The mutagenicity and anti-mutagenicity effect of aqueous leaves extract of V*inca rosea* by bacterial system (Part one)

القابلية التطفيرية والمضادة للتطفير للمستخلص المائي لنبات عين البزون *Vinca rosea* بإستخدام نظام بكتيري (الجزء الأول)

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Abstract

This study was carried out in order to determine the toxic, mutagenic and anti-mutagenic effects for aqueous extract of V*inca rosea* dried leaves against the mutagenic effect of Methotrexate (MTX) as a chemical mutagen and Ultra Violet rays (UV) as a physical mutagen. The effect was studied in a bacterial system (G-system). The used system consisted of three wild isolates G_3*Bacillus* spp., G_{12}*Arthrobacter* spp. and G_{27}*Brevibacterium* spp., The study depended on recording survival fraction (S_x) as an indicator for the induction of Streptomycin and Rifampic in resistance mutants as a genetic marker. Aqueous extract was prepared from dried leaves, gradual concentrations of plant aqueous extract was used to choose the suitable concentration which is remembered the negative control, the optimum concentration of aqueous extract was 200 µl/ml comparing with the negative control. The interactions included three types of treatments (pre-mutagen, with-mutagen and post-mutagen) in order to determine the mechanisms of this plant extracts in preventing or reducing the genotoxic effect of mutagen (MTX and UV).The results showed that the interaction effect between the optimum concentration of aqueous extract and the mutagen on survival fraction (S_x) increase the survival fraction value of the of G-system isolates to reach normal value compare with positive control (MTX), the results of the interaction between optimum concentration for extracts and the treatment with mutagen to induce resistance mutant for streptomycin and rifampicin found that the MTX had no effect to induce resistance mutant for these two antibiotics, for all types of treatment (pre-MTX, with-MTX, and post-MTX), the aqueous extract suppress or repair mutant and give 100% protection for bacterial cells, while the UV had some effects to induce resistance mutant for these two antibiotics for pre-UV and within-UV treatments and the aqueous extract showed suppressing or repairing mutant partially with post-UV treatment and gave 100% protection for bacterial cells.

Keywords:*Catharanthus roseus*, *Vinca rosea*, **Aqueous extract, Anti-mutagenicity**

الملخص

أجريت هذه الدراسة للكشف عن التأثيرات السمية و التطفيرية والمضادة للتطفير للمستخلص المائي لأوراق نبات عين البزون الجافة *Vinca rosea* ومقارنة فعاليته تجاه عقار الميثوتركسيت Methotrexate (MTX) باعتبارها مطفر كيميائي والتعرّض للأشعة فوق البنفسجية بأعتبارها مطفر فيزيائي بمعاملات متداخلة للمستخلص مع المطفر قبل ومع وبعد التعرّض للمطفر باستعمال (G-system) والذي يتضمن ثلاث أنواع بكتيرية هي : G_3*Bacillus* spp.، G_{12}*Arthrobacter*spp. و G_{27}*Brevibacterium*spp. بالاعتماد على معامل البقاء (S_x) Survival fraction لدراسة التأثيراتوحث الطفرات المقاومة للمضادين الحيويين الستربتومايسين والريفامبسين كمؤشرات وراثية Genetic markers. تم تحضير المستخلص المائي من أوراق نبات عين البزون الجافة، وحضرت تراكيز متدرجة من المستخلص المائي للنبات لمعرفة تأثير التراكيز وأختيار التركيز الأمثل المقارب بتأثيره على معامل البقاء معامل بقاء مجموعة السيطرة، وكان التركيز الأمثل للمستخلص المائي هو 200 مايكروغرام/مليلتر مقارنة مع السيطرة السالبة، شملت معاملات التداخل ثلاث أنواع هي (قبل، مع، بعد) المعاملة بالمطفر لتحديد فعالية المستخلص النباتي في منع أو تقليل سمية المطفر(MTX, UV). وأظهرت نتائج تأثير التداخل بين التركيز الأمثل للمستخلص والمطفر على معامل البقاء (S_x) ارتفاع قيم معامل بقاء عزلات النظام لتصل إلى قيم مقاربة للطبيعية مقارنة بالسيطرة الموجبة (MTX, UV)، وكما أظهرت نتائج التداخل بين التركيز الأمثل للمستخلص والمعاملة بالمطفر في حث طفرات المقاومة للمضادين الستربتومايسين والريفامبسين، أن المطفر (MTX) لم يكن له أي تأثير في حث الطفرات المقاومة للمضادين المذكورين للمعاملات قبل ومع وبعد المعاملة بالمطفر وللعزلات الثلاث وبذلك عملالمستخلص المائي لنبات عين البزون على إخماد أو تصليح الطفرات ووفر حماية 100% للخلايا البكتيرية، بينما

أظهرت نتائج التداخل بين التركيز الأمثل للمستخلص والتعرّض لأشعة UV في حث طفرات المقاومة للمضادين الستربتومايسين والريفامبسين أن التعرّض للأشعة لها تأثير في حث الطفرات المقاومة للمضادين المذكورين في المعاملات قبل ومع المعاملة بالأشعة وأن المستخلص المائي لنبات عين البزون عمل على إخماد أو تصليح الطفرات ووفر حماية 100% للخلايا البكتيرية في المعاملة بعد التعرّض لأشعة الـ UV.

الكلمات المفتاحية: *Catharanthus roseus*، عين البزون، المستخلص المائي، المضادة للتطفير

Introduction

A significant development in medical oncology is the use of cytotoxic drugs for cancer chemotherapy. Although these drugs are used to target tumor cells, most of them can also induce genotoxic, carcinogenic and teratogenic effects in non-tumor cells [1, 2]. These side effects limit the application of chemotherapeutic agents despite their high efficacy in the killing of target malignant cells. Therefore, the search for alternative or complementary drugs that are effective on cancer cells while showing minimal toxicity to normal cells is an active area of research [3]. Many of these investigations are plant-based, folkloric medicine from various societies around over the world. Moreover, a report from WHO [4] stated that about 80% of the world population is wholly or partially dependent on plant-based drugs.

The medicinal plant *Catharanthus roseus* L. G. Don, formerly *Vinca rosea* L. (Apocynaceae), has been used in traditional medicine by various societies to address diabetes, cancer, hypertension, fever or hemostasis [5,6]. *C. roseus*is of enormous pharmaceutical interest because it contains more than 400known terpenoidindole alkaloids (TIAs), and many of the alkaloids exhibit strong pharmacological activities [7]. Previous studies have identified significant active compounds in *C. roseus*, including vinblastine and vincristine (anticancer), ajmalicine (antihypertensive) and serpentine (sedative) [7, 8]. Vinblastine and vincristine are produced by the plant in small amounts [9] and are commonly used in combination with other drugs for the treatment of cancers, such as lymphomas, leukemia, Hodgkin's disease, malignant lymphomas, neuroblastoma, rhabdomyosarcoma, Wilm's tumor and other cancers [7, 10, 11]. Vincamine and vinpocetine from C. roseus, have vaso-dilating and memory enhancing properties and have been shown to alleviate vascular dementia and Alzheimer's disease [12, 13]. Its antibacterial and antidiabetic activities have also been reported [14].

In this work, we aimed to study the mutagenic and anti-mutagenic effects of *V. rosea* leaves aqueous extract were studied *in vitro* by using microbiological systems which is the more systems that used for detection the cytotoxicity of biological matter, our microbiological systems was a bacterial system called G-system which consist of three wildnon-pathogenic isolates, G_3*Bacillus* spp., G_{12} *Arthrobacter* spp.and G_{27} *Brevibacterium* spp.that have antibiotics sensitivity feature for streptomycin and rifampicin [15, 16], Table (1).

The standardized mutagens that used for mutagenic effect detection of matter with the biological systemsis Nitrosoguanidine (NTG)، 5-Bromouracil (5-BU), Acridine Orange (AO),Hydroxylamine (HA) and Methotrexate (MTX) [16, 17].

Table (1): G-system characteristic

Isolate No.	Gram stain	Sensitivity test	
		Refampicin ($_{20}$ µg/m)	Streptomycin (10 µg/m)
G3	-ve	Sensitive	Sensitive
G12	-ve	Sensitive	Sensitive
G27	-ve	Sensitive	Sensitive

The selective of this system depended on limited parameters such as resistance to streptomycin and rifampicin antibiotics considered as chromosomal features which is more stable from plasmid features that is unstable with continuous cultures, treated with chemical compounds or high temperature [15], sensitivity of samples for streptomycin and rifampicin were examined by using gradient concentration media plates [15, 16], and found that suitable concentration of streptomycin was 10 µg/ml and 20 µg/ml for rifampicin as membered in Table (1) above [15, 16].

Materials and Methods

Collection and Extraction of Plant Leaves

The extraction method was carried out according to Ahmad [18]. Fresh leaves of *V. rosea* were collected from Karbala University Gardens. They were classified by the Herbarium of Biology Department, College of Science/ University of Karbala. The leaves were dried and powdered by using coffee grinder, and 50 grams of the powdered leaves were extracted with one liter of distilled water and soaked at 40°C for 24 hours in a shaker water bath. The extract was transferred into 50 ml Falcon tubes and centrifuged at 2000 rpm at 25°C for 15 minutes. The clear supernatant was collected, and a working stock of crude aqueous extract (at a concentration of 100 mg/ml) was prepared when 100 mg of extract was dissolved with 1 ml of sterile phosphate buffer saline (PBS). The aqueous extract was filter-sterilized using a 0.2 µm filter before being liquated into dark vials. The stocks were diluted to various concentrations to be used in subsequent experiments. [18].

G-system was obtained from the Genetic Engineering and Biotechnology Institute for Postgraduate studies/ University of Baghdad.

Such evaluation included gradient concentrations of *V. rosea* leaves extract were used for detection the cytotoxicity, mutagenicity and antimutagenicity effects of the plant extract on the system cells, then interaction between optimum concentration of plant extract and optimum concentration of MTX (from Hixal Company, Germany) (50 µg/ml) according previous study [19] was tested to detect the anti-mutagenic effects of plant extract against the mutagenic effect of the optimum concentration of MTX by treating 5 ml of cells suspension (phosphate buffer pH 5.5) with:

1. The optimum concentration of aqueous extract 200 µg/ml for 15 minute at 37ºC [15, 16], then treated with the optimum concentration of MTX 50 µg/ml and incubate for 15 minute at 37ºC (Pre-treated with MTX).
2. The optimum concentration of aqueous extract 200 µg/ml with the optimum concentration of MTX 50 µg/ml and incubate for 15 minute at 37ºC (with-treated with MTX).
3. The optimum concentration of MTX (50 µg/ml) for 15 minute, then treated with the optimum concentration of aqueous extract 200 µg/ml and incubate for 15 minute (post treated with MTX).

Another interaction between optimum concentration of plant extract and optimum exposure time to UV rays (10 minute, 254 nanometer) according previous study [20] was tested to detect the anti-mutagenic effects of plant extract against the mutagenic effect of the optimum exposure time of UV rays by treating 5 ml of cells suspension (phosphate buffer pH 5.5) with:

1. The optimum concentration of aqueous extract 200 µg/ml for 15 minute at 37ºC[15,16], then treated with the optimum exposure time of UV rays [20] 10 minute incubation at 37ºCand 5 minute without exposure to UV at 37ºC(Pre-treated with UV).
2. The optimum concentration of aqueous extract 200 µg/ml with the optimum time of UV rays with the incubation for 15 minute at 37ºC (10 minute with the exposure to UV and 5 minute without the exposure to UV at 37ºC (within-treated with UV).
3. The optimum exposure time to UV rays with the incubation for 10 minute at 37ºCand 5 minute without exposure to UV at 37ºC, then treated with the optimum concentration of aqueous extract 200 µg/ml with the incubation for 15 minute (post treated with UV).

Control groups were divided to two groups:

1. Negative control that treated with the same steps of all the experiments but without treating with any mutagens.
2. Positive control that treated with the same steps of all the experiments with treating with MTX or UV mutagen.

All Tubes were incubated for 24 hours for phenotypic expression and calculate the survival index, and the induced mutation of streptomycin and rifampicin resistant.

Data are expressed as:

1. Survival index (S_x) for system cells (G_3, G_{12}, G_{27}) using the following equation

$$\mathbf{Sx} = \frac{\textbf{No. of cells obtained after the treatment}}{\textbf{No. of control cells}}$$

2. Mutant frequency (M_x) for system cells (G_3, G_{12}, G_{27}) using the following equation.

$$Mx = \frac{\text{No. of induced mutant in X concentration}}{\text{No. of control cells}}$$

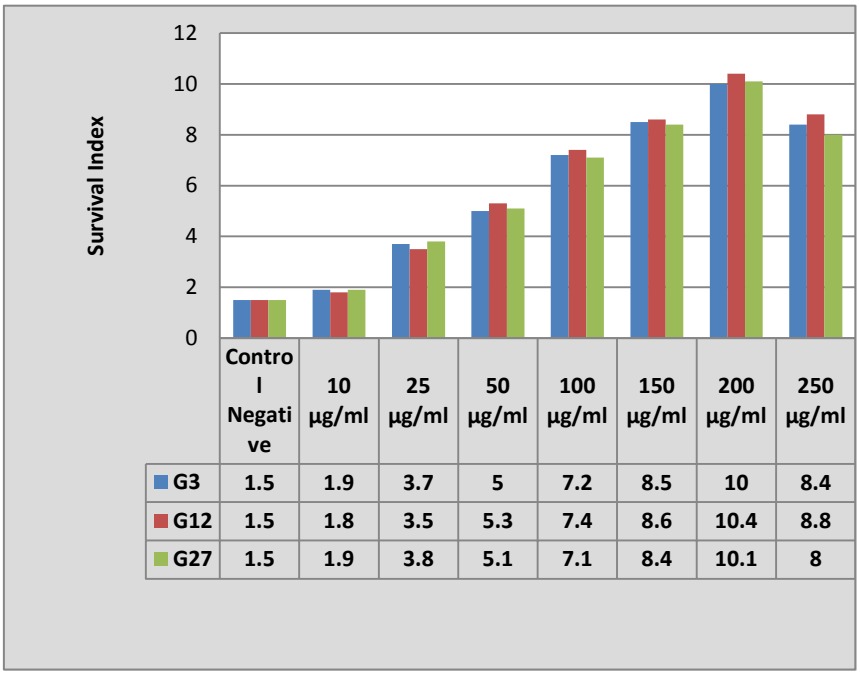
Statistical Analysis

ANOVA table was used to determine the differences between studied groups by using SPSS version 21.0. Statistical significance was considered at $P \leq 0.05$.

Results and Discussion

Free radical and repair systems were play important role in inducing and reducing cancers *in vivo*. So for the important medical features of *V. rosea* leaves extract we used the aqueous extract to study the mutagenic and anti-mutagenic effects of extract.

The present results demonstrated that the plant extract was significantly increasing the effective of survival index when was treated with different concentration of the extract, the effect of *V. rosea* leaves aqueous extract showed significant increasing with the survival index of G-system isolates when treated with the concentrations 10, 25 , 50, 100, 150, 200, 250 µg/ml comparing with the control, the concentration 10 µg/ml was showed the minimum effect of this extract on the survival index 1.9, 1.8, 1.9 for the G_3, G_{12} and G_{27} respectively, while the maximum effect of this extract on the survival index was in the concentration 200 µg/ml 10, 10.4, 10.1 for the G_3, G_{12} and G_{27} respectively. The survival index of G-system isolates was significantly decreased at the concentration 250 µg/ml comparing with the concentration 200 µg/ml, so the suitable concentration for the last experiments was 200 µg/ml, as showed in figure (1).



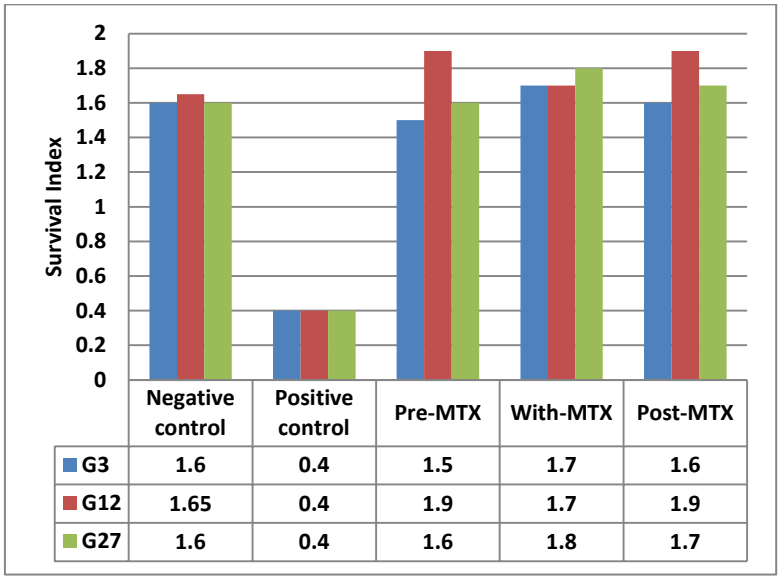
	Control Negative	10 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
G3	1.5	1.9	3.7	5	7.2	8.5	10	8.4
G12	1.5	1.8	3.5	5.3	7.4	8.6	10.4	8.8
G27	1.5	1.9	3.8	5.1	7.1	8.4	10.1	8

Fig. (1): The effect of different gradient concentration of *V. rosea* leaves aqueous extract on the survival index of G-system isolates.

The results also showed that the extract had no resistance mutation for streptomycin and rifampicin antibiotics, and such observing suggest that the *V. rosea* leaves aqueous extract was non-mutagenic agent.

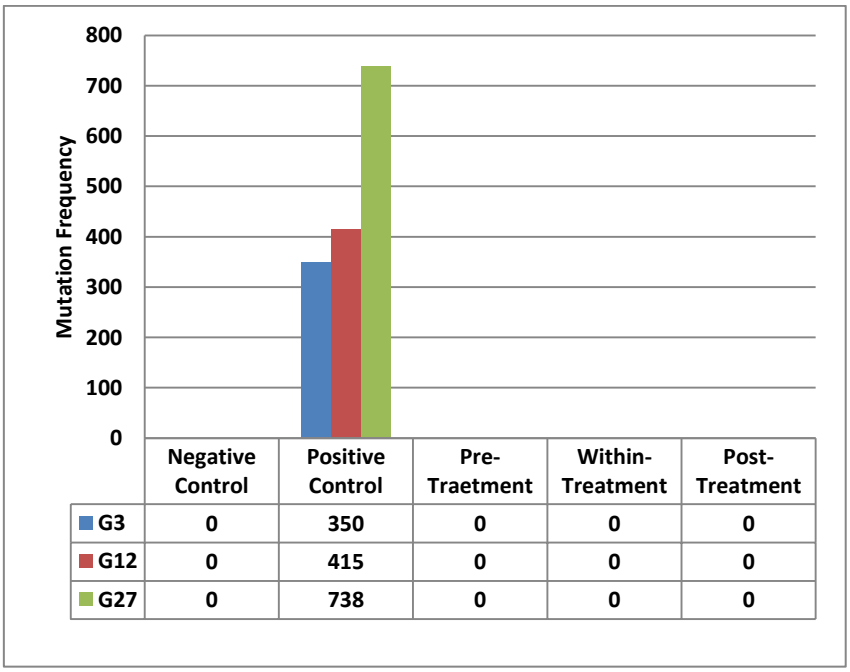
The interaction between optimum concentration that chosen from the previous step with the optimum concentration of the standard chemical mutant (MTX) (50 µg/ml) according to previous study [19] showed significant increasing of the survival index of G-system isolates that treated with the MTX with pre-MTX, within-MTX, post-MTX comparing with the control. The survival index of pre-MTX treatment was 1.5, 1.9, 1.6 for the G_3, G_{12} and G_{27} respectively, and for within-MTX treatment was 1.7, 1.7, 1.8 for the G_3, G_{12} and G_{27} respectively, finally for post-MTX treatment was 1.6, 1.9, 1.7 for the G_3, G_{12} and G_{27} respectively, as shown in figure (2).

There was no mutation resistant for streptomycin and rifampicin induced by MTX when the isolates treated with the aqueous extract for all treatment comparing with the positive control (MTX only) as shown in figure (3). So these results lead may suggest that the optimum concentration of the aqueous extract (that contain the antioxidant compounds such as flavonoids, phenols, alkaloids and others compounds) was reduced and repaired the mutations that induced from the interaction with the MTX for the three isolates.



	Negative control	Positive control	Pre-MTX	With-MTX	Post-MTX
G3	1.6	0.4	1.5	1.7	1.6
G12	1.65	0.4	1.9	1.7	1.9
G27	1.6	0.4	1.6	1.8	1.7

Fig. (2): The effect of the interaction between concentrations of *V. rosea* leaves aqueous extract and MTX on the survival index of G-system isolates.



	Negative Control	Positive Control	Pre-Traetment	Within-Treatment	Post-Treatment
G3	0	350	0	0	0
G12	0	415	0	0	0
G27	0	738	0	0	0

Fig. (3): The effect of the interaction between the concentration of *V. rosea* leaves aqueous extract and MTX to induce mutations of G-system isolates.

The interaction between optimum concentration that chosen from the first step with the optimum exposure time to the standard physical mutant (UV) (10 minute, wave length 245 nanometer) according to previous study by Al-bakri [20] showed significant increase of the survival index of G-system isolates that exposed to UV with pre- UV, within- UV, post- UV comparing with the control. The survival index of pre- UV exposure was 0.8, 0.6, 0.63 for the G_3, G_{12} and G_{27} respectively, and for

within- UV exposure was 0.84, 0.9, 0.7 for the G_3, G_{12} and G_{27} respectively, finally for post- UV exposure was1.9, 1.7, 1.4 for the G_3, G_{12} and G_{27} respectively as shown in figure (4).

There was no mutation resistant for streptomycin and rifampicin induced by UV when the isolates treated with the aqueous extract for (post- UV) comparing with the positive control (UV only), while the results showed there is some mutations appears the two treatment of exposure to UV, the treatment pre-UV 255, 195, 283 and within- UV 145, 121, 133 for the G_3, G_{12} and G_{27} respectively, as shown in figure (5). These results lead may suggest that the optimum concentration of the aqueous extract was reduced and repaired the mutations that induced from the interaction with the UV exposure, for the three isolates with the post-UV exposure treatment. While some mutations appear in the pre-UVand within-UV exposure treatments because the system isolates failed to repair all broken DNA damaged from the exposure to UV rays.

The results of the post-UV exposure treatment showed significant differences $P \leq 0.05$ with the within-UV exposure treatment and that leads to suggest that the plant aqueous extract have or play important role in reducing the number of resistant mutation for the two antibiotics.

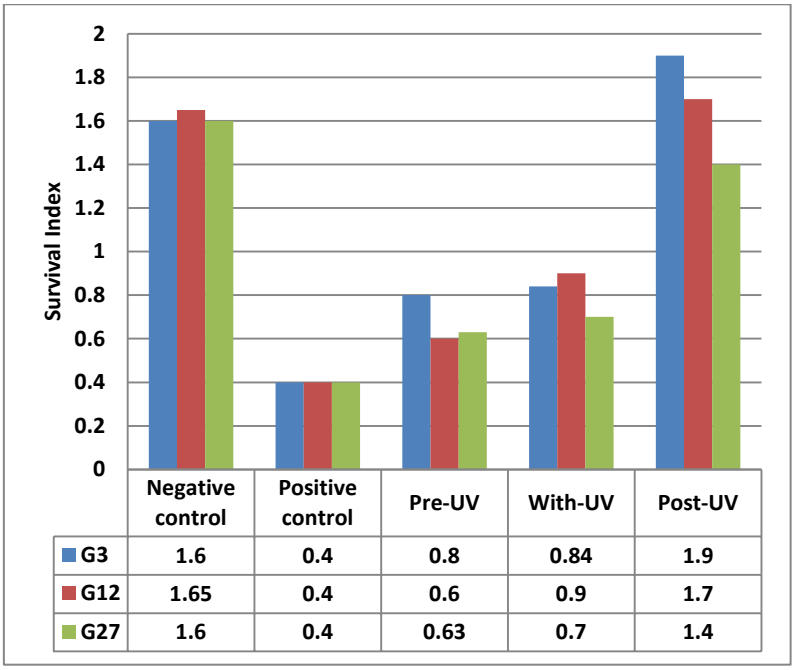


Fig. (4): The effect of the interaction between the concentration of *V. rosea* leaves aqueous extract and exposure to UV on the survival index of G-system isolates

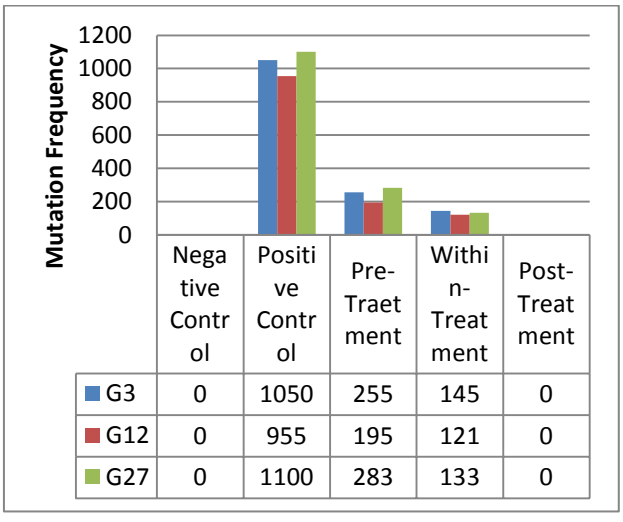


Fig. (5): The effect of the interaction between the concentration of *V. rosea* leaves aqueous extract and exposure to UVfor induce mutations of G-system isolates.

The present results demonstrated that the plant extract was significantly effective in survival index when treated with different concentration of the extract and with different mutagens which may suggest that the antioxidant compounds in the aqueous extract may play a protective role that protects the system isolates from the genotoxic effects of the mutagens [15, 16]. *V. rosea* contains about 130 alkaloids of the bisindole group out of which 25 alkaloids are dimeric in nature. Two of the dimeric alkaloids are vinblastine and vincristine, mainly present in the aerial parts, have found extensive application in the treatment of human neoplasma. Vinblastine sulphate is used particularly to treat Hodgkin's disease besides lymphocarcoma, choriocarcinoma, neuroblastoma, and carcinoma of breast, lungs and other organs in acute and chronic leukemia. Vincristine sulphate arrest mitosis in metaphase and is very effective for treating acute leukemia in children and lymphocytic leukemia. It is also used against Hodgkin's disease, Wilkins's tumor, neuroblastoma and reticulum cell sarcoma [21], the aqueous extract of *V. rosea* leaf has a wide range of biological effects; including treatment of diabetic people, and used for treatment circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow and also used to combat heart arrhythmias and to improve the blood circulation in the brain, *V. rosea* leaves extract may be responsible for the wound healing activity. Furthermore, the Folkloric application of the plant (for instance anti-oxidant, antibacterial, antifungal and anti-leukemic activity) can also be interpreted on such ground, especially it indicates that it has possibly antioxidant properties which play a crucial role in the defense against free radicals, and it had antibacterial, antifungal properties, and insecticidal activities which have confirmed by others [22, 23, 24, 25, 26, 27, 28, 29].

Another active material that founded in the *V. rosea* leaf extract that have antimicrobial properties is alkaloids, tannins, triterpenoids, and flavonoids [21].

From these findings, it is possible to suggest that the plant may protect, reduce, or repair G-system isolates from the cytotoxicity and mutagenicity effects of the two investigated mutagens, but it is too early to reach a final conclusion, and further investigations are required to cover such subject.

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