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A Comparative Study Between Lipid A Extracted from *Salmonella typhi* and *Pseudomonas Aeruginosa* to Demonstrate the Extent of its Stimulation of Immune System

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Abstract

Lipid A in the cell wall of *Salmonella typhi* and *pseudomonas aeruginosa* were extracted and analyzed by the GC/MS technique. Three concentrations were prepared (50, 100, 200) mg/100g of body weight. The sample was divided into two groups of Albino rats, consisting of three animals for each concentration. Lipid A was calculated and then injected in three doses over three consecutive days. Interleukin-2 and Interleukin-9 levels were measured and were (13.11, 3.150, 3.180),(12.183, 15.766, 36766)pg/ml respectively c compared with the controls (3.205 and 6.433)pg/ml for three concentration respectively for *Salmonella typhi* whereas *Pseudomonas aeruginosa* were (3.086, 3.159, 3.168),(12.133, 14.400, 21.100) pg/ml compared with the controls (6.337 and 0.230) pg/ml for the three concentrations, respectively. White blood cell counting and total count were (9.052, 9.2033, 9.118)*10µl in *Salmonella typhi*, and were (8.235, 9.239, 8.945)*10µl in *Pseudomonas aeruginosa*. Neutrophils, lymphocytes and monocytes were (5.408, 5.506, 6.221),(3,716, 2.882, 2.405),(0.087, 0.139, 0.1820)*10µl respectively for the three concentrations in *Salmonella typhi* and were(4.353, 5.526, 5.994),(3.944, 3.413, 3.016),(0.123, 0.143, 0.072)*10µl respectively for the three concentrations in *Pseudomonas aeruginosa*. Eosinophils and basophils were (0.342, 0.266, 0.348),(0.281, 0.004, 0.004)*10µl for the three concentrations for *Salmonella typhi* while in *Pseudomonas aeruginosa* were(0.164,0285, 0.136),(0.000, 0.000, 0.000)*10µl respectively for the three concentrations.

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1. Introduction

Lipid A is the most important part of the lipopolysaccharide composition and it is located inside of the bacterial cell wall, It is composed of a β(1-6)- linked disaccharide of glucosamine phosphorylated at the 1 and 4 positions, 2-3 acylated with R-3-hydroxy-myristoyl groups (3-OH-C_{14}) [1]. Lipid A is very toxic to humans and animals and causes septic shock, and it is found in larger quantities in gram-negative bacteria [2] in addition it acts as a powerful stimulator for the immune receptors for lipopolysaccharide [3,4]. Its toxicity depends on the number of acyl chains, which can vary in length. Usually, there are 4-6 chains depending on the type of bacteria (Fig.1) [5].The acyl chains of lipid A (sub-region of LPS) cross-link with MD2 and TLR4 molecule, leading to dimerization of the cytosolic tails of TLR4, which contain a signalling motif known as a Toll/interleukin-1receptor[6]. Immediately after a bacterial infection occurs, the immune system begins to produce many inflammatory cytokines, such as Interleukin-2 and Interleukin-9,through the pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) on innate immune cells [7]. IL-9is produced by Lymphocytes and has an effective role in many diseases such as infections, autoimmunity, allergy, and immunodeficiency-cancer [8]. IL-2 is a cytokine discovered in the 1970s as a soluble substance that stimulates the immune system via Lymphocytes of the type l (CD4) [9]. White blood cells (WBC) like Lymphocytes, Monocytes, Neutrophils, Basophils and Eosinophils act as the first line of immune defense against

pathogens. These cells are responsible for secreting immune cytokines after bacterial invasion of the body, Neutrophils are the first responders to bacterial infections and Eosinophils and involved in parasitic infections and allergic responses. [10].
Salmonella is a gram-negative bacteria with rod shape and non-motile bacteria, that can be classified in to typhoidal and non-typhoidal salmonella strains (NTS), *Salmonella typhi* causes the thermoregulatory set-point to rise at the hypothalamus, leading to a brain protein-mediated rise in body temperature [11], so, this injury stimulates the immune system to produce immune cytokines and immune cells [10] and also increases white blood cells, especially neutrophils and other types [12].

Pseudomonas aeruginosa is a gram-negative bacterium and it is characterized by being opportunistic and highly resistant to antibiotics, its danger lies in the Lipid A compound found in the endotoxin, which is part of the composition of its cell wall, as it is one of the virulence factors and leads to stimulating the immune system and production of cytokines and immune cells [13]. *Pseudomonas aeruginosa*, after growth and formation of biofilm, lipid A will be modified and become more resistant to antibiotics. Lipid A with penta-acrylate chains binds TLR2 and converts to TLR4-MD2-CD14 complex inducing a more robust inflammatory response [14; 15].

2. Materials and Methods:

2.1. Preparation of Bacteria:

2.1.1. **Pure Isolates**:

Isolated and pure *Salmonella typhi* and *Pseudomonas aeruginosa* genera were obtained from the Bank of the Department of Biology, College of Science, University of Mosul.

2.2. Bacteria Cultivation:

under study were cultured in 5 liters of Brain Heart Infusion broth medium (BHI) for dense growth, incubated at 37◦C for 48-72 hours in a shaker incubator then, cultures were centrifuged at 5000 rpm for 30 minutes, and bacterial sediments were washed twice with 5 ml of ethyl alcohol (95%), shaken well, Finally samples were centrifuged at 3000 rpm for 10 minutes, cell pellets were kept in a refrigerator at 4-8◦C until used [16].

2.3. Extraction of Bacterial Lipid A:

Bacterial cells were washed (2g) and suspended in 400ml of isobutyric acid-ammonium hydroxide (5:3) volume and placed in a water bath at 100◦C for 2 hours with continuous stirring. The solution was cooled with ice water and then precipitated at 2000 rpm for 15 min. Then it was washed twice with 400 ml of methanol and centrifuged at 2000 rpm for 15 min. Lipid A molecules are formed and kept in the freezer until use [1,17]

2.4. Determine the purity of Lipid A:

The purity of lipid A was determined using gas chromatography-mass spectrometry (GC-MS) (5973 network mass selective detector, USA) [18].

2.5. Preparation of lipid A concentrations:

0.01 of dry Lipid A dissolved in 2mlof normal saline with 0.025% of triethylamine and heated at 56 ◦C to prepare 200 mg/ml concentration. Other concentrations were also prepared by transferring 1 ml of concentrate 200 mg/ml to another tube containing 1 ml of physiological solution, to obtain concentrations of 100 and 50 mg/ml [19].

2.6. Animal injection:

Forty-eight animals(Albino-Rats)in the first months of life were distributed into two groups with three replicates for each concentration of lipid A. They were placed in appropriate environmental conditions. Then, the doses of lipid A were calculated according to the weight of the animal and injected intraperitoneal in equal doses for three consecutive days, then blood samples were taken and sera were obtained. The samples were kept in the refrigerator to conduct the tests of the current study [16].

2.7. Estimating the levels of IL-9 and IL-2:

The levels of cytokines under study were estimated using the specific kit for each type and using the ELISA technique. Kits were used from Elab Science Biotech-nology Inc.

2.8. Estimation of white blood cells:

Blood samples were collected and placed in the EDTA tubes and mixed well. WBC pipette was filled to0.5 mark and diluted with reagent solution to point 11, then mixed well and placed on a hemocytometer chip for checking the total number of W.B.C. Also, one drop of blood was fixed on a glass slide to make a thin blood smear to calculate the differential count for the samples being studied [20].

2.9. Statistical analysis

The results were analyzed using the ANOA system at a probability level of less than 0.05. Similar letters indicate there is no significant difference while the different letters indicate the presence of significance at the same probability level.

3. Results:

GC-MS analysis of lipid A extracted from *Salmonella typhi* showed that it contains many compounds in different forms (Fig.1), pretensions such as Tetradecanoicacid Hexadecanoic acid n-ethylester , 12 –Octadecadienoic acid, Cis-13-Octadecenoic acid, Octadecanoic acid as shown in (Table-1) As for the *Pseudomonas aeruginosa*, the results were the following compounds Hexadecanoic acid, 11, 14, 14-Eicosadienoic acid, cis-13-Octadecenoic, Octadecanoic acid and trans-13-Octadecenoic acid as shown in (Table -2) (Fig-2)

Table 1. Some of the *Salmonella typhi* lipid A structure

arrange	Peak#	R .Time	Area	Area%	Name	Similarity Index (SI)
1	7	16.617	184649	0.42	Tetradecanoic acid	94%
2	10	18.364	105804	0.24	Hexadecanoic acid Methyl ester	94%
3	13	20.139	399720	0.91	9, 12-Octadecadic acid Methyl ester	95%
4	14	20211	196608	0.43	Cis-13- Octadecenoic acid Methyl ester	92%
5	16	21.066	1973235	0.47	Octadecanoic acid	94%

Table 2. Some of the *Pseudomonas aeruginosa* lipid A structure

arrangement	Peak#	R.Time	Area	Area%	Name	Similarity Indwx (SI)
1	1	18.371	64567	0.83	Hexadecanoic acid,methylester	95%
2	6	20.710	286949	37.03	11,14-Eicosadienoic acid,methylester	87%
3	7	20.765	1935402	24.88	Cis-13-octadecenoic acid	92%
4	8	20.968	511954	6.61	Octadecanoic acid	93%
5	19	23.767	165616	2.14	Trans-13-octadecenoic acid	92%

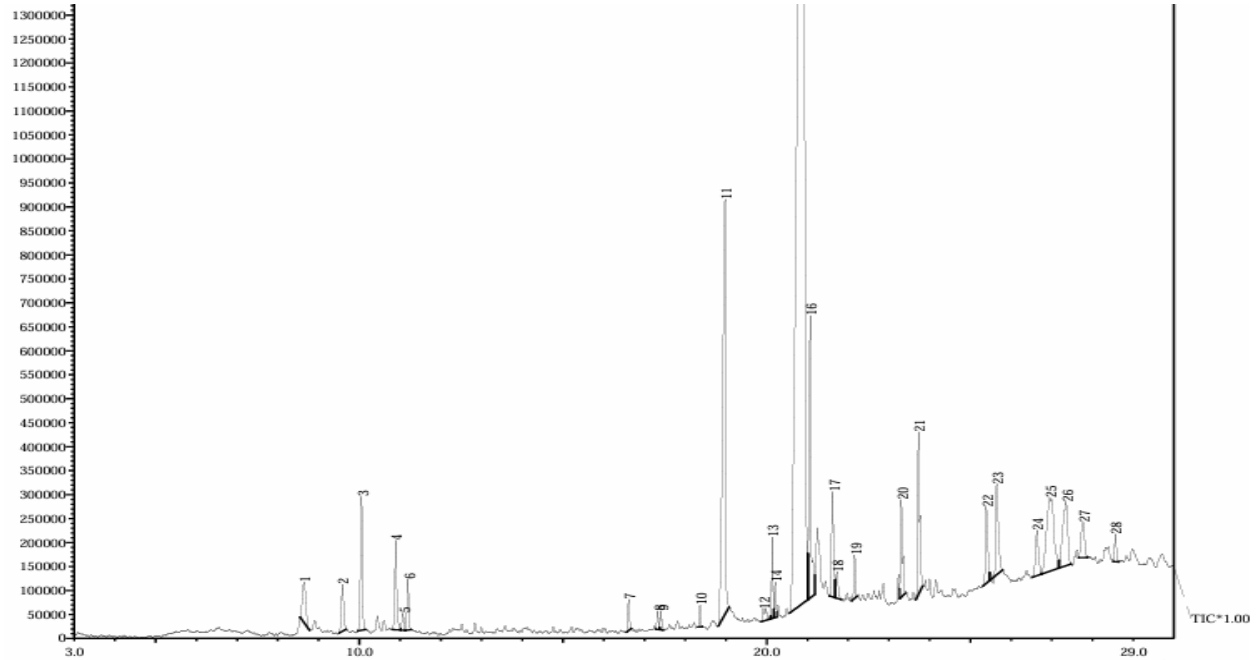

Figure 1. *Salmonella typhi* GCMS (7 Tetradecanoic acid, 10 Hexadecanoic acid Methyl ester, 139, 12-Octadecadic acid Methyl ester,14 Cis-13- Octadecenoic acid Methyl ester, 16 Octadecanoic acid)

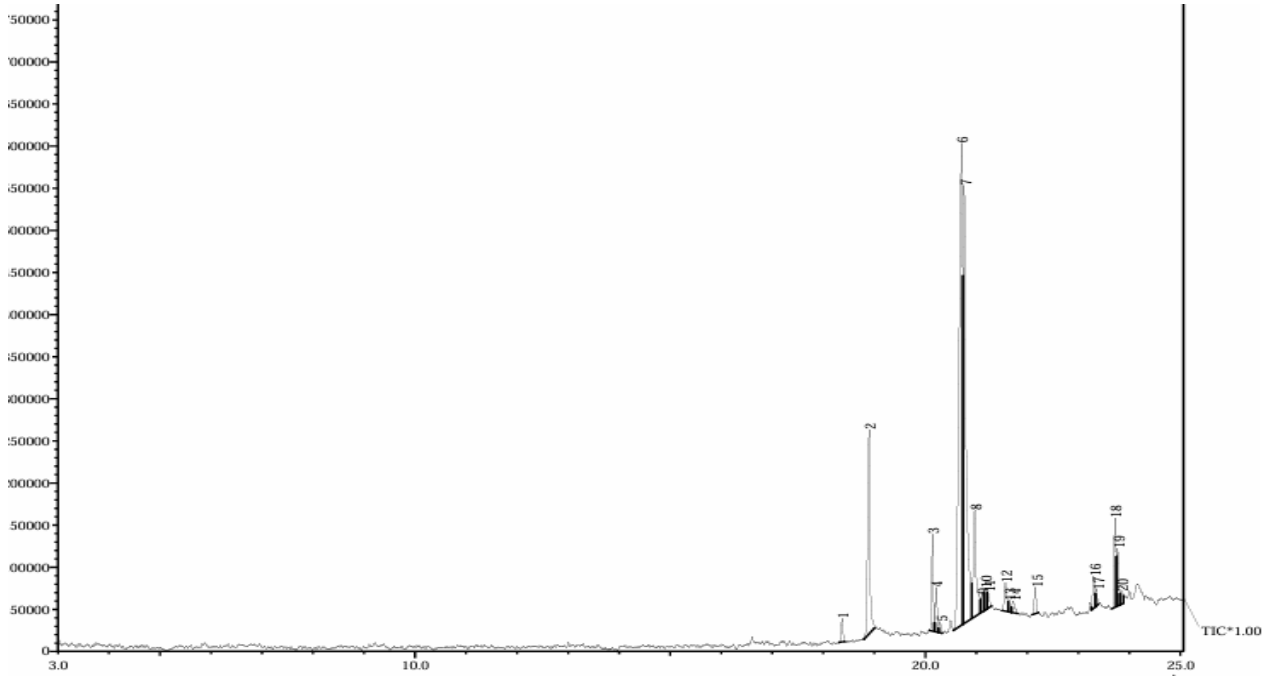

Figure 2. *Pseudomonas aeruginosa* GCMS(1 Hexadecanoic acid,methylester ,6 11,14-Eicosadienoic acid,methylester,7 Cis-13-octadecenoic acid, 8 Octadecanoic acid, 19 Trans-13-octadecenoic acid)

The study also showed the effect of bacterial lipid A on the cytokines under study IL-2 and IL-9 regarding the effectiveness of salmonella, the levels of IL-2 were (3.091, 3.150 and 3.180) pg/ml, while for IL 9 were (12.183. 15766 and 36.766) pg/ml for concentrations (50, 100, 200) mg /100g B.W., as for lipid A for *Pseudomonas aeruginosa* were (3.086, 3.159 and 3.168)pg/ml, while for IL-9 (12.133, 14.400 and 21.100)pg/mL for three concentrations (50, 100, 200) mg/100g B.W., as shown in Table-3 and Table-4.

Table 3. Levels of IL-2 and IL-9 stimulated by lipid A of *Salmonella typhi*

Concentration mg/100 B.W.		**IL-2 pg/ml**	**IL-9 pg/ml**
50	Mean	9.091 ab	12.183 d**
	N	3	3
	Std. Deviation	8.036	0.725
100	Mean	3.150 a*	15.766 c**
	N	3	3
	Std. Deviation	1.452	1.250
200	Mean	3.180 a*	36.766 a**
	N	3	3
	Std. Deviation	1.808	0.873
Control	Mean	3.205a*	6.433 e**
	N	3	3
	Std. Deviation	1.322	0.404

*similar letters indicate that there were no significant differences between the coefficients at level of $p<0.05$
**different letters indicate that there were significant differences between the coefficients at level of $p<0.05$

Table 4. Level of IL-2 and IL-9 stimulated by the lipid of *Pseudomonads aeruginosa*

Concentration mg/100 B.W.		IL-2pg/ml	IL-9pg/ml
50	Mean	3.086 ab	12. 133 d**
	N	3	3
	Std Deviation	7.016	0.3214
100	Mean	3.159 a*	14.400 c**
	N	3	3
	Std Deviation	3.927	0.556
200	Mean	3.168 a*	21.100 b**
	N	3	3
	Std Deviation	0.815	1.276
Control	Mean	2.968 b**	6.633e**
	N	3	3
	Std Deviation	0.337	2.230

*similar letters indicate that there were no significant differences between the coefficients at level of p<0.05
**different letters indicate that there were significant differences between the coefficients at level of p<0.05

The total number of white blood cells was (9.052, 9.203, 9.118) *10µl for *Salmonella typhi*, and (8.235, 9.239, 8.947)*10µl for *Pseudomonas aeruginosa*. The results of differential count for Neutrophils, Lymphocytes, Monocytes, Eosinophils and Basophils for three concentrations (50, 100, 200) mg/ml were (5.408,3.716,0.087, 0.342 and 0.281),(5.506, 2.88, 0.139, 0.266 and 0.004) and (6.221, 2.405, 0.182, 0.348 and 0.0040) for *salmonella typhi* and (4.353,3.944, 0.123, 0.164 and 0.00), (5.526, 3. 413, 0.143, 0.285 and0.00) and (5.994, 3.016, 0.072, 0.186 and 0.00) for *Pseudomonas aeruginosa* as shown in tables (5 and6).

Table 5. Total and differential Count of W.B.C. stimulated by Lipid A for *Salmonella typhi*

Concentration mg/100B.W.		Total count of W.B.C* µl	Neutrophils*10 µl	Lymphocytes*10 µl	Monocytes*10 µl	Eosinophils*10 µl	Basophils* 10 µl
50	Mean	9.052 cd*	5.408 b**	3.716d**	0.087 d**	0.342 a*	0.281 a*
	N	3	3	3	3	3	3
	Std. Deviation	0.164	0.432	0.200	0.005	0.372	0.204
100	Mean	9.203 bcd*	5.506 ab*	2.882 cd*	0.139 c**	0.266 b*	0.004 b*
	N	3	3	3	3	3	3
	Std. Deviation	0.328	0.318	0.820	0.0025	0.0323	0.006
200	Mean	9.118 bcd*	6.2219 ab*	0.182 b*	0.182 b**	0.348 b*	0.004 b*
	N	3	3	3	3	3	3
	Std. Deviation	1.068	0.288	0.104	0.010	0.042	0.006
Control	Mean	10.067 ab*	3.431d**	0.219 b*	0.219 a**	0.341b*	0.131 b*
	N	3	3	3	3	3	3
	Std. Deviation	0.217	0.460	0.019	0.019	0.039	0.037

*similar letters indicate that there were no significant differences between the coefficients at level of p<0.05
**different letters indicate that there were significant differences between the coefficients at level of p<0.05

Table 6. Total and differential Count of W.B.C. stimulated by Lipid A for *Pseudomonas aeruginosa*

Concentration mg/100B.W.		Total count of W.B.C* µl	Neutrophils*10 µl	Lymphocytes*10 µl	Monocytes*10 µl	Eosinophils*10 µl	Basophils* µl
50	Mean	8.23c*	3.944 b*	4,353 b*	0.123 c*	0.164 c**	0.00 b*
	N	3	3	3	3	3	3
	Std. Deviation	0.318	0.084	0.084	0.006	0.003	0.00
100	Mean	9.239 bc*	5,526 bc*	3.413 b*	0.143 c*	0.285 b*	0.00 b*
	N	3	3	3	3	3	3
	Std. Deviation	0.420	0.370	0.370	0.0056	0.162	0.00
200	Mean	8.945 cd*	5,994b*	3.016 b*	0.072 d**	0.186b*	0.00b*
	N	3	3	3	3	3	3
	Std. Deviation	0.334	0.258	0.258	0.033	0.002	0.00
Control	Mean	10.511 a**	6.319a**	6.319a**	0.223 a**	0.345a**	0.133 b*
	N	3	3	3	3	3	3
	Std. Deviation	6.435	6.219	0.219	0.213	0.027	0.019

*similar letters indicate that there were no significant differences between the coefficients at level of $p<0.05$
**different letters indicate that there were significant differences between the coefficients at level of $p<0.05$

4. Discussion

From observing Tables 1 and 2, it appears that there were many different fatty acids involved in the composition of lipid A, whether it is from *Salmonella typhi* and *Pseudomonas aeruginosa* in certain proportions, such as (Tetradecanoic acid 94%, Hexadecanoicacid, methyl ester 94% 9,12-Octa decadieacid, methyl ester 95%,Cis-3- Octadecenoic acid methyl ester 94%, Octadecanoic acid 94%, and trans-13- Octadecanoic acid 92% and 11,14-Eicosadienoic acid, methylester 87% that agree with [18]. The results also showed that there is a great similarity between the composition of lipid A with *Salmonella typhi* and *Pseudomonas aeruginosa*, especially compounds Hexadecanoic acid, methyester, Cis-13-Octadecadecenoic acid methyl ester and Octadecanoic acid as they are present in both genera [2], and this result is consistent with [21]. Lipid A of gram-negative bacteria could be modified depending on the nature of the bacteria and the extent of their exposure to mutagens [22].

The results also showed that there was no significant effect of lipid A on the levels of IL-2 for all concentrations under study compared to the control sample. However, a clear significant effect of lipid A was observed on the level of IL-9 compared to the control samples where a concentration 200 mg/ml showed the highest effect followed by 100mg/ml and finally concentration 50 mg/ml for *Salmonella typhi* as showed in Table-3, who proved that lipid A (antigen) activated T helper cell, B cell and Mast cell is responsible for secretion IL-9 [23; 24]. This confirms that lipid A causes septic shock due to arterial and venous dilation then, induces inflammation , fever and leukopenia and causes septicemia and shock [25]

There was a similar case with lipid A of the *pseudomonas aeruginosa*, as there was no effect on the levels of IL-2 for all concentrations, because a rapid transient process, particularly in T cells, lasting for a few hours, while there was a significant effect on IL-9, where concentration 200 mg/mL showed the highest effect, followed by concentration 100 mg/ml, then concentration 50 mg/mL, compared to control samples as showed in table-4, this result was consistent with what mentioned by researcher Steimle and his group that lipid A has the ability to induce pathological immune reaction and release immune cytokines [5], while disagreed with what the researcher [26].Our current study showed the effectiveness of lipid A on stimulating immune cells, as we observed a slight effect on the total number of white blood cells for both, *Salmonella typhi* and *pseudomonas aeruginosa* were decreased in all concentrations compared to control samples which indicates that there is immune suppression and this result can be a tribute to the repeated exposure to different doses of Lipid A [27].

Regarding differential counting, the results showed a significant increase in the number of neutrophils for all concentrations of lipid A for both bacteria under study for all concentrations compared to the control sample, this was in agreement with Sivanakar and his group, who showed that lipid A is endotoxin which interacts with myeloid differentiation -

2(MD-2) and Toll-like receptar-4 (TLR-4) and activates the cascade of cytokine and immunity cells. as Showed in Table- 3 and 4. [28].

The number of Lymphocytes decreased compared to the control samples as a result of their influence by the three concentrations of lipid A for both bacteria under study, noting that concentrations 100 mg/ml and 200 mg/ml had the strongest effect on the lymphocytes for the *Salmonella typhi*, the situation differed for the *Pseudomonas aeruginosa*, the reason for this could be due to difference in the composition of lipid A of *Salmonella typhi* which is facultative intracellular Hexa and Hepta-acylated chains of lipid A structure [29] while *Pseudomonas aeruginosa* has penta-acylated chains of lipid A [30]. Regarding the monocytes, their numbers were generally low at all concentrations and both bacteria under study compared to the control samples, but we can notice from table 5 and 6 that the concentration of 50 mg/ml was significant on monocytes as it was 0.087*10µl for *Salmonella typhi* compared to the control sample 0.219*10µl, while *Pseudomonas aeruginosa* showed a different result, as the concentration of 200 mg/ml was more effective on the cells as their number was0.072*10µl compared to the control sample(0.223*10µl). Although *Pseudomonas aeruginosa* belongs to the ESKAPE group, which causes many diseases and resistance to antibiotics, our results differed in the current study, the *Salmonella typhi* showed greater activity [14].

The study also showed that the number of Eosinophils was not affected by the lipid A of *Salmonella typhi* at concentrations of (50 and 200) mg/ml, but the concentration of 100 mg/ml had a significant effect as the number decreased to 0.266*10µl compared to the control sample 0.341*10µl, which could be due to the difference in the composition of lipid A, including the number and length of the chains [32], while all concentrations had a significant effect on the number of Eosinophils especially the concentrations of (50 and 200) mg/ml, which led to decrease in the number of Eosinophils of lipid A of *pseudomonas aeruginosa* compared to control sample were 0.345* 10µl [31].

The number of Basophils decreased significantly at all concentrations of lipid A for both bacteria under study compared to the control samples. The increase in the number of neutrophils was an abnormal condition due to the release of toxic components such as oxygen radicals and thus may lead to apoptosis of the cells [32].

5. Conclusion:

From the current study, it was concluded that lipid A activates the immune system by accelerating the production of important immune factors in the inflammatory process , such as cytokines and immune cells , and that its percentage is very important in this effect. We also concluded that, in general, there was no significant difference between lipid A found in most genera of gram-negative bacteria.

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7. Conflict of interest:

Authors do not have any conflicts of interest to declare.

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دراسة مقارنة بين الدهن A المستخلص من بكتريا *Salmonella typhi* و *Pseudomonas aeroginosa* لبيان مدى تحفيزه للجهاز المناعي

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المستخلص

استخلص الدهن A لجنسي *Salmonella typhi* و*Pseudomonas aeruginosa* وحددت نقاوته باستخدام تقنية كروماتوغرافيا الغاز – الطيف الكتلي . حضرت تراكيز من الدهن A (50، 100 , 200) ملي غرام \100 غرام من وزن الجسم وحقنت مجموعتين من الجرذان البيض السويسرية بجرع محسوبة من الدهن A لثلاث ايام متتالية ,حسبت مستويات الانترلوكينات 2و9 كانت (3,11، 3,15 ، 3,18) و (12,18، 15,76 ، 36,76)مايكروليتر/ مل على التوالي للتراكيز الثلاثة مقارنة بعينات السيطرة (3,20 و6,43) ملي غرام / 100 غرام من وزن الجسم على التوالي لبكتريا *Salmonella typhi* اما *Pseudomonas aeruginosa* كانت (3,08 ، 3,15 ، 3,16) و (12,13 , 14,40 , 21,10) مايكروليتر/ مل على التوالي مقارنة بعينات السيطرة (6,33 و 0,23) مايكروليتر/ مل. الاعداد الكلية والتفريقية لخلايا الدم البيضاء لكلا الجنسين كانت (9,052 , 9,203 , 9,118) و (8,23 , 9,23 , 9,94)×10 مايكروليتر للتراكيز الثلاثة على التوالي وكانت الاعداد التفريقية للخلايا العدلة , اللمفية , الوحيدة , الحمضة و القعدة كانت (5,40, 5,50 , 6,22) , (4,35 , 5,52 , 5,99)و (3,71 , 2,88 , 2,40) , (3,94 , 3,41 , 3,01) و (0,08 , 0,13 , 0,18) , (0,12 , 0,14 , 0,07) و (0,34 , 0,26, 0,34) , (0,16 , 0,28 , 0,13) × 10 مايكروليتر للجنسين والتراكيز الثلاثة على التوالي.