Effect of ultraviolet and ultrasonic on potential antidiabetic activity of in vitro shoot cultures of *Orthosiphon aristatus*

D. Hunaefi\(^1\)\(^,\) N.D. Yuliana\(^1\), I. Smetanska\(^3\) and N. Gruda\(^4\)

\(^1\) Department of Food Science and Technology, Bogor Agricultural University (IPB), Indonesia
\(^2\) South East Asian Food, Agricultural Science & Technology Center, Bogor Agricultural University (IPB), Indonesia
\(^3\) University of Applied Science Weihenstephan- Triesdorf, Germany
\(^4\) University of Bonn, Germany

*dashcbdk@apps.ipb.ac.id*

Abstract. *Orthosiphon aristatus* Boldingh., a native tropical plant from Indonesia, is a medicinal herb that has been reported to possess an antidiabetic potential. However, study on this functional activity is limited especially on in vitro plant culture of *Orthosiphon aristatus*. The present study sought to investigate the effect of ultraviolet (UV) and ultrasonic (US) on this potential for *in vitro* shoot cultures of *Orthosiphon aristatus* (ISCOA). Methanol (70\% v/v) extracts of ISCOA had the ability to inhibit alpha-amylase and alpha-glucosidase enzymes, dependent on the concentration of the extract. Among all treatment, highest inhibition of \(\alpha\)-amylase and \(\alpha\)-glucosidase activities were observed in combined treatment between UV for 60 minutes and US for 6 minutes. This was showed by the highest reduction by 0.4 point of IC50 inhibitory alpha-amylase and alpha-glucosidase activities in day 3 and in day 1 after exposure UV and US, respectively. The combination of UV and US has been shown to be effective in improving potential anti-diabetic properties of *in vitro* shoot cultures of Orthosiphon aristatus in which correlated with the increasing level of secondary metabolites.

1. Introduction

*Orthosiphon aristatus* is a native tropic-medicinal plant from Southeast Asia. It belongs to Lamiaceae family. This plant has been widely used as a traditional/natural alternative medicine in Indonesia, Malaysia and Thailand for the treatment of various diseases; for instance, gout, diabetes mellitus, hypertension, rheumatism, tonsillitis and menstrual disorder, and especially those affecting the urinary tract, that is for treating kidney ailments and bladder related diseases (18).

Many studies have indicated that *O. aristatus* exhibits several secondary metabolic active constituents. These compounds contribute to the plant’s potential medicinal properties. However, the pharmacologically effects of *O. aristatus* have been recommended mainly due to its phenolics compounds (16) which are the most dominant constituent in the leaf. These phenolic compounds had been reported to be effective in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems (17). One of the most important phenolic compounds in *O.
aristatus is rosmarinic acid (RA) (1). This phenolic compound was thought to be the main contributor for the therapeutic effects and antioxidant activity of this plant (1).

RA is an ester of caffeic acid and 3, 4-dihydroxyphenyllactic acid (14) and it becomes one of the most targeted phytochemicals for development functional foods (44) due to its interesting biological activities, such as antiviral, antibacterial, anti-inflammatory and antioxidant (44). The development of functional foods containing RA from medicinal plants, herbs and spices that consist of naturally occurring antioxidants is preferable (38). Furthermore, this escalating demand of RA from medicinal plants has led us to do this research to increase the production of RA in Orthosiphon aristatus and to characterize the antioxidant properties of this plant extract which may provide useful information for better formulation of functional foods application.

Indeed, plant cell cultures that accumulate RA have been proposed for production of this phenolic compound (38, 50). And plant tissue cultures using plantlets that obtained from direct shoot organogenesis are generally preferred for mass production of the targeted compound as they are more genetically stable (12). Moreover, as a native tropic-medicinal plant, to grow *O. aristatus* in sub-tropic climate area was considered to be relatively difficult. Therefore, in vitro shoot cultures of Orthosiphon aristatus was used to be the media for this study.

Other reasons of using in vitro shoot cultures of *Orthosiphon aristatus* (abbreviated as ISCOA) are (1) this in vitro system may easily control biotic and abiotic stresses on the cultivar, (2) this system allows harvest of this plant at any condition regardless of climates condition and seasons, (3) and also through this system, rapid growth mass production can be achieved as well as be combined with sustainable secondary metabolites production (12, 46).

To improve secondary metabolites production of in vitro plant cultures, several strategies such as supplementation and physical stress elicitation has been used (5), for instance, ultraviolet (UV) as physical stress elicitation had been used to improve targeted secondary metabolite production in several plant cell cultures (4). Similarly, the utilization of ultrasound/ultrasonic (US) to enhance secondary metabolites in plant cell cultures had been reported by several researchers (54). Ibdah et al. (19) showed that under ultraviolet stress with high light irradiance (1200–1500 µmol m−2 s−1) on *Mesembryanthemum crystallinum* displayed a rapid cell- in betacyanins and acylated flavonol glycosides production in the upper leaf epidermis. Lin et al. (31) reported that US had the ability to increase the biosynthesis of ginsenoside saponins in Panax gingseng cell cultures by 75% as compared to the control. Furthermore, Wu and Lin (54) also showed that US treatment for two minutes elevated volumetric taxol by 1.5- to 1.8-fold of control. However, there is no report of using both physical stresses on in vitro cultures of *Orthosiphon aritatus*.

Biosynthesis of RA is associated with the elevated of PAL activity through the phenyl-propanoid pathway (36, 47). In addition, proline-linked pentose phosphate pathway which includes the phenyl-propanoid pathway had been also proposed to regulate RA production (28, 56). That is why, for better understanding the effect of ultraviolet and ultrasonic treatment on biosynthesis of RA and phenolics in in vitro shoot cultures of *O. aristatus*, the model in Figure 1 has been intended based on previous model (43, 56).
Moreover, since antioxidant activity of plant extract are closely linked to their secondary metabolites as antioxidant component, therefore, the effect of the UV and US on antioxidant activity of ISCOA is also evaluated in our studies. One method of antioxidant activity analysis is not sufficient for the complete assessment of plant extract antioxidant potential due to the fact that antioxidant component in the plant extract may react differently to different antioxidant activity assay (8). For example, Wang and co-workers (52) found that some compounds, which have ABTS radical scavenging activity, may not show DPPH radical scavenging activity. Considering the limitation on using one antioxidant activity assay, thereby, in our experiment, determination of antioxidant activity was carried out using four different methods; DPPH free radical scavenging, Trolox Equivalent Antioxidant Capacity (TEAC), β-carotene/linoleic acid bleaching, and SOD-like antioxidant activity methods.

Lastly, the purposes of the study were (a) to investigate the effect of UV and US treatment on production of RA and other secondary metabolites with respect to inhibition activities of two enzymes: α-amylase and α-glucosidase activities and antioxidant properties of ISCOA, (b) to study the signals related to the pathway of secondary metabolites production (Figure 1) in in vitro shoot cultures such as glucose-6-phosphate dehydrogenase (G6PDH), phenylalanine ammonium-lyase (PAL), and
peroxidase (PO) activity, and proline content were investigated, and (c) to further examine the profiles of phenolic acids of this plant after the treatment applied.

2. Material and methods

2.1. Plant Materials
Orthosiphon aristatus with article number of 600646 was purchased online from Pflanzenversand Hans-Günter Röpke (http://www.pflanzenkindergarten.de) for establishment of in vitro shoot culture.

2.2. Culture Media and Chemicals for analysis
MS Medium was obtained from E-Merck (Darmstadt, Germany). Pyrogallol, gallic acid, and ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); Quercetin Dihydrate (C15H10O7·2H2O ≥99% HPLC) from Fluka, Biochemika (Neu-Ulm, Germany); Folin-Ciocalteu's phenol reagent 2 M, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). The standards of phenolic acids; chlorogenic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, sinapic acid, caffeic acid, ferulic acid, RA, cinnamic acid and gallic acid were obtained from Sigma Aldrich, Chemie GmbH (Steinheim, Germany). All used chemicals were analytical grade.

2.3. Establishment of ISCOA
Stems from the acclimatized mother plant were cut into 2.5 – 5 cm and sterilized in 70% ethanol for 20-30 seconds. They were then transferred into 10% sodium hypochlorite for 5 min, and washed three times in sterile water before being sown into 250 ml Erlenmeyer flasks on Murashige and Skoog medium (MS; Murashige and Skoog, 1962) for 4 weeks. Stems of sterile plants were cut into 1 – 1.5 cm pieces (±100 mg fresh weight.L-1) and placed in 250 ml Erlenmeyer flasks containing 50 ml liquid MS medium. ISCOA were transferred to a new medium every four weeks and maintained at 25 ± 2°C on a rotary shaker under light irradiation. The best line of ISCOA which was fourteen generation (sub-culturing) from the first establishment was used for our research.

2.4. Ultraviolet (UV) and Ultrasonic (US) treatment
As source of UV, we have used UV generator Paulmann L5/7/9/11.313 (Paulmann Licht GmbH, Springe Völkksen, Germany) with Phillips UVB lamp PL-S 9W/12/2P with emission range from 290-320 nm and peak at 302 nm (230 Volt ~ 50 Herz and 0.17 A) (Phillips Electronics, Pila, Poland). For the US treatment, ultrasound bath, Bandelin Sonorex Digital 10 P (Bandelin electronic GmbH & Co. KG, Berlin, Germany) with double half-wave 35 kHz frequency automatic and ultrasonic peak output of 600 Watt with current consumption 3.4 A was used.

For the treatment, we have used ISCOA with the similar fresh weight (5.3 ± 0.04 g), length (4.0 ± 0.05 cm), number of leaves (6.0) and number of roots (4.0) of two weeks old and from 14th subculture. These ISCOA were subjected to the UV and US treatment in different combination (Table 1).

| Combination* | UV 0 | UV 20 | UV 40 | UV 60 |
|--------------|------|-------|-------|-------|
| US 0 | Control | UV 20 | UV 40 | UV 60 |
| US 2 | US 2 | UV 20 – US 2 | UV 40 – US 2 | UV 60 – US 4 |
| US 4 | US 4 | UV 20 – US 4 | UV 40 – US 4 | UV 60 – US 4 |
| US 6 | US 6 | UV 20 – US 6 | UV 40 – US 6 | UV 60 – US 6 |

*0, 2, 4, 6, 20, 40, and 60 showed the time of the treatment in minutes and the name of the samples was named based on the time and combination physical treatment applied.
The ISCOA in 250 ml flask were transferred into sterile Petri disc and subjected to UV irradiation for certain time (Table 1) under aseptic condition in clean bench. For US treatment, the ISCOA in 250 ml flask covered with sterilized plastic caps were directly subjected to US treatment in ultrasonic bath filled water (5 L) for a definite time mentioned in Table 1. Samplings were performed 1, 3, 7 and 14 days after the day of the treatment (day 0) and namely as day 1, day 3, day 7, and day 14.

2.5. Analysis of the fresh weight of ISCOA

2.5.1. Chlorophyll a and b

Chlorophyll a and b in leaves were measured based on previous spectrophotometrically method by Lichtenthaler and Wellburn (30) using fresh weight basis. The chlorophyll content later expressed as mg per gram fresh weight (mg.g FW-1).

2.5.2. Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), phenylalanine ammonium-lyase (PAL, EC 4.3.1.5), and peroxidase (PO, EC 1.11.1.7) activities

The extraction of G6PDH and its assay were determined spectrophotometrically at the wavelength 339 nm according to the previous method by Shetty et al. (45). The enzyme quantity was measured in nanomoles per minute per milligram protein (nmoles.min-1.mg protein-1). While the extraction of PAL and PO was assayed spectrophotometrically at OD 290 nm and 420 nm, respectively. Both assays were based on previous method described by Dörnenburg and Knorr (10). The activity of enzyme was expressed as micro-Units per gram of fresh weight (mU.g FW-1).

2.5.3. Proline content

Proline content of ISCOA leaves was measured on the spectrophotometer basis of a previously described assay used for plants Bates et al. (3). Proline content was expressed as micromoles per gram of fresh weight of ISCOA materials (µmoles.g FW-1).

2.5.4. Sample preparation and extraction

The plant was harvested and weighed and immersed in liquid nitrogen to avoid any possible enzyme degradation and to prevent phenolic compound volatilization then followed with freeze-drying. The lyophilized sample was grounded by flint mill (Retsch, Retsch GmbH, Haan, Germany) of 20000 rpm and 2 min to a fine powder. The dried powder of ISCOA was weighed of 100 mg and extracted with 5 ml of 70% methanol. And then it was placed in ultrasonic bath at 55°C for 2 hours. The mixture was centrifuged at 13000 rpm for 5 minutes and the supernatants were collected in different probe. The extraction was repeated twice until the final concentration of ISCOA extract was 10 mg.ml-1. The extract was stored at -20°C until further use. All samples and determination were prepared and measured in triplicate

2.5.5. α-amylase and α-glucosidase inhibitory activities

The α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) inhibitory activities of ISCOA extracts were analyzed according to the method as previous described by Ranilla et al. 2. The α-amylase inhibitory activity was measured spectrophotometrically at 540 nm, while α-glucosidase inhibitory activity was measured at 405 nm. Percentage of inhibition was calculated according to the equation:

$$\% \text{ Inhibition} = \left( \frac{AC - AS}{AC} \right) \times 100$$

where AC is the absorbance of the control, and AS is the absorbance of the samples. Appropriate dilution of the ISCOA extracts was made to calculate the IC50 of α-amylase and α-glucosidase inhibitory activities.
3. Results

We investigated the effect of UV and US treatment on α-amylase and α-glucosidase inhibitory activities in a model system, in vitro shoot cultures of *Orthosiphon aristatus*. The difference between in vitro shoot cultures and in vitro tissue cultures had been explained by Schevchenko et al. (46).

Turning to the stresses applied, US treatment suppressed the growth of ISCOA as observed by significantly lower biomass and length appeared at day 3 post-exposure, whereas UV irradiation up to 60 min had no effect on the growth of ISCOA (Figure 2 a and Figure 2 b). Furthermore, although slightly lower in the content of chlorophyll a content of US 6 samples at day 1 after physical treatment, however, statistically, there was no significant different (p > 0.05) on the chlorophyll a and b of ISCOA leaves after the application of UV and US treatment (Figure 2 c and Figure 2 d). G6PDH, PAL, PO activities and proline content

In order to examine whether stress treatment involved in a signal transduction pathway leading to enhancement of RA synthesis. Therefore, G6PDH, PAL, PO activity and proline content were investigated. Figure 3 shows the intracellular G6PDH, PAL, PO activity; and proline content in ISCOA after the treatment was applied (1, 3, 7 and 14 days).

In term of G6PDH activity (Figure 3 a), this enzyme activity was not significant different after UV treatment as compared to control. However, the US 6 and the combined treatment of UV 60 and US 6 suppressed this enzyme activity at day 1 after treatment. The most prominent significant increase was observed on day 3 after the treatment (Figure 3 a). This enzyme activity dropped afterward and remained stable until day 14 of post-exposure. The intracellular PAL activity of ISCOA rose significantly after one day exposure to US 6 and UV 60-US 6 (Figure 3 b). It reached maximum at day 3 and declined slowly to day 14 of post-exposure. The UV treatment of ISCOA had no significant effect on PAL activity as compared to control. Whereas for peroxidase enzyme, only the combination (UV 60-US 6) treated samples showed a significant influence on PO enzyme activity. In contrast to PAL activity that reached maximum at day 3 after combined treatment UV 60 and US 6, PO activity in treated samples increased gradually and reached its maximum at day 7 (Figure 3 c).

Additionally, proline content increased (Figure 3 d) at day 1 after UV 60 treatment, while US 6 had no effect on proline content of ISCOA. The combined exposure of UV 60 and US 6 resulted in a rapid increase of proline content at day 1 and rose slightly at day 3. It dropped at day 7 and remained constant at day 14.
Figure 2. a. Biomass; b. shoot length; c. chlorophyll a; and d. chlorophyll b of ISCOA subjected to UV and US treatment.
Figure 3. G6PDH, PAL, PO activity; and proline content in ISCOA after the treatment was applied (1, 3, 7 and 14 days)

α-amylase and α-glucosidase inhibitory activities

Results showed that methanol (70% v/v) extracts of ISCOA had activities to inhibit both enzymes dependent on the concentration of the extracts (Figure 4).
Figure 4. a. Concentration dependent ISCOA extracts α-amylase inhibitory activity to calculate IC50; b. IC50 α-amylase inhibitory activity of ISCOA extracts subjected to different physical stresses; c. Concentration dependent ISCOA extracts of α-glucosidase inhibitory activity to calculate IC50; and d. IC50 α-glucosidase inhibitory activity of ISCOA extracts subjected to different physical stresses.
Lower IC50 was achieved in α-glucosidase inhibitory activity as compared to α-amylase inhibitory activity. This means that ability of ISCOA extracts to inhibit the function of α-glucosidase is higher than α-amylase (Figure 4). The inhibitory activities of both enzymes attained the minimum level of IC50 at day 3 after treatment. Furthermore, results of these enzymes inhibition showed that inhibitory activity to α-glucosidase was more responsive to the physical stresses applied than α-amylase (Figure 7d). Treatment with US 6 and UV 60 – US 6 resulted in significant lower of IC50 compared to control immediately after one day treatment while inhibitory of α-amylase appeared significantly different at day 3 post-exposure for UV 60-US 6 treatment.

4. Discussion

The inhibition of cell growth and reduction of cell viability after US treatment had been reported by several researchers (55). Similarly, several researchers reported that UV-B radiation exerts powerful stress effect on plants and it resulted in reduction of plant biomass (27). US and the combined treatment of US-6 and UV-60 treatment on biomass and shoot length was confirmed in our research. This phenomenon could be explained by morphological changes as reduced cell elongation as a result of plant adaptation to the treatment by lowering indole acetic acid (IAA) (26). Therefore, on the assumption that growth (biomass and shoot length) is an integral index of plant condition, a decrease in US and the combined of US 6 and UV 60-treated ISCOA growth due to the treatment can result from the changes in the physiological and biochemical characteristics (58).

In contrast to US effect, the biomass and shoot length was not hampered by the UV treatment alone. The similar effect of UV-B had been reported by Murali et al. (35) on total biomass and leaf area of soybean and by Bandurska et al. (2) on dry weight and water content of barley. Correspondingly, Lavola (29) reported that the exposure of UV-B for 3 hour per day on birch had no significant effect on shoot growth and did not result any leaf injuries. Interestingly, some enhancements on plant biomass had also been reported as a response to UV treatment (49). These phenomena were partially due to accumulation of secondary metabolites and leaf thickening (35) indicating that the UV induced formation of phenolic compounds had some antioxidant protective function (29).

The US and UV treatment resulted in slightly lower level (although no significant different) of chlorophyll a and b in ISCOA was noted. Similarly, Kováčik et al. (24) reported that chlorophylls a and b was found slightly lower after UV treatment. Recently, a decrease in total chlorophyll content after UV exposure was also reported by Anttila (2011). This change indicated as shift from primary metabolism to the secondary metabolism (11).

G6PDH (EC 1.1.1.49) is the key enzyme of pentose phosphate pathway. It plays important role to provide precursors in synthesis of phenolic secondary metabolite (45). And significantly higher G6PDH activity was observed in ISCOA after the combined treatment of US 6 and UV 60. The increase in G6PDH activity after UV-B radiation on turfgrasses plant had been reported by Sarkar et al. (42). However, no data was found about the changing in this enzyme activity after US exposure. Higher G6PDH activity of physical elicited samples can be explained by an activation of pentose phosphate pathway (42).

PAL (EC 4.3.1.5) had previously been shown to catalyze the incorporation of phenylalanine into phenolic compounds including RA, thus functioning as entry point enzyme in the initial step of the phenylpropanoid pathway that is responsible for the biosynthesis of these compounds (47) and the first stage in the formation of phenolics is deamination of phenylalanine catalyzed by PAL (2). In our experiments, the UV and US treatment significantly influenced the PAL activity as compared to the control. PAL activity in ISCOA leaves had the highest level at day 3 post-exposure. In Lithospermum erythrorhizon cell culture, PAL activity rose sharply after exposure to fix ultrasonic bath 38.5 KHz and reached the highest level at day 5 after exposure (31). The increase PAL activity of US-stimulated Taxus chinensis cells was also reported by Wu and Lin (54). Additional fact of induced PAL activity after the UV-B treatment was also observed on barley (2, 32) after the psychical stress treatment. PAL
activity is a key factor responsible for increased accumulation of phenolics compound on barley leaves after UV-B radiation (32).

Meanwhile, peroxidase (EC 1.11.1.7) activity in plants had been reported to increase in response to the stress applied by Kwak et al. (27). This increase of PO activity was suggested being responsible for the formation of UV protection such as phenolic compounds (27). Similarly, a dramatic increase in peroxidase after 38.5 kHz US for 2 minutes had also been reported by Wu and Lin (53) as plant biochemical adaptation to US stress. The increment of this enzyme activity was confirmed in our study.

The increase in proline content of ISCOA leaves after UV and US treatment was pronounced. Proline is known as an osmolyte that accumulates in plant cells in response to stress (40). This amino acid synthesis is hypothesized to be able to regulate the pentose phosphate pathway in erythrocytes (28). Elevated proline levels in C. nivalis, turf grasses, and wheat seedlings after UV-B exposure had been previously reported by Duval et al. (11), Sarkar et al. (42) and Yao et al. (57), respectively. It has been hypothesized that stress induced proline accumulation in plants may reduce the damage by production of free radicals in thylakoid membrane of chloroplast (11). Various compensatory mechanisms that may be stimulated proline accumulation and plant survival are attributed to mitochondrial electron transport suppression (11). Although absence effect of UV treatment on barley had recently been reported by Bandurska (2), however, most of the report using UV as stress applied reported an increase in this amino acid (23, 57).

Application of physical elicitors such as US and UV may induce distinct changes in the plant secondary metabolism. Phenolics are one of the most widespread classes of plant secondary metabolites produced from the shikimate, acetate, and potentially the proline linked pentose phosphate pathways (11) for driving precursors (erythrose-4-phosphate) toward shikimate and phenylpropanoid pathway (Figure 1) (56). The significant increase in secondary metabolites, phenolic compounds in particular, after US and UV treatment as here reported, which is not surprising, are similar to finding by many investigators using UV (2) and US as abiotic elicitors. These facts demonstrate an important role of phenolic compounds in the protection cell against the effect of UV and US-physical elicitors.

Additionally, nowadays, research priorities on dietary management of type II diabetes are becoming more prevalent. Considering diet-linked challenge of type II diabetes, consumption of food and beverage rich in α-amylase and α-glucosidase inhibitors – so called hypoglycemic foods – are receiving more attention. α-Amylase and α-glucosidase are well-known key enzymes, playing a vital role in the management of hyperglycemia linked type II diabetes 1. Therefore, characterization of ISCOA extracts on α-Amylase and α-glucosidase inhibition activities were also analyzed.

Lower IC50 was achieved in α-glucosidase inhibitory activity as compared to α-amylase inhibitory activity. Interestingly, higher glucose-6-phosphate dehydrogenase, phenylalanine ammonium-lyase activity, peroxidase and proline content were also observed after the treatment applied which indicated an activation of secondary metabolism through the proline linked pentose phosphate pathway. The results indicated that the combination of UV and US has been shown to be effective in improving antioxidant properties of in vitro shoot cultures of Orthosiphon aristatus. The effectiveness was characterized by the highest increment in the concentration of secondary metabolites accumulation which resulted in the highest antioxidant activity.

With the results obtained in our study, it can be assumed that stimulation of secondary metabolites by US related to the metabolic activity and defense response of plant cell to the mechanical stress such as inducing energy-intense hydrodynamic and acoustic captivation and micro streaming which causes mechanical damage and shear stress to the cells under US treatment 34, 75 as well as various compensatory mechanisms under UV treatment that may involved in plant survival by activation of secondary metabolites production 48, 54.

The UV and US treatment can act as abiotic elicitors to induce plant defense responses 19 and provide a significant contribution to the antioxidant activity of the plant tissue 12, 78. The treatment resulted in a significant increase phenolic compounds which might be responsible for enhancing antioxidant activity: DPPH, TEAC, SOD-like activity and β-carotene/linoleic acid bleaching.
Stimulated antioxidant activity using β-carotene/linoleic acid bleaching method in C. nivalis extract after UV light exposure had been previously reported 48. Other works describing oxidative stress in Chlorella likewise report a markedly increase antioxidant activity after UV-B treatment 79 and in blueberries significantly higher antioxidant activity was detected in all range of UV radiation 77. Similarly, elevated activity of antioxidant enzymes: SOD, reactive oxygen species (ROS) and catalase (CAT), in Scenedesmus quadricauda 58 and turfgrasses 49 was affected by UV exposure and in Taxus chinensis was influenced after 38.5 kHz US for 2 minutes 36. Additionally, Kozak et al. 76 reported that antioxidant properties and flavonoids accumulation in soybean had increased as an adaptive response to UV radiation. These increasing antioxidant activity in US and UV stimulated ISCOA is a further proof that secondary metabolites (as antioxidant components) plays an important role in the cell survival under abiotic stress condition leads to increasing antioxidant activity and protection 37. Plants also synthesize low molecular antioxidants such as phenolics which may directly or indirectly scavenge ROS 58. These phenolics compounds posse protective function, for instance, they are potent UV-B absorbing compounds and they mitigate the effects of free radicals 64 as these were showed in our results by scavenging activity of DPPH free radicals.

The metabolism of phenolic substances has been intensively studied in plant cultures derived from different plant species (6). Here we report that the enhancement of phenolics accumulation in ISCOA as model system was directly proportional to the resistance of the plant to UV and US applied stresses and reflect the ability of Orthosiphon aristatus as a native medicine-tropical plant to occupy in open habitats (49). Longer stresses applied resulted in stronger response (51). It should be noted that the enhancement in phenolics production here was accompanied by an increase in PAL activity, a pivotal steps in the phenylpropanoid biosynthesis and this enzyme is one of the key factors for increased accumulation of these substances (20, 23). This increase of PAL activity was followed by an elevated concentration in proline content. And with respect to those marked increases, these were coupled with an increase in G6PDH enzyme activity. This enzyme is the first rate limiting enzyme to pentose phosphate pathway (Figure 1) (42) and Shetty (43) proposed that proline linked pentose phosphate pathway could stimulate both the shikimate and phenylpropanoid pathways. The modulation of this pathway (Figure 1) could lead to the stimulation of phenolic phytochemicals that can behave as antioxidants by trapping free radicals in direct interaction or scavenge them through a series coupled, antioxidant enzyme defense system reactions (42). UV light exposure had suggested modulating these compounds of C. nivalis by activation biochemical pathways related to proline metabolism (11). And in the our study, higher antioxidant activity in ISCOA due to UV and US treatment were observed in different antioxidant measurements and these phenomenon were associated with higher phenolics content accumulation. Moreover, it has been suggested that antioxidant response pathway in plants relies upon key NADPH-requiring enzymes similar to proline biosynthesis (43). These defensive mechanisms are believed to work through antioxidant response pathways involving peroxidases as well as phenolics biosynthesis (43). In the present study, higher peroxidase activity in ISCOA has been achieved after the physical stress treatment, therefore, these obtained results may reflect associated role with proline linked pentose phosphate pathway in the cytosol under stresses induced condition. These interesting findings provide stimulus for further research into deeply genetic responsible for this enhancement phenomenon by UV and US in ISCOA as a model system.

5. Conclusion

In conclusion, the present work has demonstrated the beneficial effects of UV and US treatment on secondary metabolites, enzyme activity, proline content and antioxidant properties of ISCOA as model study. The combination of UV and US has been shown to be effective in improving α-amylase and α-glucosidase inhibitory activities of ISCOA. The effectiveness was characterized by the lowering IC50 of α-amylase and α-glucosidase inhibitory activities. The results indicated that UV and US which can be considered as physical elicitation stressors may activate phenolic substances production in ISCOA through a proline linked pentose phosphate pathway. This mechanism, however, remained to be
verified through further depth investigation focused on genetic expression that would be provide detail mechanism and understanding phenolics biosynthesis in ISCOA under the stresses applied.

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