in fields, lakes, rivers or sea.

Greece is one of the most prominent wine producing regions of Europe, with many small and medium size wineries in operation. A decrease in the amount of the produced waste will have positive effects. However, the high economic cost of waste management has not allow the alteration of the environmentally harmful practices that are currently followed.

Plate 2.5 Winery Byproducts that have been rejected after vinification,

2.2.5.1 Polyphenolic compounds of winery byproducts

The polyphenolic compounds present in the grapes pass to the wine more or less depending on the characteristics of the winemaking process. But independently of this transfer, and bearing in mind that the major part of these compounds is found in the grape solids, a high proportion remains in the vinification residues or wastes. The large amount of polyphenols in grape pomace, especially of red grapes, is a disadvantage for their potential use as animal feed or for composting, thus before further utilization polyphenols should be extracted.

Nowadays, industry produces grape seed and grape skin extracts that are finding increasing applications as food lipid antioxidants (Steinberg 1997) or dietary supplements for disease prevention (Keys 1995, Ross 1993) (Plate 2.5). Recently, even a new family of antioxidants has been obtained from a residual fraction of polymeric polyphenols of grape origin (Mancini *et al* 2000). Recent studies have demonstrated the beneficial effects of grape pomace polyphenols for human health (Simic 1994, Rice-Evans *et al* 1997, Lurton 2003). In particular, many reports refer to the antioxidant properties of grape pomace extract (Monagas *et al.,* 2006), and its ability to reduce cholesterol in *in vivo* experiments (Bobek P., 1999, Renaud et al 1992, Halpern *et al* 1998).

Extracts from residues left in the production of wine are used as active substance combinations for producing cosmetic and pharmaceutical compositions (Plate 2.6). Such compositions are used in skin and hair products (Henry *et al* 2001, Pykett *et al* 2001) and hemorrhoid treatments (Borod *et al* 2001). Also, a preservative solution for peeled fruits and vegetables, juices, and cut flowers has been patented which includes flavonoids provided from grape seed oil (Selleck, 2001).

Plate 2.5 Commercial nutrition supplements and cosmetics from grape extracts.

2.3 Effect of vase solutions microorganisms on cut carnation vase life

A major problem affecting the cut flower stage is the growth of microorganisms (fungi and bacteria) in vase solutions. Van Doorn (1997) reported that microorganisms multiply rapidly, under favourable conditions and can enter through vessels above the cut surface causing bacterial occlusions. Put and Van der Meyden (1988) indicated that the number of bacteria usually decreases on the way up to the stem. The presence of bacteria in the vase solution influences the vase life of cut roses and affects their longevity (Florack *et al.,* 1996). In other study, was found that vase life of 'White Sim' carnations was reduced, when the vase solution (water) that was maintained it was artificially infected with bacteria 10^8 cfu ml^{-1} (Papadimitrioy 2006)

A major cause of quality deterioration in cut flowers is the blockage of xylem vessels by microorganisms that accumulate in the vase solution or in the vessels themselves. It has been suggested that addtion of antibacterial agents improve water conductance by preventing bacterial growth and producing occlusions (Van Doorn, 1997). Knee (2000) indicated that the application of HQS (Hydroxyquinoline sulfate) increased significantly cut carnation vase life and the gain of fresh weight compared with the control. In addition to HQS, many germicides, such as silver nitrate, aluminum sulfate, copper sulfate etc have been shown to inhibit bacterial growth in cut flower stems (Van Doorn, 1997; Van Meeteren, 2000, Torre and Fjeld, 2001). Chlorine which also used to control bacteria in vase solutions (van Doorn *et al.,* 1990; Joyce *et al.,* 1996; Knee, 2000; Faragher *et al.,* 2002) is a constituent of many preservatives, such as DICA (Dichloroisocyanuric acid). DICA is a slow degradation chloride compond that maintains free chlorine availability under chlorine demand conditions.

CHAPTER THREE

EXTRACTION AND ISOLATION OF NATURAL COMPOUNDS FROM OLIVE LEAVES, OLIVE MILS WASTE WATER AND WINERY BY-PRODUCTS

CHAPTER THREE

3.1 INTRODUCTION AND OBJECTIVES

Plant polyphenols have been reported to have a variety of biological effects, including antimicrobial, antioxidant, anticarcinogenic and antiinflammatory activities Specifically some phenolic compounds such as oleuropein, resveratrol, hydroxytyrosol and a number of phenolic acids have been reported to inhibit various microorganisms (Aziz, Farag, Mousa, & Abo-Zaid, 1998; Bisignano, et al, 1998; Papdopoulou, Soulti, & Roussis, 2005).

Olive mills waste waters (OMWW) and winery by-products constitute a cheap source of phenolic compounds and are rich in such components. The recovery of high added-value products from olive leaves, olive mill waste water has been a significant issue with economic importance for the pharmaceutical and food industries. There is a great interest in the isolation and exploitation of natural antioxidants.

The aim of this study was to extract and purify natural antioxidants from olive leaves(oleuropein), OMWW (hydroxytyrosol) and grape pomace (resveratrol, quercetin) using Amberlite XAD adsorbents and medium pressure liquid chromatography (MPLC).

3.2. MATERIALS AND METHODS

3.2.1. OLIVE LEAF EXTRACT

3.2.1.1 Plant material

Oleuropein was isolated from leaves of *Olea europaea* var. koroneiki. The leaves were picked randomly from trees of an approximately 50-60 years-old olive grove in the island of Crete (Greece) during the crop season 2005–2006.

3.2.1.2 Extraction and isolation

The olive leaves were dried at room temperature and pulverised. A portion of powdered leaves (50kg) were then exhaustively extracted 3 times with 150l acetone for 24 hr (plate 3.1). After filtration through a filter paper, the solvent was removed under reduced pressure (Büchi Rotavapor R220) (plate 3.2) to give a dark green solid residue ~10kg. The residue was further treated with CH_2Cl_2/ MeOH solvent system resulting in 50-60 % oleuropein purity. Decanting the supernatant and evaporating to dryness resulted in a yellow powder of about 50-60 % oleuropein purity, and a total yield of approximately 3 – 3.5 kg.

Further, 500g of previous extract (oleuropein 50-60%) was evaporated to dryness and was subjected to chromatographic fractionation with a medium pressure liquid chromatography (MPLC) using silica gel 60H as the static phase with a CH_2Cl_2/MeOH solvent system of increasing polarity (100:0 to 0:100) (Table 3.1). 20 eluted fractions of 1 l were determined for max purity oleuropein (99%) by thin-layer chromatography (TLC) on glass pre-coated silica gel 60 F_{254} and RP-18 F_{254} sheets, with detection under 254 and 366nm UV lamps and by spraying with a methanolic solution of vanillin sulfate.

Pure oleuropein was bought from Extrasynthese (Genay France) and used as a control. Flash column chromatography was carried out using silica gel flash (Merck, 0.040–0.060 mm) with an applied pressure of 300 mbar. MPLC was performed with a Buchi model 688 Chromatography pump (Plate 3.3).

Table 3.1 MPLC solvent system of increasing polarity (CH_2Cl_2:MeOH / 100:0→0:100).

Fraction	Solvent system
1	CH_2Cl_2 / 100
2	CH_2Cl_2:MeOH / 95:5
3	CH_2Cl_2 :MeOH / 95:5
4	CH_2Cl_2 : MeOH / 92.5:7.5
5	CH_2Cl_2 : MeOH / 92.5:7.5
6	CH_2Cl_2 : MeOH / 92.5:7.5
7	CH_2Cl_2 : MeOH / 92.5:7.5
8	CH_2Cl_2 : MeOH / 92.5:7.5
9	CH_2Cl_2 : MeOH / 90:10
10	CH_2Cl_2 : MeOH / 90:10
11	CH_2Cl_2 : MeOH / 87.5:12.5
12	CH_2Cl_2 : MeOH / 87.5:12.5
13	CH_2Cl_2 : MeOH / 87.5:12.5
14	CH_2Cl_2 : MeOH / 87.5:12.5
15	CH_2Cl_2 : MeOH / 85:15
16	CH_2Cl_2 : MeOH / 85:15
17	CH_2Cl_2 : MeOH / 80:20
18	CH_2Cl_2 : MeOH / 80:20
19	CH_2Cl_2 : MeOH / 50:50
20	MeOH / 100

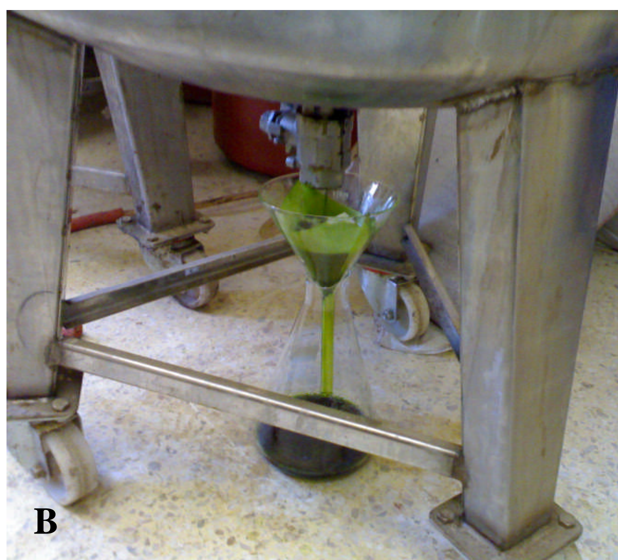


Plate 3.1 A, B. Extraction apparatus

Plate 3.2. Büchi Rotavapor R220

Plate 3.3. Büchi 688 Chromatography Pump

Plate 3.4. MPLC system

3.2.2. OLIVE MILLS WASTE WATER (OMWW) EXTRACT

3.2.2.1 Amberlite XAD polymeric adsorbents

The presence of phenol and phenolic compounds in industrial waste effluents has become an increasingly important source of stream pollution due to the direct discharge of such wastes into waterways in Crete. With increased governmental pressure to reduce phenol concentration in effluent streams to 0.1 mg/l or less it has become increasingly important to develop processes which can accomplish this objective and at the same time permit the recovery of phenol in a useful form.

It has been demonstrated that Amberlite XAD polymeric adsorbents can remove substantial quantities of phenolic compounds (Paleos *et al.* 1969) from aqueous solution and also have the added benefit of being easily separated with nonaqueous solvents or caustic solution. In many cases this permits the recovery of the phenolic material in a useable form.

The Amberlite XAD adsorbents are hard, insoluble beads of a porous polymer characterized by a spectrum of surface polarities and by a variety of surface areas, porosities, and pore size distributions (Albright 1972, Gustafson 1970). Because of the difference in surface properties, the polymeric sorbents display a wide range of sorption (via van der Waals forces) behavior and can be employed in both aqueous and nonaqueous systems.

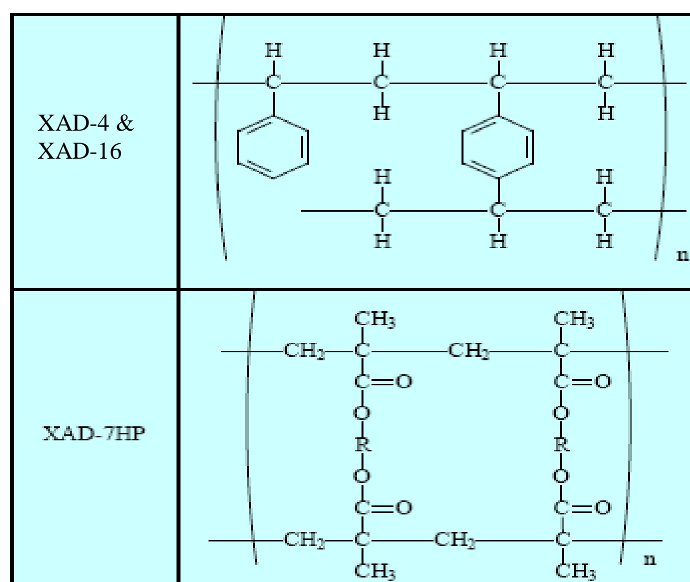


Figure 3.1 Chemical structure of polymeric adsorbents XAD-4 XAD-16 XAD-7HP

Three resins, namely, XAD-4, XAD-7HP, and XAD-16 (Rohm and Hass Co.), (Figure 3.1) were tested (Agalias *et al* and Kazantzoglou *et al* unpublished data) in the adsorption experiments. Among these adsorbents, the commercially available Amberlite XAD-4 and XAD-16 resins were reported to be an ideal adsorbent for a wide variety of organic compounds, especially for phenols.

It has been demonstrated (Agalias *et al* 2007) that both XAD-4 and XAD-16 are capable of successfully adsorpting hydroxytyrosol but that XAD-16 possesses the highest breakthrough adsorption capacity and should be considered as the most preferred resin. XAD-7 is also highly efficient, but during the experiments with OMWW, it was observed that it presented a very high affinity to the contained colorants and consequently it could not be used for selective adsorption of hydroxytyrosol.

3.2.2.2 Olive Mills Waste waters

The wastewaters were collected from a three phase olive mill in Heraklion, Crete, Greece, in February 2007. The wastewaters were stored in a tank at ambient temperature for 48 hr before filtration.

3.2.2.3 Extraction and isolation

a. Filtration.

The olive mills waste water treatment process initially involved three successive stages of filtering aimed at gradually reducing the size of suspended particles of waste waters to 25 μm. The first filtration was realized using a stainless steel Waterscreen rotating screen (plate 3.5). It was self-cleaned (a stainless steel scrapper removes solids and mud from Waterscreen filter), with rotation speed ranged between 2 and 2.5 rpm. Filter storage tank was fed with the waste water through a polyethylene pipe (1 inch) using a pump which was immersed in the tank waste. The feeding flow was regulated to 1.5 m^3/h. Afterwards the filtered liquid was piped through a centrifugal pump to a second stainless steel rotating filter, that was same in size with the precedent, but had slots (120 μm).

Plate 3.5. Stainless steel waterscreen rotating screen

Then the waste water, with centrifugal pump passed through a Pall bagfilter (with 25 μm pore sizes) (plate 3.6). The bagfilter was placed in a stainless stell container

Plate 3.6. Pall bagfilter with 25 μm pore sizes

b. Treatment of OMWW with resin.

At this stage, the filtered waste water passed through an adsorptive resin (XAD-16) to remove/detain interested substances (polyphenols, lactones).The filtered wastewater (1.5 m^3) was passed through a column which was filled with 100 l of Amberlite XAD16 resin (plate 3.7). Feed was achieved using hydropneumatic pump (WILDEN), which was connected with compressor that provides air pressure of 2.5 bar. The feed flow was 125 l/h and the procedure was completed in 4 hours. The waste was collected in a tank and passed for second time from the column in order to achieve maximum adsorption.

Plate 3.7 Absorption columns XAD16

After the procedure was completed, the resin was flushed with proper solvent (ethanol) for the receipt of components that have been absorbed and then regenerated in order to be ready to used again. Regeneration includes the following stages:

a. The column was supplied with water for 15 min. in order to remove the particles settled on the resin surface. This procedure was continued until the received water was completely colorless

b. The column was supplied with water from the bottom (output) for 5 minutes in order to rearranged the resin particles in the column

c. Regeneration of resin with feeding a mixture 50/50 (v/v) ethanol/isopropanol for about 35 minutes and flow of 250 l/h

d. Washing the resin with water for approximately 30 min to remove solvent

c. Solvent Recovery

The third stage of the process intended to recover and evaporate the solvents which obtained during the second stage. QVF thermal evaporation system (Plate 3.8) was used. This system had a 200l evaporation vessel and the evaporation rate was 50 l/h. The conditions of evaporation were 45 ^{o}C temperature and 100 mbar vacuum.

The final brown, semisolid residue obtained after the evaporation of the solvents used in the regeneration of XAD16 was 4.5 kg .

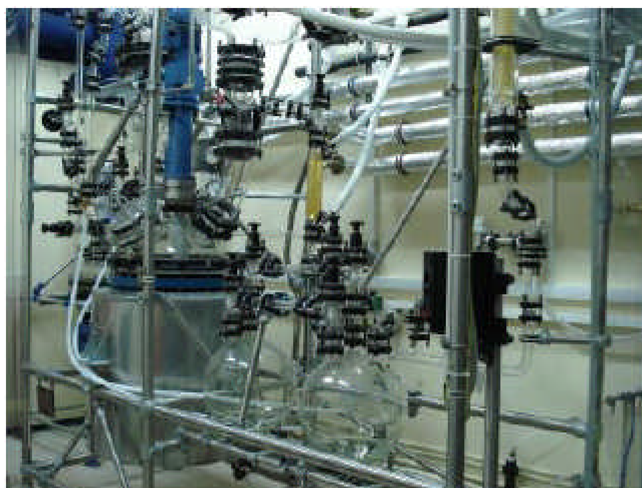


Plate 3.8. QVF evaporation system

d. HPLC analysis

A ThermoFinnigan HPLC system was used for the analysis of OMWW extract, comprised of a P4000 pump, a UV 2000 dual wavelength detector and a Rheodyne 7725i injector with an 100 μl loop.

The chromatographic separation of the substances was performed using a gradient elution program described in Table 3.2. Ammonium acetate 0.05 M buffer adjusted to pH 5.0 with glacial acetic acid. A Pinnacle ODS 3μm 150 x 4.6 mm was used with flow rate constant at 1 ml/min and the injection duration was 20 min.

Table 3.2 Gradient elution program applied to the HPLC Analysis of OMWW, flow 1 ml/min.

Time	Ammonium acetate 0.05 M	CH_3CN
0	90	10
10	70	30
15	60	40
20	90	10

3.2.3 GRAPE POMACE EXTRACT

3.2.3.1 Plant material

Red vinification by-products were of the Mandilari cultivar (*Vitis vinifera* sp.), obtained from the Miliarakis winery in the regions of Heraklion (Crete, Greece).

3.2.3.2 Extraction and isolation procedure

The first step of the treatment system included the collection, air-drying, pulverization and extraction of grape pomace. The dry grape pomace was extracted 3 times with ethanol (EtOH) with mechanical stirring for 24 hr (plate 3.10). Then the extract was separated from the powder and diluted with ten volumes of water. The resulting water-alcohol was finally filtered using a bagfilter with 25μm porosity.

The second section of the treatment procedure included the passing of the filtered water-alcohol extract through a specialised adsorbent resin (XAD-16) in order to achieve the removal/ recovery of the polyphenol content.

The XAD-16 polymeric adsorbent (plate 3.11) was preconditioned before use by passing 4-5 bed volumes of deionized water through beds of the resin and subsequently 5-7 bed volumes of ethanol.

3-25 bed volumes of filtered aqueous solution, depending upon the phenol concentration, were adsorbed on a column containing XAD-16 adsorbent resin and the column effluent was discarded. The resin was slowly rinsed with 1-5 bed volume of water to remove entrapped/adhering water soluble impurities. The adsorbed polyphenols were eluted from the column with a suitable amount of solvent, preferably 1 to 5 bed volumes of ethanol.

The filtered extract (500 l) was fed into a column, which was filled with 100 l of XAD16 resin. The extract was fed into the resin through a PVC pipe using a pump. The feeding flow rate was 125 l/hr. The resin total feeding duration was 4 hr.
Afterwards, in order to regenerate the resin, the procedure described in paragraph 3.2.2.3.b was followed again. After the completion of this procedure, the resin was

ready to be used again. The water-alcohol grape pomance waste at this stage was a light red coloured liquid, which did not contain any polyphenolic substances. All contained polyphenols had been successfully adsorbed.

The final part of the treatment procedure aimed at the recovery of the organic solvent mixture, and the obtainment of the dry enriched extract. The organic solvent mixtures that had been used in the second section, were evaporated separately in a thermal treatment system consisting of Rotary evaporators (BÜCHI R220) (plate 3.12). The organic solvents mixtures were thermally treated under conditions of 45 ^{o}C temperature and 100 mbar vacuum. The condensation of the produced organic solvents vapours was performed by using a coolant (glycol) at -7 ^{O}C.

The obtained extract after the resin treatment is a semisolid product.

Plate 3.9. Grape pomance-Winery byproduct

Plate 3.10. Processing of dry grape pomance in a solid-liquid extraction vessel

Plate 3.11. Absorption columns XAD-16

Plate 3.12. Solvent evaporation unit (Büchi Rotavapor R-200)

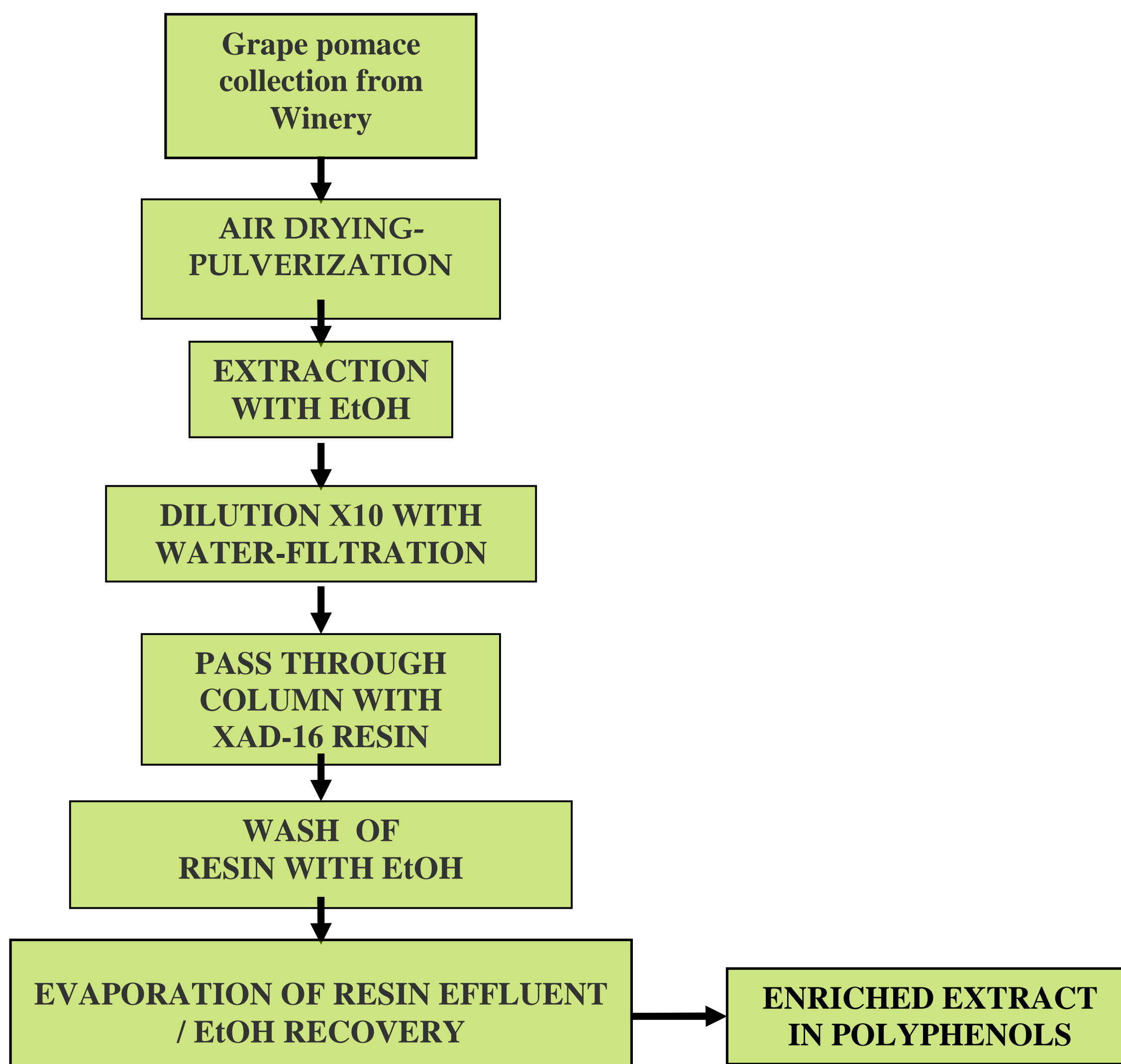


Figure 3.2 Schematic presentation of the three major steps of the polyphenol extraction from grape pomace

3.2.3.3 HPLC analysis

Polyphenols detection and identification were performed using a Thermo Finnigan 3000 system equipped with: a Quaternary pump, degasser, a Diode Array Detector (DAD) and a Rheodyne 7725i injector with an 100 μl loop. Samples were injected on a reverse phase Lichrosphere C18 column (250mm x 4.1 mm, particle size 5 μm) and a C18 guard column with a flow rate 1 ml/min. The chromatographic separation of the substances was performed using a gradient elution program described in Table 3.3. Peaks were identified by comparing their retention time (RT) and UV-Vis spectra with those of standards. The analysis was monitored at 280, 320 and 360 nm simultaneously. Data were quantitated using the corresponding curves of the reference compounds as standards.

Table 3.3 Gradient elution program applied to the HPLC Analysis of grape pomace extract, flow 1 ml/min.

Time	3% AcOH in an aqueous solution of AcONa (2 mM)	CH_3CN
0	95	5
45	20	80
60	35	65
65	10	50

3.3. RESULTS AND DISCUSSION

3.3.1. OLIVE LEAVES

From 50 kg powdered olive leaves after extraction with acetone and further treatment with CH_2Cl_2/ MeOH solvent system resulted in a yellow powder of about 50-60 % (Agalias *et al.* unpublished data) oleuropein purity with a total yield of approximately 3-3.5 kg.

Based on results of 20 eluted fractions (Table 3.1) presented on TLC after detection under 254 and 366nm UV lamps and by spraying with a methanolic solution of vanillin sulphate, it was decided to focus on fractions to 5-8. In these fractions appeared spots which absorbed in UV-lamps and stained dark yellow (oleuropein). From fractions 5–8 approximately 160g pure oleuropein (98%) was isolated. Purity of isolated oleuropein was checked using HPLC (Figure 3.3).

Conclusively from 50 kg. powdered olive leaves were isolated 3 - 3,5 kg. oleuropein 50-60% purity and 160 g oleuropein 98%.

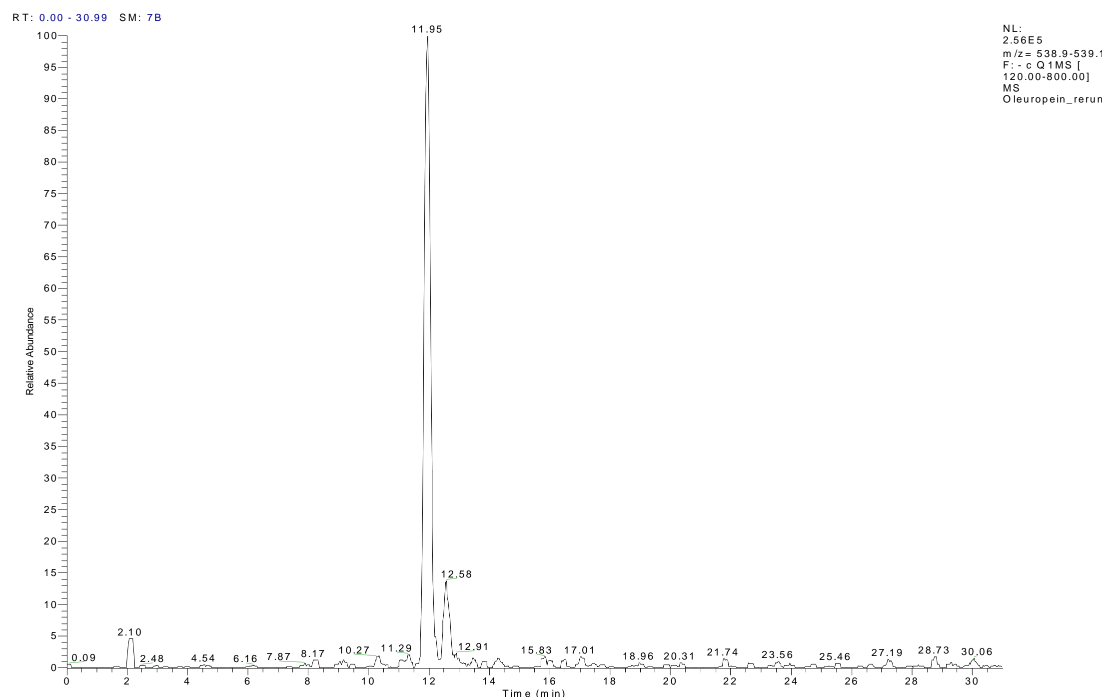


Figure 3.3 HPLC chromatogram of pure Oleuropein (98%)

3.3.2. OLIVE MILLS WASTE WATER (OMWW)

As can be observed from the HPLC results in Figures 3.4 and 3.5, treatment with resin XAD-16, adsorbed 87% of hydoxytyrosol and 100% of tyrosol, contained in non-treated with XAD-16 OMWW

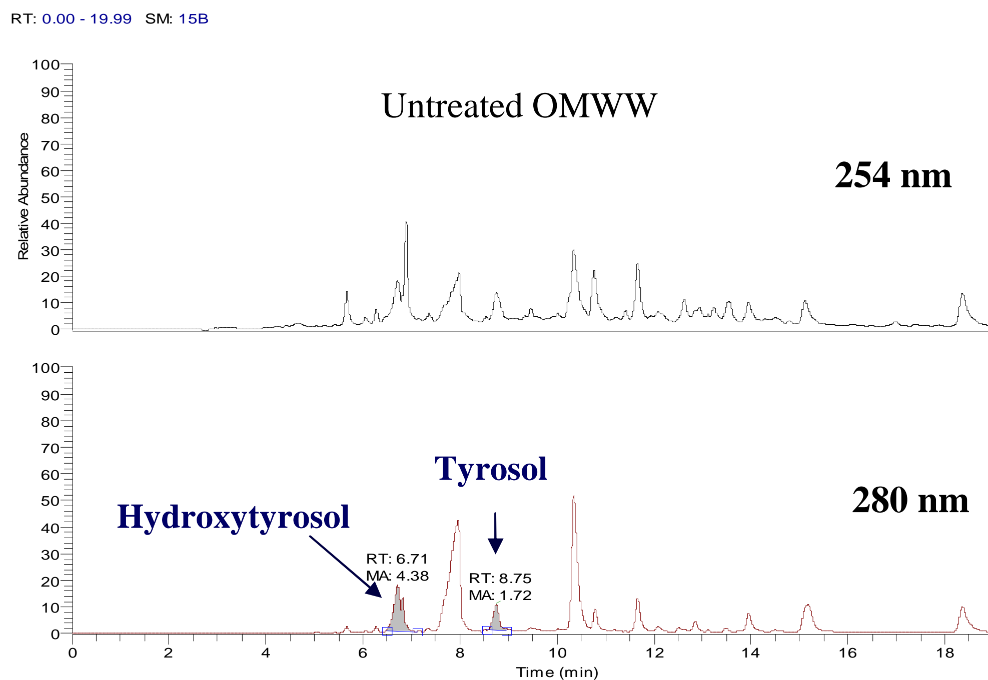


Figure 3.4 HPLC chromatograph (UV 254, UV 280) of unteated OMWW with XAD-16 resin

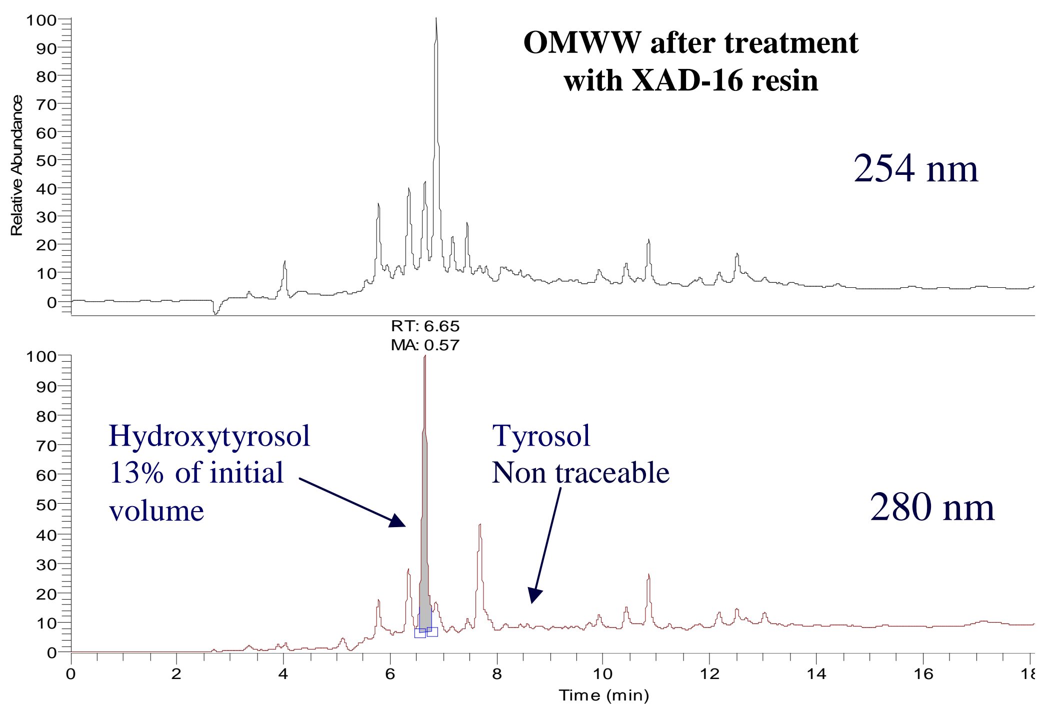


Figure 3.5. HPLC chromatograph (UV 254, UV 280) of OMWW after treatment with XAD-16 resin

The final brown semisolid residue obtained after the evaporation of the solvents used in the regeneration of XAD16 was rich in polyphenols.

3.3.3 GRAPE POMACE EXTRACT (GPE)

The obtained extract after the resin treatment was a semisolid product rich in polyphenols. HPLC chromatogram in Figure 3.6 shows the phenolic compounds from enriched red grape pomace extact that has been isolated.

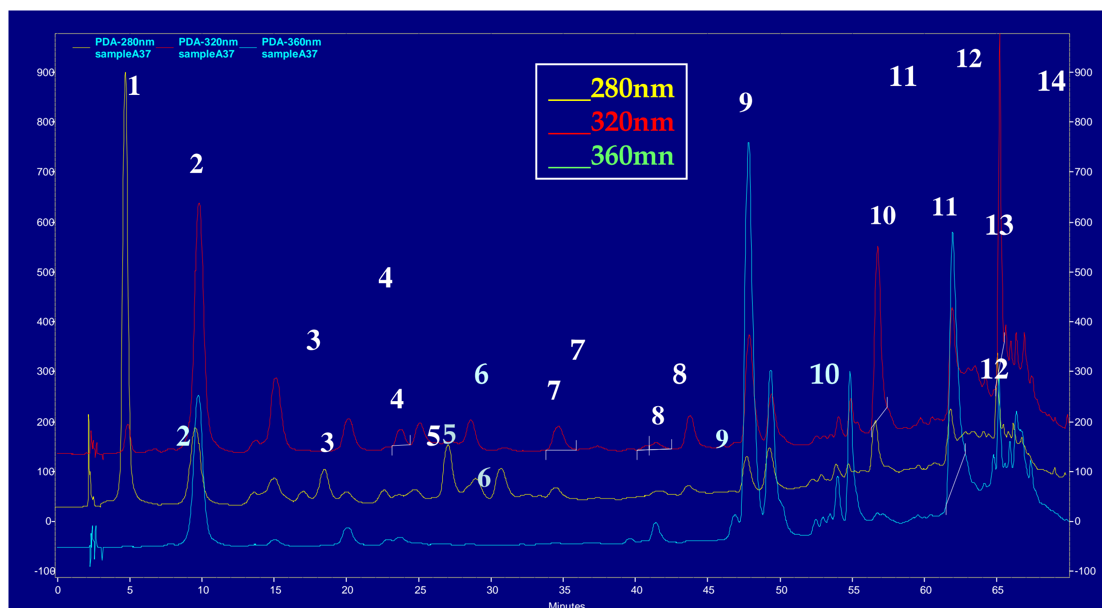


Figure 3.6 HPLC chromatogram of Phenolic compounds from enriched red grape pomace extact: 1 gallic acid, 2 caftaric acid, 3 catechin, 4 Caffeic acid, 5 syringic acid, 6 epicatechin, 7 p-coumaric acid, 8 ferulic acid, 9 quercetin glucosides, 10 trans-resveratrol, 11 quercetin, 12 viniferin.

CHAPTER FOUR

IN VITRO EVALUATION OF EXTRACTED NATURAL COMPOUNDS

CHAPTER FOUR

4.1 INTRODUCTION AND OBJECTIVES

Pure plants extracts or/and essential oils show antifungal and antibacterial activity against a wide range of fungi and bacteria. Recently there has been increasing interest in the effects of these compounds against important plant pathogens (fungi and bacteria) such as *Botrytis cinerea* as well as other fungi such as *Fusarium oxysporum fsp melonis* and bacteria such as *Clavibacter michiganensis*, *Pseudomonas syringae* and *Xanthomonas campestris.*

Although the antimicrobial activity of oleuropein, GPE and OMWW extract have been studied widely, little research has been done on screening these extracts efficiency against phytopathogenic microoraganisms. The objective of this work study was the *in vitro* investigation of isolated oleuropein, a crude natural antioxidant mix extracted from olive leaves, olive mills waste water (OMWW) and a grape pomace extract (GPE) as phytoprotective agents against economically important fungal and bacteria strains.

In particular, *in vitro* antimicrobial activity was assessed on a series of fungal pathogens including: *Botrytis cinerea,* the cause of grey mold disease, *Alternaria alternata*, causing leaf spots and moulds on several plant species, *Fusariu oxysporum fsp melonis,* causing Fusarium wilt of melon, a *Rhizopus* species (a genus causing fruit and vegetable decays), the crucifer pathogen *Colletotrichum higginsianum,* causing anthracnose leaf spot disease on several *Brassica* and *Raphanus* species and *Phytophthora parasitica* var. *nicotianae,* the causal agent of the black shank disease of tobacco.

Bacterial strains including *Clavibacter michiganensis spp. michiganensis,* the cause of bacterial canker of tomato, *Ralstonia solanacearum* causing bacterial wilt in solanaceous plants, *Pseudomonas syringae* pv. *tomato,* the cause of bacterial speck on tomato and Arabidopsis and *Xanthomonas campestris* pv. *vesicatoria* causing the bacterial spot disease of pepper and tomato were tested.

4.2. MATERIALS AND METHODS

4.2.1. Test microorganisms

P. parasitica (syn. *nicotianae*) Dastur var. *nicotianae* Tucker race 0 strain is a tobacco isolate (Bottin et al, 1999). It was grown on V-8 agar medium (50 ml of V-8 juice and 20 g agar per litre, pH 5.0) at 23°C and maintained at 15°C in the dark. Isolate IMI349063 of *Colletotrichum. higginsianum* Sacc., originating from *Brassica. campestris* subsp. *chinensis* (O'Connell et al., 2004) was grown on ANM medium (glucose 20g, malt extract 20g and peptone 1g per l) and Bannerot agar medium (glucose 2.8g, Yeast Extract 0.1mg, Peptone C 2g, $MgSO_4 \cdot 7H_2O$ 1.3g and KH_2PO_4 2.7 g per l) at 23°C for maintenance and sporulation. *B. cinerea*, *F. oxysporum fsp melonis*, *A. alternata* and *Rhizopus* sp. were obtained from the Applied Mycology Group, Biotechnology Centre, Cranfield University, Silsoe UK. Cultures of each fungal species were maintained on potato-dextrose agar (PDA) and kept at 4°C for short term storage, while conidia were dehydrated and kept at -80°C for long term storage.

Bacterial strains *Clavibacter michiganensis spp. michiganensis* (4040, tomato), *Ralstonia solanacearum* (819-6 potato), *Pseudomonas syringae pv. apii* (255a celery), *Pseudomonas syringae pv. tomato* (132 tomato), *Pseudomonas corrugata* (1157 tomato), *Pseudomonas viridiflava* (Ac.2 melon), *Xanthomonas* campestris pv. vesicatoria (5075 tomato) and *Pseudomonas savastanoi* (1266 olive) were provided by the collection of Prof. Goumas (TEI- CRETE). All bacterial strains were maintained in long-term storage in sterile 25% glycerol solution at -70 °C.

4.2.2 Fungi Radial Growth

A) Oleuropein at 50-60% and 98% purity, grape pomace extract (GPE) and olive mill waste water (OMWW) extract stocks were prepared for use in all bioassays.

Inhibition of the mycelial growth of fungi was determined by measuring the radial growth on potato dextrose agar (PDA) plates containing the natural extracts mentioned above at a range of concentrations for 4 and 7 days, respectively. The experiments were conducted in 90 mm Petri dishes, inoculated with 10 mm PDA plugs of young mycelial cultures. Three replicates in two repetitions were used per treatment; the incubation was at the optimum temperature for each pathogen in the dark. The percent radial growth and the fifty percent inhibition of radial growth (ED_{50}) compared to control were determined.

B) To assess the effects of pure resveratrol on the radial growth of *Botrytis cinerea*, 10 μl aliquots of conidial suspensions at 1×10^6 spores ml^{-1} were plated onto PDA plates, which also contained different concentrations of resveratrol (0, 60, 173, 300μgr/ml) and the Petri dishes were incubated at 25 °C for 7 days.

4.2.3. Spore germination of *Botrytis cinerea* and *Colletotrichum higginsianum*.

Spores of *Botrytis cinerea* and *Colletotrichum higginsianum* were harvested from 2 week old sporulating PDA and ANM cultures respectively. 20 ml of sterile water was added to a Petri plate culture, the spores were gently dislodged from the surface with a sterile glass rod and suspensions were filtered through sterilized gauze to remove mycelial fragments. The spores of fungi were diluted with sterile water to obtain 10^6 spores/ml (counted with a hemacytometer) and used as a stock. Suspensions were then subjected to a series of dilutions in order to determine the one that would yield 50-200 germinating spores when plated onto a Petri dish containing the appropriate nutrient medium. The two working concentrations that would yield such numbers equalled to a final concentration of 10^2 to 10^3 spores ml^{-1} for both *ascomycetes*.

To determine the effect of naturals extracts on germination of spores of *Botrytis cinerea* and *Colletotrichum higginsianum*, 200 µl aliquots of the before mentioned conidial suspensions were plated on washed, autoclaved permeable cellophane discs (Cellophane octaframe, MERCK-Eurolab, France) that was placed on top of PDA and ANM -containing Petri dishes respectively, with the inclusion of the selected natural extract at a series of concentrations (0, 0.1%, 0.5%, 1% v/v). Plates were then incubated at 23 and 25°C and in the dark. Inhibition/restriction of spore germination was measured on the basis of the number of germinating spores which, due to the emerging hyphae, gave them the appearance of a colony after 36 hours for *B. cinerea* and 48 h for *C. higginsianum*. For each treatment, three replicate plates were used and the experiments were repeated at least three times. To further investigate whether the extracts affected spore germination and germ tube and/or hyphae formation, pieces of the cellophane membrane were gently removed from the medium and observed under an inverted light and an inverted fluorescence microscope (for *C. higginsianum*) exactly as described by Haseloff *et al* 1997 using an EGFP filter (Zeiss, Oberkochen, Germany) with an excitation spectrum of 450–490 nm and at an emission band pass of 515–565 nm.

4.2.4. Bacteria

The antibacterial activity was determined by counting colonies on LB agar plates containing oleuropein of 50-60% and 98% purity, GPE and OMWW extract at a range of concentrations. Bacteria were allowed to grow up to a 0.1-0.2 optical density- OD_{600} (UV-1700, Shimadzu spectrophotometer). The bacterial population was determined by means of sequential dilutions and growth tests in LB media. Spotting tests (10μl) of the appropriate dilution (10^3) were then applied to test the bacterial growth to various concentrations of above extracts (0, 0.02, 0.05, 0.07, 0.09, 0.1, 0.13, 0.15 and 0.2%). Minimum inhibitory concentration (MIC) was determined after 1-2 days incubation. The MICs are defined as the lowest concentration of antimicrobial inhibiting the visible growth of microorganism on the agar plates.

The incubation was at 28°C in the dark and the bacterial growth was recorded after 2 or 3 days. All extracts were incorporated in each sterilized medium (LB). Three replicates were used per treatment and experiments were repeated at least twice.

Extract solubility was enhanced by using ethanol as a solvent. Ethanol concentrations did not exceed 0.5% (v/v) in the case of fungi or 0.1% (v/v) in the case of bacteria, an equal amount of ethanol was added to the control.

4.3. RESULTS

4.3.1. Fungal Radial Growth natural extracts

a. *Botrytis cinerea*

The antimicrobial activity of the tested extracts against *B. cinerea* after 4 and 7 days incubation is shown in Figures. 4.1, 4.2 and Table 4.1, 4.2. All extracts except OMWW extract can effectively inhibit the radial growth of *B. cinerea.* The most significant inhibition was observed on the plates with 1% GPE with 0% of the length of the mycelial growth compared with control (100%) after 4 and 7 days incubation.

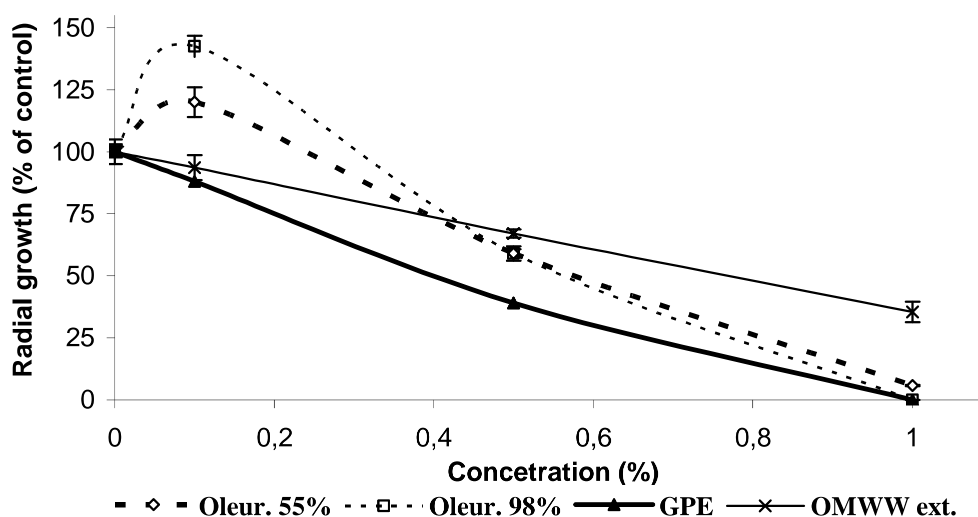


Figure 4.1. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Botrytis cinerea* after 4 days.

Semi-pure and pure Oleuropein (98%) had a similar effect in *B. cinerea* mycelial growth and plates with 1% concentration achieved also the best results. On plates with 1% semipure oleuropein the percentages of the radial growth inhibition of *B. cinerea* were 5.8% and 11.55 % compared with control after 4 and 7 days respectively while the respective percentages for 1% pure oleuropein were 0% and 15.3% compared with control, after 4 and 7 days incubation.

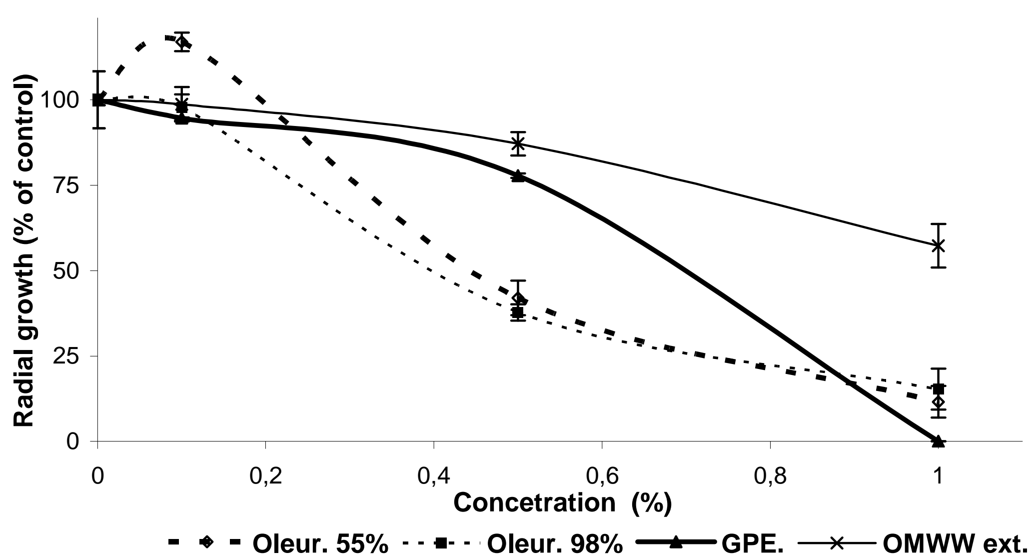


Figure 4.2. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Botrytis cinerea* after 7 days.

OMWW extract was not so effective on *B. cinerea* mycelial growth as the other three extracts. OMWW showed only some delay at high concentrations and plates with 1% concentration achieved 35.46% and 57.29% inhibition of radial growth after 4 and 7 days incubation respectively.

Table 4.1 Effect of Oleuropein 55% and 98% purity on radial growth of *Botrytis cinerea* after 4 and 7 days.

OLEUROPEIN 55 %				
BOTRYTIS	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50}[a] 4days (mg/ml, %)**	**ED_{50}[a] 7days (mg/ml,%)**
Control	100%	100%	**5.8 0.58%**	**4.5 0.45%**
Oleur 55% -0.1%	120%	117%		
Oleur 55% -0.5%	59%	42%		
Oleur 55%- 1%	5.8%	11.55%		
OLEUROPEIN 98%				
Control	100%	100%	**5.7 0.57%**	**4 0.4%**
Oleur 98% -0.1%	142.5%	97.8%		
Oleur 98% -0.5%	59%	37.8%		
Oleur 98%- 1%	0%	15.3%		

[a] ED_{50} The concentration of extract (mg/ml) causing a 50% reduction in the linear growth of the fungi on medium at 4 and 7 days respectively.

Table 4.2 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Botrytis cinerea.*

GRAPE POMACE EXTRACT				
BOTRYTIS	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} 4days (mg/ml, %)**	**ED_{50} 7days (mg/ml, %)**
Control	100%	100%	**3.9 39%**	**7.2 72%**
GPE -0.1%	88.01%	94.72%		
GPE -0.5%	39.03%	77.89%		
GPE -1%	0%	0%		
OLIVE MILL WASTE WATER EXTRACT				
Control	100%	100%	**7.8 78%**	**n.d***
OMWW ext.-0.1%	93.62%	98.74%		
OMWW ext.-0.5%	67.09%	87.19%		
OMWW ext.- 1%	35.46%	57.29%		

* n.d.: not definied

Effects of pure <u>resveratrol</u> on radial growth of *Botrytis cinerea*

In PDA plates, pure resveratrol at concentration of 60 µg/ml and 173 µg/ml delayed but did not inhibit the mycelial growth while at a concentration 300 µg/ml the mycelial growth of *B. cinerea* (plate 4.1 and figure 4.3) was completely inhibited.

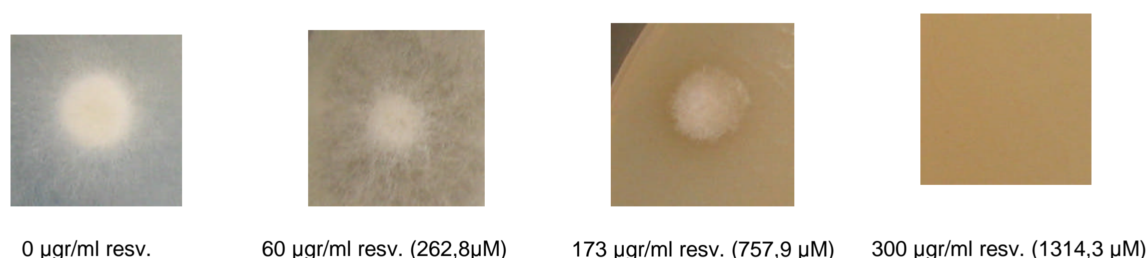


Plate 4.1 PDA dishes with different concentrations of pure resveratrol (0, 60, 173, 300µgr/ml), incubated at 25 °C for 7 days.

As can been seen in figure 4.3 mycelial growth of *B. cinerea* was greater at 4 days than 7 days incubation.

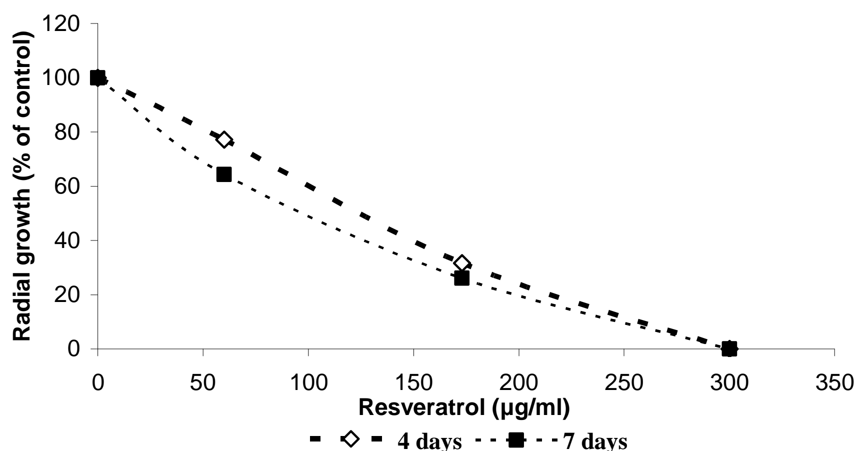


Figure 4.3 Effect of pure resveratrol on *Botrytis cinerea* radial growth after 4 and 7 days.

b. *Rhizopus spp.*

As can been seen in Figure 4.4 and Table 4.3 and 4.4, after 4 days incubation, semipure and pure oleuropein and GPE showed similar effects against *Rhizopus spp.* mycelial growth and their efficiency was not significantly different. The radial growth of *Rhizopus spp.* was inhibited by 97.46% , 98.84% and 88.1% from semipure oleuropein (2.54% of control), pure oleuropein (1.16% of control) and GPE (11.9% of control) respectively.

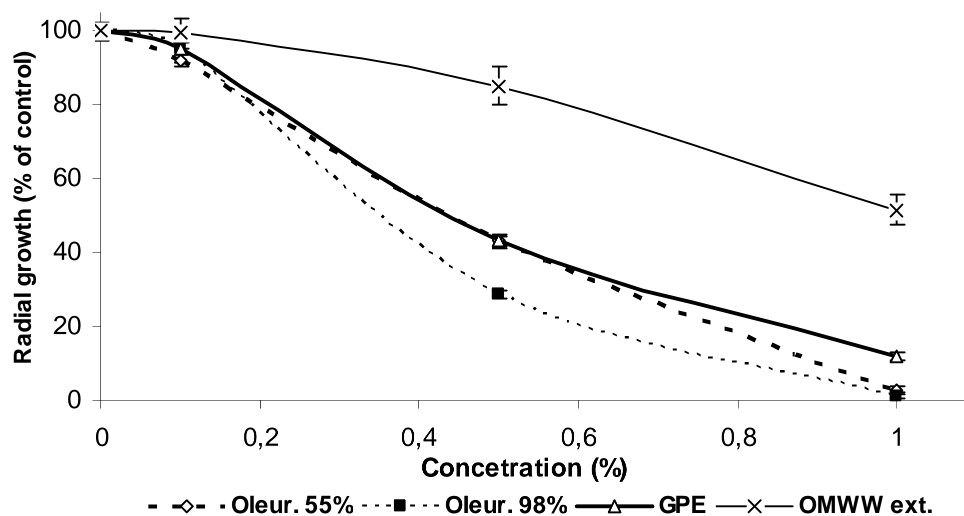


Figure 4.4 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Rhizopus* after 4 days.

After 7 days incubation of *Rhizopus spp.* the three above extracts showed again similar results (Figure 4.5 and Table 4.3, 4.4). On plates with a higher concentration (1%) of semipure, pure oleuropein and GPE, the percentages of the radial growth of *Rhizopus spp.* were 25.38%, 23.37% and 25.06% compared with the control respectively.

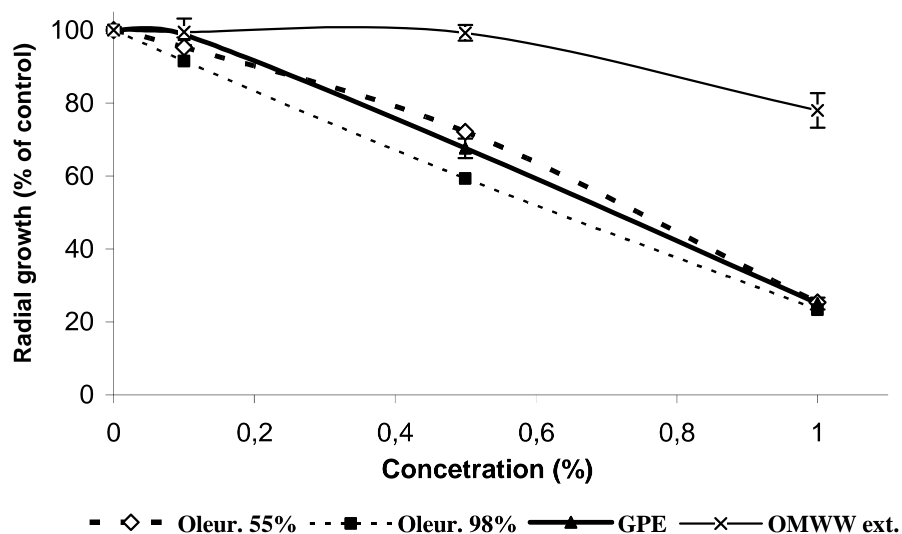


Figure 4.5. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Rhizopus* after 7 days.

OMWW extract was not as effective as the other three extracts and caused a delay in mycelial growth only at 1% concentration. The inhibition levels in *Rhizopus spp.* radial growth were 48.35% and 22.03% after 4 and 7 days incubation respectively.

Table 4.3 Effect of Oleuropein 55% and 98% purity on radial growth of *Rhizopus sp* after 4 and 7 days.

OLEUROPEIN 50-60%				
RHIZOPUS	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} [a] 4days (mg/ml, %)**	**ED_{50} [a] 7days (mg/ml, %)**
Control	100%	100%	**4.33 43.3%**	**7.5 75%**
Oleur 55% -0.1%	91.99%	95.23%		
Oleur 55% -0.5%	43.09%	72.11%		
Oleur 55%- 1%	2.54%	25.38%		
OLEUROPEIN 98%				
Control	100%	100%	**3.4 34%**	**6.23 62.3**
Oleur 98% -0.1%	95.08%	91.46%		
Oleur 98% -0.5%	28.54%	59.30%		
Oleur 98%- 1%	1.16%	23.37%		

[a] ED_{50} The concentration of extract (mg/ml) causing a 50% reduction in the linear growth of the fungi on medium at 4 and 7 days respectively.

Table 4.4 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Rhizopus sp* after 4 and 7 days.

GRAPE EXTRACT				
RHIZOPUS	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} [a] 4days (mg/ml, %)**	**ED_{50} [a] 7days (mg/ml, %)**
Control	100%	100%	**4.3 43%**	**7.1 71%**
GPE -0.1%	95.33%	98.73%		
GPE -0.5%	43.13%	67.59%		
GPE -1%	11.90%	25.06%		
OLIVE MILL WASTE WATER EXTRACT				
Control	100%	100%	**n.d***	**n.d**
OMWW extr.-0.1%	99.45%	99.49%		
OMWW extr.-0.5%	85.16%	99.24%		
OMWW extr.- 1%	51.65%	77.97%		

* n.d.: not definied

c. *Alernaria alternata*

The antimicrobial activity of the tested extracts against *Alernaria alternata* after 4 and 7 days incubation is shown in Figures. 4.6, 4.7 and Table 4.5, 4.6. All extracts except OMWW extract could effectively inhibit the radial growth of *A. alternata.* After 4 days incubation, semipure and pure oleuropein and GPE showed similar effects against *A. alternata* mycelial growth and their efficiency was not significantly different. On the plates with 1% semi pure, pure oleuropein and GPE at 1%, 100% inhibition of mycelial growth was observed at 4 days. OMWW extract at 1% showed radial growth inhibition at 38.69% compared with the control. The ED_{50} (mg/ml) values after 4 days incubation were 4.42 for semi pure oleuropein, 5.17 for pure oleuropein, 3.71 for GPE and 7.93 for OMWW extract.

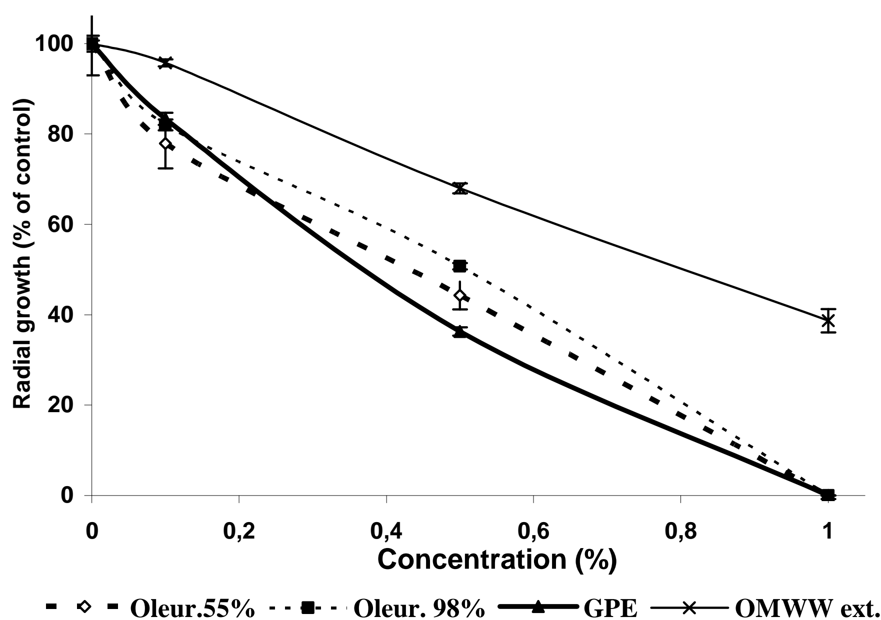


Figure 4.6 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Alternaria alternata* after 4 days.

After 7 days incubation plates with 1% GPE completely inhibited at 1% the radial growth of *A. alternata* mycelial. The pure and semi pure oleuropein also significantly reduced the radial growth of *A. alternata* by 75.07% and 81.23%, respectively, in comparison with the control. OMWW extract was not as effective as the other tested extracts in reducing the radial growth of *A. alternata* with 61.19% inhibition compared with control and ED_{50} value 8.62 mg/ml. The ED_{50} (mg/ml) values after 7 days incubation were 6.33 for semi pure oleuropein, 7.48 for pure oleuropein and 3.38 for GPE.

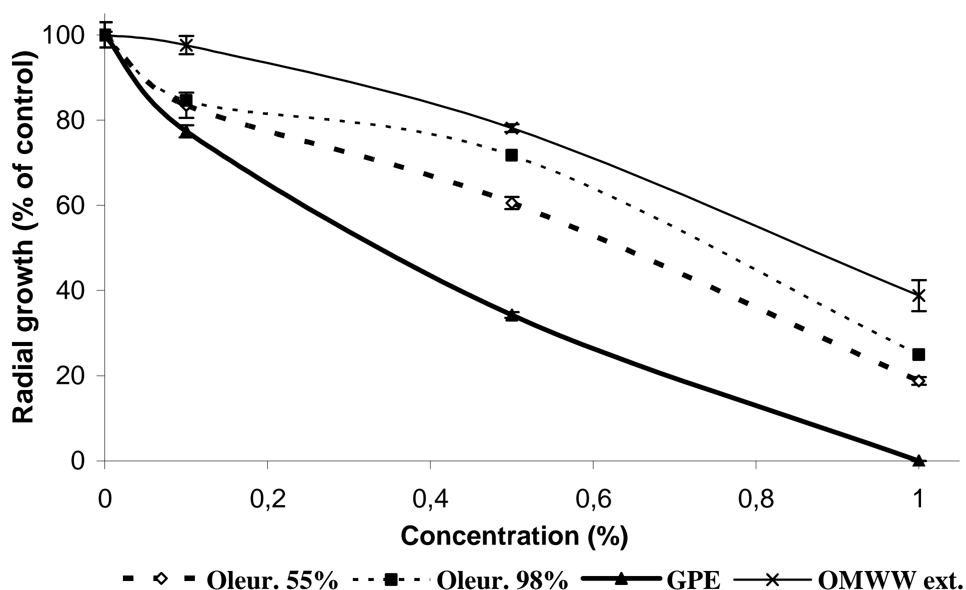


Figure 4.7 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Alternaria alternata* after 7 days.

Table 4.5 Effect of Oleuropein 55% and 98% purity on radial growth of *Alternaria alternata* after 4 and 7 days.

ALTERNARIA	Radial Growth (% of control) 4 days	Radial Growth (% of control) 7 days	ED_{50} [a] 4days (mg/ml, %)	ED_{50} [a] 7days (mg/ml, %)
OLEUROPEIN 50-60%				
Control	100%	100%	**4.42 44.2%**	**6.33 63.3%**
Oleur 55% -0.1%	77.87%	83.47%		
Oleur 55% -0.5%	44.27%	60.50%		
Oleur 55%- 1%	0%	18.77%		
OLEUROPEIN 98%				
Control	100%	100%	**5.17 51.7%**	**7.48 74.8%**
Oleur 98% -0.1%	82.02%	84.59%		
Oleur 98% -0.5%	50.71%	71.71%		
Oleur 98%- 1%	0%	24.93%		

[a] ED_{50} The concentration of extract (mg/ml) causing a 50% reduction in the linear growth of the fungi on medium at 4 and 7 days respectively.

Table 4.6 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Alternaria alternata* after 4 and 7 days.

ALTERNARIA	Radial Growth (% of control) 4 days	Radial Growth (% of control) 7 days	ED_{50} [a] 4days (mg/ml, %)	ED_{50} [a] 7days (mg/ml, %)
GRAPE EXTRACT				
Control	100%	100	**3.71 37.1%**	**3.38 33.8%**
GPE -0.1%	83.33%	77.34		
GPE -0.5%	36.31%	34.28		
GPE -1%	0%	0.00		
OLIVE MILL WASTE WATER EXTRACT				
Control	100%	100%	**7.93 79.3%**	**8.62 86.2%**
OMWW extr.-0.1%	95.71%	97.59%		
OMWW extr.-0.5%	67.98%	78.13%		
OMWW extr.- 1%	38.69%	38.81%		

d. *Phytophthora parasitica*

As can been seen in Figure 4.8 and Table 4.7, 4.8, after 4 days incubation the radial growth of *Phytophthora parasitica* was totally inhibited by pure oleuropein and GPE at 500 mg/ml (0.5%). A significant reduction (99.37%) was also recorded on plates with 0.5% semipure oleuropein, while OMWW extract at the same concentration showed 68.27% reduction in the mycelial growth of *Ph. Parasitica* compared with the control. ED_{50} values after 4 days incubation were 0.51, 0.43, 0.42, and 2.708 mg/ml for semirure oleuropein, pure oleuropein, GPE and OMWW extract respectively.

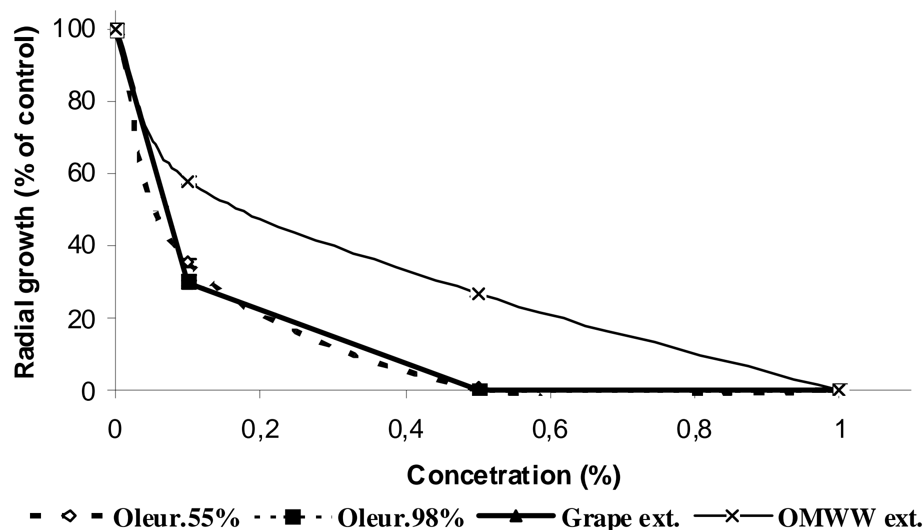


Figure 4.8 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Phytophthora parasitica* after 4 days

The results shown in figure 4.9 and Table 4.7, 4.8, after 7 days incubation show that GPE resulted in 100% inhibition of the radial growth of *Ph. parasitica* at 0.5%. Mycelial growth was totally inhibited by semipure and pure oleuropein at 1%, while on plates with 1% OMWW extract, 95.38% inhibition was observed compared with the control. ED_{50} values after 7 days incubation were 0.48, 0.78, 0.61, and 2.82 for semipure oleuropein, pure oleuropein, GPE and OMWW extract respectively.

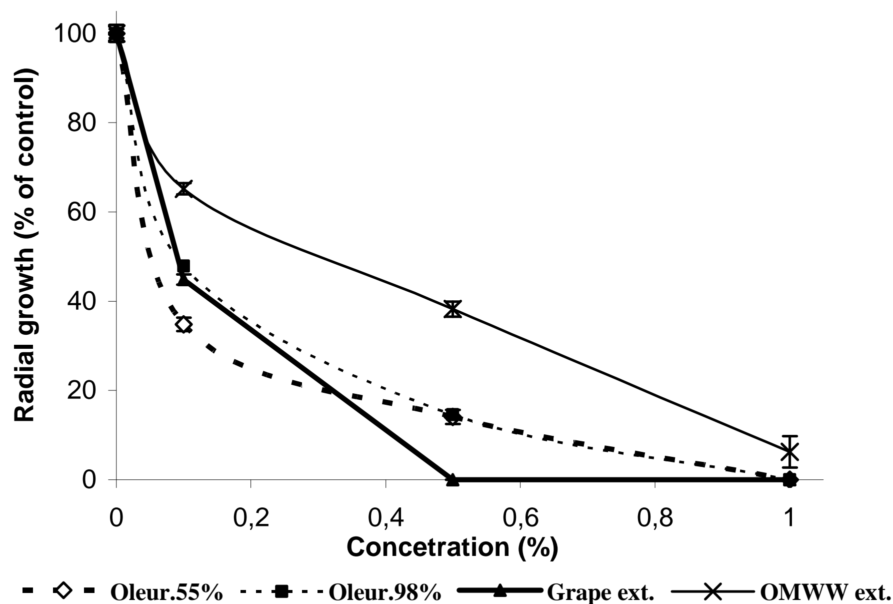


Figure 4.9 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Phytophthora parasitica* after 7 days.

Table 4.7 Effect of Oleuropein 55% and 98% purity on radial growth of *Phytophthora parasitica* after 4 and 7 days.

OLEUROPEIN 50-60%				
PHYTOPHTHORA	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} 4days (mg/ml, %)**	**ED_{50} 7days (mg/ml, %)**
Control	100%	100%	**0.51 51%**	**0.48 48%**
Oleur 55% -0.1%	34.47%	32.14%		
Oleur 55% -0.5%	0.63%	13.50%		
Oleur 55%- 1%	0%	0%		
OLEUROPEIN 98%				
Control	100%	100%	**0.43 43%**	**0.78 78%**
Oleur 98% -0.1%	30.05%	43.57%		
Oleur 98% -0.5%	0%	13.33%		
Oleur 98%- 1%	0%	0%		

Table 4.8 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Phytophthora parasitica* after 4 and 7 days.

GRAPE EXTRACT				
PHYTOPHTHORA	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} 4days (mg/ml, %)**	**ED_{50} 7days (mg/ml, %)**
Control	100%	100%	**0.42 4.2%**	**0.61 6.1%**
GPE -0.1%	29.33%	40.24%		
GPE -0.5%	0%	0%		
GPE -1%	0%	0%		
OLIVE MILL WASTE WATER EXTRACT				
Control	100%	100%	**2.71 27.1%**	**2.82 28.2%**
OMWW ext.-0.1%	67.79%	64.29%		
OMWW ext.-0.5%	31.73%	36.43%		
OMWW ext.- 1%	0%	4.62%		

e. *Colletotrichum higginsianum*

Inhibition of radial growth of *Colletotrichum higginsianum* was determined only after 7 days incubation, because of the slow on growth of this fungi. As can been seen in Figure 4.10 and Table 4.9, 4.10, radial growth of *Col. higginsianum* was totally inhibited by GPE at 0.5%, while semipure and pure oleuropein show 100% inhibition at 1% concentration. 1% OMWW extract, delayed but did not completely inhibit the mycelial growth of *Col. higginsianum*. ED_{50} (mg/ml) values after 7 days incubation were 4.15, 4.16, 1.84, and 4.9 for semirure oleuropein, pure oleuropein, GPE and OMWW extract respectively.

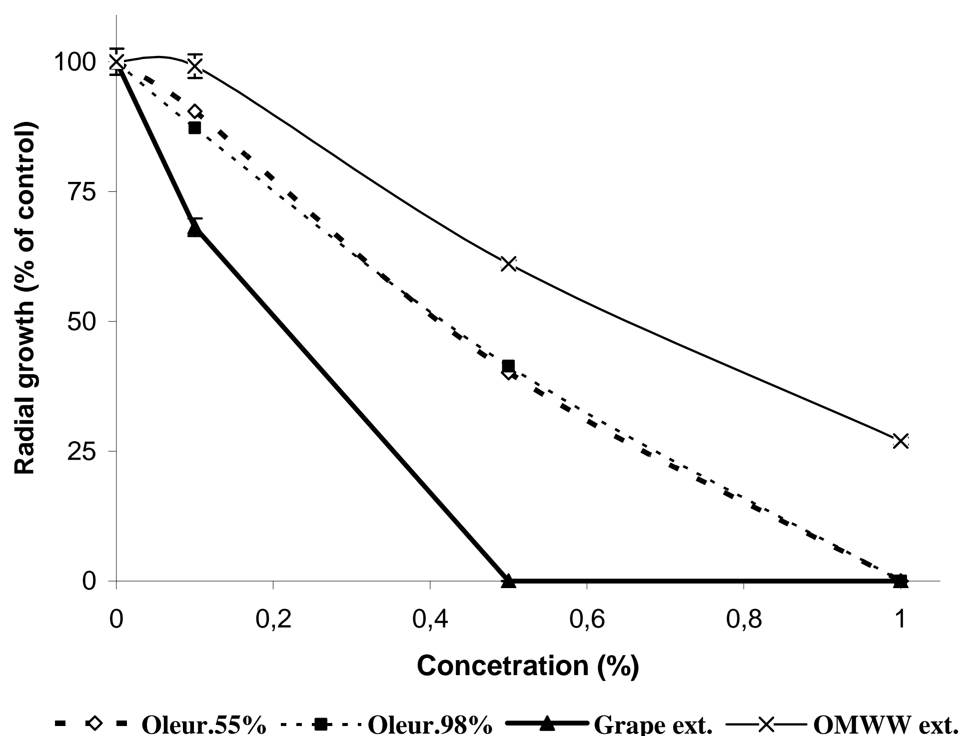


Figure 4.10 Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Colletotrichum higginsianum* after 7 days

Table 4.9 Effect of Oleuropein 55% and 98% purity on radial growth of *Colletotrichum higginsianum* after 7 days.

Colletotrichum	**Radial Growth (% of control) 7days**	**ED_{50} 4days**	**ED_{50} 7days (mg/ml, %)**
OLEUROPEIN 50-60%			
Control	100%	-	**4.15 41.5%**
Oleur 55% -0.1%	90.45%		
Oleur 55% -0.5%	40.13%		
Oleur 55%- 1%	0%		
OLEUROPEIN 98%			
Control	100%	-	**4.16 41.6%**
Oleur 98% -0.1%	87.26%		
Oleur 98% -0.5%	41.4%		
Oleur 98%- 1%	0%		

Table 4.10 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Colletotrichum higginsianum* after 7 days.

Colletotrichum	**Radial Growth (% of control) 7 days**	**ED_{50} 7days (mg/ml, %)**
GRAPE EXTRACT		
Control	100%	**1.84 18.4%**
GPE -0.1%	68.15%	
GPE -0.5%	0%	
GPE -1%	0%	
OLIVE MILL WASTE WATER EXTRACT		
Control	100%	**4.9 49%**
OMWW extr.-0.1%	91.72%	
OMWW extr.-0.5%	48.41%	
OMWW extr.- 1%	18.6%	

f. *Fusarium oxysporum*

The antimicrobial activity of the tested extracts against *Fusarium oxysporum* after 4 and 7 days incubation is shown in Figures. 4.11, 4.12 and Table 4.11, 4.12 After 4 days incubation, semipure and pure oleuropein and GPE showed similar effects against *F. oxysporum* mycelial growth and their efficiency was not significantly different. In particular at low concentration (0.1%) both oleuropein extracts showed an increase in mycelial growth compared to the control, at a level 6% and 12% for semipure and pure respectively. Oleuropein extracts delayed but did not inhibit the growth of this fungus even at a high concentration (1%) and ED_{50} values for semipure and pure oleuropein were 8.87 mg/ml and 7.45 mg/ml respectively. Similarly OMWW extract did not inhibit totally the mycelial growth at high concentration but seems to be more effective than oleuropein extracts. The inhibition observed on plates with OMWW extract was 32.15% and the ED_{50} was 5 mg/ml. On the other hand on plates with 1% GPE 100% inhibition of mycelial growth was observed, with an ED_{50} value 3.3 mg/ml

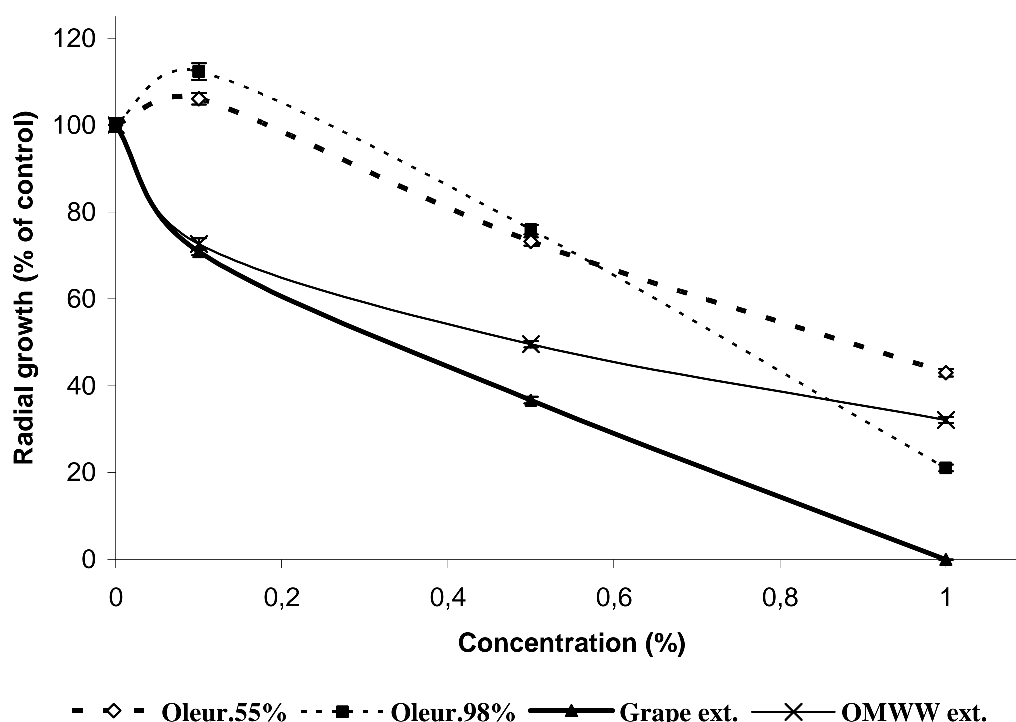


Figure 4.11. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Fusarium oxysporum* after 4 days.

As can been seen in figure 4.11, 4.12 and Table 4.11, 4.12, results were similar for *F. oxysporum* after 4 and 7 days incubation. Thus GPE showed 100% inhibition of the radial growth of *F. oxysporum* at 0.1% concentration, with ED_{50} value 2.63 mg/ml. Semipure and pure oleuropein showed an increse in radial growth at low concentration (0.1%) again and at high concentration (1%) 53.87% and 26.7% inhibition, was observed respectively, compared to control. The inhibition observed on plates with OMWW extract was 31.12% and ED_{50} was 5.1 mg/ml, approximately the same with that after 4 days incubation

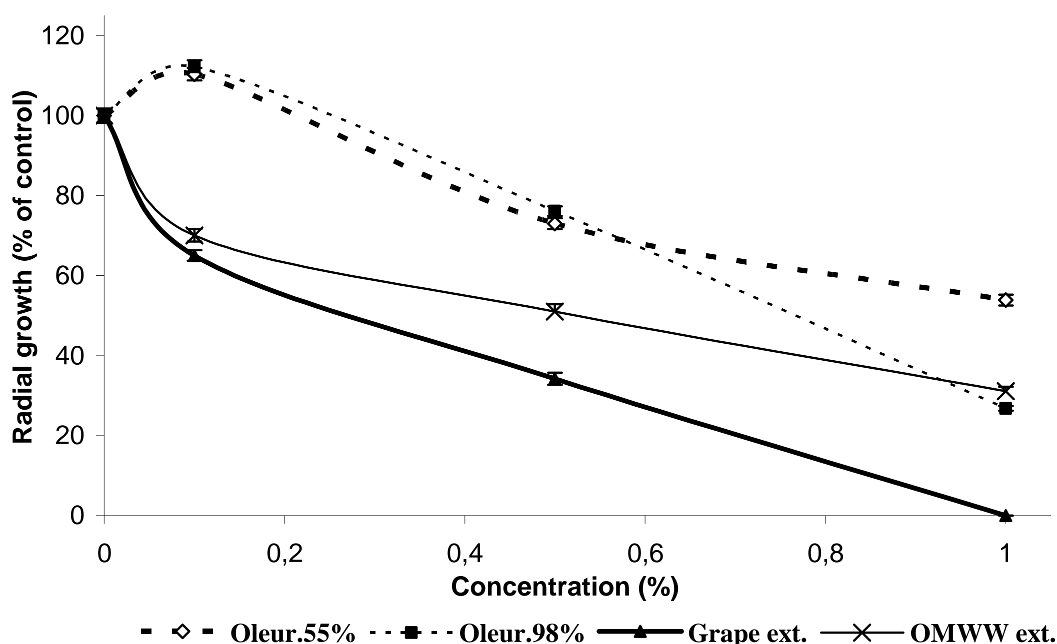


Figure 4.12. Effect of semi-pure and pure oleuropein, GPE and OMWW extract on radial growth of *Fusarium oxysporum* after 7 days.

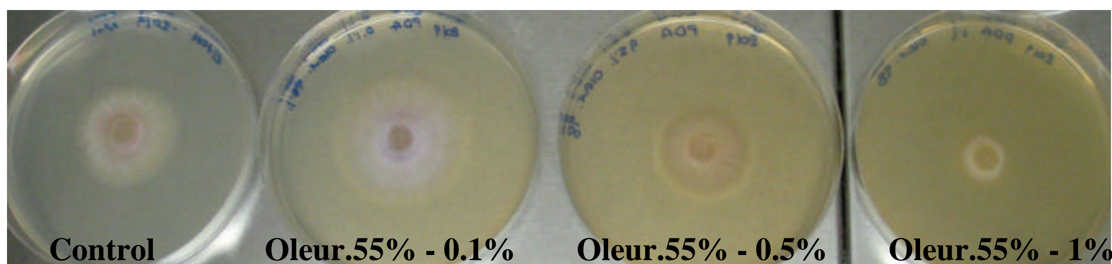


Plate 4.2 Inhibition of mycelial growth of *F. oxysporum* on plates with semipure oleuropein after 4 days incubation

Table 4.11 Effect of Oleuropein 55% and 98% purity on radial growth of *Fusarium oxysporum* after 4 and 7 days.

OLEUROPEIN 50-60%				
Fusarium	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} 4days (mg/ml, %)**	**ED_{50} 7days (mg/ml, %)**
Control	100%	100%	**8.87 88.7%**	**-**
Oleur 55% -0.1%	106.06%	110.3%		
Oleur 55% -0.5%	73.2%	72.96%		
Oleur 55%- 1%	43.01%	53.87%		
OLEUROPEIN 98%				
Control	100%	100%	**7.45 74.5%**	**7.62 76.2%**
Oleur 98% -0.1%	112.34%	112.1%		
Oleur 98% -0.5%	75.95%	76.07%		
Oleur 98%- 1%	21.08%	26.8%		

Table 4.12 Effect of grape pomace extract (GPE) and olive mill waste water extract on radial growth of *Fusarium oxysporum* after 4 and 7 days.

GRAPE EXTRACT				
Fusarium	**Radial Growth (% of control) 4 days**	**Radial Growth (% of control) 7 days**	**ED_{50} 4days (mg/ml, %)**	**ED_{50} 7days (mg/ml, %)**
Control	100%	100%	**3.3 33%**	**2.63 26.3%**
GPE-0.1%	70.89%	65.01%		
GPE- 0.5%	36.71%	34.23%		
GPE -1%	0%	0%		
OLIVE MILL WASTE WATER EXTRACT				
Control	100%	100%	**5 50%**	**5.1 51%**
OMWW ext.- 0.1%	72.59%	70.02%		
OMWW ext.- 0.5%	49.56%	51%		
OMWW ext. - 1%	32.15%	31.12%		

4.3.2 Inhibition of spore germination by natural extracts

a. ***Colletotrichum higginsianum***

Figure 4.13 shows spores germination of *C. higginsianum* after 48hr. incubation when treated with natural compounds. There are marked differences between these treatments. Spore germination was inhibited by 0.5% GPE to the greatest degree, reducing the spore germination rate to 0%, while the OMWW extract germination rate, at the same concentration, reached 26.5% and was inhibited totally (0%) at 1% concentration, compared with the control. Pure oleuropein was not so effective on *C. higginsianum* spore germination as the other two extracts. Specifically, plates with 0.5% pure oleuropein achieved 65% spore germination while on plates with a higher concentration of pure oleuropein (1%) the same percentage was 15%, compared with the control.

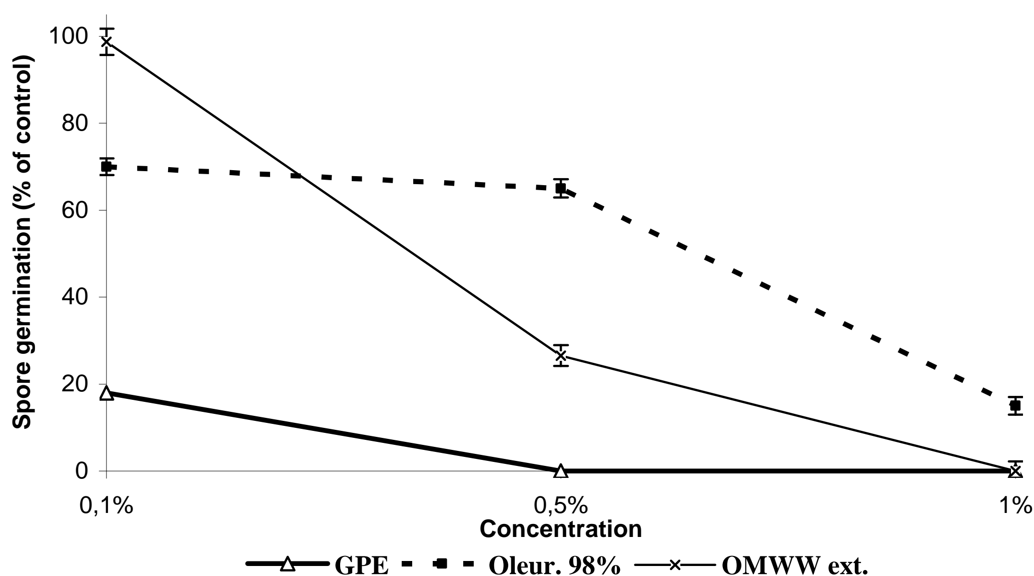


Figure 4.13. Effect of natural compounds on spore germination of *Colletotrichum higginsianum* after 48hr. incubation.

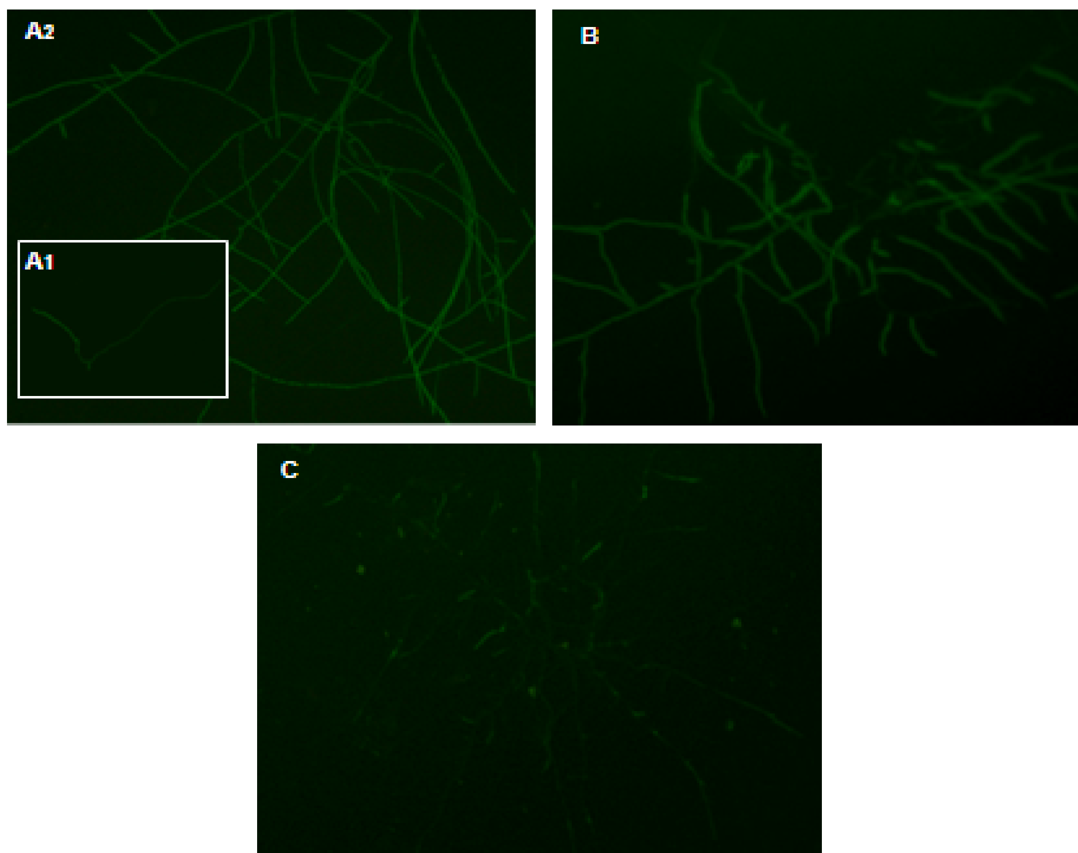


Plate 4.2: Effect of natural extracts on *C. higginsianum* spore germination Germination of conidia on substrates containing the appropriate nutrient medium and a range of concentrations (0, 0.1%, 0.5% and 1% v/v) of Oleuropein (98%), OMWW extract and GPE. Values represent % germinating conidia compared to control (medium and ethanol in levels equal to treatments): [A]. Conidial germination of a C. higginsianum strain, after a 24 hr and 48 hr incubation on control medium (A_1 and A_2 respectively) and a 48 hr incubation on a substrate containing 0.5% OMWW extact (B) and 1% Oleuropein (C).

b. ***Botrytis cinerea***

The antifungal activity of natural compounds on spore germination and germ tube elongation of *B. cinerea*, after 36 hr. incubation, is shown in Figure 4.14 and markedly in Plate 4.3 and 4.4. Spore germination and germ tube elongation of *B. cinerea* was inhibited by GPE only when the concentration was 1%. OMWW extract had approximately the same percentages of inhibition at all concentrations ranged from 34.78% (0.1%) to 18.63% (1%), compared with the control. Pure oleuropein was no effective again on *B. cinerea* spore germination and germ tube elongation, with the grater inhibitory effects at the higher concentration (1%).

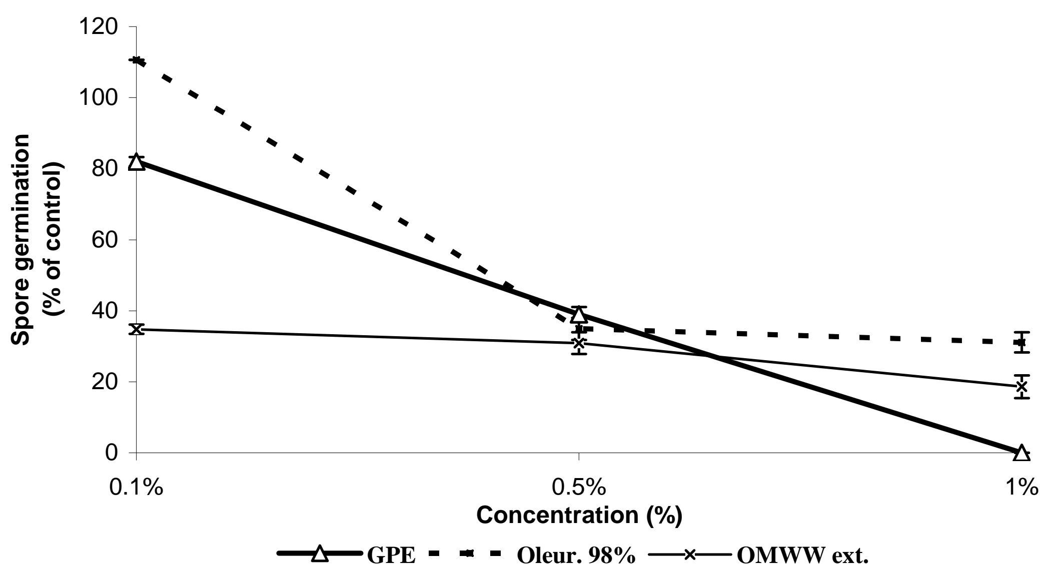


Figure 4.14. Effect of natural compounds on spore germination of *B. cinerea* after 36 hr incubation.

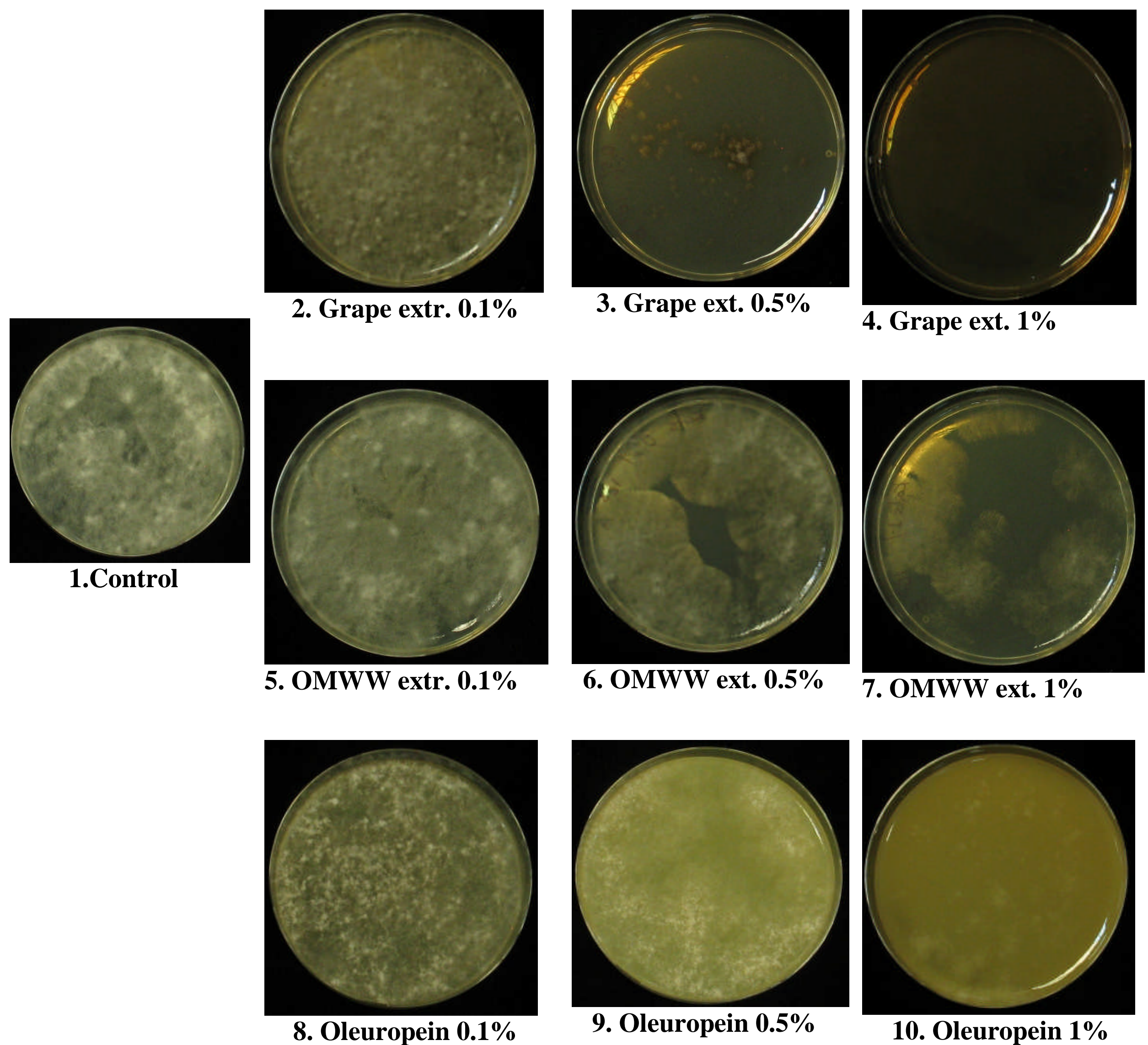


Plate 4.3. Spore germination and germ tube elongation of *B. cinerea* after 72 hr on PDA cultures with 0 (1 control), 0.1% (**2**), 0.5% (**3**) and 1% (**4**) GPE., 0.1% (**5**), 0.5% (**6**) and 1% (**7**) OMWW extract, 0.1% (**8**), 0.5% (**9**) and 1% (**10**) oleuropein (98% purity).

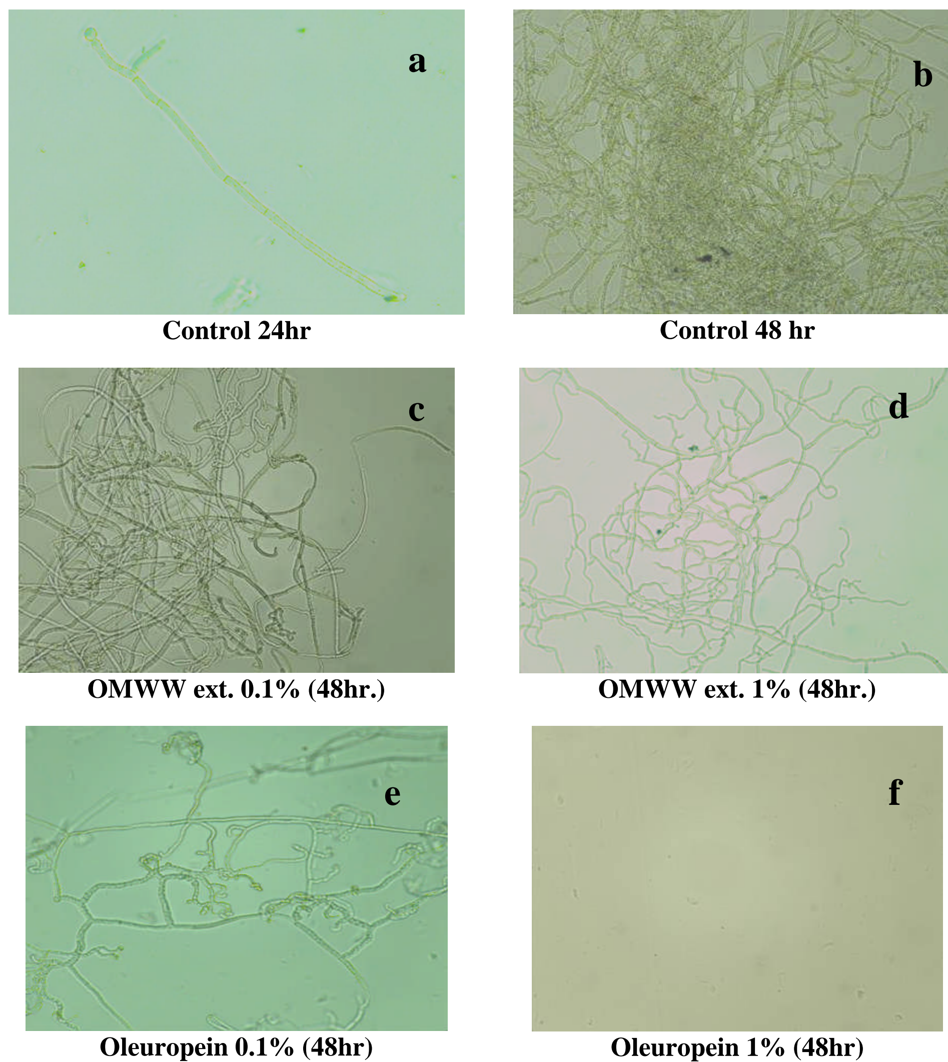


Plate 4.4 (a-f) Microscopic structural changes in *Botrytis cinerea* in response to different treatment. (a and b) Mycelium sampled from untreated cultures grown on PDA (control). (c) Mycelial sampled from cultures grown on PDA added 0.1% oleuropein.

4.3.3 Bacterial strains

a. *Clavibacter michiganensis* (4040*)*

The antibacterial activity of the tested extracts against *Clavibacter michiganensis* after 2 days and optical density (O.D.$_{600}$.) 0.137, is shown in Figure 4.15. The most significant inhibition was observed on the plates with semipure and pure oleuropein. The MIC for semipure and pure oleuropein was 0.07 mg/ml. GPE and OMWW extract was not as effective as the oleuropein against *C. michiganensis*. The MICs for OMWW extract was 0.2 mg/ml while for GPE was not defined.

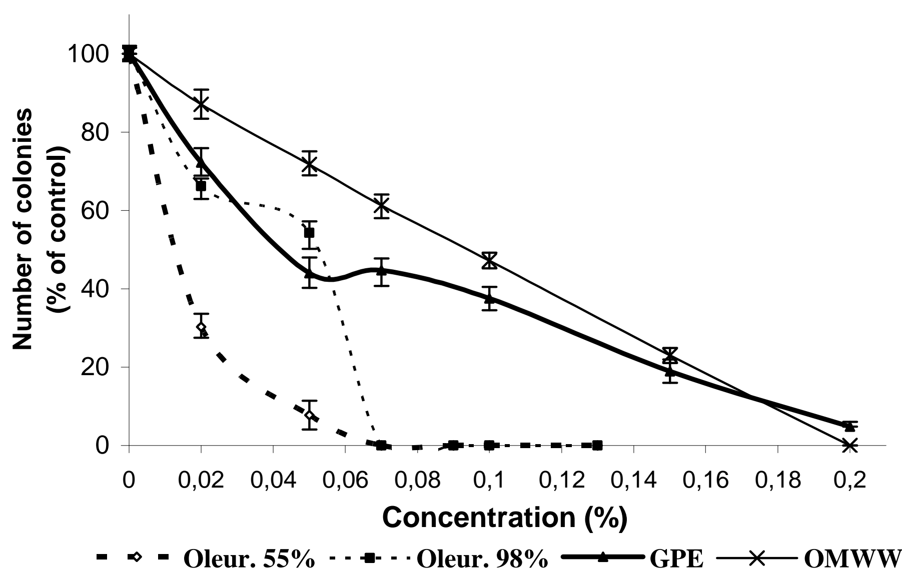


Figure 4.15 Effect of extracts on colony forming ability of *Clavibacter michiganensis* subsp. Michiganensis (4040) after 3 days incubation.

b. *Xanthomonas* campestris pv. Vesicatoria (5075)

As can been seen in figure 4.16, colony forming ability of *Xanthomonas campestris* pv. vesicatoria (5075) after 2 days incubation and optical density (O.D.$_{600}$) 0.17, was completely inhibited by semipure, pure oleuropein, GPE and OMWW extract at 0.02%, 0.02% and 1,5% and 1% respectively.

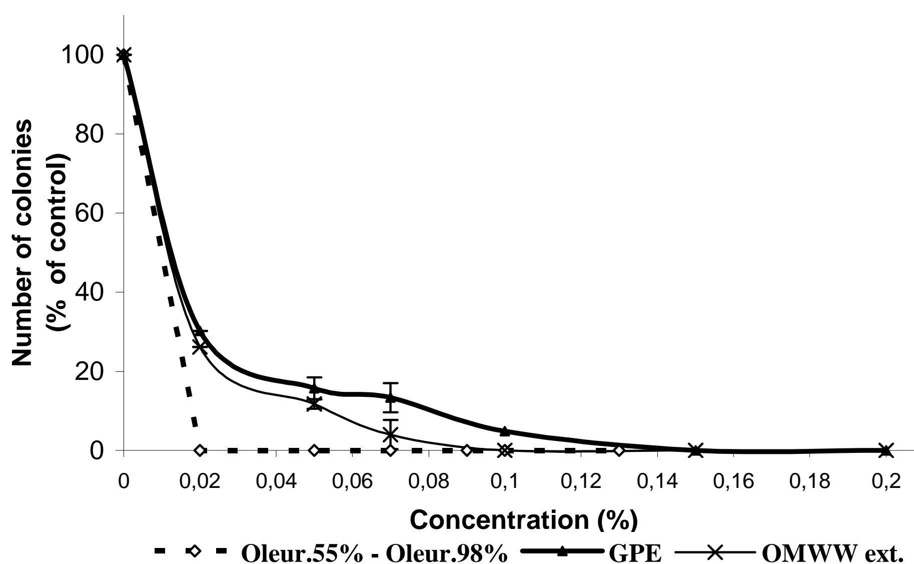


Figure 4.16 Effect of extracts on colony forming ability on colony forming ability of *Xanthomonas campestris* pv. vesicatoria (5075) after 2 days incubation.

c. *Erwinia atroseptica* (3217)

The activity of tested extracts against *Erwinia atroseptica* is shown in Figure 4.17. After 2 days incubation and optical density (O.D.$_{600}$) 0.158, colony forming ability of *E. atroseptica* was completely inhibited by semipure and pure oleuropein at 0.07% (0.07 mg/ml MIC), while GPE and OMWW extract at the same concentration showed 55.24% and 64.11% reduction in colony forming ability of *E. atroseptica* compared with the control. The MICs for GPE and OMWW extract were not defined.

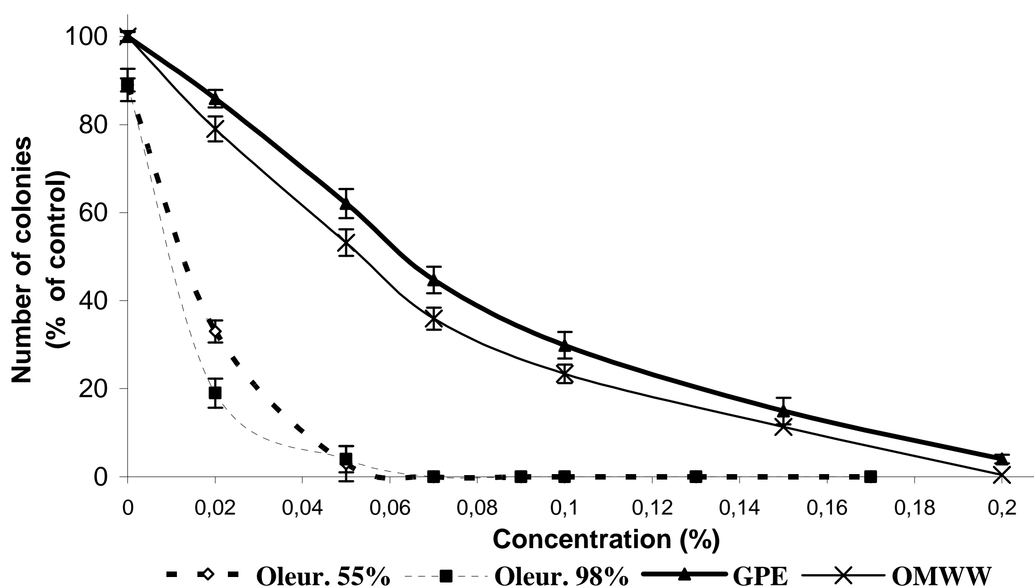


Figure 4.17 Effect of extracts on colony forming ability of *Erwinia atroseptica* (3217) after 2 days incubation.

d. ***Erwinia amylovora*** **(15)**

The results reported in Figure 4.18 after 2 days incubation time and optical density (OD_{600}) 0.11, that semipure oleuropein resulted in 100% inhibition of growth *Erwinia amylovora* at 0.07% (0.07 mg/ml MIC), while at the same concentration pure oleuropein and OMWW extract showed 67.87% and 83.46% reduction in colony forming ability of *E. amylovora* compared with the control. MICs for pure oleuropein and OMWW extract were 0.09 mg/ml and 0.2 mg/ml respectively. 2% GPE delayed but did not completely inhibit the growth of *E. amylovora*. MIC for GPE was not defined.

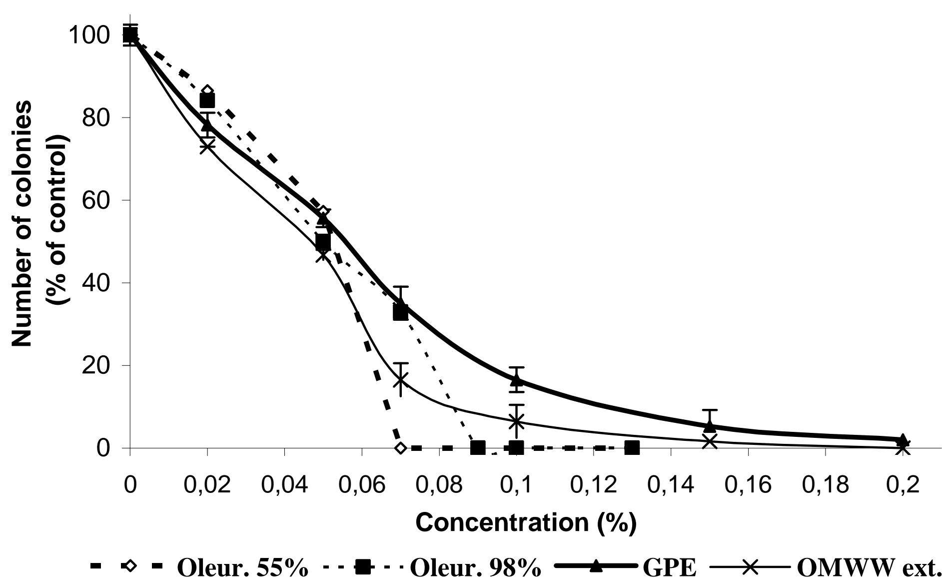


Figure 4.18 Effect of extracts on colony forming ability of *Erwinia amylovora* (15) after 2 days incubation.

e. *Ralstonia solanacearum* (819-6)

The antibacterial activity of the tested extracts against *Ralstonia solanacearum* after 3 days and optical density (O.D.$_{600}$.) 0.139, is shown in Figure 4.19. All extracts showed similar effects against *R. solanacearum* and their efficiency was not significantly different. The MIC for semipure, pure oleuropein and OMWW extract was 0,07 mg/ml while the MIC for GPE was 1000 mg/ml.

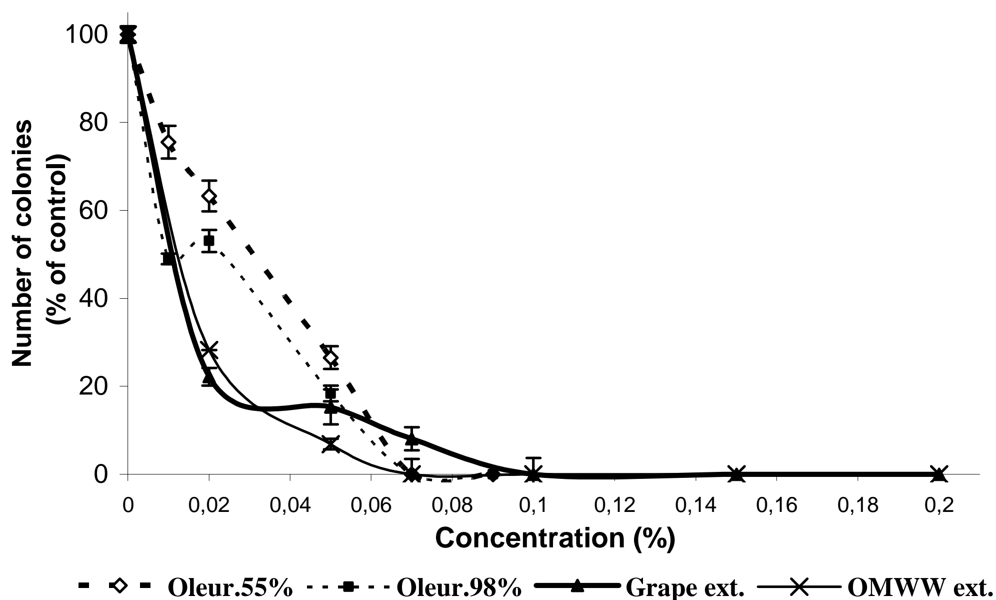


Figure 4.19 Effect of extracts on colony forming ability of *Ralstonia solanacearum* (819-6) after 3 days incubation.

f. *Pseudomonas syringae* pv. *Apii (* 255a)

As can been seen in figure 4.20, colony forming ability of *Pseudomonas syringae* pv. Apii after 2 days incubation and optical density (O.D.$_{600}$) 0.117, was completely inhibited by semipure and pure oleuropein at 0.07% (0.07 mg/ml), while OMWW extract and GPE, at the same concentration, showed 69.15% and 67.89% reduction in growth of *Ps. syringae* pv. Apii respectively compared with the control. The MICs for OMWW extract and GPE were 0.15 mg/ml and 0.2 mg/ml respectively.

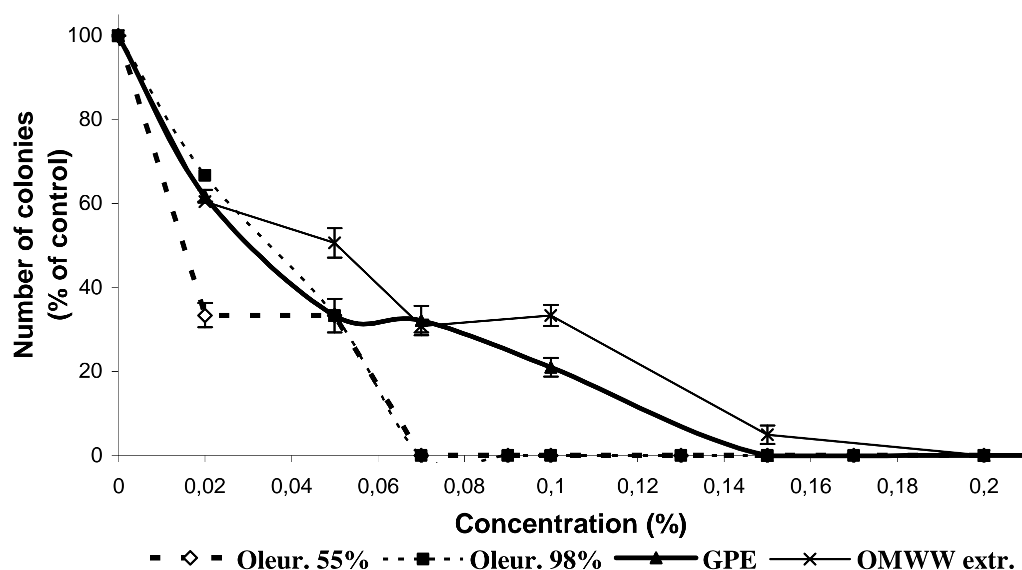


Figure 4.20 Effect of extracts on colony forming ability of *Pseudomonas syringae* pv. Apii (255a) after 2 days incubation.

g. *Pseudomonas corrugate* (1157)

The results shown in Figure 4.21 after 2 days incubation time and optical density (OD_{600}) 0.148, reported that at low concentration (0.02%) plates with semipure and pure oleuropein showed greater colony forming ability compared to the control and the most significant inhibition was observed on the plates with pure oleuropein at 0.1% concentration. GPE and OMWW extract showed similar effects against *Ps. corrugate* growth and their efficiency was not significantly different. In particular plates with GPE and OMWW extract showed that they delayed but did not inhibit the growth of *Ps. corrugate* even at a high concentration (0.2%). The MICs for GPE and OMWW extract were not defined.

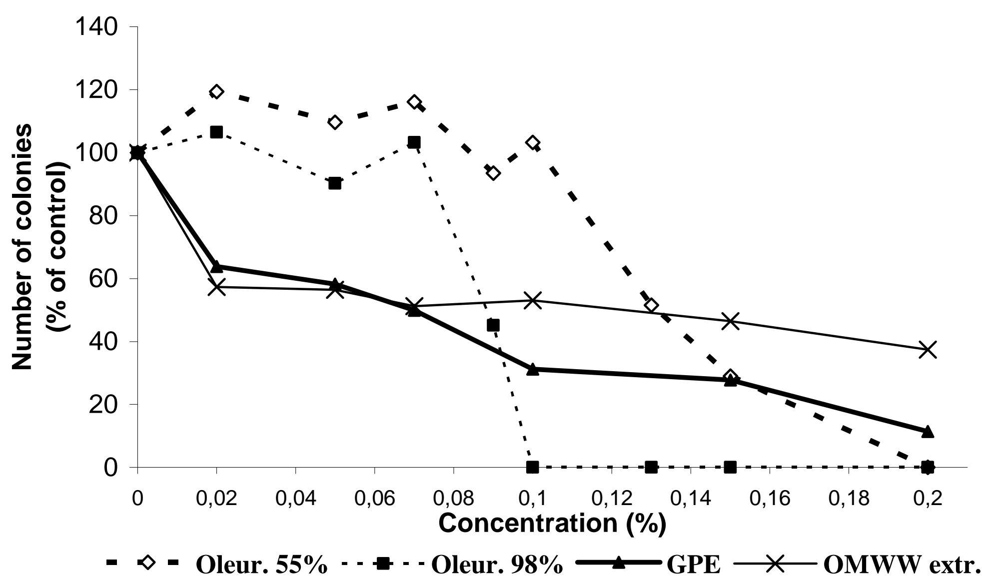


Figure 4.21 Effect of extracts on colony forming ability of *Pseudomonas corrugate* (1157) after 2 days incubation.

h. ***Pseudomonas viridiflava*** **(Acant.2)**

As can been seen in figure 4.22, colony forming ability of *Pseudomonas viridiflava* after 2 days incubation and optical density (O.D.$_{600}$) 0.125, was totally inhibited by semipure and pure oleuropein at 0.05% (0.05 mg/ml), while OMWW extract and GPE, at the same concentration, showed 83.88% and 80,65% reduction in growth of *Ps. viridiflava* respectively compared with the control. The MICs for OMWW extract and GPE were 0.15 mg/ml.

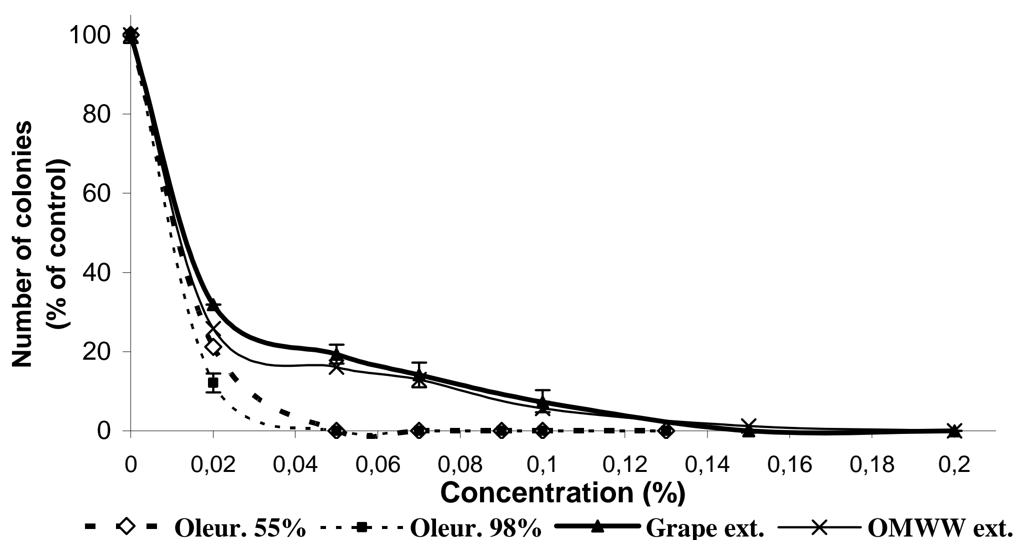


Figure 4.22 Effect of extracts on colony forming ability of *Pseudomonas viridiflava* (Acant.2) after 2 days incubation.

i. *Pseudomonas syringae* pv. *Tomato* (132)

The results reported in Figure 4.23 after 2 days incubation time and optical density (OD_{600}) 0.23, show that semipure and pure oleuropein resulted in 100% inhibition of growth *Pseudomonas syringae* pv. tomato at 0.05% (0.05 mg/ml MIC), while at the same concentration GPE and OMWW extract showed 87.23% and 84.04% reduction in colony forming ability of *Ps. syringae* compared with the control. MICs for GPE and OMWW extract were 0.15 mg/ml for both extracts.

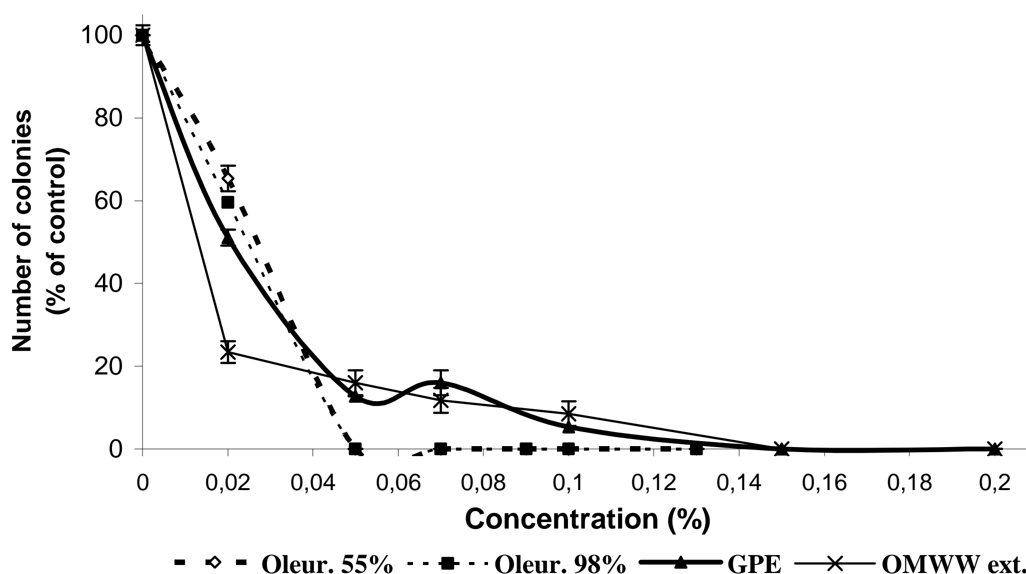


Figure 4.23. Effect of extracts on colony forming ability of *Pseudomonas syringae* pv. tomato (132) after 2 days incubation.

j. *Pseudomonas savastanoi* (1266)

The results reported in Figure 4.24 after 2 days incubation time and optical density (OD_{600}) 0.151, show that at low concentration (0.02%) colony forming ability of *Pseudomonas savastanoi* was completely inhibited by semipure and pure oleuropein, while GPE and OMWW ext., at the same concentration, showed 61.39% reduction and 4.19% increase in colony forming ability of *Ps. savastanoi* compared with the control. In particular plates with OMWW ext. showed that it delayed but did not inhibit the growth of *Ps. savastanoi* even at high concentration (0.2%). MIC for OMWW ext. was not defined while for GPE was 0.1 mg/ml.

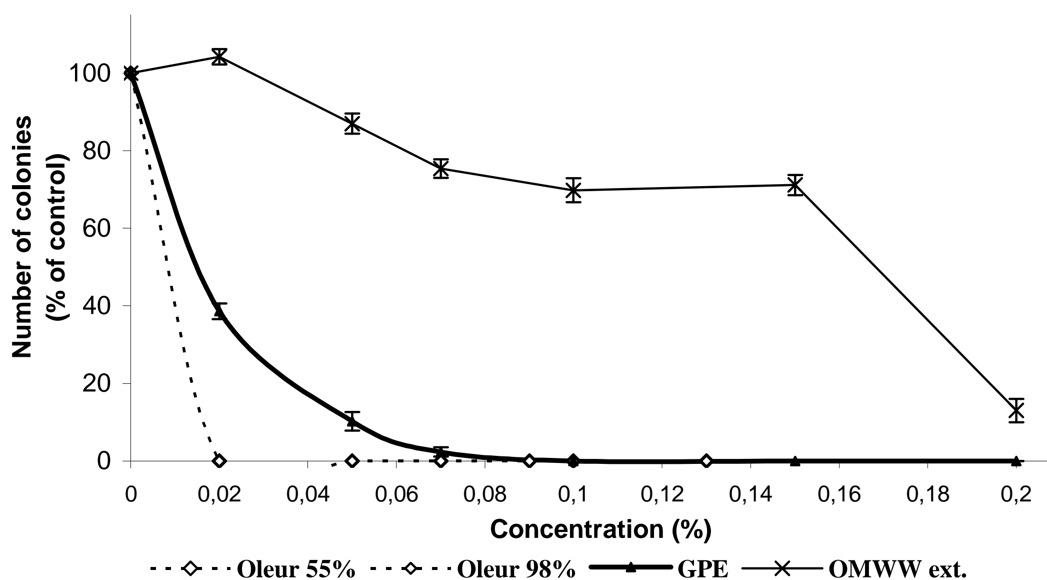


Figure 4.24 Effect of extracts on colony forming ability of *Pseudomonas savastanoi* (1266) after 2 days incubation.

4.4. DISCUSSION

Conclusively, all three natural extracts had antifungal potential which was analogous to their concentration in the solid medium. Semi-pure and pure Oleuropein had a similar effect in all pathogens tested, ED_{50}, were similar (Table 4.13 and 4.14) and their efficiency was not significantly different. Oleuropein was less efficient for *A. alternata*, *F. oxysporum* and *Rhizopus* sp. than *B. cinerea*, *C. higginsianum* and *P. parasitica*. Surprisingly, oleuropein proved to be efficient at a concentration of 0.5% for *B. cinerea*, a fungus showing tolerance to the other two extracts at these concentration levels. *F. oxysporum* proved to be the most tolerant to oleuropein with ED_{50} values after 4 days incubation were 8.87 and 7.45 mg/ml for semipure and pure oleuropein respectively. Oleuropein treatments at a low concentration slightly stimulated growth fungi such as *B. cinerea* and *F. oxysporum*.

Table 4.13 ED_{50} values (mg/ml) after 4 days incubation for all fungi we used.

	ED_{50} – after 4 days (mg/ml, %)			
	Oleur. 55%	**Oleur. 98%**	**GPE**	**OMWW ext.**
Botrytis cinerea	**5.8 (58%)**	**5.7 (57%)**	**3.9 (39%)**	**7.8 (78%)**
Rhizopus sp.	**4.33 (43.3%)**	**3.4 (34%)**	**4.3 (43%)**	**-**
Alternaria alternata	**4.42 (44.2%)**	**5.17 (51.7%)**	**3.71 (37.1%)**	**7.93 (79.3%)**
Phytophthora parasitica	**0.51 (5.1%)**	**0.43 (4.3%)**	**0.42 (4.2%)**	**2.708 (27.08%)**
Colletotrichum higginsianum	**-**	**-**	**-**	**-**
Fusarium oxysporum	**8.87 (88.7%)**	**7.45 (74.5%)**	**3.3 (33%)**	**5 (50%)**

Radial growth of all pathogens was inhibited by GPE at a concentration of 1% for all pathogens except *P. parasitica* and *C. higginsianum* where a final concentration of 0.5% was enough to inhibit growth (Fig. 4.8, 4.9, 4.10). GPE significantly inhibited radial growth of *C. higginsianum, F. oxysporum* and *P. parasitica* even at a concentration as low as 0.1%. GPE inhibited *A. alternata* growth at a level of 77% compared to control in a 0.1% concentration and had a less significant impact for *Rhizopus* and *B. cinerea*. As can been seen in Tables 4.13 and 4.14, ED50 for GPE values after 4 days were 3.9 mg/ml for *B. cinerea*, 4.3 mg/ml for *Rhizopus sp.*, 3.71 mg/ml for *A. alternata*, 0.42 mg/ml for *P. parasitica* and 3.3 mg/ml *F. oxysporum*,

while after 7 days incubation GPE ED_{50} values were 7.2 mg/ml for *B. cinerea*, 7.1 mg/ml for *Rhizopus sp*., 3.38 mg/ml for *A. alternata*, 0.61 mg/ml for *P. parasitica*, 1.84 mg/ml for *C. higginsianum* and 3.3 mg/ml *F. oxysporum*.

Table 4.14 ED_{50} values (mg/ml) after 7 days incubation for all fungi we used.

	ED_{50} – after 7 days (mg/ml)			
	Oleur. 55%	Oleur. 98%	GPE	OMWW ext.
Botrytis cinerea	4.5 (45%)	4 (40%)	7.2 (72%)	-
Rhizopus sp.	7.5 (75%)	6.23 (62.3%)	7.1 (71%)	-
Alternaria alternata	6.33 (63.3%)	7.48 (74.8)	3.38 (33.8%)	8.62 (86.2%)
Phytophthora parasitica	0.48 (4.8%)	0.78 (7.8%)	0.61 (6.1%)	2.82 (28.2%)
Colletotrichum higginsianum	4.15 (41.5%)	4.16 (41.6%)	1.84 (18.4%)	4.9 (49%)
Fusarium oxysporum	-	7.62 (76.2%)	2.63 (26.3%)	5.1 (51%)

OMWW extract was generally less efficient than the other two extracts. In particular, OMWW extract had similar effects to Oleuropein on *A. alternata* and *C. higginsianum* compared to the control, but had less affect on *B cinerea* and *Rhizopus*, where it only showed some delay in growth at high concentrations. However it was effective for *F. oxysporum*, *C. higginsianum* and *P. parasitica* where it gave a significant reduction in growth even at low concentrations. As can been seen in Tables 4.13 and 4.14, ED_{50} for OMWW extract values after 4 days were 7.8 mg/ml for *B. cinerea*, not defined for *Rhizopus sp*., 7.93 mg/ml for *A. alternata*, 2.71 mg/ml for *P. parasitica* and 5 mg/ml *F. oxysporum*, while after 7 days incubation OMWW extract ED_{50} values were not defined for *B. cinerea* .and for *Rhizopus sp*., 8.62 mg/ml for *A. alternata*, 2.82 mg/ml for *P. parasitica*, 4.9 mg/ml for *C. higginsianum* and 5.1 mg/ml *F. oxysporum*.

Inhibition of *B cinerea* and *C. higginsianum* spore germination by natural extracts is shown in Figures 4.13 and 4.14. and showed that only GPE, was sufficiently effective to inhibit spore germination and germ tube of *C. higginsianum* and *B. cinerea*. The results were in good agreement with those of radial growth of fungi Figures 4.1, 4.2 and 4.10. where mycelial growth of *B. cinerea* and *C. higginsianum* was totally inhibited by 1% and 0.5% GPE respectively. Pure oleuropein was not so effective as

the other two extracts and caused a delay in spore germination of both fungi. OMWW extract appeared to be more efficient in inhibiting spore germination *C. higginsianum* than *B. cinerea*.

Table 4.15 Minimum Inhibitory concentrations (MICs) of natural compounds against bacterial strains used in this study.

BACTERIAL STRAIN	MIC * mg/ml (mM)			
	Oleuropein 55%	**Oleuropein 98%**	**GPE**	**OMWW ext.**
Pseudomonas syringae pv.apii 255 [a]	**0.7 (1.295)**	**0.7 (1.295)**	**1.5**	**2**
Xanthmonas vesicatoria 5075	**0.2 (0.37)**	**0.2 (0.37)**	**1.5**	**1**
Pseudomonas corrugata 1157	**N.D***	**1 (2.405)**	**N.D***	**N.D***
Pseudomonas viridiflava Ac2	**0.5 (0.925)**	**0.5 (0.925)**	**1.5**	**1.5**
Ralstonia solanacearum 819-6	**0.7 (1.295)**	**0.7 (1.295)**	**1**	**0.7**
Pseudomonas syringae tomato 132	**0.5 (0.925)**	**0.5 (0.925)**	**1.5**	**1.5**
Pseudomonas savastanoi 1266	**0.2 (0.37)**	**0.2 (0.37)**	**1**	**N.D**
Clavibacter michiganensis michiganensis 4040	**0.7 (1.295)**	**0.7 (1.295)**	**N.D. ***	**2**
Erwınia Amylovora 15	**0.7 (1.295)**	**0.9 (1.665)**	**N.D. ***	**2**
Erwinia atroseptica 3217	**0.7 (1.295)**	**0.7 (1.295)**	**N.D. ***	**N.D***

*N.D.: not defined

Oleuropein (semipure and pure) showed remarkable antibacterial activity, as show in Figures 4.15- 4.24, Table 4.15 and the MICs values ranging from 0.2 mg/ml to 0.7 mg/ml This observation confirmed the findings of others studies reporting the antimicrobial properties of oleuropein (Bisignano *et al* 1999, Fleming *et al* 1973). However GPE and OMWW extract were not as effective as oleuropein against bacterial strains used in this study. MICs values for GPE and OMWW extract ranged from 1 mg/ml to 2 mg/ml (Table 4.15).

CHAPTER FIVE

IN VIVO EVALUATION OF ISOLATED NATURAL COMPOUNDS

CHAPTER FIVE

5.1 INTRODUCTION AND OBJECTIVES

There is an increasing interest in exploitation of natural compounds for potential functional use. Applications of natural compounds are gaining increasing interest for controlling plants diseases. 'It is difficult to associate the antimicrobial activity to single compounds or classes of compounds. It seems that the antifungal and antimicrobial effects are the result of many compounds acting synergistically' (Hadizadeh, *et al* 2009).

The objective of this field work study was the *in vivo* evaluation of potential antimicrobial activity of isolated pure oleuropein, OMWW extract and GPE against commercial varieties of plants and fruits. In particular *in vivo* antibacterial activity of pure oleuropein and OMWW extract was assessed in greenhouse experiments, on *Xanthomonas campestris* pv. *vesicatoria*, the cause of bacterial spot of pepper plants and *Pseudomonas syringae* pv. *tomato*, the cause of bacterial speck of tomato plants, respectively. Besides endophytic growth of *Phytophthora parasitica* var. *nicotianae,* causal agent of the black shank disease of tobacco, on tobacco leaves by measuring its radial growth inside the infected tissue, was assessed. Furthermore the potential of postharvest treatment of table grapes with the grape pomace extract (GPE) to restrain grey mould (*Botrytis cinerea*) disease incidence and development was estimated and finally was evaluated the use of pure oleuropein as antibacterial in vase solution for cut flowers (carnation). Although the antimicrobial activity of oleuropein, GPE and OMWW extract has been studied widely, little research has been done on screening the in *vivo* efficiency of these extracts against important plant diseases and postharvest treatments.

5.2. MATERIALS AND METHODS

5.2.1. Treatments on pepper plants

- **Host and pathogen cultures**

Pepper (*Capsicum annuum var. annuum*) cultivar Topacio was used as hosts in this experiment. They were obtained from Fytochem S.A.- Kronos nursery (Crete).

- **Toxicity test of Oleuropein in Peppers**

Toxicity data are needed to assess the impact of oleuropein exposure on pepper plants, and to evaluate the optimal concentration and purity of oleuropein we can use for *in vivo* experiments against bacterial spot on pepper plants without toxicity symptoms. Plants with approximately 10 leaves were used. Three fully expanded leaves of each plant were treated. Upper and lower surface of leaves were wounded with a sterile bottle brush and suspensions of Oleuropein of different concentrations and purity were sprayed onto both surface.

Infiltration: Leaf surface was pricked with a sterile pin and the Oleuropein solution forced through the openings with a syringe without needle until the intercellular spaces of leaves were fully infiltrated.
Four leaves on each of three different replicate plants were randomly selected for treatment.

Conditions: Experiments of toxicity tests were conducted in an air-conditioned greenhouse (plate 5.1) with a maximum daily temperature of 26 ^{o}C for 15 days.

Evaluations were made at 15 days after oleuropein applications. We considered affected rate of the whole plant symptoms like wilt, bleaching, deformations and necrosis. Treatments of experiment are shown in Table 5.1

Table 5.1: Treatments for toxicity test with oleuropein on pepper plants

	Treatments for toxicity test
1.	Control- Plants (3) with no treatment
2i.	Control- Plants (3) with wounded leaves
2ii.	Control- Plants (3)-Infiltered with sterile water
3i.	Plants(3)- Sprayed with Oleuropein (55%) 0,01%
3ii.	>> (55%) 0,05%
3iii.	>> (55%) 0,1%
3iv.	>> (55%) 0,5%
4i.	Plants(3)- Sprayed with Oleuropein (98%) 0,01%
4ii.	>> (98%) 0,05%
4iii.	>> (98%) 0,1%
4iv.	>> (98%) 0,5%
5i.	Plants(3)- Wounded leaves& sprayed with Oleuropein(98%) 0,01%
5ii.	>> (98%) 0,05%
5iii.	>> (98%) 0,1%
5iv.	>> (98%) 0,5%
6i.	Plants(3)- Wounded leaves& sprayed with Oleuropein(55%) 0,01%
6ii.	>> (55%) 0,05%
6iii.	>> (55%) 0,1%
6iv.	>> (55%) 0,5%
7i.	Plants(3)-Infiltered leaves with Oleuropein(55%) 0,01%
7ii.	>> (55%) 0,05%
7iii.	>> (55%) 0,1%
7iv.	>> (55%) 0,5%
8i.	Plants(3)-Infiltered leaves with Oleuropein(98%) 0,01%
8ii.	>> (98%) 0,05%
8iii.	>> (98%) 0,1%
8iv.	>> (98%) 0,5%

- **Suppressive treatments of oleuropein against bacterial spot (*Xantomonas campestris* pv. *vesicatoria* 5075) of pepper seedlings-plants.**

Conditions: Experiments were conducted in greenhouse where the temperature was adjusted to 26°C by air-conditioning and RH 90 % or more by sprinkle irrigation (plate 5.1). Plants were exposed to natural light with photoperiods ranging from 10 to 14 h with supplemental artificial lighting.

Plate 5.1 Pepper plants in greenhouse

Strain: *Xanthomonas campestris* pv. *vesicatoria* (5075) was provided by phytopathology lab (TEI- CRETE). Bacterial cells were cultured on LB broth for 1-2 days at 28 ^{o}C on a rotary shaker set at 300 rpm. Cells were collected by centrifugation at 4000 rpm for 5 min, washed twice with 10 mM MgCl and then resuspended in 10 mM MgCl. The suspension was diluted to an absorbance of 0,15 at a wavelength of 600. All bacterial strains were maintained in long-term storage in sterile 25% glycerol solution at -70 ^{o}C.

Oleuropein solution**:** Pure Oleuropein (98%) was used for treatment. A solution of 0,1% Oleuropein was prepared from a stock solution of 15% Oleuropein diluted in ethanol. Each solution used in the trials contained 0.05% (w/v) of the surfactant Triton X-100 to improve the adhesion properties.

Growth of Bacteria on Peppers: Pepper plants with 10-12 leaves approximately were used. Five fully expanded leaves of each plant were treated. The upper and lower surface of leaves were pricked with a sterile bottle brush and the bacterial suspension was sprayed on the leave surface. Assessments were made on 4 leaves with good appearance (excluding leaves that were over injured, torn etc.) of each plant. Leaves were randomly selected on plants. The experiment was repeated twice.

Symptoms developed on the third day post- inoculation (plate a,b,c) and were scored using a 0 to 4 grading in which 0 corresponds to the absence of bacterial spot

symptom, 1 from traces to 25% of affected leaf area, 2 from 26 to 50% of affected leaf area, 3 from 51 to 75% of affected leaf area and 4 from 76 to 100% of affected leaf area (leaves destroyed or fallen).

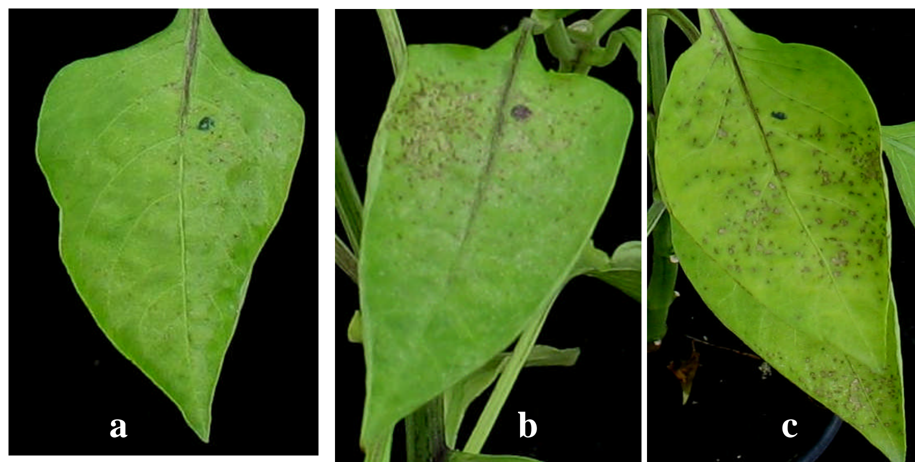


Plate 5.2 a, b, c Symptoms of *Xantomonas campestris* on pepper leaves.

Treatments of experiment are shown in Table 5.2. 3 replicates plants were used, inoculating 5 leaves on each. Inoculum density was adjusted to an OD_{600} 0,1 and 0,25. OD_{600} 0,1 was sufficient to give good infection in control (treatment No3) and was further analysed, contrary to 0,25 (OD_{600}) which developed normosensitive response and rejected.

Table 5.2 Suppressive treatments of oleuropein against *Xantomonas campestris* pv. *vesicatoria* of pepper seedlings-plants.

	Treatments
1.	Control- Plants (3), with wounded leaves
2.	Control- Plants (3), with wounded leaves and sprayed with Oleuropein 0.1%
3.	Control -Plants (3), with wounded leaves and infected with inoculum with $O.D_{600}$* 0.1.
4.	Plants, with wounded leaves, sprayed with Oleur. 0.1% 2hr before the infection.
5.	Plants, with wounded leaves, sprayed with Oleur. 0.1% 2hr before, 24hr and 72hr after infection.
6.	Plants, with wounded leaves, sprayed with Oleur. 0.1% 24hr, 48 hr and 72hr after infection.
7.	Plants, with wounded leaves, sprayed with Oleur. 0.1% 72hr after infection.
8.	Plants, with wounded leaves, sprayed with Oleur. 0.1% 72, 48, 24 2hr before, 24hr and 72hr after infection.

*Optical density was measured at 600 nm using a Shimadzu UV-1700 spectrophotometer

5.2.2 Suppressive treatments of OMWW extract against bacterial speck (*Pseudomonas syringae* pv. *tomato*) of tomato plants.

Tomatoes (*Solanum lycopersicum*) cultivar Despoina was used as hosts in this experiment. They were obtained from Fytochem S.A.- Kronos nursery (Crete). Experiments were conducted in greenhouse where the temperature was adjusted to 26°C by air-conditioning and RH 90 % or more by sprinkle irrigation. Plants were exposed to natural light with photoperiods ranging from 10 to 14 hr with supplemental artificial lighting.

Plate 5.3 a) Wound tomato leaf with a bottle brush, **b)** Spray tomato leaves with a solution (inoculum or OMWW extract)

Strain: *Pseudomonas syringae* pv. *tomato* (132) was provided by phytopathology lab (TEI- CRETE). Bacterial cells were cultured on LB broth for 1-2 days at 28 ^{o}C on a rotary shaker set at 300 rpm. Cells were collected by centrifugation at 4000 rpm for 5 min, washed twice with 10 mM MgCl and then resuspended in 10 mM MgCl. The suspension was diluted to an absorbance of 0,15 at a wavelength of 600. All bacterial strains were maintained in long-term storage in sterile 25% glycerol solution at -70 ^{o}C.

OMWW extract solution: A solution of 0,1% OMWW extract was prepared from a stock solution of 20% OMWW extract diluted in EtOH. Each solution used in the trials contained 0.05% (w/v) of the surfactant Triton X-100 to improve the adhesion properties.

<u>Growth of *Pseudomonas syringae* on Tomatoes plants</u>: Tomatoes plants with 10 leaves approximately were used. Four fully expanded leaves of each plant were treated. The upper and lower surface of leaves was pricked with a sterile bottle brush and the bacterial suspension was sprayed on the leave surface (plate 5.3 a, b). Assessments were made on 3 leaves with good appearance (excluding leaves that were overinjured, torn etc.) of each plant. Leaves were randomly selected on plants. The experiment was repeated twice.

Symptoms developed on the second day post- inoculation (plate 5.4 a, b, c) and were scored using a 0 to 4 grading in which 0 corresponds to the absence of bacterial spot symptom, 1 from traces to 25% of affected leaf area, 2 from 26 to 50% of affected leaf area, 3 from 51 to 75% of affected leaf area and 4 from 76 to 100% of affected leaf area (leaves destroyed or fallen).

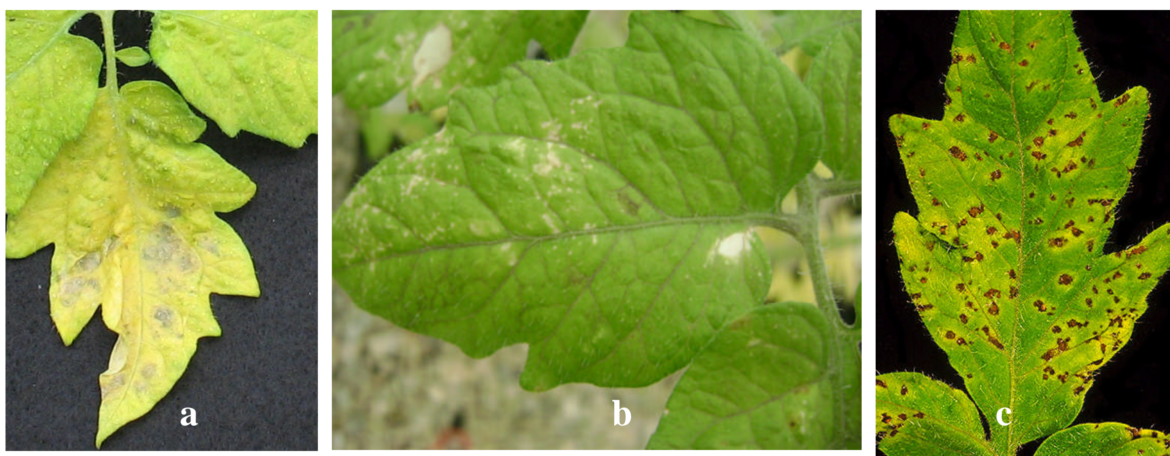


Plate 5.4 a, b, c Symptoms of *Pseudomonas syringae* on tomato leaves.

Treatments of experiment**:** Treatments of experiment are shown in Table 5.3. 3 replicates plants were used, inoculating 4 leaves on each. Inoculum density was adjusted to an OD_{600} 0,15

Table 5.3 Suppressive treatments of OMWW extract against *Pseudomonas syringae* pv. *tomato* of tomato plants.

	Treatment
1.	Control- Plants (3), with wounded leaves.
2.	Control- Plants (3), with wounded leaves and sprayed with Oleuropein 0.1%.
3.	Control -Plants (3), with wounded leaves and infected with inoculum with $O.D._{600}$* 0.15.
4.	Plants, with wounded leaves, sprayed with OMWW ext. 0.1% 2hr before the infection.
5.	Plants, with wounded leaves, sprayed with OMWW ext. 0.1% 2hr before, 24hr and 72hr after infection.
6.	Plants, with wounded leaves, sprayed with OMWW ext. 0.1% 24 hr, 48 hr and 72hr after infection with inoculum 0.1 and 0.25 ($O.D._{600}$).
7.	Plants, with wounded leaves, sprayed with OMWW ext. 0.1% 72, 48, 24 2hr before, 48hr and 72hr after infection.

*Optical density was measured at 600 nm using a Shimadzu UV-1700 spectrophotometer

5.2.3 Tobacco leaf inoculation assay

Tobacco plants (*Nicotiana tabacum* cv Xanthi) were grown under greenhouse conditions: a temperature of 25 ± 2°C, 16/8h photoperiod and 75% relative humidity. Plugs of *Phytophthora parasitica* var. *nicotianae* mycelium, growing on V8 agar, were cut out using a 15 mm diameter cork borer and placed upside-down on the abaxial leaf surface of 4 weeks-old plants. Mycelium plugs were covered with a fine layer of moisturised cotton and leaves were incubated for 7 days at 23°C, inside moistened trays that were kept tightly shut in order to ensure 100% relative humidity for the first 3 days. Leaves were allowed to a few hours of daylight and subsequently, a relative humidity of about 70% was kept in the trays. Solutions of 0.1% oleuropein (98%), 0.1% omww ext. and 0.1% GPE were stock solutions of 20% oleuropein, omww ext. and GPE respectively. Each solution used in the trials contained 0.05% (w/v) of the surfactant Triton X-100 to improve the wetting properties of the solutions. Leaves were sprayed with extracts a) 2 days and 2h before infection, b) 2h before infection c) 2h before and 2 days after infection d) 2 and 4 days after infection.

Endophytic growth of *P. parasitica* was assessed by measuring its radial growth inside the infected tissue (Plate 5.5), which developed a water-soaked appearance (Moschou *et al* 2009). Asympomatic systemic spread of the pathogen was excluded by random selection of leaf discs and light microscopy.

Plate 5.5 Endophytic growth of *Phytophthora parasitica* var. *nicotianae* on tobacco leaf.

5.2.4 Post harvest treatments of table grapes to control *B. cinerea*

Table grapes (*Vitis vinifera*) cvs Thompson Seedless were harvested from a vineyard in Heraklion, Crete Greece. No fungicides were applied prior to harvest. Mature clusters from several grapevines were collected. Only fruit of uniform size and color were selected to obtain homogeneous batches and all were chosen on the basis of absence of injuries. Trials were conducted on single detached berries and table grape clusters.

Fungi and growth conditions: *Botrytis cinerea* strains were obtained from the Applied Mycology Group, Biotechnology Centre, Cranfield University, Silsoe UK. Cultures of each fungal species were maintained on potato-dextrose agar (PDA) and stored at -80°C. PDA cultures of *B. cinerea* grown at 25 ◦C. To prepare the inoculum, an amount of 20 ml of sterile water, containing 0.05% (v/v) Triton X-100 was added to a two week old sporulating culture. Conidia were gently dislodged from the surface with a glass sterile rod and suspensions were filtered through four layers of cheesecloth to remove mycelial fragments. The suspensions were diluted with sterile water to an absorbance of 0.25 at 425 nm as determined by a spectrophotometer. This suspension contained about 10^6 conidia/ml (Mlikota Gabler and Smilanick, 2001) and was diluted with sterile water to 1.0×10^5 conidia/ml.

Antifungal substances preparation: Grape extract solution (GPE) was dissolved in distilled water to make a 0,5%, 1% and 5% (v/v) working solutions. The antifungal action of GPE was compared to that of chitosan (Sigma), a commercial natural product used to increase the ability of plants to defend against fungal infections, in a 1% (w/v) water solution. Furthermore Rovral, a commercial fungicide for *B. cinerea*, was also used in a 1% final concentration. Each solution used in the trials contained 0.05% (w/v) of the surfactant Triton X-100 to improve the wetting properties of the solutions.

Treatment of single detached berries: Berries were pulled from the rachis with pedicel detached, which exposed the berry flesh and enabled wound inoculation to occur. The berries were then immersed for 1 min in spore suspension of *B. cinerea* with 10^5 spores/ml and then left to air dry at 25 °C for 1 hr prior to treatment. Inoculated berries were kept at 25 °C in a covered plastic box for 2, 24, 2+24 and 48 h prior to treatment. Berries were immersed separately for 1 min in 0.5% GPE, 1% GPE, 5% GPE, 1.0% chitosan and 1% Rovral, left to air dry at 25 °C for 30min, kept at 25 °C for 7 days and then examined for gray mold decay at the end of storage. Approximately ten detached berries were treated in each treatment and the experiment was repeated twice times.

Treatment of table grape clusters: Entire clusters of table grapes (*Vitis vinifera*) cvs Thompson Seedless, were harvested and stored in VPE (ventilated polyethylene) bags of about 100- 150 gr. of grape each. In the first group test (**A**), grapes were immersed in 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan, 1% Rovral and in water (control) for 60 sec at room temperature. Three replicates were used per treatment. After treatment, grapes were air dried for about 1hr., placed into new VPE bags and then in commercial flimsy plastic boxes and stored for 5 weeks at 0-1°C (RH>90%). In the second group test (**B**), approximately 10^5 conidia/ml were used to infect grapes. Whole clusters of grapes were immersed for 1 min in spore suspension of *B. cinerea* with 10^5 spores/ml and then left to air dry at 25 °C for 2 hr prior to treatment. Then grapes were immersed for 1 min in water (control) or in 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. After treatment, grapes were air dried, repackaged in new VPE bags, then in commercial flimsy plastic boxes and stored for 5 weeks at 0-1°C (RH>90%). Three replicates were used per treatment and experiments performed twice.

5.2.5 Potential use of pure oleuropein as antibacterial in vase solution

Cut flowering stems of carnation *(Dianthus caryophyllus* L.) were harvested in the greenhouse at commercial maturity by cutting the stalks at soil level. Flowers were brought to the laboratory as soon as possible. Upon arrival in the laboratory the stems were recut in deionised water, removing about 3cm, giving a final stem length of 45 cm. Lower leaves were removed up to 35–45 cm from the cut stem base.

Flowers were placed in glass bottles containing 150 ml of solution at the onset of treatment. The experiments included 7 flowers per treatment, individually placed in the bottles and were held at 21°C, 60 -70% relative humidity (R.H.). Each bottle mouth was covered with aluminum foil to prevent solution evaporation and a small slit in the foil cover accommodated the stems. Thereafter, the fresh weight of the flowers (f.w.) and bottle weight (bottle + solution) were measured at the same time every four days from day 0. These data were used to determine the relative fresh weight (RFW) as percentage of initial fresh weight (% initial f.w.) (Joyce and Jones, 1992). Flowers were placed in the various vase solutions. Senescence was characterized by wilting of the petals.

Antibacterial compounds: Various concentrations, of DICA, i.e. sodium dichloroisocyanuric acid, a commercial antibacterial chemical and pure oleuropein (98% purity) were used. All vase solutions were made with deionized water. The bottles had been washed but not sterilized before use.

Treatments of experiment are shown in Table 5.4.

Table 5.4 Vase solutions was used to evaluate the use of oleuropein as antibacterial compound in vase solution.

1	Control – deionised water H_2O (7 bottles with 1 flower per bottle, 150 ml)
2	DICA 25 ppm >>
3	DICA 50 ppm >>
4	DICA 100 ppm >>
5	Oleuropein 98% - 50 ppm >>
6	Oleuropein 98% - 100 ppm >>
7	Oleuropein 98% - 200 ppm >>
8	Oleuropein 98% - 400 ppm >>

Vase life evaluation- Longevity**:** Vase life (in days), flower fresh weight and wilt score were recorded in a vase-life evaluation room. Flowers were weighed after 4, 7 and 10 days of vase life. For that purpose, flowers were taken out of water for as short a time as possible (20–30 sec). The fresh weight of each flower was expressed relative to the initial weight to represent the water status of the flower. Flower fresh weight was measured with a digital balance.

The mean wilt score was recorded daily on all flowers using the arbitrary scale: 0, bud stage; 1, open flower; 2, slightly senescent flower; 3, moderately senescent flower; 4, severely senescent flower; 5, dead flower. End of carnation vase life was determined by complete wilting of flowers (all scored with 5, dead flowers).

Determination of bacterial numbers: Vase solution which was present after ten days in bottles in which 7 freshly harvested carnation had been placed, was first used as a source of bacterial inoculum. For determination of the number of bacteria, bottle water samples (1ml per bottle) were diluted and plated on Plate Count Agar (casein 5g, yeast extract 2.5g dextrose 1g and agar 15g per 1l). Plate Count Agar is recommended for the plate count of microrganisms in food, dairy products, water and waste water. Petri dishes were kept at 28°C for 48 h before enumeration of bacteria. The optical density of samples was also measured at 600 nm using a Shimadzu UV-1700 spectrophotometer. Experiments were repeated three times.

5.3. RESULTS

5.3.1. Toxicity test with oleuropein on peppers

Toxicity results were obtained 15 days after oleuropein application in leaves of pepper plants. Treatments with plants sprayed with oleuropein (55% and 98% purity) solution at concentrations 0.01%, 0.05%, 0.1% and 0.5% did not show any toxicity symptom. A high toxicity of leaves, occurred after infiltration with 0.1% and 0.5% oleuropein for both semipure and pure oleuropein on treatments 7iii, 7iv, 8iii and 8iv (Table 5.1). Less strong toxicity symptoms also occurred on treatment 5iii (wounded leaves and sprayed with 0.1% pure oleuropein) and 6iii (wounded leaves and sprayed with 0.1% semipure oleuropein) than wounded leaves treated with 0.5% oleuropein (treatments 5iv and 6iv). Therefore oleuropein of 98% purity and 0.1% final concentration was decided to be used in further experiments with pepper plants.

5.3.2. Suppressive treatments of oleuropein against *Xanthomonas campestris* pv. *vesicatoria* of pepper seedlings-plants.

The antibacterial activity of pure oleuropein against bacterial spot of pepper plants is shown in Figures 5.1 - 5.5. As can been seen in Figure 5.1, when oleuropein application was 2h before the infection, the growth of bacterial spot on pepper leaves was effectively inhibited.

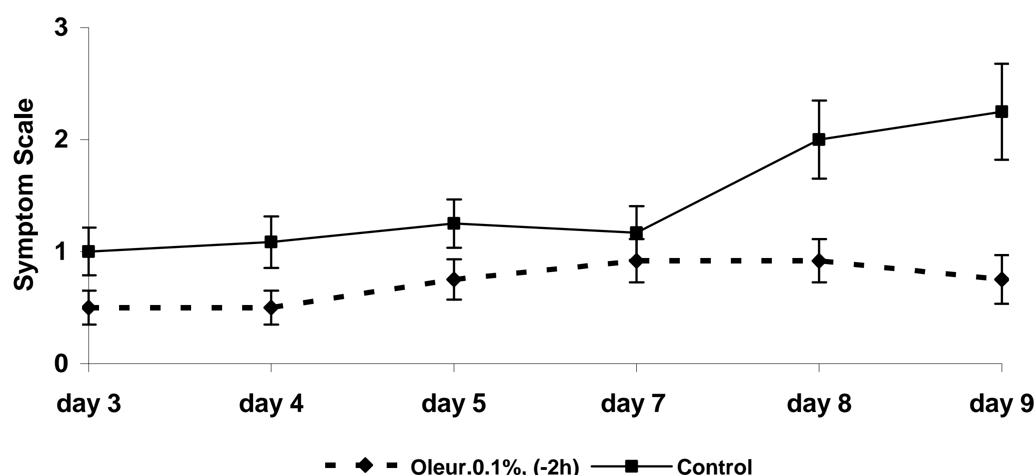


Figure 5.1 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 2h before the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean.

The results shown in Figure 5.2 were similar with these of Figure 5.1. Pure oleuropein application reduce surprisingly the incidence of bacterial spot on pepper leaves compared with the control, 2h before, 24h and 72 after the infection.

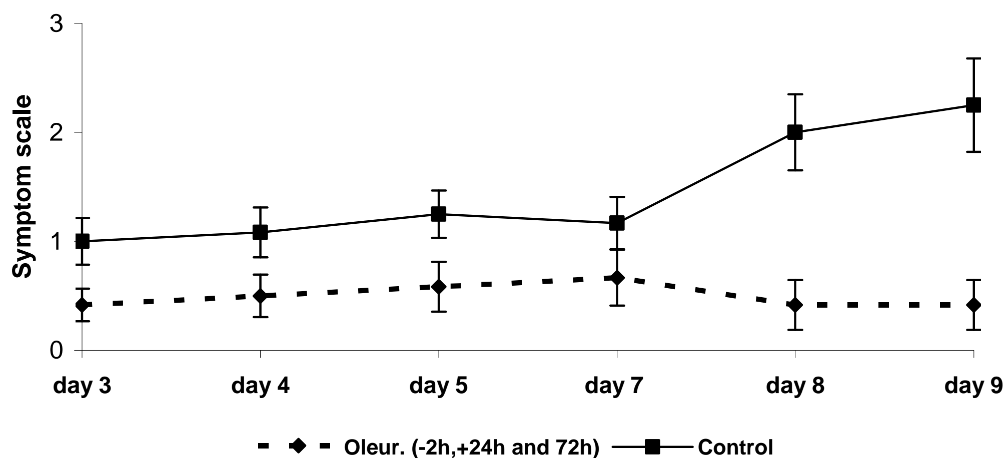


Figure 5.2 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 2h before and 24h and 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean.

However when oleuropein sprayed on pepper leaves 24h, 48 and 72h (Figure 5.3) and 72h after the infection (Figure 5.4), did not show inhibition compared with the control.

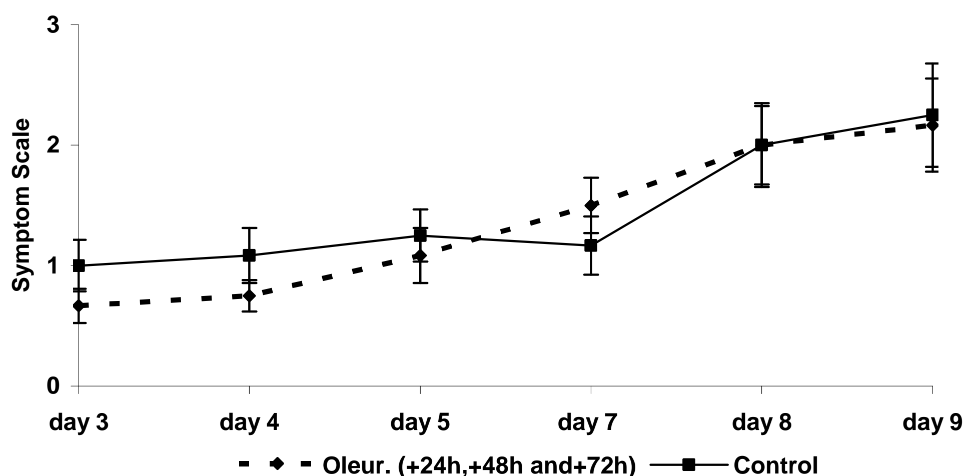


Figure 5.3 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 24h, 48h and 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean.

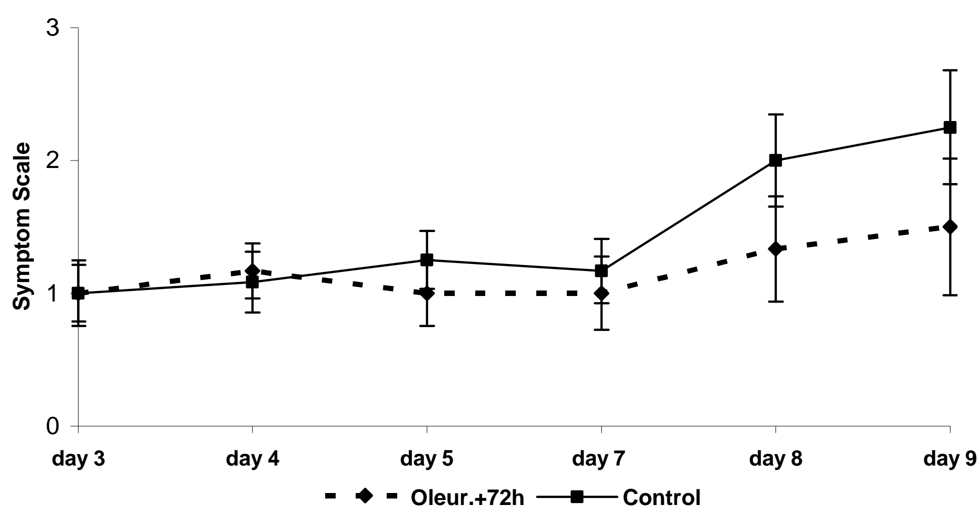


Figure 5.4 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean.

As can been seen in Figure 5.5 precautionary treatment of pepper plants leaves by means of 0.1% pure oleuropein restrain surprisingly the appearance bacterial spot symptoms on leaves .

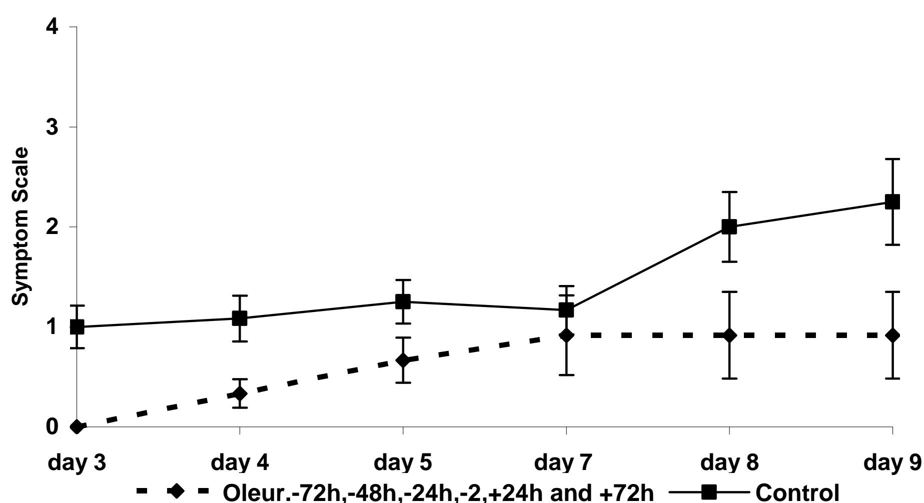


Figure 5.5 Effect of 0.1% pure oleuropein, sprayed on pepper plants leaves 72h, 48h, 24, 2h before and 24h, 72h after the infection with *Xanthomonas camestris* pv. vesicatoria. The values are the means of four replicates. Bars indicate standard errors of the mean.

As observed from Figures 5.1-5.5 maximum appearance of *Xanthomonas campestris* pv. *vesicatoria* symptoms in leaves occurred between 5-8 days after infection. It seems that 0.1% pure oleuropein (98%) had a protective effect against bacterial spot which is more obvious when oleuropein application started before infection, namely in treatment 4 (plants sprayed with oleuropein 2h before the infection), treatment 5 (plants sprayed with oleuropein 2h before the infection, 24h and 72h after the infection) and treatment 8 (plants sprayed with oleuropein 72, 48, 24, 2hr before, 24hr and 72hr after infection the infection). On the other hand oleuropein solution, did not has any protective effect against bacterial spot, when application started after the infection, in treatment 6 (plants sprayed with oleuropein 24hr, 48hr and 72 hr after the infection) and treatment 7 (plants sprayed with oleuropein 72 hr after the infection).

5.3.3 Suppressive treatments of OMWW extract against *Pseudomonas syningae* of tomato seedlings-plants.

The antibacterial activity of OMWW extract against bacterial speck of tomato plants is shown in Figures 5.6-5.9. As can been seen in Figure 5.6, when OMWW ext. application was 2h before the infection, the growth of bacterial speck on leaves effectively inhibited for the two weeks of incubation. At the end of experiment, this treatment symptoms scored 1 (0 to 4 grading).

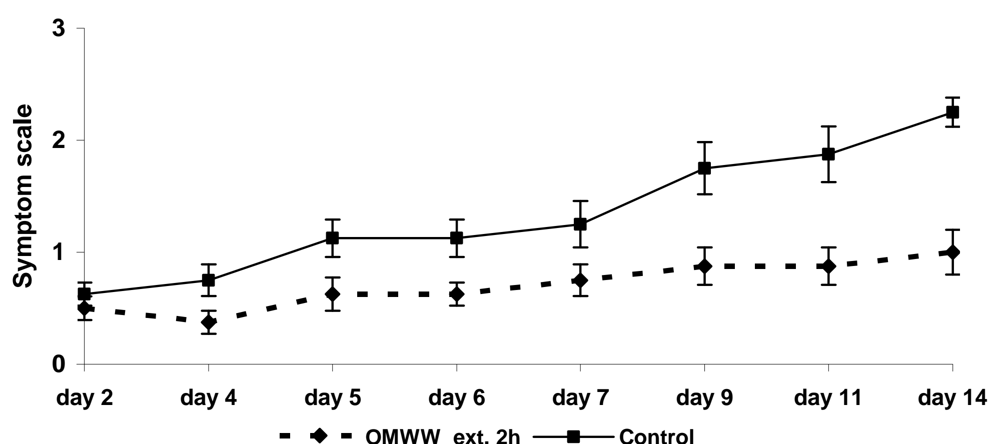


Figure 5.6 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 2h before the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean.

Figure 5.7 shows effect of 0.1% OMWW extract, sprayed on tomato plants leaves 2h before, 24h and 72h after the infection with *Pseudomonas syringae* pv. tomato. Results were similar with previous treatment. 0.1% OMWW extract solution inhibit surprisingly the occurrence of bacterial speck on tomato leaves. Treatment symptoms were scored ranged from 0.25 to 0.375 (0 to 4 grading) at the end of experiment.

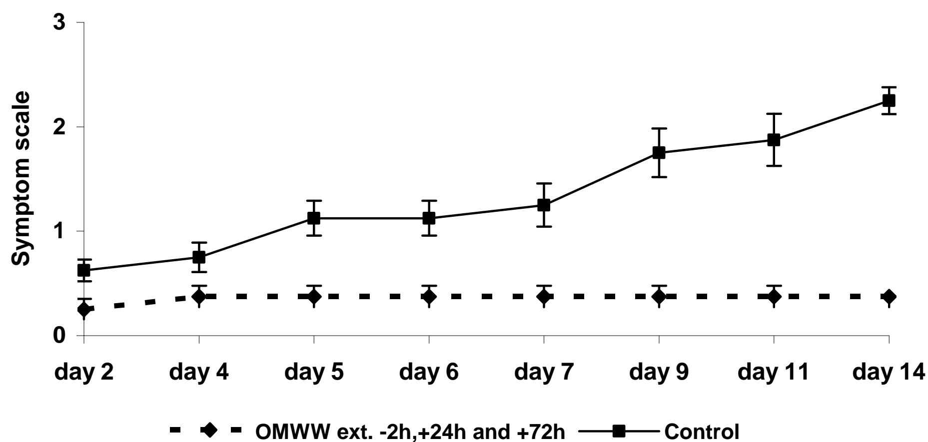


Figure 5.7 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 2h before, 24h and 72h after the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean.

The results shown in Figure 5.8 were similar with these of Figure 5.6 and 5.7. 0.1% OMWW extract application reduce terrifically the incidence of bacterial speck on tomato leaves compared with the control, 24h, 48h and 72 after the infection. Treatment symptoms were scored ranged from 0.5 to 0.667 (0 to 4 grading) at the end of experiment

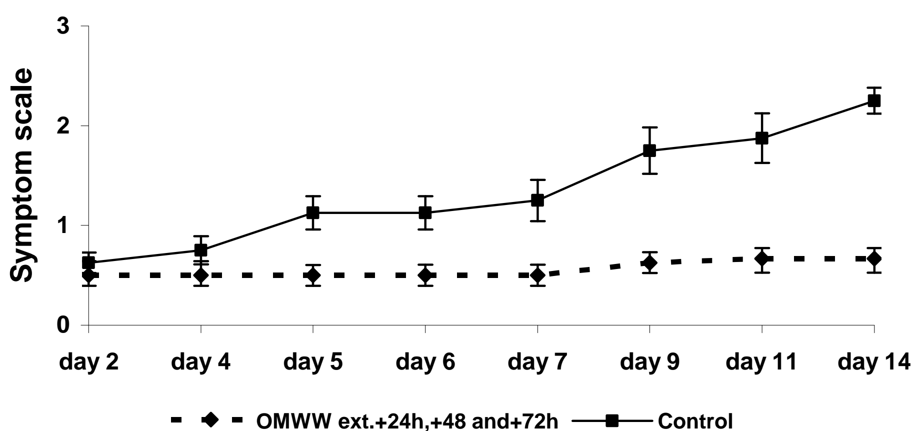


Figure 5.8 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 24h, 48h and 72h after the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean.

Figure 5.9 shows precautionary treatment of tomato plants leaves by means of 0.1% OMWW ext. When leaves treated with 0.1% OMWW ext. solution, 72h, 48h, and 2h before, 48h and 72h after the infection, the incidence of bacteria speck on leaves reduced terrifically.

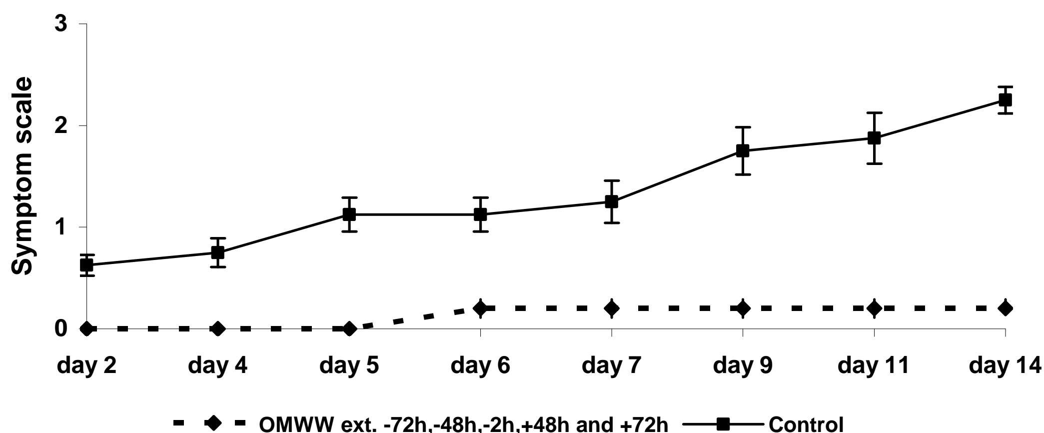


Figure 5.9 Effect of 0.1% OMWW extract, sprayed on tomato plants leaves 72h, 48h 2h before and 48h and 72h after the infection with *Pseudomonas syringae* pv. tomato. The values are the means of three replicates. Bars indicate standard errors of the mean.

As observed from Figures 5.1-5.5 maximum appearance of *Pseudomonas syringae pv. tomato* symptoms in leaves occurred between 5-14 days after infection. It seems that 0.1% OMWW extract had a protective effect against bacterial speck which is obvious in all treatments of the experiment. Especially precautionary treatment (Figure 5.9) gave the best results against bacterial speck on tomato plants leaves.

5.3.4 Effects of natural compounds on tobacco leaf infection by *P. parasitica*

The antifungal activity of 0.1% pure oleuroepein, 0.1% OMWW extract and 0.1% GPE after 7 days incubation in 25°C is shown in Figure 5.10. Oleuropein treatments had a drastic effect on *P. parasitica*. In particular, pure oleuropein 0.1% treatments in tobacco leaves two days and 2 hours before infection inhibited leaf infection by 44,1% compared to the control. A single treatment two hours before infection also restrain by 21.8% its endophytic growth compared to control. A significant inhibition (60.3%) was recorded on treatment with 0.1% pure oleuropein 2 hours before and 2 days after leaf infection. Post-infection treatment (2 days and 4 days after infection) was less effective, and endophytic growth in tobacco leaves was restrained by 19.17% compared with the control.

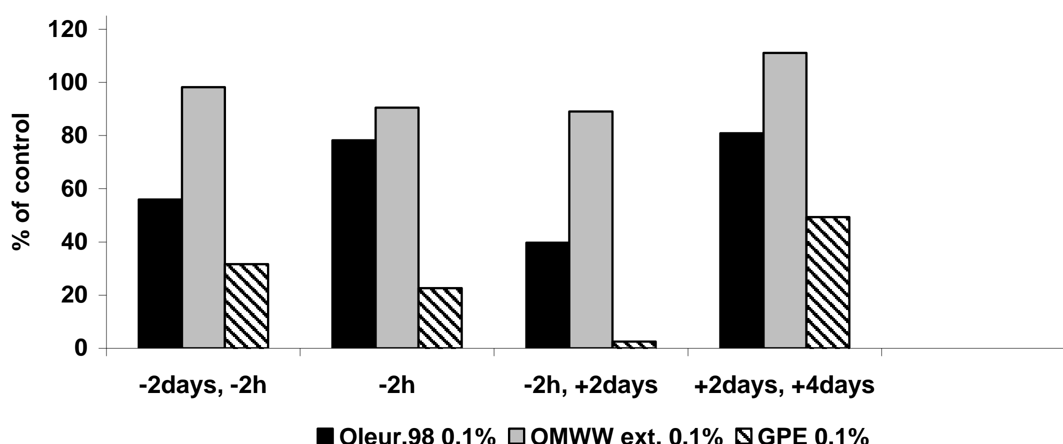


Figure 5.10 Effects of 0.1% pure oleuropein, 0.1% OMWW extract and 0.1% GPE i) 2 days and 2 h before ii) 2h before iii) 2 h and 2 days after iv) 2 and 4 days after inoculation on the adaxial surface by placing discs of *P. parasitica* mycelium cut from the edge of an actively growing colony. The values are reported in endophytic radial growth (lesion formation) % of the control.

As can been seen in Figure 5.10, 0.1% GPE showed the most significant inhibition in all treatments. In particular 0.1% GPE had approximately the same percentages of inhibition at all treatments ranged from 50.73% (+2 days, +4 days) to 77.37% (1%), compared with the control, except when GPE applied two hours before and two days after infection where the inhibition reached 97.46%. compared with the control. 0.1%

OMWW extract was the less effective on tobacco leaves application and showed negligible inhibition. OMWW ext. at post-infection treatment (2 days and 4 days after infection) showed 11.02% increase of leaves infection compared with the control.

5.3.5 Efficiency of Grape Pomace Extract on post harvest gray mold control

As can been in Figure 5.11 after 7 days incubation, GPE and the antifungal substances reduced significantly the incidence of grey mold on grape berries when applicated 2h before the infection with 10^5 spores/ml. Treatments with 5% GPE and 1% Rovral produced the strongest inhibitory effect with 3% and 2% gray mold incidence respectively. 1% GPE and 1% Chitosan also significantly reduced gray mold incidence with percentages 18% and 28% respectively, which were better than that with 0.5% GPE with 31% gray mold incidence.

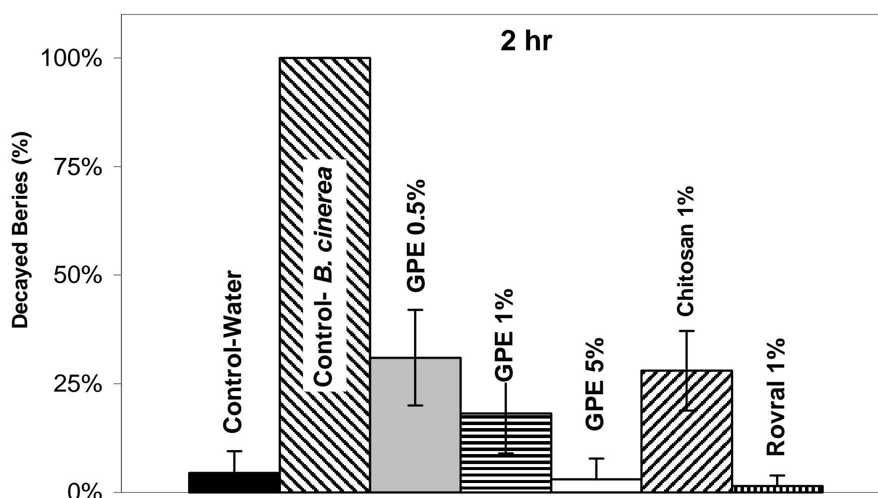


Figure 5.11 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **2h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean.

The rate of *B. cinerea* incidence increased in all treatments when the applications of GPE and antifungal substances were 24h and 48h respectively after the infection of grape berries (Figure 5.12 and 5.13).

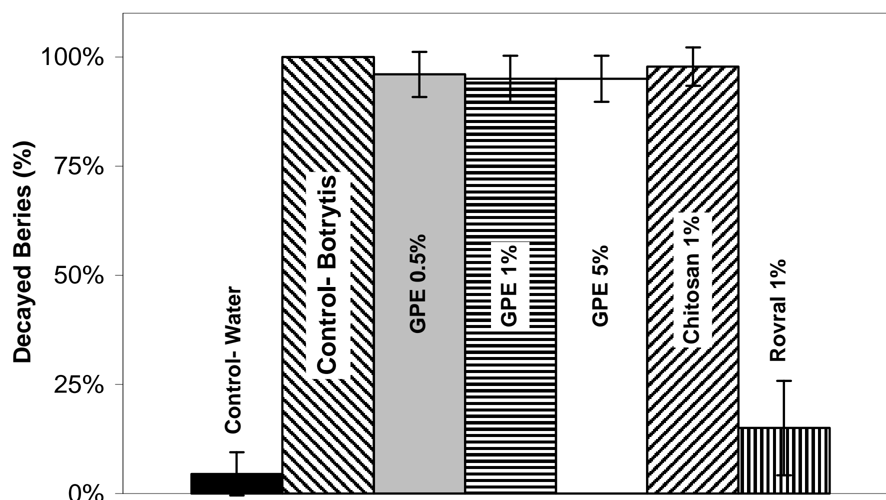


Figure 5.12 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **24h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. The values are the means of three replicates. Bars indicate standard errors of the mean.

The results illustrated in Figure 5.12 and 5.13 shows that there were not significant differences among treatments except treatment with Rovral, compared with the control, when GPE and antifungal substance application time increased (24h and 48h respectively after infection). The percentages of gray mold incidence of all treatments ranged from 95% to 100% except treatment with Rovral, where the percentages were 15% (24h after inoculation) and 52% (48h after inoculation) gray mold incidence.

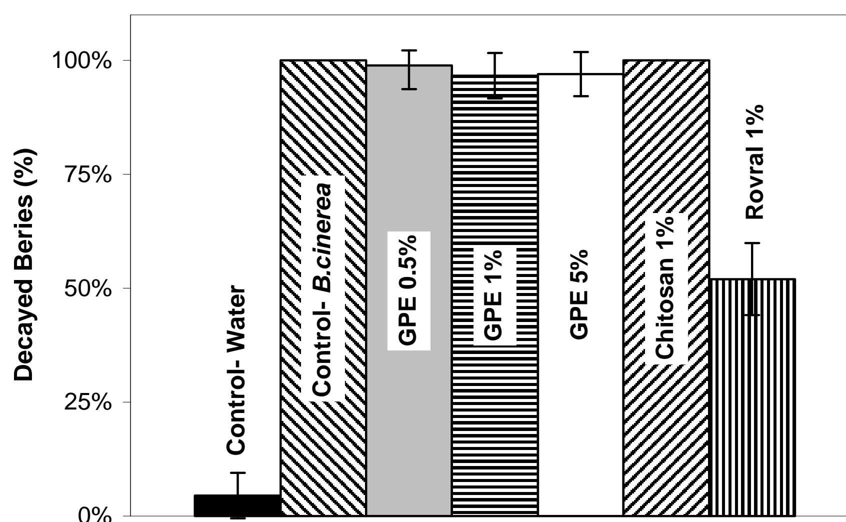


Figure 5.13 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **48h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. The values are the means of three replicates. Bars indicate standard errors of the mean.

Inhibition of gray mold incidence caused by immersion in 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral after 2h and 24h of inoculation with approximately 10^5 spores of *B.cinerea* /ml shown in Figure 5.14. Treatments with 5% GPE and 1% Rovral produced the strongest inhibitory effect with 2% and 4% gray mold incidence respectively. 1% GPE and 0.5% GPE reduced also significantly gray mold incidence with percentages 52% and 43% respectively, which were better than that with 1% Chitosan with 85% gray mold incidence.

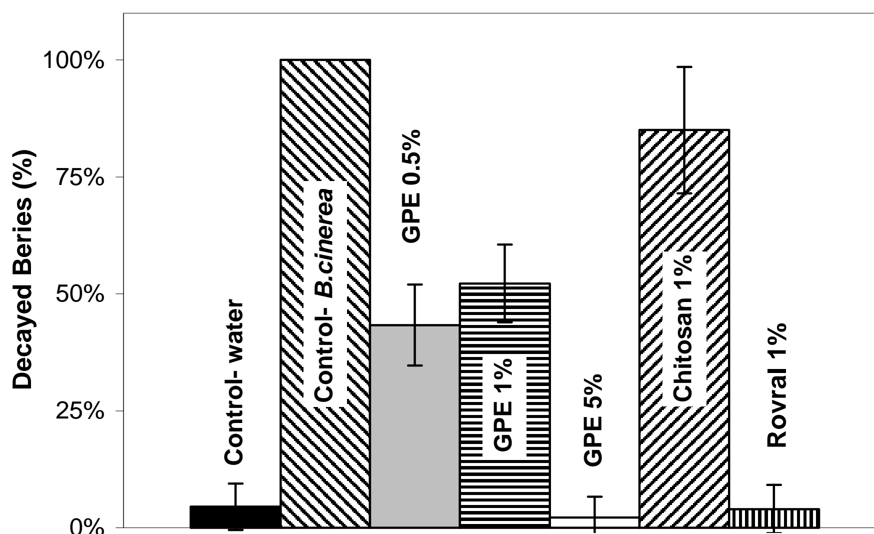


Figure 5.14 *B. cinerea* incidence on single berries incubated for 7 days at 25°C. Berries were inoculated by spraying with 10^5 spores/ml and then immersed after **2h and 24h**, in water as control, 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral. The values are the means of three replicates. Bars indicate standard errors of the mean. The values are the means of three replicates. Bars indicate standard errors of the mean.

Figure 5.15 shows the effect of water (control), 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral treatments on the decay of un-inoculated table grapes clusters during storage at 0-1°C (RH>90%). Natural incidence of decay, caused mostly by *B. cinerea* was reduced significantly by 1% GPE, 5% GPE and 1% Rovral. Immersion in 1% GPE and 5% GPE reduced the number of decayed berries per kg to 62.5 and 63.7 respectively, at the end of storage (after 5 weeks), while 1% Rovral reduced the number of decayed berries per kg to 52.53. Treatments with 0.5% GPE and 1% Chitosan showed similar results with the control. Immersion in 0.5% GPE and 1% Chitosan reduced the number of decayed berries per kg to 72.5 and 70.09 respectively, at the end of storage (after 5 weeks), while the number of decayed berries per kg after treatment with water (control) was 83.7.

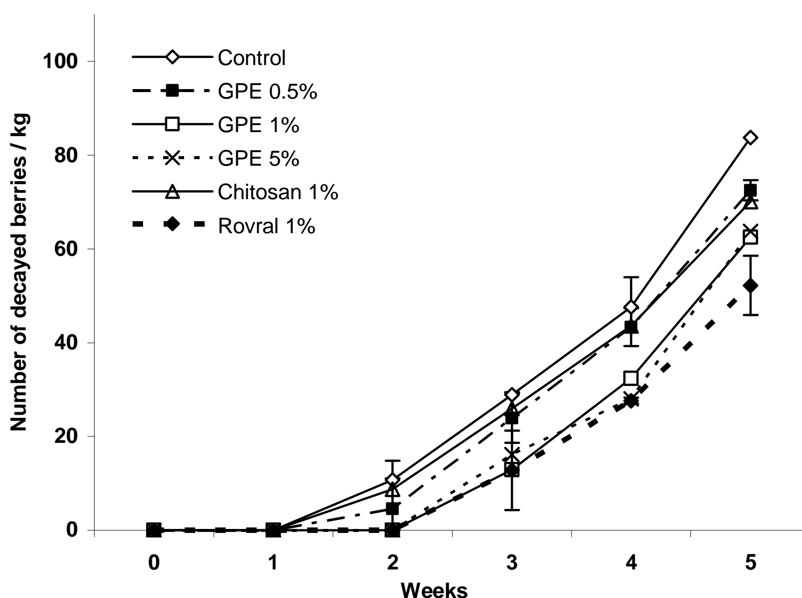


Figure 5.15 Effect of water (control), 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral treatments on the decay of un-inoculated table grapes clusters during storage at 0-1°C (RH>90%). The values are the means of three replicates. Bars indicate standard errors of the mean.

Figure 5.16 shows the effect of 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral treatments on the decay of inoculated table grapes clusters with approximately 10^5spores of *B.cinerea* /ml, 60 sec before each treatment, during storage at 0-1°C (RH>90%). Incidence of decay, caused by *B. cinerea* was reduced significantly for the two weeks after inoculation, by each all three GPE working concentrations and 1% Rovral. During this period, 5% GPE showed a better potential (8.56 decayed berries per kg, after 2 weeks) compared to Chitosan (48.64 decayed berries per kg, after 2 weeks) and in levels similar to Rovral (3.18 decayed berries per kg, after 2 weeks). Two weeks after inoculation, the only compounds that offer some protection were 5% GPE and Rovral. Following this period, grapes develop rapidly grey mould in all treatments, including that of Rovral. At the end of storage (after 5 weeks), immersion in 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral reduced the number of decayed berries per kg to 159.16, 159.6, 155.94, 162.13 and 139.49 respectively.

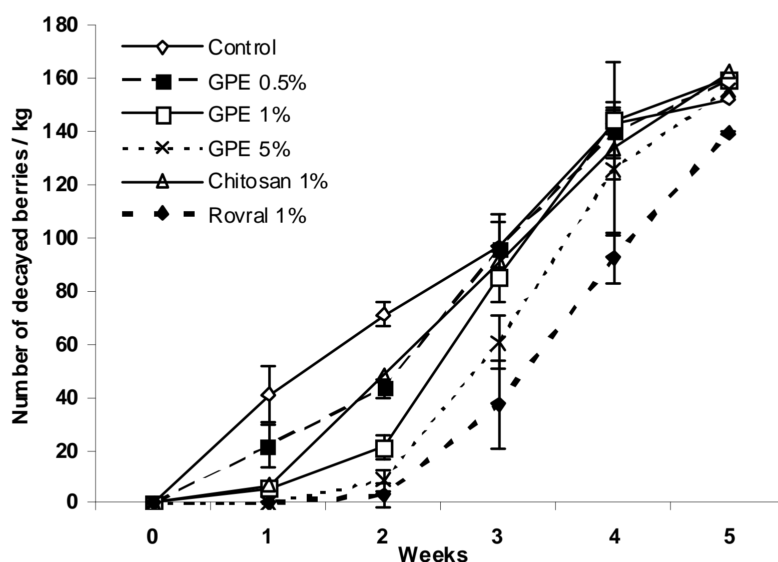


Figure 5.16 Effect of 0.5% GPE, 1% GPE, 5% GPE, 1% Chitosan and 1% Rovral treatments on the decay of inoculated table grapes clusters during storage at 0-1°C (RH>90%). Approximately 10^5spores/ml were used to infect table grapes clusters. The values are the means of three replicates. Bars indicate standard errors of the mean.

5.3.6 Effect of use of oleuropein as antibacterial in vase solution

Figure 5.17 shows the percentage change of relative fresh weight (%) of initial weight of carnation cut flowers in vase solutions with different concentrations of DICA and pure oleuropein. After four days relative fresh weight (RFW) of carnations treated with DICA 25ppm, 50ppm and 100ppm was increased 8.02%, 9.7% and 8.74% respectively, while carnations RFW treated with 50ppm, 100ppm, 200ppm and 400ppm pure oleuropein, increased 8.15%, 9.8%, 8.78%, 7.83% respectively. Carnations treated with water (control) as vase solution, after 4 days, increased 5.87% RFW.

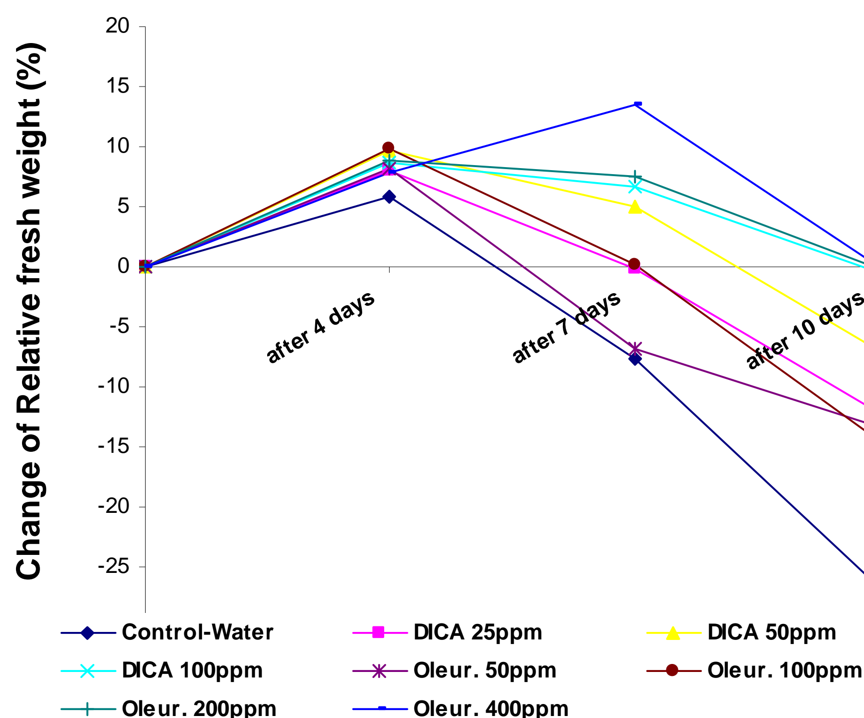


Figure 5.17 Change in relative fresh weight (%) of initial weight, of cut carnations treated with water (control), DICA 25ppm, DICA 50ppm, DICA 100ppm as vase solutions, after 4, 7 and 10 days.

However after 7 days, cut carnations treated with DICA 50ppm, DICA 100ppm, Oleur. 100ppm, Oleur. 200ppm and Oleur. 400ppm as vase solutions, increased RFW, 4.93%, 6.63%, 0.21%, 7.45% and 13.45% respectively, while carnations treated with water (control), DICA 25ppm and Oleur. 50ppm, as vase solutions,

decreased RFW, 7.63%, 0.14% and 6.89% respectively. At the end of the experiment, after 10 days, RFW of cut carnations decreased in all treatments except, this with Oleur. 400ppm where observed increase 0.02% of RFW. In particular cut carnations treated with water (control), DICA 25ppm DICA 50ppm, DICA 100ppm, Oleur. 50ppm Oleur. 100ppm and Oleur. 200ppm as vase solutions, decreased RFW 26.67%, 12.23%, 6.95%, 0.43%, 13.34%, 14.43% and 0.18% respectively.

The antibacterial activity of DICA and pure oleuropein at different concentrations, as vase solutions after 10 days, is shown in Figure 5.18. The most significant inhibition in colony forming ability was observed on the plates with DICA 50ppm, DICA 100ppm, Oleur. 200ppm and Oleur.400ppm compared with the control (water). Besides treatments with DICA 25ppm, Oleur. 50ppm and Oleur. 100ppm showed also great reduction in colony forming ability of vase solutions bacteria after ten days, with 28.5, 58 and 21.5 colonies on agar plates.

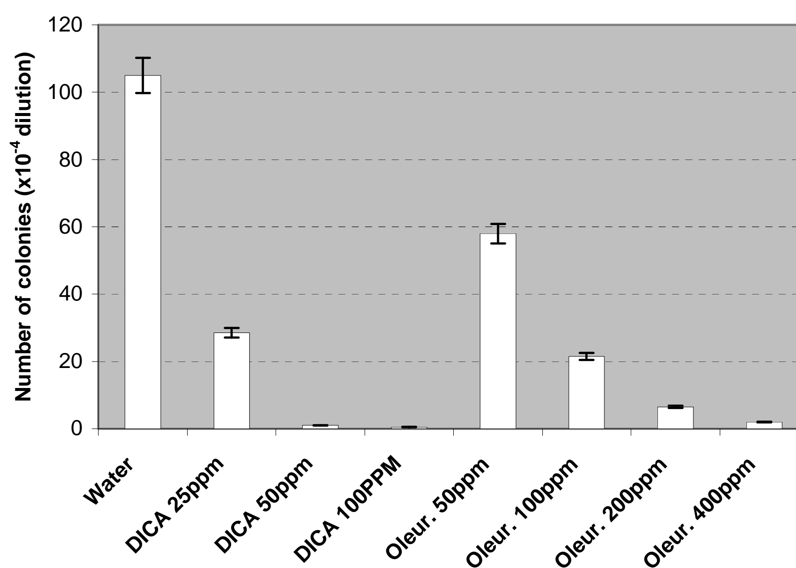


Figure 5.18 Number of colonies ($x10^4$) in cut carnations vase solutions: a) water (control), b) DICA 25ppm, c) DICA 50ppm, d) DICA 100ppm, e) Oleur. 50ppm, f) Oleur. 100ppm, g) Oleur. 200ppm and h) Oleur. 400ppm, after 10 days. The values are the means of three replicates. Bars indicate standard errors of the mean.

Figure 5.19 shows vase life (in days) of cut carnations treated with DICA and oleuropein at different concentrations. End of carnation vase life was determined by complete wilting of flowers (all scored with 5, dead flowers). As can been seen in Figure 5.19, increasing the concentration of DICA and oleuropein the vase life of cut carnations in the vase solution was improved. In particular vase life of carnations was more than doubled by using DICA 100ppm (26.5 days), Oleur. 200ppm (25 days) and Oleur. 400ppm (28.25 days), compared with control (water- 9days), while DICA 25ppm, DICA 50ppm, Oleur. 50ppm and Oleur. 100ppm as vase solution, increased also 13 days 17 days, 14.75days and 13.5 days respectively.

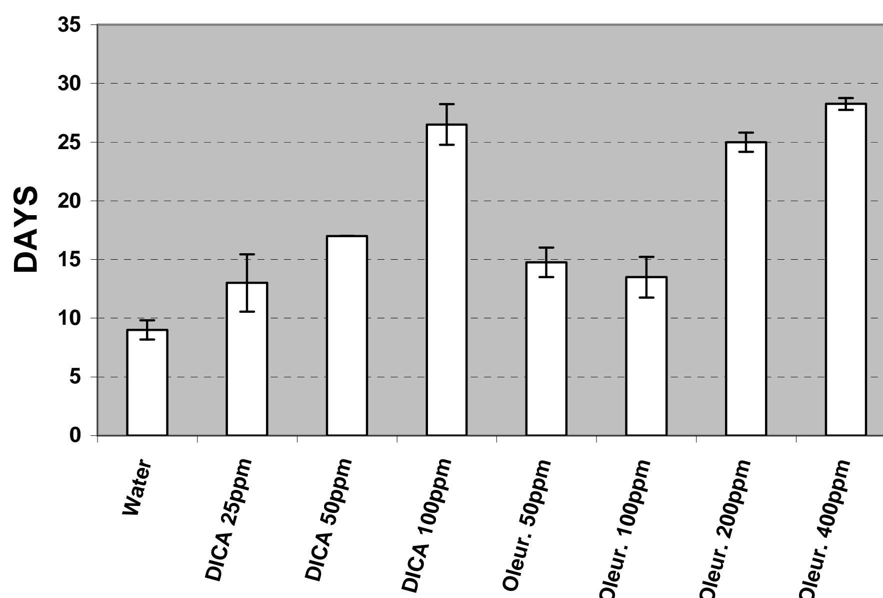


Figure 5.19 Longevity effects of water (control), DICA 25ppm, DICA 50ppm, DICA 100ppm, Oleur. 50ppm, Oleur. 100ppm, Oleur. 200ppm and Oleur. 400ppm on carnation vase life longevity. The values are the means of seven replicates. Bars indicate standard errors of the mean.

Plate 5.6 shows the effects of water, DICA 25ppm, DICA 50ppm, DICA 100ppm, Oleur. 50ppm, Oleur. 100ppm, Oleur. 200ppm and Oleur. 400ppm as vase solutions, on carnation vase life longevity after 10 days.

Plate 5.6 Effects of water (control), DICA 25ppm, DICA 50ppm, DICA 100ppm, Oleur. 50ppm, Oleur. 100ppm, Oleur. 200ppm and Oleur. 400ppm on carnation vase life longevity.

5.4 DISCUSSION

• Oleuropein toxicity test on peppers

Toxicity results obtained 15 days after oleuropein application on pepper plants leaves showed that treatments with plants sprayed with oleuropein (pure and semipure) solution at concentrations 0.01%, 0.05%, 0.1% and 0.5% did not show any toxicity symptom, while high toxicity of leaves, occurred after infiltration with 0.1% and 0.5% oleuropein. On the other hand, on wounded pepper leaves sprayed with 0.1% oleuropein (semipure and pure), less strong toxicity symptoms occurred than wounded leaves treated with 0.5% Oleuropein. Oleuropein toxicity has been widely studied. Hamdi and Castellon (2005) observed that oleuropein is a non toxic antioxidant with anti-tumor activities. Researchers after several studies failed to determine a lethal dose for animals (mice) when olive leaf extract or oleuropein was applied as nutrition supplement. Toxicity results of olive leaves extracts and oleuropein on humans were similar (Walter 1997). Olive leaf extracts have been demonstrated that is safe to use as supplement even during pregnancy and breastfeeding. On the other hand, *in vitro* studies have shown that oleuropein and its derivatives are very toxic and act as natural antibiotics against a range of gram - positive and gram-negative bacteria such as *Bacillus cereus*, *Staphylococcus aureus*, *E. Coli* etc. However no research has been done on screening the toxicity of pure oleuropein on plants.

• Oleuropein treatments against bacterial spot of pepper seedlings-plants

Thus, oleuropein of 98% purity and 0,1% final concentration was decided to be used in suppressive treatments against *Xanthomonas campestris* pv. *vesicatoria* of pepper seedlings-plants. Bacterial spot is a widespread and extremely harmful disease, for tomato and pepper plants, especially in warm climates. Copper compounds were used mainly (Azaizeh & Bashan, 1984) to reduce losses foliar bacterial diseases, but frequent use of copper possibly resulted in the appearance of copper resistance to phyto-pathogenic bacteria (Pohronezny *et al.*, 1994, Ritchie and Dittapongpitch, 1991, Jones *et al.*, 1991). Besides a EU regulation (Reg. EU n. 473/2002) restricts the use of copper in organic agriculture. Thereby there is a great interest to control important

disease with natural compounds. The use of pure oleuropein gave good results in suppressive treatments against bacterial spot of pepper seedlings-plants. 0.1% pure oleuropein had a protective effect against bacterial spot which is more obvious when oleuropein application started before infection, namely in treatment which i) plants sprayed with oleuropein 2h before the infection, ii) plants sprayed with oleuropein 2h before the infection, 24h and 72h after the infection and iii) plants sprayed with oleuropein 72, 48, 24, 2hr before, 24hr and 72hr after infection the infection. On the other hand 0.1% oleuropein solution, did not has any protective effect against bacterial spot, when application started after the infection, in treatment 6 (plants sprayed with oleuropein 24hr, 48hr and 72 hr after the infection) and treatment 7 (plants sprayed with oleuropein 72 hr after the infection).

- OMWW extract treatments against bacterial speck of tomato plants

Bacterial speck of tomato plants caused by *Pseudomonas syringae* pv. *tomato* is also a widespread, greenhouse or open field disease. Treatments with copper compounds and agronomical practises such as seeds certification were used mainly (Colin et al., 1984; Varvaro et al., 2001) to reduce losses from bacterial speck. As alternative to copper compounds OMWW extract can be used. Results were better than previous (oleuropein), when 0.1% OMWW extract was used for suppressive treatments against bacterial speck of tomato plants. This extract had impressive inhibitory effect against bacterial speck occurance on tomato leaves, which is obvious in all treatments of the experiment. Especially precautionary treatment (Figure 5.9) gave the best results against bacterial speck on tomato plants leaves. Quattrucci (2009) reported the antibacterial activity of natural extracts obtained from *Liliaceae* and *Moraceae* plants, against bacterial speck. Both *in vitro* and *in vivo* experiments confirmed their antibacterial activity. Recently Balestra *et al.* (2008) demonstrated also that extracts from *Allium sativum* and *Ficus carica fruits* reduce, *in vitro* and *in vivo*, disease incidence caused by bacteria of Kiwifruit (*Pseudomonas syringae pv. syringae, Pseudomonas viridiflava*) and of tomato (*Pseudomonas syringae pv. tomato*) plants.

• <u>Natural compounds effects on tobacco leaf infection by *P. parasitica*</u>

For the first time, the effects of natural compounds such as oleuropein, OMWW extract and GPE, against *Phytophthora parasitica* var. *nicotianae* on tobacco leaves was examined in *in vivo* experiment under greenhouse conditions. Findings displayed that 0.1% GPE showed the most significant inhibition in all treatments. Oleuropein treatments had as well a drastic effect on *P. parasitica*, especially when applicated two times before the infection. OMWW extract was the less effective on tobacco leaves application and showed negligible inhibition. These results are in good agreement with those of radial growth in *in vitro* experiments. Snook *et al*., (1992) reported the inhibition of *Phytophthora parasitica* var. *nicotianae* by aromatic acids and coumarins, while Prost *et al*. (2005) evaluated the antimicrobial activities of plant oxylipins against patogens. 43 natural oxylipins were used against a set of 13 plant pathogenic microorganisms including bacteria, oomycetes including *Phytophthora parasitica* var. *nicotianae* and fungi. Results showed that most of these compounds reduce the growth of microorganisms. Falcón *et al.* (2007) evaluated the effect of chitosan derivatives against *Phytophthora parasitica* var. *nicotianae* in vitro and in vivo experiments. Results showed that less acetylated and degraded chitosan are better for direct inhibition of oomycete growth and partially acetylated and degraded chitosan are suitable to protect tobacco against *P. parasitica* by systemic induction of plant resistance.

• <u>GPE efficiency on post harvest gray mold control</u>

Botrytis cinerea the cause of grey mold is considered the most important disease of table grapes. Several studies have been carried out to determine the antifungal activity of natural compounds against *B. cinerea* as postharvest disease on table grapes. Chitosan, a natural compound derived from cells, have been reported in many studies for its strong antifungal activity when applied either as a pre- or postharvest treatment (Reglinski *et al.,* 2005, El Ghaouth *et al.,* 2000). Romanazzi *et al.* noted that chitosan antimicrobial activities was activated, when it was dissolved in an acid it solution such as acetic acid, which was considered the best for this purpose (Romanazzi *et al*., 2005). Chitosan had strong inhibitory effect against gray and blue molds of table grapes, when dissolved in acetic acid (Romanazzi *et al*., 2006, Romanazzi *et al*.,

2002). Recenlty Xu *et al.* (2007) used grapefruit seed extract, in combination with chitosan extract, to control postharvest gray mold in 'Redglobe' table grapes. Both natural compounds, alone or combined reduced surprisingly postharvest gray mold incidence of 'Redglobe' table grapes, compared with the control. Our results indicate that GPE significantly reduced the incidence of *B. cinerea* on grape berries and the time after treatment influenced the numbers of decayed berries. In particular, treatments with 5% GPE and 1% Rovral produced the strongest inhibitory effect with 3% and 2% gray mold incidence respectively. 1% GPE and 1% Chitosan also significantly reduced gray mold incidence with percentages 18% and 28% respectively, which were better than that with 0.5% GPE with 31% gray mold incidence. The rate of *B. cinerea* incidence increased in all treatments when the applications of GPE and antifungal substances were 24h and 48h respectively after the infection of grape berries and the percentages of gray mold incidence of all treatments ranged from 95% to 100% except treatment with Rovral, where the percentages were 15% (24h after inoculation) and 52% (48h after inoculation) gray mold incidence.

On the other hand application of 0.5% GPE on table grapes did not alter significantly disease natural incidence two to five weeks after storage, similarly to control and treatment with Chitosan. However, grapes treated with higher GPE concentrations (1% and 5%) gave a significantly reduced disease incidence compared to control, their protective effect being similar to 1% Rovlar for the whole period of storage. *B. cinerea* infection was kept at significantly lower levels compared to control after treatment with each all three GPE working concentrations (0.5%, 1% and 5%) for the two weeks of incubation. During this period, high GPE concentration showed a better potential compared to chitosan and in levels similar to Rovlar. Two weeks after inoculation, the only compounds that offered some protection were 5% GPE and 1% Rovlar. Following this period, grapes developed rapidly grey mould in all treatments, including that of Rovlar. There are no prior reports describing the effect of GPE to control postharvest decay of table grapes.

- Use of oleuropein as antibacterial in vase solution

A major problem affecting the cut flower stage is the growth of microorganisms (fungi and bacteria) in vase solutions. Van Doorn (1997) reported that microorganisms multiply rapidly, under favourable conditions and can enter through vessels above

the cut surface causing bacterial occlusions. Papadimitrioy *et al* (2006), was found that vase life of 'White Sim' carnations was reduced, when the vase solution (water) that was maintained it was artificially infected with bacteria 10^8 cfu ml^{-1} It has been suggested that addtion of antibacterial agents improve water conductance by preventing bacterial growth and producing occlusions (Van Doorn, 1997). Chlorine which is also used to control bacteria in vase solutions (van Doorn *et al.,* 1990; Joyce *et al.,* 1996; Knee, 2000; Faragher *et al.,* 2002) is a constituent of many preservatives, such as DICA (Dichloroisocyanuric acid). DICA is a slow degradation chloride compond that maintains free chlorine availability under chlorine demand conditions.

Our results show that oleuropein, as antibacterial, in carnation vase solutions significantly inhibited the colony forming ability of vase solutions bacterial strains, especially solution with 200ppm and 400ppm pure oleuropein gave surprisingly inhibition compared with the control and their effect were similar to DICA. Moreover pure oleuropein was shown to lengthen the vase life of cut carnation flowers. In particular life of carnations was more than doubled by using 200ppm and 400ppm oleuropein and results were similar again with DICA.

CHAPTER SIX

SUMMARY OF CONCLUSIONS OF THE THESIS AND FUTURE CONSIDERATION

CHAPTER SIX

This chapter presents a bullet point list of conclusions of the present thesis and suggests some future consideration.

The main motivation for the current study was the exploitation of natural compounds as an alternative way to control important plant diseases.

The aim of the study was to isolate and purify natural antioxidants from olive leaves, olive mills waste waters and grape pomace (winery byproducts) and to exploit these compounds by using them as phytoprotective agents against important pathogens in Greece.

6.1 SUMMARY OF THE OUTCOMES OF THIS PROJECT

(a) *In vitro* experiments

1. All natural extracts had antifungal potential which was analogous to their concentration in the solid medium.
2. Radial growth of all pathogens was inhibited by GPE at a concentration of 1% for all pathogens except *P. parasitica* and *C. higginsianum* where a final concentration of 0.5% was enough to inhibit growth. GPE showed strong antifungal activity.
3. Semipure and pure Oleuropein had a similar effect in all pathogens tested, ED_{50}, were similar and their efficiency was not significantly different.
4. Oleuropein was less efficient for *A. alternata*, *F. oxysporum* and *Rhizopus* sp. than *B. cinerea*, *C. higginsianum* and *P. parasitica*.
5. Oleuropein treatments at a low concentration slightly stimulated growth fungi such as *B. cinerea* and *F. oxysporum.*
6. OMWW extract was generally less efficient than the other two extracts. In particular, OMWW extract had similar effects to Oleuropein on *A. alternata* and *C. higginsianum* compared to the control, but had less affect on *B cinerea* and *Rhizopus*, where it only showed some delay in growth at high concentrations.

7. OMWW extract was effective for *F. oxysporum*, *C. higginsianum* and *P. parasitica* where it gave a significant reduction in growth even at low concentrations.
8. Only GPE, was sufficiently effective to inhibit spore germination and germ tube of *C. higginsianum* and *B. cinerea*.
9. Pure oleuropein was not so effective as the other two extracts and caused a delay in spore germination of both fungi. OMWW extract appeared to be efficient in inhibiting spore germination *C. higginsianum* than *B. cinerea*.
10. Pure and semipure oleuropein showed remarkable antibacterial activity. MICs values ranged from 0.2 mg/ml to 0.7 mg/ml.
11. GPE and OMWW extract were not as effective as oleuropein against bacterial strains used in this study. MICs values for GPE and OMWW extract ranged from 1 mg/ml to 2 mg/ml.

(b) *In vivo* experiments

1. Plants sprayed with oleuropein (pure and semipure) solution at concentrations 0.01%, 0.05%, 0.1% and 0.5% did not show any toxicity symptom, while high toxicity of leaves, occurred after infiltration with 0.1% and 0.5% oleuropein.

2. On wounded pepper leaves sprayed with 0.1% oleuropein (semipure and pure), less strong toxicity symptoms occurred than wounded leaves treated with 0.5% Oleuropein.

3. Oleuropein of 98% purity and 0.1% final concentration was decided to be used in suppressive treatments against *Xanthomonas campestris* pv. *vesicatoria* of pepper seedlings-plants.

4. 0.1% pure oleuropein had a protective effect against bacterial spot which is more obvious when oleuropein application started before infection, namely in treatment which i) plants sprayed with oleuropein 2h before the infection, ii) plants sprayed with oleuropein 2h before the infection, 24h and 72h after the infection and iii) plants sprayed with oleuropein 72, 48, 24, 2hr before, 24hr and 72hr after infection the infection.

5. 0.1% oleuropein solution, did not has any protective effect against bacterial spot, when application started after the infection, in treatment 6 (plants sprayed with oleuropein 24hr, 48hr and 72 hr after the infection) and treatment 7 (plants sprayed with oleuropein 72 hr after the infection).

6. OMWW extract had impressive inhibitory effect against bacterial speck occurance on tomato leaves, which was obvious in all treatments of the experiment. Especially precautionary treatment gave the best results against bacterial speck on tomato plants leaves.

7. 0.1% GPE showed the most significant inhibition against *Phytophthora parasitica* var. *nicotianae* on tobacco leaves in all treatments.

8. Oleuropein treatments had as well a drastic effect on *P. parasitica*, especially when applied twice before the infection.

9. OMWW extract was the less effective on tobacco leaves application and showed negligible inhibition.

10. GPE significantly reduced the incidence of *B. cinerea* on grape berries and the time after treatment influenced the numbers of decayed berries. In particular, treatments with 5% GPE and 1% Rovral produced the strongest inhibitory effect.

11. 1% GPE and 1% Chitosan also significantly reduced gray mold incidence with percentages 18% and 28% respectively, which were better than that with 0.5% GPE with 31% gray mold incidence.

12. The rate of *B. cinerea* incidence increased in all treatments when the applications of GPE and antifungal substances were 24h and 48h respectively after the infection of grape berries and the percentages of gray mold incidence of all treatments ranged from 95% to 100% except treatment with Rovral.

13. Application of 0.5% GPE on table grapes did not alter significantly disease natural incidence two to five weeks after storage, similarly to control and treatment with Chitosan.

14. Table grapes treated with higher GPE concentrations (1% and 5%) gave a significantly reduced disease incidence compared to control, their protective effect being similar to 1% Rovlar for the whole period of storage.

15. *B. cinerea* infection was kept at significantly lower levels compared to control after treatment with each all three GPE working concentrations (0.5%, 1% and 5%) for the two weeks of incubation. During this period, high GPE concentration showed a better potential compared to chitosan and in levels similar to Rovlar.

16. Two weeks after inoculation, the only compounds that offer some protection were 5% GPE and 1% Rovlar. Following this period, grapes develop rapidly grey mould in all treatments, including that of Rovlar.

17. Oleuropein, as antibacterial, in carnation vase solutions significantly inhibit the colony forming ability of vase solutions bacterial strains, especially solution with 200ppm and 400ppm pure oleuropein gave surprisingly inhibition compared with the control and their effects were similar to DICA.

18. Pure oleuropein was shown to lengthen the vase life of cut carnation flowers. In particular life of carnations was more than doubled by using 200ppm and 400ppm oleuropein and results were similar again with DICA.

FUTURE CONSIDERATIONS

- In vitro elaluation of extracted natural compounds should be carried on for more plants pathogens.
- Creation of plant protection compositions, based on extracted natural compounds, which will be used against more pathogens in greenhouse and in field. In particular, the use of oleuropein or OMWW extract can be evaluated against *Pseudomonas savastanoi* pv. *oleae* the cause of tumour in olive trees. Moreover, GPE could be used as antifungal in vineyards against *B. cinerea*.
- Furthermore, it will be useful, to use these componds and especially GPE, against postharvest pathogens of other fruits and vegetables.

- Combined use of oleuropein or OMWW extract, with copper compounds to control plant diseases.
- It would be interesting, today to restrict amount of the additive sulfur dioxide (SO_2) in vinification and gradual replacement with natural antioxidants such as oleuropein and GPE.
- Evaluate the influence of storage time on olive oil quality with the addition of natural antioxidant substances that was extracted (oleuropein, OMWW ext., GPE).

CHAPTER SEVEN

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