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Effects of Short Term UVB and UVC Irradiation on Hydroxyphenylpyruvate Reductase Expression and Rosmarinic Acid Accumulation in *Orthosiphon aristatus*

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ABSTRACT

Ultraviolet (UV) light is one of the abiotic stresses experienced by plants. Generally, UV irradiation can affect the growth and development of plants and radiation above ambient level can cause the activation of plant stress responses through the self-protective secondary metabolism system. One of the products of these responses is rosmarinic acid, which is an important secondary metabolite in the medicinal plant, *Orthosiphon aristatus*. Rosmarinic acid exhibits many pharmacological and biological properties such as antioxidant, anticancer, antiviral, antibacterial, and anti-inflammatory activity, as well as other health promoting properties. The biosynthesis of rosmarinic acid involves two derived pathways, which are phenylpropanoid, and tyrosine-derived pathways. Hydroxyphenylpyruvate reductase (hppr) is an enzyme involved in the tyrosine-derived pathway. In this study, we investigated the effects of short-term UVB and UVC treatments on the biosynthesis of rosmarinic acid and *hppr* gene expression. High performance liquid chromatography and quantitative real time polymerase chain reaction were used. The results showed that rosmarinic acid content increased within one hour of both UVB and UVC exposures, and declined after one-hour post-exposure. On the other hand, *hppr* expression was down regulated upon UVB and UVC treatments. Meanwhile, the expression of *hppr* showed a negative correlation to rosmarinic acid accumulation.

Keywords: *Orthosiphon aristatus*; Rosmarinic acid; Hydroxyphenylpyruvate reductase; UVB; UVC

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1. Introduction

Depletion of the stratospheric ozone layer has caught the attention of the world. It has led to the increase of atmospheric greenhouse gases, solar ultraviolet (UV) light penetration, and global climate change. These environmental stresses such as extreme temperatures, drought, salinity, UV radiation, and pathogen spread are potentially harmful to plant communities [1]. Increased UV light is one of these abiotic stress factors, which can affect the production of secondary metabolites [2]. UV light is one component of solar radiation; most of this radiation penetrates through the ozone layer and reaches the earth's surface without a significant biological impact on living organisms due to the absorption of the UV component by the ozone layer. UV light is categorized into three types based on wavelength: UVA, UVB, and UVC. The wavelength of UVA is between 315-400 nm which is the longest; UVB about 280-315 nm, and UVC has the shortest wavelength, 200-280 nm [3]. In the past, UV radiation was generally known to have negative effects on plant productivity [4]. However, it has been discovered that low doses of UVB exerts a positive impact on medicinal plants by stimulating the accumulation of secondary metabolites [3] and increasing glycyrrhizin content in *Glycyrrhiza uralensis* after high intensity and long term exposure of UVB [5]. Zu and co-workers in 2010, showed an increase of taxol content after UVB exposure in the plant *Taxus chinensis* L. [6], meanwhile Kim and co-workers reported a positive response of *Melissa officinalis* to UVB irradiation by increasing the production of phenypropanoid related metabolites [7]. The defense or stress tolerance to UVB radiation are associated with the triggering of various signal transduction pathways, activation of DNA repair mechanisms, and generation of secondary metabolites [8, 9]. Secondary metabolites such as flavonoids, alkaloids, and lignin are compounds that provide UV protection for plant species [10]. These compounds display UVB absorption bands, which serves to protect the plant genetic material [10-11]. Meanwhile, UVC light has a higher negative impact on living organisms due to its relatively short wavelength that has higher energy. High dosage of UVC light is considered to be harmful to living organisms, causing cell damage and eventual cell death [12]. Kara in 2013 reported that seedling height, total chlorophyll, chlorophyll a and b of *Phaseolus vulgaris*, were reduced after UVC radiation exposure, adversely affecting the growth and photosynthesis of the bean plant [13]. Other findings on UVC exposure and its effects on plants include increasing secondary metabolites in potato, buckwheat, and brassica [14-15] as well as increasing levels of secondary metabolites such as carotenoids, anthocyanins, and flavonoids in durum wheat leaves and derivatives of boswellic acid [16-17]. Although currently the synthetic chemistry industry is advanced, it remains that plant secondary metabolites, especially from medicinal plants and herbs, are still important economically and pharmaceutically.

Orthosiphon aristatus, locally known as *misai kucing*, can be found in the region of South East Asia and is one of the many local herbs used as herbal medicine [18]. More than twenty phenolic compounds have been isolated from this plant which exhibit high anti-oxidant properties [19]. Previous studies have found that *O. aristatus* possesses many pharmacological and biological properties such as diuretic effects [20-21], hepatoprotective effects [22], anti-diabetic effects [23], action as a gastro-protective agent [24], antimicrobial effects [25-26], anti-inflammatory effects [27] and others. Rosmarinic acid is a caffeic acid ester of 3-(3,4-dihydroxyphenyl)lactic acid, found abundantly in the plant families of Boraginaceae and Lamiaceae [28]. It is also one of the main compounds found in *O. aristatus*, which has high medicinal value due to its biological activities and

pharmacological properties such as antioxidant, antimicrobial, anticancer, antiviral, antibacterial, anti-inflammatory, and antiphlogistic effects making it a valuable compound in the food, pharmaceutical, and cosmetics industries [29-33]. Rosmarinic acid is biosynthesized using a hydroxycinnamoyl donor and acceptor derived from the phenylpropanoid and shikimate pathways respectively. Its biosynthesis has been widely investigated and characterized in the past by Petersen and his team [28, 34, 35]. Meanwhile, hydroxyphenylpyruvate reductase (hppr) is an oxidoreductase involved in rosmarinic acid biosynthesis via reduction of hydroxyphenylpyruvate to hydroxyphenyl-lactates in dependence of NAD(P)H [34]. Due to the importance of rosmarinic acid and the involvement of hppr in its biosynthesis, research has been undertaken to increase the production of rosmarinic acid, either through chemically-synthesized means or by fermentation of suspension cultures to produce large scale quantities of metabolites [36]. However, the regulatory role of the enzymes involved in rosmarinic acid biosynthesis remains unclear. Understanding the gene regulation and metabolic pathway underlying the biosynthesis of active plant compounds will allow us to improve the production of these desired compounds. Hence, the present study investigated the effects of UVB and UVC irradiation on the accumulation of rosmarinic acid, and the correlation between *hppr* gene expression and rosmarinic acid accumulation after short-term UVB and UVC exposure.

2. Materials and Methods

2.1 Plant materials and UV treatment

The *O. aristatus* plants for this study were cultivated in a greenhouse at Universiti Malaysia Sarawak. Twenty-week old *O. aristatus* was used for exposure to UV. The plants were pretreated in darkness for a period of 24 hours prior to UV treatment and were then transferred to the modified exposure chamber with a UVB lamp (TL 20 W/01 RS, Philips, Holland) at a distance of 20 cm above the plant for a fixed period of time [37]. The plant was exposed to UVB light for a fixed period of time and leaf samples were analyzed at multiple time-points: 0 min (control), 15 min, 30 min, 45 min, 60 min, and 1 hour dark recovery. Meanwhile, the UV dosage at each time point was measured using UV Fastcheck strips (UV Process Supply, USA). Leaves were collected randomly at each time point and immediately frozen with liquid nitrogen. The samples were stored at -80 °C for further analysis or freeze-dried for RNA isolation and rosmarinic acid extraction. For analysis, the harvested leaf samples were ground into powder with a mortar and pestle, and then divided into two portions; one for gene expression analysis and the other for HPLC analysis.

2.2 Gene expression analysis

For gene expression analysis, total RNA was isolated using a plant total RNA mini kit (Geneaid, Taiwan). The RNA purity and integrity were verified by agarose gel and quantified using an Ultrospec 1100 pro spectrophotometer (Amersham Biosciences, USA). The samples with a A260/280 absorbance ratio between 1.8 to 2.1 were selected for further analysis. The total RNA was also purified by DNase I treatment (Fermentas, Lithuania) prior to the reverse transcription process. A two-step real-time PCR (RT-PCR) was performed, wherein first strand cDNA was synthesized using M-MuLV Reverse Transcriptase (Fermentas, Lithuania). Quantitative RT-PCR was performed using a Real-time PCR Applied Biosystem 7300 (ThermoFisher, USA). The target nucleic acid was quantified through the relative quantification method. RT-PCR primers were designed using PrimerQuest software (Integrated DNA Technologies) based on the sequences of the maturase K gene (*matK*) (accession number JN119569.1) and the *hppr* gene [38]. The primers used for

the target gene *hppr* were (F: 5'-GCGGATTTGTGAGTGTGATAAG-3'; R: 5'-GACTAGGATGTCACTGTTCCTC-3'), with a product size of 250 bp. Meanwhile, primers for endogenous gene was 18s rRNA primers, (F: 5'-GAACGTCTTGTGAACGTCTTTG-3'; R: 5'-CCGTTTGCACGTTTGAAAGATA-3'), with a product size of 235 bp.

Quantitative RT-PCR reactions were carried out using the Maxima SYBR qPCR green master mix (Thermo Scientific, USA) which includes cDNA template, Maxima SYBR qPCR Green master mix (Thermo Scientific, USA), *hppr* forward primer or 18S rRNA gene, *hppr* reverse primer or 18S rRNA gene, ROX solution, and nuclease free water. Thermal cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles for 95°C for 15 s and 60°C for 1 min, according to manufacturer's protocol. All samples were analyzed in triplicate with the same RNA preparation and reverse transcription. The differences in gene expression level between control and UV treated samples were calculated as RQ value or log10 RQ by the software.

2.3 Extraction and high-performance liquid chromatography analysis

Powdered leaf samples of 0.25 g were extracted with 10 mL of 50% methanol in water for 1 hour at 60°C with agitation. After centrifugation, the supernatant was filtered by using a 0.45 µm poly filter (Acrodisc Syringe Filters, USA) and analyzed by HPLC (Agilent 1260 Infinity Quaternary Liquid Chromatography, located at Clinical Research Centre, Kuching). The column used was Merck Licrochart purospher start RP18 column end-capped (250 mm, 4.6 mm i.d, 5 µm pore size). The chromatographic condition was performed based on the modified protocol from previous studies [39-40]. The detection wavelength was 330 nm with an injection volume of 20 µL. The injected sample was eluted with a gradient mobile phase comprised of phosphoric acid, eluent A (Mallinckrodt, USA), acetonitrile, and eluent B (JT Baker, Germany), at a flow rate of 1.0 mL/min at 30°C with a separation time of 35 min. The gradient used was 0-12 min, linear gradient from 15% to 25% B; 10-15 min, from 25% to 15% B, maintain at 15% B until 35 min. The concentration of rosmarinic acid in samples was calculated using a standard curve. The mean values from three independent replicates were obtained.

2.4 Quantitative analysis of rosmarinic acid

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 were 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 the rosmarinic acid standard and samples.

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). Correlation analysis was done using Pearson Correlation Coefficient.

3. Results and Discussion

3.1 Effect of UVB treatment on plant physical change

UV radiation has been shown to cause alterations in plant's physiological and biological processes, and even cause morphological changes. The response of plants to UV light includes their UV-absorbing sunscreens and the activation of antioxidant defenses [41]. In this study, *O. aristatus* was exposed to UVB and UVC radiation for fixed time periods (15, 30, 45,

and 60 min) with a UV dosage of 100 mJ/cm^2 measured by UV Fastcheck strips (UV Process, USA). In the UVB treatment, no significant changes were observed for leaf morphology. On the other hand, one hour of UVC treatment had caused significant leaf shrinkage and the development of a yellowish color (data not shown).

The current knowledge regarding the eco-physiological impacts of UV irradiation of plants have been studied using natural, or slightly higher than natural, levels of UV radiation on field analyses [12]. Several studies have indicated that UVB radiation on plants has minor inhibitory effects on growth and acclimation response [42-43]. For plants exposed to UVC radiation, prolonged exposure has been shown to decrease plant growth and the shrinking and yellowing of leaves [13, 44]. Despite the various reports on UV irradiation of plants, thus far no information is available on the effects of UVB and UVC on *O. aristatus*. Our work indicates that short-exposure time and low radiation levels allowed the plants to partially maintain their normal physiological status without any adverse damage. For this reason, a maximum of one-hour of exposure to both UVB and UVC radiation was selected, after which the plants were allowed to recover in dark.

3.2 UVB treatment

Sufficient and acceptable qualities of total RNA from *O. aristatus* leaves were yielded through the use of a commercial kit. No significant RNA degradation was observed. The overall A260/280 nm ratio obtained from the RNA of UVB treated plants was in the range of 1.7-2.2. This indicated that the extracted RNA was relatively free from polyphenol and polysaccharide contamination (Fig. 1). The RNA was then used for PCR to detect the expression of the *hppr* gene along with two control genes, 18S rRNA and MatK (Fig. 2).

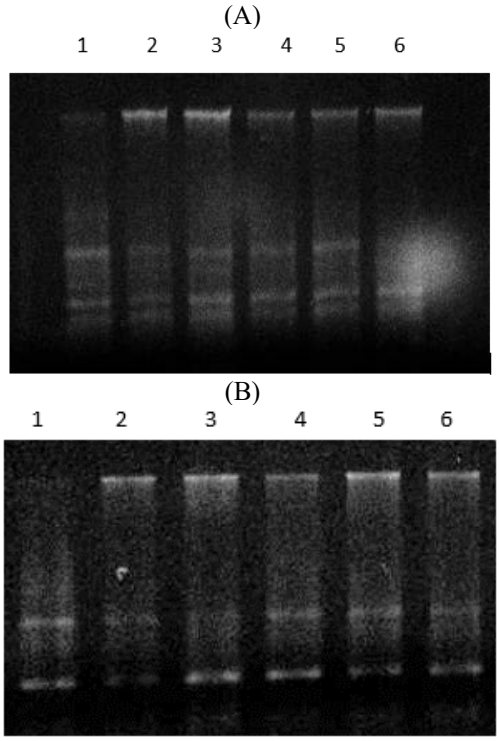

Fig. 1. Agarose gel electrophoresis of isolated total RNA from *O. aristatus* at different time points after UVB (A), and UVC (B) treatments. Lane 1 at 0 min as control, lane 2 at 15 mins, lane 3 at 30 mins, lane 4 at 45 mins, lane 5 at 60 mins, and lane 6 at 1 hr dark recovery.

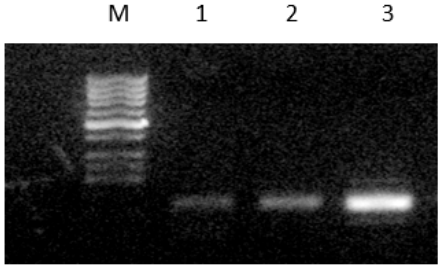

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *O. aristatus* genes. Lane 1 represents the PCR product for the *hppr* gene (~250 bp), Lane 2 is the PCR product for 18S rRNA (265 bp), and Lane 3 is the PCR product for matK (235 bp).

3.3 Determination of rosmarinic acid quantities

O. aristatus leaves were used for HPLC analysis to determine the amount of

rosmarinic acid present. The samples were extracted using 50% methanol at an extraction temperature of 60°C with agitation for one hour. A simple and effective extraction method was used in order to prevent further degradation of compounds after treatment and storage. HPLC equipped with a degasser, quaternary pump, auto sampler, and a UV/Vis DAD detector was used for analysis of rosmarinic acid content. Meanwhile, a reverse phase C18 column was used for the separation of rosmarinic acid (RA) from the crude extract of *O. aristatus*. The detection wavelength was chosen 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 of 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 Figures 3A and 3B, respectively.

The gradient method with a mobile phase comprising 0.17% phosphoric acid in water and acetonitrile was used for the RA analysis. The gradient method provides a better separation of the desired compound and a shorter elution time due to the complexity of the samples. Good separation was achieved within 20 min using the chromatographic condition with 16 minutes retention time (Figs. 4A-B). Rosmarinic acid in sample before UV treatment was determined to be 0.4961 µg/mL, which indicated that 104.25 µg/g of compound was contained in 0.25 g of *O. aristatus* leaves. These results are in contrast to previous work in *Agastache rugosa*, where rosmarinic acid was obtained from various tissues such as flowers, roots, leaves, and stems with concentrations ranging from 9.14-48.43 µg/g per 0.1 g of starting sample [39]. Meanwhile rosmarinic acid extraction from *O. Stamineus,* using different solvents from 50 g of sample was previously reported to have resulted in rosmarinic acid concentrations of 17.23 mg/g, 8.45 mg/g, and 1.1 mg/g, when using methanol, water, and ethanol, respectively [40].

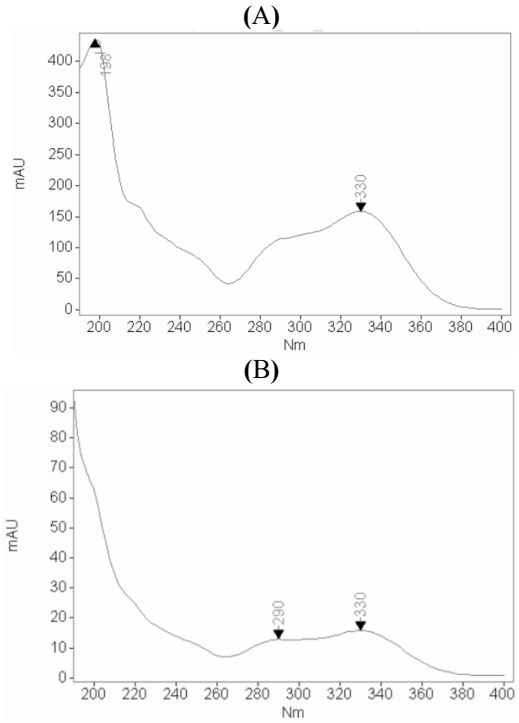

Fig. 3. Chromatogram of 20 ppm rosmarinic acid standard using Agilent ChemStation software. (A), and (B) chromatogram of rosmarinic acid identified in *O. aristatus* leaves before UV treatment.

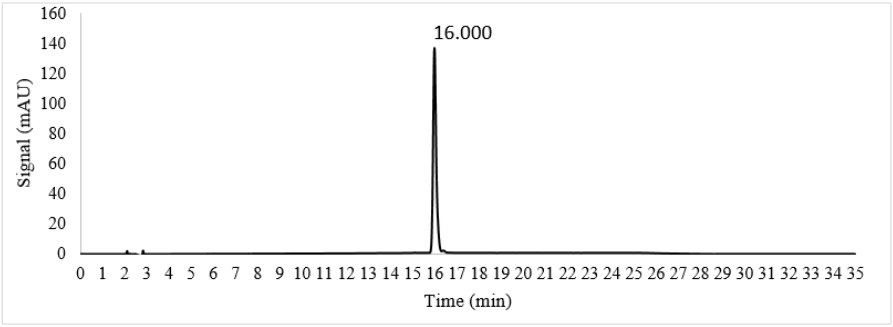

Fig. 4A. HPLC chromatogram (330 nm) of 10 µg/mL rosmarinic acid standard.

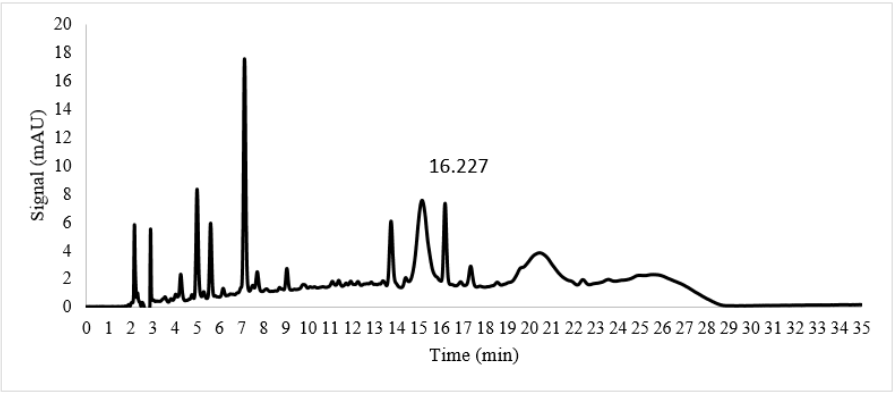

Fig. 4B. HPLC chromatogram (at wavelength 330 nm) of rosmarinic acid identified in *O. aristatus* leaves before UVB treatment and the concentration determined was 0.4961µg/mL.

3.4 Rosmarinic acid and UV exposure

Figs. 5A and 5B show the representative data of rosmarinic acid production after UVB and UVC treatments. Fluctuation in rosmarinic acid accumulation was observed after the UVB treatment. The results showed that after receiving 30 min of treatment, the rosmarinic acid content slightly increased (p<0.05) by the enhanced UVB radiation (Fig. 5A). The rosmarinic acid production increased up until reaching its maximum at 45 min of UVB treatment. The rosmarinic acid content increased significantly, with the highest concentration found being 173.23 µg/g, compared to the control's concentration of 66.36 µg/g. Rosmarinic acid production decreased after 60 min of treatment. UVB radiation was stopped after 1 hour of exposure at which point the plant was placed in the dark for one hour. The rosmarinic acid content continued to decrease after 1 hour of the dark recovery period. However, the rosmarinic acid content was found to be higher than that of the control. Similar phenomena were also observed in a study of the effect of UVB on the synthesis of glucosinolate, which found that the glucosinolate content increased slightly at the initial stage of UVB treatment and that the glucosinolate content was higher than the control after dark recovery [37].

Rosmarinic acid content at different time points under UVC radiation are shown in Figure 5B. At the first 15 min of treatment, UVC induced the production of rosmarinic acid; however, it decreased after 30 min of treatment. After 45 min of UVC treatment, the RA content increased significantly to its highest concentration of 49.084 µg/g with the control concentration

being 16.59 µg/g. Rosmarinic acid content decreased slightly after 60 min of treatment. UVC radiation was stopped after 1 hour at which point the plants were placed in darkness for 1 hour. Interestingly, the rosmarinic acid content increased after 1 hour of dark recovery. For UVC treatment, a fluctuation in rosmarinic acid content was observed. However, rosmarinic acid content increased after stopping UVC treatment, which indicated that the plant is going through a recovery process. A similar observation was also seen in a study on the impact of UVC radiation on the total anthocyanin content in the strawberry plant [45].

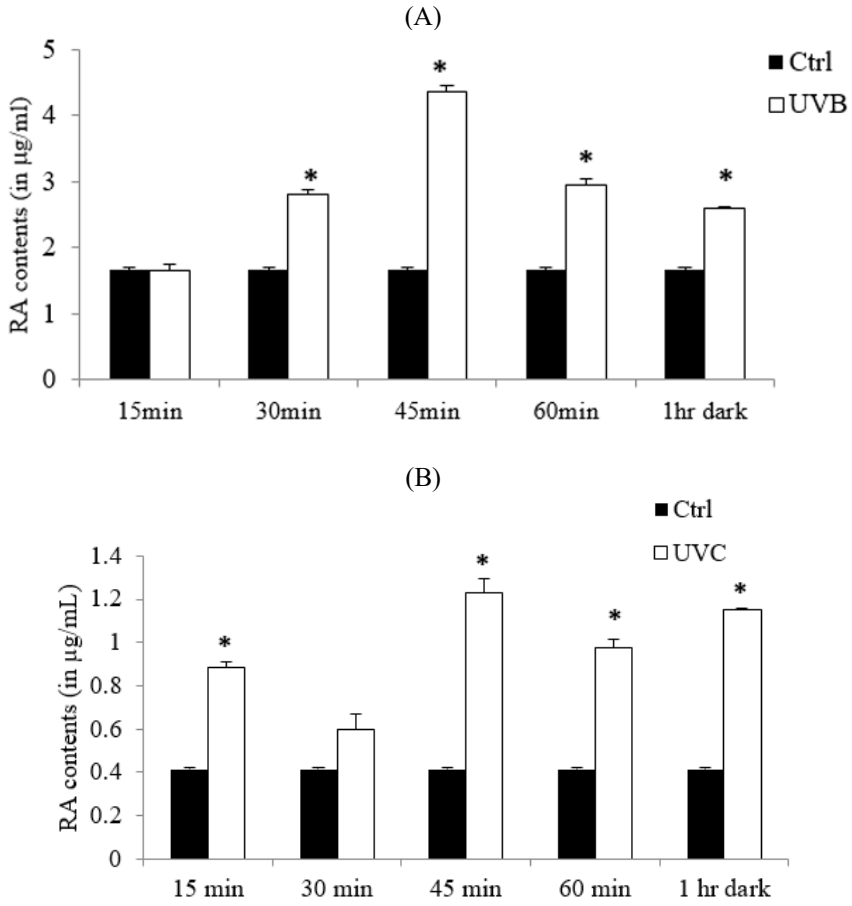

Fig. 5. Rosmarinic acid production in response to UVB (A) and UVC (B) treatments. The data represent the average of three independent biological replicates and comparison with control. The vertical bars indicate the standard errors. The statistical analysis of differences between control and each time point of treatment were performed by 2-tailed T test, $p<0.05$ is indicated with asterisk (*).

3.5 *Hppr* expression after UVB and UVC treatment

For the RT-PCR analysis, figures 6A and 6B represent the relative expression log-10 (RQ) value of *hppr* against different time points of UVB and UVC treatments compared to the control as calibrator. *Hppr* expression was up-regulated at 15 min but down-regulated after that at the 45 min time point of UVB treatment (Fig. 6A). This indicated that the plant self-protective system was initiated to contend with the UV

light stress [41]. It should be remembered that hppr is also involved in other physiological processes, such as photorespiration and NADP(H) cycling, which could explain the differences between its expression and rosmarinic acid accumulation [46]. The results showed that 1 hour of UVB light treatment on *O. aristatus* is sufficient to up-regulate *hppr* expression, which in turn increases rosmarinic acid production. Similar phenomena was also found in a study of glucosinolates in *Arabidopsis* leaves, in which UVB irradiation for 1 hour was sufficient to induce the production of glucosinolates, and that exposure for up to 12 hours inhibits in the expression of glucosinolate synthesis genes and decreases glucosinolate content [37]. On the other hand, *hppr* expression was down-regulated with the exception of UVB radiation after 1 hour dark recovery, which can be explained by the recovery of *hppr* regulation by UVB radiation [11].

Meanwhile, when exposed to UVC, *hppr* expression was down-regulated throughout the exposure (Figure 6B). Interestingly, *hppr* expression increased after 1 hour of UVB treatment and declined after 1 hour of dark recovery, while it increased after UVC treatment stopped and the plant recovered in dark. Up-regulation of *hppr* expression after the removal of UVC indicates that UVC exposure causes stress for the plant but gene expression recovered after its removal [47]. The delayed effect of UVC on the expression of *hppr* may be attributed to plant defence against the UV stress [48-49]. However, the results for UVB and UVC irradiation are in contrast to the results of exposure of *O. aristatus* to UVA, as reported by Ahmad and co-workers, where *hppr* expression in *O. aristatus* showed a positive correlation to UVA exposure [38].

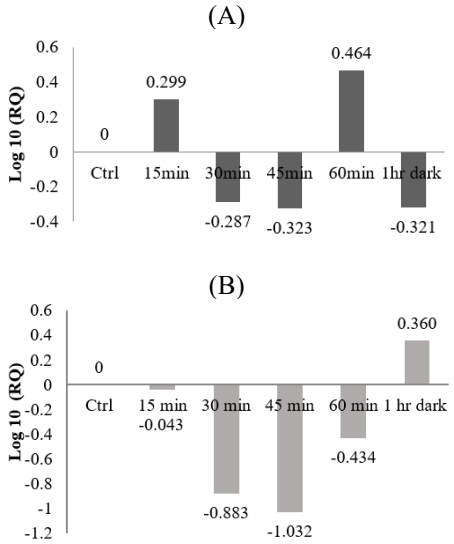

Fig. 6. The relative expression of *hppr* in *O. aristatus* after short-term UVB (A) and UVC (B) treatments. The bar graph showing the log-10 (RQ) value of *hppr* measured at 15 mins, 30 mins, 45 mins, 60 mins, and 1 hour dark recovery after UV treatment, and control (without UVB or UVC treatment, respectively).

3.6 Correlation between *hppr* gene expression and rosmarinic acid accumulation

Fig. 7 shows the relative expression of *hppr* and rosmarinic acid concentrations against time of UV exposure. Generally, a negative correlation was observed between *hppr* gene expression and rosmarinic acid accumulation after both UVB (Fig. 7A) and UVC (Fig. 7B) treatments. The correlation coefficient value was found to be in the range of -0.25 to -0.38. The results showed that the increase in *hppr* gene expression results in a co-suppression effect with the reduction of rosmarinic acid levels. When *hppr* gene expression was low, the average concentration of rosmarinic acid increased. The relative expression of the *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 treatment. Interestingly, rosmarinic acid production was the highest at 45 min of

UVB treatment (Fig. 7A). Meanwhile, short term UVC exposure on *O. aristatus* also showed a negative correlation between *hppr* expression and rosmarinic acid production, with a correlation coefficient value of 0.16 (Fig. 7B). A negative correlation was observed for all triplicates of UVC treatments, with correlation coefficient values in the range of -0.4 to 0.17. The results also showed fluctuations in the rosmarinic acid content. *Hppr* expression was down-regulated after 30 min of UVC treatment but increased after 1 hr of dark recovery.

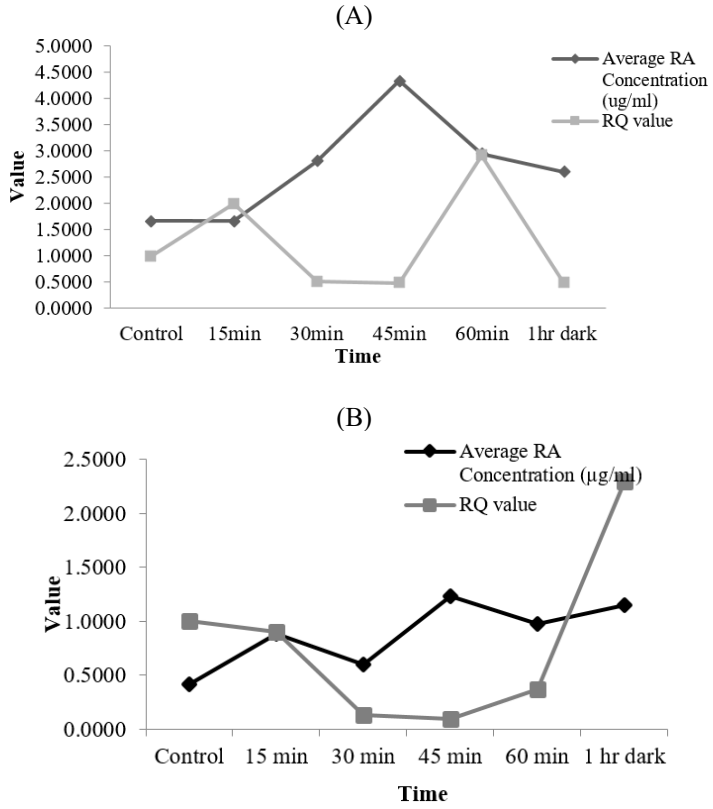

Fig. 7. Correlation between the RA production and *hppr* expression after UVB (A) and UVC (B) treatments. The average RA concentration denoted the average RA concentration of three dependent replicates, whereas the RQ value denoted the relative expression value of *hppr* gene after UVB or UVC treatments, respectively.

A similar pattern of correlation was also observed in *Salvia militiorrhiza*, in which *PAL* expression was abundant in the roots with low content of rosmarinic acid [50]. Besides that, UVB treatment was also reported to negatively affect tea plant culture growth and the size of callus forming cells, by inducing the production of phenols and flavans in tea plants [52], and anthocyanin levels in strawberry plants after UVC exposure [45]. This information suggests that the biosynthesis of rosmarinic acid in plants is also controlled by other biosynthesis pathways. Nevertheless, the results in this study show that rosmarinic acid content increased with short term UVB treatments of up to 45 min and decreased at 60 minutes UVB exposure, despite *hppr* being down regulated. This indicates that the rosmarinic acid biosynthesis pathway

does not depend solely on the expression of *hppr*, other biosynthesis pathways may also be involved. Further investigations on the control of rosmarinic acid content in *O. aristatus* might provide better knowledge on how enzymes interact with each other to produce the final product.

4. Conclusion

Our findings show that short-term enhanced UVB and UVC irradiations of *Orthosiphon aristatus* affect the biosynthesis of rosmarinic acid. Increased rosmarinic acid levels were detected upon exposure to both UVB and UVC. However, hppr activity showed an opposite reaction towards production of rosmarinic acid. This seems to indicate the involvement of a different pathway in rosmarinic acid production upon exposure to UVB and UVC radiation. These findings are particularly useful for growers, as secondary products in plants have been proven to exert beneficial effects and production of these secondary products can be increased via systems of commercially available UV light exposure.

Acknowledgement

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