



Faculty of Resource Science and Technology

**Study on the Effects of UVB and UVC Radiations on
Hydroxyphenylpyruvate Reductase Gene Expression and Rosmarinic Acid
Production in Medicinal Plant, *Orthosiphon aristatus***

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Study on the Effects of UVB and UVC Radiations on Hydroxyphenylpyruvate
Reductase Gene Expression and Rosmarinic Acid Production in Medicinal
Plant, *Orthosiphon aristatus*

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DECLARATION

I declare that this thesis is the result of my own research except as cited references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Date: 20th September 2017

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ABSTRACT

Ultraviolet radiation (UV) is one of the abiotic stress which can activate the plant stress response through the self-protective secondary metabolism system. Rosmarinic acid is one of the prominent secondary metabolites found in the medicinal plant, *Orthosiphon aristatus* which exhibits many medicinal properties. Application of UV light towards the plants especially medicinal plants can be an alternative method to induce its beneficial secondary metabolites. Here, this study investigates the differential effects of UVB and UVC treatments on the rosmarinic acid accumulation and its related gene expression in *O. aristatus* leaves. Plants were exposed under UVB and UVC light respectively for one hour. The leaves samples were collected every 15 minutes after UV treatments and the succeeding one hour dark recovery process. The concentration of rosmarinic acid determined by high performance liquid chromatography increased slightly at the initial 30 minutes of UVB treatment until the highest concentration found after 45 minutes and declined after that. Whereas, rosmarinic acid produced at lower levels in UVC treatment. However, the rosmarinic acid content increased after one hour recovery period in both treatments. The expression of hydroxyphenylpyruvate reductase (HPPR) gene measured by quantitative real time polymerase chain reaction was down-regulated upon exposure to UVB and UVC. Negative correlation was found between the expression of HPPR gene and rosmarinic acid production after both treatments. No significant different was observed between the effect of UVB and UVC treatment towards the plants. As a conclusion, the correlation findings are contrast with some others findings which can be explained by the complexity of rosmarinic acid biosynthesis pathway and different UV sensitivity in different plant species. This study could serve as starting point for future research study on the UV stress tolerance response in *O. aristatus* plants. Further studies on other related

genes involved in rosmarinic acid biosynthesis pathways against UV will provide better understanding at both molecular and metabolite levels.

Keywords: *Orthosiphon aristatus*, rosmarinic acid, UVB, UVC, hydroxyphenylpyruvate reductase

Kajian Kesan Radiasi UVB dan UVC Terhadap Ekspresi Gen Hydroxyphenylpyruvate Reductase dan Penghasilan Asid Rosmarinik daripada Tumbuhan Ubatan, Orthosiphon aristatus

ABSTRAK

Radiasi ultraungu (UV) merupakan salah satu tekanan abiotik yang boleh mengaktifkan tindak balas tekanan tumbuhan melalui sistem metabolisme sekunder pelindungan diri. Asid rosmarinik adalah salah satu metabolit sekunder yang penting di dalam tumbuhan ubatan, Orthosiphon aristatus yang memperolehi banyak sifat perubatan. Kegunaan UV terhadap tumbuh-tumbuhan terutamanya tumbuhan ubatan boleh menjadi kaedah alternatif untuk meningkatkan metabolit sekunder yang bermanfaat di dalam tumbuhan. Kesan pembezaan rawatan UVB dan UVC terhadap penghasilan asid rosmarinik dan ekspresi gen yang berkaitan di daun O. aristatus telah diselidik. Tumbuhan terdedah di bawah cahaya UVB dan UVC berasingan selama satu jam. Sampel daun dikumpulkan setiap 15 minit selepas rawatan UV dan selepas tempoh pemulihan gelap. Kandungan asid rosmarinik yang ditentukan oleh kromatografi cecair berprestasi tinggi menunjukkan sedikit peningkatan pada awal 30 minit rawatan UVB sehingga kandungan tertinggi ditunjukkan selepas 45 minit dan menurun selepas itu. Sedangkan, asid rosmarinik dihasilkan oleh rawatan UVC adalah lebih rendah. Walau bagaimanapun, kandungan asid rosmarinik meningkat pada kedua-dua rawatan selepas tempoh pemulihan gelap. Ekspresi gen hydroxyphenylpyruvate reductase (HPPR) yang diukur oleh kuantitatif PCR masa sebenar diturunkan selepas pendedahan cahaya UVB dan UVC. Hubungan negatif telah didapati antara ekspresi gen HPPR dan penghasilan asid rosmarinik selepas kedua-dua rawatan. Perbezaan yang signifikan tidak ditentukan antara kesan rawatan UVB dan UVC terhadap tumbuhan. Kesimpulannya, hasil negatif korelasi berbeza dengan penyelidikan lain dan ini dijelaskan oleh kerumitan laluan biosintesis asid rosmarinik dan kepekaan UV berbeza dalam spesies tumbuhan yang

berlainan. Kajian ini boleh menjadi titik permulaan untuk kajian penyelidikan masa depan mengenai tindak balas toleransi tekanan UV terhadap tumbuhan O. aristatus. Kajian lanjut mengenai gen lain yang berkaitan dalam laluan biosintesis asid rosmarinik terhadap UV akan memberikan pemahaman yang lebih teliti pada kedua-dua tahap molekul dan metabolit.

Kata kunci: Orthosiphon aristatus, asid rosmarinik, UVB, UVC, hydroxyphenylpyruvate reductase

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iii
ABSTRAK	iv
TABLE OF CONTENTS	v
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
CHAPTER 1: INTRODUCTION	1
1.1 Research Background	1
1.2 Research Objectives	4
CHAPTER 2: LITERATURE REVIEW	5
2.1 Ultraviolet Light	5
2.1.1 Ultraviolet-A (UVA) light	6
2.1.1.1 Impact of UVA light on secondary metabolites synthesis in plant	6
2.1.2 Ultraviolet-B (UVB) light	7
2.1.2.1 Impact of UVB light on secondary metabolites synthesis in plant	7
2.1.3 Ultraviolet-C (UVC) light	9
2.1.3.1 Impact of UVC light on secondary metabolites synthesis in plant	9
2.2 Orthosiphon aristatus	11
2.2.1 Introduction to O. aristatus	11
2.2.1.1 Physical Characteristics	12

2.2.2 Phytochemicals isolated from <i>O. aristatus</i>	13
2.2.3 Pharmacological and biological properties	14
2.3 Hydroxyphenylpyruvate reductase (HPPR)	15
2.3.1 Expression studies of Hydroxyphenylpyruvate reductase (HPPR)	16
2.4 Rosmarinic acid (RA)	17
2.4.1 General properties of rosmarinic acid	18
2.4.1.1 Physical properties	18
2.4.1.2 Chemical properties	19
2.4.2 Biosynthesis of rosmarinic acid	19
2.4.3 Pharmacological and biological activities	22
2.4.3.1 Antioxidant	22
2.4.3.2 Anti-inflammatory	22
2.4.3.3 Anticancer	23
2.4.3.4 Antimicrobial	23
2.4.3.5 Antiviral	23
2.4.3.6 Neurodegenerative disease	24
2.4.4 Increasing of rosmarinic acid production	24
2.4.5 Extraction and detection methods of rosmarinic acid	25
CHAPTER 3: METHODOLOGY	26
3.1 Overall methodology	26
3.2 Plant materials and UV light treatments	27
3.2.1 UV Treatment	27
3.2.2 Sample preparation	27
3.3 Gene expression analysis	28

3.3.1 Total ribonucleic Acid (RNA) Extraction	28
3.3.2 RNA purity and integrity	29
3.3.2.1 RNA visualization	29
3.3.2.2 RNA quantification	30
3.3.3 RNA purification	30
3.3.4 Two-step real-time RT-PCR	30
3.3.5 First strand cDNA synthesis	30
3.3.6 Primers design	31
3.3.7 Polymerase Chain Reaction (PCR)	32
3.3.8 Relative quantification	33
3.3.8.1 Quantitative Real-time PCR	33
3.4 Rosmarinic acid analysis	34
3.4.1 Sample preparation for HPLC	34
3.4.2 Chromatographic analysis	34
3.4.3 Standard solution preparation	35
3.4.4 Method validation	35
3.4.5 Statistical analysis	36
CHAPTER 4: RESULTS	37
4.1 Effect of UVB and UVC treatment on plant physical change	37
4.2 Molecular analysis	37
4.2.1 RNA extraction	37
4.2.2 Gradient PCR	39
4.2.3 Reverse transcription polymerase chain reaction (RT-PCR)	42
4.2.4 Real time RT-PCR	44

4.3 HPPR gene expression	44
4.3.1 HPPR gene expression after UVB and UVC treatment	44
4.3.2 Comparison between UVB and UVC treatments	46
4.4 Rosmarinic acid production	46
4.4.1 Determination of rosmarinic acid	46
4.4.2 Method validation	49
4.4.3 Rosmarinic acid accumulation after UVB treatment	50
4.4.4 Rosmarinic acid accumulation after UVC treatment	51
4.4.5 Comparison between UVB and UVC	52
4.5 Correlation between HPPR gene expression and level of rosmarinic acid	52
CHAPTER 5: DISCUSSION	54
5.1 Plant physical change after UV treatment	54
5.2 Two-step real-time RT-PCR	55
5.2.1 HPPR gene expression after UVB and UVC treatments	58
5.3 Rosmarinic acid production	60
5.3.1 Extraction of rosmarinic acid	60
5.3.2 Rosmarinic acid content after UVB and UVC treatment	61
5.4 Correlation between HPPR gene expression and rosmarinic acid accumulation	63
CHAPTER 6: CONCLUSIONS	66
REFERENCES	68
APPENDICES	86

LIST OF TABLES

		Page
Table 1	Effect of UVB radiation exposure on secondary metabolites biosynthesis in some plants species.	8
Table 2	Effect of UVC radiation exposure on secondary metabolites biosynthesis in plant cell cultures and some plants species.	10
Table 3	Scientific Classification of <i>O. aristatus</i> plant, information obtained from National Plant Germplasm System (1998).	12
Table 4	Primers designed for gene analysis	31
Table 5	Optimal PCR condition	32
Table 6	Two step thermal cycling condition for quantitative real-time PCR	33
Table 7	Chromatographic condition of rosmarinic analysis	35

LIST OF FIGURES

	Page
Figure 1 White flower blossom of <i>Orthosiphon aristatus</i> plant with stamens/cat's whisker extended outwards from the flower stalk.	12
Figure 2 <i>Orthosiphon aristatus</i> plant with reddish purple colour petiole, leaves arranged in opposite pairs and quadrangle stem (Wallich, 2013).	12
Figure 3 Chemical structure of rosmarinic acid from Petersen & Simmonds (2003).	18
Figure 4 Rosmarinic acid biosynthesis pathway from <i>Coleus blumei</i> suspension culture (Petersen & Simmonds, 2003).	21
Figure 5 Overall methodology	26
Figure 6 <i>O. aristatus</i> plant before UVC treatment	37
Figure 7 <i>O. aristatus</i> plant after UVC treatment	37
Figure 8 Agarose gel electrophoresis of total RNA from <i>O. aristatus</i> leaves after different time point of UVB treatment, 0 min as control (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5) and 1 hr dark recovery (lane 6).	39
Figure 9 Agarose gel electrophoresis of total RNA from <i>O. aristatus</i> leaves after different time point of UVC treatment, 0 min as control (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5) and 1 hr dark recovery (lane 6).	39
Figure 10 Agarose gel electrophoresis of gradient amplification of <i>O. aristatus</i> HPPR gene using HPPR2 primers in temperature range of 48 °C, 50 °C, 51.7 °C, 56 °C, 57.8 °C, 59.7 °C and 61.8 °C. Lane M denotes the	41

50 bp DNA ladder (Fermentas) whereas lanes 1 to 7 denote the plant sample and lane 8 denotes the negative control.

- Figure 11 Agarose gel electrophoresis of gradient amplification of *O. aristatus* 18S rRNA gene using 18S rRNA2 primers in temperature range of 48 °C, 50 °C, 51.7 °C, 56 °C, 57.8 °C, 59.7 °C and 61.8 °C. Lane M denotes the 50 bp DNA ladder (Fermentas) whereas lanes 2 to 8 denote the plant sample and lane 1 denotes the negative control. 41
- Figure 12 Agarose gel electrophoresis of gradient amplification of *O. aristatus* matK gene using matK2 primers in temperature range of 48 °C, 50 °C, 51.7 °C, 56 °C, 57.8 °C, 59.7 °C and 61.8 °C. Lane M denotes the 50 bp DNA ladder (Fermentas) whereas lanes 2 to 8 denote the plant sample and lane 1 denotes the negative control. 41
- Figure 13 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene of interest, HPPR gene and housekeeping genes, 18SrRNA gene and matK gene. 42
- Figure 14 BLAST alignment of *O. aristatus* HPPR gene (gene of interest) between sequencing product and nucleotide sequence, HPPR gene from *Orthosiphon aristatus* (Ahmad, 2015). Alignment identities are found to be 99% (204/205) and E-value of 2e-107. 43
- Figure 15 BLAST alignment of *O. aristatus* 18S rRNA gene (housekeeping gene) between sequencing product and nucleotide sequence, 18S rRNA gene from *Orthosiphon aristatus* (NCBI database). Alignment identities are found to be 99% (196/197) and E-value of 3e-103. 43

Figure 16	BLAST alignment of <i>O. aristatus</i> matK gene (housekeeping gene) between sequencing product and nucleotide sequence of truncated matK gene from <i>Orthospion stamineus</i> (NCBI database). Alignment identities are found to be 98% (235/239) and E-value of 7e-121.	43
Figure 17	The relative expression of HPPR gene in <i>O. aristatus</i> plant after short-term UVB treatment. The bar graph showing the log-10 (RQ) value of HPPR gene measured after different time points of UVB treatment and compare with control without UVB treatment as calibrator. The 15 min, 30 min, 45 min and 60 min represent the time point of UVB treatment, whereas 1 hr dark represents the 1 hr dark recovery after 60 min UVB exposure.	45
Figure 18	The relative expression of HPPR gene in <i>O. aristatus</i> plant after short-term UVC treatment. The bar graph showing the log-10 (RQ) value of HPPR gene measured after different time points of UVC treatment and compare with control without UVC treatment as calibrator. The 15 min, 30 min, 45 min and 60 min represent the time point of UVC treatment, whereas 1 hr dark represents the 1 hr dark recovery after 60 min UVC exposure.	45
Figure 19	The chromatogram of rosmarinic acid standard (20ppm) generated from Agilent ChemStation software	47
Figure 20	The chromatogram of rosmarinic acid identified in <i>O. aristatus</i> leaves before UVB treatment generated from Agilent ChemStation software	47
Figure 21	HPLC chromatogram (330 nm) of 10 µg/mL rosmarinic acid standard	48

Figure 22	HPLC chromatogram (at wavelength 330 nm) of rosmarinic acid identified in <i>O. aristatus</i> leaves after UV treatment and the concentration determined was 2.60 µg/mL.	48
Figure 23	Calibration curve of rosmarinic acid standard with R squared value of 0.997 and graph equation of $y=64.57x$.	49
Figure 24	Blank chromatogram with no sample added	49
Figure 25	The variation of rosmarinic acid production profiles response to short-term UV-B treatment. 15 min, 30 min, 45 min and 60 min represent the time point of UV-B treatment, whereas 1hr dark represents the 1 hr dark recovery after 60 min UVB exposure. The data represent the average of three independent biological replicates and comparison with control respectively. The vertical bars indicate the standard errors. The statistical analysis of differences between control and each time points of treatment were performed by 2-tailed T test, $P<0.05$ is indicated with asterix (*).	50
Figure 26	The variation of rosmarinic acid production profiles response to short-term UVC treatment. 15 min, 30 min, 45 min and 60 min represent the time point of UVC treatment, whereas 1hr dark represents the 1 hr dark recovery after 60 min UVC exposure. The data represent the average of three independent biological replicates and comparison with control respectively. The vertical bars indicate the standard errors. The statistical analysis of differences between control and each time points of treatment were performed by 2-tailed T test, $P<0.05$ is indicated with asterix (*).	51

- Figure 27 Correlation between the RA production and HPPR gene expression 53
- after UVB treatment. The average RA concentration denotes the average RA concentration of three dependent replicates, whereas the RQ value denotes the relative expression value of HPPR gene after UVB treatment. 15 min, 30 min, 45 min and 60 min denote the time point of UVB treatment, whereas 1 hr dark denotes the 1 hr dark recovery after 60 min UVB exposure.
- Figure 28 Correlation between the RA production and HPPR gene expression 53
- after UVC treatment. The average RA concentration denotes the average RA concentration of three dependent replicates, whereas the RQ value denotes the relative expression value of HPPR gene after UVC treatment. 15 min, 30 min, 45 min and 60 min denote the time point of UVC treatment, whereas 1hr dark denotes the 1 hr dark recovery after 60 min UVC exposure.

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary DNA
cds	coding DNA sequence
Chl:Iaa	chloroform:isoamylalcohol
CHPL	4-coumaroyl-4'-hydroxyphenyllactic acid
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DEPC	diethylpyrocarbonate
EDTA	ethylenediaminetetraacetic acid
g	gram
g/kg	gram per kilogram
g/L	gram per liter
g/cu cm	gram per cubic centimeter
HPPR	Hydroxyphenylpyruvate reductase
HPLC	High performance liquid chromatography
μL/mL	Microgram per microliter
μM	micromolar
M	molar
mM	milimolar
matK	Maturase K
MW	Molecular weight

MoHE	Ministry of Higher Education
NAD	Nicotinamide adenine dinucleotide
NAD(P)H	reduced form of nicotinamide adenine dinucleotide phosphate
pKa	acid dissociation constant
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
PTFE	Polytetrafluoroethylene
RT-PCR	Reverse transcription polymerase chain reaction
RP-HPLC	Reversed phase high performance liquid chromatography
rpm	Revolutions per minute
RA	Rosmarinic acid
RAS	Rosmarinic acid synthase
RNA	ribonucleic acid
RNAi	RNA interference
18S rRNA	18S ribosomal RNA
RQ	Relative quantification
TAE	Tris-acetate
UDG	Uracil DNA Glycosylase
UV	Ultraviolet light
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C

CHAPTER 1

INTRODUCTION

1.1 Research background

It is now widely known that the depletion of stratospheric ozone layer, increase in atmospheric greenhouse gases and solar ultraviolet light penetration, are part of the global climate change effects. The attention regarding global climate change has led to the growing attention in application of fundamental scientific knowledge to better utilize the environmental cues to achieve desired endpoint in crop production. A wide range of environmental stresses are potentially harmful to plants such as high or low temperature, drought, salinity, UV and pathogen infection (Ramakrishna & Ravishankar, 2011). In the past, ultraviolet (UV) radiation is generally recognized to cause negative impacts on global plant productivity (Wargent & Jordan, 2013). However, interesting phenomena are discovered when studying the effect of UV towards some plants species. For instance, lemon balm plant responds positively to UVB radiation by increasing the production of phenylpropanoid related metabolites (Kim et al., 2012) and grapevine plant respond to UVC by increasing the production of phytoalexins (Lesniewska, Adrian, Klinguer, & Pugin, 2004). The plant responses towards UV radiation may be further exploited for sustainable contribution to the strengthening of global crop production, especially in medicinal plants such as modifications in secondary metabolism, enhanced photo-protection, up-regulation of anti-oxidative responses, delayed development of decay and modification resistance to pest and disease attack (Wargent & Jordan, 2013).

Despite of advancement in synthetic chemistry, plants are still a crucial source of pharmaceuticals and other compounds of economic importance (Zhang & Bjorn, 2009). In recent years, researchers and consumers have been concerned with the health and nutritional aspects of metabolites such as phenolics, minerals, vitamins and others. It is estimated that more

than 80% of world population in developing countries are depending primarily on herbal medicines as their basic healthcare needs (WHO, 2002; Vine, 2004). The medicinal value and therapeutic effects of these plants are attributed by their bioactive secondary metabolites. In Malaysia, herbal medicines have received great attention as an alternative natural medicines or health supplements (Yam et al., 2013).

Orthosiphon aristatus, locally known as misai kucing, is one of the Malaysia local herbs that serve as a potent source of novel herbal medicine against a few critical human diseases. *O. aristatus* can be found in South East Asia countries, particularly Malaysia, Thailand and Indonesia, as it is a tropical plant. It is commonly consumed by native in the form of herbal tea to treat ailments especially kidney or bladder related disease. More than twenty phenolic compounds have been isolated from this plant and exhibited high anti-oxidant properties which supported the traditional use of *O. aristatus* plant as potential medicine and lead the plant to be important in research (Sumaryono et al., 1991). Extensive researches have studied on the pharmacological and biological properties of *O. aristatus* plant since the early 90s until lately, such as diuretic effects (Doan et al., 1992; Arafat et al., 2008; Gao et al., 2015), anti-diabetic (Sriplang et al., 2007), gastro-protective agent (Yam et al., 2009), hepatoprotective effect (Yam, Basir, Asmawi, & Ismail, 2007; Alshawsh, Abdullah, Ismail, & Amin, 2011), antimicrobial (Ho, Noryati, Sulaiman, & Rosma, 2010; Kong, Tan, & Nathan, 2014), anti-inflammatory as well as analgesic agents (Yam, Asmawi, & Basir, 2008). The pharmaceutical properties of *O. aristatus* are the main reason for many researches in order to find and develop novel medicines from the plants.

Rosmarinic acid is the prominent compound found in *O. aristatus* plant which exhibits pharmacological properties. It is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid commonly found in species belonging to the plant families of Boraginaceae and Lamiaceae

(Petersen & Simmonds, 2003) . Besides that, rosmarinic acid has high medicinal value due to its biological activities and pharmacological properties such as antioxidant, antimicrobial, anticancer, antiviral, antibacterial, anti-inflammatory and antiphlogistic effects which make it a valuable compound in the food, pharmaceutical and cosmetics industries (Ogata, Tsuruga, Matsuno, & Mizukami, 2004; Hakkim et al., 2011; Popov et al., 2013; Han et al., 2015).

In addition, rosmarinic acid can be chemically synthesized or produced by fermentation of suspension cultures. Instead of large scale production, these methods will also increase the treatment cost due to the production of large quantities of waste streams such as solvents in the extraction or highly concentrated salts solutions for regeneration of ion-exchange resins in absorption (Wilson et al., 2001). Because of the beneficial effects of rosmarinic acid, the present study was conducted to investigate the accumulation of rosmarinic acid through the plant self-protective system against short term UV radiation exposure which has a low environmental impact and operating cost.

Rosmarinic acid biosynthesis have been widely investigated and characterized in the past by Maïke Petersen and his team (Petersen, 1999; Petersen & Simmonds, 2003; Petersen et al., 2009; Petersen, 2013). Hydroxyphenylpyruvate reductase (HPPR) is the key enzyme involved in the rosmarinic acid biosynthesis by reducing the hydroxyphenylpyruvate to hydroxyphenyllactates in dependence of NAD(P)H (Kim, Janiak, & Petersen, 2004). Nevertheless, until now, the regulatory role of enzymes involved in rosmarinic acid biosynthesis is not clear. Understanding of gene regulatory and metabolic pathway underlying the biosynthesis of active plant compounds will allow us to further improve the production of desired compounds in future genetic engineering work. Hence, the correlation between HPPR enzyme and rosmarinic acid biosynthesis, the HPPR gene expression and rosmarinic acid accumulation after short term UVB and UVC radiation exposure were investigated in this study.

1.2 Research objectives

The objectives of present study are:

- (i) To quantitatively determine the *O. aristatus* HPPR gene expression in response to short term UVB and UVC radiation in *O. aristatus*
- (ii) To quantitatively determine the rosmarinic acid production in *O. aristatus* in response to short term UVB and UVC radiation
- (iii) To investigate the correlation between the rosmarinic acid production and HPPR gene expression in response to short term UVB and UVC radiation in *O. aristatus*

CHAPTER 2

LITERATURE REVIEW

2.1 Ultraviolet light

Ultraviolet light (UV) is part of solar radiation which penetrates through the ozone layer and reaches the earth surfaces without significant biological impacts on living organisms due to the absorption of UV light by the ozone layer. However, high level and prolonged exposure of UV light causes negative impacts towards living organism such as skin cancer, mutagen or death of plants.

Ultraviolet light is classified into three types: UVA, UVB and UVC. These three types of UV light are differentiated based on their wavelength. UVA has the longest wavelength between 315-400 nm, UVB wavelength is between 280-315 nm, whereas UVC has the shortest wavelength between 200-280 nm according to the International Commission on Illumination (Katerova, Todorova, Tasheva, & Sergiev, 2013). The impacts of increased UV radiation correspond to the wavelength energy, therefore, UVC light can cause injuries towards living organisms due to its short wavelength which has higher energy.

On the other hand, the growth and development of plants involve photosynthesis which requires water, carbon dioxide and sunlight. Sunlight is part of the electromagnetic radiation from sun which consists of infrared, ultraviolet and visible light (Katerova et al., 2013). Plant especially medicinal plant and herbs, are important source of pharmaceutical and economic importance on its compounds despite of the advancement in synthetic chemistry industries. Secondary metabolite are compounds present in plant but not essential for plant life (Katerova et al., 2013). Researches concerned on the production of secondary metabolites such as in genetic engineering, modification of planting condition and plant stress tolerance have been widely studied in order to improve and enhance the secondary metabolites accumulation in

plants (Wahid, Gelani, Ashraf, & Foolad, 2007; Kirakosyan, Cseke, & Kaufman, 2009; Ramakrishna & Ravishankar, 2011; Bitá & Gerats, 2013). Enhanced ultraviolet light is one of the abiotic stress factors which can affect the production of secondary metabolite (Thines et al., 2007; Pavarini, Pavarini, Niehues, & Lopes, 2012). Meanwhile, the depletion of stratospheric ozone layer has caused the increasing of ultraviolet radiations, especially UVB and UVC radiation to reach the Earth surface. The impacts of UV radiation towards living organisms are of concerned and researches focused on the UV effect are conducted (Arita et al., 2005; He et al., 2016). The impacts of UV light can provide both positive and negative effects towards plants in term of physically or biologically (Li, He, & Zu, 2006; Zu et al., 2010). Most of the past researches studied on the effects of UV radiation on plant metabolism and gene expression were conducted in greenhouse, growth chamber or laboratory conditions (Zuet al., 2004; Kara, 2013; Simioni et al., 2014).

2.1.1 Ultraviolet-A (UVA) light

UVA light is less damaging as compared to UVB and UVC light. In cosmetic industry, UVA lamp is used in sun-tanning device to produce cosmetic tan effect. Solar UVA was found to be almost completely apasses through the atmosphere without being absorbed by the ozone layer due to its long wavelength (Morales et al., 2010). As a result, UVA is the major solar UV spectrum which plants are exposed to.

2.1.1.1 Impact of UVA light on secondary metabolites synthesis in plant

UVA penetrates deeper into the leaves and produce active oxygen species as compare to others UV light (Wilson, Thompson, Huner, & Greenberg, 2001). Besides that, UVA also helps to ameliorate the UVB effect through the activation of DNA repair mechanism by photolyase

enzyme (Ibdah, Krins, Seidlitz, Heller, & Strack, 2002). It has been reported that UVA linearly increased the production of phenolic compounds in *Bentula pendula* leaves and up-regulated the related gene expression (Morales et al., 2010). UVA is also found to be important for the epidermal flavonoids formations (Ibdah et al., 2002).

2.1.2 Ultraviolet-B (UVB) light

In general, UVB radiation causes negative impact to biological life. UVB radiation can negatively alter the plant physiological processes, productivity and growth (Kumari and Prasad, 2013). However, in the past researches conducted, it has discovered that UVB radiation above ambient level exert beneficial effects by stimulating the accumulation of secondary metabolites in medicinal plants. UVB radiations are able to activate the self-protective secondary metabolism system (Gu et al., 2010). Secondary metabolites such as flavonoids, alkaloids and lignin produced are compounds that provide protection from UV radiation (Gu et al., 2010). These compounds have UVB absorption bands which protects the genetic material of plants (Gu et al., 2010; Morales et al., 2010).

2.1.2.1 Impact of UVB light on secondary metabolites synthesis in plant

High doses of UVB radiation affect the growth and development of plant, photosynthesis, other processes in plant and eventually causes cell death (Alexieva et al., 2001; Katerova et al., 2013). However, low dose of UVB or UVC radiation can help to trigger acclimation responses in plants, and the activation of enzymatics and non-enzymatics defense systems (Katerova et al., 2013). The defense or stress tolerance to UVB radiation are related to the stimulation of different signal transduction pathways, production of secondary metabolites and DNA repair mechanisms (Mackerness, 2000; Brown et al., 2005). Summary review of the effect of UVB radiation on plants secondary production, especially medicinal plants are presented in Table 1.

Table 1: Effect of UVB radiation exposure on secondary metabolites biosynthesis in some plants species.

Plant(s)	Secondary metabolites	Effects of UVB radiation	References
Arabidopsis thaliana leaves (Rosette)	Glucosinolate	Production of glucosinolate increase at first 1 hr and declined after continous 12 hrs of enhanced UVB exposure	(Wang et al., 2011)
Rosmarinus officinalis (Rosemary plants)	Rosmarinic acid	Higher yield of rosmarinic acid as compared to plant without UVB treated	(Luis et al., 2007)
Catharanthus roseus L. (Madagascar periwinkle)	Catharanthine; vindoline	Three-fold increase in cantharathine, 12-fold increase in vindoline after 5 min UVB treatment	(Ramani & Jayabaskaran, 2008)
Tropaeolum majus (monks cress)	Glucotropaeolin	Production increase up to 6 fold after UVB treatment	(Schreiner et al., 2009)
Ginkgo biloba.L (gingko)	Quercetin; Kaempferol; isorhamnetin; glycosides	Increase up to 57.2% after UVB induction	(Sun et al., 2010)
Catharanthus roseus L (Madagascar periwinkle)	lochnericine ; serpentine; ajmalicine; rhammericine	Decrease in rhammericine; Significant increase of others after 20 min of UVB treatment	(Binder et al., 2009)
Taxus chinensis L (Chinese yew)	Taxol	Taxol content increased after UVB exposure	(Zu et al., 2010)
Melissa officinalis (Lemon blam)	Phenylpropanoid related metabolites: shikimic acid; quinic acid and phenylalanine	Phenylpropanoid related metabolites significantly increase after 2 hrs of UVB radiation exposure as compared to control	(Kim et al., 2012)
Asparagus officinalis L (white asparagus)	Flavonol quercetin-4-O-monoglucoside	Increase after UVB treatment; increase of enzyme activity (phenylalaninse ammonia-lyase and peroxidase)	(Eichholz et al., 2012)
Trifolium repens (White clover)	Kaempferol	Increased in concentration of flavonol glycosides of kaempferol after UVB radiation	(Hofmann et al., 2003)

Table 1 continued

Betula pendula (silver birch)	Phenolic acids; condensed tannin; flavonoids such as Quercitrin; Hyperin; kaempferol-3- rhamnoside; myricetin-3- galactoside	No increase in phenolic acid and condensed tannin; But significantly increased of flavonoids which are important UVB protectors	(de la Rosa et al., 2001)
Glycyrrhiza uralensis (Chinese liquorice)	Glycyrrhizin	1.5-fold increase after high intensity and long term low intensity of UVB radiation exposure	(Afreen et al., 2005)

2.1.3 Ultraviolet-C (UVC) light

Application of UVC light in low doses has been commercially applied in sterilization against pathogens in hospitals or laboratories and food industries, to delay senescence for longer fruit storage (Shama, 2009; Rohanie & Ayoub, 2012; Civello, Villarreal, Lobato, & Martínez, 2014). High dosage of UVC light exposure is considered to be extremely harmful to living organism which will cause cell damage and eventual cell death (Nawkar et al., 2013). In the study of morphological and physiological effects of UVC radiation exposure on bean plant, *Phaseolus vulgaris* L, Kara found that the seedling height, total chlorophyll, chlorophyll a and b were reduced after UVC radiation exposure for 14 days thus concluding that enhanced UVC radiation adversely affect the growth and photosynthesis in bean plant (Kara, 2013).

2.1.3.1 Impact of UVC light on secondary metabolites synthesis in plant

Despite the use of high fluency UVC rate, a relatively low UVC dose will also induce the production of secondary metabolite (Katerova et al., 2013). A summary review of the effect of UVC radiation on plants secondary production in plant cell cultures and some plants are presented in Table 2.

Table 2: Effect of UVC radiation exposure on secondary metabolites biosynthesis in plant cell cultures and some plants species.

Plant(s)	Secondary metabolites	Effects of UVC radiation	References
Potato and buckwheat	Total polyphenols and phenolcarboxylic acids	The yield of secondary metabolites increased after UVC radiation exposure	(Orsák et al., 2001)
Durum wheat leaves	Carotenoids; Anthocyanins; flavonoids; proline content	Significantly increased after UVC treatment as compared to control	(Balouchi et al., 2009)
Vitis vinifera cell culture (Grapevine)	Phytoalexins	UVC radiation induce the production of phytoalexins	(Lesniewska et al., 2004)
Vitis vinifera, Cabernet Sauvignon variety (Grapevine)	Stilbene derivatives: Resveratrol; piceid; viniferins; pterostilbene	Whole range of stilbene derivatives are induced after 6 min of UVC treatment	(Schmidlin et al., 2008)
Brassica oleracea var. Italica (Broccoli florets)	4-methoxyglucobrassicin; 4-hydroxyglucobrassicin glucoraphanin	UVC tended to enhance the secondary metabolites under hermetic dose of UVC radiation exposure	(Nadeau et al., 2012)
Boswellia serrata Roxb, callus culture (Indian olibanum)	Derivatives of boswellic acid: Acetyl-11-keto-b-boswellic acid; b-boswellic acid	Acetyl-11-keto-b-boswellic acid production increased by 10-fold; b-boswellic acid increase by 7-fold after 5 min of UVC treatment	(Ghorpade et al., 2011)
Chlamydomonas nivalis cell culture (Snow alga)	Proline and phenolics	Proline increased by 3-fold after 2 days of UVC radiation exposure; Whereas, phenolics increased by 12-24% after 7 days of UVC radiation exposure	(Duval et al., 2000)
Scenedesmus quadricauda (Chlorophyceae)	Phenols and Flavonoids	No significant increased after 1 hr of UVC treatment	(Kováčik et al., 2010)

2.2 Orthosiphon aristatus

Recently, herbal medicine has been concerned as alternatives medicines in Malaysia which are commercialized in form of dietary supplement. *Orthosiphon aristatus* is one of the Malaysia local herbs which serve as a potent source of novel herbal medicine for critical human diseases. *O. aristatus* can be found in tropical countries. Besides that, Sumaryono and his team had successfully isolated more than twenty phenolic compounds from this plant which exhibited high anti-oxidant properties and lead the plant to the concern of researches (Sumaryono et al., 1991). Several pharmacological properties of *O. aristatus* are further reviewed in this chapter. However, the genetic studies on *O. aristatus* are limited.

2.2.1 Introduction to *O. aristatus*

O. aristatus is herbaceous plant belonging to the family Lamiaceae which are widely grown in tropical area, especially in the South East Asia countries. The leaves and stem tips of the plant are usually dried and served in tea form. It is traditionally used to treat several human ailments. *O. aristatus* is a fast growing plant that is also used as ornamental plant and garden decoration purpose. The plant has several scientific synonyms and alternative common names. They are commonly known as Cat's whisker plant, Java Tea, Misai Kucing and Kumis Kucing. Meanwhile, other scientific names are such as *Orthosiphon stamineus*, *Orthosiphon spicatus* or *Orthosiphon blaetter*. The taxonomy of *O. aristatus* are shown in Table 3.

Table 3: Scientific Classification of *O. aristatus* plant, information obtained from National Plant Germplasm System (1998).

Scientific Classification	
Binomial name:	<i>Orthosiphon aristatus</i> (Blume) Miq.
Kingdom:	Plantae
Order:	Lamiales
Family:	Lamiaceae
Subfamily:	Nepetoideae
Tribe:	Ocimeae
Genus:	<i>Orthosiphon</i>
Species:	<i>O.aristatus</i>

2.2.1.1 Physical Characteristics

The plant grows about the height of 1.5 m with leaves arranged in opposite pairs (Basheer & Majid, 2010). The leaf apices are acuminate with an acute leaf base. The petiole is relatively short and in reddish purple colour as shown in Figure 2. Besides that, the stem is quadrangle, erect with profuse branching and in reddish colour. The stamens extended from the flower stalk in purple or white colors looking like cat's whiskers as shown in Figure 1, therefore, giving the name in Western countries or misai kucing in Malaysia.



Figure 1: White flower blossom of *Orthosiphon aristatus* plant with stamens/cat's whisker extended outwards from the flower stalk.



Figure 2: *Orthosiphon aristatus* plant with reddish purple colour petiole, leaves arranged in opposite pairs and quadrangle stem (Wallich, 2013).

2.2.2 Phytochemicals isolated from *O. aristatus*

Earlier studies reported that more than 20 phenolic compounds were isolated and identified from *O. aristatus* which included lipophilic flavones, flavonol glycosides and caffeic acid derivatives such as rosmarinic acid and 2,3-dicaffeoyltartaric acid, quantified through HPLC analysis (Sumaryono et al., 1991). The group found that the extraction of caffeic acid derivatives were higher when using hot water extract method which was comparable to herbal tea.

Phytochemicals such as staminolactones A, staminolactones B and norstaminol A, three highly oxygenated staminane-type diterpenes were isolated from *O. stamineus* and showed mild cytotoxic activities against colon cancer (Stampoulis et al., 1999). Methylripariochromene A was isolated from the plant and found to be a potential hypertension agent to lower blood pressure (Matsubara et al., 1999, Ohashi et al., 2000). Two novel migrated pimarane-type diterpenes, neoorthosiphols A and B were isolated from the plant in the year of 2000 (Ohashi et al., 2000). In the early 2001, another five novel highly oxygenated diterpenes were isolated from the aerial part of *O. stamineus* such as orthosiphols K, L, M, N and norstaminone A (Awale et al., 2001). In addition, four novel highly oxygenated isopimarane-type diterpenes, siphonols A-D were isolated and found to be strong nitric oxide inhibitors (Awale et al., 2003).

Other bioactive compounds such as pentacyclic triterpenes betulinic acid, oleanolic acid, urosolic acid and β -sitosterol were successfully isolated from the leaves extract of the plant (Tezuka et al., 2000). Qualitative HPLC method was developed to determine the major phytochemicals in the extract of *O. stamenius* such as sinensetin, eupatorin, 3-hydroxy-5,6,7,4-tetramethoxyflavone and rosmarinic acid and reconfirmed the antioxidative potency of *O. stamenius* (Akowuah et al., 2004).

2.2.3 Pharmacological and biological properties

Research studies on *O. aristatus* medicinal potentials are conducted since early 90s. The beneficial effects of the plant have received attention by researchers to improve or manufacture novel drugs which can treat human critical diseases or common diseases such as diabetes, hypertension and kidney stones.

In early 90s, *O. stamineus* and other Vietnamese herbal remedies were claimed to increase diuresis when examined under standardized condition in placebo controlled double-blind crossover model (Doan et al., 1992). Improvement in diuretic action is important for curing kidney stones. When the volume flowing through kidney increases, it will help to dissolve the stones and assist in flushing them out from body without further retention or deposition (Basheer & Majid, 2010). Hydroalcohol extract of *O. stamineus* has also found to increase the urination and urinary sodium excretion in rats (Beaux et al., 1999, Olah et al., 2003). Moreover, significant increase of urine volume and electrolytes excretion were significantly increase after treating the Sprague-Dawley rats with single oral dose of 0.5g/kg methanol: water (1:1) *O. stamineus* extract (Arafat et al., 2008). The diuretic and hypouricemic effects of *O. aristatus* extract has again led it as a remedy towards kidney stones. Thus, the findings above validate the traditional use of *O. aristatus* for kidney stone treatment.

Besides that, *O. stamineus* leaves extract also showed anti-hyperglycemic effect on streptozotocin induced diabetic rat which significantly reduced of blood glucose level on glucose-loaded fasting rats of 150 mg/kg body weight was observed (Mohamed et al., 2011). Anti-ulcerogenic activity was examined against ethanol-induced ulcers in rats administrated with *O. stamineus* methanolic extract and significant reduction on ulcer index was observed (Yam et al., 2009). Furthermore, study by Yam et al. (2007) reported that *O. stamineus* exhibited hepatoprotective effect in rats due to its high anti-oxidant and free radical scavenging

properties. Recent study by Yam et al. (2009) has reported another interesting property of *O. aristatus* which is fever-reducing potential of the plant extract. The methanol-water extract of plant significantly reduced the yeast-induced elevation in body temperature of tested rats within four hours treatment (Yam et al., 2009).

O. aristatus can be also served as remedy to cure pain and inflammation (Yam et al., 2008). In addition, *O. stamineus* leaf extracts was found to be a promising anti-infective agent against *Staphylococcus aureus* infection through modulating the immune response in the infected host (Kong et al., 2014). *O. stamineus* extract were tested against selected food-borne bacteria in vitro and found that 50% methanolic extract demonstrated inhibition against *Vibrio parahaemolyticus* (Ho et al., 2010).

The toxicity effect of *O. aristatus* has been concerned and widely studied in rodents. However, no significant toxic effects were observed from the research studies (Chin et al., 2008). In conclusion, *O. aristatus* is considered safe to be consumed under optimal consumption amount.

2.3 Hydroxyphenylpyruvate reductase (HPPR)

Hydroxyphenylpyruvate reductase (HPPR) is the key enzyme involved in the biosynthesis of rosmarinic acid by reducing the hydroxyphenylpyruvate to hydroxyphenyllactates in dependence of NAD(P)H. The detail role of HPPR enzyme in rosmarinic acid biosynthesis pathway is further discussed in Chapter 2.4. Due to the crucial role of HPPR enzyme in the biosynthesis of rosmarinic acid, studies on HPPR gene and correlation between genes to metabolite network are widely conducted in the past. However, the studies are limited to plant species such as *Coleus blumei*, *Orthosiphon aristatus*, *Salvia miltiorrhiza* and *Salvia officinalis*. The HPPR-cDNA from *Coleus blumei* was first isolated and heterologously expressed in *Escherichia coli* with open reading frame contained 939 bp, 313 amino acids residues (EMBL

accession number AJ507733) (Kim et al., 2004). According to Kim et al. (2004), HPPR belongs to the family of D-isomer-specific 2-hydroxyacid dehydrogenase.

Recently, a full-length cDNA for HPPR was isolated from *Orthosiphon aristatus*. The open reading frame of HPPR-cDNA from *Orthosiphon aristatus* contained 924 nucleotides encoding a protein of 307 amino acids residues (Ahmad, 2015). The deduced protein sequence was found to contain D-isomer specific 2-hydroxyacid dehydrogenases NAD-binding signature and a putative catalytic domain which also showed that the sequence belongs to D-isomer-specific 2-hydroxyacid dehydrogenases.

2.3.1 Expression studies of Hydroxyphenylpyruvate reductase (HPPR)

Through understanding of the gene regulatory and metabolic pathway underlying the biosynthesis of active plant compounds, will allow us to further improve the production of desired compounds. Via genetic engineering, gene of interest can be introduced via in vitro culture technique to enhance the accumulation of targeted secondary metabolites. The HPPR gene expression was first correlated with rosmarinic acid production in hairy roots of *S. miltiorrhiza* (Xiao et al., 2009). The real-time quantitative PCR findings from the research found that *Smhppd* was constitutively expressed in roots, stems and leaves of *S. miltiorrhiza*, with the high expression especially in roots. Xiao et al. (2011) then overexpressed and co-expressed the cinnamic acid 4-hydroxylase, tyrosine amino transferase and hydroxyphenylpyruvate dioxygenase genes in *S. miltiorrhiza* hairy root cultures and observed higher rosmarinic acid accumulation in the co-expression of tyrosine aminotransferase/HPPR (Xiao et al., 2011).

Besides that, HPPR was expressed in cell suspension cultures of *Salvia officinalis* showed an increase in rosmarinic acid yield (Barberini et al., 2013). In addition, the

involvement of HPPR in rosmarinic acid was confirmed by RNAi suppression and overexpression studies of HPPR and rosmarinic acid synthase (RAS) genes in hairy root cultures of *Coleus blumei* (Hucherig & Petersen, 2013). The study found that rosmarinic acid accumulation was strongly reduced during RNAi suppression of HPPR and RAS genes and rosmarinic acid level increased during the overexpression (Hucherig & Petersen, 2013). Besides that, the HPPR transcript level was equally varied with RAS during overexpression and RNAi suppression. This explained the key role and correlation of HPPR to the rosmarinic acid biosynthesis.

2.4 Rosmarinic acid (RA)

Rosmarinic acid is a common caffeic ester found in plants particularly in many aromatic herbs, for instance, rosemary, lemon balm, sage, lavender and others. Due to the high phenolic compound content, most of these herbs are used as folk remedies to maintain health.

Rosmarinic acid and other similar compound have been named as “Labiatergerbstoffe” even before the identification of rosmarinic acid chemical structure. The naming of RA is later named by two Italian chemists from the plant, *Rosmarinus officinalis*, which pure RA was first isolated (Petersen & Simmonds, 2003). The biosynthesis of RA has been well-defined in the past which involve L-phenylalanine and L-tyrosine pathways. The biogenetic studies was began by scientists, Ellis and Towers which they had discovered the incorporation of two aromatic amino acids precursors, phenylalanine and tyrosine in RA biosynthesis (Ellis & Towers, 1970). The chemical synthesis of rosmarinic acid was conducted in the early 90s and the enzyme involved in RA biosynthesis was actively investigated by Petersen (Petersen et al., 2009).

Rosmarinic acid (RA) is an important polyphenolic compound found in aromatic herbs with pharmacological properties. RA was first isolated and characterized in the late 1950s by

two Italian chemists from the herbal plant, Rosemary (Petersen & Simmonds, 2003). RA exhibits various biological activities and pharmacological properties such as antioxidant, antimicrobial, antiviral, antibacterial, antiinflammatory and antiphlogistic effects which make it a valuable compound for food, pharmaceutical and cosmetics industries (Ogata et al., 2004). These beneficial properties of RA have led to its research and the development of alternative methods to increase the production of RA.

2.4.1 General properties of rosmarinic acid

RA is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid (Petersen & Simmonds, 2003). The molecular formula of rosmarinic acid is $C_{18}H_{16}O_8$ with molecular weight of 360.3 g/mol (Zelić et al., 2005). The chemical structure of rosmarinic acid is showed in Figure 3. Derivatives of rosmarinic acid have been identified in plants such as lithospermic acid which is a conjugate of rosmarinic acid and caffeic acid; and lithospermic acid B, a dimer of rosmarinic acid (Petersen & Simmonds, 2003).

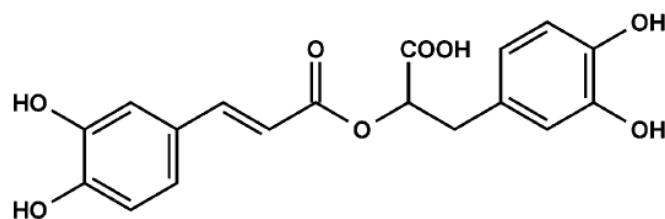


Figure 3: Chemical structure of rosmarinic acid from Petersen & Simmonds (2003).

2.4.1.1 Physical properties

Rosmarinic acid exist as cream crystalline solid at 25 °C and its melting point is between 171-175 °C (Zelić et al., 2005). According to U.S. National Library of Medicine (2013), the density of RA is 1.547 g/cu cm; its dissociation constants is $pK_a = 3.57$ (est); its vapor pressure is

1.1×10^{-13} mm Hg at 25 °C and the maximum UV absorption is at the wavelength of 221 nm, 291 nm and 332 nm.

2.4.1.2 Chemical properties

Based on U.S. National Library of Medicine (2013), solubility of rosmarinic acid is found to be soluble in ethanol, dimethyl sulfoxide or dimethyl formamide to approximately 25 mg/mL.

2.4.2 Biosynthesis of rosmarinic acid

The rosmarinic acid biosynthesis have been widely identified and characterized by Maïke Petersen and his team since 1990s. The biosynthesis of rosmarinic acid involves two pathways with amino acids, L-phenylalanine and L-tyrosine as the precursor. Caffeic acid in RA is formed from phenylalanine pathway, while 3,4-dihydroxyphenyllactic acid is from the tyrosine pathway (Ellis & Towers, 1970, Petersen & Simmonds, 2003, Ogata et al., 2004). The enzymes in RA biosynthesis pathways have been unraveled and studied in suspension cultures. The RA biosynthesis pathway, as determined by Peterson from *Coleus blumei*, as shown in Figure 4.

Phenylalanine pathway begins with the deamination of l-phenylalanine to t-cinnamic acid by phenylalanine ammonia-lyase (PAL), following by the hydroxylation of t-cinnamic acid and formed 4-coumaric acid by cytochrome P450 monooxygenase cinnamate 4-hydroxylase and finally form a coenzyme A thioester, 4-coumaroyl-CoA (Petersen & Simmonds, 2003). On the other hand, the second precursor is formed through tyrosine pathway.

Tyrosine pathway begins with the transamination of L-tyrosine with 2-oxoglutarate to 4-hydroxyphenylpyruvate and glutamate by pyridoxalphosphate-dependent transaminase, tyrosine aminotransferase (TAT). The 4-hydroxyphenylpyruvate is initially formed from tyrosine prior to the other reaction in tyrosine pathway as it is not an intermediate of aromatic

amino acid in plant biosynthesis (Petersen & Simmonds, 2003). Besides that, 4-hydroxyphenylpyruvate also acts as an important precursor for homogentisic acid production. For RA biosynthesis, 4-hydroxyphenylpyruvate is reduced to corresponding hydroxyphenyllactate by hydroxyphenylpyruvate reductase (HPPR) in dependence of NADPH. The resulting hydroxyphenyllactate is the only isomer accepted by rosmarinic acid synthase (RAS) (Petersen & Simmonds, 2003). The condensation of 4-coumaroyl-CoA and 4-hydroxyphenyllactic acid is catalyzed by RAS to form 4-coumaroyl-4'-hydroxyphenyllactic acid (CHPL) where it is then hydroxylated and catalyzed by two distinct cytochrome P450 and converted to RA (Ogata et al., 2004).

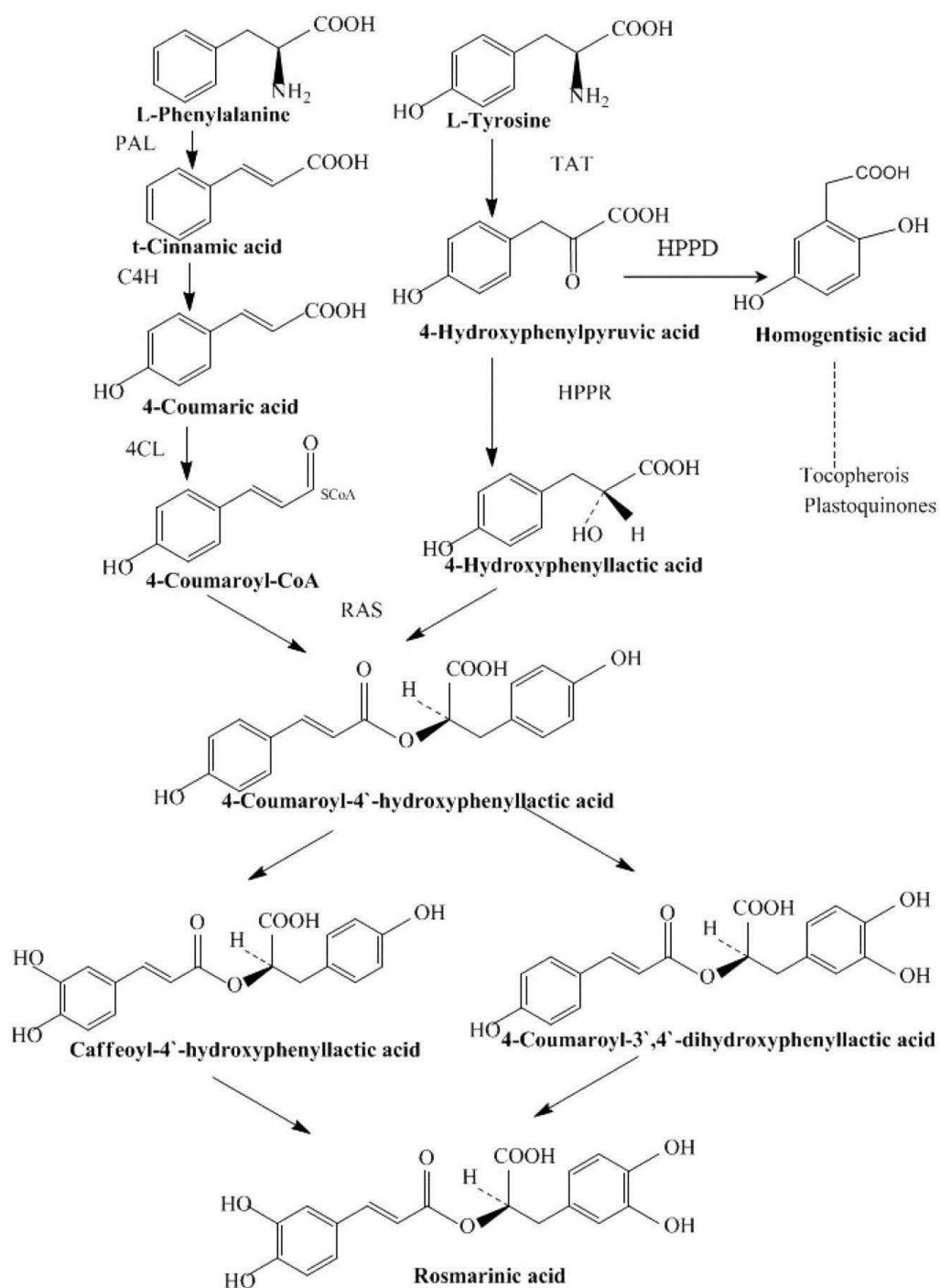


Figure 4: Rosmarinic acid biosynthesis pathway from *Coleus blumei* suspension culture (Petersen & Simmonds, 2003).

2.4.3 Pharmacological and biological activities

Researches in natural products have been increasing due to the chemo-preventative and chemo-therapeutic ability of secondary metabolites found in herbal plants. RA is one of the secondary metabolites which exhibit several pharmacological and biological properties such as antioxidant, anti-inflammatory, anticarcinogenic, angiogenesis, antimicrobial, antiviral properties and neurodegenerative disease (Iuvone, Filippis, Esposito, D'Amico, & Izzo, 2006).

2.4.3.1 Antioxidant

RA compound consist of four phenolic hydrogens which contribute to its ability to control free radical oxidation (Bhatt, Mishra, & Bansal, 2013). This was supported by Popov et al. (2013) that the high antioxidant activity of RA was due to the capability in neutralization of reactive oxygen species and the generation of luminal radicals. Besides that, RA showed significant preventive effect on sertolli cells apoptosis and increased antioxidant levels in rats after exposure to electromagnetic fields (Hajhosseini, Khaki, Merat, & Ainehchi, 2013).

2.4.3.2 Anti-inflammatory

RA is suggested to be useful compound in the pharmacological modulation of injuries associated to inflammation. Study by Rocha et al. (2015) showed that RA with dose of 25 mg/kg were able to reduced paw oedema in rat model at 6 hr by over 60% and significantly reduce multi-organ dysfunction markers by modulating NF-kappa B and metalloproteinase-9 in thermal injury model (Rocha et al., 2015). Besides that, significant anti-inflammatory effect of RA in carrageenan-induced paw edema and cotton pellet-induced granuloma formation in mouse models were determined which suggested the rational use of RA in treatments of pain and inflammatory disorders (Boonyarikpunchai, Sukrong, & Towiwat, 2014).

2.4.3.3 Anticancer

RA has been proposed to be a potential preventative agent against both cancer and nonmalignant mammary epithelial cells by antagonizing AP-1-dependent activation of cyclooxygenase-2 gene (COX-2) expression in colon cancer HT-29 cells and nonmalignant breast epithelial cell line (MCF 10A) (Scheckel, Denger, & Romagnolo, 2008). The expression of pro-inflammatory COX-2 gene is regarded as one of the risk factor in tumor development (Scheckel, Denger, & Romagnolo, 2008). RA have also been suggested as a potential therapeutic agent for suppressing the Warburg effect in gastric cancer cells through inflammatory pathway, which Warburg effect is a universal property of most cancer cells (Han et al., 2015).

2.4.3.4 Antimicrobial

Study of RA extract from eelgrass showed nematicidal and antibacterial effects against pine wood nematode and bacteria such as *Klebsiella* sp., *Stenotrophomonas maltophilia*, *Streptomyces* sp. and *Pantoea agglomerans* which suggested RA could be added in the preparation of pesticide against pine wilt disease (Wang et al., 2012).

2.4.3.5 Antiviral

RA shows antiviral effect in an experimental murine model infected with Japanese encephalitis virus (JEV) (Swarup, Ghosh, Ghosh, Saxena, & Basu, 2007). RA extract of *Salvia miltiorrhiza* showed potential antiviral activity against enterovirus 71 infections which EV71 IRES-mediated translation was suppressed at the concentrations of 30 µg/mL (Chung et al., 2015).

2.4.3.6 Neurodegenerative disease

Alzheimer's disease is a common disease found in elderly people. RA extracts of sage (*Salvia officinalis*) has found to induce neuroprotective effect on the study of Alzheimer amyloid- β peptide (A β)-induced toxicity in cultured rat pheochromocytoma (PC12) cells (Iuvone et al., 2006).

2.4.4 Increasing of rosmarinic acid production

Alternative methods have been developed to induce the production of rosmarinic acid. In vitro studies to enhance RA production on plant cell suspension cultures have been widely conducted such as cell suspension culture of *Coleus blumei*, *Orthosiphon aristatus*, *Salvia officinalis*, *Mentha* and others. Different types of inducers or condition are applied to increase the RA synthesis, chemically or biologically.

For instance, addition of growth regulators, such as 2,4 dichlorophenoxyacetic acid and kinetin, to *Ocimum sanctum* (L.) cell suspension cultures has been showed to increase the production of rosmarinic acid by two-fold compared to cell suspension without supplement (Hakim et al., 2011). Other research include the study of the accumulation of RA in *Litospermum erythrorhizon* cell cultures (Ogata et al., 2004) and in vitro sprout culture of *Orthosiphon aristatus* (Hunaefi & Smetanska, 2013) with the addition of elicitor, yeast extract or methyl jasmonate resulted in a drastic increase of RA production. Addition of amino acid precursor, tyrosine and phenylalanine also showed increases in the RA production without significant increase in biomass of *Mentha* in vitro RA enhancement analysis (Roy & Mukhopadhyay, 2012). Whereas, study by Hunaefi & Smetanska (2013) showed that the effect of *Orthosiphon aristatus* tea fermentation showed that longer tea fermentation reduced the rosmarinic acid content.

2.4.5 Extraction and Detection methods of rosmarinic acid

Analysis of rosmarinic acid has been widely preformed using reversed phase high performance liquid chromatography (RP-HPLC). The common detection wavelengths of this compound are 300 nm to 340 nm, depending on the detector used. Various solvents have been selected to extract rosmarinic acid from plant samples such as water, ethanol and methanol in varying concentration of 50% (v/v) to 70% (v/v).

Meanwhile, gradient chromatographic conditions are commonly used for rosmarinic acid compound separation. Binary solvent systems are found to be more efficient in polyphenolic compounds extraction due to their relative polarity (Dent et al., 2013). Study also showed that RA content was found to be 5-10% (w/w) in 70% ethanolic extracted at 60 °C and better separation in binary system, water-acetonitrile mixture (Lau et al., 2014). Solvents such as acetonitrile, methanol, and acetic acid in water or phosphoric acid in water are used for rosmarinic acid chromatographic analysis.

CHAPTER 3

METHODOLOGY

3.1 Overall Methodology

The summary of methodology was shown in Figure 5.

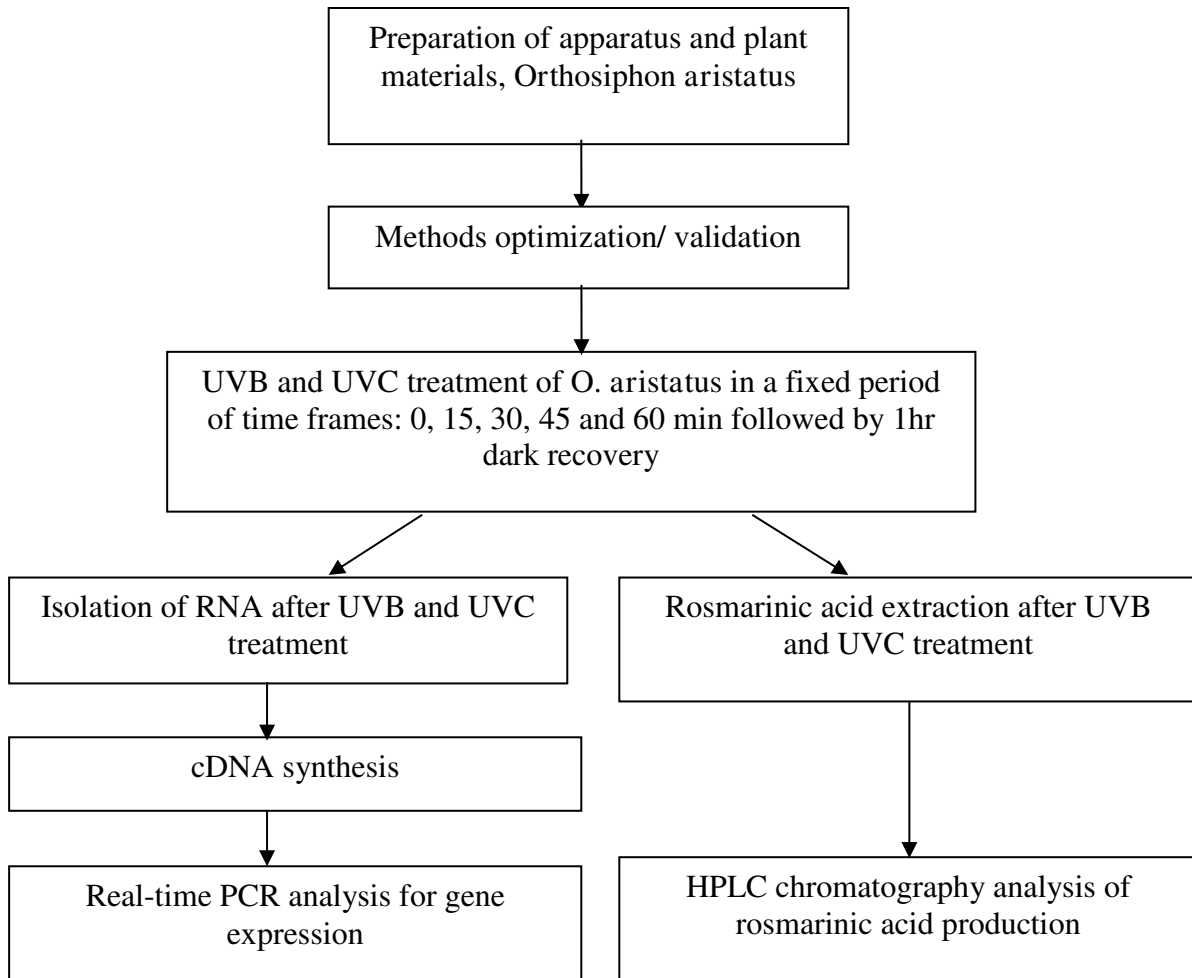


Figure 5: Overall methodology

3.2 Plant materials and UV light treatments

O. aristatus plants were grown from cuttings for a period of 20 weeks in order to obtain sufficient samples. *O. aristatus* plant was chosen as raw material because of its medicinal value and the ease for cultivation. The plant of 20 weeks old was pretreated in dark for a period of 24 hrs before treated with UVB and UVC light.

3.2.1 UV treatment

The 20 weeks old plant was transferred to the exposure chamber suspended with UVB lamp (Model: TL 20 W/01 RS, Philips, Holland) or UVC lamp (Model: TUV C30 W/G30 T8, Philips, Holland) with a distance of 20 cm above the plant for a fixed period of duration (Wang et al., 2011; Kara, 2013). The plant was treated with continuous UVB/UVC light exposure up to 1 hr. The samples were then collected at 0 min (control without UV treatment), 15 min, 30 min, 45 min, 60 min, and after 1 hr dark recovery. Triplicate treatments were conducted for each UV light exposure respectively. The UV dosage after each time frame was measured by using the commercial UV Fastcheck stirps (UV Process Supply, USA).

3.2.2 Sample preparation

A total of ten leaves were collected randomly at each time frame of UVB/UVC treatments. The samples were pre-sterilized with 70% ethanol, rinsed with distilled water and wiped dry with sterilized tissues. The leaves were then immediately frozen with liquid nitrogen and stored at -80°C for further analysis - freeze-dried for RNA isolation and rosmarinic acid extraction. The harvested leaves samples were ground into powder with mortar and pestle. The powder was divided into two sub-samples, one for gene expression analysis and another for HPLC analysis.

3.3 Gene expression analysis

In gene expression analysis, methods optimization was prior performed on total RNA extraction, cDNA synthesis, primer selection, real time qPCR and PCR condition. Total RNA were isolated from commercial kit following by DNase treatment and two-step real-time RT-PCR.

Meanwhile, the selected gene of interest in this study was hydroxyphenylpyruvate reductase gene which played an important role in rosmarinic acid biosynthesis. Two genes were selected as housekeeping genes which are 18S rRNA and maturase K genes. Optimal primers and housekeeping genes were selected for further analysis.

3.3.1 Total ribonucleic Acid (RNA) Extraction

All solutions and reagents were prepared using diethylpyrocarbonate (DEPC)-treated water. All glassware was treated in 0.1% DEPC water overnight and autoclaved on the next day prior to use. Mortar and pestle were kept in a fridge before they were used.

The total RNA was isolated from *O. aristatus* using the plant total RNA mini kit (Geneaid, Taiwan) according to the manufacturer's protocol. The extraction process involves five steps: 1) pre-step of plant tissue dissociation, 2) cell lysis, 3) RNA binding to the specific column, 4) washing of contaminants and 5) RNA elution.

At the initial step, the powdered UV-treated leaves of 0.10 g were transferred to a 1.5 mL microcentrifuge tube. A total of 500 μ L PRB Buffer and 5 μ L of beta-mercaptoethanol was added to the sample. PRB buffer was added to ensure efficient cell lysis for plant species containing high polysaccharide. The sample was then mixed through vortexing and incubated at 60 °C for 5 min. The incubated sample mixture was transferred to the filter column with a

collection tube and centrifuged for 1 min at 1,000 x g. The filtrate was then transferred to a new 1.5 mL microcentrifuge tube.

In step 2, RNA binding, absolute ethanol of half volume to the filtrate was added and mixed vigorously. The sample mixture was transferred to the RNA binding column and centrifuged at 12,200 x g for 1 min and the flow-through was discarded.

In the washing step, a total of 400 μ L of W1 buffer was added to the column and centrifuged at 12,200 x g for 1 min. The flow-through was discarded and 600 μ L of wash buffer was added to the column. The column was centrifuged at 12,200 x g for 1 min and the flow-through was discarded. A 600 μ L of wash buffer was added again to the column and centrifuged at 12,200 x g for 1 min. The flow-through was then discarded and the column was centrifuged at 12,200 x g for 3 min to dry the column matrix.

In the final step of RNA elution, the dried column was placed in a new 1.5 mL microcentrifuge tube and 50 μ L of RNase-free water was added to the column matrix. The column was allowed to stand for 2 min to ensure complete absorption of RNase-free water. The purified RNA was eluted through centrifugation at 12,200 x g for 1 min. The extracted RNA was stored at -80°C for DNase treatment.

3.3.2 RNA purity and integrity

3.3.2.1 RNA visualization

The RNA integrity was verified through gel-electrophoresis on 1% agarose gel pre-treated with 1 μ g/mL ethidium bromide (Promega, United Kingdom) at 100 V for 25 min in Tris-acetate (TAE) buffer (Bio-rad, USA). The gel was visualized under UV light.

3.3.2.2 RNA quantification

The purity of total RNA was quantified through spectrophotometer (Ultrospec 1100 pro, Amersham Biosciences, England) at wavelength 230, 260 and 280 nm with dilution factor of 100. The samples with ratio of absorbance A260/280 between ~1.8 to 2.1 were selected for the following analysis.

3.3.3 RNA purification

The total RNA with DNA contaminated were purified by DNase I treatment (Fermentas, Lithuania) according to manufacturer's instructions prior to reverse transcription. A total reaction mixture with 2 µL DEPC treated water, 1 µL 10X reaction buffer with magnesium chloride, 14 µL RNA, 1 µL ribonuclease inhibitor and 1 µL DNase I RNase free enzyme were incubated at 37 °C for 30 min followed by 10 min incubation at 65 °C with 1 µL 25 mM EDTA.

3.3.4 Two-step real-time RT-PCR

Two-step real-time RT-PCR was performed. First strand cDNA was synthesized from purified RNA, followed by real-time PCR.

3.3.5 First strand cDNA synthesis

Reverse transcription was performed using M-MuLV Reverse Transcriptase (Fermentas, Lithuania,). Reaction mixture of 20 µL consisted with template RNA, 10 µM random hexamer, DEPC treated water, 5X reaction buffer, ribolock RNase inhibitor, 10 µM dNTP mix and reverse transcriptase enzyme are incubated at 25 °C for 10 min followed by 60 min incubation at 42 °C. The reaction was terminated at 70 °C for 10 min. The cDNA template was stored at -20 °C for further analysis.

3.3.6 Primers design

Primers were developed through Primer Quest tool from Integrated DNA technologies (<https://sg.idtdna.com>). Several primers were designed for gene of interest, HPPR gene and housekeeping genes, 18S rRNA and maturase K genes, as presented in Table 4. Nucleotide sequence of HPPR gene was obtained from Ahmad et al. (2015), whereas the nucleotide sequence of 18S rRNA (Accession no.: JX974580.1) and maturase K genes (Accession no.: JN119569.1) were obtained from National Center for Biotechnology Information (NCBI). The gene sequences were shown in Appendix I. The primers were evaluated by gradient PCR and conventional PCR prior to quantitative real-time PCR for suitable primers selection and optimization of amplification condition.

Table 4: Primers designed for gene analysis

Primer		Primer Sequences	Length	Tm	Product Length (bp)
HPPR1	Forward	5'-GCG GAT TTG TGA GTG TGA TAA G-3'	22	61	115
	Reverse	5'-AAT TCT GCC CAA TCC AAT GAT AC-3'	23	62	
HPPR2	Forward	5'-GCG GAT TTG TGA GTG TGA TAA G-3'	22	61	250
	Reverse	5'-GAC TAG GAT GTC ACT GTT CCT C-3'	22	61	
HPPR3	Forward	5'-ATC AAG TGC AAG GAG AAG GG-3'	20	62	93
	Reverse	5'-AGC CAA CAT CAA CCC GAT AG-3'	20	62	
18srRNA1	Forward	5'-GTG ACG GGT AAC GGA GAA TTA G-3'	22	62	108
	Reverse	5'-CCG TGT CAG GAT TGG GTA AT-3'	20	62	
18srRNA2	Forward	5'-GAA CGT CTT GTG AAC GTC TTT G-3'	22	62	235
	Reverse	5'-CCG TTT GCA CGT TTG AAA GAT A-3'	22	62	
MatK1	Forward	5'-TGA TTG CGA AAT TGG CTA AAG AG-3'	23	62	112
	Reverse	5'-TAC GCC CAA ATC GGT CAA TAA TA-3'	23	62	

Table 4 continued

MatK2	Forward	5'-GAA CGT CTT GTG AAC GTC TTT G-3'	22	62	265
	Reverse	5'-CCG TTT GCA CGT TTG AAA GAT A-3'	22	62	

3.3.7 Polymerase Chain Reaction (PCR)

Commercial PCR master mix, 2x GoTaq green master mix (Promega, United Kingdom) were used with total reaction of 25 µL including GoTaq green master mix, forward and reverse primers, cDNA template and nuclease-free water. Negative control was included in the application. Optimal amplification condition was shown in Table 5. The PCR fragments were separated on a 2.0% v/v ethidium bromide pre-stained agarose gel for 25 min and visualized under UV illumination. The amplified products were sequenced and suitable primers were selected for further analysis. Sequencing service was carried out by First BASE Laboratories Sdn Bhd. The sequences results were confirmed by aligning with the selected genes sequences through Basic Local Alignment Search Tool (BLAST) program (<https://www.ncbi.nlm.nih.gov.com>).

Table 5: Optimal PCR condition

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	2 min	1
Denaturation	94	30 sec	35
Annealing	50	30 sec	35
Extension	72	1 min	35
Final extension	72	5 min	1
Hold	4	-	-

3.3.8 Relative quantification

Target nucleic acid was quantified through relative quantification. The ratio between the target gene and endogenous gene in the sample was determined. Quantitative Real-time PCR was performed using Real-time PCR Applied Biosystem 7300 (Applied Biosystem, USA). Data were generated and obtained from the specified software, Applied Biosystem 7300 SDS software. The gene expression level between control and UV treated samples were calculated as RQ value or $\log_{10}$ RQ by the software.

3.3.8.1 Quantitative Real-time PCR

Quantitative real-time PCR was performed using Maxima SYBR qPCR green master mix (Thermo Scientific, USA). The primers used for target gene was HPPR2 primers, whereas for endogenous genes was 18s rRNA2 primers. Real-time PCR reaction of 25 μ L containing cDNA template, Maxima SYBR qPCR Green master mix (2X), forward primer for HPPR gene or 18S rRNA gene, reverse primer for HPPR gene or 18S rRNA gene, ROX solution and nuclease free water were used for the analysis. All samples from different time frame of UVB/ UVC treatment were analyzed in triplicate with the same RNA preparation and reverse transcription. The optimal thermal cycling condition was presented in Table 6.

Table 6: Two step thermal cycling condition for quantitative real-time PCR

Step	Temperature, °C	Time	Number of cycles
UDG Pre-treatment	50	2 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing/Extension	60	1 min	40

*UDG- Uracil DNA Glycosylase

3.4 Rosmarinic acid analysis

Rosmarinic acid was chosen as the target component because of its known bioactive properties. The rosmarinic acid was determined using the reverse phase high performance liquid chromatography (HPLC). Methanol extraction method was chosen to extract rosmarinic acid in *O. aristatus* due to its better selectivity (Jamshed & Ismail, 2011; Malahubban et al., 2013). All reagents and solvents were analytical and HPLC grade (Merck). The standard of rosmarinic acid (purity $\geq 98\%$ w/w from *Rosemarinus officinalis* L, Sigma, USA) was used as the external standard.

3.4.1 Sample preparation for HPLC

Powdered leaves samples of 0.25 g were extracted with 10 mL of 50% v/v methanol in water for 1 hr at 60 °C with agitation. The samples were then centrifuged at 13,000 rpm for 10 min and the supernatant was filtered by using PTFE syringe filter. The filtrate was subjected to HPLC analysis. They were stored at $-20\text{ }^{\circ}\text{C}$ before analysis.

3.4.2 Chromatographic analysis

High performance liquid chromatography (Model: Agilent 1260 Infinity Quaternary Liquid chromatography, Agilent Technologies, Germany). equipped with degasser, quaternary pump, auto sampler and UV/Vis DAD detector was used for determination of rosmarinic acid. The column used was Merck Licrochart purospher (Germany) start RP18 column endcapped (250 mm, 4.6 mm i.d, 5 μm pore size). The chromatographic condition was performed based on the modified protocol (Tuan et al., 2012; Malahubban et al., 2013). The wavelength of UV was set at 330 nm with the injection volume of 20 μL . The injected sample was eluted with a gradient mobile phase comprising of 0.17% (v/v) phosphoric acid in water (mobile phase A),

(Mallinckrodt, USA) and acetonitrile (mobile phase B), (JT Baker, Germany). The elution was carried out at a flow rate of 1.0 mL/min at 30 °C and a separation time of 35 min. The gradient mixture of mobile phase A and B were presented in Table 7. The concentration of rosmarinic acid in the samples was determined based on the standard calibration curve. Mean values of three independent replicates was calculated.

Table 7: Chromatographic condition of rosmarinic analysis

Time (min)	0.17% v/v Phosphoric acid in H ₂ O(A)	Acetonitrile (B)
0-12	85%	15%
12-25	75%	25%
25-35	85%	15%

3.4.3 Standard solution preparation

An accurately weighed rosmarinic acid reference was mixed in 10 mL volumetric flask with 50% v/v methanol in water. The concentration of the solution prepared was 10,000 µg/mL. A range of different concentrations of rosmarinic acid (Sigma, USA) at 1 µg/mL, 5 µg/mL, 10 µg/mL and 20 µg/mL were prepared through stepwise dilution from the stock solution with 50% v/v methanol.

3.4.4 Method validation

The specificity of the method was determined by running a procedural blank which no reference compound was added to 50% v/v methanol. The standard calibration curve was established by plotting the peak area against the concentrations of rosmarinic acid. The data was analyzed by linear regression analysis. The linearity between the peak area and the expected concentrations

was demonstrated by the standard calibration curve of rosmarinic acid. The specificity was determined through spectrum comparison between rosmarinic acid standard and samples.

3.4.5 Statistical analysis

The samples were analyzed in triplicate and the mean value was calculated. The mean data was compared by pair-t-test and one-way ANOVA using SPSS version 13.0 (IBM Corporation, US).

CHAPTER 4

RESULTS

4.1 Effect of UVB and UVC treatment on plant physical change

In present study, *O. aristatus* plant was treated with UVB and UVC radiations respectively for 15 min, 30 min, 45 min, 60 min and 1 hr dark recovery. The UV dosage after each time frame of UV treatment was measured through the commercial UV strip (UV Fastcheck strips from UV Process Supply, USA). UVB dose was found to be 100 mJ/cm² and UVC dose was 150 mJ/cm². In UVB treatment, no significant changes were observed for the change in leaves morphology. On the other hand, one hour UVC treatment had significantly caused the leaf to shrink and developed yellowish colour in plants as shown in Figure 6 and Figure 7. The treated plants were observed for one week after UV treatment and no further plant death were found.



Figure 6: *O.aristatus* plant before UVC treatment



Figure 7: *O.aristatus* plant after UVC treatment

4.2 Molecular analysis

4.2.1 RNA Extraction

Commercial kit, plant total RNA mini kit (Geneaid, Taiwan) was used to extract the total RNA from *O. aristatus* leaves after fixed period of UVB/UVC treatment. The total RNA were extracted from 0 min of UVB/UVC treatment as control, followed by 15 min, 30 min, 45 min

and 60 min, and after 1 hr of dark recovery. Basic precautions were practiced prior to RNA extraction, consumables such as glassware, mortar pestle and centrifuge tubes were pre-treated with 0.1% DEPC water for overnight and autoclaved. The kit involved five steps of extraction processes, pre-step of plant tissue dissociation, followed by cell lysis, RNA binding to the specific column, washing of contaminants and final step RNA elution. Meanwhile, instead of RB cell lysis buffer, PRB cell lysis buffer was used in the first step due to the high polysaccharide content in medicinal plant, *O. aristatus*. Through the protocol, adequate and acceptable qualities of total RNA from *O. aristatus* leaves were yielded.

The RNA integrity was analyzed on 1% agarose gel pre-treated with 1 $\mu\text{g/mL}$ ethidium bromide as shown in Figure 8 and Figure 9 where 28S and 18S bands are clearly visible along with smear showing the presence of additional rRNAs. No significant RNA degradation was observed. The overall A260/280 nm ratio of 1.7-2.2 from RNA of UVB and UVC treated *O. aristatus* leaves (Appendix III) indicated the RNA purity is relatively high. Meanwhile, the overall A260/230 nm ratio for both UVB and UVC treated samples were in between of 1.7 to 2.3 (Appendix III). This indicated that the RNA extract are relatively free from polyphenols and polysaccharides contamination. The RNA yield was in the range of 30 $\mu\text{g/g}$ to 50 $\mu\text{g/g}$ of fresh leaves samples of 100 mg. Total RNA were purified by DNase I treatment (Fermentas, Lithuania) prior to reverse transcription in order to avoid false positive amplification in PCR or real-time qPCR.

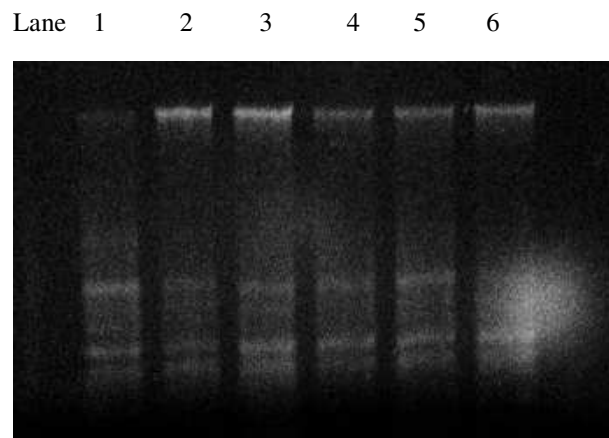


Figure 8: Agarose gel electrophoresis of total RNA from *O. aristatus* leaves after different time point of UVB treatment, 0 min as control (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5) and 1 hr dark recovery (lane 6).

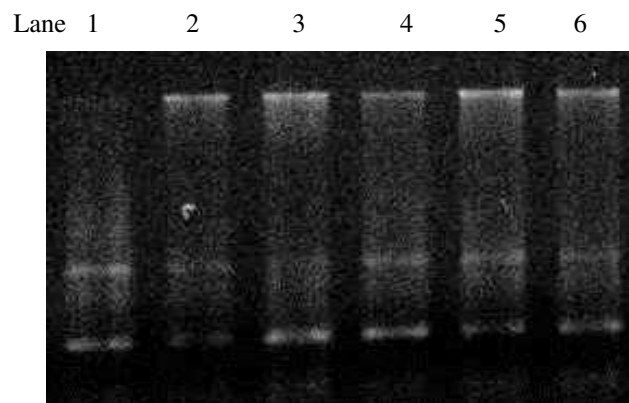


Figure 9: Agarose gel electrophoresis of total RNA from *O. aristatus* leaves after different time point of UVC treatment, 0 min as control (lane 1), 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5) and 1 hr dark recovery (lane 6).

4.2.2 Gradient PCR

Prior to real-time RT-PCR, conventional RT-PCR and gradient PCR were performed in order to select the suitable primers and optimal PCR condition for gene expression analysis. Primer optimization was performed through gradient PCR to determine the optimal annealing temperature for further analysis. Several primers for gene of interest, HPPR gene and housekeeping genes, 18S rRNA and maturase K (matK) were designed from the existing gene

database. The primers specificities are stated in Chapter 3.3.6.

Primer pairs, HPPR2, matK2 and 18S rRNA2, were selected and used for further analysis due to their stability in certain annealing temperature. Primer pair HPPR2 (forward and reverse) produced PCR bands with product size of approximately 250 bp (Figure 10) at gradient temperature range from 48 °C to 61.8 °C. The optimal annealing temperature was at 50 °C. The primers were used for the amplification of gene of interest in RT-PCR.

Besides that, gradient PCR in temperature range from 48 °C to 61.8 °C, revealed that primer pair 18S rRNA2 (forward and reverse) produced bands with product size of approximately 235 bp (Figure 11). The optimal annealing temperature was at 50 °C. The primers were used for the amplification of housekeeping gene in RT-PCR. In addition, gradient temperature ranges from 48 °C to 61.8 °C revealed that primer pair matK 2 (forward and reverse) produced bands with product size of approximately 265 bp (Figure 12). The optimal annealing temperature was at 50 °C. The primers were used for the amplification of housekeeping gene in RT-PCR.

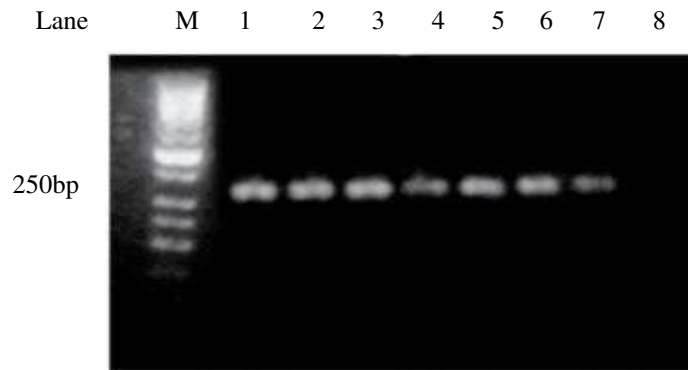


Figure 10: Agarose gel electrophoresis of gradient amplification of *O. aristatus* HPPR gene using HPPR2 primers in temperature range of 48 °C, 50 °C, 51.7 °C, 56 °C, 57.8 °C, 59.7 °C and 61.8 °C. Lane M denotes the 50 bp DNA ladder (Fermentas, Lithuania) whereas lanes 1 to 7 denote the plant sample and lane 8 denotes the negative control.

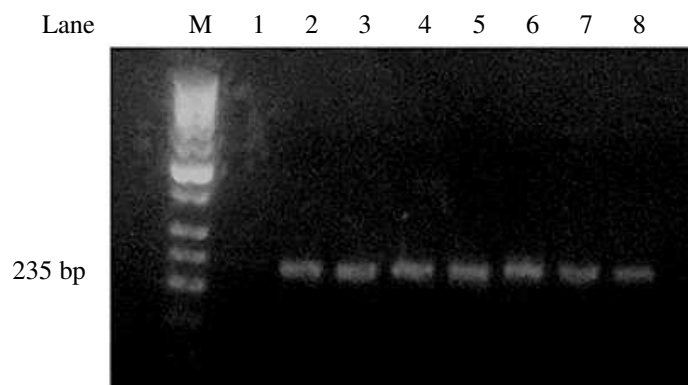


Figure 11: Agarose gel electrophoresis of gradient amplification of *O. aristatus* 18S rRNA gene using 18S rRNA2 primers in temperature range of 48 °C, 50 °C, 51.7 °C, 56 °C, 57.8 °C, 59.7 °C and 61.8 °C. Lane M denotes the 50 bp DNA ladder (Fermentas, Lithuania) whereas lanes 2 to 8 denote the plant sample and lane 1 denotes the negative control.

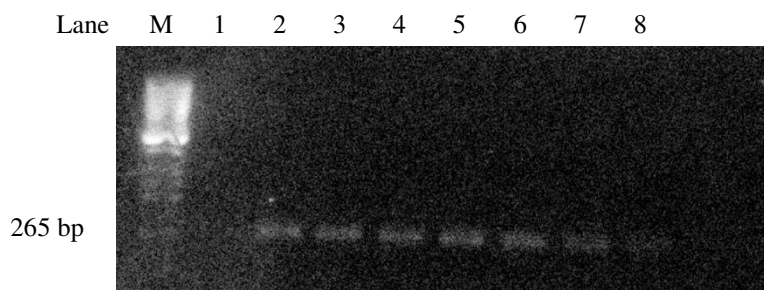


Figure 12: Agarose gel electrophoresis of gradient amplification of *O. aristatus* matK gene using matK2 primers in temperature range of 48 °C, 50 °C, 51.7 °C, 56 °C, 57.8 °C, 59.7 °C and 61.8 °C. Lane M denotes the 50 bp DNA ladder (Fermentas, Lithuania) whereas lanes 2 to 8 denote the plant sample and lane 1 denotes the negative control.

4.2.3 Reverse transcription polymerase chain reaction (RT-PCR)

Amplification of HPPR, 18S rRNA and matK genes under optimum PCR condition of samples from *O. aristatus* leaves are shown in Figure 13. Clear and single bands were successfully amplified. The PCR products were sent to First Base Laboratories Sdn. Bhd for sequencing. The sequence result of HPPR gene was aligned with online NCBI Basic Local Alignment Search Tool (BLAST). The alignment results revealed a similarity of 99% (204/205) and E-value of $2e-107$ when aligned with previously reported full length HPPR sequences (Zuliza, 2014), Appendix I. The BLAST alignment is shown in Figure 14.

Meanwhile, the PCR product of 18S rRNA was sequenced and aligned with online NCBI Basic Local Alignment Search Tool (BLAST). The alignment results revealed a similarity of 99% (196/197) and E-value of $3e-103$ when aligned with partial nucleotide sequence of 18S rRNA gene from *Orthospion aristatus* (GenBank: JX974580.1, NCBI), Appendix II. The BLAST alignment is shown in Figure 15. The PCR product of matK gene was sequenced and aligned with online NCBI Basic Local Alignment Search Tool (BLAST). The alignment results revealed a similarity of 98% (235/239) and E-value of $7e-121$ when aligned with nucleotide sequence of truncated maturase K (matK) gene from *Orthospion stamineus* (GenBank: JN119569.1, NCBI), Appendix II. The BLAST alignment is shown in Figure 16.

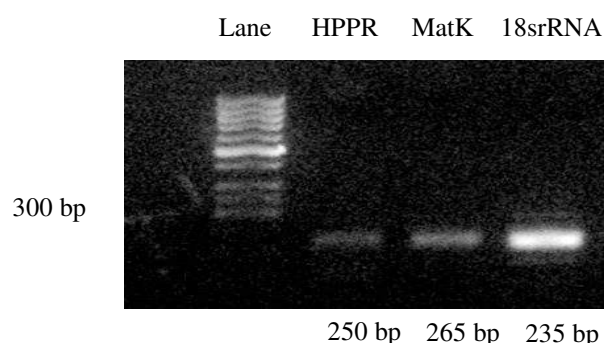


Figure 13: Reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene of interest, HPPR gene and housekeeping genes, 18SrRNA gene and matK gene.

```

Query 378 ATTTGGCGACTTCAAGTTGACGACTAAGTTTCAGCGGCAAAAGAGTTGGTATCATTGGATT 437
          |||
Sbjct 19  ATTTGGCGACTTC-AGTTGACGACTAAGTTTCAGCGGCAAAAGAGTTGGTATCATTGGATT 77

Query 438 GGGCAGAATTGGCTTAGCAGTTGCTGAGCGAGCAGATGCATTGATTGTCCCATAAATTA 497
          |||
Sbjct 78  GGGCAGAATTGGCTTAGCAGTTGCTGAGCGAGCAGATGCATTGATTGTCCCATAAATTA 137

Query 498 CTAAGATCCAAGAAACCAACACAACTACAAGTACTACAACAGCGTTGTTGAATT 557
          |||
Sbjct 138 CTAAGATCCAAGAAACCAACACAACTACAAGTACTACAACAGCGTTGTTGAATT 197

Query 558 GGCGAGGAACAGTGACATCCTAGTC 582
          |||
Sbjct 198 GGCGAGGAACAGTGACATCCTAGTC 222

```

Figure 14: BLAST alignment of *O. aristatus* HPPR gene (gene of interest) between sequencing product and nucleotide sequence, HPPR gene from *Orthosiphon aristatus* (Ahmad, 2015). Alignment identities are found to be 99% (204/205) and E-value of 2e-107.

```

Query 147 ACGGGCTCTGCCCGTTGCTGCGATGATTCATGATAACTCGACGGATCGCACGGCCTTTGT 206
          |||
Sbjct 11  ACGGGCTCTG-CCGTTGCTGCGATGATTCATGATAACTCGACGGATCGCACGGCCTTTGT 69

Query 207 GCCGCGACGCATCATTCAAATTTCTGCCCTATCAACTTTTCGATGGTAGTGTAGTGGCCT 266
          |||
Sbjct 70  GCCGCGACGCATCATTCAAATTTCTGCCCTATCAACTTTTCGATGGTAGTGTAGTGGCCT 129

Query 267 ACCATGGTGGTGACGGGTAACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAA 326
          |||
Sbjct 130 ACCATGGTGGTGACGGGTAACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAA 189

Query 327 ACGGCTACCACATCCAA 343
          |||
Sbjct 190 ACGGCTACCACATCCAA 206

```

Figure 15: BLAST alignment of *O. aristatus* 18S rRNA gene (housekeeping gene) between sequencing product and nucleotide sequence, 18S rRNA gene from *Orthosiphon aristatus* (NCBI database). Alignment identities are found to be 99% (196/197) and E-value of 3e-103.

```

Query 1  GGACATTCGGGCGA--CTATGGTTGGTCGAGG-ACCCTGCATGCATTATATTAGGTATCA 57
          |||
Sbjct 355 GGACTTTCGGGCGAACCTATGGTTGGTCGAGGAACCTGCATGCATTATATTAGGTATCA 414

Query 58 AGGAAAATCCATTCTGGCTTCAAAAGGGACATCTCTTTTCATGAATAAATGGAAATTTTA 117
          |||
Sbjct 415 AGGAAAATCCATTCTGGCTTCAAAAGGGACATCTCTTTTCATGAATAAATGGAAATTTTA 474

Query 118 CCTTGTCACCTTTTGGGAATGGCATTTTTTGGTGTGGTTTCATCCAAGAAGGATTGTAT 177
          |||
Sbjct 475 CCTTGTCACCTTTTGGGAATGGCATTTTTTGGTGTGGTTTCATCCAAGAAGGATTGTAT 534

Query 178 AAACCAATTTTCCAAGCATTCCTTGAAATTTTGGCTATCTTTCAAACGTGCAAACGG 236
          |||
Sbjct 535 AAACCAATTTTCCAAGCATTCCTTGAAATTTTGGCTATCTTTCAAACGTGCAAACGG 593

```

Figure 16: BLAST alignment of *O. aristatus* matK gene (housekeeping gene) between sequencing product and nucleotide sequence of truncated matK gene from *Orthosiphon stamineus* (NCBI database). Alignment identities are found to be 98% (235/239) and E-value of 7e-121.

4.2.4 Real time RT-PCR

In this study, the results were analyzed through relative quantification with the selected housekeeping genes, 18S rRNA and matK genes were normalized against the target gene, HPPR. Trial experiments are conducted by using the two selected housekeeping genes and normalized with the gene of interest, in order to determine the stability of housekeeping genes expression after UV treatments. From the results obtained, it was observed that the matK expression was unstable where the expression was varied at different time point of UV treatment and unidentified expression was observed. The amplifications specificity of the samples was prior confirmed by the presence of a single band of expected size for each primer pairs in agarose gels following electrophoresis. No primer dimers or other products were observed for non-specific amplification. No results were detected in the minus RT and no-template controls. The real-time PCR condition was used for later analysis.

4.3 HPPR gene expression

4.3.1 HPPR gene expression after UVB and UVC treatment

Figure 17 shows one of the representative data of the relative expression log-10 (RQ) value of HPPR gene against different time point of UVB treatment when comparing with control as the calibrator. The HPPR gene expression increased after 15 min of UVB radiation and eventually decreased until 45 min of UVB treatment. Interestingly, the HPPR gene expression increased after 1 hr of UVB radiation exposure and declined after 1 hr of dark recovery. Whereas, short term UVC treatment on *O. aristatus* plant shows negative expression of HPPR gene. The relative expression of HPPR gene, log-10 (RQ) against different time point of UVC treatment when compared with control as the calibrator is shown in Figure 18. The figure represented one of the representative data obtained. HPPR gene expression decreased linearly until 45 min and

low HPPR gene expression was observed at 1 hr of UVC treatment. Interestingly, the HPPR gene expression was up-regulated after 1 hr dark recovery with exclusion of UVC.

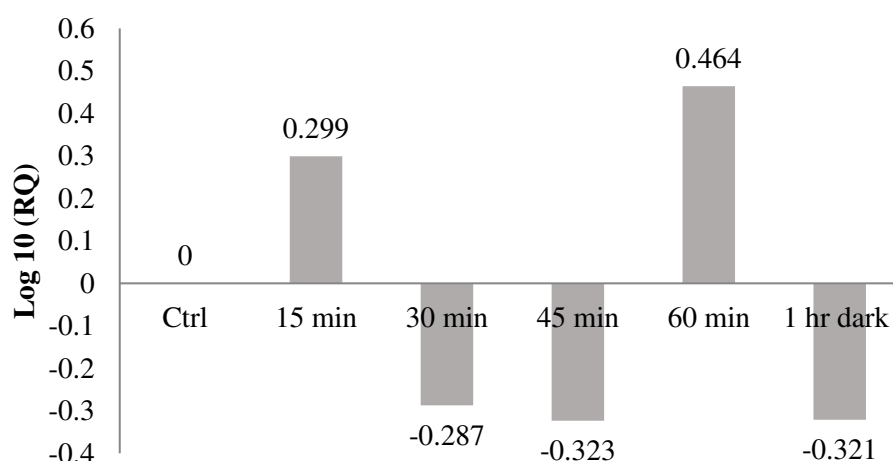


Figure 17: The relative expression of HPPR gene in *O. aristatus* plant after short-term UVB treatment. The bar graph showing the log-10 (RQ) value of HPPR gene measured after different time points of UVB treatment and compare with control without UVB treatment as calibrator. The 15 min, 30 min, 45 min and 60 min represent the time point of UVB treatment, whereas 1 hr dark represents the 1 hr dark recovery after 60 min UVB exposure.

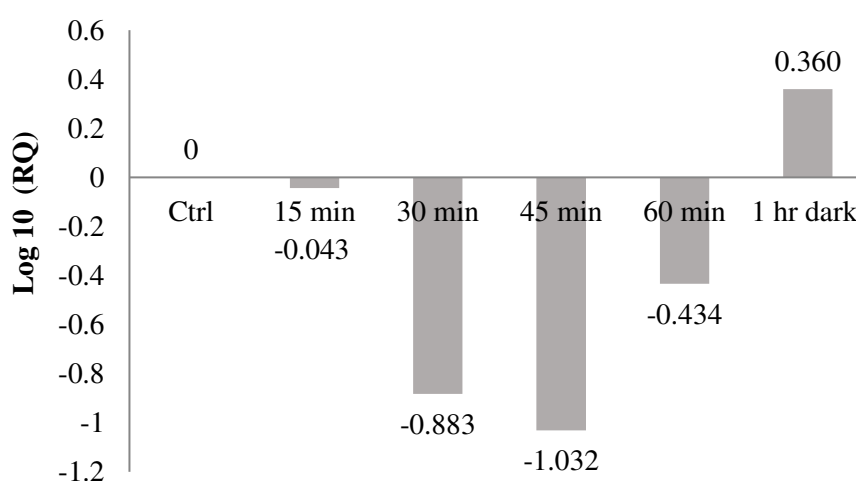


Figure 18: The relative expression of HPPR gene in *O. aristatus* plant after short-term UVC treatment. The bar graph showing the log-10 (RQ) value of HPPR gene measured after different time points of UVC treatment and compare with control without UVC treatment as calibrator. The 15 min, 30 min, 45 min and 60 min represent the time point of UVC treatment, whereas 1 hr dark represents the 1 hr dark recovery after 60 min UVC exposure.

4.3.2 Comparison on HPPR gene expression between UVB and UVC treatments

To compare the effect of both treatments, ANOVA analysis was conducted. ANOVA showed that the average expression values differed between both UV treatments for the genes HPPR ($P>0.05$). None of the UV treatment showed significant changes on HPPR gene expression.

4.4 Rosmarinic acid production

4.4.1 Determination of rosmarinic acid

The present study was conducted to determine the effect of short term UV treatment on the production of RA in *O. aristatus* leaves. The same plants samples used for quantitative real-time PCR were used for HPLC analysis of RA. Simple extraction method by using 50% methanol at extraction temperature of 60 °C with agitation for one hour was applied. A simple and effective extraction method was used in order to prevent the degradation of compound in UV treated sample after prolong storage. The extraction information was discussed in Chapter 3.4.1. In this study, high performance liquid chromatography (HPLC) equipped with degasser, quaternary pump, auto sampler and UV/Vis DAD detector was used for analysis of RA. Meanwhile, a reversed phase C18 column was used for the separation of RA from the crude extract of *O. aristatus*. The choice of detection wavelength was determined by conducting a screening with 20 ppm of rosmarinic acid standard in methanol by using the pre-installed UV/Vis DAD detector. The UV spectrum was found to be in the range from 280-340 nm and exhibited maximum wavelength at 330 nm. The chromatograms of 20 ppm RA and the control sample prior to UVB light treatment are shown in Figure 19 and Figure 20, respectively.

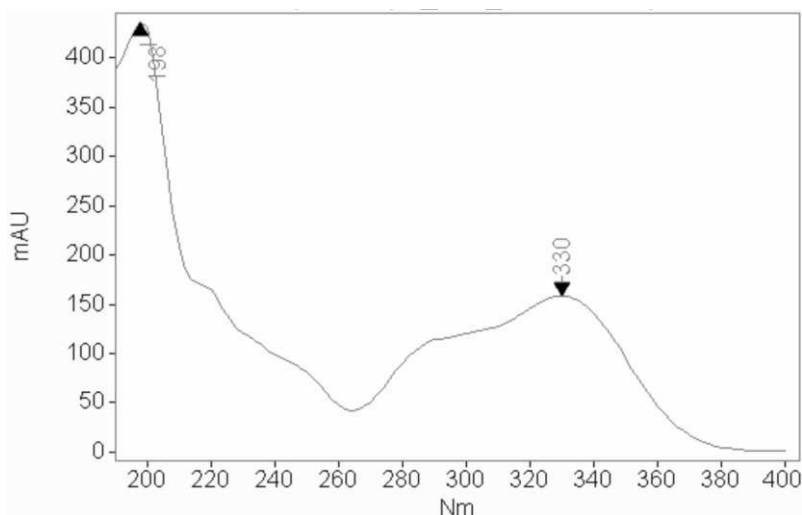


Figure 19: The chromatogram of rosmarinic acid standard (20ppm) generated from Agilent ChemStation software

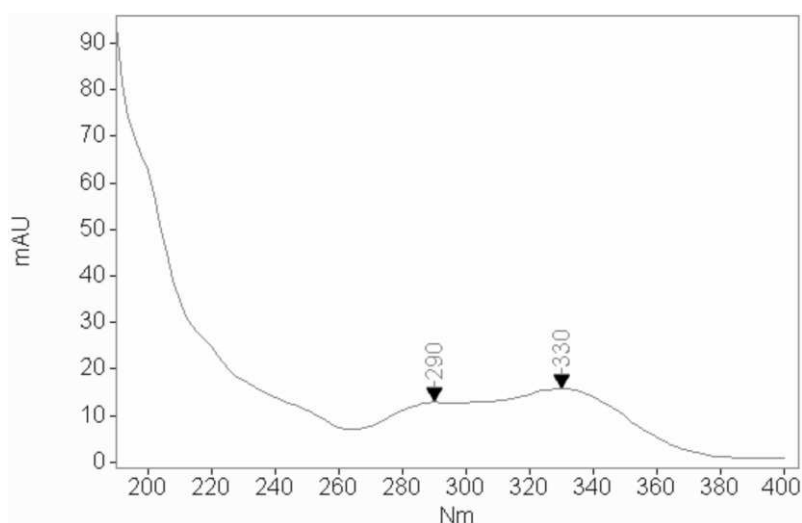


Figure 20: The chromatogram of rosmarinic acid identified in *O. aristatus* leaves before UVB treatment generated from Agilent ChemStation software

The gradient method with mobile phase comprising of 0.17% phosphoric acid in water and acetonitrile was used for the RA analysis. Gradient method provides a better separation of desired compound and shorter elution time due to the complexity of the samples. It can be seen that a good separation can be achieved within 20 min using the chromatographic condition as shown in Figure 21 and Figure 22. The retention time was found to be around 16 min. The

remainder of the gradient conditions was to ensure the efficient column washing. The quantification of rosmarinic acid in sample before UV treatment was 0.4961 $\mu\text{g/mL}$ which also indicated 104.25 $\mu\text{g/g}$ of compound contained in 0.25 g of *O. aristatus* leaves based on the dried raw as presented in Figure 22. The HPLC chromatogram for UVB and UVC treated samples were shown in Appendix V and Appendix VI.

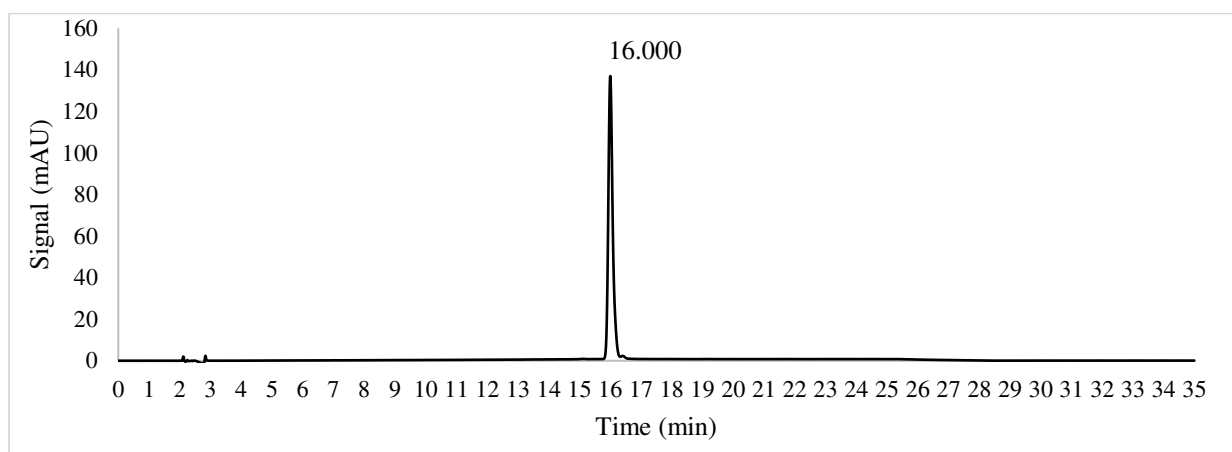


Figure 21: HPLC chromatogram (330 nm) of 10 $\mu\text{g/mL}$ rosmarinic acid standard

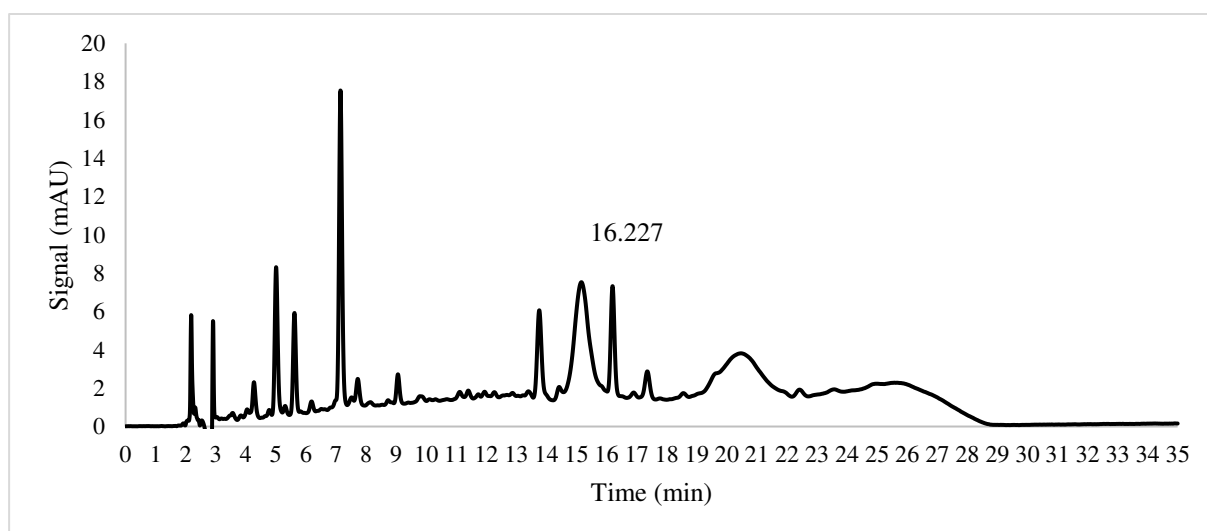


Figure 22: HPLC chromatogram (at wavelength 330 nm) of rosmarinic acid identified in *O. aristatus* leaves before UVB treatment and the concentration determined was 0.4961 $\mu\text{g/mL}$

4.4.2 Method validation

The specificity of the method was evaluated by analysis of blank, standard and sample chromatograms as shown in Figure 23 and Figure 24. The calibration curve of RA was constructed using five levels of concentrations which covered the concentrations range expected in the samples as shown in Figure 23 presenting one of the calibration curve plotted. The linearity was evaluated by the square correlation coefficient (R^2). All the values of the calibration curve constructed showed the R^2 range from 0.990 to 0.998. Calibration curve was constructed and blank was injected in each HPLC analysis. The HPLC results of sample after UV treatment are discussed in the following sub-chapters.

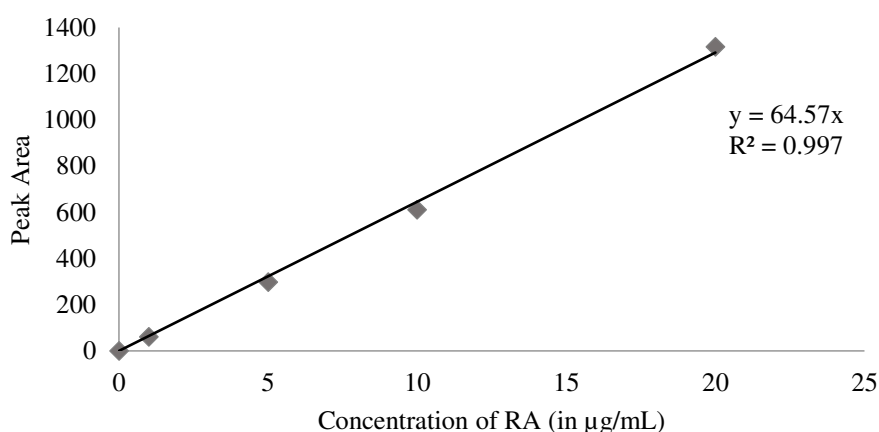


Figure 23: Calibration curve of rosmarinic acid standard with R squared value of 0.997 and graph equation of $y=64.57x$.

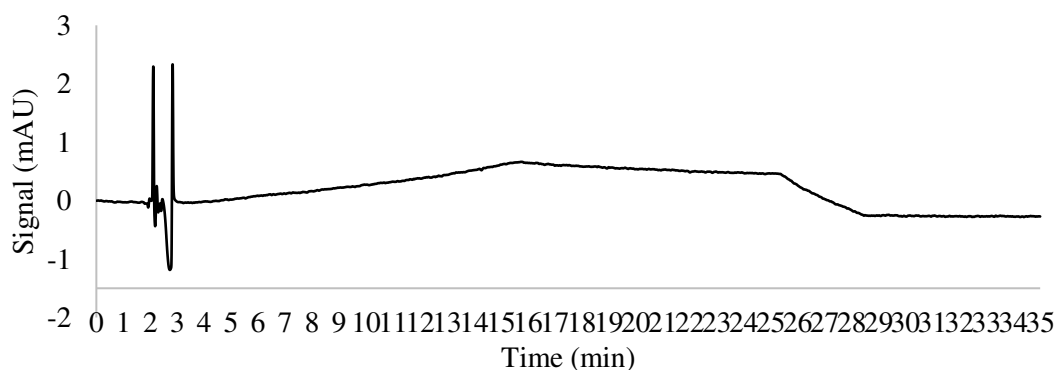


Figure 24: Blank chromatogram with no sample added

4.4.3 Rosmarinic acid accumulation after UVB treatment

The contents of rosmarinic acid varied discriminately during 45 min of UVB exposure and the dark recovery process. Results (Figure 25) showed that at the initial 30 min of treatment, enhanced UVB radiation induced the rosmarinic acid content to slightly increase ($p < 0.05$), whereas there were significant different when compare between control and treatment samples. After 45 min of UVB treatment, the rosmarinic acid content increased significantly with highest concentration found, 173.23 $\mu\text{g/g}$ comparing with control of 66.36 $\mu\text{g/g}$. Slight decreased in the RA content after 60 min of treatment was observed. UVB radiation was stopped after 1 hr of UVB radiation in order to investigate the metabolism of rosmarinic acid in plants. The plants were placed in darkness for 1 hr. Interestingly, the RA content increased slightly up to the level of control after 1 hr of dark recovery.

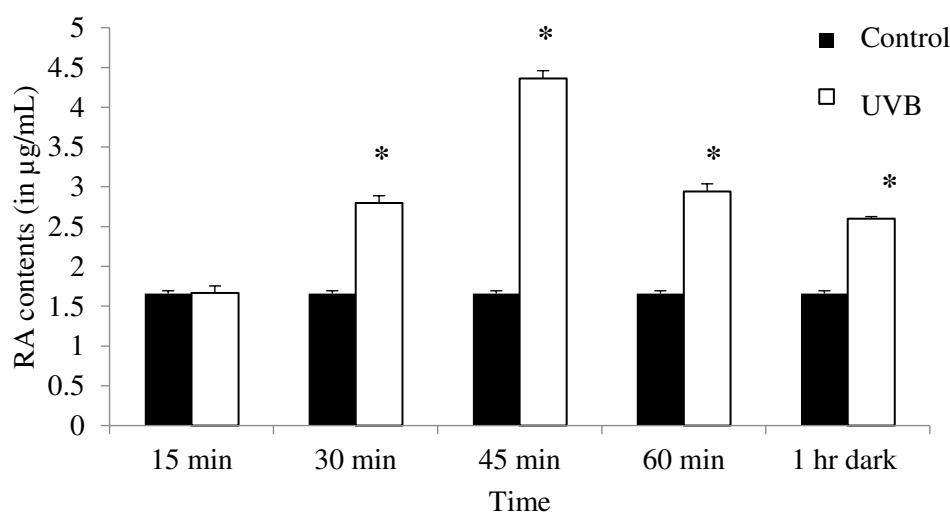


Figure 25: The variation of rosmarinic acid production profiles response to short-term UV-B treatment. 15 min, 30 min, 45 min and 60 min represent the time point of UV-B treatment, whereas 1hr dark represents the 1 hr dark recovery after 60 min UVB exposure. The data represent the average of three independent biological replicates and comparison with control respectively. The vertical bars indicate the standard errors. The statistical analysis of differences between control and each time points of treatment were performed by 2-tailed T test, $P < 0.05$ is indicated with asterix (*).

4.4.4 Rosmarinic acid accumulation after UVC treatment

Rosmarinic acid contents produced at different time frame under UVC radiation are shown in Figure 26. At the first 15 min of treatment, UVC induced the production of RA, however, the content was decreased after 30 min of treatment. After 45 min of UVC treatment, the RA content was significantly increased ($p<0.05$), the highest concentration among the triplicate treatments was found to be $49.084\text{ }\mu\text{g/g}$ with control concentration of $16.59\text{ }\mu\text{g/g}$. Rosmarinic acid content was slightly decreased after 60 min of treatment. UVC radiation was stopped after 1 hr of UVC radiation in order to investigate the metabolism of RA in plants. The plants were placed in darkness for 1 hr. Interestingly, the RA content increased after 1 hr of dark recovery. Minor difference was observed in the accumulation of RA content among the triplicate treatments.

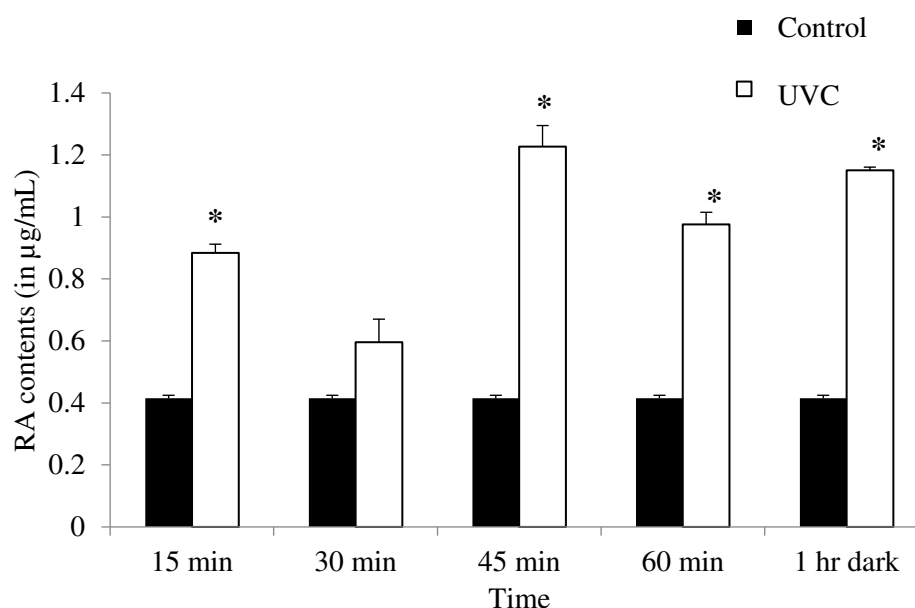


Figure 26: The variation of rosmarinic acid production profiles response to short-term UVC treatment. 15 min, 30 min, 45 min and 60 min represent the time point of UVC treatment, whereas 1hr dark represents the 1 hr dark recovery after 60 min UVC exposure. The data represent the average of three independent biological replicates and comparison with control respectively. The vertical bars indicate the standard errors. The statistical analysis of differences between control and each time points of treatment were performed by 2-tailed T test, $P<0.05$ is indicated with asterix (*).

4.4.5 Comparison on rosmarinic acid content between UVB and UVC treatment

ANOVA showed that no significant difference in the amount of RA produced between both UVB and UVC treatment ($p>0.05$). However, UVB treatment showed better induction in the rosmarinic acid content compared to UVC treatment.

4.5 Correlation between HPPR gene expression and level of rosmarinic acid

Figure 27 shows the relative expression of HPPR and RA concentrations against time of UV exposure with correlation coefficient value of -0.27. Negative correlation was also observed for all triplicate UVB treatments with correlation coefficient value in the range of -0.25 to -0.38. The result showed that when the HPPR gene expression was low, the average concentration of rosmarinic acid increased. The relative expression of HPPR gene was denoted as RQ value after normalization with the selected housekeeping gene. The HPPR gene expression was lowest after 45 min of UVB treatments. Interestingly, the RA production was the highest after 45 min of UVB treatments.

Besides that, short term of UVC treatment was applied on *O. aristatus* plant. Figure 28 showed negative correlation of HPPR gene expression and rosmarinic acid production after short term of UVC radiation exposure with correlation coefficient value of 0.16. Negative correlation was observed for all triplicate UVC treatments with correlation coefficient value in the range of in the range of -0.4 to 0.17. The results also showed fluctuation in the rosmarinic acid contents. HPPR gene expression was down-regulated after 30 min of UVC treatment and increase dramatically after 1 hr of dark recovery.

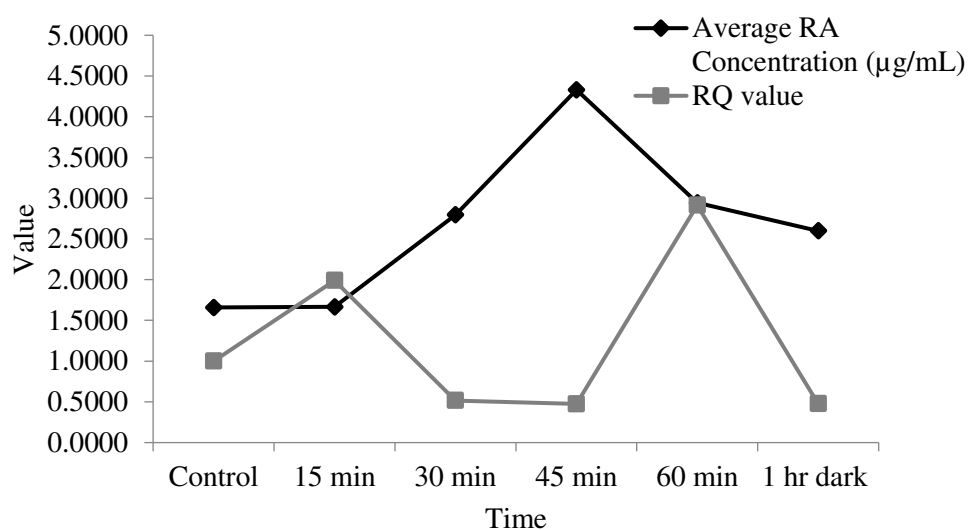


Figure 27: Correlation between the RA production and HPPR gene expression after UVB treatment. The average RA concentration denotes the average RA concentration of three dependent replicates, whereas the RQ value denotes the relative expression value of HPPR gene after UVB treatment. 15 min, 30 min, 45 min and 60 min denote the time point of UVB treatment, whereas 1 hr dark denotes the 1 hr dark recovery after 60 min UVB exposure.

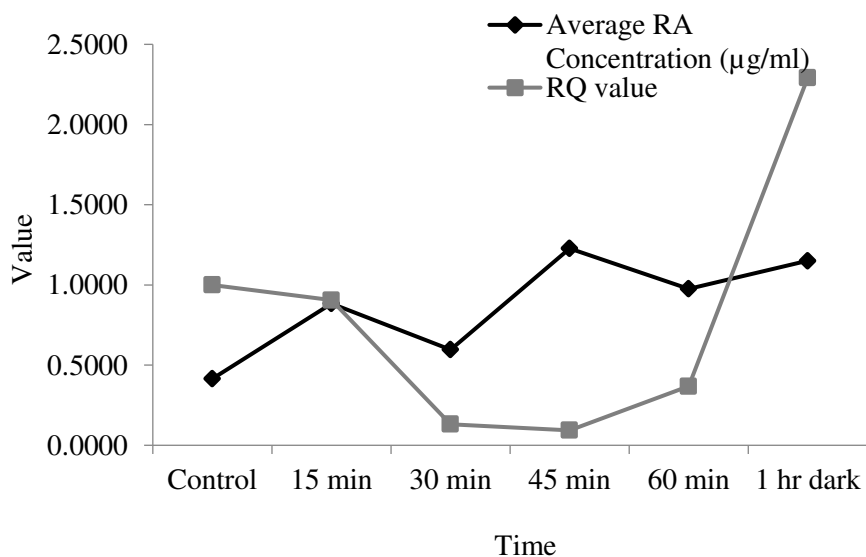


Figure 28: Correlation between the RA production and HPPR gene expression after UVC treatment. The average RA concentration denotes the average RA concentration of three dependent replicates, whereas the RQ value denotes the relative expression value of HPPR gene after UVC treatment. 15 min, 30 min, 45 min and 60 min denote the time point of UVC treatment, whereas 1 hr dark denotes the 1 hr dark recovery after 60 min UVC exposure.

CHAPTER 5

DISCUSSION

5.1 Plant physical change after UV treatment

Plants are photosynthetic organisms which depend on sunlight for energy. Plants respond toward light through different photoreceptors and exhibit photomorphogenic development (Nawkar et al., 2013). Plants are exposed to photosynthetically active radiation (PAR) and ultraviolet light (UV). Ultraviolet light is divided into three types: UVA, UVB and UVC. Each types of UV light are classified based on their wavelength, such as UVA (315-400 nm) UVB (280-315 nm) and UVC (200-280 nm) according to the International Commission on Illumination (Katerova et al., 2013). The negative impact of UV radiation is based on its wavelength. Hence, UVC light can cause higher injuries towards living organisms due to its shorter wavelength thus higher energy. Besides that, UV radiation has also been shown to cause alterations in plant physiological and biochemical processes and causes morphological changes. However, plants respond to UV radiation commonly by reducing the penetration of radiation based on their UV-absorbing sunscreens and activation of antioxidant defense (Mazza et al., 2000).

The results show that no plant morphological alteration after UVB treatment. Whereas, there are changes in plant leaf structure and colour after UVC treatment. The current knowledge regarding to the ecophysiological impacts of UV radiation on plants has conducted largely through field experiments using natural or moderately higher level of UVB radiation (Nawkar et al., 2013). However, the results from all these experiments summarized that UVB radiation has minor inhibitory effects on plant growth, activation of acclimation response and interference between plants and herbivores (Ballare et al., 2001; Caldwell et al., 2003; Caldwell et al., 2007).

Meanwhile, numerous studies have investigated the effects of enhanced UVC treatment on plants and have shown diverse range of responses such as changes in physiological, morphological and biochemical levels. Bean plants treated with UVC radiations exposure showed significantly leaves shrink, leaves size decreased and seedling height decreased (Kara, 2013). UVC radiation exposure on young tomato plant for 60 min and 120 min was characterized by senescence with a general stem and leaf yellowing (Castronuovo et al., 2014). The author also concluded that high UVC doses caused irreversible damages, both at physiological and morphological levels, which will lead to plants death, whereas lower irradianations (up to 60 min) allowed plants to partially recovery their normal physiological status. Prolonged UVC radiation exposure could adversely affected the growth and photosynthesis in *O. aristatus* plant.

There are differences between species as regards to UV radiation sensitivity (Teramura, 1983) but no information on the effects of UVB and UVC on *O. aristatus* plants, that is instead considered as potential medicinal plant. The results indicated that high UVC doses (60 min and above) will cause damages to plants which prolong treatment may lead the whole plant to death. By contrary, lower irradianations allowed the plants to partially maintain their normal physiological status. Next steps foresee physiological, genetic and molecular investigations and studies on the possible tolerance mechanisms of *O. aristatus* plants to face UVC radiation.

5.2 Two-step real-time RT-PCR

In molecular studies of plant biology, RNA extraction from specific plant organs or tissue is the preliminary step. A high quality of RNA with sufficient quantity is mandatory requirement for gene expression studies such as reverse transcriptase, real-time quantitative PCR, construction of cDNA libraries or microarray analyses (Cardillo et al., 2006). However,

isolation of high quality RNA is often difficult due to the presence of polyphenols, polysaccharides and the secondary metabolites accumulation in certain plant tissues especially in recalcitrant plants or from plant reserve-rich organs such as fruits and seeds (Zamboni et al., 2008). These contaminants causes the RNA to be susceptible to RNase degradation as contaminants co-purify and co-precipitate with RNA in the presence of alcohols during extraction, remain in the final extract and interfering in the downstream applications (Salzman et al., 1999). Furthermore, plants under abiotic or biotic stress can enhanced the difficulty in plant RNA extraction because of the biosynthesis and accumulation of secondary metabolites (Winkel-Shirley, 2002; Chaves et al., 2003). No standardize method of RNA isolation can be used in all plant species due to the presence of varieties and expressive quantities of secondary metabolites. Extensive modifications of extraction protocols are required. Nevertheless, due to the advancement in biotechnology, commercial RNA extraction kits are developed and successfully extract RNA from many tissues including tissues rich in polysaccharides and polyphenols (Kiefer et al., 2000). Based on results obtained, an acceptable quality of total RNA were yielded with relative high purity and relatively free from polyphenols and polysaccharides contamination. The total RNA was then further used in RT-PCR and real time RT-PCR. RT-PCR and gradient PCR are common technique used in molecular biology analysis. In this study, two-step RT-PCR was performed followed by real time RT-PCR.

In plant stress responses studies, gene expression is commonly analyzed before and after the stress treatments. Real-time RT-PCR is a sensitive and precise method for the detection of low abundance mRNA (Nathalie et al., 2005). The results of real-time PCR were measured through relative quantification method. Meanwhile, housekeeping genes serve as an internal reference between different samples and help to normalize for experimental error. An ideal housekeeping gene should expressed at a constant level across different conditions and its

expression is assumed to be unaltered by experimental parameters (Thellin et al., 1999; Schmittgen & Zakrajsek, 2000). The common housekeeping genes are such as 18S ribosomal RNA, β -actin, cyclophilin or glyceraldehydes-3-phosphate dehydrogenase (Tong, Gao, Wang, Zhou, & Zhang, 2009). Based on the results, 18S rRNA was selected as the single reference gene in this study for normalization against the gene of interest because of its constant expression after UV treatment. The 18S and 28S ribosomal subunits are examples of commonly used internal controls (Burleigh, 2001; Gonzalez et al., 2002; Nathalie et al., 2005). When compare to other housekeeping genes, their expression level was extremely stable (Sturzenbaum & Kille, 2001). In addition, the use of 28S or 18S rRNA as internal standards for mRNA quantification studies was recommended because of its low mRNA variations and poor modification to the total RNA level (Thellin et al., 1999).

However, housekeeping gene, *matK* is not selected for further analysis because of the unstable and unidentified expression due to absence or too low abundance of the gene. Light stress could be one of the factors towards an unstable expression of *matK* gene as the *matK* RNA and protein levels are regulated by light and developmental stage (Barthet & Hilu, 2007). Study has found that maturase K has an unusual evolutionary mode and tempo for protein coding gene, including relatively high substitution rates at both nucleotide and amino acids levels (Barthet & Hilu, 2007). These evolutionary characteristic have raised questions about the function of *matK* and some researchers suggest that *matK* may not be functional in some plants (Kores et al., 2000; Whitten et al., 2000; Hidalgo et al., 2004). However, the use of *matK* as housekeeping gene is recommended for other research studies as the expression of it in different tissues, plants or others stress responses are still unknown (Keyser, Riek, & Van Bockstaele, 2004). Further research in the study of *matK* function in *O. aristatus* plant can contribute to better knowledge of the gene and further to the gene response to UV treatment.

As a result, the primer pairs, HPPR2 (target) and 18S rRNA2 (housekeeping) are selected. The optimum annealing temperature is at 50 °C and PCR product size approximately 250 bp and 235 bp respectively. The sequence results of PCR products were aligned with NCBI (BLAST) with similarity of 99%. The gene expression after UVB and UVC treatments are further discussed in the following sub-chapters.

5.2.1 HPPR gene expression after UVB and UVC treatments

In this work, the expression profiles of rosmarinic acid metabolism related gene, HPPR in response to short term UVB and UVC radiation are analyzed by quantitative real-time PCR. The real time PCR data were analysed using the Applied Biosystem 7300 SDS software, version 1.3.1. The gene expression level as calculated by the software between control and UV treated samples are calculated as RQ value or $\log_{10}$ RQ. The gene expression levels of the plants irradiated by short term UVB and UVC are compared with control plant at the same time point. The *O. aristatus* plant samples were treated with UVB and UVC for 15 min, 30 min, 45 min, 60 min and 1 hr dark recovery respectively. Triplicate UVB/UVC treatments were conducted and similar expression patterns were observed for each treatment. The information of HPPR gene involved in rosmarinic acid biosynthesis pathway was determined based on the NCBI database, from previous researches (Ellis & Towers, 1970; Petersen & Simmonds, 2003; Ogata et al., 2004; Zuliza, 2014).

Changes in HPPR gene expression was observed after UVB treatment which indicated that the plant self-protective system was initiated to tolerate with the UV light stress (Mazza, et al., 2000). The initial high level of HPPR after 15 min of UVB exposure might also be attributed to its involvement in other physiological processes such as photorespiration or NADP/NADPH cycling, which could explain the non-complete synchronicity between its expression and

rosmarinic acid accumulation (Hucherig & Petersen, 2012). Meanwhile, the results show that 1 hr of UVB light treatment on *O. aristatus* plant are efficient to up-regulate the expression of HPPR gene which in turn increases the rosmarinic acid production. For instance, short time of UVB radiation exposure (1 hr) was sufficient to induce the production of glucosinolates in *Arabidopsis* leaves and over exposure up to 12 hrs causes inhibition in the expression of glucosinolates biosynthetic genes and decreasing glucosinolate contents (Wang et al., 2011). On the other hand, HPPR expression was down-regulated with the exclusion of UVB radiation after 1 hr dark recovery which indicates the regulation of HPPR gene by UVB radiation (Morales et al., 2010).

Low expression of HPPR gene was observed after 1 hr of UVC treatment which indicated that HPPR gene was down-regulated when plants were exposed to UVC radiation. Whereas, up regulation of HPPR gene expression was found after the removal of UVC light which indicated that UVC treatments causes stress towards the plant but the gene expression recovered after the removal of UVC radiation exposure (Rai et al., 2011). The delay of UVC regulation on the expression of HPPR gene might be attributed to plant defense against the UV stress (Kunz et al., 2008; Piofczyk et al., 2015). It may be noteworthy to analyse the effect of prolonged UVC radiation to better understand the effect of UVC towards plant enzyme activity in greater detail. However, prolonged UV treatment can cause cell apoptosis and eventually cell death (Castronuovo et al., 2014).

Besides that, none of the UV treatment showed significant changes on HPPR gene expression. This indicated that the rosmarinic acid biosynthesis pathway involved complex response mechanism which requires further analyses to determine the enzyme response towards the abiotic stress, UV light. Both UV treatments showed negative expression at initial UV radiation and eventually increase after 1 hr of UV radiation exposure. The present data lead to

the assumption that short term UVB treatment of 1 hr is sufficient to up-regulate the HPPR gene expression and further analysis of UVC treatment may require for the HPPR gene expression analysis. Further investigations on HPPR protein levels and enzyme activity may be helpful to elucidate its role in the rosmarinic acid biosynthesis pathway in the plant.

5.3 Rosmarinic acid production

5.3.1 Extraction of rosmarinic acid

Orthosiphon aristatus is an important medicinal plant in Malaysia. It has been widely used as traditional herbal medicine for various ailments. The high medicinal value of *O. aristatus* appears to be related to its content of phenolic compounds, amongst which rosmarinic acid (RA) is one of the main constituent found in *O. aristatus*. Rosmarinic acid is a simple phenolic compound. Several different analytical methods were described in the literature to determine its content in Lamiaceae species such as gas chromatography, thin-layer chromatography, UV-Vis spectrophotometry and HPLC (Lamaison et al., 1991; Azmad & Ismail, 2009; Tuan et al., 2012; Petersen, 2013). RA is relatively polar, thus, it tends to be eluted from non-polar C18 column when polar solvent was used as the mobile phase.

Meanwhile, methanol was chosen as solvent due to its better selectivity for rosmarinic acid (Malahubban et al., 2013; Jamshed & Ismail, 2011). Solvent plays a crucial role in fractionation or extraction of phenolic compounds from the crude extracts. Based on Hildebrand solubility parameter approach, the difference in compound solubility value less or equal to 3.4 units are likely to be miscible (Reichardt, 2006). Polar solvent, methanol instead of water is used for extraction as the difference in solubility parameter between methanol and RA is less than water (Lau et al., 2014). RA is more dissolvable in methanol than water. It is found that the methanol:water (50:50) extract was rich in RA corresponding to the previous findings

(Jamshed & Ismail, 2011) In addition, methanol was chosen as the solvent for *O. aristatus* extraction in clinical study as the extract was found to be very effective in many pharmacological activities such as antipyretic, anti-inflammatory and analgesic effects as well as protective agent against alcohol-induced gastropathy and liver damage (Yam et al., 2007; Yam et al., 2008; Yam et al., 2009; Yam et al., 2013).

5.3.2 Rosmarinic acid content after UVB and UVC treatment

Applications of abiotic stress such as relative low UVB dose on plant in order to induce changes in plant's secondary metabolism have been widely studied. However, this is limited to UVC treatment on plants. Applications of UVC radiation on plants are mainly focused on the postharvest treatment towards the harvested fruits or vegetables.

Rosmarinic acid (RA) is crucial phenolic compound found in aromatics herbs. The rosmarinic acid profiles have been well characterized by Petersen and his research team (Petersen & Simmonds, 2003; Petersen, 2013). Rosmarinic acid content was found to 2-fold increased after UVB treatment in medicinal plant, rosemary (Luis et al., 2007). The effect of UVC radiation on rosmarinic acid production in plants is limited. Phenolic compounds exhibit several health-promoting properties such as anticancer and antioxidant properties (Holst & Williamson, 2004; Schreiner et al., 2009). Due to the beneficial properties of phenolic compounds, it was widely studied in term of its structure, medicinal value and methods to increase its production in plants species. Studies have found the increase of phenolic compounds corresponded to the antioxidant activity which phenolics moderate the effects of oxidative stress by mediating the redox status (Kumari et al., 2009). In addition, phenolic compounds can act as UV protectors due to their ability to absorb light from the range of 270 to 290 nm (Kumari & Prasad, 2013).

In this study, the impact of short term UVB and UVC radiation on rosmarinic acid synthesis in *O. aristatus* leaves were determined and discussed. For UVB treatment, all triplicate treatments showed the similar patterns of RA production. The RA content increased gradually to the highest RA produced after 45 min of UVB exposure and the amount declined after that until 1 hr of dark recovery. However, the amount of RA after 1 hr dark recovery was found to higher as compare to the control. This may due to the insufficient recovery period for the plant to be recovered. We suggested a longer recovery period may be required. Similar phenomena was observed in the study of UVB treatment on the synthesis of glucosinolate content which the glucosinolate content increase slightly at the initial stage of UVB treatment and the glucosinolate content was higher than control after dark recovery (Wang et al., 2011). For UVC treatment, fluctuation in RA accumulation was observed. However, the RA produced was higher as compare to control. This indicated that short term UVC caused stress towards the plant but the inconsistent respond towards the stress may require further study on it. However, interesting results was found that RA content increased after removal of UVC exposure which indicated the recovery process in the plant. Similar phenomena was also observed in the study of the impact of UVC radiation toward the total anthocyanin content produced in strawberry plant (Erkan et al., 2008). The synthesis of RA content in this study was inconsistent in the initial stage with significant increased after 45 min of UVC treatment and declined after that. There are differences between species on the sensitivity towards the UV radiation but there is limited information on the effect of UVC on the production of RA in *O. aristatus*.

No significant different in the RA production between UVB and UVC was found. However, the overall amount of RA produced after UVB treatment was found to be higher than UVC treatment. This can be explained that UVB may be a better elicitor to induce the RA production in *O. aristatus* plant. Each individual compounds might be differentially regulated

by UV radiation at different stages of their leaf development and also their contributions towards UV protection might also vary (Morales et al., 2010). Meanwhile, RA biosynthesis pathway involved complex mechanism and the effect of UVB and UVC radiation exposure towards the RA production in *O. aristatus* plant were limited. Further researches have to be carried out in order to have better knowledge on how UV treatments would affect the metabolism of *O. aristatus* plant in the production of RA.

5.4 Correlation between HPPR gene expression and rosmarinic acid accumulation

Rosmarinic acid biosynthesis involved two pathways with amino acids as the precursor, L-phenylalanine and L-tyrosine. It has been reported that both phenylalanine ammonia lyase and 4-hydroxyphenylpyruvate reductase, are entry point enzymes in phenylpropanoid pathway and tyrosine pathway respectively. Researches had found that they were responsible for elicitor-enhanced RA accumulation in *L. erythrorhizon* cell cultures (Mizukami et al., 1993) and in *Coleus blumei* suspension cultures respectively. (Szabo et al., 1999).

Hydroxyphenylpyruvate reductase (HPPR) is considered the key enzyme responsible to the metabolic biosynthesis of RA which reduces hydroxyphenylpyruvate to corresponding hydroxyphenyllactate in dependence of NAD(P)H (Petersen & Simmonds, 2003; Kim et al., 2004). The correlation between RA production and HPPR gene expression was first reported in the hairy roots of *S. militirrhiza* (Xiao et al., 2009). The RA production was enhanced by overexpression of the cinnamic acid 4-hydrolase and hydroxyphenylpyruvate dioxygenase genes in *S. militirrhiza* (Xiao et al., 2011). Besides in vitro methods, applying alternative strategies such as abiotic or biotic stresses towards plant could also be potential ways to enhance the production of secondary metabolites in plants (Xiao et al., 2011; Khojasteh, et al., 2014; Ejtahed et al., 2015)

Flavonoids and hydroxycinnamic acids are reported to be the two main groups of phenolics that acts as sunscreen in leaf epidermis to protect the inner cells from harmful radiations (Bassman, 2004). In addition, rosmarinic acid, caffeic acid and narigin are also efficient radical scavengers where the scavenging efficiency was greatly increased by high UVB exposure in rosemary plant (Zhang et al., 2009). Since the RA biosynthesis was found to be controlled by the HPPR gene encoding enzyme in the metabolites pathways (Xiao et al., 2011), this study aims to determine the correlation between HPPR transcript accumulation and rosmarinic acid profiles in *O. aristatus* leaves.

In this study, *O. aristatus* plants were treated with short exposures of UVB and UVC and the profiles generated were analysed. Negative correlation between HPPR gene expression and RA production was found after both UVB and UVC treatments. The increase in HPPR gene expression results in co-suppression effect with the reduced of RA levels. The findings were contrast with previous research findings which found that when HPPR gene was overexpressed, the rosmarinic acid production was also induced (Xiao et al., 2011; Ejtahed et al., 2015). The difference may due to the different nature or behavior of plant and especially after stress applied. Most of the previous studies was conducted by using in vitro methods with addition of elicitors to induce the production of secondary metabolites. Whereas, a raw plant samples may have different behavior responding to the abiotic stress applied due to the complexity of plant mechanism. Meanwhile, the complexity was extended to the UV-mediated gene regulation which was demonstrated in the study of *A.thaliana* plant against chronic and low fluencies rates of UVB light exposure. The research found that under these condition, several phenylpropanoid pathway genes were down-regulated although the polyphenol content had increased in the plant; chlorophyll biosynthesis genes expression was up-regulated but the chlorophyll-bindings proteins down-regulated and many genes related to stress responses were

also down-regulated (Hectors et al., 2007). According to Killian et al. (2007), the complex response pattern may contribute the fact that some UV-induced genes are only transiently expressed and the others remain activated for long periods.

In addition, similar correlation pattern was also observed in *S. militiorrhiza* after UVB treatment which PAL expression was abundant in the roots with low content of RA (Hu et al., 2009). Apart from that, UVB treatment was also found to negatively affect tea plant culture growth and size of callus forming cells, via induction of phenolic and flavans metabolites in the tea plants (Zagoskina et al., 2003). Whereas, similar phenomena were found in the study of total anthocyanin production in strawberry plant after UVC treatment which fluctuation in secondary metabolites was observed with down regulation of the gene responded. Based on the above information, it is suggested that the biosynthesis of rosmarinic acid in plants is controlled by a complex mechanism. Nevertheless, in this study, the results showed that rosmarinic acid content was increased with short term of UVB treatment. Further studies on the rosmarinic acid biosynthesis pathway especially in tyrosine pathway might provide better knowledge on how the enzyme interacts with each other to produce the final product.

CHAPTER 6

CONCLUSIONS

Recently, the depletion of stratospheric ozone layer has catalyzed and lead to more solar ultraviolet radiation to reach the earth's surface. UV radiation acts as an environmental stress which triggers various responses in the growth and development of plants. The study of plant stress responses is a vital issue today in view of the threat from global warming. Despite advancement in synthetic chemistry, plants still contribute as an important resource for the pharmaceuticals industries and other compounds of economic importance. Secondary metabolites produced by plants are important compounds particularly in pharmaceutical field due to its therapeutic value and act as a plant defense compounds. Meanwhile, UV radiation is one of the environmental stresses which can stimulate the production of secondary metabolites.

O. aristatus is a well-known remedy for various health disorders. Malaysia with its tropical climate of high temperature and rainfall provides a suitable environmental condition for plant which flourish extensively and making it easy for commercial cultivation. Owing its beneficial health properties to the predominant phenolic compound, rosmarinic acid, therefore alternative method to increase rosmarinic acid production is necessary. Thus, this work investigated the effect of short term UV treatment towards rosmarinic acid production in *O. aristatus*.

In summary, the key findings of this study include firstly, the ability to increase secondary product, rosmarinic acid, within one hour of UVB radiation exposure and slight increment after UVC radiation exposure as compare to control. We suggested that UVB light exposure may be a better stress inducer to enhance rosmarinic acid production than UVC light exposure.

Second finding of the project relates to the correlation between levels of rosmarinic acid produced and HPPR gene expression. Negative correlation was observed in both UVB and UVC treatments, which are varied from other findings. These differences can be explained by the complexity of rosmarinic acid biosynthesis pathway and different sensitivity of plant towards UV light exposure. Our work clearly has some limitations. Nevertheless, we believe our work could serve as a base or starting point for future research study on the UV stress tolerance response in *O. aristatus* plants.

In addition, we hope that our findings will be useful particular for *O. aristatus* plants' breeders as secondary products in these plants have been proven to exert beneficial effects and these products can be induced via exposure under UV lights which are commercially available and cost affordable. Our results had also provided evidence that short term of UVB and UVC radiation exposure could affect the biosynthesis of RA. Nevertheless, further studies such as on the different duration of UV radiation exposure, genes related to rosmarinic acid biosynthesis and field experiments instead of laboratory condition, would provide better information or alternative to improve crop quality and increase the use of medicinal plants in pharmaceutical industries.

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APPENDICES

Appendix I

Nucleotide sequence of hydroxyphenylpyruvate reductase (HPPR) gene from *Orthosiphon aristatus*, linear DNA, 924 bp (Ahmad et al., 2015)

```
1      ATGGATGCCA TCAGCGTTCT GATGATGTGC CCCATGAGCG ACTACTTGGA GCAAGAGCTC
61     GACAAGCGCT TCAAGCTCTT CCGTTACTGG ACTCAGCCGC AGTTTCCGGC CGAGTCAATC
121    CGCGCAGTGG TCGGTAACTC CACCGCCGGC GCCGACTCCG ACTTGATCGA TGAAGTCCCG
181    AAATTGGAGA TTGTATCTTG CTATAGCGTG GGTCTCGATA AAGTCGACTT GATCAAGTGC
241    AAGGAGAAGG GGGTTAGGGT TACCAACACG CCCGATGTGC TGACGGATGA CGTGGCGGAT
301    TTGGCTATCG GGTTGATGTT GGCTGTTTTG AGGCGGATTT GTGAGTGTGA TAAGTATGTG
361    AGGAGTGGGG CGTGGAATTT TGGCGACTTC AAGTTGACGA CTAAGTTCAG CGGCAAAAGA
421    GTTGGTATCA TTGGATTGGG CAGAATTGGC TTAGCAGTTG CTGAGCGAGC AGATGCATTT
481    GATTGTCCCA TAAATTACTA CTCAAGATCC AAGAAACCCA ACACAAACTA CAAGTACTAC
541    AACAGCGTTG TTGAATTGGC GAGGAACAGT GACATCCTAG TCGTAGCATG TGCCCTGACT
601    CCAGAAACAA CTCACATTGT GAATCGAGAA GTAATCGATG CATTGGGTCC AAAGGGAATT
661    CTCATCAACA TCGGAAGGGG ACCTCATGTC GATGAAACAG AGTTGGTGTT AGCTCTTGTTG
721    GAGGGCCGTC TGGGTGGCGC GGGTCTTGAT GTCTTCGAAA GGAACCGGA GGTACCGGAA
781    CAGCTCTTTG GGCTCGAGAA TGTTGTCCTG TTGCCACATG TAGGGAGTGG CACTGTGGAA
841    ACGCGCAAGG TTATGGCTGA CCCTGTTCTG GGAAATTTGG AAGCTCACTT TTCCAGCAAG
901    CCTCTGTTAA CTCCTGTGGT TTGA
```

Appendix II

Nucleotide sequence of truncated maturase K (matK) gene from *Orthospion stamineus*, complete cds, 862 bp (GenBank: JN119569.1, NCBI)

```
1      TTTTACATT TAAATTTTGT GTTAGGATAT ACTAATACCT CACTTTGTCC ATGTGGAAAT
61     CTTGATTCAA ACTCTTCGCT ATTGGGTAAA AGATGTTTCT TCTTTGCATT TATTACGAGT
121    TTTTCTCAAC CAATATTGTA GTCTTATTAC TTCAAAGAAA GTCAGCTCCT CTTTGTCAAA
181    AAGAAATCAA AGATTCTTTT TTTTCTTATA TAATTCTCAT GTATGTGAAT ACGAATCTAT
241    TTTCGTCTTT CTACGTAACC AAACCTTTTCA TTTACGATCA ACATCTTCTG GAGTTCTTCT
301    TGAACGAATC TATTTTTTATA TAAAAATAGA ACGTCTTGTG AACGTCTTTG TTAAGGACTT
361    TCGGGCGAAC CTATGGTTGG TCGAGGAACC CTGCATGCAT TATATTAGGT ATCAAGGAAA
421    ATCCATTCTG GCTTCAAAAG GGACATCTCT TTTTCATGAAT AAATGGAAAT TTTACCTTGT
481    CACTTTTTTGG GAATGGCATT TTTTGGTGTG GTTTCATCCA AGAAGGATTT GTATAAACCA
541    ATTTTCCAAG CATTCCCTTG AAATTTTTTGG CTATCTTTCA AACGTGCAAA CGGGCCCTTC
601    CGTGGTACGG AGTCAGATTT TAGAAAATGC ATTTCTAATC AATAATGCTA TTAAGAAGCT
661    CGATACCCCT TGTTCCAATT ATTCTCTGA TTGCGAAATT GGCTAAAGAG AAATATTTGT
721    AACGTATTGG GGCATCCCAT TAGTAAGCCG ATTTGGGCTG ATTTATCAGA TTCTAATATT
781    ATTGACCGAT TTGGGCGTAT ATGCAGAAAT ATTTCTCATT ATCATAGCGG ATCTTCCAAA
841    AAAAAGAATT TGTATCGAAA AA
```

Partial nucleotide sequence of 18S ribosomal RNA gene (18S rRNA) gene from *Orthospion aristatus*, 749 bp (GenBank: JX974580.1, NCBI)

```
1      ACTGTGAAAC TGCGAATGGC TCATTAAATC AGTTATAGTT TGTTTGATGG TAATTGCTAC
61     TCGGATAACC GTAGTAATTC TAGAGCTAAT ACGTGCACCA AACCCCGACT TCTGGAAGGG
121    ACGCATTTTAT TAGATAAAAG GTCGACACGG GCTCTGCCCG TTGCTGCGAT GATTCATGAT
181    AACTCGACGG ATCGCACGGC CTTTGTGCCG GCGACGCATC ATTCAAATTT CTGCCCTATC
241    AACTTTTCGAT GGTAGTGTAG TGGCCTACCA TGGTGGTGAC GGGTAACGGA GAATTAGGGT
301    TCGATTCCGG AGAGGGAGCC TGAGAAACGG CTACCACATC CAAGGAAGGC AGCAGGCGCG
361    CAAATTACCC AATCCTGACA CGGGGAGGTA GTGACAATAA ATAACAATAC CGGGCTCTTA
421    GAGTCTGGTA ATTGGAATGA GTACAATCTA AATCCCTTAA CGAGGATCCA TTGGAGGGCA
481    AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTTA AGTTGTTGCA
541    GTTAAAAAGC TCGTAGTTGG ATTTTGGGTT GGGTTCGGCCG GTCCGCCATT CGGTGTGCAC
601    CGGTCTGCTC GTCCCTTCTG CCGGCGATGC GCTCCTGGTC TTAAGTGGCC GGGTCTGTGCC
661    TCCGGCGCTG TTAATTTGAA GAAATTAGAG TGCTCAAAGC AAGCCTACGC TCTGGATACA
721    TTAGCATGGG ATAACATTAT AGGATTTTCG
```

Appendix III

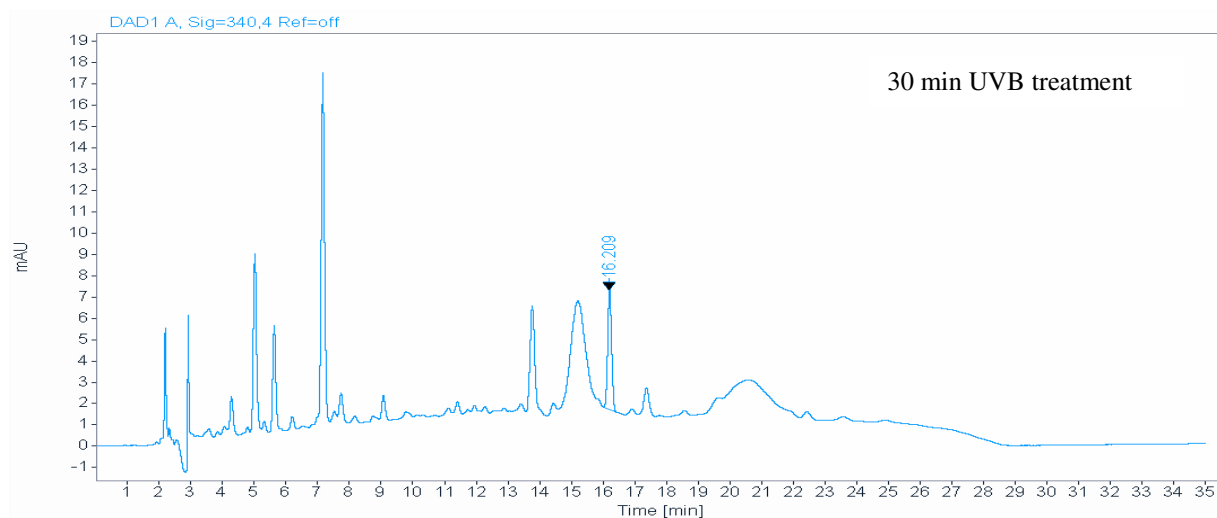
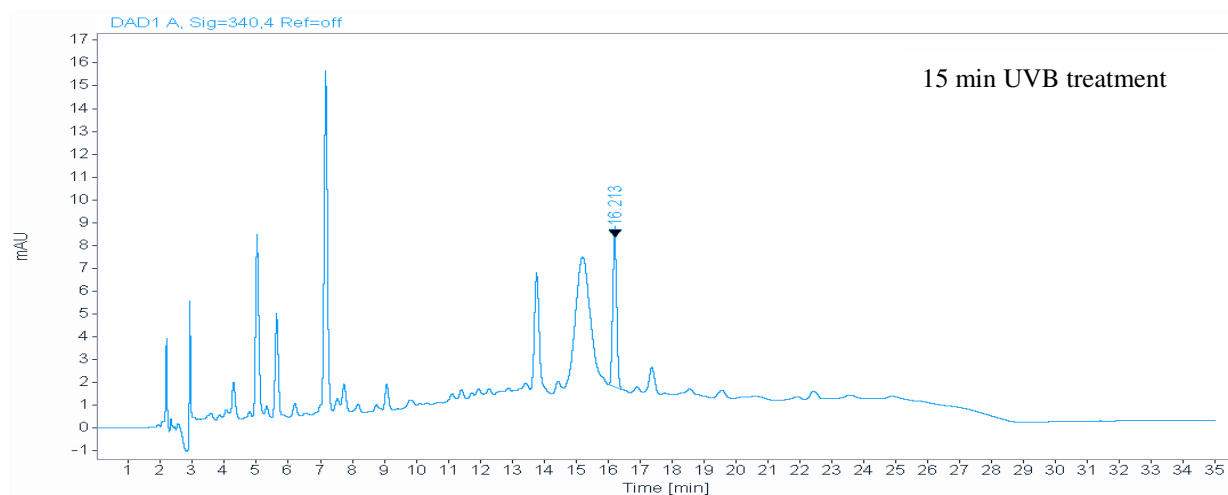
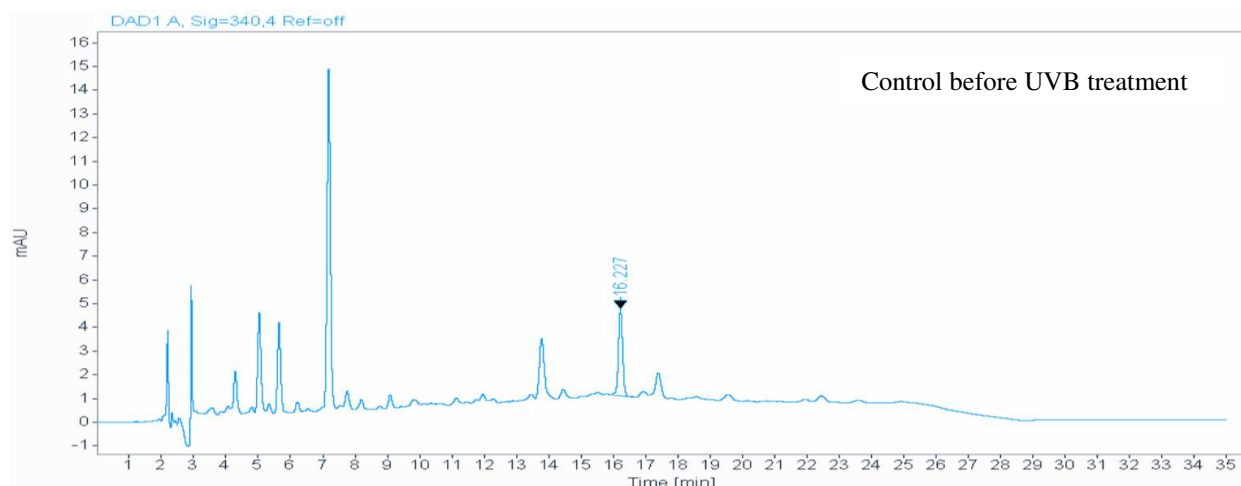
Summary of absorbance ratio, A260/280 and A260/230 from RNA of *Orthosiphon aristatus* leaves samples before and after UVB/UVC treatment.

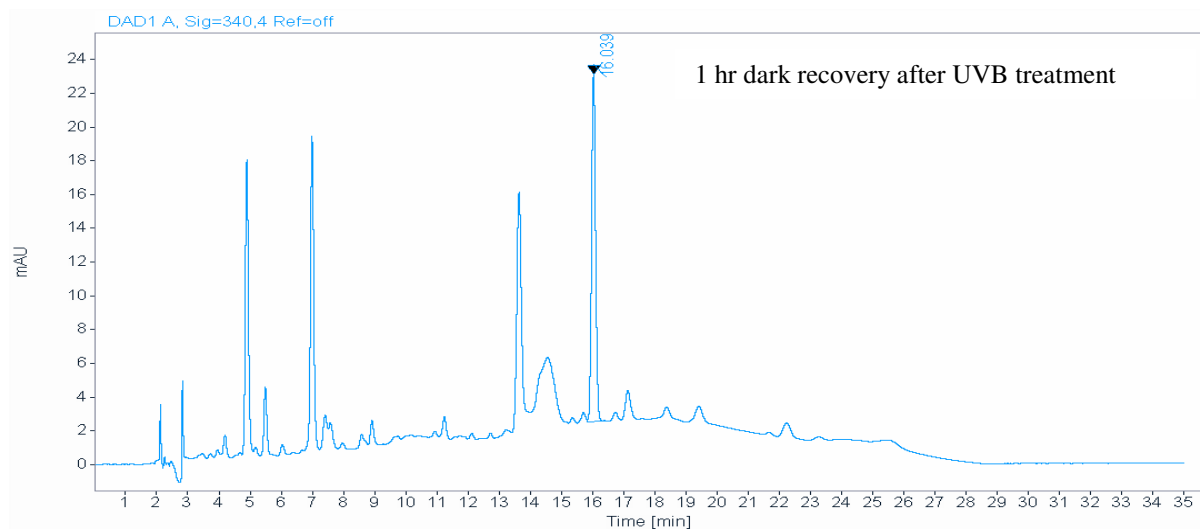
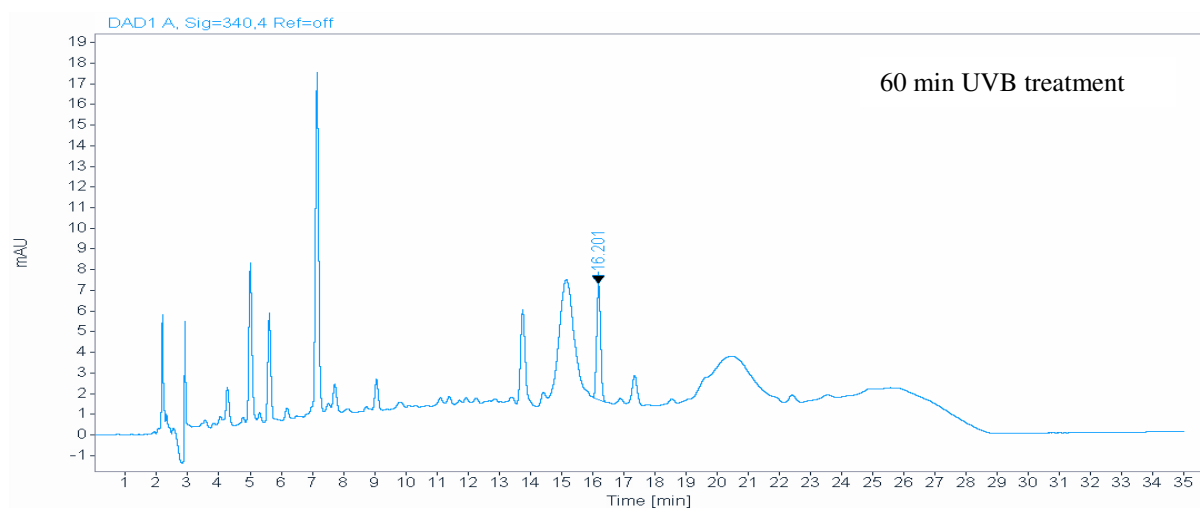
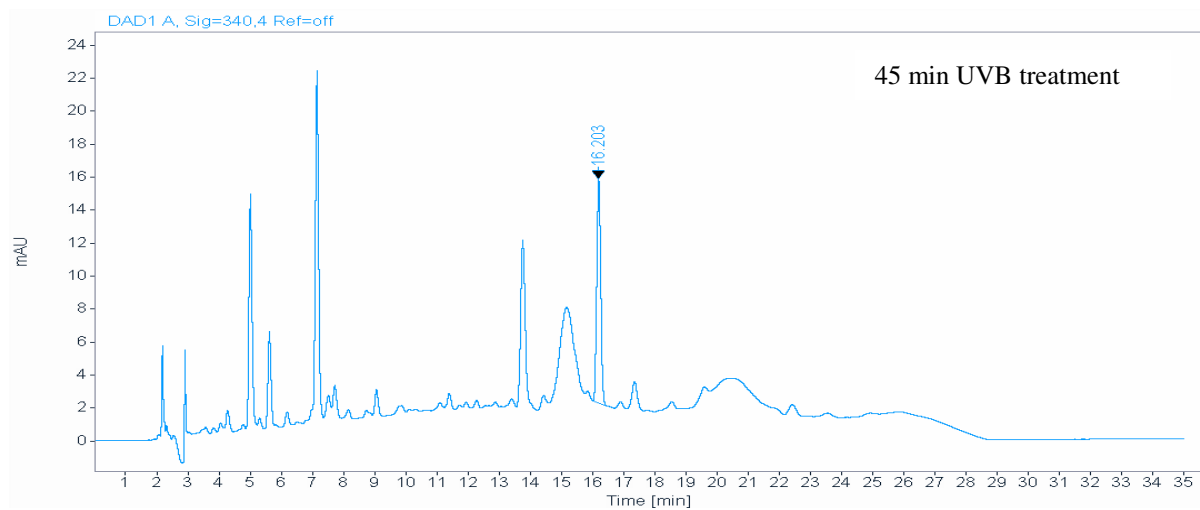
Treatment	Time	A260/280 ratio	A260/230 ratio
UVB 1	Control	1.983	2.070
	15 min	1.783	2.103
	30 min	1.986	2.012
	45 min	1.898	1.999
	60 min	1.988	1.968
	1 hr dark	1.848	2.013
UVB 2	Control	1.898	2.301
	15 min	2.000	2.100
	30 min	1.988	2.000
	45 min	1.887	1.986
	60 min	2.011	2.111
	1 hr dark	1.969	1.987
UVB 3	Control	2.121	2.119
	15 min	2.011	2.264
	30 min	1.786	1.989
	45 min	2.011	1.999
	60 min	1.959	2.105
	1 hr dark	2.000	2.121

Treatment	Time	A260/280 ratio	A260/230 ratio
UVC 1	Control	1.968	1.798
	15 min	1.986	2.000
	30 min	1.999	2.135
	45 min	1.798	1.898
	60 min	2.011	1.999
	1 hr dark	1.769	2.011
UVC 2	Control	2.199	2.168
	15 min	1.898	1.997
	30 min	2.015	1.989
	45 min	1.997	2.201
	60 min	1.986	2.001
	1 hr dark	1.999	2.138
UVC 3	Control	1.998	2.133
	15 min	1.798	2.203
	30 min	1.799	1.988
	45 min	2.008	2.169
	60 min	1.978	1.998
	1 hr dark	1.896	2.301

Appendix IV

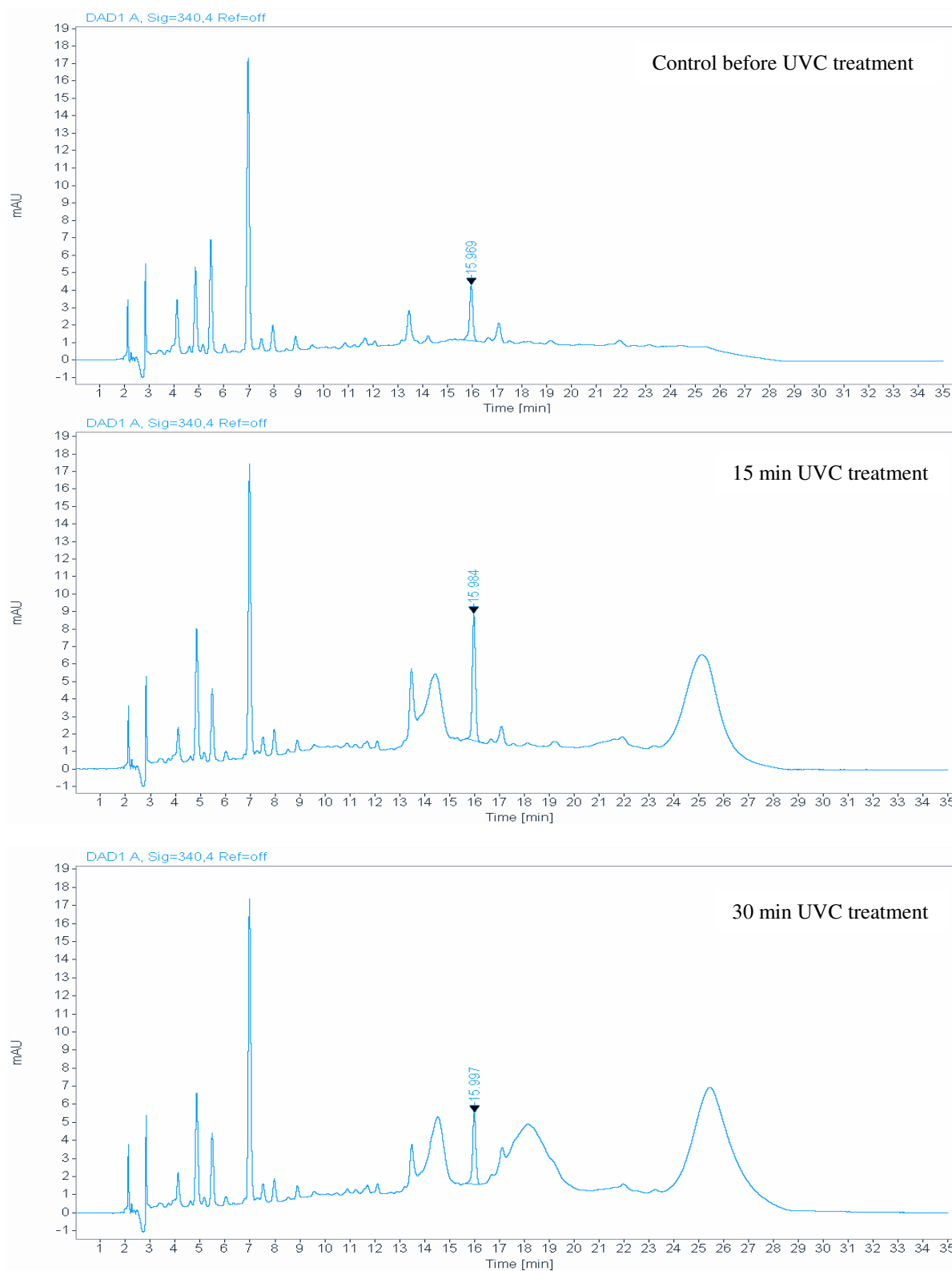
HPLC chromatograms of *Orthosiphon aristatus* samples after each time point of UVB treatment. Data information were generated and obtained from Agilent ChemStation software.

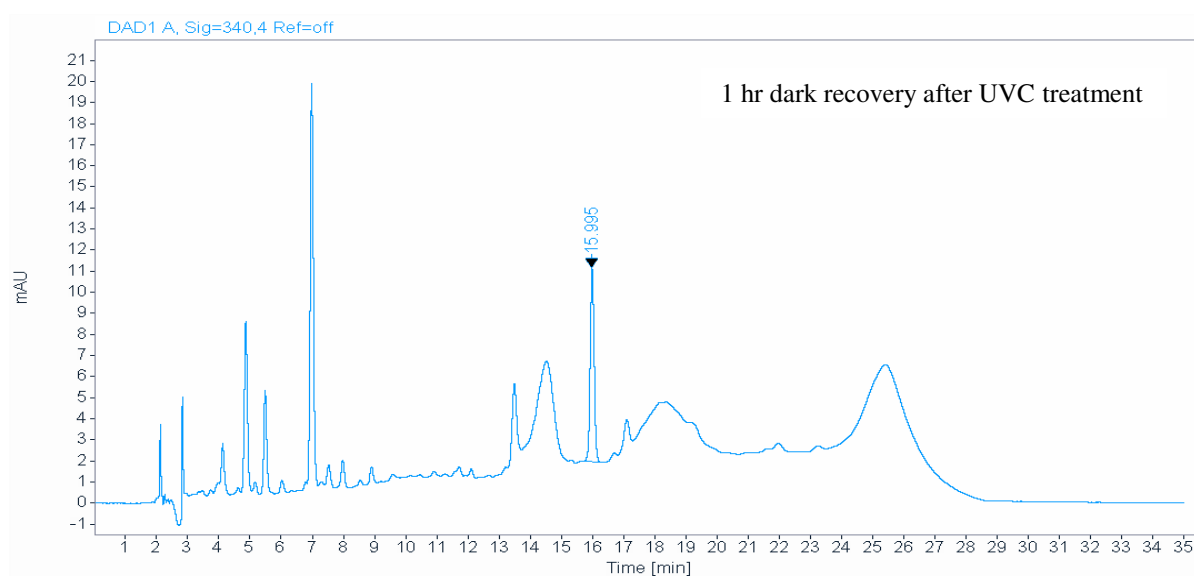
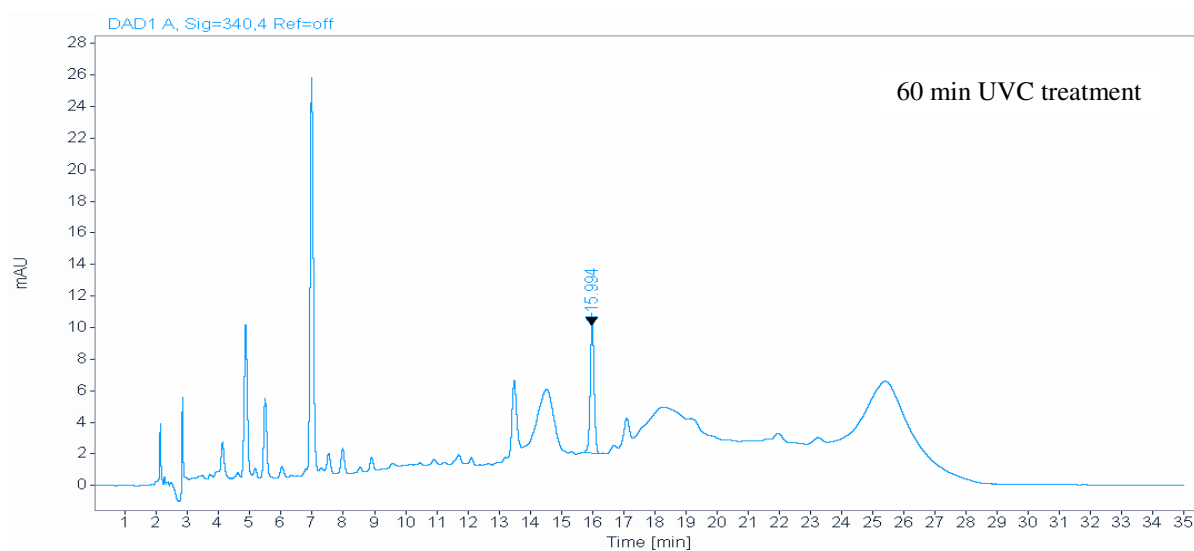
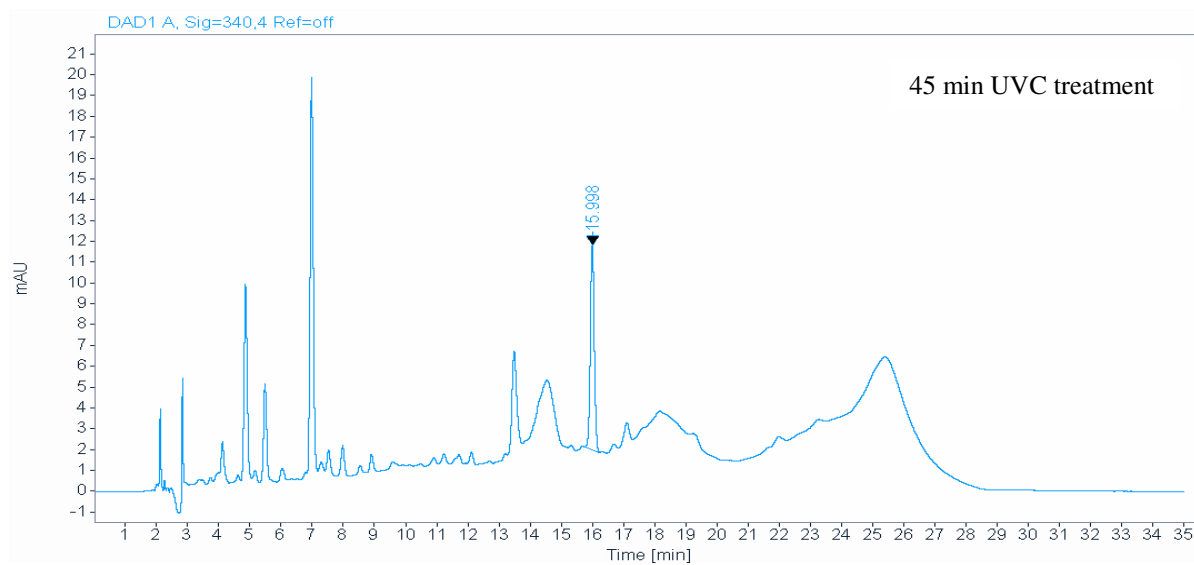




Appendix V

HPLC chromatograms of *Orthosiphon aristatus* samples after each time point of UVC treatment. Data information were generated and obtained from Agilent ChemStation software.





Appendix VI

Summary of relative expression of HPPR gene and changes in RA production after different time-point of UVB treatment. Data information were generated and obtained from Applied Biosystem 7300 SDS software and Agilent ChemStation software respectively.

Treatment	Time	Average RA (µg/mL)	Std	RQ	Log 10(RQ)
UVB 1	Control	1.6590	0.0346	1	0
	15 min	1.6655	0.0888	1.990	0.299
	30 min	2.7962	0.0916	0.516	-0.287
	45 min	4.3307	0.0973	0.475	-0.323
	60 min	2.9425	0.0948	2.913	0.464
	1 hr dark	2.5992	0.0269	0.477	-0.321
UVB 2	Control	0.4961	0.0483	1	0
	15 min	0.7933	0.0979	0.087	-1.060
	30 min	0.6986	0.0541	0.078	-1.108
	45 min	1.8005	0.0520	0.113	-0.947
	60 min	0.7396	0.0787	0.130	-0.886
	1 hr dark	2.8616	0.0598	0.12	-0.921
UVB 3	Control	0.4801	0.0334	1	0
	15 min	0.7104	0.0730	0.538	-0.269
	30 min	0.6090	0.0959	0.814	-0.089
	45 min	1.1057	0.0936	0.990	-0.004
	60 min	0.9786	0.0711	0.491	-0.309
	1 hr dark	1.5143	0.0341	0.605	-0.218

Appendix VII

Summary of relative expression of HPPR gene and changes in RA production after different time-point of UVC treatment. Data information were generated and obtained from Applied Biosystem 7300 SDS software and Agilent ChemStation software respectively.

Treatment	Time	Average RA ($\mu\text{g/mL}$)	Std	RQ	Log 10(RQ)
UVC 1	Control	0.3515	0.0010	1	0
	15 min	0.6164	0.0202	0.163	-0.788
	30 min	0.6601	0.0488	0.276	-0.559
	45 min	0.5281	0.0334	0.516	-0.287
	60 min	0.5090	0.0479	0.384	-0.416
	1 hr dark	0.7437	0.0632	0.557	-0.254
UVC 2	Control	0.5312	0.0647	1	0
	15 min	0.5653	0.0494	0.839	-0.076
	30 min	0.4355	0.0135	1.128	0.052
	45 min	0.4743	0.0736	0.569	-0.245
	60 min	0.5591	0.0646	0.304	-0.517
	1 hr dark	0.5198	0.0493	0.508	-0.294
UVC 3	Control	0.4147	0.0100	1	0
	15 min	0.8836	0.0287	0.905	-0.043
	30 min	0.5960	0.0743	0.131	-0.883
	45 min	1.2271	0.0678	0.093	-1.0315
	60 min	0.9761	0.0390	0.368	-0.434
	1 hr dark	1.1504	0.0104	2.292	0.360