Extraction and purification of Lipopolysaccharide from *Salmonella typhymurium* and its cytotoxicity on cervical cancer cell line (Hela)

Moutab W.Q.[1], Abdul-Rahman Ibrahim A.[2] and Aziz L.M.[3]*
1. College of Education for Pure Science, University of Anbar, IRAQ
2. College of Applied sciences, University of Fallujah, IRAQ
3. College of Veterinary Medicine, University of Fallujah, IRAQ
*Biotechnology_2017@yahoo.com

Abstract

This study included extract of Lipopolysaccharide from Salmonella typhimurium followed by partial purification and evaluation of its cytotoxicity on cervical cancer cell line. Sensitivity test against eleven antimicrobial agents was done for all of the Salmonella isolates as well as the isolates identification on molecular level through used of three genes (invA, STM4497 and Stn) to detect the strains of Salmonella typhimerum. The isolate S259 was selected because it was more resistant to antimicrobial agents as well as having the three studied genes. The LPS was extracted from 10.66 g of dry weight of bacteria by hot phenol method. After partial purification, the concentration of LPS in phenolic phase was 435.57µg/ml while in aqueous phase it was 381.76 µg/ml. Molecular weight of LPS was estimated and it was equivalent to 93325 and 71614 Dalton for phenolic and aqueous phases respectively.

Different concentrations of lipopolysaccharide have been used to study the effect of cytotoxicity in the cervical cancer cell line. LPS was found to exhibit cytotoxicity on the cervical cancer cell line Hela in both phenolic and aqueous phases of LPS. The results showed the highest rate of inhibition of cervical cancer cells at the concentration of 350 µg/ml by 48.30% in the aqueous phase while the rate of inhibition was 58% in the same concentration in the phenolic phase.

Keywords: Lipopolysaccharide, *Salmonella typhimurium*, Hela cell line, cytotoxicity.

Introduction

Lipopolysaccharide is the main component of the outer membrane of Gram-negative bacteria, which forms 75% of the surface[1] and 5-10% of the total dry weight of the bacteria[2]. This component is the most virulence factor, important of these bacteria in causing the disease and septicemia in both human and animal[3]. The toxicity of the LPS is due to lipid A , which is attributed to the effectiveness of LPS biological, where the LPS compound is not a toxic molecule in itself but its toxic effect is through the effectiveness of immune cells which are believed to play an important role in pathophysiology of the toxicity of gram negative bacteria through production of biologically active molecules like: pro-inflammatory cytokines, free radicals mediators and prostaglandins[4].

The presence of lipopolysaccharide molecules in the outer layer of the outer membrane is of great importance in the resistance of bacteria against antibiotics, detergents and solutions in addition to its role in the lethal toxicity, lowering blood pressure and fever, leukocytosis and platelet aggregation[5].

However, some internal toxins act to stimulate the immune response[6]. LPS has been found to have a beneficial effect of enhancing B cell response that increases the resistance of viral and bacterial infections and stimulates interferon production by T lymphocytes, complements and stimulation of interleukin- 1[7].

Some researchers have suggested that lipopolysaccharide is one of the new treatments. It is one of the compounds characteristic of the Gram-negative cell wall[8]. It has been found to be stimulated of TLR-4 with LPS to promotion of migration and the invasion of lung cancer cells[9]. Recent studies have shown that lipopolysaccharide can increase the migration of human esophageal cancer through stimulation of TLR-4 [10]. On the other hand, it was found that the use of lipopolysaccharide as a catalyst enabled the treatment of colorectal cancer in addition to the glibolastoma multiforme[11].

Cancer is a very serious disease and causes mortality in around of the world, so studies are of interest in development of treatments for this disease. Due to the modicum of available studies in the field of the use of LPS extracted from bacteria in the inhibition and killing of cancer cells, the study aimed to extract and partially purification the LPS compound from selected isolate of *Salmonella typhymurium*. Anticancer property of LPS was determined against cervical cancer cell line (Hela).

Material and Methods

Sample collection: Total of 201 diarrheic patient samples were collected from of patients at the Fallujah educational hospital. The specimens were directly inoculated onto S.S and MacConkey agar (oxoid) and were incubated at 37C for 24 hours.

Identification of Salmonella typhimurium by PCR assay: PCR was done for identification of *Salmonella typhimurium* by using three primers. The primers were used to amplify:

1. 211bp of *invA* gene.
F- 5'ATCAGTACCAGTCGTCTTATCTTGAT 3'
R- 5'TCTGTTTACCGGGCATACCAT 3' [12]
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, $MgCl_2$ 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.

2. 523 bp of *STM4497* gene
F- 5' GGAATCAATGCCCGCCAATG 3'
R-5' CGTGCTTGAATACCGCCTGTC 3' [12].
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 01µl, $MgCl_2$ 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. 617 bp *Stn* gene
F-5' TTGTCTCGCTATCACCC 3'
R-5' ATTCGTAACCCGCTCCTGTCC 3'[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, $MgCl_2$ 1.5µl and Genomic DNA 3 µl. Amplification was performed in a programmed thermal cycler 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.

Antibiotics susceptibility: Antimicrobial susceptibility tests of the isolates were determined by the disc diffusion technique on Muller Hinton agar using method of Bauer et al[14]. The bacteria were grown on the Muller-Hinton agar plate and then the antibiotic tablets were placed by sterile forceps. The plate was incubated at 37°C for 18-24 hours. The diameter of each zone of inhibition was measured in millimeters using a meter rule on the underside of the plate. The zone diameter of each isolate was compared with National Committee of Clinical Laboratory Standards (NCCLS) 2010[15]. Results were recorded as susceptible, intermediate susceptible or resistant, based on the zone size of each antimicrobial disc used.

Extraction of lipopolysaccharide
Culturing of bacterial isolates: The selected bacterial isolate was cultured on S.S. agar (Oxoid) and incubated at 37˚C for 24 hours under aerobic condition. The isolate that has grown was harvested by phosphate buffer saline pH 7 using spreader and then washed twice by same buffer. The cells were precipitated using cooling centrifuge at 3000 rpm/min for 15 min at 4ºC. The pellet of cells was re-suspended in PBS buffer and centrifuged again for 10 min. the cells were dried by cooled acetone (1:10) in ratio.

Destruction of bacterial cells: Destruction of bacterial cells was done by using enzymes depending on the method of Johnson et al[16] summarized 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: Depending on the method of Westphal et al[17], the suspension of bacteria was preheated in a water bath at 70°C and add an equal volume of 90% of phenol solution, previously heated at 70°C. then the mixture was placed in the magnetic stirrer with a heating unit at 70°C for a quarter of an hour. 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 minutes.

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[18] at 490 nm to estimate the carbohydrate concentration[19] and measuring the absorbance at 260 nm for detecting the nucleic acids[20].

Proteins were measured[21] 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.

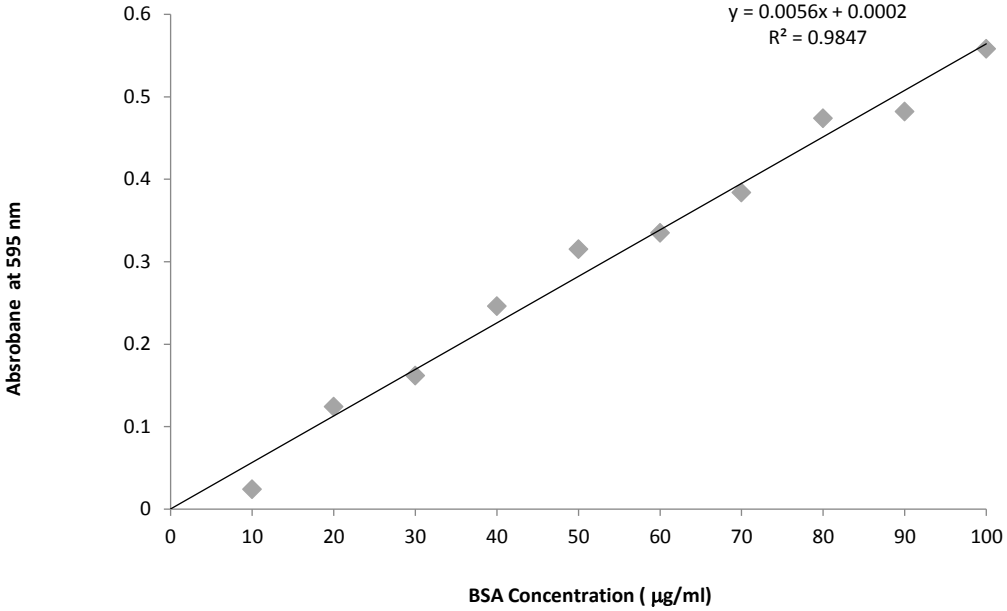

Figure 1: Standard curve of bovine serum albumin

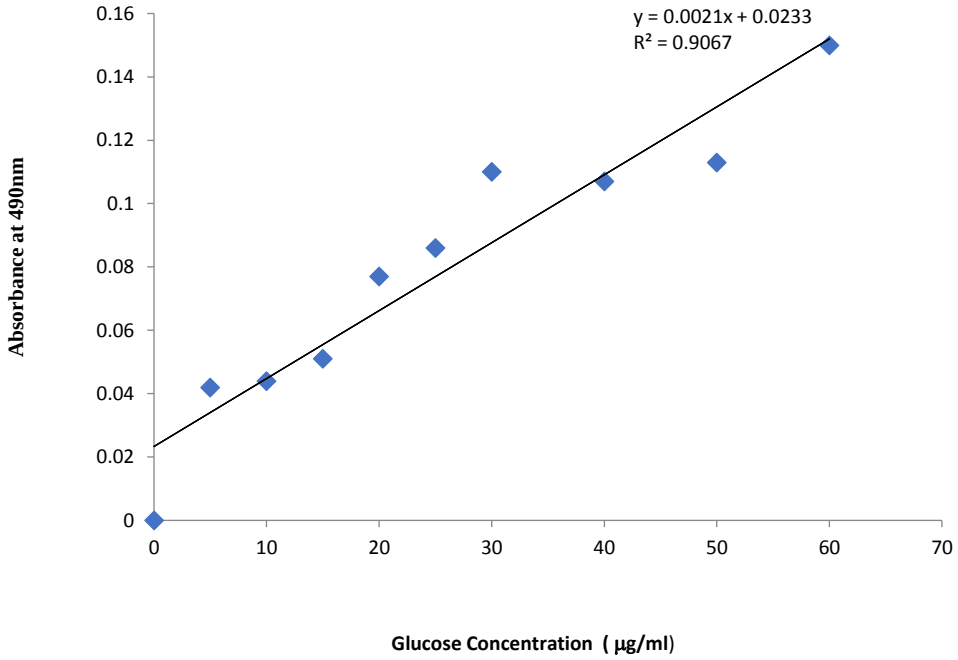

Figure 2: Standard curve of glucose

Determination of molecular weight for Lipopolysaccharide: Molecular weight of lipopolysaccharide was determined by gel filtration chromatography using Sphacryl S – 200. Blue dextran 2000 was used to determine the void volume, which is equal to 43 ml. Determination of molecular weight for 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 cervical cancer cell line (Hela): The cytotoxic of LPS against on cervical cancer cell line (Hela) was studied by MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide)[22]. The cells were plated on 96 wells at 37ºC for 24 hours at a density of 10^4 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 for an hour and a half at 37ºC, 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 according to the following equation:

Inhibition rate = A-B / A* 100

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

Results and Discussion

Salmonella typhimurium used in this study was isolated from 201 stool samples collected from the patients of Al-Fallujah Educational Hospital and the reviewers of the external consultation clinics for both sexes and different ages for males and females. Black colored colonies were obtained on Salmonella Shigella agar. The morphological and biochemical characterization revealed the isolated organism to be *Salmonella* (table 1).

Antibiotics sensitivity of *Salmonella* species: The clinical isolates of *Salmonella* were tested for antimicrobial sensitivity against eleven antimicrobial agents. The resistance of Rifampin was 100%, followed by the Doxycycline resistance at 94.74% while the average resistance of Cefixime, Cefotaxime and Cephalexin was 52.63%, 42.1% and 42.1% respectively.

Most effective antibiotics against *Salmonella* were Amikacine, Imipenem, Aztreonam and Ciproflaxacin with a sensitivity percentage 100, 100, 94.74 and 94.74% respectively as in figure 3. The results showed that all of isolates were sensitive to Ciproflaxacin except the isolate S259 which appeared multi-resist against different antibiotics including Ciproflaxacin, Rifampin, Gentamycin, Ceftriaxone, Doxycycline, Cephalexin and Cefixime. These results guideline depended on[15].

Identification of Salmonella isolates by PCR technique: Ten isolates of Salmonella which were the most resistant antibiotics were selected for the diagnosis using polymerase chain reaction (PCR). The genes (*invA, STM4497* and *Stn*) were used to detect the strains of *Salmonella typhimerum.* The results showed that all the selected strains contained *invA* gene (211bp) as in figure 4. The *invA* gene is responsible for the susceptibility of the *Salmonella* bacteria to tissue invasion[23]. The result was consistent with what was stated[24].

The results of the diagnosis using the gene *STM4497* (523bp) to detect *Salmonella typhimuium* showed that only seven isolates gave a positive result of the presence of this gene as in figure 5. The results were consistent with[24], because isolates gave specific bonds to the gene.

While the result of the diagnosis was the use of *Stn* gene (617bp), showed that only three isolates possessed this gene, which is encoded for the production of intestinal toxin of *S. typhimurium*, as shown in figure 6. The results of the current study agreed with[25] where a *Stn* gene was reported in Salmonella but differed in proportions from the current study.

Selection of *S.typhimurium* isolate: The selected organism was the most resistant to antimicrobial agents as well as having the three studied genes used for the extraction and purification of LPS.

Extraction of lipopolysaccharide: The yield of obtained bacterial growth was 10.66 gm as dry weight of selected *Salmonella typhimurium* isolate. LPS was extracted by hot phenol method[16]. The concentration of the lipopolysaccharide was determined using the standard glucose curve. Its concentration in the aqueous phase was estimated at 381.76 µg/ml while the concentration of LPS in the phenolic phase was estimated at 435.57 µg /ml.

Some researchers have used the phenolic phase because it contains a large amount of lipopolysaccharide and may be 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 and therefore accumulate LPS in the phenolic phase[26]. The distribution of LPS in both aqueous and phenolic phases depends on the important factor which is hydrophilic bond due to formation of side bond O[27].

Partial purification of lipopolysaccharide: Both of aqueous and phenolic phases were partial purified by gel filtration chromatography using Sphacryl S-200 gel which is

separation of high molecular weight protein and complex sugar. The flow rate was 34 ml / hour and the parts were read on the wavelength 600 nm and the void valume was equal to 43 ml. Forty-five fractions were collected and assessed for both aqueous and phenolic phases by measuring the carbohydrate amount[19] at a wavelength of 490 nm whereas the amount of protein linked LPS was measured at 289 nm (figures 7 and 8).

Table 1
Biochemical characterization of local isolates of *Salmonella*

Test Samples	Urease	Indol	MR	VP	Motility	Catalase	Oxidase	H_2S	Gram stain
S4	-	-	+	-	+	+	-	+	-
S 6	-	-	+	-	+	+	-	+	-
S30	-	-	+	-	+	+	-	+	-
S65	-	-	+	-	+	+	-	+	-
S71	-	-	+	-	+	+	-	+	-
S75	-	-	+	-	+	+	-	+	-
S129	-	-	+	-	+	+	-	+	-
S134	-	-	+	-	+	+	-	+	-
S192	-	-	+	-	+	+	-	+	-
S253	-	-	+	-	+	+	-	+	-
S255	-	-	+	-	+	+	-	+	-
S256	-	-	+	-	+	+	-	+	-
S258	-	-	+	-	+	+	-	+	-
S259	-	-	+	-	+	+	-	+	-
S282	-	-	+	-	+	+	-	+	-

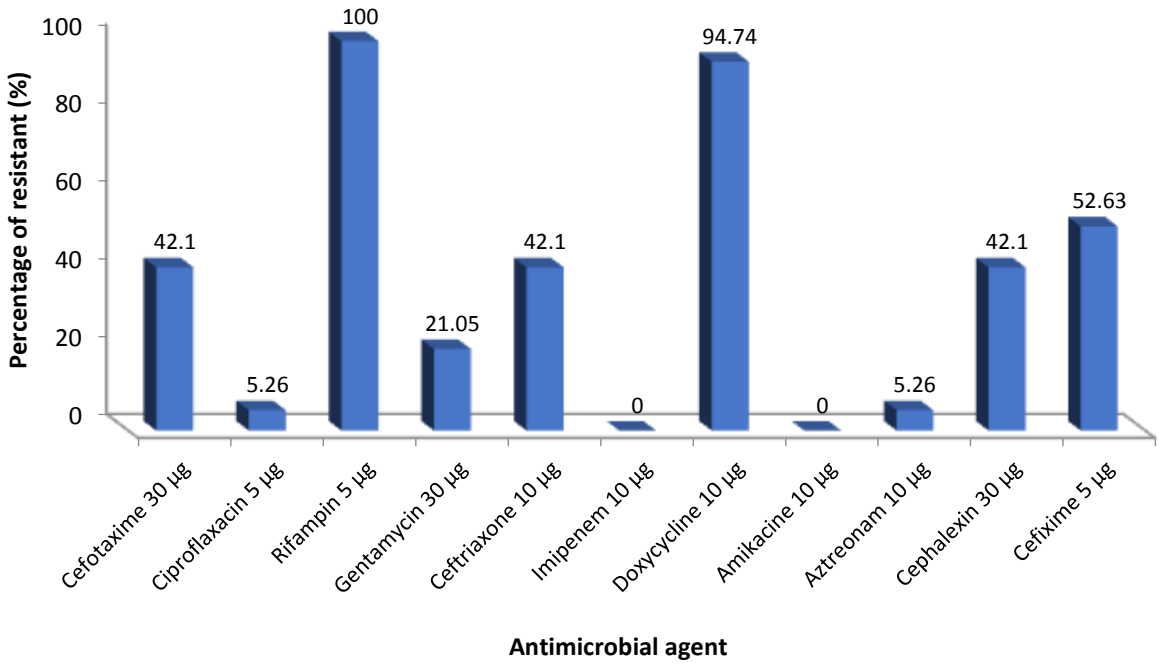

Figure 3: Susceptibility of *Salmonella* species

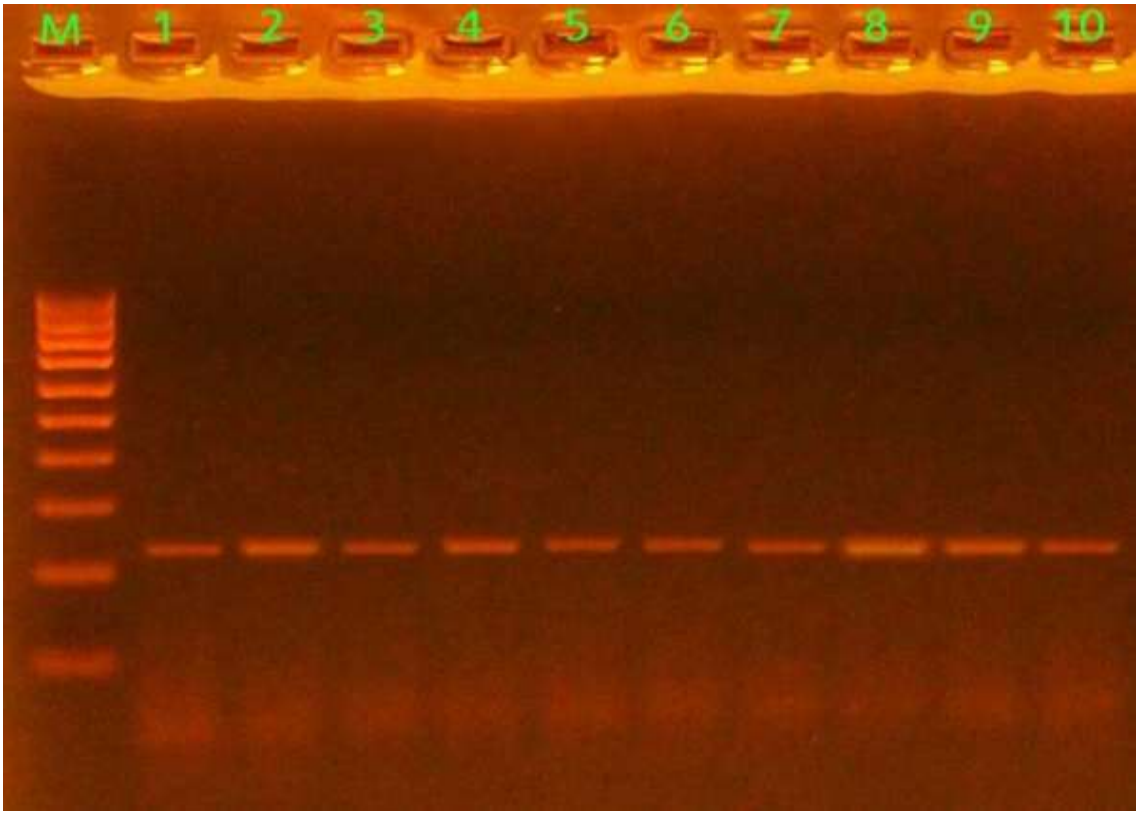

Figure 4: Agarose gel electrophoresis of the PCR products amplified. Lane M: DNA 100-bp ladder marker. Lane 1-10: DNA of *Salmonella* isolates amplified by *invA* gene. Lane 9: S259 isolate. 1.5% agarose gel, 5V/cm at 1.5 hr.

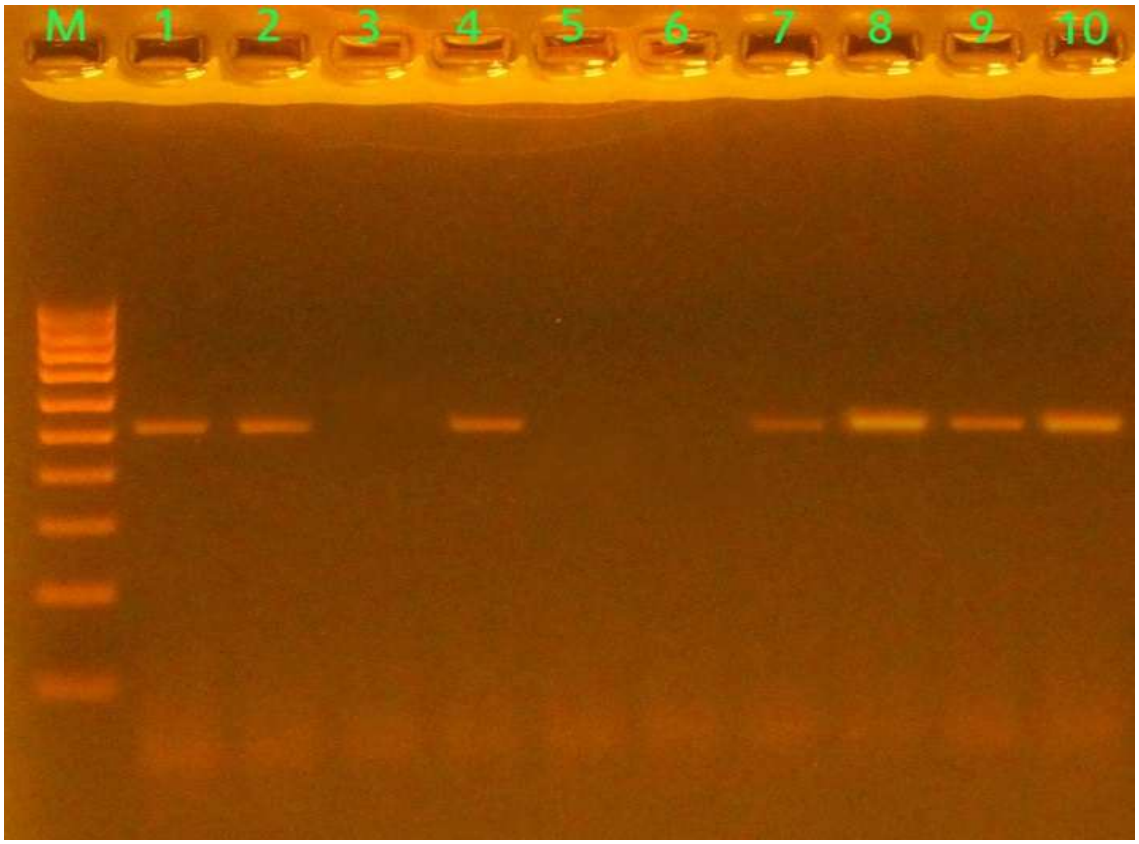

Figure 5: Agarose gel electrophoresis of the PCR products amplified. Lane M: DNA 100-bp ladder marker. Lane 1-10: DNA of *Salmonella* isolates amplified by *STM4497* gene. Lane 9: S259 isolate. 1.5% agarose gel, 5V/cm at 1.5 hr.

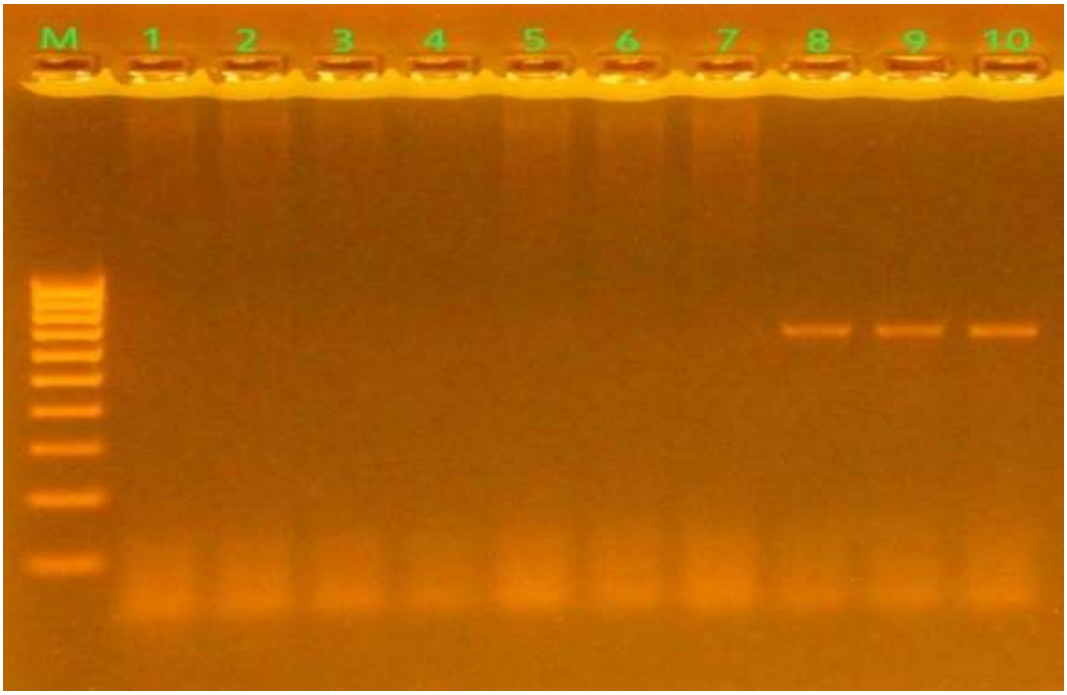

Figure 6: Agarose gel electrophoresis of the PCR products amplified. Lane M: DNA 100-bp ladder marker. Lane 1-10: DNA of *Salmonella* isolates amplified by *Stn*. Lane 9: S259 isolate. gene 1.5% agarose gel, 5V/cm at 1.5 hr.

The results showed that in phenolic phase there was one peak of carbohydrate whereas there were two peaks of protein large and small one linked to lipopolysaccharide and difficult to separate. And in aqueous phase there was one peak of carbohydrate while there were three peaks of protein: two large and small one linked to the carbohydrate. Some researchers have concluded that the extraction and purification of LPS can determine the condition of disease, so the researcher[34] indicated that extraction of purified LPS from E. coli isolates of UTI patients' urine samples can be an important step to understand the UTI disease conditions.

Determination of lipopolysaccharide molecular weight: Determination of LPS Molecular weight was done by gel filtration chromatography using Sphacryl S - 200. Blue dextran 2000 was used to determine the void volume which equals to 43 ml whereas the elution volumes (Ve) to each standard protein were measured. As a result, estimation of molecular weight for LPS in aqueous phase was equivalent to 71614 Dalton while the molecular weight in phenolic phase was equal to 93325 Dalton as shown in figure 9 and table 2. 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 oligosaccharide (long or short)[28].

The results of the Molecular Weight Assessment were close to reported[29]. The molecular weight of Lipopolysaccharide (70794 Dalton) was estimated and this result was close to the results obtained in this study.

Cytotoxicity of LPS on cervical cancer cell line (Hela): The cytotoxicity effect of LPS extracted from the local isolation of *S. typhimurium* in its phenolic and aqueous phases, was examined to reduce the growth and proliferation of cancerous cervical cells outside the living cells (*in vitro*). The cervical cell line was treated with different concentrations of lipopolysaccharide including: 0, 100,150, 250, 350 mg / mL for both aqueous and phenolic phases and within 72 hours at 37°C. The cell density was estimated by measuring the absorbance at a wavelength of 492 nm using ELISA.

The results showed a significant cytotoxic effect of different concentrations of lipopolysaccharide for phenol and aqueous phases in the growth of Hela cells. This effect starts from the low concentration to continue towards the high concentrations when compared with the control treatment for each phase. Results showed the role of LPS in reducing the density of cancerous cervical cells. A difference was also observed in cytotoxic effect of lipopolysaccharide on cancer cells in aqueous phase from its effect in the phenolic phase.

Table 3 showed that there was a clear effect of lipopolysaccharide in its aqueous phase, where inhibition rate of cervical cancer cells was 53.33% at concentration of 350 µg ml and the inhibition ratio from 100 µg/ml to 250 µg/ml was as follows: 17.33%, 20.33% and 36.21% respectively compared to the control treatment with a 0% inhibition ratio, whereas the inhibition ratios of the lipopolysaccharide extract in its phenolic phase were 18.4, 25, 42.33, 58%, respectively. Also, it is noted from table 3 that the effect of the lipopolysaccharide extract in the phenolic phase was having more effect from its aqueous phase at any of the concentrations used in the study as in figures 10 and 11.

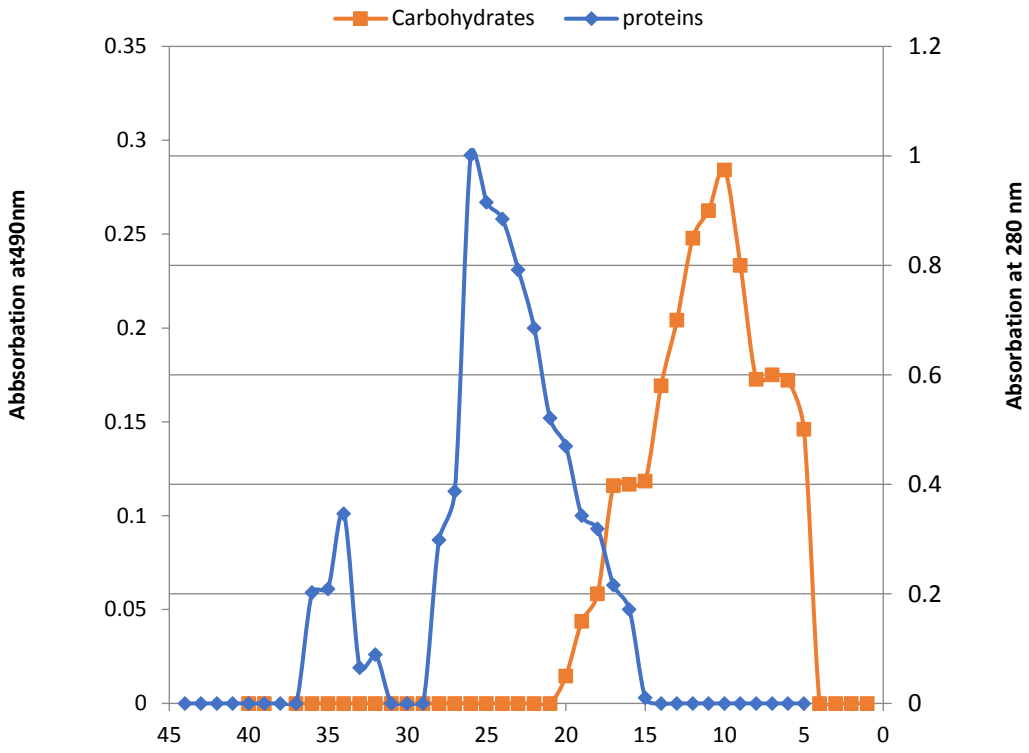

Figure 7: Gel filtration chromatography of *S.typhimurium* (phenolic phase) lipopolysaccharide by using Sphacryl S – 200, the column dimensions were (1.5 x 70 cm) and elution was done with phosphate buffer saline pH 7.2 at flow rate 34 ml/h.

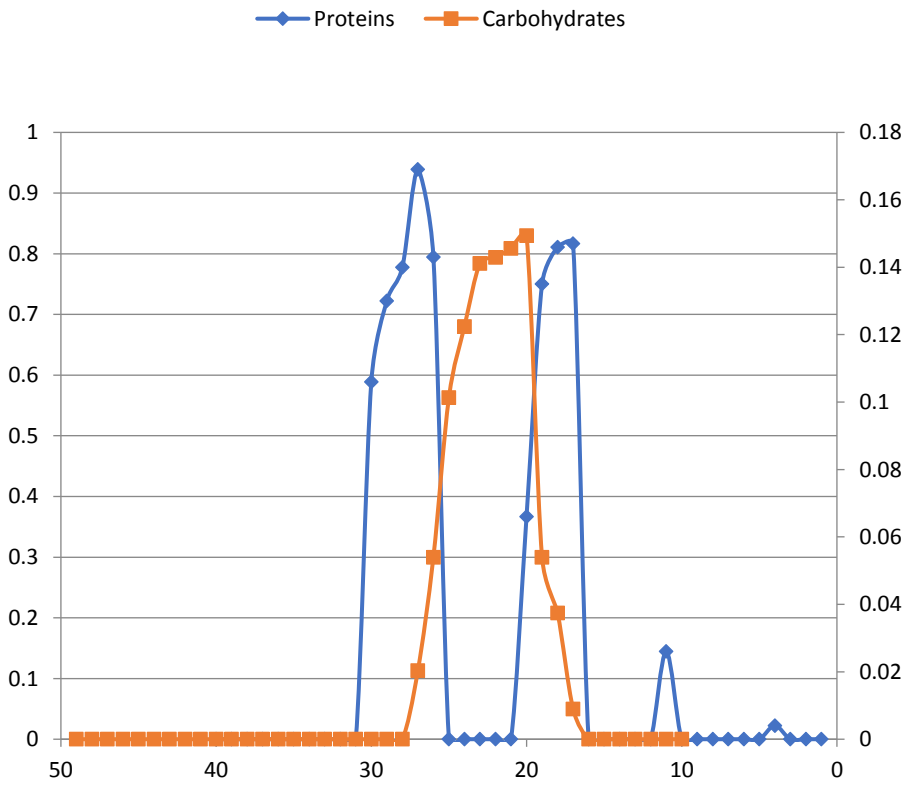

Figure 8: Gel filtration chromatography of *S.typhimurium* (aquatic phase) lipopolysaccharide by using Sphacryl S – 200, the column dimensions were (1.5 x 70 cm) and elution was done with phosphate buffer saline pH 7.2 at flow rate 34 ml/h

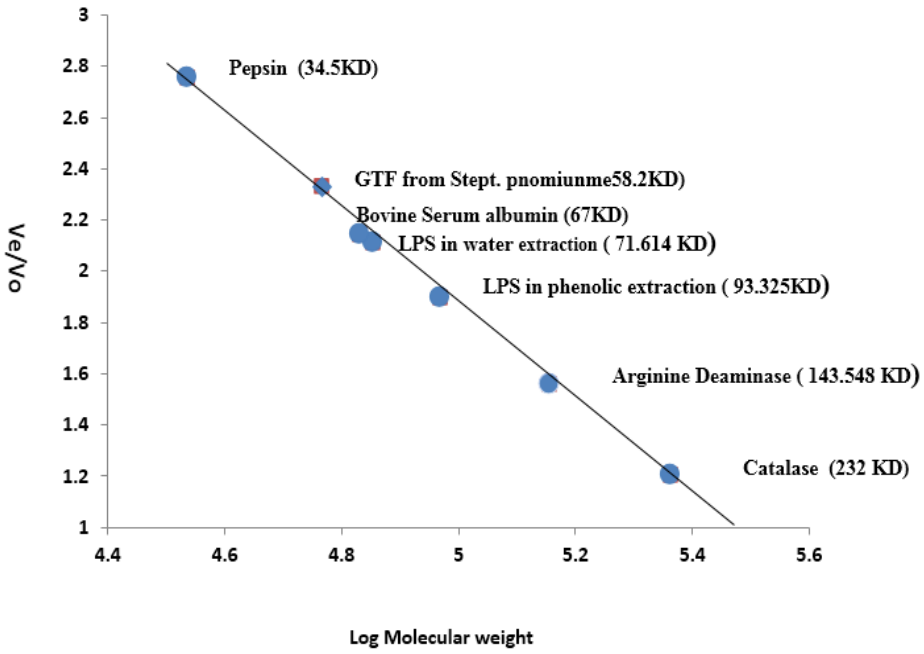

Figure 9: Molecular weight of LPS for both aquatic and phenolic phases by using gel filtration chromatography (Sphacryl S – 200) the column dimensions was (1.5 x 70 cm) and elution was done with phosphate buffer saline pH 7.2 at flow rate 34 ml/h

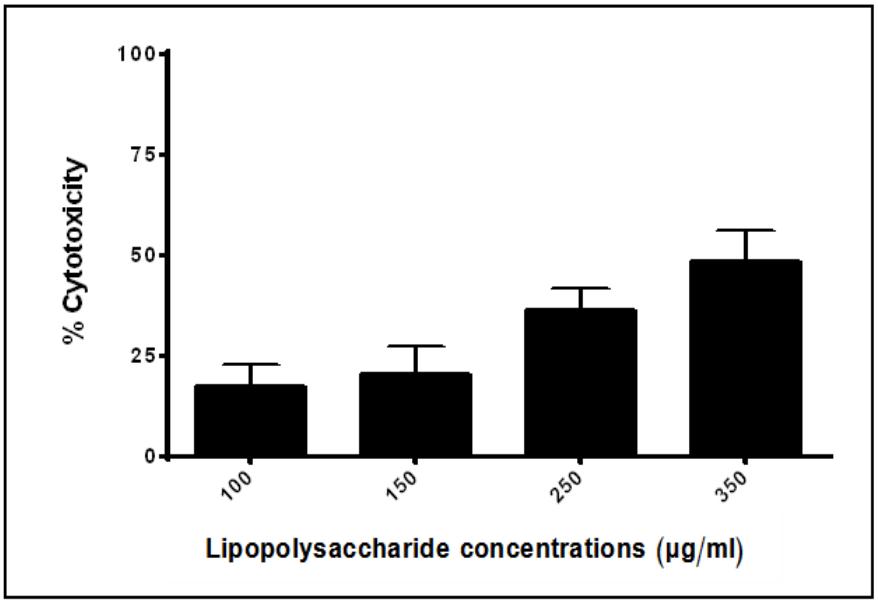

Figure 10: Cytotoxicity of LPS (aquatic phase) in cervical cell line (Hela)

Table 2
Standard protein and standardization of LPS from *S. typhimurium* for both aqueous 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 aqueous phase	71.614	2.142
LPS in phenolic phase	93.325	1.904
Bovine serum albumin	67	2.15

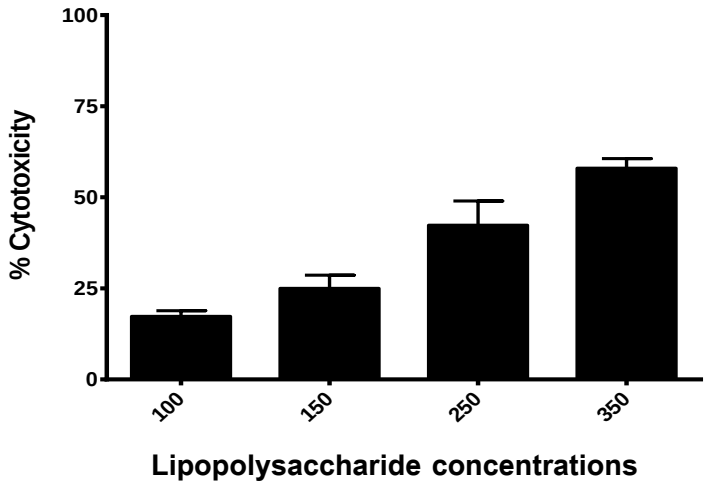

Figure 11: Cytotoxicity of LPS (phenolic phase) in cervical cell line (Hela)

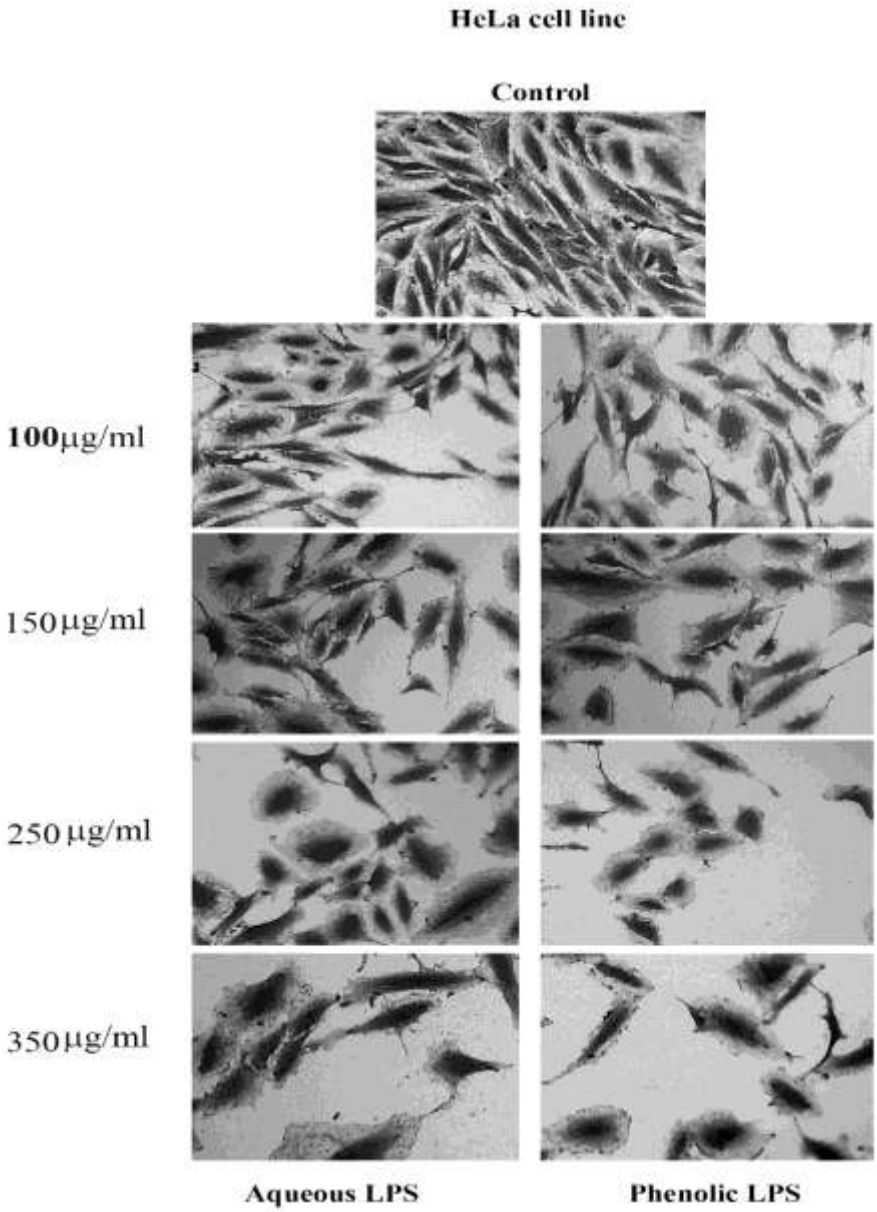

Figure 12: Cytotoxicity of LPS (phenolic and aqueous phases) in cervical cell line (Hela)

Table 3
The percentages of inhibition rates of cervical cancer cells (Hela) treated with different concentrations of lipopolysaccharide in its aqueous and phenolic phases.

Concentration μg/ml	phenolic phase	aqueous phase
	Percentages of inhibition (%)	
0	0	0
100	18.4	17.33
150	25	20.33
250	42.33	36.21
350	58	48.30

The figure 12 showed gradual change in the shape and number of cancer cells with change of lipopolysaccharide concentrations compared with the control treatment with 0 μg/ml concentration of lipopolysaccharide and 0% of cancer cells inhibition in both phenolic and aqueous phases.

In recent studies, it was found that the lipopolysaccharide has a cytotoxicity effect in reducing the density and growth of cancer cells whether *in vitro* or *in vivo* by introducing the cancer cell in programmed death stage (apoptosis). The researcher[31] suggested that lipopolysaccharide enhances programmed apoptosis of breast cancer cells by inhibiting receptors known as Toll-like receptors (TLRs) which have been linked to the development of cancer.

Another study suggests that lipopolysaccharide can stimulate the killing of cancer cells by enhancing the immune response by stimulating the transcription of the genes which encoded the proteins responsible for the release of cytokines that are associated with cyclooxygenase-2 enzymes that play a role in inhibition of cancer cells[31].

The results in the present study were in agreement with[32] who found a cytotoxicity effect of LPS in oral and esophageal cancer cell lines when used the LPS in high concentrations. The results also agree with[33] who studied the cytotoxicity by treating the NCI-H69 lung cancer cell line with different concentrations of LPS, including 100, 150, 250 mg / ml, they observed there was gradually reduction in the growth density of lung cancer cells and they found that the lowest survival rate of the cancer cells was 48.88% at the concentration of 250 mg / ml of the LPS extract, a result similar to that found in the current study.

The results of the study showed that there is a different effect of LPS between in the cancer cell lines (Hela and mcf-7) which may be due to each cancer cell line having metabolic behavior different from the other in addition to the difference of receptors on the surface of cells causing a different response to external factors including exposure to the LPS. This result was identical to that obtained[32] but using different cancer cell lines.

Conclusion

We concluded from this study that LPS was found to exhibit cytotoxicity on the line of cervical cancer cells in different concentrations used. So, the ratio cell viability was found to be less at 350 mg/ml. Also, we concluded that the LPS of *Salmonella typhimurium* has cytotoxic properties which can be used in development of drugs for cancers.

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