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Mutagenic and anti-mutagenic effect of alcoholic extract of *Citrullius colocynthis* seeds using bacterial system (G-system)

التأثير التطفيري والمضاد للتطفير للمستخلص الكحولي لبذور نبات الحنظل *Citrullius colocynthis* بأستخدام نظام بكتيري (G-system)

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Abstract

Methanolic extract (80% methanol) of *Citrullius colocynthis* dried seeds was evaluated for its mutagenic and anti-mutagenic effect *in vitro* using bacterial system (G-system). The study depended on recording survival index (S_x) as an indicator for study the cytotoxicity effects and the induction of Streptomycin and Rifampicin resistance mutants as a genetic marker.Alcoholic extract of 80% methanol was prepared from dried seeds, and then the extract was dried, then 5 ml of distilled water was used to dissolve the powder, gradual concentrations of plant seeds alcoholic extracts were used to choose the suitable concentration which is resemble the negative control.

The interactions included three types of treatments (pre-mutant, with-mutant and post-mutant) between optimum methanolic extract of dried seed (125 µg/ml)and optimum concentration of methotrexate drug (MTX) (50 µg/ml)as a chemical mutagen and the optimum exposure time of ultra violet ray (UV) (10 minutes, 254 nanometer) as a physical mutagen in order to determine the mechanisms of this plant extract in preventing or reducing the genotoxic effect of MTX and UV.

The results showed that a significant increasing in survival index(S_x) of treated cells which is resemble the negative control when compared with positive control, and the methanolic seed extract of *C. colocynthis*had no effect to induce resistance mutant for the two antibiotics for all treatment. So these findings suggest that the plant extract have no genotoxic effect on the cells system.

The results of interaction between optimum concentration for extract 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 the three types of treatment (pre-MTX, with-MTX, and post-MTX), while the result of interaction between optimum concentration of extract and the treatment with UV shows that UV had no effect to induce resistance mutant for these two antibiotics, for the two types of treatment (with-UV, and post-UV), the alcoholic extract suppresses or repair mutant from the treatment with MTX and UV ray and give protection 100% for bacterial cells, while the percentage of pre-UV treatment was (90%).

الخلاصة

تم تقييم تأثير المستخلص الميثانولي لبذور نبات الحنظل *Citrullius colocynthis* الجافة خارج الجسم الحي من خلال أستخدام نظام بكتيري (G-system) بالأعتماد على معامل البقاء Survival index (S_x) لدراسة التأثيراتوحث الطفرات المقاومة للمضادين الحيويين الستربتومايسينوالريفامبسين كمؤشرات وراثية .

تم تحضير المستخلص الكحولي (80 % ميثانول) من بذور نبات الحنظل الجافة ، جُفف المستخلص وذُوب بـ 5 مليلتر من الماء المقطر ، وحضرت تراكيز متدرجة من المستخلص الكحولي للنبات لمعرفة تأثير التراكيز، وكان التركيز الأمثل للمستخلص الكحولي هو 125 مايكروغرام/مليلتر مقارنة مع السيطرة السالبة ، شملت معاملات التداخل ثلاثة أنواع هي (قبل ، مع ، بعد) المعاملة بالمطفر بين التركيز الأمثل للمستخلص النباتي والتركيز الأمثل لعقار الميثوتركسيت (50 µg/ml) كمطفر كيميائي ، والتركيز الأمثل للمستخلص النباتي والوقت الأمثل للتعرّض للأشعة فوق البنفسجية (10 دقائق ، وطول موجي 254 نانومتر) كمطفر فيزيائي ، لتحديد فعالية المستخلص النباتي في منع أو تقليل السمية للمطفرين . وأظهرت نتائج تأثير التداخل بين التركيز الأمثل للمستخلص وعقار الميثوتركسيت على معامل البقاء (S_x) ارتفاعاً معنوياً في قيم معامل بقاء عزلات النظام لتصل إلى قيم مقاربة للطبيعية مقارنة بالسيطرة الموجبة ، كما أظهرت نتائج التداخل بين التركيز الأمثل للمستخلص والمعاملة بالمطفر في حث طفرات المقاومة للمضادين الستربتومايسينوالريفامبسينوأن عقار الميثوتركسيت لم يكن له أي تأثير في في حث الطفرات المقاومة للمضادين المذكورين للمعاملات قبل ، مع وبعد المعاملة بعقار الميثوتركسيتوللعزلات الثلاث ، بينما الأشعة فوق البنفسجية لم يكن لها أي تأثير في حث الطفرات المقاومة للمضادين المذكورين للمعاملات مع وبعد المعاملة بالمطفروللعزلات الثلاث وبذلك عملالمستخلص الكحولي لبذور نبات الحنظل على إخماد أو تصليح الطفرات ووفرت حماية 100% للخلايا البكتيرية ، في حين كانت نسبة المعاملة قبل التعرّض للأشعة فوق البنفسجية (90%).

Keywords:*Citrulliuscolocynthis*, Methanolic extract, Anti-mutagenicity.

Introduction

Citrulluscolocynthis (Cucurbitaceae), commonly known as 'bitter apple', 'colosynth', 'vine-of-Sodom' and 'tumba' is a tropical plant that grows abundantly in the Arabian countries and widely in other parts of the world [1]. In traditional medicine, this plant has been used to treat constipation [2], diabetes [3], odema, fever, jaundice, bacterial infections and cancers, and it is also used as an abortifacient [1]. Further studies have implanted that *C. colocynthis* is rich in compounds that have antioxidant and free radical scavenging potentials [4,5]. Preliminary phytochemical screening of the plant showed the presence of large amounts of phenolic and flavonoids [6]. Flavonoids and phenolic compounds are widely distributed in plants, and have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic, and others [7]. Accordingly, the present study was planned with aims to study the mutagenic and anti-mutagenic effects of *C. colocynthis* seed alcoholic extract *in vitro* by using Microbiological systems -that is include bacteria, fungi and yeast- 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 is consist of three isolates, G_3*Bacillus* spp., G_{12}*Arthrobacter* spp.and G_{27}*Brevibacterium* spp., that was have antibiotics sensitive feature for streptomycin and rifampicin [8, 9], table (1) .

Nitrosoguanidine (NTG)، 5-Bromouracil (5-BU),Acridine Orange (AO)andHydroxylamine (HA) was standardized mutagens used for detection of mutagenic effects of matter [9, 10].

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 was 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 [10], sensitivity of samples for streptomycin and rifampicin were examined by using gradient concentration media plates [8, 9], and found that suitable concentration of streptomycin was 10 µg/ml and 20 µg/ml for rifampicin as membered in table (1) above.

Materials and Methods

Plant Seed Collection and Extraction:The extraction method was carried out according to [11], Dried seeds of *C. colocynthis* were collected from a local store of herbal medicine from Karbala City, and they were certified by the Herbarium of Biology Department, College of Science (University of Baghdad). The seeds were powdered using coffee grinder, and 500 grams of the powder was subjected to successive extraction (twenty four hours) in a Soxhlet apparatus, using 500 ml of methanol 80 % as solvent at 50°C. The extract was then evaporated on a rotaryevaporatorand dried, then the powder was dissolved by using 5 ml of distilled water, then the extract was filtrated by millipore filter 0.22 µm[11].

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

Such evaluation included gradient concentrations of *C. colocynthis* seed extract were used for detection the cytotoxicity and mutagenicity 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 [12] was tested to detect the anti-mutagenic effects of plant extract against the mutagenic effect of the optimum concentration of MTX by treat 5 ml of cells suspension (phosphate buffer pH 5.5) with:

1. The optimum concentration of alcoholic seed plant extract (125 µg/ml) for 15 minute at 37 Cº [8, 9], then treated with the optimum concentration of MTX (50 µg/ml) and incubate for 15 minute at 37 Cº (Pre-treated with MTX).
2. The optimum concentration of alcoholic seed plant extract (125 µg/ml) with the optimum concentration of MTX (50 µg/ml) and incubate for 15 minute at 37 Cº (with-treated with MTX).
3. The optimum concentration of MTX (50 µg/ml) for 15 minute, then treated with the optimum concentration of alcoholic seed plant extract (125 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 [13] was tested to detect the anti-mutagenic effects of plant extract against the mutagenic effect of the optimum exposure time of UV rays by treat 5 ml of cells suspension (phosphate buffer pH 5.5) with:

1. The optimum concentration of alcoholic seed plant extract (125 µg/ml) for 15 minute at 37 Cº [8, 9], then treated with the optimum exposure time of UV rays [13] 10 minute incubation at 37 Cº and 5 minute without exposure to UV at 37 Cº(Pre-treated with UV).
2. The optimum concentration of alcoholic seed plant extract (125 µg/ml) with the optimum time of UV rays with the incubation for 15 minute at 37 Cº (10 minute with the exposure to UV and 5 minute without the exposure to UV at 37 Cº) (within-treated with UV).
3. The optimum exposure time to UV rays with the incubation for 10 minute at 37 Cº and 5 minute without exposure to UV at 37 Cº, then treated with the optimum concentration of alcoholic seed plant extract (125 µg/ml) with the incubation for 15 minute (post treated with UV).

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 by:

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

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

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

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

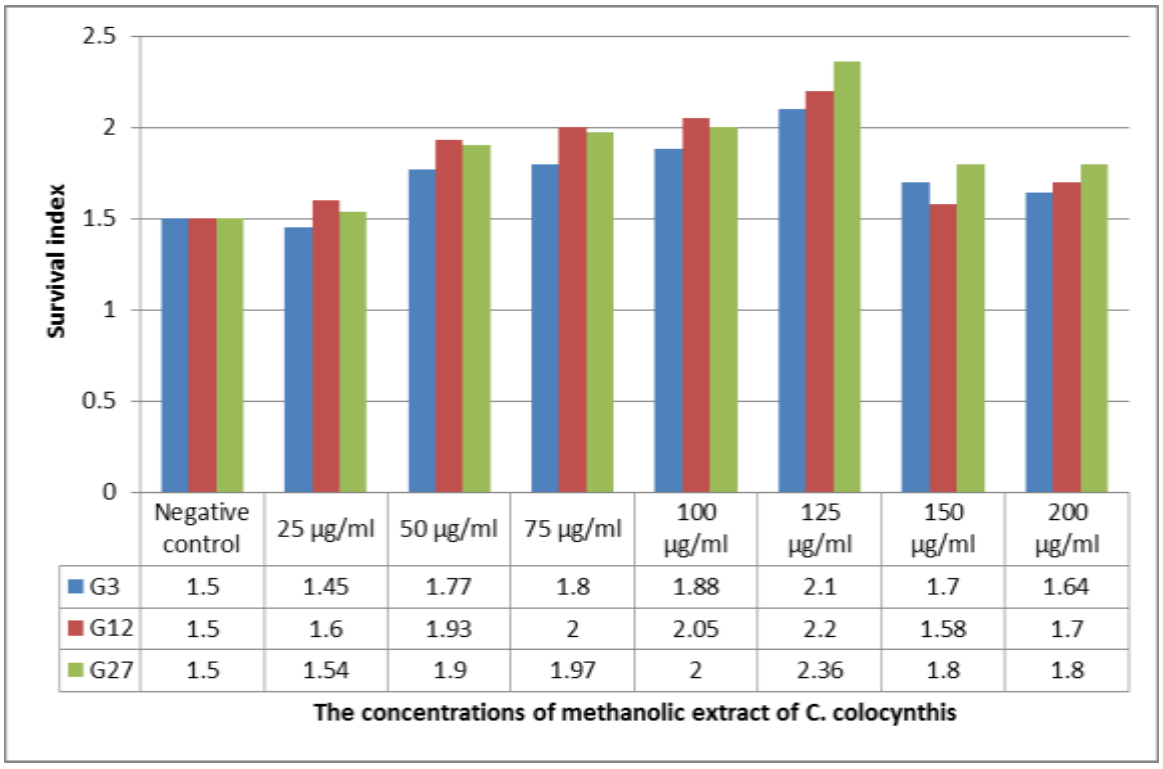
Statistical Analysis:

ANOVA table was used to determine the differences between studied groups by using the computer program SPSS version 13.0. Statistical significance was considered at $P \leq 0.05$.

Results and Discussion

Free radical and repair systems were play important role in induce or reduce cancers *in vivo*.So for the important biological features of *C. colocynthis* seed extract we used the alcoholic extract to study the mutagenic and anti-mutagenic effects of extract.

The present results demonstrated that the plant extract was significantly effective in survival index when treated with different concentration of the extract, the effect of *C. colocynthis* seed alcoholic extract showed significant increasing with the survival index of G-system isolates when treated withthe concentrations (50, 75 , 100, 125, 150, 200 µg/ml) comparing with the control, the concentration 25 µg/ml was showed the minimum effect of this extract on the survival index (1.45, 1.6, 1.54) 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 125 µg/ml (2.1, 2.2, 2.36) for the G_3, G_{12} and G_{27} respectively, the survival index of G-system isolates was significantly decreased at the concentration 150 – 200 µg/ml comparing with the concentration 125 µg/ml, so the suitable concentration for the last experiments was 125µg/ml, as showed in figure (1).



	Negative control	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	125 µg/ml	150 µg/ml	200 µg/ml
G3	1.5	1.45	1.77	1.8	1.88	2.1	1.7	1.64
G12	1.5	1.6	1.93	2	2.05	2.2	1.58	1.7
G27	1.5	1.54	1.9	1.97	2	2.36	1.8	1.8

Figure (1): The effect of different gradient concentration of *C. colocynthis* seed alcoholic extract on the survival index of G-system isolates.

Our results showed that the extract had no resistance mutation for streptomycin and rifampicin antibiotics, these lead to suggest that the *C. colocynthis* seed alcoholic 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 [12] 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 (1.55, 1.51, 1.56) for the G_3, G_{12} and G_{27} respectively, and for within-MTX treatment was (1.54, 1.52, 1.55) for the G_3, G_{12} and G_{27} respectively, finally for post-MTX treatment was (1.57, 1.53, 1.56) for the G_3, G_{12} and G_{27} respectively, as shown in figure (2).

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

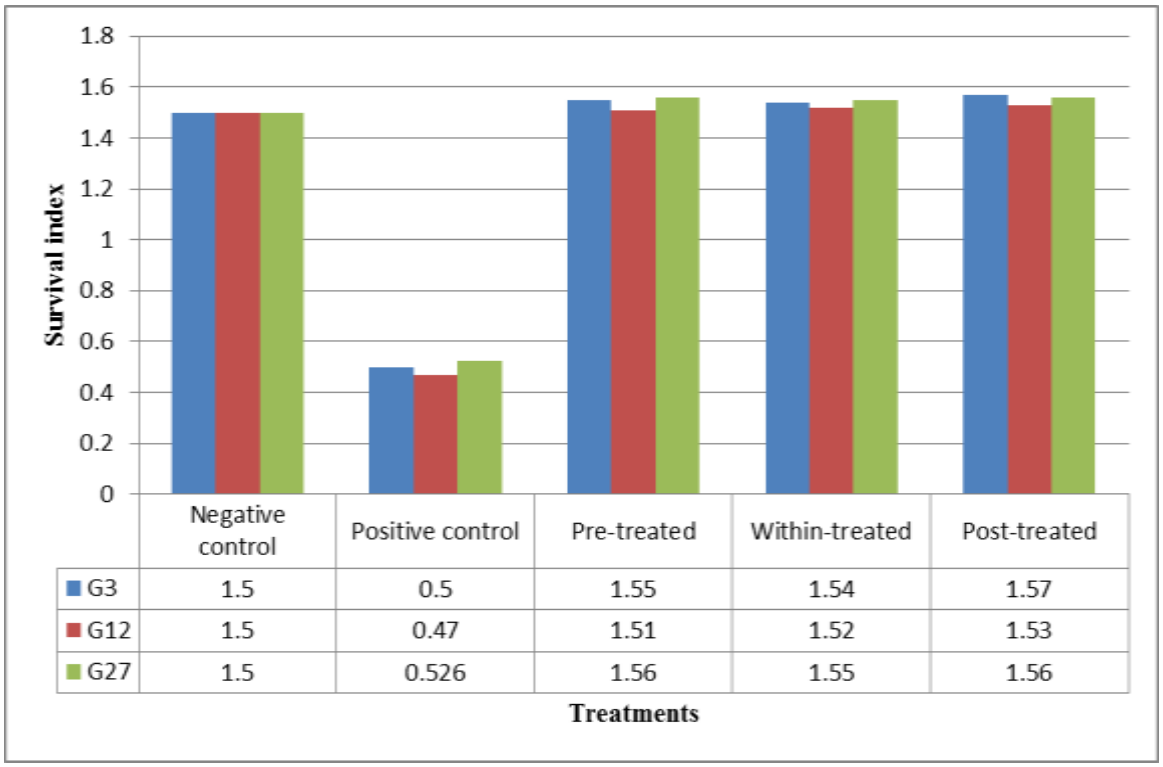


Figure (2): The effect of the interaction between the optimum concentration of *C. colocynthis* seed alcoholic extract and MTX on the survival index of G-system isolates.

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 [13] showed significant increasing 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 (1.54, 1.52, 1.5) for the G_3, G_{12} and G_{27} respectively, and for within-UV exposure was (1.56, 1.56, 1.54) for the G_3, G_{12} and G_{27} respectively, finally for post-UV exposure was (1.51, 1.52, 1.51) for the G_3, G_{12} and G_{27} respectively as shown in figure (4).

There is no mutation resistant for streptomycin and rifampicin induced by UV when the isolates treated with the alcoholic extract for the two treatment of exposure to UV (within-UV, post-UV) comparing with the positive control (UV only), while the results showed there is some mutations appears with the treatment pre-UV (12, 15, 13) for the G_3, G_{12} and G_{27} respectively, as shown in figure (5), so these results lead us to suggest that the optimum concentration of the alcoholic extract was reduced and repaired the mutations that induced from the interaction with the UV exposure for the three isolates with the within-UV and post-UV exposure, while appear some mutation in the pre-UV exposure because the system isolates failed to repair all it's broken DNA damaged from the exposure to UV rays.

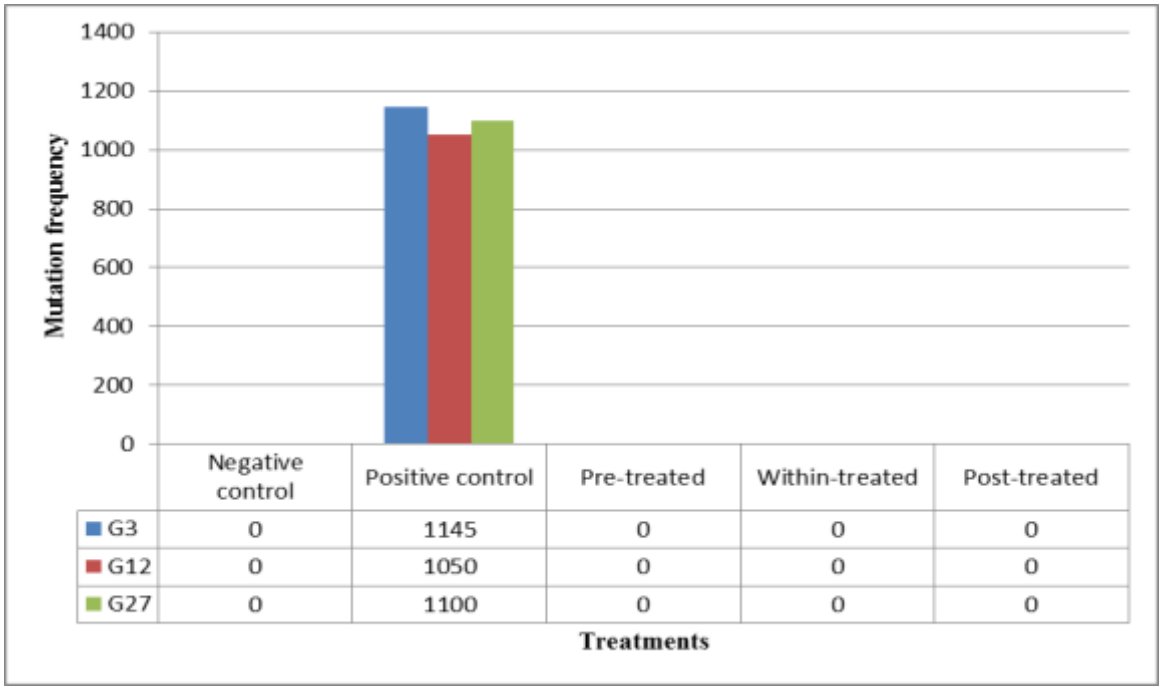


Figure (3): The effect of the interaction between the optimum concentration of *C. colocynthis* seed alcoholic extract and MTX to induce mutations of G-system isolates.

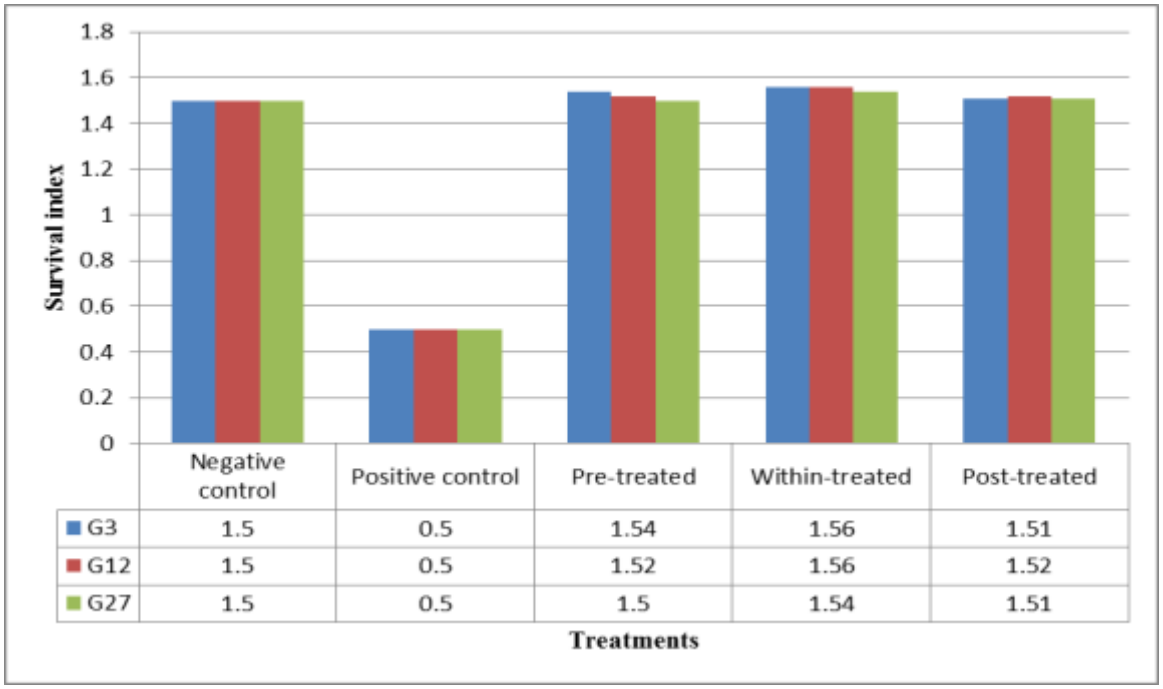


Figure (4): The effect of the interaction between the optimum concentration of *C. colocynthis* seed alcoholic extract and exposure to UV on the survival index of G-system isolates.

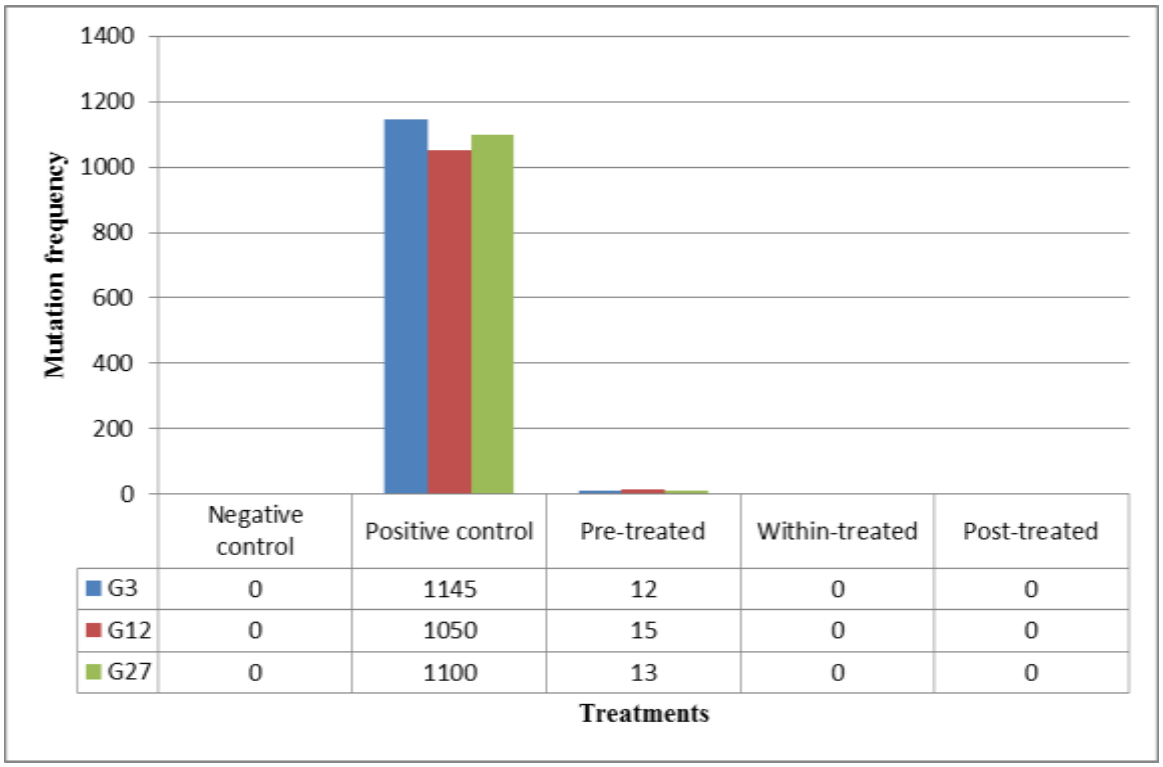


Figure (5): The effect of the interaction between the optimum concentration of *C. colocynthis* seed alcoholic extract and exposure to UV to induce mutations of G-system isolates.

The present results demonstrated the plant extract was significantly effective in survival index when treated with different concentration of the extract and with different mutagens, these leads to suggest that the extract may contain chemical compounds that protect G-system isolates from the genotoxic effects of the mutagens reviewing the literature revealed that *C. colocynthis* is rich in three flavonoids glycosides (isosaponarin, isoviterxin and isoorientin 3'-O-methyl ether), protein (rich in isoleusine, leusine and tryptophan amino acids), lipids, saponins, flavonoids (such as apigenin and quercetin and leuteolin), and a considerable amount of ions (potassium, phosphorus and iron) such constituents have been demonstrated to have immunomodulatory effects [6, 14, 15, 16, 17, 18]. Another active material that founded in the *C. colocynthis* seed extract that have antimicrobial properties is Alkaloids, tannins, steroids, pigments and iridoids [19].

In agreement with such augmentation [6, 14, 15, 16, 17, 18] were able to demonstrate that these constituents have a wide range of biological effects; including treatment of diabetic people in Mediterranean countries, and used for treatment many inflammatory disease, constipation, odema, fever, jaundice, leukemia, bacterial infections, cancer, used as abortifacient and it had antibacterial and antifungal properties, which have confirmed by others [1, 2, 3, 19, 20].

Furthermore, the Folkloric applicationof the plant (for instance anti-oxidant, antibacterial, antifungal and anti-leukemic activity) can also be interpreted on such ground, especially it use consider the anti-oxidant potential of the plant or these active ingredient [4, 5, 6, 7, 21, 21,23, 24]. From the findings, it is possible to suggest that the plant may be protected, reduced, or repaired G-system isolates from the cytotoxicity and mutagenicity effects of the two mutagens, but it is too early to reach a final conclusion, and further investigations are required to cover such subject.

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