Nat. Volatiles & Essent. Oils, 2021; 8(4): 12701-12715

NVEO
Natural Volatiles &
Essential Oils

Extraction And Purification Of Lipid A From Lipopolysaccharide Extracted From Proteus Mirabilis

Shaymaa Jabbar Hassoon , Aida B. Allawe

Department of microbiology -Collage of Veterinary medicine -University of DIYALA,Iraq

Department of microbiology -Collage of Veterinary medicine -University of Baghdad,Iraq

ORCID ID 0000-0002-0582-1006

Abstract

Current study included three parts: the first part is the isolation and identification ofOne case of Proteus mirabilis mixed with Pseudomonas and the other mixed with E.coli spp. And 1 case of Proteus mirabilis in pure form.from 50 samples were taken from infected ear of sheep. These isolates were identified by morphological examination and biochemical tests and vitak2 system. In the second part of this study, lipopolysaccharide (LPS) was extracted from Proteus mirabilis by using hot phenol method ,and after that crude extracted LPS purified by gel filtration chromatography using sephadex G-200 gel . 4 g. of partial purified LPS were prepared from cell of P. mirabiliscell , chemical analysis of LPS in 1 ml of crud and partial purified LPS showed that the carbohydrate percentages were (8, 17) % respectively ,while protein percentage were (14, 6) % respectively. The third part of this study Lipid A was separated from extracted lipopolysaccharide **by** A combination of mild-acid hydrolysis and solvent extractions (Bligh-Dyer) are used to liberate lipid A from covalently attached polysaccharide**,**830 mg of partial purified Lipid A were prepared from 1.5 g of lipopolysaccharide ..

Key words : Proteus mirabilis , lipid A , lipopolysaccharide

استخلاص وتنقية الدهون أ من عديد السكريد الشحمي المستخلص من بكتريا المتقلبة الرائعة

شيماء جبار حسون ,عايدة برع علاوي

فرع الاحياء المجهرية –كلية الطب البيطري – جامعة ديالى – العراق

فرع الاحياء المجهرية –كلية الطب البيطري جامعة بغداد – العراق

الخلاصة

تضمنت الدراسة الحالية ثلاث محاور :المحور الاول عزل وتشخيص حالة واحدة من بكتريا المتقلبة الرائعة مختلطة مع الزوائف الزنجارية واخرى مختلطة مع الاشريشيا القولونية وعزلة واحدة من المتقلبة الرائعة بشكل نقي من خمسين عينة

تم اخدها من ادان الاغنام المصابةهذهالعزلاتشخصتبالاعتمادعلىالفحص الشكليوالفحوصاتالبيوكيماوية ونظام الفايتك اما المحور الثاني من التجربة تضمن استخلاص عديد السكريد الشحمي من بكتريا المتقلبة الرائعة بطريقة الفينول الساخن ثم نقى جزئيا بواسطة كروموتوكرافيا الترشيح الهلامي باستخدام هلام السيفادكس تم تحضير 4 غم من عديد السكريد الشجمي المنقى جزئيا من خلايا بكتريا المتقلبة الرائعة اظهر التحليل الكيميائي في 1 مل من عديد السكريد الشحمي الخام والمنقى جزئيا حيث بلغت نسبة الكاربوهيدرات (17,8) على التوالي بينما البروتينات كانت (6,14) على التوالي .الجزء الثالث من هده الدراسة تم فصل الدهون أ من عديد السكريد الشحمي عن طريق مزيج من التحلل المائي الحمضي الخفيف واستخلاص بالمذيبات(بلاي –دير) المستخدمة لتحرير الدهون أ من عديد السكريات المترابطة باصرة تساهميه. 830 ملغم من الدهون أ حضرت من 1.5 غم من عديد السكريد الشحمي .

الكلمات المفتاحية / المتقلبة الرائعة , الدهون أ, عديد السكريد الشحمي

Introduction

The genus Proteus encompasses rod-shaped Gram-negative bacteria that belong to the family Enterobacteriaceae ,which collected with Providenciaand Morganellastrains create the tribe Proteeae(1).

Bacterium Proteus mirabilis is one of the most significant species belonged to the genus Proteus spp. are Gram-negative aerobic bacteria.. They are named based on their ability to undergo morphological changes of colonies. With peritrichouse flagella(2).

Proteus mirabilis possesses a numerous of virulence factors that would support to the events of inflammation factors such as the production hemolysin fimbria expression, flagella , urease production , movement in waves (swarming) ,the capacity of attaching to epithelial cell and amino acid deaminases in addition to the expression of lipopolysaccharide (LPS) antigens and capsular polysaccharides (CPSs), have been described in many studies (3)

The lipopolysaccharide (LPS) is a central component of the outer membrane in Gram-negative bacteria and frequently plays a key role in pathogenesis (4). The classical LPS molecule has a tripartite structure comprising (i) lipid A, the hydrophobic moiety that anchors LPS to the outer leaflet of the outer membrane; (ii) core oligosaccharide (herein core), which together with lipid A, contributes to maintain the integrity of the outer membrane; and (iii) O antigen polysaccharide or O antigen, which is connected to the core and consists of a polymer made of repeating oligosaccharide units in direct contact with the external milieu (4).. .

The LPS molecules only including lipid A and core oligosaccharides, are generally referred to as ‘rough’ and often called lipooligosaccharides, while the complete LPS capped with O antigen is called ‘smooth. (**5**).

Lipid A is, in normal circumstances, a phosphorylated glucosamine disaccharide decorated with multiple fatty acids. These hydrophobic fatty acid chains anchor the LPS into the bacterial membrane, and the rest of the LPS projects from the cell surface. The lipid A domain is responsible for much of the toxicity of Gram-negative bacteria.,(6).

The Lipid A moiety is a very conserved component of the LPS., However Lipid A structure varies among bacterial species and Lipid A structure defines an overall host immune activation. Discovery of lipid A is present mainly in Gram-negative bacteria; however, a recent study indicates it might exist in plants (7)

Proteus lipid A contains glucosamine disaccharide substituted with phosphate residues and fatty acids. It also contains 4-amino4-deoxy-L-arabinopyranose (L-Arap4N), which quantitatively substitutes the ester-linked phosphate residue of the glucosamine backbone. LPS forms of bacteria contributes to their resistance against bactericidal action of the serum (8).

Material And Method

Samples collection

Fifty samples were taken from infected ear of sheep ,right and left ears from different region of baqubah city , Samples were collected from different age groups in the period 1/12/2020 to 10/12/2021 After collection, all samples were cultured on blood agar media as enriched media and MacConkey agar as differentiated media and incubated at 37 ° C for 24 hr.

Identification of the isolates

Identification of suspected isolates was done according to the colony morphology,microscopic examination and biochemical tests (9). then confirmed by Vitek2 system .

LPS. Extraction:

LPS was extracted by hot phenol-water Method according to (10).

Purification of LPS by Gel filtration chromatography:

Partial purification of crude LPS was done by gel filtration chromatography using sephadex G-200 column the partial purified was applied to a sephadex G-200 column (3 by 50 cm) previously equilibrated with 0.025 M phosphate buffer, fractions were collected and

endotoxin was recovered by detecting the carbohydrate contents for each fraction according to the method of Dubois et al., (11) at 490 nm.

Isolation and Purification of Lipid A From LPS. (Proteus mirabilis)

A combination of mild-acid hydrolysis and solvent extractions (Bligh-Dyer) are used to liberate lipid A from covalently attached polysaccharide. Isolation of lipid a molecules from whole bacteria involves the extraction of LPS from the bacterial cell surface. Lipid A was isolated by mild hydrolysis of the water phase deriving LPS The hydrolysate was cooled and then converted into a two-phase Bligh-Dyer system, i.e., chloroform/methanol/ water , 2:2:1.8 (v/v/v), by adding adequate amounts of chloroform and methanol. The chloroform phase containing lipid A was separated by centrifugation and then washed twice with the aqueous phase from a freshly prepared two-phase Bligh–Dyer mixture [12]. The lipid A were dried byand then stored at – 20 C in chloroform/methanol (3:1, v/v). fig.(1).

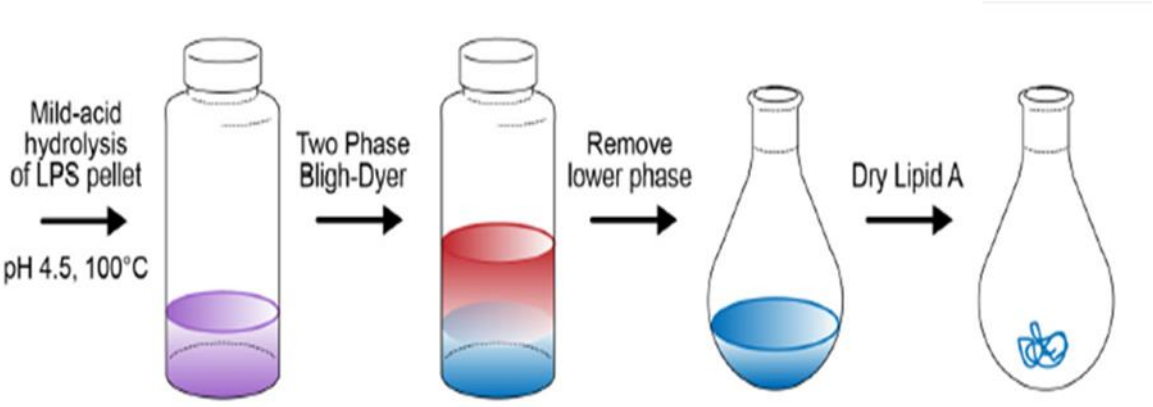


Fig.1 Schematic of lipid A isolation procedure.

FTIR (Fourier Transformed Infrared) Analysis

Nature and chemical structure of the active compound were examined using the Fourier transformed infrared spectroscopy (FTIR) in order to characterized the chemical nature of compounds. FTIR spectrometry, an advanced type of infrared spectrometry, which give the functional groups that are found in the compound in order to propose a chemical structure of the extract.

Results and discussion

Isolation of Proteus mirabilis

The results of current study showed that from 50 swabs collected from infected ear of sheep ,. Most samples showed growth of more than one species of bacteria on different

media.One case of Proteus mirabilis mixed with Pseudomonas and the other mixed with E.coli spp. And 1 case of Proteus mirabilis in pure form.table 1

Table (1): Number of Proteus mirabilis isolates from infected ear of sheep and goat .

origin	Sp.	No.
Ear	Proteus mirabilis. Mixed with Pseudomonas	1
	Proteus mirabilis	1
	Proteus mirabilis . mixed with E.coli	1

Pugh ,(13) reported that little is known about otitis in goat and sheep compared with the information available on cattle and horses, and many factors can predispose sheep to otitis including the anatomic orientation of the ear canal itself, the skin lining of the external ear canal has a large numbers of gland, these include modified porcine gland which produce large amounts of secretions which provide good suitable media for irritation and infection (14).

This result was agree with,(15) Who isolated Proteus mirabilis from infected right and left ear in sheep.In addition, (16) who was isolated Proteus mirabilis from ear of sheep and disagree with (17) who was no isolated Proteus spp. From sheep's otitis media .This may be due to the season of collecting samples and the possible medication taken before sampling.

4-1-2 Identification of Proteus mirabilis :-

Isolates which were identified as Proteus appeared as small pale colonies, little convex and circular with smooth edges on MacConkey's agar plates and were lactose non fermenter, so they turned the media to yellow . MacConkey agar was used for growing Proteus strains because it differentiates it from other Gram negative species and it contains all required nutrients for Proteus growth. In addition, Proteus culture has a special smell (fish smell). On blood agar Proteus isolates showed swarming motility which it is very significant character to differentiated it from other enterobacteriaceae family. Fig.(2)

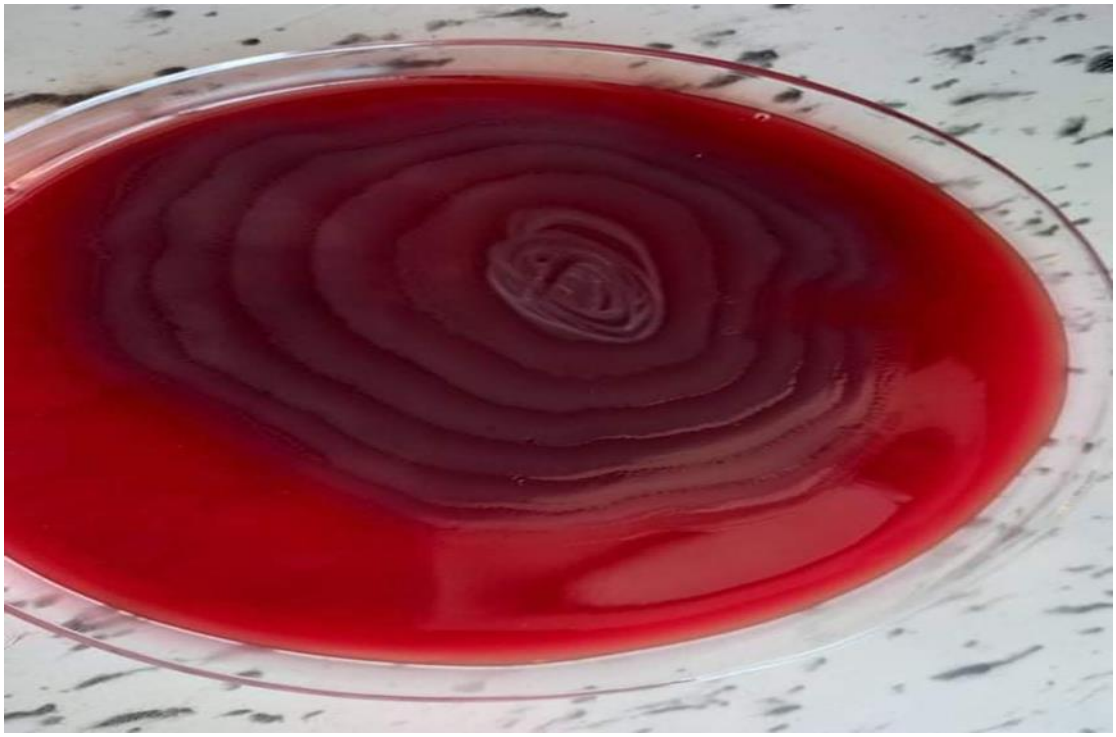


Fig.(2) Proteus mirabilis isolates showed swarming phenomena on blood agar

Several biochemical tests were achieved to identify the isolates of Proteus mirabilis , results mentioned in table (2) show that these isolates were positive for catalase, urease , Phenylalanine and gelatin but gave negative results for citrate utilization, oxidase and indole tests .

This bacteria showed its ability for H2S production when it was cultured on Triple sugar iron (TSI) agar while the slant turn to red, and its bottom was yellow this is because of the ability of ferment glucose only .

Table (2): Biochemical tests for Identification of Proteus mirabilis Result

Biochemical tests	**Results**
Indole	-ve
Catalase	+ve
citrate utilization	-ve
Oxidase	-ve
Gelatin	+ve
Urease	+ve
Phenylalanine	+ve
TSI	k/A H2S +

	Gas -

Confirmation diagnosis

For confirmation of the results, VITEK 2 system (ID) GN cards were used and the results are indicated in Table 3.

Table (3): Vitek 2 compact system test for proteus mirabilis isolates.

APPA	-	ADO	-	PyrA	-	IARL	-	dCEL	-	BGAL	-
H2S	+	BANAG	-	AGLTp	-	dGLU	+	GGT	+	OFF	-
BGLU	-	dMAL	-	dMAN	-	dMNE	-	BXYL	-	BAIap	-
ProA	-	LIP	-	PLE	-	TyrA	+	URE	+	dSOR	-
SAC	-	dTAG	-	dTRE	+	CIT	+	MNT	-	5KG	-
ILATk	-	AGLU	-	SUCT	+	NAGA	-	AGAL	-	PHOS	+
GlyA	-	ODC	+	LDC	-	IHISa	-	CMT	+	BGUR	-
O129R	+	GGAA	-	IMLTa	-	ELLM	-	ILATa	-		

(–) a negative result, (+) a positive result

4-2extraction and purification of lipopolysaccharide

According to the hot phenol method the extraction of LPS was prepared . Harvest of 4 g. of partial purified LPS were prepared from cell of P. mirabilis.. Chemical analysis of the LPS extracted from Proteus mirabilis were accomplished by calculating the carbohydrate contents according to Dubois method (11), and calculate the protein contents according to Bradford method (18).

The crude LPS was partially purified by gel filtration chromatography via sephadex G-200 column The 40 collected fractions were first subjected for the determination of protein through reading the absorbance of each fractions at 280 nm as submitted by (19). Afterward, each fraction was handled by a method of phenol-sulphuric acid (11) to calculate carbohydrate content, then the absorbance was read at a wave length of 490 nm. The correlation between absorbency and fractions number of both concentration (protein and carbohydrate) was drawn figure (3).

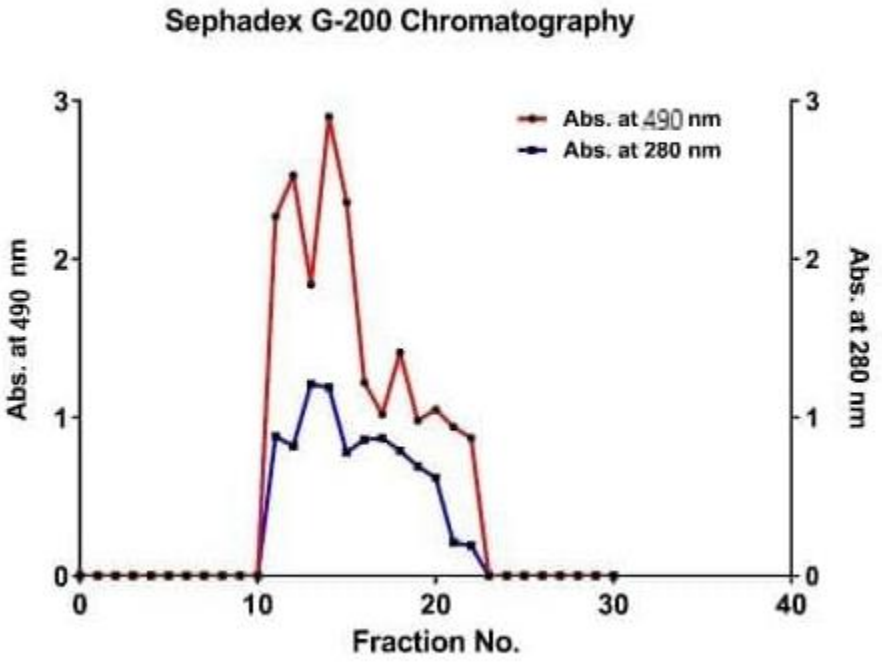


Figure (3) shows relationship between absorbency and fraction number of each component (protein and carbohydrate) .

in the current study the concentration of carbohydrates in the crude lipopolysaccharide is 55 (8%)mg\ml and in the partially purified lipopolysaccharide is 120 (17%) mg\ml table (4)

Figure(3) shows that at 490 nm one peak (red color) were saw for carbohydrate. This peak has protein components associated with LPS(blue color) and difficult to separation from it

Table (4): The biological activity of carbohydrate and protein contents of crude and partially purified LPS.

LPS	Protein Concentration mg/ml	Charbohy. Concentration mg/ml
Crude LPS	**112 (14 %)**	**55 (8%)**

Partially Purified	**40 (6%)**	**130(17%)**

The extraction introduced by the hot phenol method is still the most frequent procedure employed for LPS extraction not only for its simplicity of application, but is one of the few methods that can extract lipopolysaccharide from smooth and rough bacteria and this method of extraction provided high-quality LPS (20) .

The presence of protein with carbohydrate in the LPS extract in results of current study was close to what many researchers have found, and that the presence of protein with LPS is an attribute inherent to and difficult to separate from (21)

The differences in the protein and carbohydrate percentages in the purified LPS may be related to the differences in the bacterial strains and their content of LPS, the differences in the methods used in extraction and purification of LPS and the experiments circumstances(22).

.Most studies are in favor of that the percentage yield of carbohydrates and proteins following endotoxin purification may vary widely.

Al-Muslemawi, (23) who found the concentration of carbohydrates in the crude LPS is 300 mg\ml and in the LPS partially purified is 315mg\ml a high percentage of carbohydrates were observed in partially purified extract, as it reached 86% while it was in crud77%.

These results also close to a number of local studies conducted on lipopolysaccharide extracted from other bacterial types.(24) found that the percentage of carbohydrates in the crude and partially purified extracts of the LPS of **Citrobacterfreundii**was 12% and 16%, respectively.(25) found that the amount of carbohydrates in the crude extract of LPS for Proteus mirabilis 153 mg\ml and in the partially purified extract 221mg\ml. The increase in the percentage of carbohydrates in the purified extract indicates the efficiency of the purification process used in removing other cellular contaminant or debris, which has a role in increasing the degree of purity.

In our studythe protein concentration it was found that the percentage of proteins in the partial purified LPS 6% while in crud LPS 14%. (23) who found that the percentage of protein decreased in the purified LPS from what it was in the crude extract of LPS, as it reached in

the LPS partially purified 13.1% and crud 22%, while (24) found that the percentage of protein in the crude and partially purified extracts of the LPS of **Citrobacterfreundii**was 22% and 9 %, respectively. (26) found that the endotoxin of Pseudmonas aeruginosa contains 72% carbohydrates, 5.8% proteins and 2.2% of nucleic acids, knowing that it did not use DNase enzymes RNase and Protease in the extraction and purification process.

Extraction of lipid A from LPS

A combination of mild-acid hydrolysis and solvent extractions (Bligh-Dyer) are used to liberate lipid A from covalently attached polysaccharide.mild acid treatment of LPS splits it into polysaccharide and lipid A moieties TNF-inducing activity was co-fractionated with lipid A in the precipitated fraction. , in contrast , Alkaline hydrolysis of LPS, which cleaves the ester bond and releases fatty acid from the lipid A moiety of LPS and decreased the TNF-inducing activity to LPS.((27). this result agreement with (28) who extraction of lipid A from LPS by mild acid hydrolysis.

In our result Harvest of 830 mg of partial purified Lipid A were prepared from 1.5 g. of lipopolysaccharide .The amounts of lipid A extraction and isolation can also be adjusted depending on the bacterial strain you are working with.

Bligh-Dyer extractions have been historically utilized for the isolation of whole lipid species from various types of preparations (e.g., animal tissue, plant tissue, etc.). The Bligh-Dyer method consists of multiple solvent extractions using chloroform, methanol and water to extract the lipid species in the organic phase. Unlike other methods, this does not select for rough- or smooth-types of LPS, providing optimal recovery of lipid A species. (29).

The amount of lipid A extract depended on bacterial strain . For instance, V. cholerae requires at least 200 ml of culture in order to obtain high quality mass spectra; however, More starting material (larger culture volumes) is required for some bacterial species .A near result was recorded by(**30**) who obtained 36 mg. of lipid A from 120 mg. of LPS.

Fourier Transformed Infrared (FTIR) Analysis

The FTIR spectrum used to identify the functional group of the active compound present in the extracts of the lipid A based on the peaks value in the region of IR radiation.(31). When the lipid A extract passed into the FTIR, the functional groups of components separated based on its peaks ratio. Table (5), fig (4) there are many active group present in lipid A extract which include :

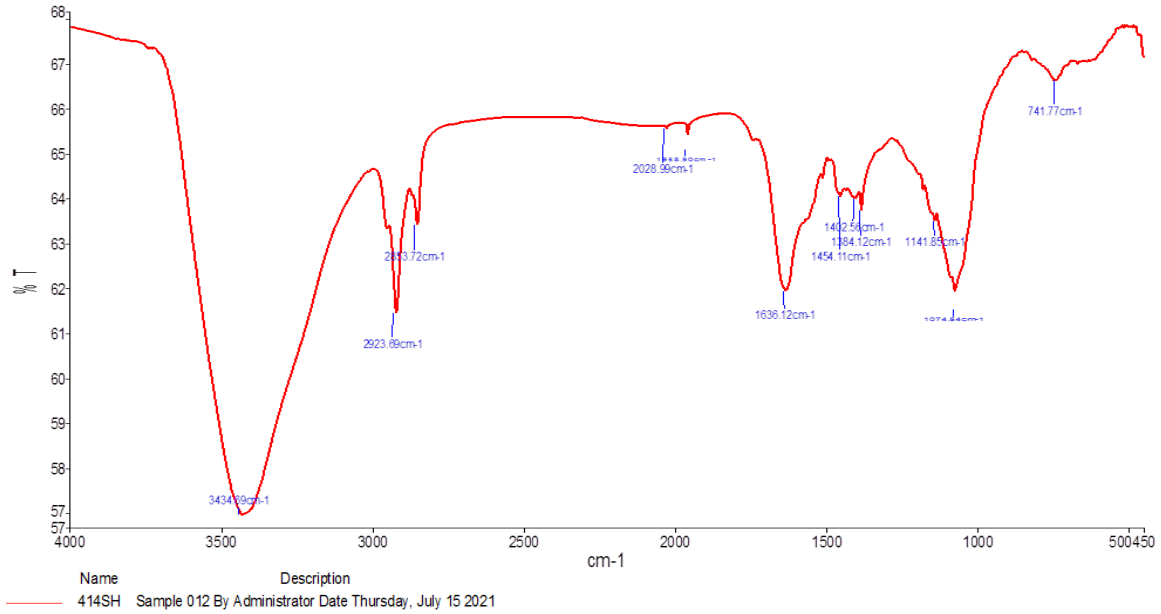
The active functional group (2923.69 cm−1) this belong to C-H stretching bands in malignant and normal tissues, 2853.72 Asymmetric CH2 stretching mode of the methylene chains in membrane lipids(32).the active group 1636.12 belongs to carbonyl group (C=O) , but the peak value of1636.12 represented to C–O–C (CH3) asymmetric bending vibration lipids. The other active component (1344.12) which represented the organic phosphates (P=O stretch) , broad absorption band in the range of between 3650 and 3250 cm-1 this band confirms the existence of hydroxyl (-OH) (33).

The lipid A is represented by stretching vibrations of C–H (2820–2940 cm−1; 1460–1470 cm−1) , Organic phosphates (P=O stretch) (1350-1250 cm−1) (31)

Asymmetric CH2 stretching mode of the methylene chains in membrane lipids (2853-2860 cm−1) .show a relative increase in fatty acid.(34).

(Table 5) FTIR Peaks Values and Functional of lipid A extract.

Peak Number	X (cm-1)	Y (%T)
1	3434.69	56.95
2	2923.69	61.48
3	2853.72	63.46
4	2028.99	65.59
5	1958.90	65.46
6	1636.12	61.97
7	1454.11	64.08
8	1402.56	64.04
9	1344.12	63.77
10	1141.85	63.54
11	1074.54	61.96
12	741.77	66.67



(Fig.4) FTIR analysis of lipid A extracts

CONCLUSIONS

In this study, P. mirabilis is the species implicated in otitis media in sheep and isolation of lipid A from extracted Lipopolysaccharide for to use in other research because it known as the immune modulator and trigger the immune response

Acknowledgements

My sincere thanks and respect to my supervisor, and I thank diyala university for helping me .

References

1-Penner, J. L. “Genus XXIX. Proteus,” in Bergey's Manual of Systematic Bacteriology. The Proteobacteria: Part B, the Gammaproteobacteria, 2nd Edn., eds(2005).

3-Drzewiecka, D. Significance and roles of Proteus spp. bacteria in natural environments. Microb. Ecol.(2016) 72, 741–758. doi: 10.1007/s00248-015-0720-6

2-Schaffer, J. N., & Pearson, M. M.. Proteus mirabilis and urinary tract infections. Urinary Tract Infections: microbial spectr October (2015) 3(5) 383-433doi 10.1128/microbiolspec.UTI-0017-2013

4-Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem. (2014);83:99–128 .https://doi.org/10.1146/annurev-biochem-060713-035600.

5-Casey A, Fox EM, Schmitz-Esser S, Coffey A, McAuliffe O, Jordan K. Transcriptome analysis of Listeria monocytogenes exposed to biocide stress reveals a multi-system response involving cell wall synthesis, sugar uptake, and motility. Front Microbiol. 2014;5:68.doi.org/10.3389/fmicb.2014.00068

6-VishnuPriyaSampath. Bacterial endotoxin-lipopolysaccharide; structure, function and its role in immunity in vertebrates and invertebrates. Agriculture and Natural Resources Volume 52, Issue 2, April 2018, Pages 115-120https://doi.org/10.1016/j.anres.2018.08.002

7-Brade H, Opal SM, Vogel SN, Morrison DC, editors. Endotoxin in Health and Disease. Marcel Dekker, Inc.; New York: 1999. p. 950..

8-kaca, w., arabski, m., fudała, r., holmstrom, e., sjoholm, a., weintraub, a., futoma-kołoch., b., bugla-płoskońska, g., doroszkiewicz, w.. Human complement activation by smooth and rough Proteus mirabilis lipopolysaccharides. Arch. Immunol. Ther. Exp.(2009) 57: 383–391DOI:10.1007/s00005-009-0043-8

9-Betty, A. F., D. F. Sahm and A. S. Weissfeld.. Bailey and Scott's Diagnostic Microbiology. 12th ed. Mosby, Inc., Elsevier Inc., Missouri.(2007)

10-Westphal O., Luderitz O., Eichenberger E. and Keiderling W Bacterial Lipopolysaccharide: Extraction with phenol –water and further application of the procedure, Methods Carbohydrate Chemo., (1952) 5, 83-91**https://doi.org/10.1021/ed040pA394**

11-Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A. and Smith, F Colorimetric methods for determination of sugars and related substance. Anal. Chem.,(1965) 28, pp: 350-356.https://doi.org/10.1021/ac60111a017

12-Komaniecka, I.; Choma, A.; Lindner, B.; Holst, O. The structure of a novel neutral lipid A from the lipopolysaccharide of Bradyrhizobiumelkanii containing three mannose units in the backbone. Chem. Eur. J.(2010) 16, 2922–2929.doi: 10.1002/chem.200902390.

13-Pugh DG. Sheep and Goat Medicine. 4th ed. Philadelphia: WB Saunders Com2000.

14-Radostits OM, Gay CC, Blood DC and Hinchcliff KW. Veterinary medicine: A textbook of the diseases of Cattle, Sheep, Pigs, Goats and Horses. 9th ed. Philadelphia: WB Saunders Com2000.

15- AL-Hayyawi S.M. Comparison of microbial isolates isolated from external ear canal of sheep and their susceptibility to antibiotics Proceeding of the Eleventh Veterinary Scientific Conference(2012)pp. 41-48 **DOI:** https://doi.org/10.30539/iraqijvm.v36i0E.379

16-TiberiuConstantin,KassemHoushaimy and DorinŢogoe Medical and Surgical Management of Otitis in Sheep-case Report . Agriculture and Agricultural Science Procedia Volume 10, 2016, Pages 390-395https://doi.org/10.1016/j.aaspro.2016.09.079

17- Al-Farwachi M. I and Al-Hassan M. M.(2008) Clinical and microbiological study of otitis externa in sheep .Iraqi Journal of Veterinary Sciences, Vol. 22, No. 1, 2008 (43-48). DOI:10.33899/ijvs.2008.5669

18-Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annu. Biochem.,(1976) 72, pp: 248-254.DOI: 10.1006/abio.1976.9999

19- Bruck, C.; Portetelle , D.; Jlineur, C.andBollen, A. One-step purification of mouse monoclonal antibodies from ascetic fluid by DEAE affinity gel blue chromatography. J. Immunal. Methods.(1982), 53:313-19.doi/10.1016/0022-1759(82)90178-8

20-Rezania,S.; Amirmozaffari ,N.; Tabarraei ,B.; Jeddi-Tehrani ,M.; Zarei,O.; Alizadeh ,R.; Masjedian ,F. and Zarnani ,A.H Extraction, Purification and Characterization of Lipopolysaccharide from Escherichia coli and Salmonella typhi. Avicenna J. Med. Biotech .(,2011) 3(1): 3-9. https://pubmed.ncbi.nlm.nih.gov/23407691/

21-Raghad, Q. M. and May,T. F. Extraction and purification of lipopolysaccharide from KlebsiellaAoxitoka and study of its role in the their pathogenicity. Iraq. J. Phys.(2012), 53(2) 271-278.

22-Farah T. O. Immunogenic activity of Liposome incorporated Lipopolysaccharide Antigen of Salmonella entericaserovarTyphimurium in Mice Master Thesis .College of Science, University of Baghdad.(2005)

23- Al-Muslemawi, J.A.T. . Study of some biochemical, biological and pathological properties of lipopolysaccharide extract from Citrobacterfreundii. (Ph.D. dissertation). College of Science. University of Baghdad. Iraq.(2007).

24- Roua J.M. Isolation and Identification of Citrobacterfreundii From Sheep with Investigation the Immunological Responses in Rabbits.(Ph.D. dissertation). College of veterinary medicine . University of Baghdad. Iraq(2021)

25- Malik ,S.N.Extraction and Purification of Lipopolysaccharide of Localy Isolated Proteus and Study of its effect to Prevent Urinary tract infections in an animal model (Master Thesis.) thesis University of Baghdad.(2006)

26-Pier, G.B., Sidberry, H.F. and Sadoff, J.C. (1981). High molecular weight polysaccharide antigen from Pseudomonas aeruginosa, Immunotype 2. Infect. Immun. 34(2): 461-8.DOI: 10.1128/iai.34.2.461-468.1981

27-Nowotny, A., Behling, U.H. & Chang, H.L. Relation of structure to function in bacterial endotoxins. VIII. Biological activities in a polysaccharide-rich fraction. J. Immunol.,(1975) 115, 199-203.https://pubmed.ncbi.nlm.nih.gov/239055/

28-Katsuyuki Haranaka, Nobuko Satomi, Akiko Sakurai and Otomko Kunii Role of Lipid A in the Production of Tumor Necrosis Factor and Differences in Antitumor Activity between Tumor Necrosis Factor and lipopolysaccharide, Tohoku J. exp. Med., 1984 144, 385-396https://www.jstage.jst.go.jp/article/tjem1920/144/4/144_4_385/_pdf

29-Jessica V. Hankins, James A. Madsen, Brittany D. Needham, Jennifer S. Brodbelt, and M. Stephen Trent .) the Outer Membrane of Gram-negative Bacteria: Lipid A Isolation and Characterization Methods Mol Biol.(2013) ; 966: 239–258. doi:10.1007/978-1-62703-245-2_15.

30- IwonaKomaniecka 1,* , KatarzynaSusniak 1 , Adam Choma 1, Holger Heine 2 and Otto Holst**The Lipid A from the Lipopolysaccharide of the Phototrophic Bacterium Rhodomicrobiumvannielii ATCC 17100 Revisited**

Int J Mol Sci. 2021 Jan; 22(1): 258doi: 10.3390/ijms22010258

31-Sanjai J. Parikh, Jon ChoroverInfrared spectroscopy studies of cation effects onlipopolysaccharides in aqueous solution Colloids and Surfaces B: Biointerfaces 55 (2007) 241–250.doi: 10.1016/j.colsurfb.2006.12.014.

32-. Schulz, H., and Baranska, M. Identification and quantification of valuable plant substances by IR and Raman spectroscopy. Vibrational Spectroscopy, (2007)43(1), 13-25.doi.org/10.1016/j.vibspec.2006.06.001

33- Coates, J. Interpretation of infrared spectra, a practical approach. Encyclopedia of analytical chemistry, (2000)12, 10815-10837.https://analyticalscience.wiley.com/do/10.1002/sepspec.10120education/full/i97dca9608c7bfa88fcf79f9b29f68226.pdf

34-Fung, M. F. K., Senterman, M. K., Mikhael, N. Z., Lacelle, S., and Wong, P. T.. Pressure-tuning fourier transform infrared spectroscopic study of carcinogenesis in human endometrium. Biospectroscopy, (1996)2(3), 155-165.**https://doi.org/10.1002/(SICI)1520-6343**