

Growth and pathogenesis of Enteropathogenic *E.coli* as affected by bacteriocin produced and purified from *Lactobacillus* isolates with or without *Quercus infectoria* (*Manjakani*) extract.

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Summary

The antimicrobial activity of *Lactobacilli* has been widely exploited for prevention of food-borne pathogens e.g.: *Escherichia coli* being the major cause of diarrhea especially in children, because of bacteriocin activity and the importance of herbal drugs, hence this study was designed to evaluate the synergistic effect of plant extract and bacteriocin produced by *Lactobacillus* on the growth and pathogenesis of Enteropathogenic *Ecoli*.

1. The Plantaricin production was induced by adding the mutagenic agent Mitomycin C.

2. Purification of Plantaricin was made by heating crude plantaricin at 80°C for 10min and then purified by two steps method including extraction with n-butanol followed by gel filtration chromatography on Sepharose 6B column. The results showed that the specific activity was 1600 AU/mg protein with 8 purification folds and 12% recovery yield.

3. The antibacterial activity of *Quercus infectoria* with concentration 300 mg/ml was showed highly antibacterial activity in vitro and in vivo.

4. The result showed synergistic effect of Plantaricin with *Quercus infectoria* extract after experimental infection that induced by orally dosing with *Escherichia coli* in vivo. A result of histopathological study was recorded recovery of tissue.

تأثير البكتريوسين المنتج والمنقى من عزلات العصيات اللبنية بوجود أو عدم وجود مستخلص العفص في امراضية جرثومة الاشريشيا القولونية المرضية

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يعد استخدام النشاط الضد ميكروبي للعصيات اللبنية أمراً شائع الاستخدام لحفظ الأغذية وحمايتها من المسببات المرضية مثل الاشريشيا القولونية المسببة للإسهال خاصة عند الاطفال وبسبب نشاط البكتريوسين والانتشار الواسع للأدوية ذات المنشأ النباتي لذلك صممت هذه التجربة لمعرفة الدور التآزري للمستخلصات النباتية مع البكتريوسين الذي تنتجه العصيات اللبنية في نمو وامراضية الاشريشيا القولونية المرضية.

1. تم أستحداث إنتاج البلانتريسين من خلال إضافة مادة Mitomycin C المطفرة.
2. تمت في هذه الدراسة أيضا تنقية البلانتريسين اذ سخن المستخلص الخام للبلانتريسين بدرجة 80°م لمدة 10 دقائق قبل البدء بالتنقية والتي تضمنت خطوتين الاولى هي الاستخلاص بالمذيب العضوي (البيوتانول) والثانية الفصل بطريقة كروماتوغرافيا الترشيح الهلامي باستخدام عمود (Sepharose 6B). وقد أظهرت النتائج الحصول على البلانتريسين بفعالية نوعية 1600 وحدة/ملغم بروتين وبعدد مرات تنقية بلغ 8 وبحصيلة نهائية 12% .
3. أظهرت نتائج دراسة الفعالية الضد بكتيرية لمستخلص العفص بتركيز 300 ملغم / مل بامتلاكه فعالية ضد بكتيرية عالية في الزجاج و داخل الجسم الحي.

4. أظهرت الدراسة وجود تأثير تآزري للبلانتريسين مع مستخلص العفص بعد أن تم أحداث إصابة تجريبية بالاشريشيا القولونية المرضية داخل الجسم الحي إذ سجلت نتائج التقطيع النسيجي عودة النسيج إلى وضعه الطبيعي تحت هذه المعاملة .

Introduction

Bacteriocins are extracellularly released peptides or proteinaceous antimicrobial compounds, which exhibit a bactericidal effect against closely related bacteria. Bacteriocins of lactic acid bacteria are considered as safe natural preservatives or biopreservatives, as it is assumed that they are degraded by the proteases in gastro intestinal tract .Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which the important ones are Nisin, Bacteriocin, Diplococcin, Acidophilin, Bulgaricin, Helveticins, Lactacins and Plantaricins . The bactericidal activity of bacteriocins is attributable to destabilization of the functions of the cytoplasmic membrane of the target cells, altering the permeability properties of the membrane. *Lactobacillus* species are primarily used as probiotics, but can also be used as starter cultures in various fermented foods. (7).The galls of *Quercus infectoria* Olivier (Fagaceae) are locally known as “*biji manjakani*” in Malaysia, and it is one of the most popular traditional medicines in Asia. The galls have been used to treat diarrhea, dysentery, internal hemorrhages, gonorrhoea, impetigo, tonsillitis, and menorrhagia . Pharmacologically, the galls have been reported to possess activities such as anti-diabetic, antibacterial, antiviral, antifungal, larvicidal, anti-inflammatory, anti-amoebic, and wound healing (3).*Escherichia coli* is one of the most common causes of morbidity and mortality in children with diarrhea all over the world particularly in developing countries .(6)

Materials and Methods

Plant Materials

Manjakani fruits were purchased from a local market in AL-Kut city .Later the plant (fruits) was washed under tap water, and then dried in room temperature at shade . The dried leaves and fruits were crushed to affine powder by an electrical grinder.

Preparation of crude organic solvent extract of *Manjakani* plants

Organic solvent extraction of the *Manjakani* fruit was carried out by using ethanol (95%) which is considered as a very effective in extracting the active ingredients of the plant according to the method described by (5) This was done by using soxhlet apparatus, which consists of an electric heater with a thermostat regulator upon which around bottom glass flask placed that fitted to an extraction unit .The extracting unit contains the solvent and cellulose (thumble) located inside it that contains the dry plant powder .A distiller unit is fitted on to the extraction unit .For condensation of vapor solvent , 50g . of plant powder was put inside the thumble and 500 ml of 95% ethanol was put inside the flask .The extraction was carried out for 24 hrs by heating temperature that kept the solvent at 50-60 C^o until a clear & colorless solvent appeared in the extracting unit . After that, the extract was dried by using an electric oven at temperature 40-45 C^o. The dry extract was placed in an incubator under 38-40 C^o for complete dryness. The final extract was kept frozen at -20 C^o until use.

Preparation of Different Concentration of Plants Extract:

Three serial dilutions 100, 200, 300 mg/ml of *Manjakani* fruits were prepared by suspending 1, 2, 3 gm respectively in 10 ml of Dimethylsulphoxide (DMSO). Each concentration was mixed then filtered by using filtered paper 0.45μ, and kept in sterile test tube at 4°C until used.

Induction for Plantaricin production The qualified producing strains was incubated into the medium then incubated at optimal temperature for optimal time, then the Mitomycin C (2 μg per ml) was added to the broth .

Purification of Plantaricin

1 - Extraction of Plantaricin

Medium was inoculated with bacterial isolate and incubated at optimal conditions for production. Cells were harvested by centrifugation at 6000 rpm for 15 minutes. The cell-free supernatant that was referred to as crude plantaricin extract (CPE) was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15minutes (12).

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The supernatant was mixed thoroughly with n-butanol at a ratio 1:1. The mixture was centrifuged at 4000 rpm for 10 minutes to achieve phase separation. The organic phase was evaporated at 80°C by rotary evaporator, then the sediment was re-suspended in 20mM sodium citrate buffer (pH 5) and referred to as partial purified plantaricin (PPP) (1). The antimicrobial activity of plantaricin and protein concentration was determined.

2 - Gel Filtration Chromatography

Gel filtration chromatography by sepharose 6B was used as a second step for plantaricin purification.

I. Preparation of Sepharose 6B

This gel was prepared according to Pharmacia catalogue as in the following:

Sepharose 6B was washed several times with 20mM sodium citrate buffer (pH 5), degassed for 10 minutes and poured gradually in a column by using glass rod to avoid forming bubbles. Gel was left to settle down and packed well to give column with (1.5 × 64) cm dimensions; column was equilibrated with 20mM sodium citrate buffer (pH 5) overnight with flow rate 40 ml per hour.

II. Loading of Sample

Partial purified plantaricin (PPP) was loaded slowly over the sepharose 6B gel, which was prepared above. The plantaricin was eluted from the column by 20mM sodium citrate buffer (pH 5) and flow rate was adjusted to give 40 ml per hour. 5 ml for each fraction was collected. Activity and absorbance at 280nm of each fraction was determined, the active fractions were mixed. Activity and protein concentration was determined.

In vitro antibacterial activity of *Manjakani* & Plantaricin

Plantaricin was examined for inhibitory activity against EPEC strains of bacteria using the Agar Well Diffusion (AWD) assay (8).

Nutrient agar was seeded with indicator organisms and poured into sterile petri dishes. Wells of 6 mm diameter were cut into the agar and filled with 50 µL of Alcoholic extract. Plates were incubated at 37°C for 24 hr. The plates were afterwards examined for clear zones in the agar surrounding the wells. The experiment was done in two replicates. (2).

Experimental Design

A - Mice were dosed 0.25ml of DMSO per os every day for 21days, served as negative control group.

B- Mice were left without any treatment which served as control positive group.

c - Mice were infected with EPEC in dose about 0.25ml/mice which contained 1.5×10^8 for 7 days, then was treated for 14 days with different way. Mice of this group were treated orally with mixture of plantaricin 320Au / ml and *Manjakani* alcohol extract (300mg/ml) which served as synergistically effect.

Results

Effect of Mitomycin – C on Inducing Plantaricin

The results effect of Mitomycin – C on inducing Plantaricin and Acidocin showed increased of protein concentration and specific activity with used of Mitomycin –C as shown in table (1) from *Lactobacillus Plantarum*.

The results came in agreement with Laftah who used Mitomycin –C with 2 µg/ml for induced Klebocin from *Klebsiella Pneumonia* while (11) recorded production of klebocin with used 0.25µg/ml of Mitomycin –C.

Table (1): Effect of Mitomycin – C on Inducing Plantaricin

Plantaricin	AU/ml	Protein concentration mg / ml	Specific activity AU / mg
No mitomycin – C	80	0.75	106.66
Mitomycin – C	160	0.8	200

Gel Filtration Chromatography

The partial purified plantaricin (PPP) was applied on Sepharose 6B column with (1.5×64) cm dimensions. This column had been equilibrated with 20mM sodium citrate buffer (pH 5) and which later served as the eluant. Each eluted 5ml fraction read at 280nm and the curve was plotted between the absorbance and fraction number which gave 3 protein peaks as shown in figure (1).

The maximum activity of plantaricin was observed in the fractions (27). The specific activity for these fractions was 1600 AU/mg protein with 8 purification folds and 12% yield (table 2).

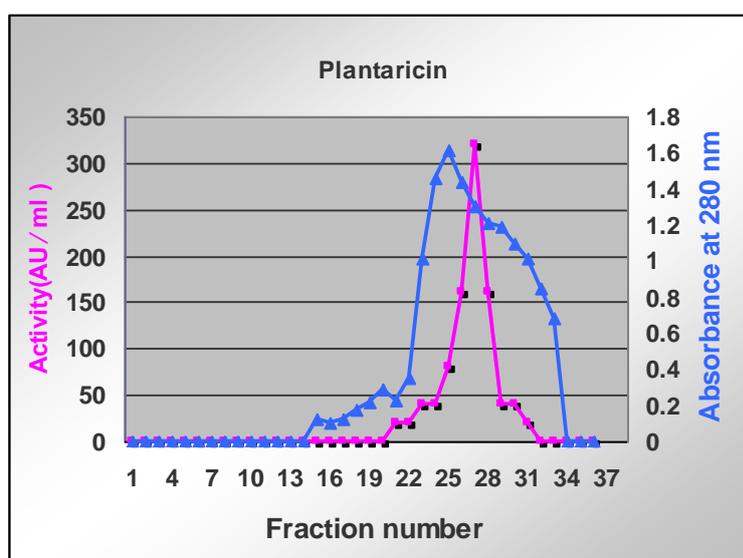


Figure (1) : Purification of plantaricin by gel filtration chromatography), using Sepharose 6B column with dimensions (1.5x64) cm, that equilibrated and eluted by 20mM sodium citrate buffer (pH 5), flow rate was 40ml/hour, with 5ml for each fraction.

Table (2): Steps of purification of plantaricin produced by *Lactobacillus plantarum*

Purification steps	Volume (ml)	Activity (AU/ml)	Protein concentration (mg/ml)	^a Specific activity (AU/mg)	^b Total activity (AU)	^c Purification fold	^d Yield (%)
(CPE)	250	160	0.8	200	40000	1	100
heating (80°C/10min)	250	160	0.6	266.66	40000	1.333	100
Extraction with butanol (1:1)	25	640	0.65	984.61	16000	4.923	40
Gel filtration (Sepharose 6B)	15	320	0.2	1600	4800	8	12

^aSpecific activity (AU/mg): represents plantaricin activity divided by protein concentration.

^bTotal activity (AU): represents Activity (AU/ml) × Volume (ml).

^cPurification fold: represents specific activity of purified fraction divided by specific activity of crude extract.

^dYield (%): represents (total activity of purified fraction divided by total activity of crude extract) × 100 (%).

In vitro antibacterial activity of *Manjakani* & Plantaricin

The results showed that plantaricin of the *Lactobacillus plantarum* have an antibacterial activity against the growth of EPEC (Fig 2), the antibacterial activity of Plantaricin against EPEC showed (40 mm ± 0.0).

The result has showed that ethanolic extract of *Quercus infectoria* extract 100 mg/ml, 200 mg/ml and 300 mg/ml affected the growth of the EPEC (Fig 3) and other bacterial strains. The 300 mg/ml showed highly inhibition zone (26 mm ± 0.0) for *E.coli*. (Table 3).

Table (3): In vitro antibacterial activity of *Manjakani* & Plantaricin

Bacteria	Mean value of inhibition zone in mm ± SE				
	Plantaricin	Manjakani 100	Manjakani 200	Manjakani 300	DMSO
<i>E .coli</i>	b 40 ± 0.0 B	b 22 ± 0.0 C	b 24 ± 0.0 B	b 26 ± 0.0 A	0±0 D

Differences small and capital letters revealed significant differences between the groups at significant level (p≤0.05).

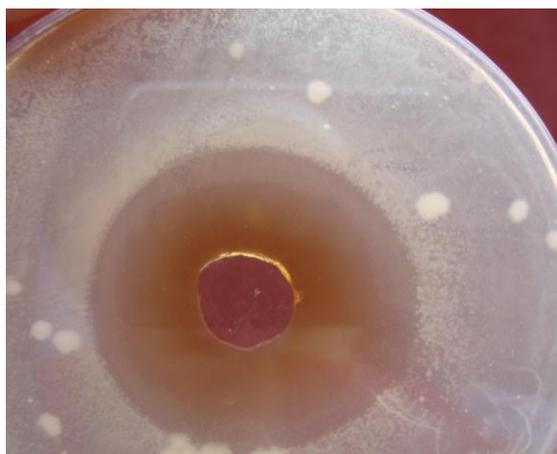


Figure (2): Antibacterial effect of Plantaricin against *EPEC*.



Figure (3): Antibacterial activity of *Manjakani* against EPEC

Synergistic effect of Plantaricin & *Manjakani* invivo

the histological examination in control positive indicated that the intestine showed mononuclear cell infiltration in sub epithelial layer and goblet cell hyperplasia and erosion of epithelial lining of villi Fig (4).while Results show the synergistic effect of plantaricin with *Manjakani fruits extract* , showed that no clear pathology lesion except mononuclear cell in lamina properia (Fig 5).

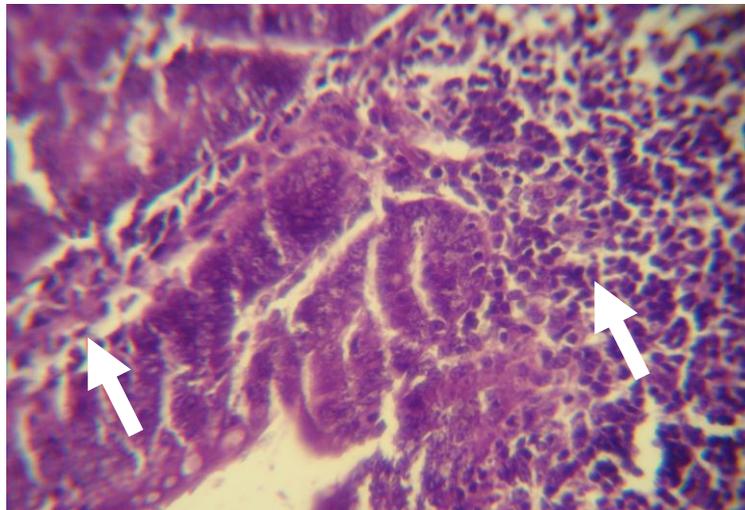


Fig (4): Histological section in intestine of mice in control positive show mononuclear cell infiltration in sub epithelial layer and goblet cell hyperplasia and erosion of epithelial lining of villi ← (H&E 40 X).

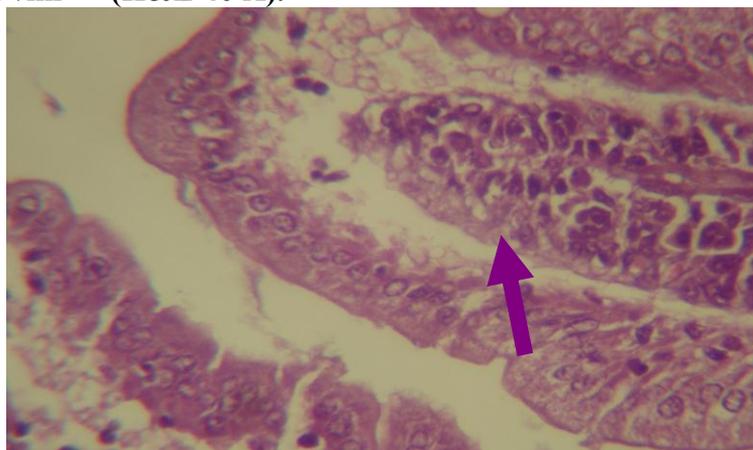


Fig (5): Histological section of mice intestine in treated group there was no clear pathology lesion except mononuclear cell in lamina properia ← (H&E 40 X).

Results showed the synergistic effect of plantaricin with *Manjakani fruits extract*, gave highly effective because ability to stimulate immune system. Bacteriocins such as plantaricin are proteinaceous antibacterial compounds and exhibited bactericidal activity (10).

Flavonoids were widely found in *Manjakani* fruits and possess many function including antiviral, anti-allergic, anti- platelet, anti-inflammatory, anti-tumor and antioxidant activities (4).

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