The impact of UV-B radiation on some metabolites and pigments of *Carum Copticum* under in vitro culture

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Abstract

Carum copticum L. is a medicinal plant of the Apiaceae family with medicinal properties. In this study, the effects of UV-B radiation on the photosynthetic pigments and essential oils as well as phenols and anthocyanin of the seedling and callus of *C. copticum* were investigated under in vitro conditions. For this purpose, the experiment was set up in a completely randomized design, with three replicates per treatment. The seedlings and calli of *C. copticum* were categorized in two groups: the first group served as control and the second group was exposed to artificial UV-B radiation for 30 min at 15 days. The results indicated UV-B treatment reduced fresh weight about 34% and 27% compared with control in seedlings and calli respectively. The amount of chlorophyll a, b and total chlorophylls as well as carotenoids decreased about 36%, 42%, 38% and 14% in seedling and 24%,32%, 26% and 14% in callus of *C. copticum* respectively under UV stress. The lowest and highest amounts of flavonoids and anthocyanin were observed at UV-B treatment and untreated plants respectively. In addition, The UV-B stress changed the compounds of the essential oil both in the seedlings and the calli of *C. copticum*. Analysis of the essential oil constituents showed thymol and gamatherpinen, the main essential oil components of *C. copticum*, increased under UV-B radiation. While, p-cymene concentration decreased under UV treatment Therefore, *C. copticum* is useful for the in vitro production of pharmaceuticals and other beneficial substances by plant tissue culture techniques under UV-B treatment.

Keywords: anthocyanin, light stress, phenols, ultraviolent, thymol, γ-therpinen.

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Introduction

It is well known that plants consistently synthesize, accumulate, and use a bewildering range of secondary metabolites as a part of their overall defense strategy in response to abiotic and biotic stress. Many of these metabolites have been used around the world as medicines for various human health problems. The demand for production of higher level of secondary metabolites such as phenols, alkaloids, essential oil, terpenoids and glycosides has been increased in recent years. These components have often been obtained in cell and callus culture when ultraviolet (UV) radiation has been applied. Moreover, depletion of stratospheric ozone due to anthropogenic sources has led to an increase in

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solar UV radiation reaching the earth's surface, which has potentially deleterious consequences for agricultural production and natural plant ecosystems (Booij-Jameset al., 2000, Zhang and Björn, 2009, Razavizadeh and Komatsu, 2018). Approximately, nine percent of the sun's spectrum consists of UV rays which are divided into three categories: UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (200–280 nm). Increased UV radiation causes direct and indirect effects including degradation and conformational changes in DNA, protein and lipids and alterations in photosynthesis, growth and morphology of plants (Booij-James et al., 2000). In plants, wide inter- and intraspecific differences have been reported in response to UV-B irradiation with respect to growth, production of dry matter and biochemical changes (Alexieva, et al., 2001). Exposure to UV decreased plant height, leaf area and increased dry weight, auxiliary branching and leaf curling in some plants. The mechanism of signaling UV-B in plants is not well defined. However, the generation reactive oxygen species (ROS), nucleotide dimer formation and two specific UV-B receptors have been considered at UV signaling in plants (Alexieva, et al., 2001).

Carum copticum L., commonly known as Ajwain, is an important plant member of the Apiaceae family with various essential oils and produces a number of secondary metabolites. The *C. copticum* grows in arid and semiarid fields in different countries such as India, Iran, Pakistan. The different therapeutic applications of this plant have been described. Its biological activities are attributed to their bioactive compounds, such as phenolics (carvacrol), flavonoids, thymol, terpinene, p-cymene, and β-pinene (Cimanga et al., 2002). Essential oils, generally derived from main parts such as leaves, seeds and bark, are popular as perfume ingredients, cosmetics, and household cleaning products as well as flavoring agents in foods and drinks (Boskabadyet al., 2014). The biosynthetic pathway of thymol, cymene and carvacrol has been clarified recently (Crocoll et al., 2010). Thymol and carvacrol (phenolic monoterpenes) are biosynthesized from isopentenyl diphosphate and dimethylallyl diphosphate from the methyl erythritol phosphate pathway (MEP). Briefly, Geranyl diphosphate synthase (GDS), a key enzyme in this biosynthetic pathway, produce geranyl diphosphate (GDP) as the universal precursor of monoterpenes. Then, γ-terpinene synthase which is a member of the monoterpene synthase family produces γ-terpinene through cyclization of Geranyl diphosphate. Subsequently, enzymes such as CYP71D178, CYP71D180 and CYP71D181 belonging to the cytochrome P450 (CYP) monooxygenases are also involved in further modifications of γ-terpinene backbone to yield thymol and carvacrol (Fig.I) (Crocoll et al., 2010). Interestingly, recent studies have shown the

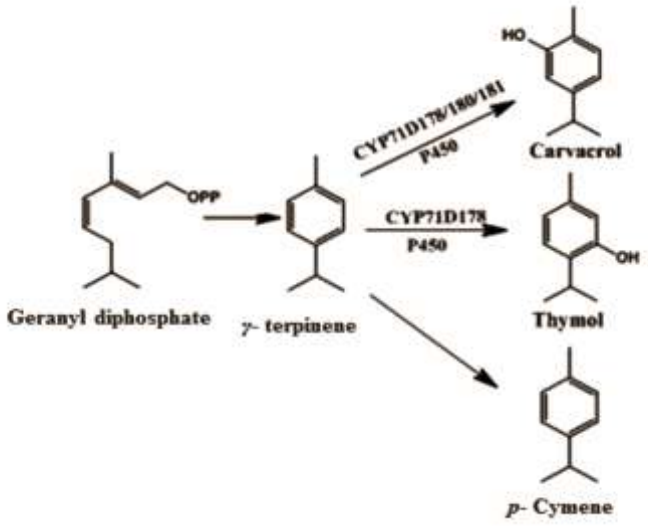

Fig. I. The biosynthetic pathway of thymol, carvacrol p-Cymene from γ-terpinene.

positive response of UV radiation on essential oils of medicinal plants (Ioannidis et al., 2002, Fedina, et al., 2010, Morales et al., 2010, Gai et al., 2016, Zhang et al., 2017). Mild doses of UV-B radiation increased the biosynthesis of essential oils in many plants and it seems that UV-B radiation have an important role to production secondary metabolites (Gai et al., 2016).Since the secondary metabolites mainly produced in response to adverse environmental conditions or in particular developmental stages, the aim of this study was investigation of important metabolites of *C. copticum* as a valuable medicinal herb with different pharmacological activities in response to UV-B radiation under in vitro conditions. Also, in this study, we investigated the effect of UV-B treatment on some physio-biochemical parameters of *C. copticum*.

Materials and Methods

Plant and callus culture

The fresh seeds of *C. copticum* L. were supplied from the oilseed cultivation company (PakanBazr, Isfahan, Iran). Seeds were surface-sterilized for 20 secs in 70% ethanol, followed by 20% sodium hypochlorite solution for 10 min. Seeds were then washed three times using sterilized distilled water and transferred into jars containing 30 mL of MS-basal medium and kept in the growth chamber (16 h light/8 h dark) at 25 °C and 95% relative humidity. After 4 weeks, the seedlings were treated by UV-B radiation.

In order to produce callus, 0.5 cm of stem explant from four-weeks-old growing seedlings were cultured on MS media containing 1 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 4 µM of benzyl amino purine (BAP) (Razavizadeh et al., 2017). After 4 weeks, callus explants were subcultured on the same medium and kept at 25 ± 2 °C for 4 weeks. The callus subculture was repeated monthly. After 3 subcultures, calli were treated by UV-B.

Experiment design and UV treatment

The experiment was set up in completely randomized design, with three replicates per treatment. The seedling and callus of *C. copticum* were categorized in two groups: the first group served as control and the second group was exposed to artificial UV-B radiation for 30 min for 15 days. The UV-B was artificially provided by UV-B lamps (Actinic BL model, Philips, Poland). The lamps were held in a plexiglass box containing two UV-B 8W lamps. Data were analyzed for the significance of differences between means using Duncan's test at $P < 0.05$ (ANOVA). SPSS software (version 21) was used for Statistical analysis of the data and results were expressed as mean ± standard error (SE).

Fresh weight

Fresh weight (FW) of seedlings and calli were measured after 15 days.

Photosynthetic pigments

Chlorophylls and carotenoids were determined according to Lichtenthaler's method (1983). The 0.2 g of seedling leaves and callus were ground in 15 mL of 80% cold acetone and centrifuged at 5000g for 10 min. The absorbance of each sample with three replications was measured at 646.8, 663.2, and 470 nm using a spectrophotometer (U-6305 model; Jenway, Staffordshire, UK) and pigments' concentrations were calculated based on the following formula and expressed as mg/g FW.

$$\text{Chl a (mg/g)} = [12.25\ (D663.2) - 2.79\ (D646.8)] \times V/1000 \times W$$

$$\text{Chl b (mg/g)} = [21.50\ (D646.8) - 4.68\ (D663.2)] \times V/1000 \times W$$

$$\text{Total Chl a+b (mg/g)} = [20.2\ (D646.8) + 8.02\ (D663.2)] \times V/1000 \times W$$

$$\text{Carotenoid} = [1000\ (D470) - 1.82\ (Chla) - 85.02\ (Chlb)] / 198$$

Flavonoids content

The contents of flavonoids were measured by adapting Kotzé and Eloff (1998) method. Samples were ground in a mortar containing ethyl alcohol and glacial acetic acid (1:99 respectively). Extracts were put in 80 °C hot bath after centrifuging for 10 minutes and the absorption rate was measured at 270, 300, 330 nm.

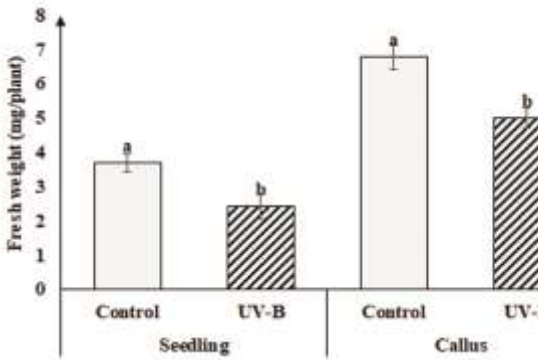

Fig. II. The effect of UV-B radiation on FW of *C. copticumin* seedling and callus. The values are means of three replicates, ±SD. Common letters in each growth stage are not significant ($p<0.05$).

Anthocyanin content

Total anthocyanin was determined according to modified Wagner's method (1979) using acidified ethanol (Methanol: HCl 99:1 v/v). A portion (0.05 g) of frozen shoot homogenized in 5 mL of acidified ethanol and then kept at 25 °C for 24 h in the dark. The extract centrifuged at 4000 X g for 10 min at room temperature. The absorbance of each supernatant was measured at 550 nm using the spectrophotometer. The extinction coefficient (ε) 33000 ($mM^{-1}cm^{-1}$) was used to calculate (Absorbance= ε× concentration × pathlength) the amount of total anthocyanin which was expressed as µM/g FW.

Absorbance= ε× concentration × pathlength

Essential oil

The gas chromatography-mass spectrometry (GC/MS) was used for the identification of essential oil components in *C. copticum*. A Hewlett–Packard 5890 GC (Hewlett Packard, Waldbronn, Germany), equipped with a flame ionization detector (HP-5970 mass-selective detector) and a 50 m × 0.20 mm HP-5 (cross-linked phenyl–methyl silicon) column with 0.25 µm film thickness, was used for this study. The FID was maintained at 250 °C. In addition, ionization energy was 70 eV. The temperature program was 100 –250 °C with changes of 4 °C min-1. Helium was used as a carrier gas, the flow through the column was 1 mL min^{-1}, and the split ratio was set to 100:1. Identification was based on sample retention time and mass recorded (Davies 1990).

Results

Fresh weight of seedling and callus of *C. copticum* were measured under UV-B treatment (Fig.II). The UV-B radiation decreased the FW of seedling shoot and callus. The results showed UV-B treatment reduced FW about 34% and 27% compared with control in seedlings and calli respectively.

Analysis of data indicated that chlorophyll a, b and total chlorophylls as well as carotenoid contents were reduced significantly by UV-B radiation. As shown in the Fig. III, the amount of chlorophyll a, b and total chlorophyll as well as carotenoids decreased about 36%, 42%, 38% and 14% in seedling and 24%,32%, 26% and 14% in callus of *C. copticum* respectively under UV stress compared with control.

Application of UV-B altered flavonoids and anthocyanin content significantly. The highest value of flavonoids and anthocyanin content were at UV-B treatment in both callus and seedling. While, the lowest content of these components detected in control callus and seedling. As compared to control, UV-B radiation increased flavonoid content at 270,300 and 330 nm in callus and seedling significantly (Fig.IV). Similarly, the anthocyanin content of callus and seedling plants improved statistically with UV-B treatment. With regard to anthocyanin concentration, a remarkable increase by 32% and 59% was observed in the seedling and callus under UV-B radiation respectively (Fig. V).

In this study, UV-B treatment induced alteration in the oil components of *C. copticum*. As can be seen in Fig. VI, UV-B increased the amount of thymol more than 47% in both callus and seedling. Moreover, UV-B treatment induced a significant increase in the amount of γ-terpinene by about 63% and 59% in seedling and callus of *C. copticum* respectively. However, radiation of UV-B caused a decrease (about 77%) in p-cymene content of seedling and callus

Discussion

The UV-B treatment may change growth and metabolite pathway in plants, as salt and

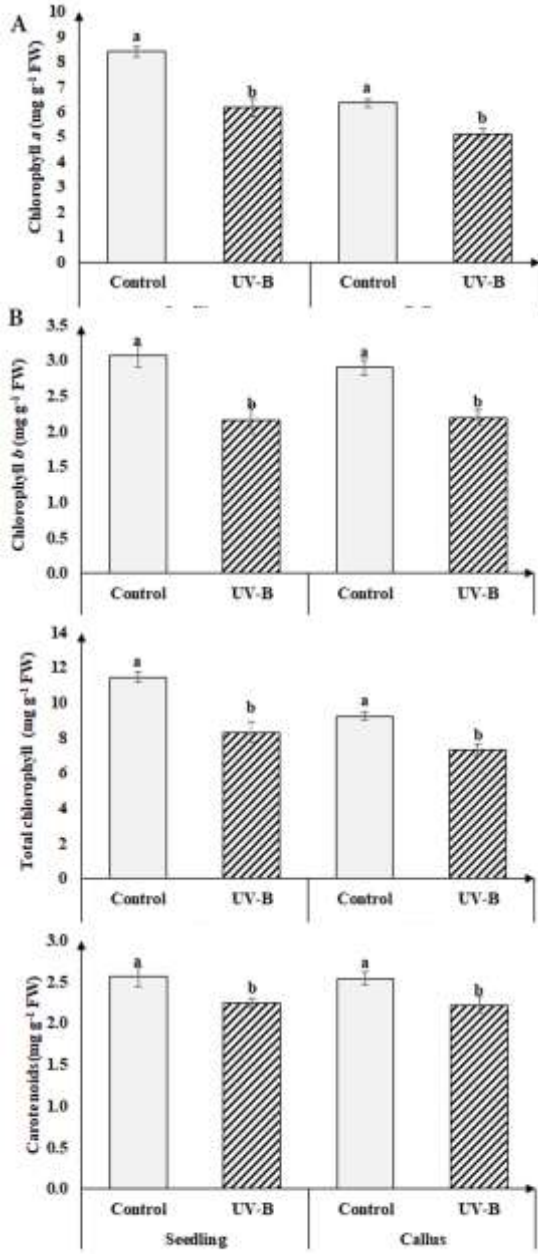

Fig. III. The effect of UV-B radiation on chlorophyll a (A), b (B), total chlorophyll (C) and carotenoids of *C. copticumin* seedling and callus. The values are means of three replicates, ±SD. Common letters in each growth stage are not significant ($p<0.05$).

drought stress. Previous studies suggested that UV-B radiation inhibited, promoted or had no effect on plant growth which indicated the impact of UV-B radiation on plant growth appeared to be species-specific (Zlatev et al., 2012). However, in the general pattern, most species are sensitive and plant biomass decreased in response to UV-B stress such as rice and maize (Fedinaet al., 2010, Zlatevet al., 2012). As a general response to UV-B radiation, plant growth is negatively affected with reduced leaf area, chlorophyll content, photosynthesis and Rubisco activity (Zhang et al., 2017). Therefore, the reduction of chlorophyll content may lead to decrease of FW in *C. copticum*.

Recent studies have reported detrimental effects of UV-B radiation on photosynthetic pigments (Zlatev et al., 2012). Similar with our results, photosynthetic pigments of *Prunella vulgaris* reduced significantly by UV-B radiation (Zhang et al., 2017). Some evidence has been shown that the reduction of chlorophyll pigments under UV radiation is associated with improving the activity of Chlorophyllase. Moreover, increasing level of ethylene and damage of enzymes involving in chlorophyll synthesis under UV-B radiation led to reduction content of chlorophyll (Booij-James et al., 2000, Fedina et al., 2010, Zlatev et al., 2012, Zhang et al., 2017).Takeuchi et al. (2002) concluded that in sensitive rice species, UV-B and UV-C significantly decreased chlorophyll contents, primarily because UV-B destroyed the structure of chloroplast, inhibited the synthesis of chlorophyll and increased the rate of chlorophyll degradation. According to our results, the reduction of chlorophyll pigments may lead to reduce photosynthesis which associated with reduced biomass.

Flavonoids are the general UV-B regulated compounds in response to UV-B radiation. In the barely, a mutant with diminished flavonoids was more sensitive than the original variety under UV-B radiation (Reuber et al., 1996). Anthocyanin, which also belongs to the flavonoid group, has been showed to play an important role in response to UV radiation. Induction of anthocyanin synthesis under UV-B radiation has been reported in many other plants (Zhang et al., 2017). It seems that UV-B radiation induces accumulation of flavonoids and anthocyanin as an important protective mechanism in plants (antioxidant function) and they act as 'sun-screen' by preventing the absorption of excess light (Reuber et al., 1996, Fedina et al., 2010, Zlatev et al., 2012).

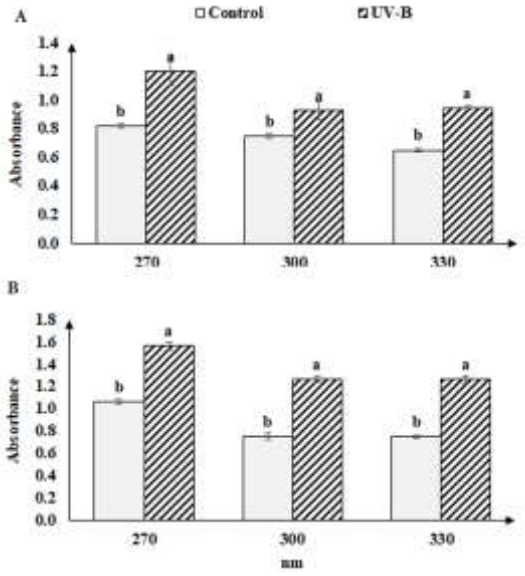

Fig. IV. The effect of UV-B radiation on flavonoids of *C. copticumin* seedling(A) and callus(B). The values are means of three replicates, ±SD. Common letters in each absorbance are not significant ($p<0.05$).

In this study, the UV-B treatment changed the compounds of the essential oil both in the seedlings and the calli of *C. copticum*. Thymol and γ-terpinene increased under UV-B radiation while p-cymene concentration decreased under UV stress. This is in agreement with previous study on *C. copticum* under drought stress (Razavizadeh and Komatsu, 2018). Thymol, p-cymene, and γ-terpinene have been well-known as the main essential oil components of seedling and callus of C.copticum (Razavizadehet al., 2017, Razavizadeh and Komatsu, 2018). Recent works stablished that UV-B radiation is a critical regulator of plant secondary metabolites, including constituents of essential oils (Gai et al., 2016). Also, several reports indicated that the yield and the composition of essential oils in many plants are increased by exposure to UV-B radiation (Ioannidis et al., 2002, Fedina, et al., 2010, Morales et al., 2010, Gai et al., 2016, Zhang et al., 2017). Hence, the elevated levels of compounds belonged to terpenoids could be due to UV-B effects on enzymes activities and metabolism improvements (Crocollet al., 2010). With regard to accumulation of γ-terpinene and thymol in this study, UV-B radiation might be due to increase activity or overexpression of geranyl diphosphate synthase and cytochrome P450 monooxygenases (Limaet

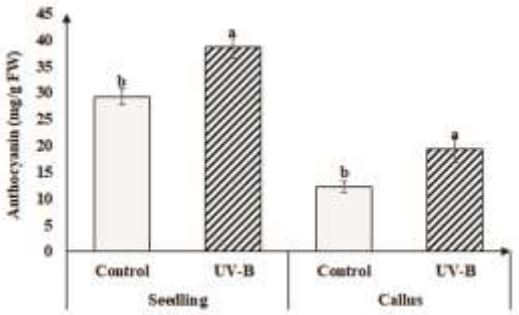

Fig. V. The effect of UV-B radiation on anthocyanin of *C. copticumin* seedling and callus. The values are means of three replicates, ±SD. Common letters in each growth stage are not significant ($p<0.05$).

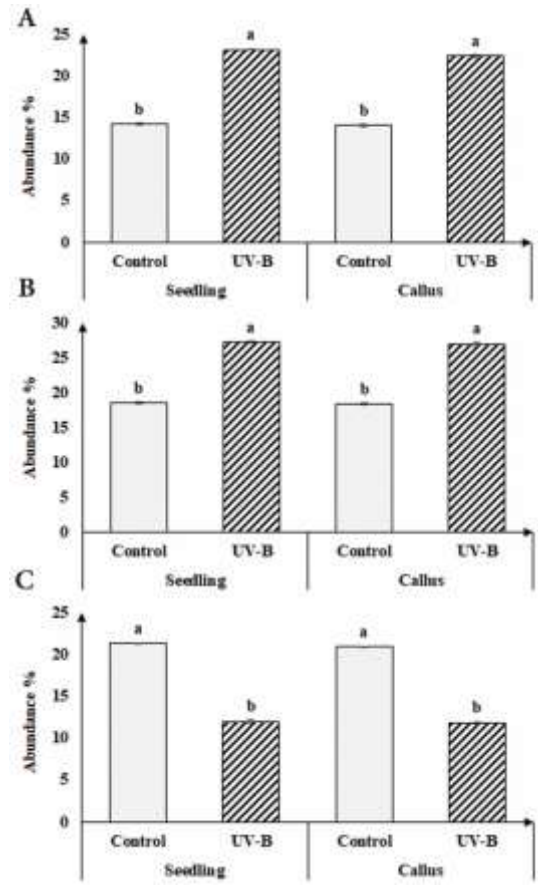

Fig. VI. The effect of UV-B radiation on γ-terpinene, thymoland p-cymene of *C. copticumin* seedling and callus. The values are means of three replicates, ±SD. Common letters in each growth stage are not significant ($p<0.05$).

al., 2013, Majdi et al., 2017) in *C. copticum*. Therefore, the decrease in p-cymene in seedlings and calli could be due to the change of synthesis direction towards production of thymol (Cimanga

et al., 2002, Majdi, Malekzadeh-Mashhady et al. 2017) in *C. copticum* . However, more detailed mechanisms for the regulation of secondary metabolites under UV-B radiation remain to be investigated.

Conclusion

According to the results, UV-B radiation was effective for enhancing total phenol, anthocyanin content and decreasing fresh weight, chlorophyll pigments and p-cymene content of *C. copticum*. Moreover, pharmaceutical compounds of *C. copticumsuch* as thymol and γ-terpinen increased significantly under UV-B treatment. Therefore, *C. copticum* is useful for the in vitro production of bioactive compounds such as thymol and γ-terpinen by plant tissue culture techniques under UV stress. The callus culture is also an efficient method of producing medicinal compounds.

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