CHARECTERIZATION OF ENTEROTOXIN PRODUCED BY LOCAL ISOLATE OF *YERSINIA ENTEROCOLITICA* AND STUDY OF THE INHIBITORY EFFECT OF *ERUCA SATIVA* ON ITS ACTIVITY

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Abstract

Enterotoxin produced by local isolate of *Yersinia enterocolitica* was purified from the culture supernatant .The purification procedure involved precipitation by ammonium sulfate (50% saturation) and analyzed by sodium dodecyl sulfate- poly acrylamide gel electrophoresis (SDS-PAGE), (159.4) fold purification was achieved with yield of (17.9)%. The molecular weight of purified enterotoxin was 11,000 Dalton.

The purified enterotoxin was heat stable at 100 ^{o}C for 15 min, and the activity was lost after incubation with 3mM dithiotheritol as reducing agent. Enterotoxic activity of *Y. enterocolitica* was neutralized by antiserum from mice immunized with the purified enterotoxin .The inhibitory activity of *Eruca sativa* extracts was tested, both seed and leave extracts had inhibitory activity in a dose- dependent manner.

توصيف السم المعوي المنتج من العزلة المحلية ***Yersinia enterocolitica*** ودراسة التأثير التثبيطي لنبات الجرجير (***Eruca sativa***) على فعاليته

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المستخلص

تم تنقية السم المعوي المنتج من العزلة المحلية *Yersinia enterocolitica* من راشح المزروع البكتيري . تضمنت عملية التنقية الترسيب بكبريتات الامونيوم بتشبع 50% والتحليل بوساطة الترحيل الكهربائي باستخدام هلام صوديوم دودسيل سلفات – بولي اكريل امايد (SDS-PAGE) وبلغ عدد مرات التنقية 159.4 مرة وبحصيلة بلغت 17.9%. قدر الوزن الجزيئي للسم المعوي بـ 11,000 دالتون.

ابدى السم المعوي النقى ثباتا حراريا عند درجة 100 م ولمدة 15 دقيقة وتم فقدان الفعالية بعد الحضانة مع العامل المختزل dithiotheritol 3mM. تم معادلة الفعالية السمية المعوية باستخدام المصول المضادة للفئران الممنعة بالسم المعوي المنقى. اختبرت الفعالية التثبيطية لمستخلصات نبات الجرجير (*Eruca sativa*)، كان لكل من مستخلص البذور ومستخلص الاوراق فعالية مثبطة للسم المعوي واعتمدت درجة التثبيط على تركيز الجرعة المستخدمة.

Introduction

Yersinia enterocolitica is an important cause of bacterial gastroenteritis in children; pathogenic strains are characterized by a chromosomally encoded ability to invade cultured mammalian cells and the possession of a virulence plasmid PYV, that may facilitate bacterial survival within host tissue (1, 2, 3). *Y.enterocolitica* invasive serobiogroups produced heat stable enterotoxin , known as YST(4) , encoded bythe chromosomal *yst* gene (5). Animal studies with *yst* deletion mutants suggest that the toxinis important in causing *Yersinia*- associated diarrhea (6). *Y. enterocolitica* enterotoxin is similar to *Escheriacha coli* heat stable (ST) enterotoxin with respects to sucking mouse activity and accumulation of cyclic guanosine 3, 5 monophosphate (GMP) in the intestine (7, 8).Although the *Y.enterocolitica* ST and *E. coli* ST are immunologically cross reactive , the heat stability and molecular weight of *Y.enterocolitica* ST are different from those of *E. coli* ST(9).

Eruca sativa is one of the medical plants that has several antioxidant constituents including glucosinolates, flavonoids, carotenoids and others (10).Various solvent extracts of *Eruca sativa* (aerial and root) and seed oil have antimicrobial activity against antibiotic resistant gram negative bacteria (*E. coli , Pseudomonas aeroginosa and Shigella flexneri*) and gram positive bacteria (*Staphylococcus aureus and Bacillus subtilis*) (11).

Eruca sativa seed oil ameliorate the harmful effect of aflatoxin (12).This study aimed to characterized purified enterotoxin produced from local isolate of *Y.enterocolitica* and detect the inhibitory effect of *Eruca sativa* on its enterotoxic activity.

Materials and methods

Bacerial strain: *Y.enterocolitica* was isolated from patient with diarrhea in a previous study (13).

Enterotoxin production: *Y.enterocolitica* was grown aerobically in Tryptic soy broth supplemented with 0.6%(wt/vol) yeast extract , the inoculated medium was incubated with shaking (250 r.p.m) at 28˚C and 37˚Cfor 48 h (14). To study the production of enterotoxin at PH of 7.5 at 37˚C (the conditions found in the ileum) , the bacteria were inoculated in brain heart infusion broth supplemented with 5Mm $CaCl_2$ and 0.1 M NaCl and incubated at 37 ˚C for 48 h . The culture supernatant was obtained by centrifugtion at 12000xg for 15 min at 4 °C and sterilized by membrane filtration (0.2µm Millipore) (15).

Assay for enterotoxin activity:

Enterotoxic activity of culture filtrate and purified toxin was assay in infant mice. Groups of two to five Swiss mice 2-4 days old were inoculated intra-gastrically with 100µl of the test filtrate in wich 0.01% Evan blue dye was incorporated as a marker. After 3h, mice were scarified and the ratio of the intestinal weight to the remaining body weight was determined and considered indicative of enterotoxin production. One unit of enterotoxic activity is defined as the minimal amount giving an intestine weight body ratio of 0.083 at 3 h after administration of the toxin containing sample (16).

Purification of *Y.enterocolitica enterotoxin:*

The sterile culture filtrate was concentrated by ammonium sulfate (25-90% saturation). the precipitate was dissolved in 10 mM Tris –HCl buffer (PH7.2) and dialyzed against distilled water , then the sample was analyzed by sodium dodecyl sulfate- poly acrylamide gel electrophoresis (SDS-PAGE) using 12.5% gel. After electrophoresis, some of the gels were stained with Coomassie brilliant blue R-250, and others were cut into 3mm- thick sections and each sections was extracted with 0.5 ml of 10 mM Tris –HCl buffer (PH7.2) for about 40 h so that the enterotoxin and other proteins will be diffused from the gel to the buffer (17, 18), the enterotoxic activity in each extract was examined as described above.

Protein determination: The concentration of protein was determined in each extract by method of Lowery *et al*. (20), with Bovine serum albumin as a standard.

Molecular weight determination: The molecular weight of the purified entertoxin was determined by Sephadex G100 gel filtration.The colum (2× 85 cm) was eluted with 10mM Tris – HCl buffer (pH7.2). Lysozyme (MW. 14,700 Dalton), Chymotrypsinogen A (MW 25,000 Dalton), Egg albumin (MW 45,000 Dalton) and Bovine serum albumin (MW 68,000 Dalton) served as marker for the estimation of molecular weight (17).

Stability test:

Heat stability: The purified enterotoxin (5µg/ml) was heated at 100˚C and 121˚C for 15 min. The enterotoxic activity was assayed in infant mice as described above.

Treatment with 3 mM dithiothreitol:
The purified enterotoxin (5µg/ml) was incubated at 37˚C for i5 min in 1 ml of 10 mM Tris –HCl buffer (PH7.2) containing.

3mM dithiothreitol. The enterotoxic activity was assayed in infant mice.

Antiserum preparation: Antiserum against *Y.enterocolitica* enterotoxin was prepared in female adult (25-30g) Swiss mice by subcutaneous administration of a water in oil emulsion (0.2ml) composed of equal parts of boiled purified enterotoxin and Freund incomplete adjuvant .The mice received injection on day 0, and at weeks 5, 9, 11, and one week after the last injection the sera were collected and heat treated (56˚C, 30 min) and stored at 4˚C(6). Neutralization of enterotoxic activity of purified enterotoxin and culture filtrate of *Y.enterocolitica* were incubated with an equal volume of prepared antisera at 37˚C for 1 h, and then assayed in infant mice. Normal mouce serum was used as a negative control (19, 21).

Preparation of *Eruca sativa* extracts: Ten grams of air dried plant material (seed and leave) were crushed seperatly in electrical grinder then extracted in 100 ml of distilled water for 6h at 40 ˚C. Every two hour it was filtered and centrifuged at 5000 xg for 15 min, than the supernatant was collected. The procedure was repeated twice and after 6 hour the supernatant was concentrated to make the final volume. The extract was then stored at 4˚C in air tigh bottles (22).

Inhibitory activity: Inhibitory activity of plant extracts (10, 20, 40 vol/vol of each extract) against purified *Y.enterocolitica* enterotoxin was tested, 50 µl the enterotoxin was incubated an equal volume of each extract concentration at 37˚C for 1 h, then assayed in infant mice.

Result and Discussion

Enterotoxn production: The local isolate of *Y.enterocolitica* produced enterotoxin at 28˚C and for 48h and showed enterotoxigenicity in infant mice, and this isolate showed enterotoxic activity at PH of 7.5 (the PH present in the ileum), but failed to produce enterotoxin at 37˚C for 48h (Table 1), the bacteria failed to produce enterotoxin when it incubated at 37˚C for 48h under normal conditions because under these conditions the PH of culture medium was allowed to change with growth of bacteria (23).

Table 1: Production of enterotoxin by *Y.enterocolitica* under various conditions

Culture condition	Enterotoxin production
Normal condition at 28˚C	+
Normal condition at 28˚C	+
PH of 7.5 at 37˚C	+

+: Showed enterotoxic activity
- : No enterotoxic activity

Purification and characterization of enterotoxin: Crude culture of *Y.enterocolitica* was concentrated by ammonium sulfate, various concentrations of ammonium sulfate were examined (25, 50, 75, 90% saturated). The optimal concentration was 50% which yielded optimal amount of toxin with specific activity about 0.564 mg/ml Table 2. The specific activity of purified enterotoxin by SDS-PAGE increased about 159.4 with a yeild of 17.9% as shown in (Table 2).

The precipitation step removed contaminating proteins and large amount of non protein contaminants (e.g. media pigments) from the toxin preparation (19). Different proceuders were used for *Y.enterocolitica* enterotoxin purification including ammonium sulfate and DEAE- Sephacel column, hydroxyl apatite column and Sephacryl S-200 gel filtration as final step, the yield was about 12.5% and when the ion exchange chromatography by DEAE-Sephadex column as fifth step and Bio-Gel column as final step the yield was 8.9% (17, 21). The molecular weight of the purified enterotoxin was determined by G-100 gel filtration, singe peak appeared as shown in

Table 2: Recovery of *Y.enterocolitica* enterotoxin purification

Fraction	Vol. (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yeild (%)
Crude culture filtrate	100	0.1096	5.86	0.0187	10.96	1	100
Ammonium sulfate (50%) precipitate	10	0.7626	1.35	0.564	7.626	30.2	69.58
SDS-PAGE	1	1.372	0.46	2.98	1.372	159.4	17.9

Figure 1 and the molecular weight was 11,000 Dalton (Figure 2).

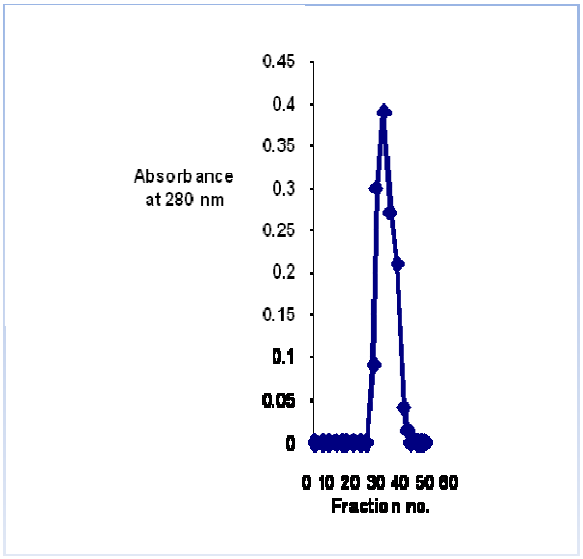

Figure 1: Gel filtration of purified *Y. enterocolitica* enterotoxin by Sephadex G100 column (2× 85 cm), the column was eluted with 10mM Tris –HCl buffer (PH7.2).

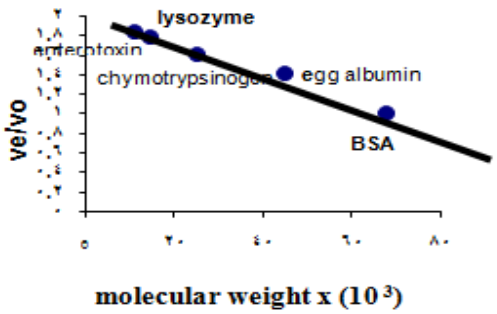

Figure 2:Molecular weight determination of purified *Y.enterocolitica* enterotoxin by Sephadex G-100 gel filtration.

The purified enterotoxin was stable to heating at 100°C and 121 °C for 15 min (Table 3). The molecular weight of *Y.enterocolitica* enterotoxin was about 9,700 Dalton by Sephadex G- 75 super fine gel filtration gel and the toxin remain active after boiling for 15 min , thus the toxin was called heat stablile enterotoxin (17). The enterotoxic activity of purified and crud culture filtrate was lost after incubating with 3mM dithiothreitol (Table 4), which suggests that disulfide bonds may be critical for the activity of *Y.enterocolitica*enterotoxin(19).

Table 3: Heat stability of *Y. enterocolitica* enterotoxin

Treatment	Activity of purified enterotoxin (U/ml)	Residual activity (%)
Intact toxin	1.372	100
100°C (15 min)	1.369	99.8
121°C (15 min)	1.362	99.27

Table 4: Treatment of *Y.enterocolitica* enterotoxin with 3mM dithiothreitol

sample	Activity (no treatment) (U/ml)	activity with 3mM dithiothreitol (U/ml)
Crude culture filtrate	0.109	0.07
Purified enterotixin	1.37	0.051

Neutralization exprtiments with antisera prepared against the purified enterotoxin indicated that the enterotoxic activity of crude culture filtrate and purified enterotoxin was

neutralized by antisera but not by normal saline Table 5 . Okamoto *et al*. (20) showed that antibody could be elicited in guinea pigs immunized with purified *Y.enterocolitica* ST and that the antibody was able to neutralized Y.enterocolitica STs and *E.coli* ST.

Table 5 : Neutralization of *Y. enterocolitica* enterotoxin

Enterotoxin	Activity with normal saline (U/ml)	activity with prepared antiserum (U/ml)
Crude culture filtrate	0.108	0.05
Purified enterotixin	1.29	0.043

Inhibitory activity of *Eruca sativa* extracts: Inhibitory activity of *Eruca sativa* extracts (10, 20, 40, vol/vol of each extract) was tested on purified *Y .enterocolitica* enterotoxin, the result showed that both seed and leave extract had inhibitory activity in dose dependent manner (Table6), the seed extract was more effective than leave extract. It produced 65.4% inhibition of activity at conc. 40 vol/vol. Seed oil of *E. sativa* has promising pharmacological efficacies and ensures the presence of bioactive components responsible for their beneficial effects (11). *E. sativa* seed oil ameliorates the harmful effect of aflatoxin by reduction of both oxidative damage and bioavailability of aflatoxin (12).

Table 6: Effect of *Eruca sativa* extracts on purified *Y.enterocolitica* enterotoxin

Treatment	Activity (U/ml)	Inhibition (%)
control (saline)	1.37	----
Seed extract vol/vol)(		
10	1.017	25.8
20	0.835	39.1
40	0.585	65.4
Leave extract vol/vol)(		
10	1.269	7.38
20	1.122	18.16
40	0.807	31.1

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