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Reflex of Oleandrin Production and Molecular Changes on the *Nerium oleander* Cell Suspension Culture under UV-A Radiation Stress Effect

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THE AIM of this study is to determine the oleandrin concentrations in the *Nerium oleander* cell suspension culture after being exposed to UV-A (365nm), at different time intervals (0 controls, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50min). The results showed that the maximum time limits for carrying N. oleander cells suspension culture to exposure of UV-A is 30min.

The present study also aims at investigating changes of the protein bands, peroxidase isozyme and oleandrin concentration at the same condition. Oleandrin was separated and quantified by HPLC. The investigation of protein changes was conducted using SDS PAGE electrophoresis techniques. The results revealed that the changes of the protein bands depend on different time intervals of exposure to A-UV radiation. Thus, the changes of the oleandrin production were consequent to gene expression changes. Peroxidase isozyme analysis, showed that exposure to UV-A for the 5, 15 and 20min had a clear impact on the peroxidase isozyme, but the greatest impact was in the sample exposed to UV-A for a period of 15 minutes (0.093±0.01) µg/ml, the same sample showed the highest concentration of oleandrin compared to the control (0.022±0.001) µg/ml. However, the other samples were less affected by the exposure to UV-A.

Keywords: *Nerium oleander* L., Oleandrin, UV-A, SDS-PAGE electrophoresis, Peroxidase isozyme.

Introduction

The *Nerium oleander* plant belongs to the Dogbane family Apocynaceae, it is an evergreen shrub or small tree typically grows to 2–6m in height, and is cultivated all over the world, particularly in temperate climates. Flowers grow in clusters in terminal branches, each 2.5–5cm, funnel-shaped with five lobes, fragrant, and show various colors from pink to red, white, peach, and yellow (Dey & Chaudhuri, 2014).

Oleandrin (C32H48O9), is of a glycoside content and the mainly toxin. The concentration of oleandrin in *Nerium oleander* tissues is 0.08% (Schvartsman, 1979). Skin care products were extracts from *N. oleander;* its benefits include antibacterial, antiviral, immune, and even antitumor properties associated with topical use (Lin et al., 2008). Yellow oleander has its own unique cardiac glycoside content that includes compounds such as Thevetia.

Plant peroxidases isozymes are involved in various physiological processes **(**Passardi et al., 2005). Class III plant peroxidases, are plant-specific oxidoreductases, and lignification participate (Lewis, 1999), wound healing and abiotic stress (Bernards et al., 1999), and aspects of plant growth regulator action (Gazarian et al., 1998).

Ultraviolet (UV) can induce tryptophan and tyrosine radicals by illumination (Creed, 1984). Heme groups are also excellent UV absorbers. UV illumination might, in some cases, induce enzyme inactivation (Saha, 1997).

The present study aims at finding out changes in the peroxidase isozyme by exposure to UV and its relation to oleandrin productivity in the *N. oleander* cell suspensions culture in order to produce high productivity cellular lines of oleandrin substance which are used widely in the pharmaceutical compounds.

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Materials and Methods

Plant material

Sampling

Nerium oleander are plants of local cultivar, with pink rose flowers. This cultivar was localized in Egypt. To obtain young plant material, plant seeds were germinated in test tubes containing 20ml of basal medium (BM). The cultured tubes were incubated in a growth chamber at (26±2˚C) at day and 22˚C at night under a photoperiod of 16h/day of 2000 LUX intensity.

Basal medium (BM) consisting of [major and minor salts (Murashige & Skoog, 1962) MS, B5 with vitamins (pantothenic acid) (Gamborg et al., 1968), and 3.0% sucrose] was provided. The pH was adjusted to 5.8 and the medium was solidified with 0.7% agar before being autoclaved for 20 min at 120˚C (Niu et al., 2008).

Callus induction:

Apical shoot tips (1 to 2mm in length) from seedlings (15-day-old) of seedling-derived cultures were maintained by transferring shoot apices to glass vessels containing MS medium supplemented with 2,4-Dichlorophenoxyacetic acid (2, 4-D) 2mg/L and Kineten 1mg/L. The cultured jars were incubated in a growth chamber at (26±2˚C) at day and 22˚C at night under photoperiod of 16 h/day of 2000 LUX intensity (Murashige & Skoog, 1962).

Cell suspension preparation and treatments

The induced callus was divided and transferred to vessels containing 40ml of the above mentioned media which were used in callus induction, but without agar, then the vessels were placed on gyratory shakers (130) rpm incubated under the same conditions used in callus induction. After two weeks cell suspension were irradiated by UV-A (365nm) using Vilber Lourmat lamb model VL-4.LC, at different intervals (5, 10, 15, 20, 25, 30, 35, 40, 45 and 50min), in addition to an untreated sample that was considered a control sample (0 times).

Determinations and analysis

pH medium measuring and cell survivor

Alkinazation of Media was measured by pH meter after exposure to UV-A (365nm), for different intervals (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50min), time zero represents untreated control sample. Also the changes on pH of media within 120min (every10min) for each sample were studied.

The cell survivor measured throw Trypan Blue, Trypan Blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells which do not take up certain dyes, whereas dead (non-viable) cells do (Liesche et al., 2015).

SDS-PAGE protein electrophoresis:

Eight samples extracted from crude were prepared by homogenized (1g frozen biomass) in a pre-cooled blender by liquid nitrogen, sodium dodecyl sulfate polyacrlamid gel electrophoresis (SDS- PAGE) was performed according to the method described by Laemmli et al. (1970) and modified by Studier et al. (1973).

Peroxidase isozyme analysis

Native – polyacrylamide gel electrophoresis (Native – PAGE) was used to identify the effect of various treatments on peroxidase isozyme finger prints. Their finger prints of peroxidase isozymes were determined according to Jonathan & Wendell (1990)

Oleandrin HPLC analysis

The samples were extracted according to Tittel & Wagner (1981) method, briefly, 10g frozen biomass from each sample in a blender pre cooled by liquid nitrogen was extracted with 15ml of methanol water (90:10) using a homogenizer for 5min. Oleandrin extract from *N. oleander* cell suspensions were analyzed in reverse phase HPLC (Shimadzu, model SPD-20A), equipped with a diode array detector (DAD), using a reversed phase Eclipse C_{18}, (150×4.6mm i. d.; 3.5µm). All samples (10ml) were filtered through 0.22µm filters (Millipore) prior to use; and 20µl were loaded for each run. For all methanol extracts, Acetonitrile: Water (60:40) was used as mobile phase at 220nm, 30ºC temperature of the column, and at 1.5ml/min flow rate.

Value and Peak Value were calculated, and results were analyzed using SPSS 22 software one way ANOVA test, sigma plot program was used to establish statistical histograms.

Results and Discussion

Medium alkalization and cell survivor

Table 1 shows that pH values of the *N. oleander* cell suspensions culture exposed to UV-A ray, increased gradually by increasing the time of exposure, a gradual increase in the pH values was associated with increasing duration of exposure to UV-A ray.

As it is shown that in zero time sample (control), no increasing in the pH values occurred after 120min of measuring, but after five minutes of the *N. oleander* cell suspensions culture exposed to UV-A ray, the pH was increased gradually from 5.8 to 6. Also, the pH values were increased by increased time of exposure to UV-A ray and the passage of time until it reached the highest values 6.7 in 30min corresponding 120min when the medium was alive. The colors of mediums until 30min were yellow. On the other hand, the pH values were rated higher than 6.7, it has reached that 8, but the mediums died and the color was brown.

N. oleander cell suspensions culture exposed to UV-A ray with a time higher than 30min resulted in a significant mortality of 100% percentage.

In zero time sample (control) and after five, ten and fifteen minutes of exposing the *N. oleander* cell suspensions culture to UV-A ray, the unstained cells (alive) were 4, 4, 3 and 3 cells, respectively, with 100% percentage survivor in 10µl of cell suspensions culture. The stained cells were zero cells. The color of a medium was yellow (Table 2), the total number of cells in one ml of cells suspensions culture was 40000 cells approximately.

The unstained cells (alive) after twenty, twenty five and thirty minutes of exposing the *N. oleander* cell suspensions culture to UV-A ray, were 3, 2 and 1 cells with percentage 75, 67 and 33% survivor respectively in 10µl of cell suspensions culture. The stained cells (died) were 1, 1 and 2 cells, respectively. The color of medium to all the above treatments were yellow (Table 2), the total numbers of cells in one ml of cells suspensions culture were approximately 40000, 30000 and 30000 cells, respectively. On the other hand, the *N. oleander* cell suspensions culture after thirty five, forty, forty five and fifty minutes of exposing the *N. oleander* cell suspensions culture to UV-A ray died, and were free from unstained cells (alive).

The colors of media to all the above treatments were brown. Accordingly, no data were obtained after exposing cell suspensions to the UV-A ray after 30min.

TABLE 1. pH values detected after exposed *N. oleander* cell suspension to A-UV rays for different period the measuring was every 10min.

No.	Period of exposure to A-UV (min.)	0	10	20	30	40	50	60	70	80	90	100	110	120
1	0	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
2	5	5.8	5.8	5.8	5.8	5.8	5.8	5.8	6	6	6	6	6	6
3	10	5.8	5.9	5.9	5.9	6.0	6.0	6.1	6.1	6.1	6.2	6.2	6.3	6.4
4	15	5.8	5.9	6	6	6.1	6.1	6.1	6.2	6.3	6.3	6.4	6.4	6.5
5	20	5.8	5.9	6	6.2	6.2	6.2	6.2	6.2	6.3	6.4	6.4	6.4	6.5
6	25	5.8	5.9	6	6	6.2	6.2	6.3	6.3	6.4	6.4	6.5	6.5	6.6
7	30	5.9	6.0	6.0	6.1	6.2	6.3	6.3	6.4	6.4	6.5	6.5	6.6	6.7
8	35*	7	7	7	7	7	7	7	7	7	7	7	7	7
9	40*	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.3
10	45*	7.1	7.7	7.8	8.0	8.0	8.1	8.0	8.0	8.0	8.0	8.0	8.0	8.0
11	50*	7.1	7.7	7.8	8.0	8.0	8.1	8.0	8.0	8.0	8.0	8.0	8.0	8.0

*Died cell suspension

TABLE 2. Counting of cells which were dyed and non-dyed by Trypan Blue solution throw hemacytometer as well as percentage of survivor of the cells.

No.	Period of exposure to A-UV (min.)	No. of unstained cells (live)	Percentage of a live cells %	No. of stained cells (died)	Percentage of a died cells %	Total No. of cells in 10µl cells suspension	Color of cells suspension	T. C.	G. S.
1	0	4	100	-	-	4	Yellow	40000	L
2	5	4	100	-	-	4	Yellow	40000	L
3	10	3	100	-	-	3	Yellow	30000	L
4	15	3	100	-	-	3	Yellow	30000	L
5	20	3	75	1	25	4	Yellow	40000	L
6	25	2	67	1	33	3	Yellow	30000	L
7	30	1	33	2	67	3	Yellow	30000	L
8	35	-	-	1	100	1	Brown	10000	D
9	40	-	-	-	-	-	Brown	-	D
10	45	-	-	-	-	-	Brown	-	D
11	50	-	-	-	-	-	Brown	-	D

The dark cells in table refer to died cell suspension
G. S.: The general state of the *N. oleander* cell suspensions culture
L: Live cell suspensions D: Died cell suspension
T. C.: Total No. of cells in 1ml of cells suspension

The results showed the relationship between the pH values and vital cells in the *N. oleander* cell suspensions culture after exposure to UV-A rays. *N. oleander* cell suspensions culture after thirty five, forty, forty five and fifty minutes from exposing the cell suspensions culture to UV-A ray may result in damaging cell membranes and cell contents getting out the medium, and this supports the hypothesis of cells disappearance during microscopic examination. However, in the case of exposure to UV-A ray less than 40min, the cell membrane becomes more susceptible to the exchange of ions, without being destroyed, causing a small increase in the pH values.

These results are in agreement with those of the Ramani & Chelliah (2007) who studied plants belonging to the same family Apocynaceae and found that the pH values of *C. roseus* cell suspension increased after irradiation with UV rays for 5min and that the value decreased after that. They reported that the increasing of the pH values affects directly the charged and polarity of the cell surfaces and that activate Myelin Basic Protein Kinase (MAPK) enzyme. This enzyme could increase the indole alkaloids through RNA transcription in the cell nucleus.

SDS - PAGE protein electrophoresis

The data shown in Table 3 and Fig. 1 reveals that the maximum numbers of bands were seven in all lanes, but the minimum shows a unique band in the lane No. 6 corresponding with 25min of exposure to A-UV was observed.

The proteins zymograms isolated from the *N. oleander* cell suspension culture of the different six time intervals of exposure to A-UV were subjected to SDS-PAGE analysis and a total bands of 36 was observed (Table 3). The bands No. one, two, three, four, five and six corresponding with 250, 200, 150, 100, 70 and 50kDa, respectively, were present in all the treated samples and control, but this band was absent in lane No. 6 with corresponding 25min. The band no. 7 corresponding with 20kDa was present in all lanes, butthis band was present as a unique band in treatment of 25min from the cell suspension culture of *N. oleander* exposed to UV-A ray.

In Fig. 1 difference in protein bands densities shows differences depending on the time of exposure to UV-A ray. The bands from 1 to 7 were in intensities ranging between the very faint and dark after exposure to UV for 5, 10, 15 and 20min,

compared to control, butin the unique band No. 7, the only treatment that was exposed to UV-A rays for 25min appeared very dark similar to the band in the control.

On the other hand, the bands from 1 to 7 were very dark in intensity after 30min from exposure to UV–A ray. It is therefore possible to infer a change in gene expression due to exposure to UV-A rays.

The current results are in agreement with Kumar et al. (2011) who studied the effect of different varieties of *Nerium oleander* L. on the protein bands, through SDS-PAGE electrophoresis technique and found that the protein bands may differ greatly in intensity, number and molecular weight depending on the different varieties. According to the results mentioned, the exposure to UV-A rays for different time intervals may affect the genetic material, and therefore changing gene expression in the cells followed by a change in the protein bands.

Peroxidase isozyme

The results of electrophoretic patterns of peroxidase isozyme extracted from *N. oleander* cell suspension cultures of the six time exposure of UV-A ray are shown in Fig. 2, the maximum number of bands were three. In zero time of exposure to UV-A ray (lane 1) showed one band with the highest intensity. Lanes (2 and 5), after five and twenty minutes of exposure to UV-A ray showed two bands; the band No. one was the highest intensity and the band No.2 was lowest intensity, the lowest band was absent in control. Lanes (3 and 7), after ten and thirty minutes of exposure to UV-A ray showed one band with medium intensity compared the control. Lane (4), after fifteen minutes of exposure to UV-A ray showed three bands; the band No. one was of high intensity and the band No. two and three were of lower intensities, the bands two and three were absent in thecontrol. This treatment gives the highest number of bands among all treatments. Lane (6), after twenty five minutes of exposure to UV-A ray showed one band; this band was of lower intensity. This band was totally different compared to the control.

From the results obtained, it was observed that the effect of exposure to UV-A rays revealed differences in the occurrence of peroxidase isozyme bands in *N. oleander* cell suspension culture compared to the control. From these results, it is clear that the differences in the peroxidase enzyme in samples exposed to UV compared to the sample that was not subjected to irradiation due to increase in the activities of the respective enzymes is due to induced gene transcriptional and de novo synthesis of proteins, or due to post translational modification of existing protein.

TABLE 3. Molecular weights of SDS-PAGE protein in *N. oleander* L. cell suspension culture under UV-A radiation stress at different intervals 0, 5, 10, 15, 20, 25 and 30min.

No. of bands	Markers kDa	0	5	10	15	20	25	30
1	**250**	+	+	+	+	+	-	+
2	200	+	+	+	+	+	-	+
3	**150**	+	+	+	+	+	-	+
4	**100**	+	+	+	+	+	-	+
5	70	+	+	+	+	+	-	+
6	**50**	+	+	+	+	+	-	+
7	**40**	-	-	-	-	-	-	-
8	**30**	-	-	-	-	-	-	-
9	20	+	+	+	+	+	+	+
10	**15**	-	-	-	-	-	-	-
11	**10**	-	-	-	-	-	-	-
12	5	-	-	-	-	-	-	-
Total	**12**	**7**	**7**	**7**	**7**	**7**	**1**	**7**

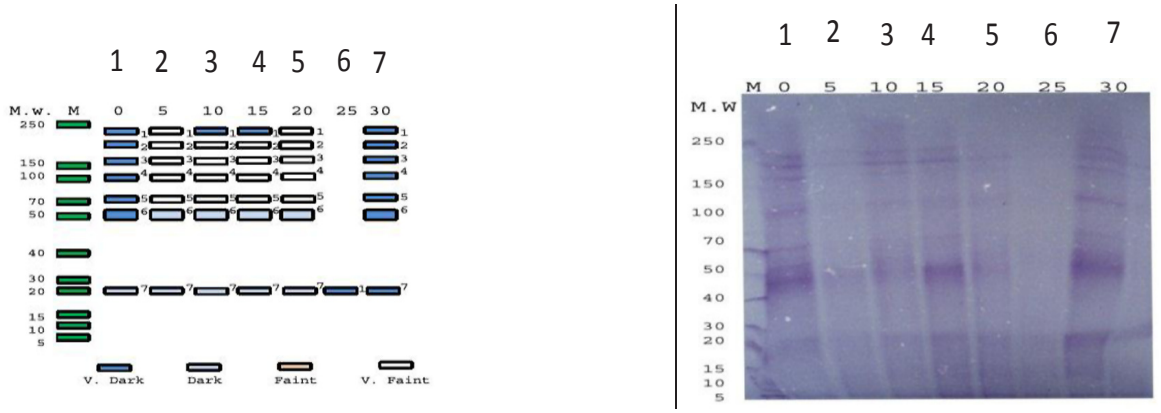

Fig. 1. SDS-PAGE protein electrophoresic pattern and histogram of the *N. oleander* L. cell suspension culture under UV-A radiation stress at different intervals 0, 5, 10, 15, 20, 25 and 30min.

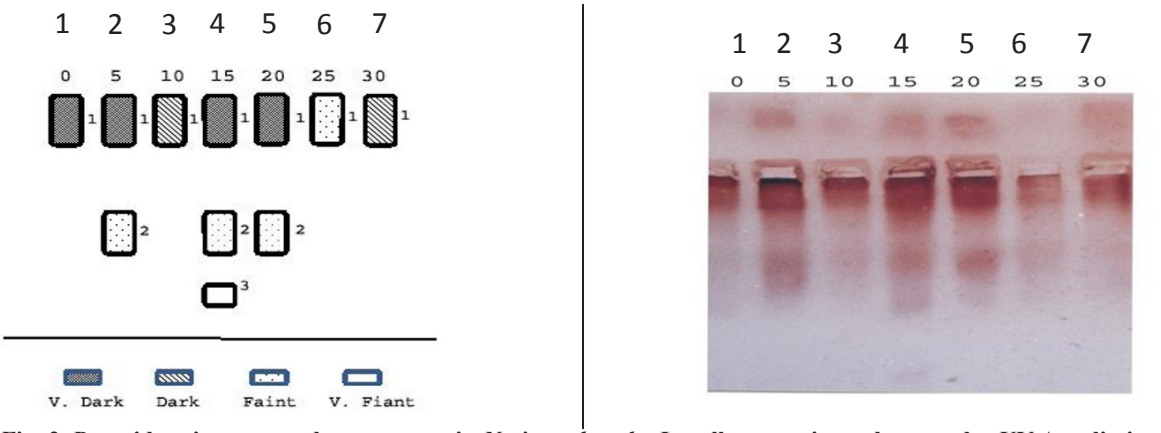

Fig. 2. Peroxidase isozyme and zymograme in *Nerium oleander* L. cell suspension culture under UV-A radiation stress condition at different intervals 0, 5, 10, 15, 20, 25 and 30min.

The results of this study are in agreement with Owk et al. (2013) who studied differences among the cultivars of *Catharanthus roseus* which belong to the same family Apocynaceae using biochemical markers through polyacrylamide gel electrophoresis (SDS-PAGE). The study included three types of enzymes, esterase (EST), peroxidase (POD) and polyphenol oxidase (PPO) isozymes. It has been found that the produced a total of 16 bands with polymorphism ranged from 66.6-100%.

Oleandrin analysis

The results of HPLC chromatograms of oleandrin which were extracted from *N. oleander* cell suspension cultures of the six time intervals of exposure to UV-A ray. Table 4 shows an increase in the concentration of oleandrin accumulation, accompanied by an increase in the duration of exposure to A-UV. The highest value of oleandrin concentration was 0.093µg/ml, and a steady increase ocurres gradually until reaching the exposure period of 15min. Then the concentration of oleandrin accumulation decreases gradually and the lowest value for the concentration of oleandrin 0.02µg/ml appeared at 25min of exposure to UV-A (Table 4). Analysis of variance (ANOVA) using LSD test at P values less than 0.05 (P> 0.05) showed statistically significant differences in oleandrin concentration in the *N. oleander* cell suspension cultures of the six time intervals of exposure to UV-A ray.

The results of the present study are in agreement with the Taha (2016) who studied the impact of abiotic effect on some secondary compounds in callus of *N. oleander* plant and found that the effect of a ($CdCl_2$ and KNO_3) increases the concentration of oleandrin. This confirms a correlation between environmental impact and the accumulation of secondary products in the plant cells.

TABLE 4. HPLC analysis of oleandrin substance which were extracted from *N. oleander* cell suspension cultures of the six time exposer of UV-A ray 10, 15, 20, 25 and 30min.

No.	Time exposer by (min)	Area	Retention time by (min)	Oleandrin concentration µg/ml
1	0	31.1212	3.690	0.022±0.001
2	5	43.5697	3.600	0.031±0.001
3	10	99.5879	3.626	0.071±0.001
4	15	130.7091	3.728	0.093±0.001
5	20	43.5697	3.722	0.031±0.001
6	25	24.8970	3.783	0.020±0.001
7	30	49.1715	3.811	0.035±0.001

Conclusion

Oleandrin concentrations in *N. oleander* cell suspension cultures after the exposure to UV-A ray for six different time intervals were increased. The UV-A radiation has an impact on changing the protein bands and the peroxidase isozymes. Ultraviolet radiation can be used at appropriate doses to improve the productivity of oleandrin, which is used in many pharmaceutical industries.

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إستجابة إنتاجية الأولندرين والتغيرات الجزيئية في مزارع خلايا نبات الدفلة تحت التأثير الإجهادي للإشعة الفوق بنفسجية

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إن الغرض من هذة الدراسة هو تحليل تركيزات مادة الأولندرين في مزارع المعلقات الخلوية لنبات الدفله وذلك بعد تعريضها للإشعه الفوق بنفسجية عند طول موجي (365) نانو ميتر وذلك لفترات زمنيه مختلفه (0 فترة ضابطه، 5، 10، 15، 20، 25، 30، 35، 40، 45 و50) دقيقة وأظهرت النتائج أن أطول فترة تعرض للإشعة الفوق بنفسجيه يمكن أن تتحملها مزارع المعلقات الخلوية هي 30 دقيقة بحد اقصى.

كما تم دراسة التغيرات في الحزم البروتينية، مشابهات الأنزيمات (البيروكسيديز) وكذلك تركيز مادة الأولندرين عند نفس الظروف من التعرض للإشعة الفوق البنفسجية وكذلك تم فصل مادة الأولندرين وتقدير كميتها بواسطة جهاز (التحليل الكروماتوجرافي للسوائل عالى الاداء)، كما تم التحقق من التغيرات في الحزم البروتينية من خلال تكنيك التفريد الكهربي للبروتين (SDS – PAGE) وقد أظهرت النتائج أن هناك تغيرات في الحزم البروتينية أعتمادا على الأختلاف في طول فترة التعرض للإشعة الفوق بنفسجية وقد ترجع هذه النتيجه إلى التغير في التعبير الجيني في الخلايا ولذلك التغيرات في إنتاجية مادة الأولندرين قد ترجع بدورها إلى التغير في التعبير الجيني.

أظهرت التحاليل الخاصة بمشابهات الأنزيمات (البيروكسيديز) أن التعرض للإشعة الفوق بنفسجية لفترات (5، 15 و20) دقيقه كان لها أثر واضح في مشابهات إنزيم البروكسيديز وأن الاثر الاكبر في العينة التي تعرضت للإشعه الفوق بنفسجية لفترة 15 دقيقة ولوحظ أن نفس العينة كانت ذات تركيز أعلى في مادة الأولندرين (0,093 ± 0,001) ميكرو جرام/مللي مقارنة بالعينه الضابطه (0,022 ± 0.001) ميكرو جرام/مللي, بينما كانت باقي العينات ذات تأثير أضعف للتعرض للإشعة الفوق بنفسجية.