Cytotoxicity of lipopolysaccharide extracted from *Salmonella enterica serovar Typhimurium* on Breast Cancer Cell Line mcf-7

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Abstract

Lipopolysaccharide was selected to investigate its effect on cell line of breast cancer *in vitro*, therefore lipopolysaccharide was extract from *Salmonella enterica serovar Typhimurium* followed by evaluation of its cytotoxicity. The experiment was divided into three parts. The first part included isolation and identification of *S. enterica serovar Typhimurium* from 302 samples included 201diarrheic patient samples and 101 food samples of poultry meat and as well as the isolates identification on molecular level through used of three genes (*invA*, *STM4497* and *Stn*) genes detected using the technique of PCR, the results shown there were seven bacterial isolates diagnosed as *S. enterica serovar Typhimurium*. The second part of the experiment was extract of LPS from 10.66 g of dry weight of bacteria by hot phenol method. The concentration of LPS in phenolic phase was 435.57µg/ml while in aqueous phase was 381.76 µg/ml. The LPS molecular weight was estimate which was equivalent to 93325 for phenolic and 71614 Dalton for aqueous phases. The third part of experiment was to study the effect of cytotoxicity in cell line of breast cancer by used different concentrations of lipopolysaccharide. Lipopolysaccharide showed cytotoxicity on the breast cancer cell line mcf-7 in both phenolic and aqueous phases of LPS. The results showed the highest rate of inhibition of breast cancer cells at the concentration of 350 µg/ml by 61.67% in the phenolic phase while the rate of inhibition was 53.33% in the same concentration of the aqueous phase.

Keywords: *Lipopolysaccharide, S. enterica serovar Typhimurium, MCF-7cell line, cytotoxicity.*

Introduction

Lipopolysaccharides its endotoxins responsible for many of the biological properties of gram negative bacteria particularly (phosphoglycolipid, lipidA) the lipid component[1]. These toxins are the most importance among virulence factors for that bacteria and causing the diseases in both animals and human [2]. There are especially effects for lipopolysaccharide, including lethal toxicity, low blood pressure, fever, and leukocytosis, which has a role in activation of leukocytes and platelets aggregation [3].

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The cancer is one of the highest causes of disease and death in the world [4]. Breast cancer is one of the most common types among women in the world, as it is considered the main cause of the death of women in 15%. Breast cancer accounts for 26% of all cancer cases diagnosed among women worldwide [5].

Some bacterial properties have mechanisms that stimulate the immune system to enhance defenses against malignant tumors [6]. Among the most important of these characteristics is lipopolysaccharide, which is the main component of the outer membrane of the cell wall of the Gram-negative bacteria, that has an assistant effect in stimulating the immune response [7].

The LPS compound have cytotoxic effect through its effectiveness of immune cells which are believed to play an important role in the cytotoxicity of gram negative bacteria through production of biologically

active molecules for example:free radicals mediators, prostaglandins, and pro-inflammatory cytokines. So, the LPS compound is not a toxic molecule in itself [8].

Lipopolysaccharide increases the resistance of bacterial and viral infections by enhancing of B cell response and stimulates interferon production by T lymphocytes [9]. Some researchers have shown that can increased the migration of human esophageal cancer through stimulation of TLR-4 by LPS [10], and it's can act important role as a catalyst enabled the treatment of glibolastoma multiforme and colorectal cancer.[11]

Because the modicum of available studies in the use of LPS extracted from bacteria in the inhibition and killing of cancer cell line, the study aimed to extract and partially purification the LPS compound from selected isolate of *Salmonella enterica serovar Typhimurium.* Cytotoxicity effect of lipopolysaccharide was determined against breast cancer cell line mcf-7.

Materials and Methods

Bacterial isolation:

A total of 302 samples included 201diarrheic patient samples were collected from of patients at the Fallujah educational hospital and 101 food samples included poultry meat collected from local markets in Fallujah city, and the consulting office at the college of veterinary medicine in university of Fallujah. The specimens were directly inoculated onto MacConkey and Samonella Shigella agar (oxoid) and were incubated at 37C for 24 hours.

Identification of *S. enterica serovar Typhimurium* by PCR assay:

Identification of *S. enterica serovar Typhimurium* was done by using of PCR assay with three primers. The primers were used to amplify the following genes:

1. 617 bp *Stn* gene

F-5' TTGTCTCGCTATCACCC 3'

R-5' ATTCGTAACCCGCTCCTGTCC3' [12]

The amplification was done by using 20 µl of reaction mixture with Taq DNA polymerase 1 U/ 20 µl, dNTP mix 250 µM, Primer F 1 µl, Primer R 1µl, MgCl2 1.5µl and Genomic DNA 3 µl. The program amplification of thermal cycler was performed at initial denaturation 94ºC for 1min followed by 35 cycle of 94ºC for 1 min, 59ºC for 1 min, 72ºC for 1 min and final extension 72ºC for 10 min. Then amplified DNA fragments were examined by utilizing electrophoresis in agarose gel (1.5%). Gels have been stained with ethidium bromide and were photographed by using gel documentation system with UV light.

2. 523 bp of *STM4497* gene

F-5'GGAATCAATGCCCGCCAATG 3'

R5'CGTGCTTGAATACCGCCTGTC3' [13]

The amplification was carried out using 20 µl reaction mixture containing from Taq DNA polymerase 1 U/ 20 µl, dNTP mix 250 µM, Primer F 1 µl, Primer R 1µl, MgCl2 1.5µl and Genomic DNA 3 µl. Amplification was performed in a programmed thermal cycler were initial denaturation 94ºC for 5min followed by 35 cycle of 94ºC for 1 min, 68ºC for 2min, 72ºC for 2min, final extension 72ºC for 10 min.

3. 211bp of *invA* gene.

F5'ATCAGTACCAGTCGTCTTATCTGAT 3'

R5'TCTGTTTACCGGGCATACCAT3' [13]

The amplification was carried out using 20 µl reaction mixture containing Taq DNA polymerase 1 U/ 20 µl, dNTP mix 250 µM, Primer F 1 µl, Primer R 01µl, MgCl2 1.5µl and Genomic DNA 3 µl. Amplification was performed in a programmed thermal cycler at initial denaturation 94ºC for 5min followed by 35 cycle of 94ºC for 1 min, 60ºC for 2min, 72ºC for 2min and final extension 72ºC for 10 min.

Antibiotics susceptibility:

Antimicrobial susceptibility tests using method of [14] by the disc diffusion technique on Muller Hinton agar . The zone diameter of each isolate was compared with National Committee of Clinical Laboratory Standards (NCCLS)[15]. Results were recorded as susceptible, intermediate susceptible or resistant, based on the inhibition zone size of each antimicrobial disc used.

Extraction of lipopolysaccharide:

The selected bacterial isolate was cultured under aerobic condition on S.S. and MacConkey agar (Oxoid) and incubated for 24 hours at 37°C . The isolate was harvested using spreader with phosphate buffer saline pH 7 and then washed twice by same buffer. The cells were precipitated using cooling centrifuge at 3000 rpm/min at 4ºC for 15 min . The pellet of cells was resuspended in PBS buffer and centrifuged again for 10 min. the cells were dried by cooled acetone (1:10) in ratio [16].

Destruction of bacterial cells:

Depending on the method of [17], destruction of bacterial cells was done by using enzymes as follows: The dried cells were suspended in PBS pH 7 (0.05M EDTA and 0.05 sodium azide) with ratio 1:10. The lysozyme enzyme was added with ratio 0.1 mg / g from weight of bacteria and the suspension was put in the magnetic stirrer in refrigerator for 18 hours, then the suspension was incubated in water bath at 37°C for 20 min and place the suspension in the magnetic stirrer for 3 min. The volume of the strand was then diluted by adding an equal volume of magnesium chloride solution (0.02M), then add DNAase and RNAase enzymes with a final concentration of 1 mg / ml. Finally, the suspension was incubated at 37°C for 10 min and then incubated at 60°C for 10 min.

Extraction of lipopolysaccharide by phenol:

The suspension of bacteria was preheated at 70°C using a water bath and add 90% of phenol solution in equal volume, previously heated at 70°C [18], then the mixture was placed in the magnetic stirrer with a heating unit at 70°C for 15 min. The mixture was put directly in a snow bath to the temperature of 20°C and then centrifuged in capacity of 18000 g and 3000 rpm for 15 min. After the centrifugation, the separation of four phases from top to bottom were observed as follows: aqueous phase, interphase, phenolic phase and sediment. The aqueous and phenolic phases were separated with a Pasteur pipette (both on one side) and then re-extract the remainder by adding three volumes of distilled water and placing the mixture in the magnetic stirrer for 5 min. then the mixture was centrifuged at the same speed above and separate the floating liquid and add to the aqueous phase. Finally, the phases were dialyzed against D.W. for several times and several days to remove phenol's odor. Partial purification by gel filtration Sephacryl s 200: One hundred and one milliliters of Sephacryl s-200 gel (Pharmacia) were washed with D.W., then were washed by phosphate buffer saline pH 7.2, degassed under vacuum. Subsequently the suspension was poured into a glass column (1.5× 90cm) and allowed the matrix to settle down. The gel was equilibrated with PBS pH 7.2 with flow rate (4ml/7min). LPS sample were added to the column and washed with PBS buffer, the fractions were collected and the absorbance at 280 nm was measured for detecting of contaminating proteins within fractions [19] at 490 nm to estimate the carbohydrate concentration [20] and measuring the absorbance at 260 nm for detecting the nucleic acids [21]. Proteins were measured [22] at a wavelength of 595nm whereas the molecular weight of LPS was determined according to standard proteins (Pepsin 34.5KD, GTF from Sterpt. pneumonia 58.2KD, Bovine Serum albumin 67KD, Arginine Deaminase 143.548 KD and Catalase 232 KD) which were added to column and the ratio of Ve/V_o was determined to the standard proteins and they were used as molecular weight markers. Blue dextran was also used for the determination of the column void volume (v_o) and Ve/V_o was measured for LPS of Salmonella typhymurium isolate. The logarithm of the molecular weight of each standard protein was plotted to obtain standard curve.

Determination of molecular weight for Lipopolysaccharide:

Determination the molecular weight of lipopolysaccharide was done by gel filtration chromatography using Sphacryl S – 200. Blue dextran 2000 was used to determine the void volume, which is equal to 43 ml. Molecular weight of LPS was estimated for both phenolic and aqueous phases by using standard protein and drawing the relationship between the logarithm of standard protein molecular weight and the recovery volume/ void volume (Ve/Vo).

Cytotoxicity of LPS on cell line of breast cancer mcf-7:

The cytotoxic of lipopolysaccharide on breast cancer cell line (mcf-7) was studied by MTT (3-(4,5-dimethylthiazol-2-yl)[23]. The cells were plated on 96 wells at 37ºC for 24 hrs. at a density of 104 cell per well, then add different concentrations of LPS 0, 100, 150,

250, 350 µg/ ml for both phenolic and aqueous phases. After 72 hours at 37ºC of incubated, the cells viability was determined. The medium was removed by addition of 28 µg/ ml of MTT and the cells were incubated at 37ºC for an one hour and a half, then the MTT solution was removed. The remaining crystals in the wells were dissolved by adding 100 mg/ml of DMSO (Dimethle Sulphoxoide) and after that incubated by a shaking incubator for 15 minutes at 37ºC. The absorption was determined using the ELISA at a wavelength of 492 nm with three repeats per concentration. The inhibition rate was calculated by the following equation:

Inhibition rate = A-B / A* 100

where **A**= control and **B**= density of the cells treated with

LPS.

Results and Discussion

S. enterica serovar Typhimurium used in current study was isolated from 302 samples included 201diarrheic patient samples were collected from patients at the Fallujah educational hospital and 101 food samples included poultry meat collected from local markets in Fallujah city.

The results shown there were 19 (6.29%) isolates of *Salmonella* spp. included 7 (36.84%) isolates diagnosed as *S. enterica serovar Typhimurium* depending on evidences of morphological and biochemical characterizations

Antibiotics sensitivity of *Salmonella* species:

Antibiotics sensitivity for clinical isolates of *Salmonella* was done against eleven antimicrobial agents. The results showed that all isolates were resistance to Rifampin at100% followed by the Doxycycline at 94.74% while the average resistance of Cephalexin, Cefotaxime and Cefixime was 42.1%, 42.1% and 52.63% respectively.

Ciproflaxacin, Aztreonam, Imipenem and Amikacine antibiotics were most effective against *Salmonella* isolates with a sensitivity percentage 94.74%, 94.74%, 100% and 100% respectively (figure 1).

Aslo, the results showed that all *Salmonella* isolates were susceptible to Ciproflaxacin except the isolate S259 which appeared multi-resist against different antibiotics.

Identification of *Salmonella* isolates using PCR assay:

Most ten of *Salmonella* isolates resistance to antibiotics were selected for the diagnosis by PCR assay using of *invA*, *STM4497* and *Stn* genes to detect the strains of *S. enterica serovar Typhimurium*.

An attempt was made to localize the gene responsible for the susceptibility of the *Salmonella* bacteria to tissue invasion.

Accordingly these *invA* gene (211bp) were amplified using specific primers with PCR cycler and optimized specific program. The results showed that all the selected strains contained *invA* gene, the specificity primers were agreement with stated [24, 25].

The results identification of *STM4497* (523bp) gene to detect *S. enterica serovar Typhimurium* showed that seven isolates gave a positive result of the presence. The specificity were reported on earlier study by[(25)], because isolates gave specific bonds to the gene.

Also the gene *Stn* (617bp) was detected, which is encoded for the production of intestinal toxin of *S. enterica serovar Typhimurium* the results shown that three isolates only possessed this gene, which agree with [26], but differed in proportions from the current study.

Also, the [27] diagnosed *S. enterica serovar Typhimurium* by PCR technique using a *fimC* gene.

According to the previous results, The selected organism which used for the extraction and purification of LPS was the most resistant to antimicrobial agents as well as having the three studied genes.

Extraction of lipopolysaccharide

The dry weight of selected *S. enterica serovar Typhimurium* isolate was 10.66 mg as the yield of obtained bacterial growth. The method which used to extracted of LPS by hot phenol was depend on [17]. determination the concentration of the lipopolysaccharide was done by using the standard glucose curve. The concentration of LPS in the phenolic phase was estimated at 435.57 µg / ml, while the concentration in the aqueous phase was

estimated at 381.76 µg/ml.

Because the phenolic phase contains a large amount of lipopolysaccharide also due to the structure of the LPS which containing a large amount of N and O-acetylated-6-deoxyhexose in the side chain O which are hydrophobic bonds, some researchers have used that method and therefore accumulate LPS in the phenolic phase [28]. So, The distribution of LPS in both phenolic and aqueous phases depends on the important factor which is hydrophilic bond due to formation of side bond O [29].

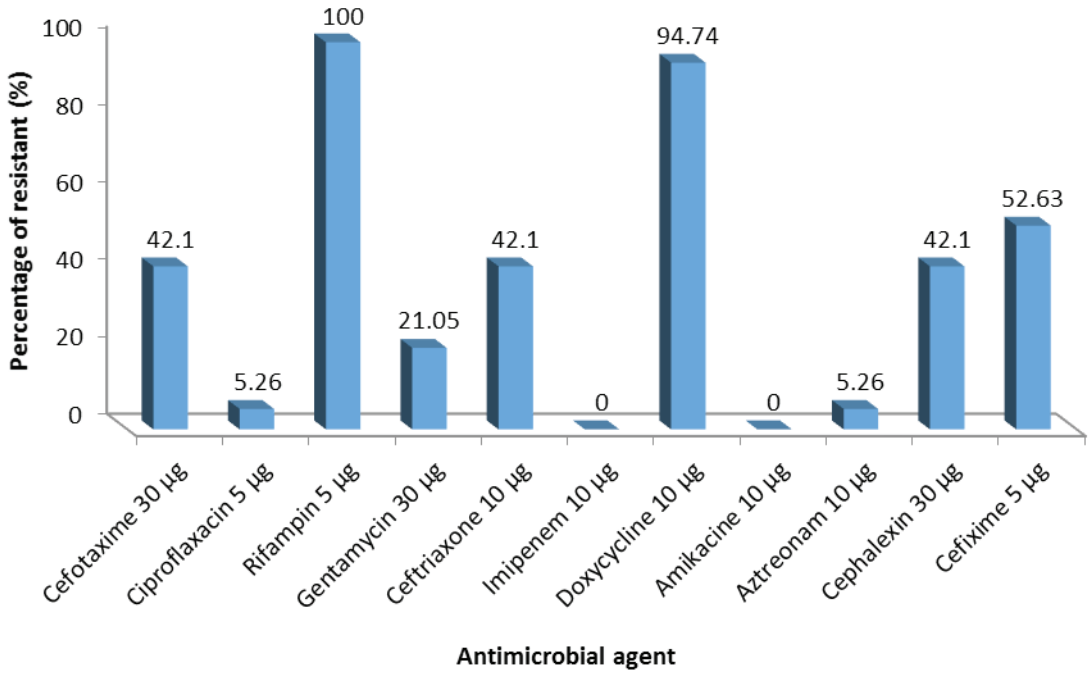

Figure 1: Susceptibility of Salmonella species

Partial purification of lipopolysaccharide:

Partial purification of lipopolysaccharide for both of phenolic and aqueous phases were done by gel filtration chromatography to separate of high molecular weight protein and carbohydrates . The parts were read on the wavelength 600 nm with flow rate was 34 ml / hour and the void volume was equal to 43 ml.

The measuring of protein amount was done by collection of forty-five fractions and assessed for both phenolic and aqueous phases at a wavelength of 289 nm whereas the amount of carbohydrate linked LPS was measured at 490 nm[20] (figures 2 and 3). The results showed that there were two peaks of protein large and small one linked to lipopolysaccharide and difficult to separate whereas phenolic phase there was one peak of carbohydrate . While in aqueous phase there were three peaks of protein: two large and small one linked to the carbohydrate and there was one peak of carbohydrate.

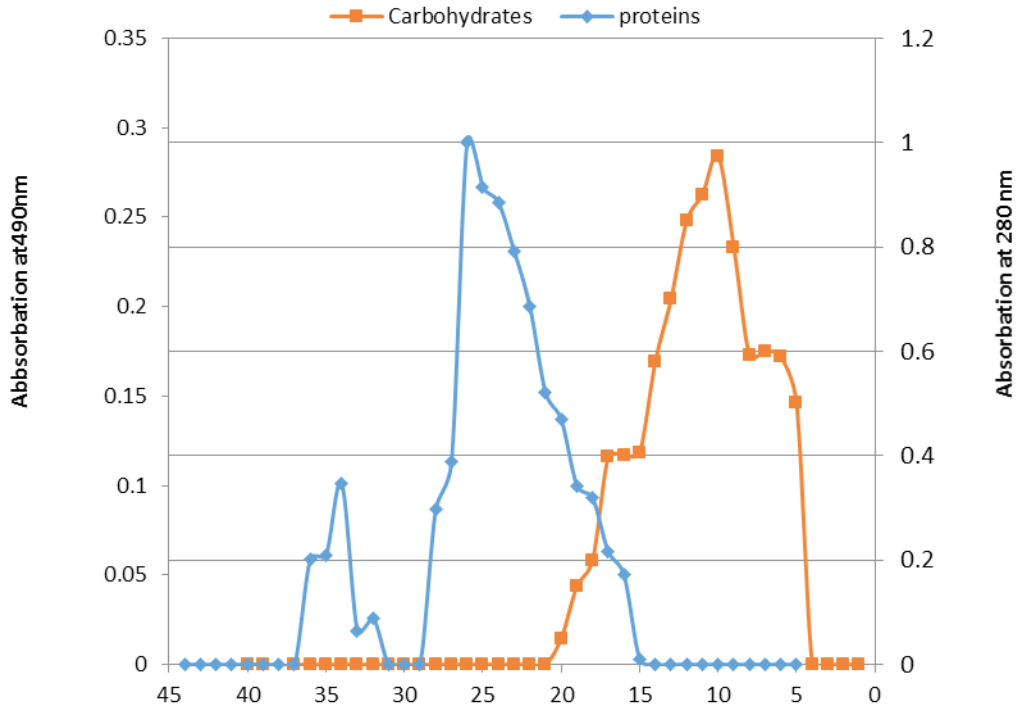

Figure (2): Gel filtration chromatography of *S.typhimurium* (phenolic phase) lipopolysaccharide by using Sphacryl S – 200, the column dimensions was (1.5 x 70 cm) and elution was done with phosphate buffer saline pH 7.2 at flow rate 34 ml/h

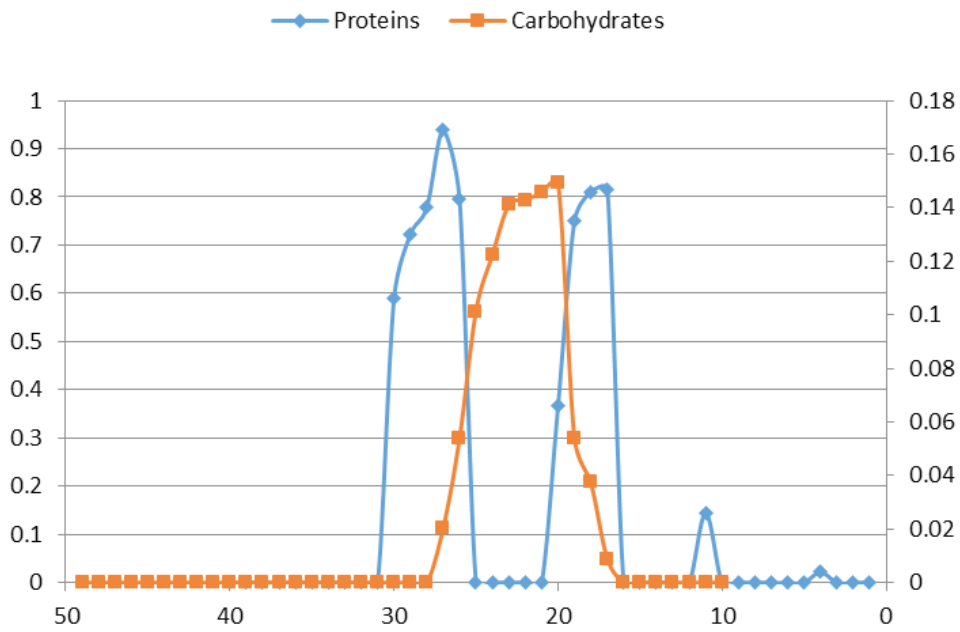

Figure (3): Gel filtration chromatography of *S.typhimurium* (aquatic phase) lipopolysaccharide by using Sphacryl S – 200, the column dimensions was (1.5 x 70 cm) and elution was done with phosphate buffer saline pH 7.2 at flow rate 34 ml/h

Determination of lipopolysaccharide molecular weight:

Results for determinate of lipopolysaccharide molecular weight showed that the molecular weight for LPS in phenolic phase was equal to 93325 Dalton and in aquatic phase was equivalent to 71614 Dalton as shown in figure (4) and table (1). The molecular weight of the lipopolysaccharide is based on its structure, such as its dependence on the oligosaccharide in its molecular weight. In addition, there are two types of oligosaccharid (short or long) [30].

The researcher [31] estimated the molecular weight of Lipopolysaccharide to (70794 Dalton). So, this result was close to the results obtained in this study.

Cytotoxicity of LPS on cell line of breast cancer (mcf-7):

Cytotoxicity of the lipopolysaccharide extracted and partial purified from the local isolate *S. enterica serovar Typhimurium* S259 was tested in an aquatic phase and phenolic phase which was purified for studying its effect on mcf-7 breast cancer cell line (*in vitro*).

The results obtained that there was a noticeable toxic effect for different lipopolysaccharide concentrations in phenolic and aqueous phase in the growth of cancerous breast cells mcf-7. That effect starts from the low concentration to toward high concentrations when compared with the control treatment for each phase.

Also the results shown ability of LPS extract for each phases to reduce the density of developing cancer cells. And observed a difference of cytotoxic effect for lipopolysaccharide between the phenolic phase and its cytotoxic effect of aqueous phase.

The statistical analysis results of lipopolysaccharide effect in the phenolic phase showed that there were significant differences between the groups of treatments and the control group. Where the fourth group, represented by concentration 350 µg/ml, recorded maximum of significant differences which was (1700±250) over the other groups comparison with control group (table 2).

As well as the figure (5) shown that the killing rate of cancerous breast cells was 61.67% at the highest concentration of lipopolysaccharide. The figure (6) also shown a gradual variation in the inhibition rates

of lipopolysaccharide in the phenolic phase, where the inhibition levels in the concentrations 100 µg / ml to 250 µg/ml were as the following: 17%, 30.67%, 46.67%, respectively.

The results also showed significant differences in effect of lipopolysaccharide for both phases on cell line of breast cancer mcf-7, as the results showed the highest significant differences of the phenolic phase than aqueous phase in affectivity on the number and shape of cancer cells line.

The results showed in current study that the cytotoxic effect of lipopolysaccharide was evident in the growth of cancer cells outside the living body during 72 hours of exposure for different concentrations of aqueous and phenolic phases when treated with cancer cell line (mcf-7).

Several studies have accorded to the importance of bacterial toxins in treating various cancer diseases by killing or reducing the growth of cancer cells through changing the cellular processes that control the spread, differentiation and programmed death stages (apoptosis) of living cells [32].

In several studies have been found that lipopolysaccharide has a toxic effect in reducing the density of developing cancer cells, whether in vitro or in vivo through the introduction of the cancerous cell in the stages of programmed death (apoptosis). The researcher [(33)] indicated that the lipopolysaccharide enhance the programmed death of breast cancer cells in humans, which inhibits receptors known as TLRs (Toll-like receptors) that have been found to be linked to the development of breast cancer.

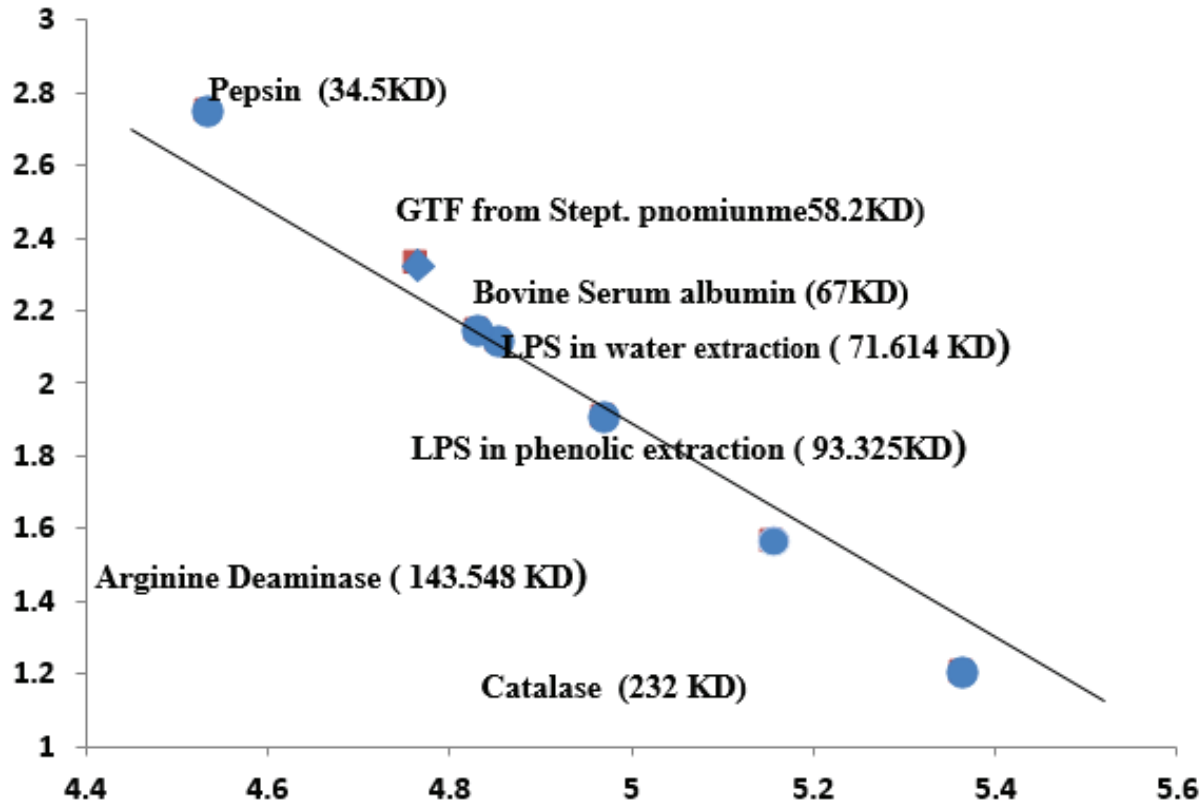

Figure (4): Molecular weight of LPS for both aquatic and phenolic phases by using gel filtration chromatography (Sphacryl S – 200) the column dimensions was (1.5 x 70 cm) and elution was done with phosphate buffer saline pH 7.2 at flow rate 34 ml/h

Table (1): Standard protein and standardization of LPS from *S. enterica serovar Typhimurium* for both aquatic and phenolic phases according to the ratio of Void volume and Elution volume (Ve/Vo) ratio.

Standard protein and purified LPS	Molecular weight (KD)	Ve / Vo
Catalase	232	1.2
Arginine deaminase	143.548	1.58
(GTF)glucotransferas	58.2	2.3255
pepsin	34.5	2.75
LPS in aquatic phase	71.614	2.142
LPS in phenolic phase	93.325	1.904
Bovine serum albumin	67	2.15

Table (2) Effect of lipopolysaccharide in its aqueous and phenolic phases on breast cancer cell line (mcf-7)

Phase	Control	Group 1 100 µg/ml	Group 2	Group 3	Group 4 350 µg/ml
			150 µg/ml	250 µg/ml	
Phenolic	10000+0a	6166+642b	4666+480c	3066+348d	1700+250e
Aqueous	10000+0a	5333+520b	4100+503c	2400+472d	1733+250d

Different letters mean significant differences between groups at a probability level (P <0.05) depending on the X^2 test.

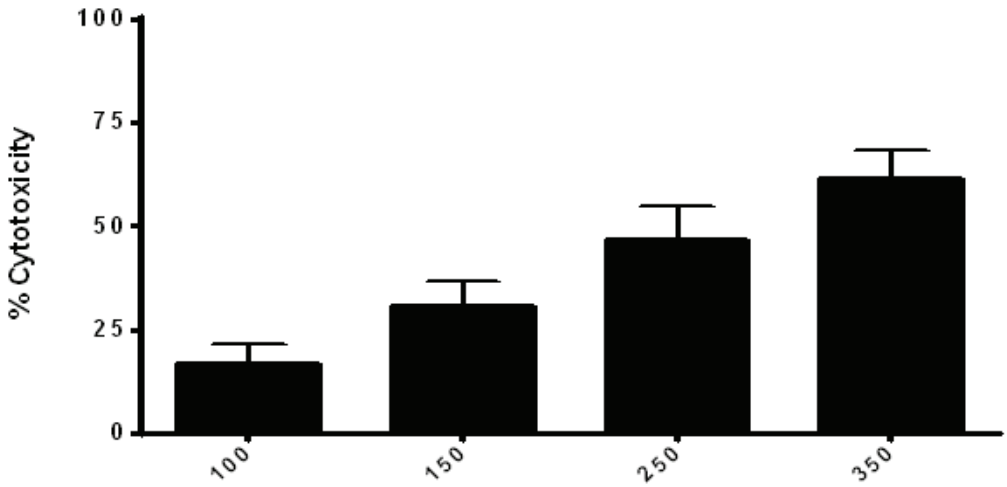

Figure (5): Cytotoxicity of LPS (phenolic phase) in breast cell line (mcf-7)

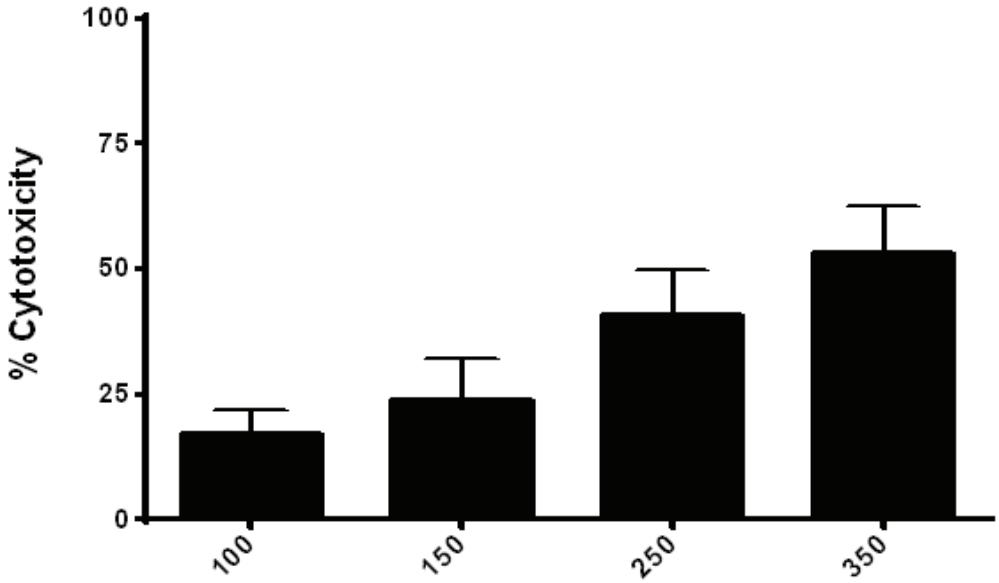

Figure (6): Cytotoxicity of LPS (aquatic phase) in breast cell line (mcf-7)

Also[34] found that exposing the pancreatic cancer cell line to lipopolysaccharide reduced its growth by approximately 50% by stopping the process of DNA replication at the G1 stage during the life cycle of the cell.

Another study indicated that lipopolysaccharide can stimulate killing of cancer cell by enhancing the immune response through stimulating transcription of encoded genes of proteins which responsible for releasing cytokines that are associated with cyclooxygenase-2 cyclooxygenase that have a role in inhibiting cancer cells [35].

The current study were consistent with [36], where he found a cytotoxic effect of lipopolysaccharide in the cell line of cervical cancer when used in high concentrations and the rate of killing of cancer cells were depended on the concentration of LPS.

The study also agreed with [37] who found that a cytotoxic effect of lipopolysaccharide on esophageal cancer and oral cells when used high concentrations of LPS.

The results also were agreement with [38], who analyzed the cytotoxicity of lipopolysaccharides by treating the lung cancer cell line - NCI - H69 with different concentrations, including: 100, 150, 250 µg/ml. His results showed A gradual reduction in the growth density of lung cancer cells. He also found that the lowest density of the cancer cells was 48.88% at a concentration of 250 µg/ml of LPS extract, which was closed result to the present study.

Among the total metabolic processes resulting from the transformation of the normal cell into a cancerous cell is the formation of Free radicals in large quantities [39]. It is believed that the interaction of free radicals with DNA can cause genetic mutations that increase the risk infection of cancer.

Addition to the oxidative stress reduces programmed cell death and increases proliferation, and growth of cancer cells [40]. Therefore, lipopolysaccharide may selectively remove free radicals and thus adversely effect on the growth and proliferation of cancer cells. Also, there are some active factors in cancer cells known as the (nuclear transcription factor) that have a fundamental role in organizing the cell cycle by coding about cytokines and other growth factors which necessary for cell life, as the presence of these factors increases the resistance of the cancer cells to chemotherapy, so inhibiting these factors will lead to an imbalance in increasing the density of the

developing cells and thus entering the cell to the stage of programmed death (apoptosis) and improving the ability to treatment of cancer disease [41].

Also, [42] provided the ability of LPS as potential anticancer agent in breast cancer that ability inducing apoptosis and could be used LPS for wide biomedical applications and could be offer new drug instead of chemotherapy in treatment of various types of cancer disease.

Conclusion

We concluded from this study that LPS have an obvious cytotoxic effect on the line of breast cancer cells in different concentrations Also, we concluded that the LPS of *S. enterica serovar Typhimurium* has cytotoxic properties which can be used in development of drugs for treatment of cancer disease.

Conflict of Interest: Nil.

Funding: Self-funding

Ethical Clearance: The Research Ethical

Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

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