

**SEPARATION, PARTIAL PURIFICATION AND MITOGENICITY OF
ENTERIC *VIBRIO FLUVIALIS* AND *AEROMONAS HYDROPHILA*
LIPOPOLYSACCHARIDES IN CHICKEN AND RAT**

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ABSTRACT

Crude smooth LPS preparations were made from *Vibrio fluvialis* and *Aeromonas hydrophila*. Then these preparations were processed in gel filtration using sepharose 50 resin which give two fractions from each of the test isolates that reach the limits of partial purity. Test for an in vivo mitogenicity were carried out using chicken and rat models. The assessments were performed through skin induration and bone marrow lymphocyte blastogenicity. Fraction 1 and fraction 2 from each of the LPS preparations were proved to be mitogenic, Though F2 of the two LPS preparations were of higher mitogenicity than fraction 1. Such mitogenic potentials were stable and crossing the species barriers.

KEY WORDS: Crude, gel filtration ,purity ,LPS, induration, blastogenicity, mitogenicity.

INTRODUCTION

Bacteria outstandingly separated into negative staining entity and positive staining entity in accordance with pioneered staining procedure which is the gram stain. Hence they are gram positive and gram negative. Gram positive have in their outer membrane teichoic and lipoteichoic acids. While, gram negative contain lipopolysaccharide And lipoproteins⁽¹⁾. The lipopolysaccharide, however, are of two morphotypes, the smooth and the rough. The smooth form has polysaccharide region, while the rough lacks the polysaccharide region thus is known as lipooligosaccharide LOS. Both of these forms contained lipid A⁽²⁾. In smooth form LPS, the core OS region is replaced by polysaccharide and in other cases are replaced by the enterobacterial common antigen. The polysaccharide region in S-LPS represented most oftenly by somatic O antigen⁽³⁾.

The LPS as an immunogen act as potent activator of macrophage, granulocytes, dendritic or mast cells and releases a large spectrum of proinflammatory and inflammatory cytokins which are essential for instructing the adaptive immune responses⁽⁴⁾. *Vibrio fluvialis* and *Aeromonas hydrophila* LPS interacted with the humoral and cellular mediators systems⁽⁵⁾. LPS may act as B lymphocyte and T lymphocyte mitogens⁽⁶⁾. The objective of the present work was to investigate separation, partial purification and fractionation of LPS from smooth *V. fluvialis* and *A. hydrophila* and study their mitogenicity *in vivo* in bird and rat.

MATERIALS AND METHODS

Bacterial growth

Fifty-handred (500) ml of brain heart infusion broth was inoculated by *A. hydrophila* and *V. fluvialis* strains ⁽⁷⁾ and incubated at 37°C for 24 hrs , bacterial suspension was examined with gram stain ,then 100 petridishes of sterile brain heart infusion agar were prepared which inoculated with suspension of *A. hydrophila* and *V. fluvialis* (5ul to each petridish) and incubated 37 C° for 24 hrs .Then bacterial growth was harvested with sterile PBS (pH 7.2), then centrifuged the suspension by cooled centrifuge 3000 rpm for 30 minutes ,after that the cells were washed three times with PBS (PH 7.2)and centrifuged by cooled centrifuge 2000 rpm for 10 minutes , bacterial cells were suspended with formalinized PBS 18 hrs for 4 C° , centrifuged by cooling centrifuge 3000 rpm for 30 minutes, then washed three times with the same solution and the precipitate was collected and frozen.

Bacterial lysis by enzymes

According to^[6]. Then added DNase, RNase mcg/1 ml suspension and the suspension was incubated at 37 C° for 10 minutes, after that repeated incubation at 60 C° for 10 minutes.

Extraction with hot phenol: according to ^[8].

LPS purification by Gel filtration Chromatography

A- Column Prepared According to ^[6]: Sephadex G50 gel was washed many times with distilled water ,then it was washed by solution (0.12M Tris-HCL(pH 8.1))and air bubbles were removed by vacuum pump ,a column (1× 51 cm) was filled by the mixture and the column was equilibrated by solution (0.12M Tris-HCL(pH 8.1) with flow rate speed at 20 ml /60 minutes . Void volume (Vo) was evaluated by using 3 ml of blue dextran solution and 3 ml of the output solution/tube was collected from the bottom of column , then the absorbant values of the collected fractions was estimated by using spectrophotometer 600 nm and the curve between the optical density and these fractions values were made , finally the gel was

washed with solution (0.12M Tris-HCL (pH 8.1)). B-Adding and replacement of crude LPS : according to^[9]: Crude LPS (15 mg) was dissolved in 3ml of solution (0.12M Tris-HCL (pH 8.1)) and added gently on column wall and it replaced as the same of blue dextran solution replacement 2ml /tube , then the absorbance of collected fractions were examined at waves length : 260 nm for nucleic acids^[10]. 280 nm for proteins conc.^[11].

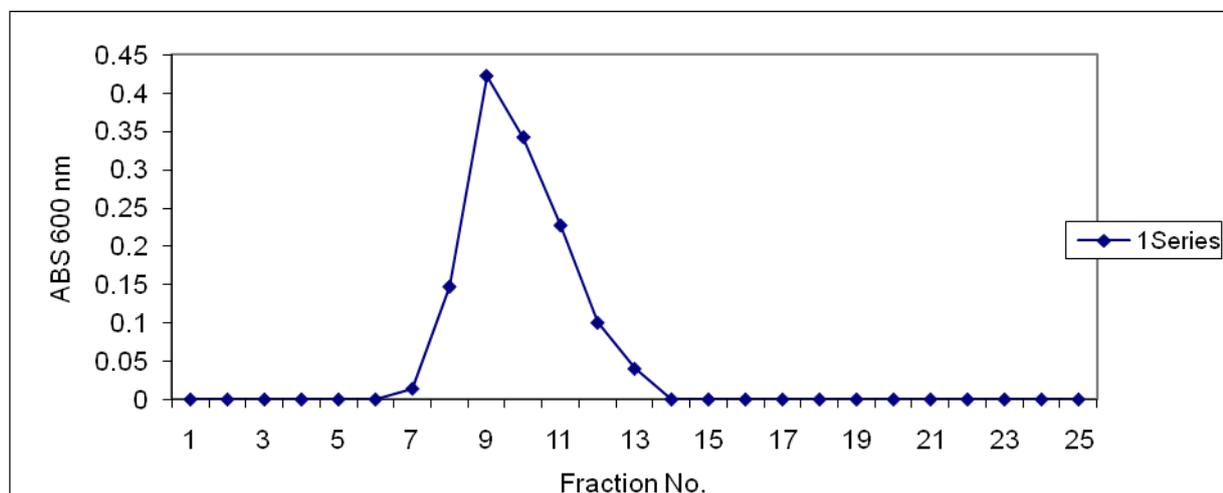


Figure (1) Chromatographically of gel filtration to dextran blue 2000 of sephadex G50 column (1×51 cm) flow rate 15ml/hr.

Chemical analysis of purified LPS

Carbohydrate estimation in LPS^[12]. Protein estimation in LPS : according to Bradford method^[13].

Methods for in vivomitogenicity

In bird

Thirty birds, their weight (20-25gm) divided into six groups each one of groups included 5 bird Group I-(*V.fluvialis* F1) Included 5 birds which inoculated with LPS as 2.5mcg/gm of bird in wing s/c at dose 0.2 ml / bird .

Group II -(*V.fluvialis* F2) Included 5 birds which inoculated with LPS as 2.5mcg/gm of bird in wing s/c at dose 0.2 ml / bird.

Group III -(*A.hydrophila* F1) Included 5 birds which inoculated with LPS as 2.5mcg/gm of bird in wing s/c at dose 0.2 ml / bird.

Group IV -(*A.hydrophila* F2) Included 5 birds which inoculated with LPS as 2.5mcg/gm of bird in wing s/c at dose 0.2 ml / bird.

Group V and VI :- were positive and negative group respectively.

In rat

Eighteen rats, their weight (gm) divided into six groups each one of groups included 3 rat :
Group I-(*V.fluviialis* F1) Included 3 rat which inoculated with LPS as 2.5mcg/gm of rat in pad at dose 0.2 ml / rat .

Group II -(*V.fluviialis* F2) Included 3 rat which inoculated with LPS as 2.5mcg/gm of rat in pad at dose 0.2 ml / rat.

Group III -(*A.hydrophila* F1) Included 3 rat which inoculated with LPS as 2.5mcg/gm of rat in pad at dose 0.2 ml / rat.

Group IV-(*A.hydrophila* F2) Included 3 rat which inoculated with LPS as 2.5mcg/gm of rat in pad at dose 0.2 ml / rat .

Group V and VI :- were positive and negative group respectively.

Blastogenicity assay in vivo

the indurations were measured 18 hrs post injection .To stop cell cycle ,100mg/ ml cholchicine in a rate of 0.25 ml per each animal was injected intramuscularly .One hour later ,femur bone was tremmed from both ends and 5 ml of sterile saline injected for bone marrow collection.Thick bone marrow smears were made and Giemsa stained for each animal ^[14].

RESULTS**Extraction and partial purification of Lipopolysaccharide from *A.hydrophila* And *V.fluailis***

The crude extracts of lipopolysaccharide (300µg) were extracted from 20gm (wet weight) of bacterial cells from each of *A.hydrophila* And *V.fluailis* ,separately by hot phenol water method. After that the crude extractes were partialy purified by sephadex G 50 column .The results revealed that the presence of two peaks of LPS for each isolates figures (2) and (3).Then the carbohydrate amounts and protein concentrations were estimated for each peaks .tables (1) and (2) .The results were showing the presence combination between carbohydrate and protein . While the DNA and RNA contents of lipopolysaccharide in the separated parts at a wave length of 260 nm were fallowed up and results of this study showed that their rate was not significant

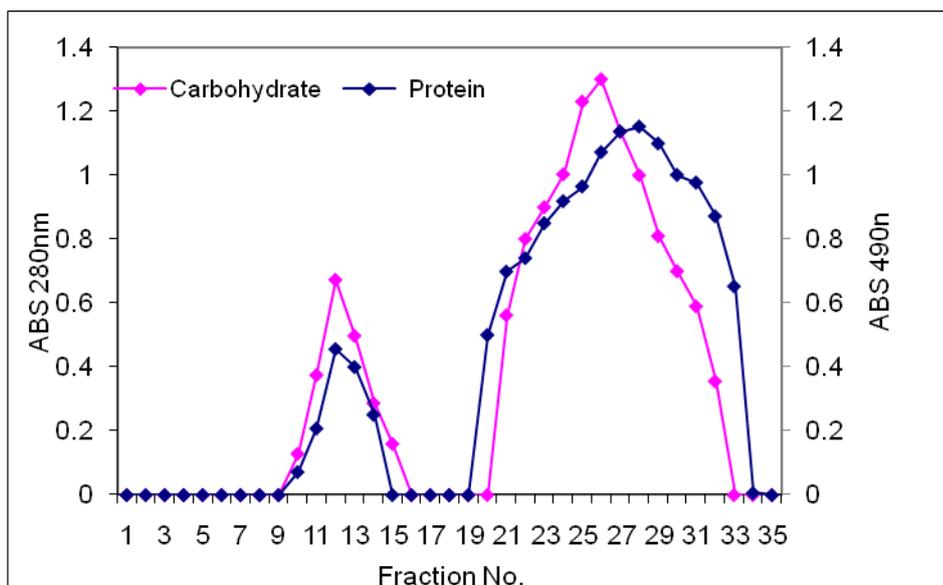


Figure (2) Gel filtration of lipopolysaccharide extraction from *Vibrio fluvialis* with flow speed 17 ml/hr on the basis of 2 ml/part

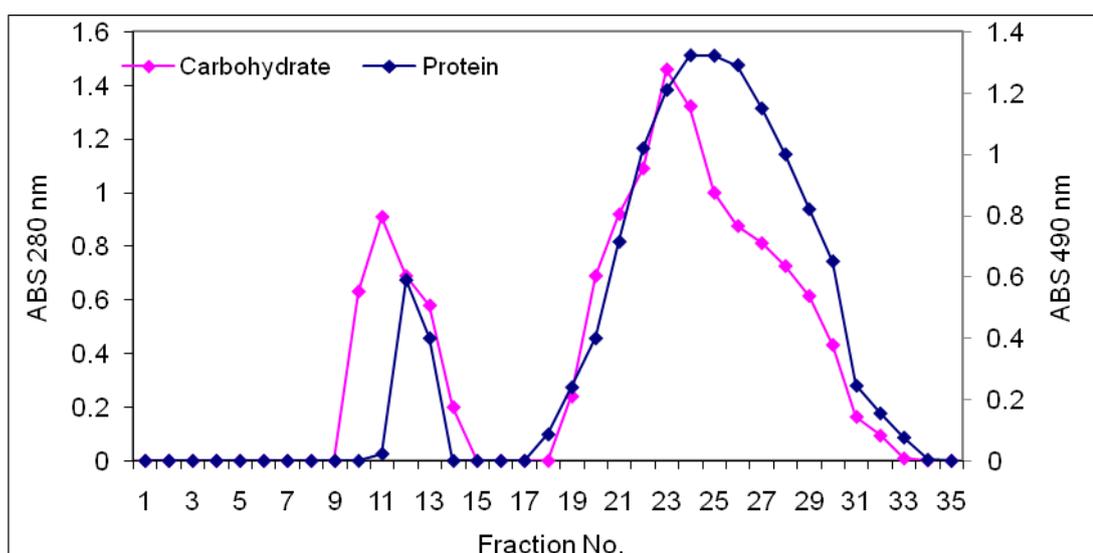


Figure (3) Gel filtration of lipopolysaccharide extraction from *A. hydrophila* with flow speed 17 ml/hr on the basis of 2 ml/part.

Carbohydrates

The concentration of carbohydrates (as compared to glucose standard) in crude lipopolysaccharide of *A. hydrophila* was 26.8485 $\mu\text{g/ml}$ while it's concentration in partially purified lipopolysaccharide in primary peak was 0.703 $\mu\text{g/ml}$ and in secondary peak was 1.051 $\mu\text{g/ml}$ figure (4). Also showed that the concentration of carbohydrates in crude lipopolysaccharide of *V. fluvialis* was 24.8993 $\mu\text{g/ml}$. While it's concentration was in partially purified lipopolysaccharide in primary peak was 0.458 $\mu\text{g/ml}$ and in secondary peak was 1.094 $\mu\text{g/ml}$. (table 1).

Table (1) the concentration of crude LPS and partial purified LPS for *A. hydrophila* and *V. fluvialis* ($\mu\text{g/ml}$).

Bacteria spp	Crude LPS concentration $\mu\text{g/ml}$	Partial purified LPS concentration	
		Primary peak $\mu\text{g/ml}$	Secondary peak $\mu\text{g/ml}$
<i>A. hydrophila</i>	26.8485	0.703	1.051
<i>V. fluvialis</i>	24.8993	0.458	1.094

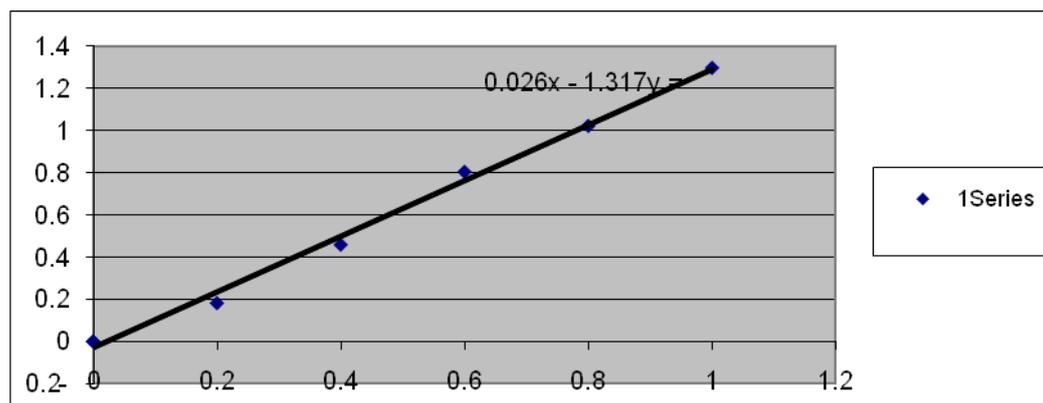


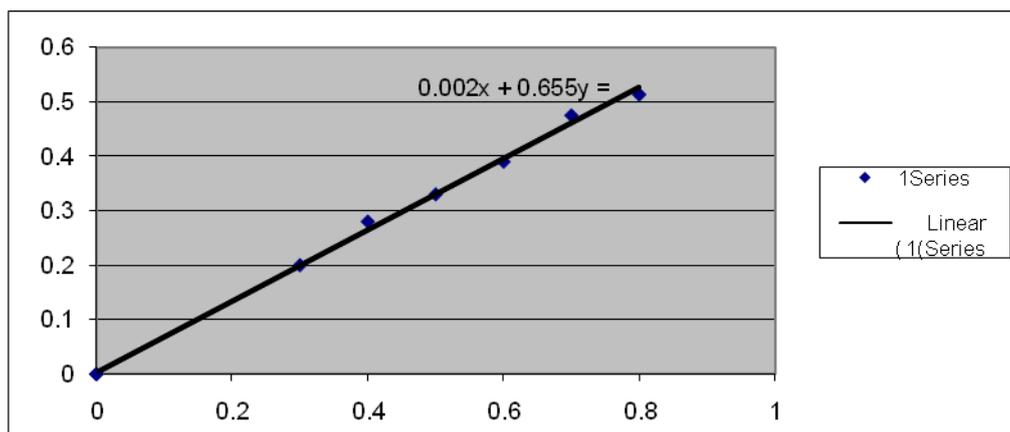
Figure (4) standard curve for carbohydrate using glucose Dubois *et al.*, (12) .

Estimation of protein

The protein concentration of crude lipopolysaccharide of *A. hydrophila* was $9.18 \mu\text{g/ml}$ while it's concentration in partially purified lipopolysaccharide in primary peak was $0.277 \mu\text{g/ml}$ and in secondary peak was $1.085 \mu\text{g/ml}$ figure (5). Whereas, the estimation study showed that the protein concentration of crude lipopolysaccharide of *V. fluvialis* was $8.8 \mu\text{g/ml}$ and it's concentration in partially purified lipopolysaccharide in primary peak was $0.449 \mu\text{g/ml}$ and in secondary peak was $0.948 \mu\text{g/ml}$ as compared to protein standard curve Table 2.

Table (2) the concentration of crude LPS and partial purified LPS for *A. hydrophila* and *V. fluvialis* .

Bacteria spp	Crude LPS concentration $\mu\text{g/ml}$	Partial purified LPS concentration	
		Primary peak $\mu\text{g/ml}$	Secondary peak $\mu\text{g/ml}$
<i>A. hydrophila</i>	9.18	0.277	1.085
<i>V. fluvialis</i>	8.8	0.449	0.948



Bradford (13). Figure (5):- standard curve for protein using bovine serum albumin

Mitogenicity

The mitogenicity of *A. hydrophila* LPS in bird was shown through skin induration and blastogenicity percent for both fraction 1 and fraction 2. The skin indurations were 0.8 ± 0.18 , 0.95 ± 0.11 for fraction 1 and fraction 2 LPS respectively. While blastogenicities were 1.95 ± 0.6 , 1.825 ± 0.4 for fraction 1 and fraction 2 LPS accordingly Table 3.

Table(3) :- the mitogenicity of F1 , F2 LPS of *A. hydrophila* in bird through skin induration and blastogenicity.

Test modulant	Skin induration	blastogenicity
<i>A. hydrophila</i> F1 LPS	0.8 ± 0.18	1.95 ± 0.6
<i>A. hydrophila</i> F2 LPS	0.95 ± 0.11	1.825 ± 0.4
Control tuberculin 0.05 IU Size 0.1	2.2 ± 0.199	0.06 ± 0.00004

Table(4) :- the mitogenicity of F1 , F2 LPS *V. fluvialis* in bird through skin induration and blastogenicity .

Test modulant	Skin induration	blastogenicity
<i>V. fluvialis</i> F1 LPS	1.2 ± 0.14	2.4 ± 0.9
<i>V. fluvialis</i> F2 LPS	1.6 ± 0.18	2.725 ± 0.18
Control tuberculin 0.05 IU Size 0.1	2.2 ± 0.199	0.06 ± 0.00004

The mitogenicity of LPS of *V. fluvialis* in bird was measured through skin induration and blastogenicity percent for both fraction 1 and fraction 2. The skin indurations were 1.2 ± 0.14 , 1.6 ± 0.18 for fraction 1 and fraction 2 LPS respectively. While blastogenicity were 2.4 ± 0.9 , 2.725 ± 0.18 for fraction 1 and fraction 2 LPS accordingly. Table (4)

The mitogenicity of *A. hydrophila* LPS in rat was shown as skin induration and blastogenicity percent for both fraction 1 and fraction 2. The skin indurations were 2.2 ± 0.7 , 2.32 ± 0.1 for

fraction 1 and fraction 2 LPS respectively. While blastogenicities were 3.60 ± 0.91 , 4.70 ± 0.9 for fraction 1 and fraction 2 LPS accordingly. **Table (5)**

Table 5:- the mitogenicity of F1, F2 LPS *A. hydrophila* in rat through skin induration and blastogenicity percent .

Test modulant	Skin induratin	blastogenicity
<i>A.hydrophila</i> F1 LPS	2.2 ± 0.7	3.60 ± 0.91
<i>A.hydrophila</i> F2 LPS	2.32 ± 0.1	4.70 ± 0.9
Control tuberculin 1IU Size 0.1	2.742 mm	3.532

The mitogenicity of LPS *V. fluvialis* in rat was showed the compared to skin induration and blastogenicity for both fraction 1 and fraction 2. The skin indurations were 2.7 ± 0.12 , 2.66 ± 0.13 for fraction 1 and fraction 2 LPS respectively. While blastogenicity percent were 3.8 ± 0.8 , 4.125 ± 0.8 for fraction 1 and fraction 2 LPS accordingly. **Table (6).**

Table 6 :- The mitogenicity of F1 , F2 LPS of *V. fluvialis* in rat was through skin induration and blastogenicity percent of the rat .

Test modulant	Skin induration	blastogenicity
<i>V.fluvialis</i> F1 LPS	2.7 ± 0.12	3.8 ± 0.8
<i>V.fluvialis</i> F2 LPS	2.66 ± 0.13	4.125 ± 0.8
Control tuberculin 0.05 IU Size 0.1	2.742 mm	3.532

DISCUSSION

The LPS are known as to be an essential component of the outer membrane of all gram negative bacteria which are often classified on the basis of the complex polysaccharides found on their surface^[1]. The standard hot phenol –water extraction method described by^[8] was used to extract LPS from approximately 20 gm (wet weight) of *A. hydrophilla* and *V.fluvialis* and that yield of 300 mg of crude LPS and to assess the purity of LPS after gel-chromatography filtration, Figures (2)(3) shows two peaks of carbohydrates which that in contrast to [16] who showed only one peak of carbohydrate, also in contrast to^[17], and^[18], then fractions of peak 2 was selected depending on their high CHO levels with less level of proteins and nucleic acids as contaminant. The first peak (PSI) is high in hexose as well as Kdo content. This may be due to the presence of material coming from the complete LPS molecule whereas the second peak (PS II) with low hexose and high Kdo content may be from the incomplete LPS molecule having only the core oligosaccharide^[19] this may be due to their existence in multiple forms i.e. those consisting of complete LPS with lipid A, Core oligosaccharide, O-antigen and those LPS lacking the O-antigen portion^[20].

Using of EDTA and Lysozyme for lyses the cell wall in LPS extraction led to separate the cat ion like Ca^{+2} and Mg^{+2} which fixed LPS to other cell wall component and added RNase and DNase enzymes led to degrade the nucleic acids which all increased the purity of extracted LPS.

Chemical analysis of the fractions of peak 1,2 were appeared having CHO in purified LPS *A.hydrophila* 0.703 ,1.051 which was lower than in crud LPS 26.8485% while Chemical analysis of fractions of peak 1,2 were appeared that CHO in purified LPS *V.fluvialis*0.458 ,1.094 which was lower than in crud LPS 24.8993% Table 1. Some LPS molecules are highly attached to proteins and peptidoglycan which were not passed through gel filtration, and hence the purified LPS quantity lower than in crude LPS. The contaminated molecules in purified LPS were lower as much as 0.277, 0.449 for *A.hydrophila* and *V.fluvialis* protein respectively and no nucleic acids, these smaller amounts of protein as indication of accuracy and efficacy of gel filtration, and these results were in agreement with ^[16] who in a work concerning the range of protein in purified LPS has been extracted by hot phenol –water method. Small amount of residual proteins in purified LPS due to un used ultracentrifugation as well as some proteins which have low molecular weight and was highly attached to LPS molecule can passed through gel ^[1]

Mitogenicity can be assessed through an in vitro approach using cell culture methods with the classical blastogenicity slide test or radioactive thymidin assays^[15]. While on using the in vivo approaches workers have been putting forward number of animal models, like phytohaemagglutin in bird wing test, mouse foot pad, bat wing flap and rat foot pad [13,15,16,17], In these cases mitogenicity have been assessed as skin induration ,mitotic index and blastogenicity percentages^[14].

Mitogens are extracts from plants and bacteria that can stimulate lymphocytes to proliferate and undergo blast transformation. Some lectins, such as phytohemagglutinin A (PHA) and concanavalin A (Con-A) are mitogenic for T cells, but not for B cells. B lymphocytes are usually activated by high molecular weight substances with repeating units, e.g. LPS ^[20]. The mitogenic actions are related to the lipid A moiety of the LPS molecule. LPS exhibits mitogenic activity and induces polyclonal antibodies of murine B lymphocytes as it enables B cells to differentiate and multiply ^[21]. Lictin-like LPS skin test ,Tables (3), (4) (5),(6) were showed the mean thickness of skin of foot pad in LPS which increased significantly after 18 hrs than control group , this result was similar to that gained by ^[22,23] . Lictin-like

LPS skin reaction depend on ability and activity of lymphocyte to recognize antigen and secrete IL-1 which enhanced proliferation and differentiation of other T-cell into Th-cells which secrete IL-2 as a chemoattractive factor to attract macrophage around the area of activated T-cell which also secrete INF- gamma that enhancing the cytolytic activity of accumulated macrophages leading into skin thickness ^[24]. LPS was an excellent mitogen for B-lymphocyte as well as activated macrophage to secrete IL-1 which in turn enhance Th2 to release IL-4 and IL-5 to provoke B-lymphocyte proliferation and differentiation to plasma cell and producing antibodies ^[25].

Smooth LPS F1 and F2 of *V.fluvialis* and *A.hydrophila* were found mitogenic in bird wing test and rat foot pad reactions they cause induration of the skin and bone marrow lymphocyte blastogenicity percentages higher or within the control limits. These F1, F2 preparations were T lymphocyte mitogens in the bird model, T and B lymphocyte mitogens in rat model. Hence, they were being lymphocyte mitogens not for bird but also for mammals. Apotentials that can be considered as crossing the species barriers^[12,25,28,29].

CONCLUSIONS

1-Fraction 1 and fraction2 were purified to the limits of partial purity from both of smooth *V.fluvialis* and *A.hydrophila* clinical isolates.

2-F1 and F2 of these organisms were found lymphocyte mitogens in an invivo avian and murine models.

3-Mitogenicity in chicken model is of T lymphocyte nature and parallel to T cell potency.

4-Mitogenicity in rat is of T and B cell types

5-This mitogenicity is stable and crosses the species barrier.

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