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# CYTOGENETIC EFFECTS OF PARTIAL PURIFIED PLANTARICIN OF *LACTOBACILLUS PLANTARUM* ON STEM CELLS MICE BONE MARROW

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**ABSTRACT**
The present study was intended to appreciate the cytogenetic effects (Chromosome aberration and Micronucleus) of Plantaricin of *lactobacillus plantarum* on mice bone marrow stem cells. Mice were divided into four groups: Group (A): control (not treated group), Group (B): Animals were administrated orally 160 AU / ml of plantaricin for 7 days ,Group (C): Animals were administrated orally 160 AU / ml of plantaricin for 15 days, and Group (E): Animals were administrated orally 160 AU / ml of plantaricin for 21 days. At the end of experiment, bone marrow samples were obtained from each mouse in all groups for cytogenetic analysis (CA and MN). After statistical analysis of the data, the results showed that Plantaricin of *lactobacillus plantarum* led to an increase in chromosomal aberrations, micronucleus ($P < 0.05$). This percentage increases in conjunction with the length of the treatment period. Different antimicrobials in plantaricin which leads to an increase in chromosomal aberration (CAs) and increase in the formation of micronucleus (MN).

**KEY WORDS:** Partial purified Plantaricin of *lactobacillus plantarum*, chromosomal aberrations,and micronucleus.

## INTRODUCTION

Lactic acid bacteria have been historically linked with food fermentations, as acidification inhibits the growth of spoilage microorganisms. The antimicrobial activities of the LAB have been known to play important roles in food fermentations, food preservation and intestinal ecology[1]**.** The basis of LAB's protection of foods is mainly due to their production of organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds such as free fatty acids or phenyllactic acid, antibiotics such as reutericyclin and bacteriocins[2]. Bacteriocins are generally defined as ribosomally synthesized peptides produced by bacteria that have bacteriostatic or bactericidal activity against other related and unrelated microorganisms [3, 4]. The bacteriocins from LAB have attracted significant attention because of their potential use as non-toxic and safe additives for food preservation and prevention of food spoilage by food borne pathogenic bacteria [5,6]. The antimicrobial action of bacteriocins involves increased permeability of the cytoplasmic membrane of the target cells, which leads to the release of small cytoplasmic particles, depolarization of the membrane potential and eventually to cell death[7]. Chromosomal aberration are used to determine the genotoxicity of some chemical agents .Micronuclei analysis also can be used to verify mutagenicity of different chemicals [8].To ensure safety of plantaricin consumption; the current study performs cytogenetic assessment on mice bone marrow stem cell.

## MATERIALS & METHODS

### Bacterial isolate

The bacterial isolate used for plantaricin production was *Lactobacillus plantarum* which isolated from fermented vegetables and culture medium which used for production plantaricin of *Lactobacillus plantarum* was BGM [9].

### Plantaricin activity assay

Plantaricin activity was achieved by serial two fold dilutions of cell-free supernatant (crude plantaricin)[10]. Dilutions were used to check the antibacterial activity of plantaricin against indicator bacteria using the well diffusion method as described by[11] and AU was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator bacteria, AU was calculated as:
(1000 / 100) × D, where 1000: constant, 100: volume of supernatant in a well (µl) and D: the dilution factor. It was calculated by described method [12]. plantaricin concentration was estimated according to [13].

### Extraction and partial Purification of Plantaricin

Basal Growth Medium was inoculated with *Lactobacillus plantarum* and incubated at 12 hours under anaerobic conditions [9]. Cells were harvested by centrifugation at 6000 rpm for 15 minutes. The cell-free supernatant that was referred to as crude plantaricin extract (CPE) was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15minutes [14].
The supernatant was mixed with n-butanol at a ratio 1:1then centrifuged at 4000 rpm for 10 minutes to achieve phase separation .The organic phase was evaporated at 80°C by rotary evaporator, then the sediment was re-suspended in 20mM sodium citrate buffer (pH 5) and referred to as partial purified plantaricin (PPP). The protein concentration of plantaricin of *Lactobacillus plantarum* was estimated.

### Experimental Design

Fourty mice were obtained from the national center for drug control and research, Baghdad. Their ages ranged between (8-12) weeks and weighting (25 ±2) gm. They were divided into four groups; ten mice in each group

were put in a separated plastic cage Group (A): control (not treated group), Group (B): Animals were administrated orally 160 AU / ml of plantaricin for 7 days, Group (C): Animals were administrated orally 160 AU / ml of plantaricin for 15 days, and Group (E): Animals were administrated orally 160 AU / ml of plantaricin for 21 days. At the end of experiment, bone marrow samples were obtained from each mouse in all groups for cytogenetic analysis (CA and MN).

## CYTOGENETIC EXPERIMENTS

***Chromosome preparation from somatic cells of the mouse bone marrow***

The experiment was done according to Allen *et al.*[15]. Colchicine was injected 2hr. before sacrifice. Mice were sacrificed by cervical dislocation. It was dissected and both of femur bones were excised. Bone marrow was aspirated by flushing with phosphate buffer saline (PBS) in the centrifuge tube. The suspension was flushed in the tube properly to get good cell suspension and centrifuged for 10 min at 2000 rpm. Supernatant was discarded and the pellet was treated with pre-warmed (37°C) KCl (0.56%) and shaken well. Suspension incubated in a water bath at 37°C for 20 min. Pellet was treated with freshly prepared fixative solution (Methanol: Glacial Acetic Acid, 3:1) and shaken well then centrifuged for 10 min at 2000 rpm. Fixative was repeated 3 times to get debris free white pellet. Few drops from the tube were dropped vertically on the slide. Slides were kept overnight to dry then stained with (Giemsa's stain) and observed under microscope in 40 x and then in 100x magnifications. A total of 100 well spread metaphase plates were scored for chromosomal aberrations) gap, chromatid break, polyploidy, acentric fragment, ring and fragmentation (were counted and data of scoring was expressed as percentage chromosomal aberrations.

**Cytogenetic analysis**

***Chromosomal aberration (CA) assay***

The prepared slides were examined under the oil immersion lens (100x) of light microscope for 100 divided cells per each animal, and the cells should be at the metaphase stage of the mitotic division where the chromosomal aberrations were clear and the percentage of these aberrations could be estimated.

***Micronucleus MN assay***

This experiment was done according to method of Schmid[16] as follow: -The femur bone cleaned from tissues and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe, (1 ml) of human plasma (heat inactivated) was injected so as to wash and drop the bone marrow in the test tube. Then the test tubes were centrifuged at speed of 1000 rpm (5 min).The supernatant was removed, and one drop from the pellet was taken to make a smear on a clean slide. The slides were kept at room temperature for (24 hr.). The slides were fixed with absolute methanol for (5 min.), then stained with Giemsa stain for (15 min), then washed with D.W and left to dry. Two slides for each animal were prepared for micronucleus test. The slides were examined under the oil immersion lens, and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus. The micronucleus index was obtained using the following equation:

$$\text{Micronucleus Index} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}}\right) \text{x } 100$$

**Statistical Analysis**

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (Two-sample T-test) using the computer program Minitab release (14.12) discovery Copyright 2004. The difference was considered significant when the probability value was less than $p<0.05$.

## RESULTS & DISCUSSION

Partial purification of plantaricin was performed by extraction with n-butanol in a 1:1 ratio.

**TABLE 1:** Purification of plantaricin produced by *Lactobacillus Plantarum*

| Purification steps | Volume (ml) | Activity (AU/ml) | Protein concentration (mg/ml) | aSpecific activity (AU/mg) | bTotal activity (AU) | [CC]Purification fold | dYield (%) |
|---|---|---|---|---|---|---|---|
| Crude plantaricin extract (CPE) | 250 | 160 | 0.7 | 228.57 | 40000 | 1 | 100 |
| After heating (80 °C/ 10min) | 250 | 160 | 0.62 | 258.06 | 40000 | 1.129 | 100 |
| Extraction with butanol (1:1) | 25 | 640 | 0.8 | 800 | 16000 | 3.500 | 40 |

aSpecific activity (AU/mg): represents plantaricin activity divided by protein concentration.
bTotal activity (AU): represents Activity (AU/ml) × Volume (ml).
cPurification fold: represents specific activity of purified fraction divided by specific activity of crude extract.
dYield (%): represents (total activity of purified fraction divided by total activity of crude extract) × 100 [18].

Plantaricin was removed from the aqueous phase and could be recovered from the organic phase this suggests that at least part of the plantaricin molecule had a hydrophobic character and shares this property with other bacteriocins [17]. The specific activity of plantaricin recorded 800 AU/mg proteins with 3.500 purification folds and 40% yield as shown above in table (1).

**TABLE 2:** Percentages of different types of chromosomal aberrations (CA) in mice bone marrow for control (I), and treatment groups (Mean ± SE)

| Experimental Groups | Types of chromosome aberration % | | | | | Total % |
|---|---|---|---|---|---|---|
| | Acentric Fragment % | Ring % | Polyploidy % | Break % | Fragment % | |
| control I | 1.2±0.10 | 0.00±0.00 | 0.00±0.00 | 0.56±0.09 | 0.64±0.11 | 2.4±0.1 |
| Treatment I | 9.52±0.400 | 0.28±0.13 | 0.28±0.10 | 1.56±0.37 | 3.84±0.492 | b*15.4±0.80 |
| Treatment II | 15.08±0.79 | 0.32±0.10 | 0.04±0.04 | 3.08±0.44 | 4.16±0.30 | c*22.6±1.15 |
| Treatment III | 18.12±0.71 | 0.48±0.16 | 0.12±0.08 | 2.92±0.59 | 6.48±0.54 | d*28.12±1.77 |

a control I Vs. treatment groups b,c,d *Significant at (P<0.05).

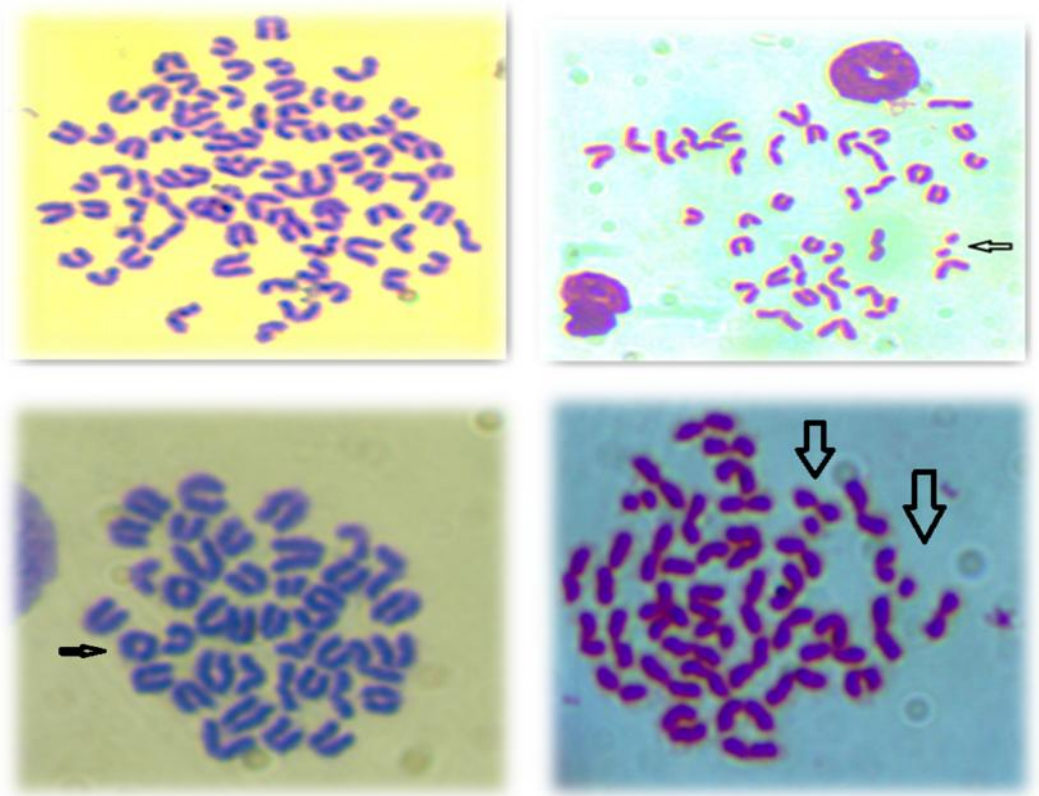
**TABLE 3:-** Percentages of micronuclei (MN) in bone marrow of mice for control group (I), treatment groups (Mean ± SE).

| Experimental Groups | MN % |
|---|---|
| control I | a3.1± 0.08 |
| Treatment I 7 day | b7±1.3 |
| Treatment II15 day | c11±0.63 |
| Treatment II21 day | d11.8±0.49 |

a control I Vs. b, c, d treatment groups *Significant at (P<0.05).

Result of micronuclei (MN) showed the frequency of MN in control differed significantly (P<0.05) as compared with treatment groups. The treatment groups showed a significant (P<0.05) increasing in CAs and MN. The reason of these results was related to the cytotoxic impact of Plantaricin which has damaging effect on DNA of mice bone marrow cells. The results indicated that the Plantaricin causes a high structural CAs instead of numerical CAs. The chromatid type aberrations like breaks and gaps are produced during the S or G2 stage (i.e. during or after replication).

In all three treatment groups, we can increase of CA and MN. MN can be formed during mitosis through the loss of either whole chromosomes or a centric chromosome fragments from the nucleus, which are separate from the nucleus of the cell. The difference in micronucleus (MN) rates was statistically significant in comparison with the values before and after treatment of plantaricin (p<0.05). This study proved that plantaricin increases the frequency of chromosomal aberration and therefore it increases the micronucleus as showed in figure (2).



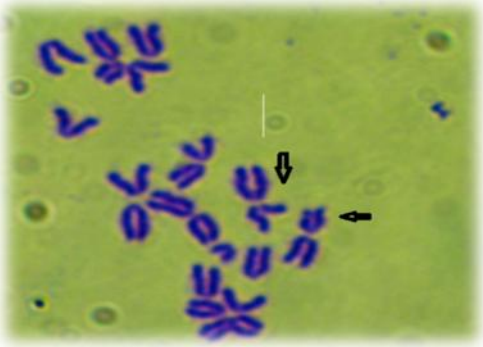


**FIGURE1:** showed different chromosomal aberrations in mice bone marrow (100X) injected only with plantaricin. Polyploidy, a centric fragment, ring, fragment, break respectively.

From the results of the presented study, it is evident that the treatment with increased the frequency of micronucleus where there was a positive correlation between the increased drug time and the induction of MN in the bone marrow cells of mice. In our study we noticed that the damage that has occurred in chromosomes resulting from the chemical composition of plantaricin in different antimicrobials, such as lactic acid, acetic acid, hydrogen peroxide, carbon dioxide and bacteriocins produced by these lactic acid bacteria, can inhibit pathogenic and spoilage microorganisms. One important attribute of LAB is their ability to produce antimicrobial compound called bacteriocin such as plantaricin[19]. These different antimicrobials lead to increase chromosomal aberration and the results came in agreement with [20] who showed $H_2O_2$ induced DNA damage and its prevention by Pippali (Piper longum) aqueous extract. The higher increase in $H_2O_2$-induced chromosomal aberrations in Fanconi's anemia compared with normal peripheral blood lymphocytes[21]. Concluded glucose deprivation with lactic acidosis – two tumor micro environmental factors can induce cancer cell chromosomal instability (CIN) potentially via 3 ways, damaging DNA, up regulating mitotic checkpoint genes, and amplifying centrosome [22].

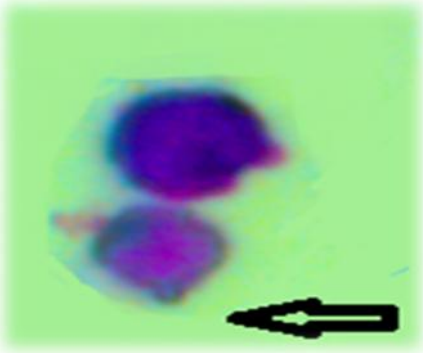


**FIGURE 2:** plantaricin formation MN at (100x).

## CONCLUSION

We can conclude that plantaricin has genetic toxicity effects on dividing cells in the bone marrow of mice. The genetic toxicity of plantaricin leads to an increase in chromosomal aberration (CAs), an increase in the formation of micronucleus (MN). The time of treatment have various effects on dividing cells in the bone marrow. That the Pis a toxic contrast agent, which shows significant genotoxicity effects on mouse bone marrow stem cells and there is a significant increasing in CA and MN, which gives evidence for the genotoxicity effect of Pin mouse bone marrow stem cells.

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