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In vivo antibacterial activity of alkaline phosphatase isolates from *Escherichia coli* isolated from diarrhea patients against *Pseudomonas aeruginosa*

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Abstract
A hundred and fifty samples of stools were collected from patients suffering diarrhea. Hundred and thirty bacterial isolated (86.7%) were identified 84 (64.6%) belonged to *Escherichia coli*, 23(17.7%) to *Klebsiella pneumoniae*, 10 (7.7%) *Enterobacter cloacae*, 7 (5.4%) to *Shigella dysenteriae*, 4(3.1%) to *Salmonella typhi* and 2(1.5%) to *Pseudomonas aeruginosa.* The ability of *E.coli* isolates to produce alkaline phosphatase enzyme was tested on MM agar medium along with pNPP. ALP was extracted, the crude extract activity and specific activity was 1.86 unit/ml and 20.44 unit/mg protein respectively. The enzyme purified by precipitating with ammonium sulfate (50-75%) saturation then using ion-exchange chromatography in DEAE-Cellulose ionic exchange column and gel filtration chromatography by using sepharose-6B gel filtration column, to obtained pure ALP enzyme with specific activity 272.73unit/mg, with 13.34 fold purification and yield of enzyme 56.45%. The *in vivo* investigation for the effect of ALP enzyme on the infection with *P. aeruginosa* was done using BLBC mice. The results shows that ALP degreased the number of bacteria in liver, spleen and lung with significant differences (P<0.05). Also the results shows a significant differences (P<0.05) in elevated levels of cytokines (IL-6, IL-10 and IL-12) compering with control group.

Keywords: ALP, *E.coli*, diarrhea

1. Introduction
Alkaline phosphatase (EC 3.1.3.1) belongs to the class of hydrolases and acts on phosphate groups. This enzyme catalyzes the hydrolysis of almost every phosphomonoester to give inorganic phosphate and the corresponding alcohol, phenol or sugar, and also catalyzes trans phosphorylation reactions in presence of large concentration of a phosphate acceptor [1].
Bacterial alkaline phosphatase is usually located in *periplasmic space* and fairly stable to resist inactivation, denaturation and degradation, and also has a higher rate of activity [2].
Alkaline phosphatases play a vital role in DNA sequencing analysis and molecular cloning, it hydrolyzes phosphate groups from monophosphate esters and oligonucleotides [3].
Alkaline phosphatase use in enzyme-labeled antigens and antibodies of enzyme immunoassay and enzyme-linked immunosorbent assay to detect the biological molecules [4].
Previous reports have suggested that ALP promotes several beneficial effects to the intestinal health of mammals, including prevention and reduction of intestinal inflammation and bacterial translocation, regulation of calcium absorption, and modulation of intestinal bacterial growth and local intestinal pH [5- 8].
ALP activity have positively modulates the growth of commensal bacteria by inducing adenosine triphosphate (ATP) dephosphorylation, leading to increased competition with potential pathogens. Therefore, this increased competitiveness by commensal bacteria may directly reduce the production of enterotoxins by pathogenic/opportunistic bacteria [8].
The purpose of this study was to evaluate *in vivo* antibacterial activity of *Escherichia coli* alkaline phosphatase against *Pseudomonas aeruginosa*.

2. Materials and Methods
2.1 Collection of stool Samples and cultured
A total of hundred and fifty (150) samples of stool were collected with aseptic technique in sterile cups from patients suffering from diarrhea who had certain clinical symptoms. Stool samples were taken from the patients and inoculated on MacConkey agar and blood agar plates by direct streaking method [9].

2.2 Isolation and identification of bacteria
Bacteria isolated as pure colonies on MacConkey agar and blood agar then bacterial isolates were examined and identified by cultural, microscopic, biochemical test and Api-20E system [10].

2.3 Screening of alkaline phosphatase
The isolates were screened by streak plate on the MM agar medium along with pNPP and the selected isolates were separately confirmed for their abilities to produce alkaline phosphatase [11].

2.4 Production of enzyme
Escherichia coli used for enzyme production was grown at 37 °C for 36 hr in a rotary shaker at 150 rpm in alkaline phosphatase production medium as described by [12]. ALP activity was measured by using Stopped Spectrophotometric Rate Determination method [13].

2.5 Extraction of alkaline phosphatase
The crude alkaline phosphatase was extracted from the selected isolate after growing the isolate in alkaline phosphatase production medium (12), the harvesting of the cells was done by centrifugation and pellets were washed with 10 mM Tris-HCl buffer pH 8.0. The suspension was further centrifuged and pellets were dissolved in 30 mM Tris-HCl, 20% sucrose buffer (pH 8.0). 0.8 mL of 10 mM EDTA and 80 µL of lysozyme (10 mg lysozyme/1 mL Tris-HCl buffer (30 mM, pH 8.0) were mixed with the bacterial suspension. The whole mixture was than centrifuged at 8,500 rpm for 15 min at 4 ºC. Supernatant was collected and pellets were finally dissolved in small volume of sucrose Tris-HCl buffer and stored at 4 ºC.

2.6 Determination of protein concentration
The protein concentration was determined using Bradford method [14] with bovine serum albumin as the standard protein.

2.7 Purification of alkaline phosphatase
The cells collected was subjected to different steps of purification including ammonium sulphate (NH4)2SO4 precipitation, dialysis, DEAE-cellulose ion-exchange chromatography and gel filtration by using gradient elution buffer.

2.8 Effect of alkaline phosphatase on Pseudomonas aeruginosa *in vivo*
Twenty healthy BALB/c mice were obtained from Animal house, college of medicine-Baghdad University. The animals were kept in the animal house of the University. Mice were (4-5) weeks old, weighting approximately (18-20gm). They were housed under standard conditions of light and dark cycle. The mice were anesthetized by intra peritoneal I.P. injection of 0.2 ml of 0.65% sodium pentobarbital before bacterial inoculation. Mice were challenged intraperitoneally with 0.5 ml of Pseudomonas aeruginosa equal to (1.5×108) CFU/ml adjusted to 0.5 Macfarland turbidity tube.
The animals were divided randomly in to three groups, three mice in each group as following:

2.8.1 Un treated control: mice injected with phosphate buffer saline only after infection for three days.

2.8.2 Treatment group: mice injected with partial purified alkaline phosphatase (1mg/20gm) after infection for three days.

2.8.3 Protective group: mice injected with partial purified alkaline phosphatase (1mg/20gm) for three days before and after the infection.
Animal were absorbed and evaluated in the 3rd day and killed by cervical dislocation, blood samples were obtained by cardiac puncture. During the necropsy, the spleen, liver and lung were excised by sterile technique. The tissue were then weighed and diluted 10-fold in PBS and homogenized for up to 30 second. The homogenates and serial dilutions of the homogenates were plated on TSA agar, and viable bacterial counts were enumerated after 24hr of incubation at 37 ºC.

2.9 Cytokine assay
The level of cytokines [interleukin-10 (IL-10), IL-6 and (IL-12)] in the sera were determined using Duo set ELISA kit.

2.10 Statistical Analysis
Data were statistically analyzed using one-way ANOVA procedure of SPSS and expressed as mean ± SD. All these experiments were performed in triplicate and within each replication analyses were carried out in duplicate. The differences among means were tested. Data were considered statistically significant when $p<0.05$ [15].

3. Results and Discussion
The result showed that a total of 130 (86.7%) samples gave positive cultures and 20 (13.3%) negative cultures. From hundred and thirty bacterial isolates (86.7%) were identified 84 isolates (64.6%) belonged to Escherichia coli and 23 isolates (17.7%) were found belong to Klebsiella pneumoniae while Enterobacter cloacae and Shigella dysenteriae found in 10 (7.7%), 7 (5.4%) isolates respectively, whereas Salmonella typhi contributed only into 4 isolates (3.1%), Pseudomonas aeruginosa was two isolates (1.5%). (16) found E.coli as the most predominant in diarrheal patients with 24% followed by *S. typhi* 23%, *K. pneumoniae* 15%, A. hydrophila, *E. cloacae* 9%, S. dysenteriae 8% and P. shigelloides, V. cholerae 5%. Furthermore, [17] considered Escherichia coli as the main causal agent of diarrhea, from the total of 100 isolate who found 47% was E.coli followed by *K. oxytoca* 22%, *C. braakii* 15%, Proteus spp.10% and Salmonella
spp., Shigella spp. and Pseudomonas aeruginosa were 3%, 2% and 1% respectively.

3.1 Screening the alkaline phosphatase producing isolates
Escherichia coli (84) isolates were screened by streaked the isolates on the MM agar medium along with pNPP and the ALP producing isolates showed yellow color surrounding the colonies compering with non-producer isolates which were non colored figure (1). The results showed 60 isolates (71.4%) were produce alkaline phosphatase while 24 isolates (28.6%) were could not produce alkaline phosphatase figure (2).

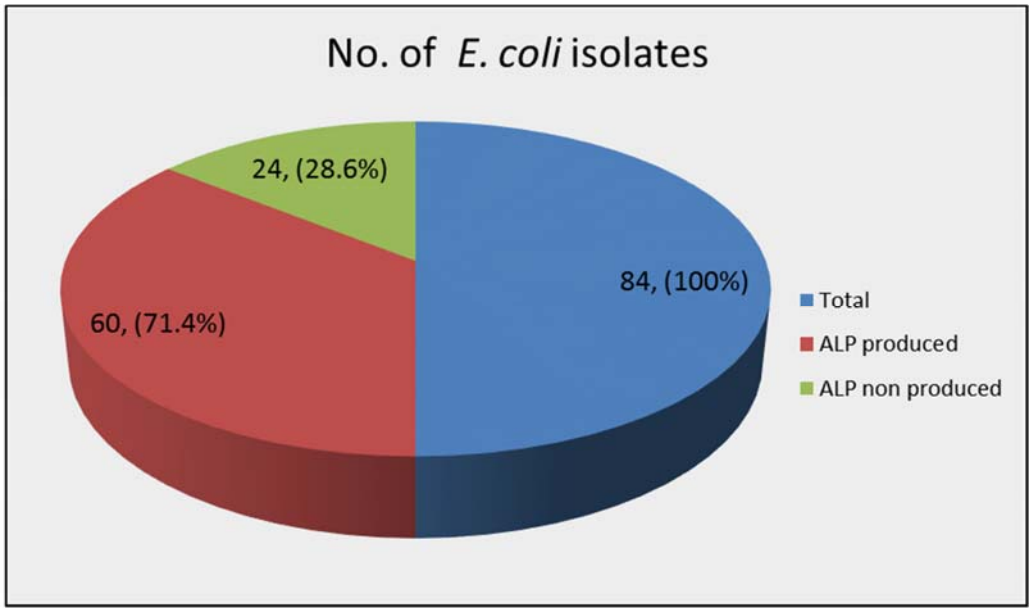

Fig 2: Screening of alkaline phosphatase production by *Escherichia coli*.

3.2 Extraction of the enzyme

The crude alkaline phosphatase activity and specific activity was 1.86 unit/ml and 20.44 unit/mg protein respectively.

3.3 Purification of alkaline phosphatase

After extraction, the pellet was taken for (50-75%) ammonium sulphate precipitation. The alkaline phosphatase activity and specific activity was (3.1unit/ml) and (39.24 unit/mg) protein respectively. The sample was subjected to DEAE cellulose column by linear gradient of NaCl (0.4 – 0.6 M). The results showed one protein peak in wash elution and three peaks in gradient elution. Only one peak among the gradient elution peaks represented enzymic activity (tubes 13-19). The fractions pooled and tested for specific activity (91.43unit/mg) a fold purification of (4.47) and enzymic yield of (60.02%) figure (3). [18] reported that specific activity of alkaline phosphatase extracted from *Escherichia coli* was 30.1 U/mg and enzymic yield was 86%.

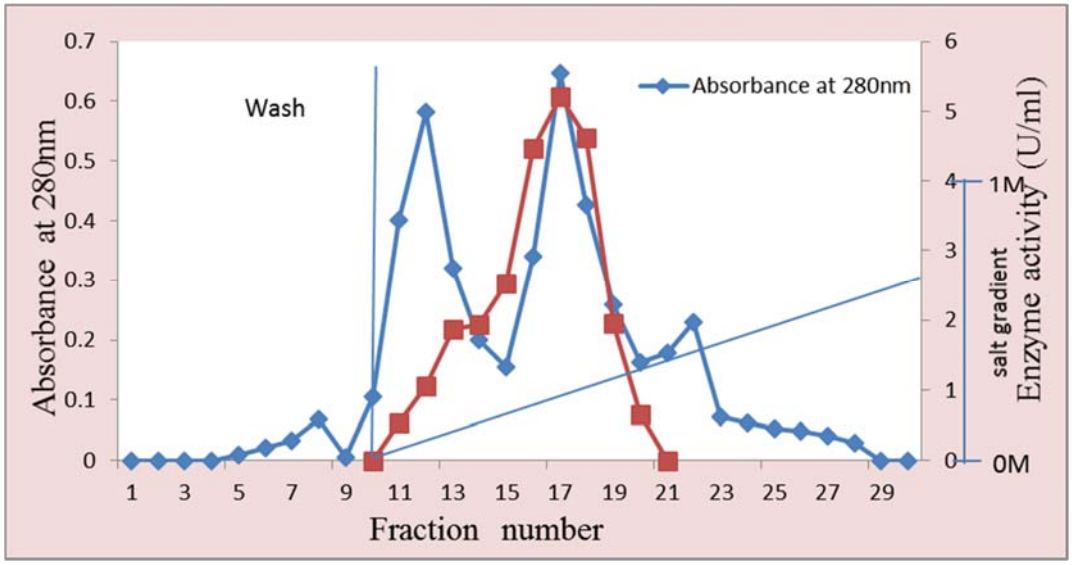

Fig 3: Ionic Exchange Chromatography for alkaline phosphatase enzyme from *Escherichia coli* through DEAE-Cellulose column (1.5 x17.5) cm. The column was calibrated with 0.1M Tris-HCl pH 9.8, flow rate 60ml/hrs and 5 ml fraction.

Further purification carried out by a gel filtration using Sepharose-6B. Enzymic fractions from DEAE-cellulose were pooled and passed through gel filtration column. The fractionation yielded two protein peaks as absorbance reading at 280nm. The second peak (fractionation tubes 29–35) contained alkaline phosphatase activity (3unit/ml), protein concentration (0.11mg/ml) with specific activity (272.73unit/mg) and the purification fold was (13.34) with yield of enzyme (56.45%) as mentioned in figure (4) and table (1). [19] used gel–filtration with sepharose to purified alkaline phosphatase enzyme extracted from *Escherichia coli* and they obtained the enzyme with specific activity 13.81unit/mg with yield of enzyme 25.39% and purification fold 72 time [20]. Recovered 68% of alkaline phosphatase from *Cobetia marina*.

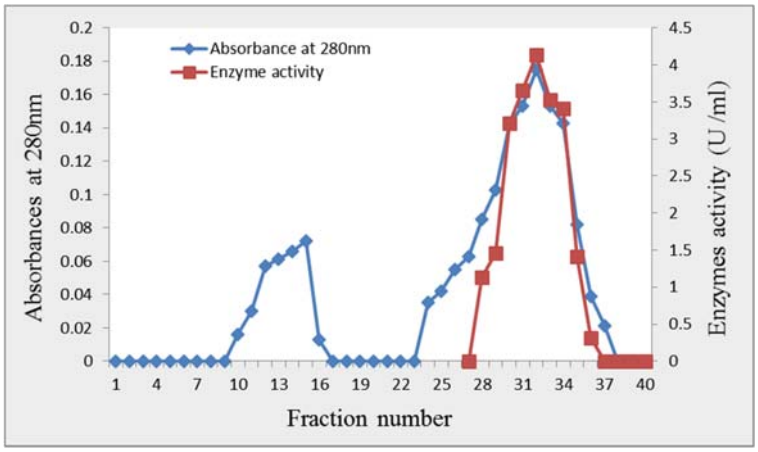

Fig 4: Gel filtration chromatography for purified alkaline phosphatase from *Escherichia coli* by using Sepharose-6B column (1.5x80) cm. The column was calibrated with 0.1M Tris-HCl pH 9.8; flow rate 30 ml/hrs and 5 ml/fraction.

Table 1: Purification steps of alkaline phosphatase from *Escherichia coli* isolated from diarrheal samples

Steps	Volume (ml)	Enzyme activity (U/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (U/mg)	Fold	Yield (%)
Crud extract	100	1.86	186	0.091	20.44	1	100
Ammonium Sulfate (50-75%)	40	3.1	124	0.079	39.24	1.92	66.67
DEAE-Cellulose	35	3.2	112	0.035	91.43	4.47	60.02
Gel-filtration Sepharose- 6B	35	3	105	0.011	272.73	13.34	56.45

3.4 Effect of alkaline phosphatase on *pseudomonas aeruginosa in vivo*

Results in table (2) shows that the second group (treatment) was infected with *P. aeruginosa* and treated with ALP was degreased in the number of bacteria and this degreasing was significant differences (P<0.05), and in comparison ALP was more effect on the third group (protective), when it made reduction the number of bacteria in liver, spleen and lung comparing with control group which was not treated with alkaline phosphatase.

Table 2: Effect of alkaline phosphatase on *Pseudomonas aeruginosa in vivo*

groups	(mean ± SD) Log 10 Cfu/g tissue *		
	Spleen	lung	liver
Group (1) control : Infected with *P. aeruginosa* and treated with PBS	6.79±1.33 P	5.22±1.17 p	4.86±0.81 p
Group (2) treatment: Infected with *P. aeruginosa* and treated with alkaline phosphatase (1mg/20g)	2.81±1.52 q	3.66±1.07 q	3.09±0.95 q
Group (3) protective: treatment with ALP before and post infection with *P. aeruginosa*	1.95±2.12 d	0±0 d	2.11±0.37 d

p-d: Data with different letters within a column are significantly different at P< 0.05
Each value in the table is (mean±SD) for 3 data

Alkaline phosphatase is able to reduce inflammation through dephosphorylation and thereby detoxification of endotoxin (lipopolysaccharide), which is an important mediator of sepsis and adenosine triphosphate, released during cellular stress caused by inflammation has detrimental effects but can be converted by alkaline phosphatase into adenosine with anti-inflammatory and tissue-protective effects [21].

On the other hand, the immunomodulatory of partial purified ALP was showed in table (3), the results showed that there were significant differences (P<0.05) in elevated levels of cytokines (IL-6, IL-10 and IL-12) in both of treatment and protective groups when contrast with control group that was non treated with alkaline phosphatase. This differences in elevated levels of cytokines may be due to inducing of immune response by ALP, and this elevated in the cytokines may be also lead to decreasing the number of the bacteria.

Table 3: Cytokines in the serum of mice infected with *Pseudomonas aeruginosa* and treated with alkaline phosphatase

groups	Cytokines concentration Pg/ml (mean ± SD) *		
	IL-10	IL-6	IL-12
Group (1) control Infected with *P. aeruginosa* and treated with PBS.	171.18 ± 9.75 a	59.22±11.03 a	75.08±6.92 a
Group (2) treatment: Infected with *P. aeruginosa* and treated with alkaline phosphatase (1mg/20g)	201.29±10.51 b	142.53±13.92 b	181.47±7.84 b
Group (3) protective**:** treatment with ALP before and post infection with *P. aeruginosa*.	247.33±19.53 e	216.72±8.39 e	234.19±9.67 e

a-e: Data with different letters within a column are significantly different at P<0.05
Each value in the table is (mean±SD) for 3 data

In all infectious diseases, besides the virulence of the pathogen, both the natural and the specific immune responses of the host are crucial for determining the outcome of the infection. The immune system has evolved different defence mechanisms against pathogens. The first defensive line is provided by "natural" immunity, including phagocytes, T cell receptor (TCR) γδ+ T cells, natural killer (NK) cells, mast cells, neutrophils and eosinophils, as well as complement components and pro-inflammatory cytokines, such as interferons (IFNs), interleukin (IL)-1, IL-6, IL-12, IL-18 and tumor necrosis factor (TNF)-α [22].

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