



## EFFECT OF UV-C STRESS ON TOTAL PHENOLICS, TOTAL FLAVONOIDS AND KHELLIN CONTENTS IN *AMMI VISNAGA* (L.) LAM. FRUITS

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*Ammi visnaga* fruits (L.) Lam., is widely used for its antispasmodic and vasodilating effects. These effects are attributed to its main content of khellin. In our research, we studied the effect of UV-C stress on the metabolite production of the fruits. Fruits were subjected to UV-C treatment for ½, 1, 2, 3, 4 and 5 hr. The change in the metabolic profile was studied by the quantification of total phenolics, total flavonoids and khellin in the methanolic extracts of the fruits applied to stress and extracts of control fruits. Total phenolics and total flavonoids were quantified colorimetrically using appropriate reagent for each. Folin-Ciocalteu reagent was applied for the quantification of total phenolics while 2% AlCl<sub>3</sub> solution was applied for the quantification of flavonoids. On the other hand, khellin was quantified by HPLC analysis. Our results showed that the concentrations of total phenolics, total flavonoids and khellin contents of the fruit extracts are significantly increased by exposing the fruits to UV-C stress with maximum production at 1 and 2 hr.

**Keywords:** *Ammi visnaga*, Folin-Ciocalteu reagent, Khellin, UV-C stress, Total phenolics, Total flavonoids.

### INTRODUCTION

*Ammi visnaga* L. Lam., known as Khella Baladi or toothpick weed, is an annual herbaceous plant indigenous to Egypt especially in the Delta region, and it surrounds the Nile River. The fruits are the main source of the medicinally used metabolites<sup>1</sup>. It is known for its content of furanochromones mainly khellin and visnagin, flavonoids and essential oil<sup>2</sup>. Khellin, the major metabolite of *A. visnaga*, is used for the treatment of vitiligo<sup>3-5</sup>, urolithiasis<sup>6</sup> and angina pectoris<sup>7</sup>. In addition, both khellin and visnagin are reported as environmentally safe bioherbicides<sup>8</sup>.

Exposing plants to stress conditions either biotic stress (e.g., bacteria and fungi) or abiotic stress (e.g., heavy metals, UV irradiation, salinity, drought, excessive heat or cold) can affect the metabolic profile of the plant. For

example, microbial polysaccharides (chitosan, chitin and pectin)<sup>9-11</sup>, as well as, yeast extract, jasmonic acid, *Aspergillus niger* and *Aspergillus flavus* fungal mycelium homogenates<sup>12</sup> have elicited anthraquinone production on *Morinda citrifolia* cell culture. Exposing *Capsicum annuum* leaves to UV and salt stress significantly have increased the accumulation of the flavonoid cynaroside and graveobioside, respectively<sup>13&14</sup>.

Few studies concerning the effect of different stress conditions on the production of *A. visnaga* metabolites were reported. Earlier data have showed that the exposure of *A. visnaga* cell culture to the abiotic stress benzo(1,2,3)-thiadiazole-7-carbothionic acid *S*-methyl ester (BION1) have induced higher production of visnagin (3 times more than control samples). Both khellin and visnagin production have been increased (5 and 2.5

times higher than control) by the biotic elicitor scleroglucan<sup>14</sup>. In addition, stress growing conditions have changed the metabolite profile of *A. visnaga* umbel methanolic extract. Growing the plant under controlled hydroponic conditions have stimulated higher production of khellin and visnagin (88 and 48 times) more than the detectable amounts in the methanolic extracts of wild plants. While growing under hypoxic and saline conditions (25 mM NaCl) have induced higher production of prim-*O*-glucosyl cimifugin<sup>15</sup>. Recently, the effect of drought stress as well as foliar spraying with salicylic acid on *A. visnaga* metabolites have been recorded. Growing *A. visnaga* under drought stress conditions have increased the production of total phenolics and visnagin in the fruits, aerial parts and roots, while khellin was significantly have increased in the aerial parts and umbels<sup>16</sup>.

According to our knowledge there is no report concerning the effect of UV stress on the metabolites of *A. visnaga*. In this work, the effect of UV stress on the metabolic profile of *A. visnaga* fruits was studied. Total phenolics, total flavonoids and khellin were quantified in the methanolic extracts of *A. visnaga* fruits exposed to UV-C stress ( $\lambda=254$  nm) for different time intervals and compared with control methanolic extract not exposed to UV stress.

## MATERIALS AND METHODS

### General

NMR spectra were recorded in CDCl<sub>3</sub> using Bruker Avance III spectrometers (400 MHz for <sup>1</sup>H NMR; 100 MHz for <sup>13</sup>C NMR, Faculty of Pharmacy, Cairo University, Egypt). UV-VIS spectra were recorded on UV-VIS spectrophotometer (Pharmacia Biotech, Cambridge, England). Analytical TLC was conducted on precoated aluminum sheets of silica gel 60 GF<sub>254</sub> (0.25 mm, E. Merck) and the spots were detected by ultraviolet irradiation (254 and 366 nm) followed by spraying with 10% v/v H<sub>2</sub>SO<sub>4</sub> reagent in methanol and heating at 110 °C for 5 min. Solvents: petroleum ether, EtOH and MeOH (El-Nasr Pharmaceutical and Chemical Co., Delta Egypt Chem. and Hawamdia Co., Egypt). Authentic quercetin was obtained from Department of Pharmacognosy, Assiut University as authentic

sample isolated from plants. Its identity was confirmed by <sup>1</sup>H NMR and <sup>13</sup>C NMR and it was applied for the quantification of flavonoids. Tannic acid, sodium hydroxide (NaOH) and sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) were purchased from Al-Gomhouria Co. (Assiut, Egypt). Folin-Ciocalteu reagent was purchased from Qualikems Fine Chem Pvt. Ltd. (Nandesari, India).

### Plant material

*A. visnaga* plants were cultivated at the faculty of Pharmacy farm, Assiut University, Assiut, Egypt. Fruits were harvested on December 2017 as they are fully grown. The collected fruits were air dried and stored at room temperature for the use.

### UV radiation stress and preparation of plant extracts

Twenty-one samples (each of 5 g) of *A. visnaga* fruits were subjected to UV-C irradiation for different periods of time: 0, 1/2, 1, 2, 3, 4 and 5 hr under laminar flow cabinet (Forma Scientific,  $\lambda=254$  nm) at room temperature. The fruits were placed in Petri dishes on a very thin layer and about ~60 cm below the UV Lamp. Each time was presented by three samples. After UV application, each dish content was transferred to Falcon tube, sonicated with methanol for 2 hr and the process was repeated two more times. The solution was filtered then concentrated under vacuum. The obtained extract was stored in vials.

### Isolation of khellin

*A. visnaga* fruits (300 g) were grinded and extracted with water (700 ml × 3 times) then filtered and concentrated under vacuum. The residue (95 g) was extracted with petroleum ether (1 L × 3). The concentrated petroleum ether extract was dissolved in methanol and khellin was crystallized to give 250 mg khellin. TLC of isolated khellin gave yellow spot, R<sub>f</sub> value (0.3, dichloromethane: methanol 9.5:0.5). <sup>1</sup>H NMR (400 MHz; CDCl<sub>3</sub>):  $\delta$ , ppm 7.63 (d, *J*= 2.2 Hz, H-2'), 7.03 (d, *J*= 2.2 Hz, H-3'), 6.11 (*s*, H-6), 4.22 (*s*, 8-OCH<sub>3</sub>), 4.08 (*s*, 4-OCH<sub>3</sub>), 2.42 (*s*, 2-CH<sub>3</sub>) (Table S1 & Fig. S1). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz):  $\delta$ , ppm 178.28 (C-4), 164.13 (C-2), 148.91 (C-7), 147.43 (C-5), 147.16 (C-8a), 145.52 (C-2'), 129.94 (C-8),

119.48 (C-6), 113.70 (C-4a), 110.61 (C-3), 105.21 (C-3'), 62.38 (C-8 -OCH<sub>3</sub>), 61.55 (C-5 -OCH<sub>3</sub>), 20.15 (C-2 -CH<sub>3</sub>) (Table S2 & Fig. S2).

### Quantification of flavonoids

All experiments were conducted in triplicate. In a test tube 2 ml of *A. visnaga* methanolic extract (1 mg/ml) was transferred and mixed with 2 ml of AlCl<sub>3</sub> (2% in methanol). The mixture was incubated for 60 min at room temperature. The developed yellow colour, indicating the presence of flavonoids, was spectrophotometrically measured at  $\lambda_{\max}$  420 nm. Using known concentrations of quercetin, a standard calibration curve was built at  $\lambda_{\max}$  = 420 nm. The total flavonoid content was calculated as the quercetin equivalent (mg/10 g of sample)<sup>17</sup>.

### Quantification of total phenolics

Each extract (0.2 ml, 1x10<sup>-3</sup> mg/ml) was transferred into a 10 ml glass tube and diluted to 3 ml with distilled water. In each tube, 0.5 ml Folin-Ciocalteu reagent (1:1 with water) and 2 ml Na<sub>2</sub>CO<sub>3</sub> (20%) were sequentially added. The developed blue colour was measured spectrophotometrically at  $\lambda_{\max}$  759 nm. A standard calibration plot was built at  $\lambda_{\max}$  = 759 nm using known concentrations of tannic acid. The concentrations of phenols in the test samples were calculated from the calibration plot and expressed as mg tannic acid equivalent of phenol / 10 g of sample<sup>17</sup>.

### HPLC analysis

HPLC analysis was carried out with Thermo high performance liquid chromatography instrument equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190-600 nm), degasser, a column having an in-line filter. Each sample was dissolved in methanol and analyzed using TSKgel ODS-100z column no. H0018 (5  $\mu$ m particle size silica, 250  $\times$  4.6 mm I.D.). The mobile phase consisted of linear gradient of H<sub>2</sub>O-CH<sub>3</sub>CN (75:25 to 25:75 in 35 min, and then to 0:100 in 5 min) and a flow rate of 1.0 ml/min. A standard calibration plot for khellin was done using the following concentrations (1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 mM). Khellin's retention time  $t_R$  was recorded at 14.5 min.

### Statistical analysis

The collected data were analyzed by Excel sheet (Microsoft office 2019). The Experimental results were expressed as the mean  $\pm$  standard error of the mean. The significance of the differences between experimental and control groups were calculated using Student's t-test (using Excel sheet, Microsoft office 2019). A *p* value of < 0.05 vs control was considered statistically significant (\*). A *p* value of < 0.001 vs control was considered statistically highly significant (\*\*).

## RESULTS AND DISCUSSION

### Results

The effect of UV-C stress on the metabolic profile of *A. visnaga* fruits was studied by the quantification of total phenolics, total flavonoids and khellin in the methanolic extracts of the fruits exposed to UV- stress for 1/2, 1, 2, 3, 4 and 5 hr, as well as, the methanolic extracts of the control fruits.

### Quantification of flavonoids

Total flavonoids were quantified using AlCl<sub>3</sub> method. This method depends on the colorimetric quantification of the yellow colour resulting from the interaction of the keto group of flavonoids with AlCl<sub>3</sub>. The intensity of the yellow colour is directly proportional to the concentration of the flavonoids and it can be measured colorimetrically at  $\lambda_{\max}$  420 nm. In this study, the concentration of flavonoids in control samples was found to be 0.82 mg/g fruit. Exposing fruits to UV stress seems to significantly increase (*p* value < 0.05) the production of flavonoids at 1/2, 1 and 2 hr in comparison to control. Maximum values were detected in samples exposed to 1 h and 2 h UV stress: 1.1  $\pm$  0.08 and 1.2  $\pm$  0.10 (mg/g fruit), respectively. (Table 1 & Fig. 1).

### Quantification of total phenolics

Further, total phenolics were estimated using Folin-Ciocalteu reagent colorimetric method. This method depends on the interaction of phenolic compounds with Folin-Ciocalteu reagent in basic media created by the presence of sodium carbonate 20% solution. This interaction results in the formation of blue

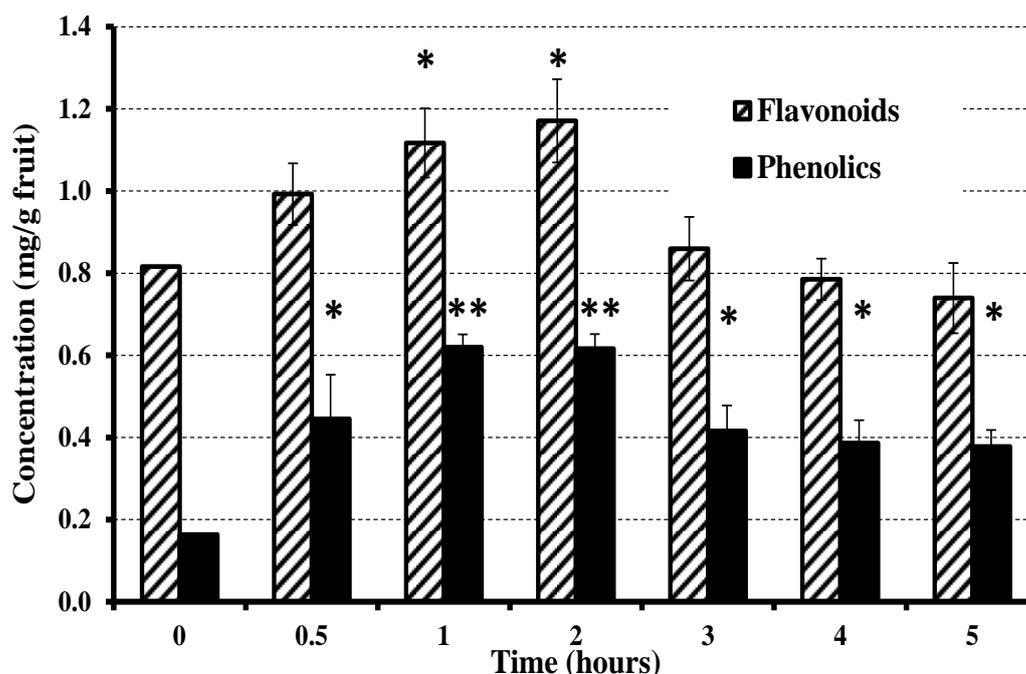
coloured complex. The intensity of the blue colour is directly proportional to the concentration of phenolic compounds and it can be measured colorimetrically at  $\lambda_{\text{max}}$  759 nm. Similar to flavonoids, the concentrations of total phenolics increased gradually upon UV stress. The concentration of total phenolics in control samples was found to be  $(0.16 \pm 0.0$  mg/g fruit). Maximum values were detected in samples exposed to 1 hr. and 2 hr UV stress:  $0.62 \pm 0.03$  and  $0.62 \pm 0.04$  mg/g fruit, respectively. The concentrations of total

phenolics started to decrease gradually in methanolic extracts of fruits exposed to 3, 4 and 5 h of UV stress, but still higher than control:  $0.42 \pm 0.62$ ,  $0.39 \pm 0.06$ ,  $0.39 \pm 0.04$  mg/g fruit, respectively (Table 1 & Fig. 1). Statistical analysis indicated that the increase of the phenolics production at 1 and 2 hr is highly significant ( $p$  value < 0.001), while of the phenolics production at ½, 3, 4 and 5 hr is significant ( $p$  value < 0.05) in comparison to control (Table 1 & Fig. 1).

**Table 1:** Total flavonoids and total phenolics (mg/g fruit)  $\pm$  S.D. in methanolic extracts of *Ammi visnaga* L. Lam. fruits after UV-C stress for different times.

Elicitation time (hr)	0	0.5	1	2	3	4	5
Amount of total flavonoids (mg/g fruit) $\pm$ S.D.	$0.82 \pm 0$	$0.99 \pm 0.08^*$	$1.1 \pm 0.08^*$	$1.2 \pm 0.10^*$	$0.86 \pm 0.08$	$0.79 \pm 0.05$	$0.74 \pm 0.09$
Amount of total phenolics (mg/g fruit) $\pm$ S.D.	$0.16 \pm 0$	$0.45 \pm 0.10^*$	$0.62 \pm 0.03^{**}$	$0.62 \pm 0.04^*$	$0.42 \pm 0.62^*$	$0.39 \pm 0.06^*$	$0.39 \pm 0.04^*$

\* $p$  value of < 0.05 vs control (0 time) was considered statistically significant, \* $p$  value of < 0.001 vs control was considered statistically highly significant (\*\*).



**Fig. 1:** Effect of UV-C stress on concentration of total flavonoids and total phenolics in methanolic extracts of *Ammi visnaga* L. Lam. fruits after UV stress for different times. Error bars represent standard deviations of one experiment conducted in triplicate. \* $p$  value of < 0.05 vs control (0 time) was considered statistically significant, \* $p$  value of < 0.001 vs control was considered statistically highly significant (\*\*).

### Quantification of khellin

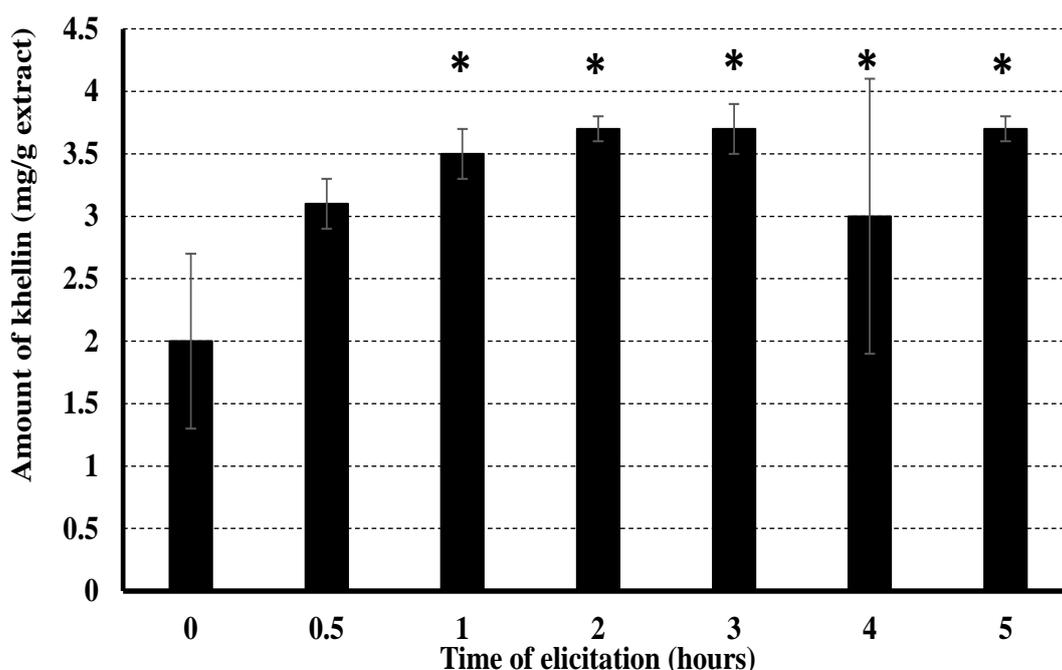
Khellin was quantified by HPLC-DAD analysis. Authentic khellin was isolated from *A. visnaga* fruits as described in the experimental part. Its purity and identity were confirmed by the comparison of its <sup>1</sup>H NMR and <sup>13</sup>C NMR with published data<sup>8,18&19</sup> (Fig.1S, Fig. 2S; Table 1S& Table 2S). The calibration curve was built using isolated khellin as described under the experimental part (Fig. 3S). The comparison of khellin concentration in the methanolic extracts of treated and control *A. visnaga* fruits showed that khellin concentration is increased by increasing the time of UV exposure. The

increase in the production of khellin is significant (*p* value < 0.05) in methanolic extracts of samples exposed to 1, 2, 3, 4 and 5 hr UV stress. The concentration of khellin in control samples was found to be (21.1 ± 8.1 mg/g extract). The highest levels were detected in samples exposed to 1 hr and 2 hr exposure periods: 39.9 ± 5 and 40.1 ± 4.2 mg/g extract, respectively. The concentrations of khellin started to decrease gradually in methanolic extracts of fruits exposed to 3, 4 and 5 hr of UV stress but still significantly higher than control: 33.6 ± 1.3, 27.3 ± 9.1, 33.6 ± 1.3 mg/g extract, respectively (Table 2 & Fig. 2).

**Table 2:** Khellin content in methanolic extracts of *Ammi visnaga* L. Lam. fruits after UV-C elicitation for different times

Elicitation time (h)	0	0.5	1	2	3	4	5
Amount of khellin (mg/g extract) ± SD	21.1 ± 8.1	28.3 ± 3.2	39.9 ± 5*	40.1 ± 4.2*	33.6 ± 1.3*	27.3 ± 9.1*	33.6 ± 1.3*

\**p* value of < 0.05 vs control (0 time) was considered statistically significant



**Fig. 2:** Effect of UV-C stress on concentration of khellin in methanolic extracts of *Ammi visnaga* L. Lam. fruits after UV stress for different times. Error bars represent standard deviations of one experiment conducted in triplicate. \**p* value of < 0.05 vs control (0 time) was considered statistically significant

## Discussion

In this work the effect of UV-C irradiation on phenolics, flavonoids and khellin contents of *A. visnaga* fruits was studied. Based on the wavelength, UV light is classified into 3 categories UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm)<sup>20</sup>. Both UV-B and UV-C are reported to induce the metabolic changes and the defense mechanisms of plants. Although, most of UV-C radiation is filtered out by ozone layer, it is known to be more effective than UV-B in inducing the plant defense mechanisms<sup>20& 21</sup>. In addition, UV-C has several applications in food technology. Exposing harvested fruits to UV-C increases their shelf-life due to its germicidal activity and because it delays the food softening by inhibiting several cell-wall degrading enzymes<sup>22-27</sup>.

Results showed the significant increase of total flavonoids, total phenolics and khellin in response to UV irradiation. Plants can response to UV stress by increasing the production of phenolics and flavonoids as a protective mechanism against the harmful effect of UV light<sup>20</sup>. It seems that the role of total phenolics and khellin in protecting *A. visnaga* fruits against UV stress is much more than flavonoids. This because flavonoids showed significant increase up to 2 hr. only, while the significant increase in the concentrations of total phenolics and khellin lasts up to 5 h stress with maximum value at 1 to 2 hr.

The observed increase in total phenolics, flavonoids and khellin contents in *A. visnaga* fruits treated with UV can be explained by increasing the activities and the up-regulations of the corresponding biosynthetic enzymes and genes involved in the phenylpropanoid pathway. The main phenylpropanoid key enzymes are: phenyl alanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and cinnamic acid-4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), *p*-coumaric acid-3-hydroxylase (C3H), caffeic acid *O*-methyltransferase (COMT) and ferulic acid-5-hydroxylase (F5H)<sup>21,28&29</sup>. For example, exposing carrot slices to UV-C light for 15 minutes have increased the production of total phenolics 111 % in comparison to carrot slices not exposed to UV light (control). This increase is correlated to the induction of PAL activity that have been increased 30 folds.

Several phenolic compounds have been increased by UV light in different percentage: chlorogenic acid, ferulic acid, dicaffeoylquinic acid, isocoumarin, *p*-hydroxybenzoic acid, *p*-coumaric acid derivative and 3,5-dicaffeoylquinic acid<sup>21</sup>. Treatment of *Vigna mungo* (L.) Hepper seeds with UV have resulted in significant accumulation of flavones, total soluble phenols and anthocyanins, which is correlated to the increase of PAL and TAL activities<sup>28</sup>. Under UV stress of barley seedlings phenolic acids were significantly accumulated, as well as, the activity of the corresponding biosynthetic key enzymes and their genes have been increased<sup>29</sup>. Alothman *et al.*<sup>30</sup> explained the increase in phenolic contents in some tropical fruits after UV irradiation to increasing their extractability as UV initiates depolymerization and dissolution of cell wall polysaccharides. However, exposing plants to UV light is known to increase the strength and to decrease the elasticity of plant cell wall<sup>31&32</sup>. Further work to estimate the possible mechanism(s) involved in the accumulation of total phenolics, flavonoids and khellin in *A. visnaga* fruits after exposure to UV is to be done.

## Conclusion

To the best of our knowledge, it's the first time to study the effect of UV light on the production of secondary metabolites of *A. visnaga* fruits. Our study showed that the concentration of total flavonoids, total phenolics and khellin increase with increasing the stress time. The highest effects were recorded after 1 and 2 hr exposures to UV stress. These results indicate the possible role of these metabolites in protecting the plant against UV stress.

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## نشرة العلوم الصيدلانية جامعة أسيوط



### تأثير الأشعة فوق البنفسجية على الفينولات الكلية والفلافونويد الكلي والخلين في ثمار الخلة البلدي

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تستخدم ثمار الخلة البلدي على نطاق واسع لتأثيراتها المضادة للتشنج والمغص. تُعزى هذه التأثيرات إلى محتواها الرئيسي من الخلين. في هذا البحث، قمنا بدراسة تأثير الأشعة فوق البنفسجية على إنتاج المواد الأيضية في هذه الثمار. خضعت الثمار للأشعة فوق البنفسجية لمدة نصف ساعة وساعة وساعتين وثلاث وأربع وخمس ساعات. تمت دراسة التغير في الشكل الأيضي من خلال القياس الكمي لمركب الفينول الكلي والفلافونويد الكلي والخلين في المستخلصات الميثانولية للفاكهة التي تعرضت للضغط ومستخلصات الفاكهة التي لم تتعرض لضغط الأشعة فوق البنفسجية. تم قياس كمية الفينولات الكلية والفلافونيدات الكلية بطريقة كالوريمترية باستخدام كاشف محدد لكل منهما. تم تطبيق كاشف الفولين سيكالتو لتقدير إجمالي الفينولات بينما تم تطبيق ٢٪ محلول كلورايد الالمونيوم لتقدير مركبات الفلافونويد. من ناحية أخرى، تم قياس كمية الخلين بواسطة تحليل جهاز الكروماتوجرافي السائل عالي الأداء أظهرت نتائجنا أن تراكيز الفينولات الكلية ومحتوى الفلافونويد الكلي والخلين لمستخلصات الفاكهة تزداد بتعريض الثمار لضغط الأشعة فوق البنفسجية بأقصى إنتاج عند ساعتين.