

Cytotoxic, antimicrobial and DNA breaking activity of Salgam

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Salgam is one of the traditional fermented beverage that was produced and consumed by Turkish people. It is a sour-soft beverage which is red in colour. Therefore, this study aimed to determine the cytotoxic, antimicrobial and DNA breaking activity of Salgam. Here, the cytotoxic effect of Salgam was studied by MTT assay using K562 (human bone marrow cells) cell line. Genotoxic effect of Salgam was studied by testing the effect of the substance on super coiled double helix DNA. In addition, antibacterial effects of Salgam were investigated using *Klebsiella pneumoniae* and *Staphylococcus aureus* strains.

K562 cells were treated with Salgam concentrations of 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10% for 24 h, after that cytotoxic effect of Salgam was studied by MTT test. Three methods were used to determine the antibacterial effect of Salgam; Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Disc Diffusion Assay. DNA damaging effect of four concentrations of Salgam against pET22b circular DNA also investigated.

Salgam inhibited proliferation of K526 cells at highest concentration for 24 h treatment period. It had no effect on *Staphylococcus aureus* and *Klebsiella pneumoniae* and the result of pET22 plasmid DNA breaking analysis revealed that Salgam did not affect the pET22b. Therefore, it concluded that Salgam can be considered as a safe beverage for human cells and bacterial flora.

Keywords: Antibacterial effect, Genotoxicity, Minimum bactericidal concentration (MBC), Minimum inhibitory concentration (MIC)

Fermented herbal products are prospective nutrients in scientists view due to lactic acid that is created from fermentation^{1,2}. Fermentation changes the flavor, nutritional value, and shelf life of drinks and foods³. Ayran, kanji, kefir, tarhana, koumiss and Salgam are some of the fermented products that are produced by around the world⁴.

Salgam is one of the traditionally fermented beverages that produced and consumed in Turkey. Salgam is a reddish and sour-soft beverage. It is made of the lactic acid fermentation of black carrot (*Daucus carota ssp.*), sourdough, turnip (*Brassica rapa L.*) salt, bulgur flour, and water. Black carrot (*Daucus carota ssp.*) plays an important role in lactic acid fermentation. The reddish color of this beverage is from black carrot which contains anthocyanin pigments³⁻⁵. There is no standard production technique for Salgam as the procedures and fermentation period vary from one method to another. The Salgam contains *Lactobacillus buchneri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*,

Lactobacillus brevis and *Pediococcus pentosaceus*. Along with bacterial strains, yeast (*Saccharomyces*) plays important role in fermentation of juice but in lower levels⁵. The fermentable sugars of black carrot and turnip are sucrose, glucose and fructose^{6,7}. The fermentation products of Salgam are acetic acid, lactic acid, ethanol (in lower level) volatile acids, lactones, esters, some carbonyl compound and volatile phenol. Shelf life of Salgam is 3 months (4°C). The shelf life of this juice can be extended up to 1-2 years adding some preservatives or using pasteurization.

A review of literature indicates that there is a paucity of research regarding the antibacterial, cytotoxic and genotoxic effects of Salgam. The frequent use of Salgam considered as the major role of DNA damages and other diseases, the aim of this study was to specify the effects of Salgam on DNA breaking. This study also investigated the antibacterial effects of Salgam against *Klebsiella pneumoniae* and *Staphylococcus aureus*. In addition, the cytotoxic effect of Salgam on K526 cells was studied by MTT assay.

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Material and Methods

Salgam juice preparation

Traditional and manufacturing techniques are two methods for preparing Salgam. The traditional technique which was presented by Erten *et al.* 2008 was used with some differentiation in this study. The prepared Salgam had two fermentation stages including fermentation of sourdough (first fermentation) and fermentation of black carrot (second fermentation). Here, fermentation of sourdough is performed by adding 0.4 g sourdough, 2 g sugar, and 10 g pea flour to five liters of water and incubating at room temperature for overnight. After this period, 7.5 g Salt, 1 kg black carrot, 5 g *Echium amoenum* and 200 g turnip were added to the mixture for the second fermentation. The mixture was incubated at room temperature for two weeks. After this time, a red radish colored Salgam juice was obtained by filtration. For sterilization of the substance, Salgam juice was filtered through a 0.2 µM sterilizing filter.

Assessment of cytotoxicity

MTT test is used as a cytotoxicity analysis persecute in toxicology. For the aim of this study, MTT assay was performed according to the method introduced by Mosmann (1983). To conduct this test, K526 cell line was used. The cells were cultured into 96 well microplates at 37°C in humidified 5 % CO₂. After 24 h, culture medium was replaced by 90 µL of RPMI1640 combined with six different concentrations of 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10% Salgam, each concentration was repeated eight times. The cells incubation continued for 24 h at 37°C. Afterward, ten microliters of 5 mg/mL MTT solution, prepared in phosphate buffered saline (PBS, pH=7.4), was added to each sample and the cells were incubated for five h. Next, unreacted dye was removed. The insoluble formazan crystals were dissolved in 200 µL well Dimethyl sulfoxide and measured spectrophotometrically in Medispec Esr-200 spectrophotometer at 570 nm. The cytotoxic effect of Salgam (%) was calculated using the following equation:

$$(A_{570nm}(\text{Salgam})/A_{570nm}(\text{untreated control}) \times 100)$$

Statistical analysis was executed using Student's *t*-test. In this research, statistical tests were performed using Minitab software.

Analysis of DNA damaging activity

The major cause of cancer and cell death is DNA damages⁸. Plasmid is a DNA with a circular double strand form which is separated in some bacterial cells. Plasmids can appear in three forms in bacteria. The first form is a covalently closed circular (CCC) form that is the most prevalent of all. The second one is the open circular (OC) form which is created by breaking one of DNA strands. The last form of the plasmid is a linear form made by breaking the double strands DNA. The covalently closed circular (CCC) DNA molecules migrate so faster than open circular (OC). The linear form of plasmid DNA migrates so faster than OC form of DNA (Norizadeh Tazehkand^{9,10}). In this research, DNA breaking effect of Salgam was investigated on pET22b plasmid DNA. DNA damaging effect was analyzed using four concentrations of Salgam. Concentrations of Salgam were selected by the diluting method (1:1- 2:1- 3:1- 4:1- pET22b to Salgam concentration). The experiments were performed in a 50 µL micro tube containing 6 µL pET22b plasmid DNA (150 µg/mL) and 6 µL of Salgam. In this study, untreated pET22b DNA was used as negative control. Then, the plasmids were treated with the test substance and incubated at room temperature for 30 min. Afterward, 2 µL of loading dye was added to test tubes, and the reactions were loaded in 1% agarose gel. So, DNA electrophoresis was performed in 120 volts for 45 min. The gel was stained in Ethidium bromide and photographed with a gel doc system¹¹.

Antibacterial effect study

The antibacterial effect of Salgam juice was studied on two clinical bacterial strains (*Staphylococcus aureus* and *Klebsiella pneumoniae*). In this study three methods were used to determine antibacterial effect of Salgam; Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and disc diffusion assay¹².

Minimum inhibitory concentration (MIC) is the minimum dose of the substance which prevents the microbial growth. Minimum Inhibitory Concentration (MIC) of Salgam were assayed by serial dilution method. The Salgam juice was diluted in concentrations between 1.95 to 750 µL /mL (1:10 to 1:1). Different concentrations of Salgam juice were added to different tubes with 4 mL of Mueller Hinton broth medium. A microbial suspension of 0.5 McFarland was prepared from *Staphylococcus aureus* and *Klebsiella pneumoniae*. The bacterial strains were

cultured in Mueller Hinton broth containing different Salgam concentrations^{13,14}. Then, the samples were incubated at 37°C for 24 h. The Minimum inhibitory concentrations (MIC) value was identified as the lowest concentration at which no visible microbial growth was recorded. Determination of minimum bacteriocidal concentration was performed using inoculation of 50 µL of each dilution with no conspicuous growth in MIC in Mueller-Hinton agar. Afterward, the samples were incubated 37°C for 24 h. One possible result could be the presence of bacterial colonies which is considered as an evidence of bacteriostatic activity. However, the other result could be the absence of bacterial colonies which demonstrates the bactericidal action of the test substance². For disc diffusion analysis, 150 µL of microbial suspension of 0.5 McFarland was spread by glass spreader on a Mueller-Hinton agar in plastic Petridishes. The 6 mm paper was soaked in 10, 15 and 20 µL of Salgam and placed on the bacterial culture then inoculated into Petri dishes at 37°C for 24 h. Eventually, the diameters of inhibition zones were measured¹⁵.

Result

The effects of salgam on K526 cell proliferation

In order to do MTT assay, the cells were treated with different concentrations of Salgam (0.3125%, 0.625%, 1.25%, 2.5%, 5%, and 10%) and incubated for 24 h. After that, the cytotoxic effect of Salgam was assayed by spectrophotometer (570 nm). The cytotoxic effect of Salgam on K526 cell line is depicted in (Table 1). In this research, the 24 h treatment of K526 cell line by Salgam in concentrations of 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10% indicated a decrease in cell proliferation up to -2.66%, -0.06%, 5.13%, 7.73%, 3.70%, and 7.21%, respectively. Only the highest Salgam concentration significantly inhibited cell proliferation when it was compared with untreated samples. In the positive control group that was treated with NaOCl, the decrement was 60.88%.

The genotoxic effects of Salgam on pET22b

The electrophoretic pattern of pET22b DNA indicated two bands on 1% agarose gel electrophoresis on a control sample namely, open supercoil and covalently closed circular DNA. pET22b plasmid DNA was treated with four doses (1:1-2:1-3:1-4:1- pET22b to Salgam concentration) of Salgam which showed two bands similar to the

negative control comprising covalently closed circular and open supercoil plasmid DNA. Therefore, the Salgam did not break the pET22b plasmid DNA (Fig. 1).

Antibacterial effect of Salgam

The minimum inhibitory concentration value and the minimum bactericidal concentration value of Salgam on *Staphylococcus aureus* (Gram-positive) and *Klebsiella pneumoniae* are shown in (Figs. 2 & 3). The minimum inhibitory concentrations of Salgam against *Klebsiella pneumoniae* and *Staphylococcus aureus* were 250 and 500 µL/mL, respectively. The MBC value of Salgam against *Klebsiella pneumoniae* was 500 µL/mL and against *Staphylococcus aureus* was

Table 1 — Cytotoxic effect of Salgam in K526 cell line

Test Substance	Treatment	
	Conc. (%)	Mean value (%) ± SE
Control	--	0 ± 1.81 b ₃
Positive Control (NaOCl)	0.1 mg/mL	60.88 ± 4.85 a ₃
Salgam	0.3125	-2.66 ± 3.44 b ₃
	0.625	-0.6 ± 2.60 b ₃
	1.25	5.13 ± 3.29 b ₃
	2.5	7.73 ± 3.29 b ₃
	5	3.70 ± 2.91 b ₃
	10	7.21 ± 2.01 a ₁ b ₃

Data are expressed as the mean values (±SE) obtained from 8 repeat; (n=8). A: significant from untreated control; a₁b₁: P< 0.05; a₂b₂: P< 0.01; a₃b₃: P< 0.001

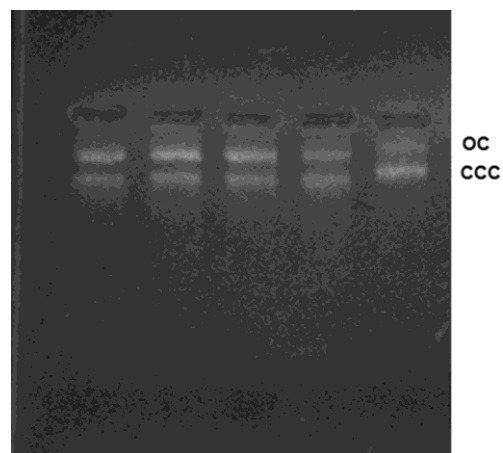


Fig. 1 — DNA Gel Electrophoresis of pET22b after treatment with Salgam. Lane 1: pET22b DNA (untreated control); Lane 2: pET22b DNA treated with Salgam (4:1); Lane 3: pET22b DNA treated with Salgam (3:1); Lane 4: pET22b DNA treated with Salgam (2:1); and Lane 5: pET22b DNA treated with Salgam (1:1). (OC= open supercoil DNA, CCC= covalently closed circular DNA)

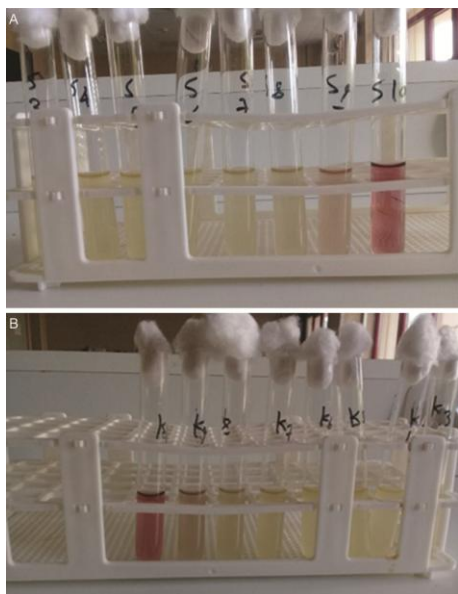


Fig. 2 — (A) The MIC result of Salgam against *Staphylococcus aureus*; & (B) *Klebsiella pneumoniae*

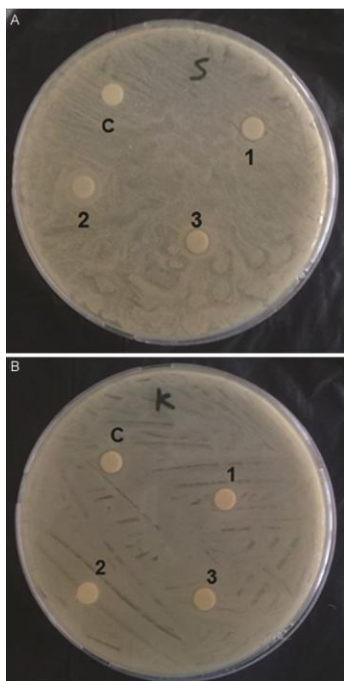


Fig. 3 — (A) The disc diffusion assay result of Salgam against *Staphylococcus aureus*; (B) *Klebsiella pneumoniae*

750 $\mu\text{L}/\text{mL}$. The MIC and MBC values of Salgam were different on the 2 g negative and gram positive bacterial strains.

In disc diffusion assay, the 6 mm paper was soaked in 10, 15 and 20 μL of Salgam and placed on the *Staphylococcus aureus* (Gram-positive) and *Klebsiella pneumoniae* culture, Then, inoculated in

Petri dishes at 37°C for 24 h. The diameter of inhibition zone for the two bacterial strains at 10, 15 and 20 μL of Salgam concentration was zero mm. The result of disc diffusion analysis showed that Salgam did not inhibit the growth of *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Discussion

To our knowledge, this research is the first study that describes the potential Cytotoxic, genotoxic, and antibacterial effects of Salgam on K526 Cell, pET22b plasmid DNA, and two bacterial strains (*Staphylococcus aureus* and *Klebsiella pneumoniae*). Short term genotoxicity tests are used to study carcinogenic agents¹⁶. In our study, we investigated the effect of Salgam on pET22 DNA. The result obtained from this research showed that the prepared Salgam did not have genotoxic activity on the pET22b plasmid. DNA gel electrophoresis of pET22b treated with Salgam at all concentrations showed two bands, untreated control comprised of open supercoil (OC) and supercoil DNA (CCC).

This research revealed that Salgam has no cytotoxic effect on K526 cells. Food colorings, natural or synthetic, are one of food additives that we used in food industry. In addition, Salgam contains anthocyanin which does not have a cytotoxic effect on K526 cells. There are some drinks that contain other food colorings which are proved to have cytotoxic or genotoxic effects. Some of soft drinks which use caramel coloring contain 4-Methylimidazole¹⁷. Cola drinks may contain 100 μg of this compound per 12 ounces serving¹⁸. National Toxicology Program reported the carcinogenic effect of 4-Methylimidazole in animal studies¹⁹. Tazehkand *et al.* reported that 4-MEI has genotoxic and cytotoxic effect on different cells²⁰. The azo dye is generally used in food industry²¹. Abe and Sasaki claimed that azo dyes induced chromosomal aberrations in Chinese hamster ovary cells²². Another popular food coloring is Tartrazine which is a synthetic lemon yellow color, in an article about genotoxic effect of Tartrazine, Patterson and Butler reported that Tatrazine induced chromosomal aberrations in lymphocytes of *Muntiacus muntjac*²³.

The natural antimicrobial agents have a significant role not only in food protection but also in the treatment and control of human illness²⁴. In this research, the MIC of Salgam against *Klebsiella pneumoniae* was 250 $\mu\text{L}/\text{mL}$ and for *Staphylococcus*

aureus was 500 µL/mL. Also, the result of this research indicated that the MBC of Salgam for *Klebsiella pneumoniae* was 500 µL/mL and for *Staphylococcus aureus* was 750 µL/mL. The result obtained from disc diffusion assay showed that Salgam did not inhibit the growth of *Staphylococcus aureus* and *Klebsiella pneumoniae* because the diameter of inhibition zone for the two bacterial strains at 10, 15 and 20 µL concentrations was zero mm. According to the findings of our research, it can be said that Salgam does not have an antibacterial and genotoxic effect on DNA and the two bacterial strains. In contrast to Salgam, a number of research concerning the antibacterial activity and genotoxicity of some other drinks and beverages have been published. For example, Rayes investigated the antibacterial effect of Cola flavored soft drink on microbial strains. The result revealed that Cola soft drink has antimicrobial activity against *Bifidobacterium bifidum* and *Lactobacillus acidophilus* and has harmful effects on the normal bacterial flora of the gastrointestinal tract²⁵. Caramel is a major substance in Cola drinks that has shown mutagenic effect on *Salmonella typhimurium* TA 100²⁶. Dag and et al. claimed that Cola has antimicrobial activity on *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Yersinia enterocolitica* bacteria and *Salmonella enteritidis*²⁷. In another research, Lemon balm beverages and heat denatured beverages were proved to have antibacterial effects against 9 type of bacterial strains²⁸.

On the other hand, 4-methyl imidazole is a food dye that is used in some of the beverages such as Cola soft drinks. Some researchers claimed genotoxic, cytotoxic, anti-proliferative and antioxidant effect of 4-MEI in different test systems^{9,17,20}. Some of the beverages did not have the antimicrobial and genotoxic effect on different bacterial cells. Thai Fermented Plant Beverage is a type of beverage made from fruit, cereals and vegetables. The study of Kantachote *et al.*²⁹ showed that fermented plant beverage did not have antibacterial activity on *Escherichia coli* and *Salmonella sp.* On the other hand, results obtained by Cabrera *et al.* who studied the effect of pomegranate juice on *Staphylococcus epidermidis* revealed that pomegranate juice did not have an antimicrobial effect on *Staphylococcus epidermidis*³⁰.

Conclusion

Salgam inhibited proliferation of K526 cells only at the highest concentration in the 24 h treatment period.

Salgam does not have an antibacterial effect on *Staphylococcus aureus* and *Klebsiella pneumoniae*. In addition, the result of pET22 plasmid DNA breaking analysis revealed that Salgam does not have any effect on pET22b breaking. Moreover, the result obtained from this research indicated that Salgam does not have cytotoxic, genotoxic and antibacterial activity in three test systems. It can be concluded that Salgam might not be a potential risk for human cells and bacterial flora. However, more studies are needed to be done about it.

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