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Exopolysaccharide vaccine extracted from *C.freundii* induce immunity against infectional pathogens

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Abstract

Eleven isolates of Citrobacter. *freundii* were isolated from100 stool and urine specimens taken from diarrhea and recurrent –urinary tract infections (UTI) patients attended to AL-Sadar Hospital. specimens were cultured on specific media, then bacterial isolates were identified depending on morphological, biochemical and VITK-2. The results showed that the C. *freundii* comprise 11 / 78 (14.1 %) of total of positive bacterial growth on macConky agar from which 10.2 % isolated from patients suffering from diarrhea and 3.8 % isolated from UTIs . The results of determine efficient isolate in exopolysaccharide(EXP) production revealed that C. *freundii* were isolated from recurrent -UTI was more mucoidy colonies and appeared highly biofilm formation with absorbancy 0.890 . The results of LD50 observed that a live suspension of C. *freundii* was 1×10^7 ,while HK *E,coli* was 2.3×10^8 finally EXP was 390 mg/ml .The immunomodulatory activity appear that EXP relaease moderate levels of proinflammatory biomarker (IL-6 , TNF-α and IL-12) that enable immune system to clear pathogen . While HK *E.coli* and whole bacteria release higher proinflammatory biomarker especially during post-dose that lead to increased the hypersensitivity and hyperresponses of immune system. In other hand EXP stimulate release moderate concentration of anti-inflammatory biomarker IL-10 and higher eleveated during post dose but HK *E.coli* and whole bacteria higher concentration during primary dose while in post dose lower than EXP vaccine. A significant immunogenicity and effective protection was observed in immunized groups with EXP challenged with 100 of LD50 of a live C. *freundii* compared with control group of mice which died within first days .

Key word: exopolysaccharide , *Citrobacter* infection , immunomodulater activity , cytokine

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INTRODUCTION

Citrobacter freundii is a gram-negative motile facultative anaerobic bacteria belonging to genus *Citrobacter* a members of the *Enterobacteriaceae* family. widely spread in environment such as water, soil and food, usually regarded as commensal species in intestinal tracts of animals and humans(1) . *C. freundii* were previously considered non-pathogenic colonizers but known to be opportunistic pathogens, they can cause serious infections of respiratory tract , urinary tract and blood stream, endocardium, meninges especially in high-risk groups such as infants and immunocompromised adults (2,3,4) . Associated with well-documented nosocomial outbreaks (5,6)

Increase the prevelance of *C. freundii* in nosocomial and community acquired infections as well as emerging of multidrug-resistant (MDR) strains increase attention on *Citrobacter* (7). So to avoid failure to prevent recurrent infections and allergic reaction to certain antibiotics lead to seeking about a new therapeutics as alternative therapy to controlling on infection caused by *C. freundii* which has become a high priority of drug development research.

Vaccination by antigenic materials extracted from pathogenic bacteria that stimulated immunity for prevention diseases caused by pathogens are one of the most promising approaches against infections (8). The activity of cell free culture extracted from *C. freundii* in induce both systemic immunity and humeral immunity was confirmed by (4) . Other study demonstrated the ability of Vi-polysaccharide extracted from *C. freundii* as a new vaccine to protect mice against challenge with Vi- positive *salmonella* enterica serovar Typhimurium (9). Different vaccines have been developed using Enterotoxigenic *E.coli* strains ,these vaccines may consist of bacterin (10) or purified fimbriae (11) as therapeutic strategies against severe diarrhea . Also, mucoid polysaccharide vaccine prepared from *P.aerogenosa*

to treatment chronic pulmonary infection ,this material characterized as active immunomodulater materials, safe and non-toxic (12)

Microbial polysaccharides represent essential components that transformed planktonic (free-living) bacteria to biofilm (aggregate) form by adherence bacteria to host cells and subsequently facilitate pathogen persistence (13) . Biofilm is a complex structure made of aggregates of microbial cells within a matrix of extracellular polymeric substances (EPS) which provides the biofilm with mechanical stability through viscoelastic properties and polysaccharides form the backbone of EPS where other components like proteins, nucleic acids and lipids can be included (14) . Polysaccharides play role as immunomodulatory activity through moderate improve in both cellular and humoral immune responses (15). .So , the study aimed to evaluate the immunomodulater activity of exopolysaccharide (EXP) extracted from C. *freundii* to prevent infection and protect the infected host from hyperimmune response that lead to destruction of infected tissue.

MATERIALS AND METHODS

Bacterial Characterization

A total of 100 stool and urine specimens (50 for each) were collected under aseptic condition from patients attending to Al-Sadar Medical City in AL_Najaf provinace were inoculated on MacConkey agar and XLD agar (Oxoid Cambridge, UK) and incubated at 37 C° for 24 h. The morphological characteristics of the colonies including size, shape, colour, were recorded , the suspected *Citrobacter* were relevant by biochemical test (16) , finally confirmed by using Vitek-2 Compact(Bio Mérieux,France,). *E.coli* obtained from microbiology laboratory college of science , Kufa university and reidentifecation by Vitek-2 system to use in heat killed vaccine preparation.

2- Detection efficient isolate in EXP production

The efficient isolate was selected based on ability to form biofilm which tested by tissue culture method TCP (17). As well as the mucoidal morphology that regard as fundamental screening for isolated EPS producing bacteria (12) .

3- extraction of exopolysacharide (EPS)

According to (13) the chosen isolate incubated at 37 C° overnight in Brain heart infusion (BHI) broth, then utilized to inoculate a fresh broth culture (1:100 v=v), that incubated in shaking incubater at 37 C^0 for 72 h. Then , centrifuged at 5000 rpm at 4 C° for 20 min. After that , each 10 ml of pellet were mixed with 60 ml of formaldehyde (36.5% solution) to prevent cell lysis during extraction process . The mixture was incubated at room temperature in a rotary shaking incubater (100 rpm) for 1 h. 50 mg of Trichloroacetic acid (20 % w: v) was added for each 1000 ml of suspension to precipitate protiens and nucleic acid , then incubated at room temperature, with shaking, for 3 h to extract EXP vaccine. Cell suspensions centrifuged at 16,000 xg for 20 min at 4 C°. The supernatant containing soluble EXP vaccine was precipitated with 3 volumes of cold ethanol to precipitate lipids , the mixature was centerfuged 16,000 xg for 20 min at 4 C° and the pellet suspended in water . filtered through a 0.2 mm filter (Corning) and dialyzed against distilled water using a 12–14 kDa molecular weight cut-off (MWCO) membrane for 24 h at 4 C°. Quantification of total carbohydrate levels was performed according to (18) using a phenol-sulfuric acid method in microplate format.

E.coli was cultured at 37 for 24 h after that , centrifugation in 3000 rpm for 15 min , then washed 3 times in phosphate –buffered salin (PBS) and adjusted to concentration 1× 10^8 , finally killed by heating in water bath at 80 C° for 1 h (19).

Sterility was determinated by culturing of both vaccines on blood and nutrient agar , while to detect safety by injected 3 rats with 1 ml of each vaccine for 1 week and monitoring clinical feature in rats finally the toxicity of EXP was detected according to (20)

Determine of Median lethal dose 50%(LD_{50}) for *C. freundii* suspension

The viable count of *C. freundii* was done by bacterial plate count method in six fold dilution (10^{-5}-10^{-10}) according to (21). LD_{50} were determined by using 30 albino rats each (20-25) g, which divided to six groups each group has five rats. Each group injected intraperitoneally (i.p.) with one of each bacterial suspension concentration (10^5,10^6,10^7, 10^8, 10^9 and 10^{10}) cfu /ml. In addition to five mice injected with 1ml of PBS as negative control. After 5 days LD_{50} was determined according to (22). The same methods were used to estimation LD_{50} for HK *E.coli* . The equation for calculate LD_{50}

Proportion distance = 50 – mortality below 50% / mortality above 50% - mortality below 50%

Determine of LD_{50} for EXP extract

The LD_{50} of EXP was determined according to (23) . EXP was calculated using karber method depended on this equation .

LD_{50} = Least lethal dose – (Σ a*b / n)

A : dose difference between groups , b : mean of dead rats between 2 groups

Immunological program

The experiments were designed to assess the immunological efficiency of EXP , HK *E.coli* and *C. freundii* suspension in albino rats, This occur through evaluate the humeral and cellular immune response, evaluation were carried out through the following desigen :

Forty healthy albino rats aged 16 to 18 weeks were divided equally into four groups then immunized i.p. with 1 ml from appropriate vaccine . The first group was immunized with 1/10 LD_{50} of EXP vaccine twice at two weeks intervals with a dose containing 39 mg/ml. The second group was immunized with 0.1 LD_{50} of HK *E. coli* antigen at the same time, Third group was injected with *C. freundii* suspension at 0.1 LD50 concentration finally , fourth control group was injected with 1 ml of PBS. Blood were collected after 2nd week of first and post dose . Sera were separated and stored at -20 ºC until use for analysis by enzyme-linked immunosorbent assay ELISA (IL- 6 , IL-12 , TNF-α and IL-10) according to the manufacturing company (Elabscience, USA) , while the remaining blood put in EDTA tube for using in phagocytic activity (24) and skin test to evaluate DHS (25) .

Challeng test

After two weeks of post dose of immunization for each type of vaccines ten rats from each group were challenge with i.p. of 1ml containing 100 LD_{50} of a live *C.freundii* suspension in addition to 10 rats received challenge dose only and regard as control group , then the relative degree of protection provide by vaccine was assessed by calculated the No. of survival rats after 14 days of injection .

Ethical Approved

This study was approved by the ethical and research committee of College of Medicine /University of Kufa / Minstery of High Education And scientific research.

Statistical analysis

The results are presented as means ± standard error (S.E) and statistical analyzed using one-way analysis of variance (ANOVA) test . Using Graphpad prism 5.04 . $P < 0.05$ was considered significant.

RESULTS

1- Characterization of *C.freundi*i

The results showed that 78 specimens (78%) out of 100 stool and urine specimens gave positive results for bacterial growth on MacConky agar medium . Among the positive growth and depending on characteristics of the microscopic , morphological , biochemical tests and Vitek 2system, only 11 (14.1 %) isolates were *C.freundii*, 8 (10.2%) isolates were isolated

from watery diarrhea and 3 (3.8%) isolates were associated with recurrent-UTI. The remaining isolates 67 (75.65%) showed growth of *C. koseri* , *Klebsiella* spp, *E.coli*, *Pseudomonase spp* , *Proteus* spp.

The results appear that *C.frundii* a gram -negative bacilli , colonies appear pink small convex on MacConkey agar and yellow, smooth ,flat and round on XLD agar. Regarding to biochemical tests , all the 11 isolates of *C.frundii* were lactose fermenting , motile and given positive test for catalase , methyl-red, citrate, and negative results for Indole , oxidase , Voges-Proskuaer, also have ability to ferment glucose on kliglerś iron agar gave (Acid/Acid). The results demonstrate with ID message confidence level excellent by VITEK-2 compact system .

2- Determine of optimum bacterial isolate in EXP production

The results illustrated that *C.freundii* isolated from recurrent-UTI patient had greater biofilm formation depending on their ability to give highest O.D (0.890) as well as exhibiting highest mucoid appearance on agar media and this isolate has been chosen to complete exopolysacharied extraction. The other 10 isolates give range of absorbance of biofilm formation (0.554-0.812)

3-LD $_{50}$ for a live suspension of *C. freundii*

The results revealed that LD $_{50}$ of *C. freundii* was (1×10^{7}) cfu/rat as showen in Table (1) and Fig (1) and the LD_{50} of heat killed *E.coli* was $2.3\times 10^{8.}$, While the lethal dose that killed half number of rats that injected with EXP was 390 mg/rat Table (2)

LD_{50} = 1600- (6.050/ 5) = 390 mg/ml

Table (1) : LD_{50} of live *C.freundii*

Con .of bacteria	No. of rat	Die Rat	survived Rat	Accumlated number of survived and dead Rat				
				D	S	D+S	D/D+S	%mortelit
10^{10}	5	5	0	17	0	17	17/17	100 %
10^{9}	5	4	1	12	1	13	12/13	92.3 %
10^{8}	5	4	1	8	2	10	8/10	80 %
10^{7}	5	3	2	4	4	8	4/' 8	50 %
10^{6}	5	1	4	1	8	9	1 / 9	11.1 %
10^{5}	5	0	5	0	13	13		0

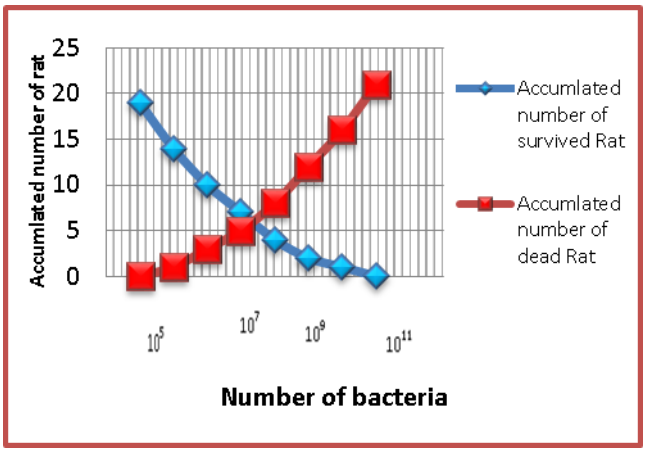


Fig (1) LD50 Value of Live *C.freundii*

Table(2): LD_{50} for exopolysaccharides extracted from ***C.freundii***

No of group	Dose	Dose differec (A)	Die	Mean B	A *b
1	1600	-	5	-	-
2	800	800	5	5	4000
3	400	400	3	4	1600
4	200	200	1	2	400
5	100	100	0	0.5	50

Immunological parameter

a. Phagocytic activity

The phagocytic activity was increased significantly (P < 0.05) in the rats after treatment with EXP , Killed antigen and whole bacteria in compare to control group , Also the results explain that EXP – vaccine were significantly elevated the mean of phagocytosis (73 and 80) respectively in rats treated after first and post dose that revealed highest increase in phagocytosis in compare with rats treated with HK *E.coli* and whole bacteria the results recorded that mean of phagocytic activity for Killed bacteria was (56 and 60) respectively after first and post doses in compare with control group (30) as showen in Fig(2)

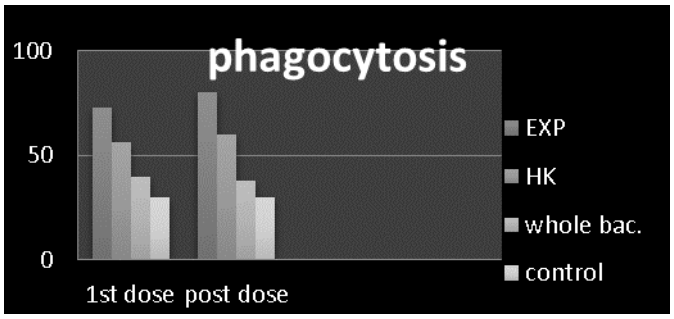


Fig (2): Phagocytic activity in rat treated with Exopolysacharied , HK-*E.coli* ,whole *C.freundii* and control

DTH Reactions

The results illustrated that EXP and HK immunogen as well as bacterial suspension increased the foot-bad thickness with mean (2.97, 3.335, 1.88) after 24 h in compare with control group with mean (1.24) .The mean of post dose shown in Fig (3)

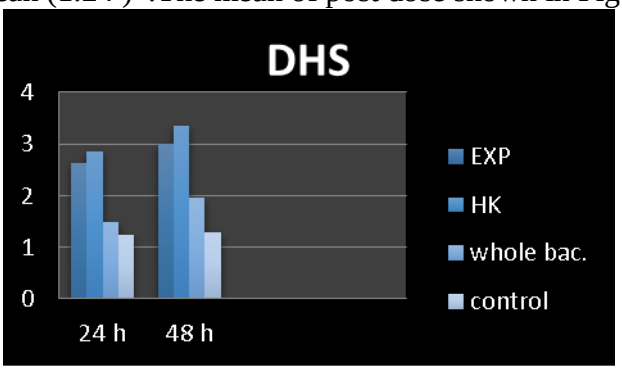


Fig (3): Delayed hypersensitivity in rat treated with Exo-polysacharied , HK-*E.coli* ,whole *C.freundii* and control

cytokine profile

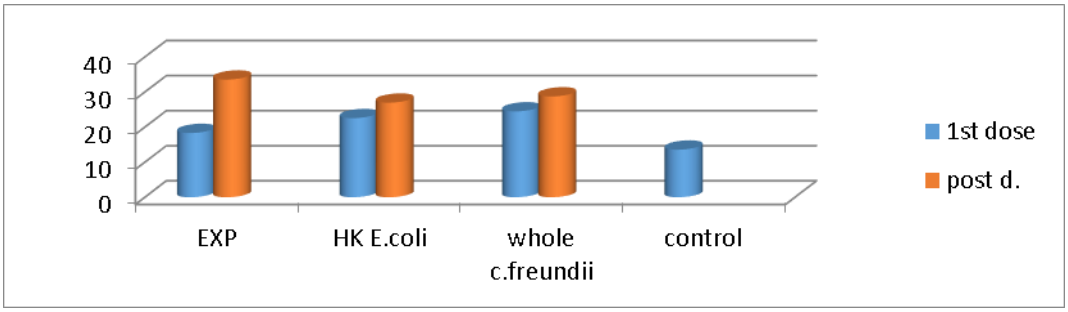
The results appear that both antigens were elevated the level of proinflammatory biomarker (TNF-α , IL-6 , IL-12) in compare with control group . Heat killed *E.coli* immunogen and bacterial suspension showed highly increased in the production of TNF-α (219 , 198) and the EXP immunogen revealed moderate increased (168) in compare with control group that recorded (120). IL-6 were appeared highly elevated in HK *E.coli* and bacterial suspension (190 ,178) ,but little elevation in EXP group (154) compared with control(112) as showen in Table (3) . this increase were significant (P<0.05) .

During post dose as show in Table(3) the results illustrated that exopolysaccharide group stimulate the release proinflamatory biomarker with high significant production (P<0.05) but lower than HK and bacterial suspension.

Table (3): TNF- α ,IL-6 and IL-12 serum level in rat treated with Exo-polysacharied , HK-*E.coli* ,whole *C.freundii* and control

		TNF- α	IL-6	IL-12
EXP	**D1**	168 ± 32	154 ± 25	17 ± 1.02
	D2	200 ± 43	198 ± 36	24 ± 5.22
Heat Killed bacteria	**D1**	219 ± 26	190 ± 18	22 ± 4.87
	D2	259 ± 47	230 ± 20	47 .6 ± 3.66
Bacterial suspension	**D1**	198 ± 42	178 ± 11	19.5 ± 7.21
	D2	227 ± 36	204 ± 27	36 ± 8.59
Control		120 ± 30	112 ± 35	14 ± 2.8
L.S.D				

The results showed high significance increased ($p<0.05$) in IL-10 concentration in post dose of EXP (33.83) and moderate elevation in HK *E.coli* and *C.freundii* suspension (21,98 , 23.26) respectively as compared with control group 12.76 as showed in Fig(4)



Fig(4) : IL-10 serum level in rat treated with Exo-polysacharied , HK-*E.coli* ,whole *C.freundii* and control

Challenge test

The results explored that EXP vaccine provide 90% protection , HK provide 60% while control group lossed all animals tested as show in table (4)

Table(4): Challenge test in the immunized (EXP , HK*E.coli*) and control groups

groups	No. of rats	No.of survival rats	Perce. Of survival rats
EXP	10	9	90 %
HK*E.coli*	10	6	60 %
control	10	0	0 %

DISCUSSION

This study revealed that *C. freundii* was most comman infectional agents recovered from diahrrea specimens . In the same line,(3) founded that *C. freundii* is the most common pathogen in frequency 6.6% in diarreatic patients , 2% of UTI patient. (26) observed that *C. freundii* cause a variety of infections like UTIs, diarrhea and wound infections and revealed that *C. freundii* was resistant to multiple classes of antibiotics .

C. freundii that isolated from patient suffering from recurrent –UTI which exhibited greater biofilm formation and mucoidal morphology selected to extract EXP this result agree with (27) they noticed a correlation between production of exopolysacharide and biofilm density that lead to [illegible] (28) suggested that selected optimum

bacteria based on development of mucoid morphology because it was one of the fundamental screening for isolation EXP producing bacteria . Similarly , (12) they chosen mucoid strain of *P.aeruginosa* to prepared mucoid exopolysaccharide- alginate conjugate vaccine .

The result of LD_{50} nearly consistence with (29) they observed that Median lethal dose of *C.freundii* suspension that killed half of animal group was 2.04×10^7 . While , (3) founded that 50% lethel dose for *C.freundii* suspension was 3.16×10^6.

C.freundii isolated from different clinical source produce different LD_{50} value for example LD_{50} from feces of patient in USA was 1.5×10^7 , while 9×10^7 from skin ulcer in spain patient (30)

The results of LD_{50} for HK *E.coli* was nearly acoording to (31) who found that LD_{50} HK *E.coli* was 0.83×10^8 and (19) they found that HK *E.coli* was 1×10^8 .

Elisk and Allam (32) suggested that LD_{50} for PS extracted from *aeromonase hydrophila* was 100 mg / ml and used 10mg/ml to study the immunomodulatory activity of PS . The difference in LD value in *C.freundii* isolates may related to source of bacterial isolation , virulence factors that posses

The current study confirmed a significant increased in phagocytic activity after treatment with EXP which agree with (33) they observed that exopolysacharide that immunized white mice show increased phagocytosis activity and increased digestion of *S.aureus* that injected after immunizes with EXP. (12) illustrated that mucoid exopolysacharide increased the killing activity by phagocytic cells and the post dose was highly increased opsophagocytosis than first dose . (34) explain that mucoid exopolysaccharide increased the killing activity in compare with whole bacteria and killed bacteria ,also revealed that high dose and large molecular weight of MEP elicited opsonic killing antibody . The result agree with (4) who illustrated that cell free culture from *citrobacter* was increased the phagocytosis activity more than *citrobacter* suspension and both of them were high significant compare with control group . Similarly , (365) they observed that OMP , LPS and Killed antigens that extracted from *C. freundi*i showed highly increased in phagocytic activity and increased the DHS in compare to control and mention that both antigen increased the phagocytic activity more than killed bacteria **.**

The results of DHS reaction agree with local study done by (36) who suggested increased the thickness of foot-bad that stimulated with EPS extract in compare with foot-bad stimulated with normal saline . in line with this results (4) founded that antigens that extracted from *C.freundii* stimulated the DHS more than control group . (37) they explain that DHS enhancement cellular immunity which characteristic by swelling , redness that occur as a result of infiltration of macrophage , neutrophil and lymphocyte at site of inflammation that recognize antigen and secrete IL-1 that enhanced proliferation and differentiation of other T-cell into Th-cells which secrete IL-2 as a chemoatrractent factor to attract macrophage around the area of activated T-cell and also secrete INF-γ that enhancing the cytolytic activity of accumulated macrophages leading to skin thickness .

Cytokine profile revealed immunomodulatory activity of EXP through maintenance the cytokines levels .This results agree with study done by (38) they observed that exopolysacharide extracted from Bifidobacteria have immunomodulatory effect by slightly elevated the levels of biomarkers whereas the bacterial suspension and heat killed antigen highly increased these cytokine and suggested that exopolysaccharide prevent commensal bacteria from evoking a strong adaptive immune response withen local host. Also, (36) illustrated that EPS extracted from *pantoea* spp a member of Enterobactereace was elevated the concentration of proinflammatory cytokine .

(39) mention that exopolysacharied play an immune-modulatory role by effect on cytokine responses on macrophage and dendritics cells isolated from tissue culture and bone marrow-derived cells . Similarly (40) revealed that exopolysaccharide was decisive in influencing the immune response to proinflammatory cytokine especially (IL-6 , TNF- α) and confirmed that exopolysaccharide from *B.longum* induced relatively low levels of proinflamatory cytokine

secretion from human dendritic cells, whereas an isogenic exopolysaccharide-negative mutant derivative (EPSneg) induced vastly more cytokines and emphasis that this immunogen is required to prevent a potent tissue damaging as a result from proinflammatory cytokine response to a commensal bacterium .

The current study observed that HK and whole bacteria induce higher cytokine production .In same line (41) demonstrated that EPS effectively induces the production of macrophage cytokines, especially TNF-a, IL-6 and IL-12 and this stimulatory activity was significantly lower than that of intact bacterial cells and lower than that of LPS that lead to elicited produce pronflammatory cytokines subsequently these cytokines develop fever, redness and other symptoms assocciated with systemic inflammatory response syndrome and tissue damage . The compounds of biofilm extracellular polysaccharide stimulated the mouse peritoneal leukocytes to increased the production of cytokines (TNF-α , IL- 6, IL-10, MCP-1 and MIP-1), and explain that biofilm components possessing the immunomodulatory properties and prevent biofilm infection (42) .

This study in accordance with study done by (31) they revealed that HK *E.coli* antigen increased the IL-12 level in rats serum treated with this antigen and founded that second dose recorded highly increased in IL-12 production . In same line(43) illustrated that gram negative bacteria such as *E.coli* , *serracia merrsense* , *Salmonella* induced significantly higher levels of proinflammatory cytokines production of IL-12 , TNF- α and INF- which associated with cell mediated immunity response and suggested that increased proinflammatory as a results of cell wall components of these bacteria .

In this study the results appear that lower IL-10 production in rats treated with exopolysacharide in apposite to rats treated with HK and bacterial suspension which recorded highly significant in IL-10 production .In this regard Kozakova (44) explain that PS extracted from *L.casei* induce the production of IL-10 and IL-12 , but emphasis that whole bacteria in compare with their bacterial compounds are highly stimulate immune markers . Gorska *et al* (45) demonstrated that exopolysacharide antigen slightly increased the IL-10 levels in compare with formaldehyde –inactivated *E.coli* and whole bacteria.

Several studies confirmed the distinguished role of exopolysaccharide as immunomodulater therapy in different disease like , (46) they observed that cystic fibrosis patients who naturally acquired mucoid exopolysaccharide specific opsonic antibodies have been associated with resistance to infection. Asker(47) they suggest that polysaccharide isolated from *Bacillus* sp. may be a good natural source for Alzheimer disease therapy and reduced all oxidative stress parameters in brain tissue , also observed no toxicity was observed for MEP in sub-chronic toxicity study for 90 days in all rats organs .Simillrly , (48) explain that exopolysaccharides produced by *E. cloacae* showed a significant anti-inflammatory and anti-diabetic effects, through the effects of EXP on the alleviation of fat in inflammation high-fat-diet (HFD) induced-diabetic mice to . Finally , The study revealed that the vaccination with EXP vaccines protected rat in frequent 90% against challenge with 100 LD50 *C.freundii* suspension while HK *E.coli* provide 60% protection and all naïve rat were died after challenge .

CONCLUSIONS

EXP-immunogen can stimulate immune response against pathogen infection by stimulate cellular immunity (phagocytosis and DHS) and humeral immunity (cytokine profile) . Also can be used to avoid immune – mediated destruction from microbial infection that causes highly released proinflammatory biomarker as well as provide highly protection against challenge dose . So this compound evoke moderate production in proinflammatory biomarker.In other hand the administration of molecules consider safer than whole bacteria or their toxins that may cause sepsis or bacteremia and cheaper than other type of vaccine like

DNA , recompenent vaccine . addition study were required to evaluate the EXP immunogen in prevent several type of infections .

Abbreviation . EPS .extracellular polymear substance , EXP.exopolysacchariedd

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