

**In Vivo Study for Measuring the Toxicity of Heat Stable Enterotoxin (a)
Produced by Enterotoxigenic *Escherichia coli* in Mice**
دراسة داخل الجسم الحي لقياس سمية السم الثابت حراريا (أ) المنتج من قبل بكتريا ايشيريشيا القولون السمية
المعوية في الفئران

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Abstract

This research was conducted to measure the safety of heat stable enterotoxin a (STa) produced by enterotoxigenic *Escherichia coli*, through studying its toxic effect on mice since it showed a promising effect in reducing the proliferation of colorectal cancer cells. The cytogenetic effect was determined after giving five different doses (100, 200, 400, 800 and 1600)µg/Kg in comparison with negative (phosphate buffer saline / PBS) and positive (mitomycin C/ MMC, at doses of 2 and 5µg/Kg) controls on mouse bone marrow cells by employing the following parameters: mitotic index, chromosomal aberrations and micronucleus, also, the serum level of liver functional enzymes (GOT, GPT, ALP) was recorded. In addition, lethal dose 50 (LD 50) with certain clinicopathological changes in five organs (colon, kidney, liver, stomach and lung) was also determined after oral administration of STa for ten successive days and at two doses (500 and 1000) µg/Kg. Results showed that, none of the five different doses of STa caused any significant changes in the three examined cytogenetic parameters in the mouse bone marrow cells; precisely, neither the low dose nor the high one of STa caused reduction or induction in these parameters. In fact, clear effect in decreasing mitotic activity and increasing spontaneous frequencies of both chromosomal aberrations and micronucleus was revealed after MMC treatment. Furthermore, significant differences in mouse serum level of the three enzymes were not seen at any doses of STa, while significant reduction in the levels of these enzymes was noticed after treatment with the two doses of MMC. In this study the LD 50 test was used to investigate the lethal effect of the partially purified STa, and it was shown to be not lethal to mice at both doses of (500 and 1000) µg/Kg, since death was not recorded, moreover, no clinicopathological effects were indicated in the all examined mouse tissues, however the only noticed clinical sign was diarrhea with all doses, which was observed after three days of STa treatment.

Keywords: heat-stable enterotoxin a, *Escherichia coli*, cytogenetic.

المستخلص

تم دراسة سلامة السم المعوي الثابت حراريا (أ) (heat stable enterotoxin/ STa) المنتج من قبل ايشيريشيا القولون السمية المعوية (enterotoxigenic *Escherichia coli*) من خلال قياس سميته على خلايا الدم الحيوي للانسان حيث اظهر هذا السم تأثير واضح في خفض معدل انقسام خلايا سرطان القولون. تم تقييم التأثيرات الوراثية الخلوية للمعاملة بـ (STa)، من خلال استخدام خمس جرعات مختلفة (100, 200, 400, 800, 1600) مايكروغرام/كغم و بالمقارنة مع سيطرة سالبة (داري الفوسفات الملحي) و سيطرة موجبة (ميتومايسين (MMC)، عند الجرعتين (5:2) مايكروغرام/كغم، في خلايا نقي العظم للفئران (داخل الجسم) بالاعتماد على المعايير التالية: معامل الانقسام الخيطي و التغيرات الكروموسومية و تكوين النوى الصغيرة. ايضا معدل انزيمات وظائف الكبد (GOT, GPT, ALP) في المصل قد تم قياسه بعد خمسة ايام متلاحقة من المعاملة بـ (STa). ايضا شملت الدراسة حساب الجرعة القاتلة (Lethal dose 50) مصحوبة بالتغيرات المرضية السريرية في خمسة اعضاء (القولون، الكلية، الكبد، المعدة، الرئة) بعد المعاملة بـ (STa) لمدة عشرة ايام متلاحقة و عند الجرعتين (500, 1000) مايكروغرام/كغم. نتائج هذه الدراسة اظهرت ان اي من الجرعات الخمس المختلفة لـ (STa) لم تسبب اي تغيرات معنوية في المعايير الثلاثة للوراثة الخلوية في خلايا نقي العظم للفئران؛ وبشكل ادق، لا الجرعة القليلة ولا الجرعة العالية قد سببت تحفيز او اختزال في هذه المعايير. ولكن كان هنالك انخفاض ملحوظ في معامل الانقسام الخيطي وزيادة معنوية في نسب كل من التغيرات الكروموسومية وتكوين النوى الصغيرة بعد المعاملة بـ الميتومايسين. اضافة الى ذلك، لم يتم حدوث اي اختلافات معنوية في معدل انزيمات وظائف الكبد الثلاثة في مصل الفئران بعد المعاملة بـ (STa). هذه الدراسة ايضا تضمنت استخدام فحص الجرعة القاتلة من اجل التحري عن التأثير القاتل لـ (STa) و قد وجد انه غير قاتل للفئران وعند كلا الجرعتين، بما انه لم يتم تسجيل اي حالة وفاة.

ايضا لم يتم العثور على اي تغيرات مرضية سريرية في جميع انسجة الفأرة المفحوصة و لكن العلامة السريرية الوحيدة التي تم ملاحظتها كانت الاسهال والذي لوحظ بعد ثلاثة ايام من المعاملة بـ (STa).

الكلمات المفتاحية: سمية السم الثابت حراريا (أ)، *Escherichia coli* ، التأثيرات الوراثية

Introduction

Colorectal cancer is considered to be one of the main causes of death all around the world. Various methods and strategies have been used to treat such kind and others of cancer. The traditional methods include: radiation, chemotherapy and surgery. Recently new approaches have been suggested and developed; one of these using *Escherichia coli* toxin, for the treatment of colorectal cancer [1].

It was found that *E. coli* produces a toxin, which mimics a natural colon process and provoked diarrhea. However, the toxin also causes a flood of calcium into the affected cells, stopping colorectal cancer cells from replicating rapidly. Three groups of *E. coli* are associated with diarrheal diseases. One of these groups is *E. coli* strains that produce enterotoxins, which is called enterotoxigenic *Escherichia coli* (ETEC) [2].

Unfortunately there is unexplained inverse relationship between the incidence of colorectal cancer and ETEC infection. The toxin that produced by ETEC, which is heat stable enterotoxin a (STa), that cause one of the serious forms of food poisoning may be used in the treatment of one of the most deadly types of cancer [3]. The authors provided a convincing evidence for the presence of a novel intracellular signaling pathways initiated by STa that prevented the proliferation of colorectal cancer cells.

Cytogenetic analyses allow for the objective evaluation of genetic material damages and it is a method that permits direct image analysis for the chromosomal damage. It can be measured by different parameters: a) mitotic index (MI), which represent the percentage of dividing cell at the metaphase [4], b) Micronucleus (MN), which is a cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomes lagging at anaphase or from acentric chromosomal fragments, and MN test can be expressed by the number of MN in (1000) cells of polychromatic erythrocytes (PCE) [5] and c) chromosomal aberrations (CAs), which serves to detect structural chromosomal aberrations, as may be induced via DNA breaks by various types of mutagens, it measured by calculating these aberration in (100) cells at the metaphase [6].

This study aims to open up a new approach in the development of anti-cancer drugs. An attempt to provide anti-cancer agent (STa) for the treatment of colorectal cancer with less or no cytotoxic effect on normal cells and with more cytotoxic effect on cancer cells. Thus, we have to determine the safety of this approach by testing the toxicity of this toxin in mice.

Materials and Methods:

All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH (England).

Experimental Animals

Ninety five Albino Swiss BALB/c female mice, which were obtained from the Biotechnology Research Center/ AL-Nahrain University, were used in this study. They were used for the cytogenetic analysis, determination of liver enzymes level, and determination of lethal dose 50 (LD 50) in addition to histopathological examination. Their ages were ranged between (8-12) weeks and weighting (25-30) gm. They were divided into subgroups, and each group was putted in a separate plastic cage. The cages were kept in a room with (23-25) C° temperature. The animals were fed with a suitable quantity of water and complete diet.

Animals treatment with STa

The animals in this experiment were treated with a cumulative dose of STa in a short time. The main aim of this experiment was to evaluate the acute effect of STa in normal mice.

Ten groups of mice, in which (95) mouse were used in this experiment and treated as follows: for the first seven groups only, one half of each group was used to determine MI and CA, while the other half was used for the determination of MN and liver enzymes level. These groups are:

Group I: Negative control (10mice), treated with (0.1ml) of PBS.

Group II: Positive control (20mice), (10mice) treated with (0.1 ml) of MMC (2µg/Kg) and (10mice) treated with (0.1ml) of MMC (5µg/Kg).

Group III: STa treatment (6mice), treated with (0.1 ml) of STa (100µg/Kg).

Group IV: STa treatment (6mice), treated with (0.1ml) of STa (200µg/Kg).

Group V: STa treatment (6mice), treated with (0.1ml) of STa (400µg/Kg).

Group VI: STa treatment (6mice), treated with (0.1ml) of STa (800µg/Kg).

Group VII: STa treatment (6mice), treated with (0.1ml) of STa (1600µg/Kg).

The PBS, MMC and STa were given orally for five successive days, and then the mice were sacrificed at the sixth day. Bone marrow and blood samples were taken and cytogenetic analysis, liver enzyme determination were carried out as described later.

Group VIII: Negative control (5mice), treated with (0.1ml) of PBS.

Group IX: STa treatment (10mice), treated with (0.1ml) of STa (500µg/kg).

Group IIX: STa treatment (10mice), treated with (0.1ml) of STa (1000µg/kg).

These three groups were used for the determination of the LD 50, which was assayed by orally giving (0.1ml) of STa and PBS for ten successive days. The mice were observed daily for hand-leg paralysis and death. Death between day 1 and 10 inclusively were tabulated, and then histopathological effects were detected by examining five organs (lung, liver, kidney, colon and stomach). The LD50 was calculated by using the method of [7].

Preparation of STa: STa was obtained as partially purified extract from previous study [8].

Biochemical Tests: These tests were performed according to [9].

Chromosomal preparation from somatic cells of the mouse bone marrow: This experiment was done according to [10]. Each animal was injected with 0.25ml of colchicine with a concentration of (1mg/ml) intraperitoneally (I.P) 2hr before sacrificing the animal, Then the animal was sacrificed by cervical dislocation and fixed on its ventral side on the anatomy plate and the abdominal side of the animal and its thigh region were swabbed with 70% ethanol. The femur bone was then taken and cleaned from the other tissues and muscles and gapped from the middle with a forceps in a vertical position over the edge of the test tube, and by sterile syringe 5ml of PBS were injected so as to wash and drop the bone marrow in the test tube. The test tube was taken and centrifuged at speed of 2000 rpm for 10min. After that the supernatant was removed and 5ml of potassium chloride (0.075) M was added as a hypotonic solution, then the test tubes were left for 30min in the water bath at 37C ° and shaken from time to time. The tubes were then centrifuged at 2000 rpm for 10min and the supernatant was removed and the fixative solution was added (as drops) on the inside wall of the test tube with the continuous shaking, the volume was fixed to 5ml and the content shaken well. The tube was kept at 4C° for 30min to fix the cells. After that the tubes were centrifuged at 2000 rpm for 10min. The process was repeated three times and the cells were suspended in 2ml of the fixative solution. By a pasture pipette, few drops from the tube were dropped vertically on two chilled slides from a height of 3 feet at a rate of (4-5) drops to give the chance for the chromosomes to spread well. Later the slides were kept to dry at room temperature, and then stained with Giemsa stain and left for 15min and washed with distilled water. The mitotic activity is expressed by the MI which is the number of dividing cells in (1000) cell. Moreover, chromosomal aberrations can be also recorded from the same slides, which represent the number of aberrations in (100) dividing cells at the metaphase.

Micronucleus test in mouse bone marrow cells: This assay was adapted from that described by [10]. The femur bone was cleaned from tissue and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube, and by a sterile syringe 1ml of human serum was injected so as to wash and drop the bone marrow in the test tube. Then the test tube was centrifuged at 1000 rpm for 5min. Later the supernatant was removed, and a drop from the pellet was taken to make a smear on clean slides and the slides were kept at room temperature for 24hr. Finally the slides were fixed with absolute methanol for 5min, then stained with Giemsa stain for 15min and washed with D.W. and left to dry. Two slides for each animal were prepared for micronucleus test. MN can be counted in (1000) cells of polychromatic erythrocytes (PCE) in mice.

Histological Examinations: This was performed by using method of [11]. At the time of death, mouse organs ovaries were taken for histopathological examination. The perfuse-fixed ovaries placed in Bouin fluid overnight, and processed for routine paraffin embedding. The ovaries were cut into 5-µm sections. Three serial sections per ovaries were mounted on slides, deparaffinized, rehydrated, and stained with

hematoxyline - eosin stain. Sections of the ovaries were examined by light microscopy; primary and secondary follicles and corpus luteum diameters were assessed in each ovary using a previously calibrated micrometer eyepiece.

Enzymatic Assays: Blood was collected from the heart of the mice and used for biochemical examination after the separation of serum. GOT and GPT were measured according to [12], while ALP was measured according to GPT tests. To estimate the activity of ALP enzymes, this method was adapted according to [13].

Statistical Evaluation: Data were analyzed by 1-way analysis of variance with ANOVA- test. Data are presented as means \pm SE. The level of significance was $P < .05$. [14].

Results and Discussion

Cytogenetic effect of STa on mouse bone marrow cells

STa effect on mitotic index (MI): The treatment effect of five doses of STa and two doses of MMC (as a positive control) in addition to PBS (as a negative control) on mitotic index of mouse bone marrow cells was shown in Table (1). There were no significant differences ($P > 0.05$) for all STa doses after five days of treatment (6.933, 6.133, 6.30, 7.0 and 6.233%) at doses of (100, 200, 400, 800 and 1600 $\mu\text{g}/\text{Kg}$) respectively as compared with negative control 6.60%. However, a significant decrease ($P < 0.05$) in the MI was seen after treatment with two doses of MMC (2.866 and 0.942%) at doses of (2 and 5) $\mu\text{g}/\text{Kg}$ respectively. The reduction in the MI after MMC treatment has been also demonstrated by many authors [15]. There are other chemotherapeutic drugs that also cause MI reduction, like methotrexate (MTX) [16] and tamoxifen (TAM) [17]. Reduction in the MI may be related to several factors: first of all, the proteins required for mitosis may not be produced in the same quantities, or the code did not reach the cells to induce it for proliferation or the drugs may caused death of bone marrow cells or due to the effect occurred in the mitotic spindles composition during cell division [18]. At the same time, MI was shown to increase after treatment with other kinds of agents, such as antioxidants (e.g., vitamin C which is the active constituent of many plants), they act by inducing cell division by acting as mitogens [19].

The non significant differences in MI that were seen after STa treatment, may be due to that STa does not have any effect on the proteins or enzymes that have correlation with cell division, or it does not have the activity of many mitogenic agents.

STa effect on micronucleus (MN) induction

The mouse bone marrow micronucleus assay holds a key position in all schemes for detecting potential carcinogens and mutagens as shown in figure (1). Micronucleus frequency of polychromatic erythrocytes from negative control mice was 0.566%. This percentage was not significantly differed ($P > 0.05$) after treatment with STa 0.30, 0.533, 0.433, 0.50, 0.590% at doses of 100, 200, 400, 800, 1600 $\mu\text{g}/\text{Kg}$ respectively, as compared with the negative control. A significant increase $P < 0.05$ in MN frequency was detected after treatment with MMC (2.733, 5.133) % at (2, 5) $\mu\text{g}/\text{Kg}$ respectively as represented in Table (1). However, similar results for induction of MN frequency in MMC-treated mice were denoted by [20]. The increase in MN number may be suggested that, micronuclei may be originated from acentric chromosome fragments, either from the double-stranded DNA damage before cell division or after breakage of anaphase bridges. It was found that MMC act as a clastogenic agent and a micronucleus inducer in both mouse and human.

Although, there are agents that cause increased MN frequency, at the same time several plant extracts cause decreased MN frequency. A number of plants were shown to contain active constituents such as rutin and quercetin which protect DNA from damage by their antimutagenic and detoxification activities [21]. STa neither causes DNA damage nor protects DNA from damage and therefore, no significant differences in MN frequency were reported after treatment with it.

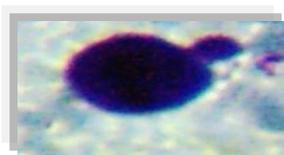


Fig (1): Micronucleated bone marrow cell from mouse

STa effect on chromosomal aberrations (CAs)

The spontaneous frequency of chromosomal aberrations in untreated mouse bone marrow cells was 0.276% which considered as negative control Table (1) Figure (2). In this study, the types of chromosomal aberrations which have been observed were; ring, gap, acentric, dicentric, chromosome break, chromatid break and deletion. Neither the low nor the high doses of STa caused a significant differences $P > 0.05$ in total and in all types of CAs frequency in comparison to the negative control. Results of Table (1) indicated that treatment with different doses of STa did not reduce or induce spontaneous CAs 0.325, 0.309, 0.286, 0.274, 0.322% at doses of 100, 200, 400, 800, 1600 $\mu\text{g}/\text{Kg}$ respectively as compared with negative control.

A significant increase in total and in all types of CAs was seen after treatment with MMC 0.907, 1.558% at doses of 2,5 $\mu\text{g}/\text{Kg}$ respectively when compared with the negative control, except with chromosome break which showed no significant difference with the 2 $\mu\text{g}/\text{Kg}$ dose only Table (1). These results came in agreement with [16], who had reported that CAs frequency increased in mouse bone marrow cells after treatment with different doses of MMC, and it was dose dependent. MMC was shown to increase CAs frequency through inhibition of DNA repair system, or it may act on topoisomerase II and leading to more DNA damage.

On the other hand, the reduction in CAs frequency was noticed after treatment with plant extracts that contain vitamins which act as scavengers for free radicals in the cells and they may also act as antimutagens for many mutagenic drugs in mouse bone marrow cells [22]. STa neither caused an increase in CAs frequency because it does not bind to DNA or affect DNA repair system, nor caused a decrease in CAs frequency because it does not have scavenger or antimutagenic properties.

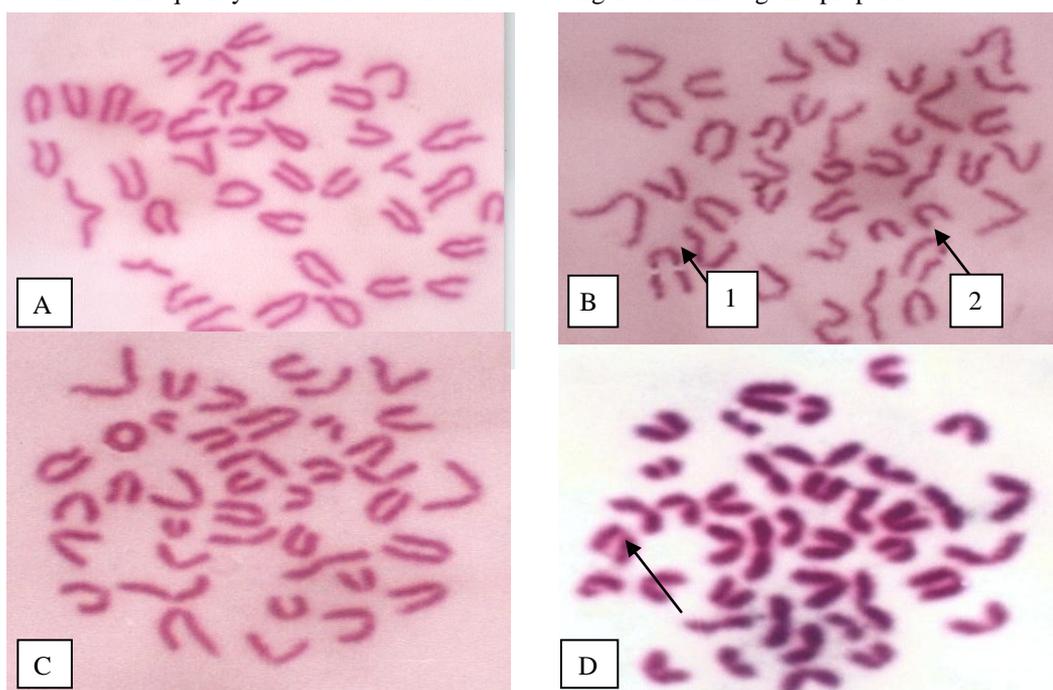


Fig (2): Metaphase of mouse bone marrow cells showing; normal chromosomes (A), 1.gap 2.chromosome break (B), ring (C), deletion (D).

Clinicopathological Effects of STa on Mouse (Mouse lethality assay)

In this study the LD 50 test was used to investigate the lethal effect of the partially purified STa, and it was shown to be not lethal to mice at both doses of 500 and 1000 $\mu\text{g}/\text{Kg}$, since death was not recorded. Ten mice were used for each dose and after ten successive days, the earliest, dominant and only clinical sign was diarrhea which was noticed after three days of oral inoculation, while mice that were orally inoculated with PBS (negative control) survived without developing any significant symptoms. This result came in agreement with [23], who found that *E. coli* (that secreting STa) treated mice suffered from diarrhea but without death in comparison with *Compylobacter jejuni* treated mice which suffered

from diarrhea that followed by death. Sections from (colon, kidney, liver, stomach and lung) were taken from both STa treated mice and controls. No histopathological abnormalities were detected in any of these tissues as shown in Figure (3). The lack of significant pathological changes in kidney, liver, stomach and lung can be explained by the absence of GC-C receptors in these organs, and since STa bind only to these receptors [24], so no pathological changes were seen. But the only explanation for the lack of significant pathological signs in the colon after STa treatment may be due to that STa caused only functional abnormalities (diarrhea) in this tissue without visible pathological disturbance. These results have documented that STa has no pathological effect and can be used in a safe way and unlike other *E. coli* toxins, such as verotoxin, which has been investigated by some investigators in a try to use it in the treatment of brain cancer [25].

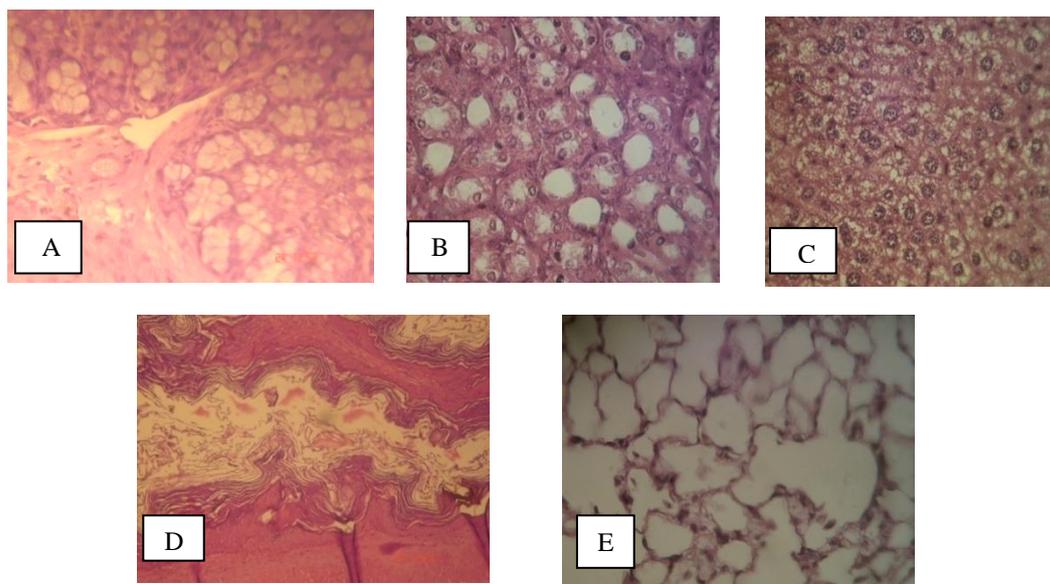


Fig (3): Histological sectioning showing normal (unaffected) mouse tissue after STa treatment at 1600 µg/ml. (Hematoxyline & Eosin) at 400X. A) colon B) kidney C) liver D) stomach E) lung.

Effect of STa on liver functional enzymes (GOT, GPT, ALP) levels in mouse

In this study the effect of STa on GOT (glutamate oxaloacetate transaminase), GPT (glutamate pyruvate transaminase) and ALP (alkaline phosphatase) levels have been investigated. Results in Table (2) showed that no significant differences $P > 0.05$ in GOT level was seen after treatment with STa when compared to that of the negative control 190.33 U/L, and the recorded levels were (186.33, 196.0, 194.0, 188.67, 192.67)U/L at doses of (100, 200, 400, 800, 1600)µg/Kg respectively. While significant increases $P < 0.05$ were seen after treatment with MMC which reached up to (231.33, 270.0) U/L at doses of (2, 5)µg/Kg respectively, as compared with the negative control.

Regarding GPT level, STa treatment did not cause any significant changes $P > 0.05$ as compared with that of the negative control 62.33 U/L. The GPT levels were (63.67, 57.0, 59.0, 58.33, 62.67)U/L at STa doses of (100, 200, 400, 800, 1600)µg/ml respectively. On other hand significant increases $P < 0.05$ were seen after MMC treatment which reached to (83.33, 98.0)U/L at doses of (2, 5)µg/Kg respectively in comparison with negative control.

At the same time, no significant differences $P > 0.05$ in ALP level were indicated after STa treatment, as compared with the negative control 65.33 U/L. They were (66.33, 68.33, 63.0, 63.33, 62.0)U/L at doses of (100, 200, 400, 800, 1600)µg/Kg respectively. Significant increases $P < 0.05$ were also seen after MMC treatment which reached to (87.67, 105.33)U/L at doses of (2, 5)µg/Kg respectively, as compared with the negative control. The levels of GOT, GPT and ALP enzymes in the serum were increased after treatment with several chemotherapeutic drugs including the MMC. This increase may be due to that, these drugs have cytotoxic effect on liver cells and this leads to increase the permeability of liver cell membrane or to cause damages in liver tissue and thus causing the release of high quantity of these enzymes into blood [26]

Table (2): STa effect on liver functional enzymes (GOT, GPT, ALP)

Treatment	GOT m±SE	GPT m±SE	ALP m±SE
PBS	a 190.33±7.51	a 62.33±6.03	a 65.33±4.51
STa 100 µg/ml	a 186.33±7.09	a 63.67±5.03	a 66.33±3.51
STa 200 µg/ml	a 196.00±7.55	a 57.00±2.00	a 68.33±3.21
STa400 µg/ml	a 194.00±6.00	a 59.00±4.51	a 63.00±1.53
STa800 µg/ml	a 188.67±5.13	a 58.33±5.51	a 63.00±2.00
STa1600 µg/ml	a 192.67±4.04	a 62.67±6.11	a 62.00±5.00
MMC2 µg/ml	b 231.33±6.51	b 83.33±5.51	b 87.67±5.69
MMC5 µg/ml	c 270.00±6.24	c 98.00±6.08	c 105.33±4.51

*Significant different from control values. P<0.05

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