Escherichia coli Isolated from Horses and Study the Effect of the Peganum harmaline Extract In Vitro and In Vivo and Antibiofilm Effect In Vitro

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ABSTRACT

Out of a hundred horses, fecal samples *Escherichia coli* was isolated from 37 samples of different ages, The isolated samples had been used to examine the effect of *peganum harmaline* on isolated *E.coli* in vitro and in vivo.

The goal of this research was to determine the antimicrobial activity of *peganum harmaline* extract by means of (ethanol: methanol 1:1) in opposition to *Escherichia coli* at various 40,20, 10, 5, 2,5, 1,25 and 0,625 mg/ml concentrations in each plastic and glass tube. The values of MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) for the extract against *E.coli* were equal to (0.625 mg/ml) for MIC and (10 mg/ml) for MBC on bacteria which cultured on glass tube while the MIC value was 40 mg/ml and MBC was10 mg/ml on plastic tube. The effect of *Peganum harmaline* on the formation of *E.coli* biofilm was investigated and the biofilm inhibitory concentrations were 40-6.25mg / ml in vitro. In vivo, a group of laboratory mice used, the LD50 of *peganum harmaline* extract was tested orally by up and down method and found to be 1030 mg/kg body weight, the extract used as 103 mg/kg bodyweight treatment after causing *E.coli* infection at 1×10^8 CFU/ml in laboratory mice the treated persist for two weeks in group one and three weeks in the second group as well as three week treated in the third group from the second days after infection while the control group left without treated and the mice sacrificed after second, third and fifth days of infection. The result shows histopathological changes in all treated groups, especially in the third group and that refers to the antibacterial effect of the *peganum harmaline* ethanolic extract when compared to the infected control group.

Keywords: *Escherichia coli*, *peganum harmaline,* antibacterial, antibiofilm, LD50, histopathological lesion.

INTRODUCTION

Escherichia coli infection is one of the most important diseases that affected many animal species that can cause diarrhea, dysentery or urinary tract infection depend on pathotype of *E.coli* [1, 2].

There was an extensive study of the virulence factors and mechanisms by which *E.coli* strains can cause diarrhea in humans, pigs, and calves [3].

In recent years the bacteria become resist many kinds of drug [4] so that we found other sources as antibacterial. There are many natural plants in nature, have antibacterial activity depend on their chemical composition [5]. *Peganum harmaline* contain alkaloid that is making the plant antimicrobial effectiveness [6].

There are many studies around the world on *P.harmaline* effect in many diseases Parkinson's disease [7], anti leishmanial [8].

This study aimed to isolate the *E.coli* bacteria from horses and used of *P.harmaline* as an antibacterial and antibiofilm drug in vitro and in vivo.

MATERIAL AND METHOD

1. Samples

Fecal samples had been accumulated from horses at different ages in a septic disposable plastic container after that the sample transport to the bacteriology laboratory of zoonotic diseases unit/college of veterinary medicine/Baghdad university.

2. Bacterial isolation

Each one gram of samples diluted with three ml of sterile phosphate buffered saline, then one loopful cultured on MacConkey's agar after 24hr of incubation at $37C^0$ the pink colony cultured on eosin methylene blue agar then confirm the isolate by *API 20E* [9].

3. Plant extraction

The seed was percushed from the local market, then grinding and the extract obtained as describe in [10].

4. Antibiotic susceptibility testing

4.1. Agar well diffusion for *P.harmaline*

A density of 10^8 cells/ml of 0.5 McFarland standard was prepared for bacterial broth cultivation, The liquor was distributed evenly over the Muller Hinton agar By swab of sterile cotton, Equal distance holes with a disinfected 5 mm in diameter, cork borer, 2 mm from the edge of the plate, were made on each plate as describe by [11]. Various concentrations were prepared from crude *P. harmaline, 150*,100,50,20,10 and Five micro liters of each plant extract (500 mg / ml) were inserted aseptically into the respective well of the agar, after that incubated the agar plate overnight at $37C^0$, then read the zone of inhibition in diameter.

4.2. Detection of Minimum inhibitory concentration of *P.harmaline* (MIC)

In a sequence of eight test tubes, the extracts of *P.harmaline* was diluted double fold (2:2) with Muller Hinton broth. Each tube as well as control tube weres inoculated with an aliquot of 1ml of the bacterial suspension ($1x10^8$) cfu/ml, With the same quantity of Muller Hinton broth, except the last tube use as control injected with 1 ml of 10^8 CFU/ml bacterial culture plus 1 ml of Muller Hinton broth. All the tubes have been incubated for 24hrs at 37°C. The determination of the MIC assay by bacterial growth visualization by using the salt (P-iodonitrotetrazolium violet) (INT) (0.04mg / ml) was added to each tube with as 0.5 ml and all tubes were incubated at room temperature for 6 hours. The tubes have been checked for change of color and the first transparent tube showed the MIC, which did not change to a red color compared with control tubes [12].

4.4. Detection of Minimal bactericidal concentration of *P.harmaline* (MBC)

MBC has been performed by subculturing a loopfull of each MIC exhibit no growth (clearance).MBC was taken as the largest dilution (smaller concentration) that did not produce a single colony of bacteria.

4.5. *P. harmaline* extract's effect on the production of bacterial biofilms

The development of biofilms in plastic and glass sterile test tubes has been measured with two-fold dilution of plant extract with muller hinon broth started from (20) ended with 0,312 mg/ml) concentrations , clinical isolate of *E.coli* cultured in broth and Standardizing as 0.5 McFarland turbidity put 1ml in each tube and incubated the tube for 4-5 hr at 37^0C Allowing bacteria to adherence thereafter apply 1ml of each concentration to each tube furthermore incubated the tube 24 hours at 37°C, control tube contain bacteria with muller hinton only.

4.6. Bacteria's capacity to bind in tubes

By utilizing of 1 % crystal violet staining to measure the adhered biomass of the cells each tube wash three times with phosphate buffere as described by [12].

4.7. LD50 dose of *P.harmaline* in laboratory mice

Oral LD50 was established as an initial attempt to assess the toxicity of alkaloids using the up-and-down approach [13]. Based on the toxicity level chart, the alkaloids tested were considered to be marginally toxic to mice (1070 mg / kg body weight [14], Sub-lethal doses (500 and 1000 mg / kg body weight) were survived mice,exhibit lethargy and prostration, lack of appetite, trembling, difficulty walking and sometimes loss of consciousness until recovery or death. *P.harmaline* extract LD50 was (1030 mg / kg B.W) and the dose treated was 103 mg / kg B.W orally.

4.8. Infective dose of *E.coli* in laboratory mice

Isolated bacteria cultivate in the tryptic soya broth overnight at $37C^0$, bacterial count done by Miles and Misra approach to get 1×10^8 CFU/ml [15,16], A group of laboratory mice 3-4 month of age divided into four groups all mice administrated with 0.2 ml of 1×10^8 CFU/ml *E.coli* suspension except the control group administered normal saline as control negative. First group: Animals were administered daily for ten days orally 0.2 ml of *P.harmaline* extract, then the animals were sacrificed and collect the internal organ for histopathology, second group treated for twenty days then sacrificed else third group treated for along thirty days the animals were sacrificed .

RESULTS

1-Isolation

E.coli was isolated from 37 out of hundred horses fecal samples obtained in Baghdad's Al-Furusia group, which are listed in Table1.

Table 1: Number of positive *E.coli* from horses fecal samples collected in Al-Furusia club of Baghdad

	Number of samples	Number of positive	Percente of isolate
Horses fecal samples	100	37	37%

2-*P.harmaline* susceptibility testing in agar well diffusion test

Crude extracts of *P.harmaline* demonstrate inhibition of bacterial growth of 150 percent, 100 percent, 50 percent, 20 percent, and 10 percent against *E.coli*, with no inhibition of 5 percent (table 2), The plant has inhibited bacterial growth from 44 mm to 7 mm in diameter .

Table 2: Explain the concentrate extract of *P.harmaline* and zone of inhibition against *E.coli*

P.harmaline extract concentrate	Zone of inhibition /mm
150	44mm
100	41mm
50	35mm
20	33mm
10	7mm
5	No zone

3-Minimum inhibitory concentration and Minimum bcteriocidal concentration of *P.harmaline* (MIC and MBC)

The result of the MIC of *P.harmaline* extract was comparable to that obtained in the agar-well diffusion technique, all plant extract concentrated (started with 20 to 0.625 mg/ml) has MIC activity against *E.coli* except 0.312 mg/ml in glasses tubes, whereas plant extract concentrated from 20 to 1.25 mg/ml the MIC activity against *E.coli* in plastic tube.

Whereas the findings have been reported for minimum bactericidal concentration (MBC) was 10mg/ml in glasses tube and 20 mg/ml in plastic tube.

4- Antibiofilm

Anti-adhesion tests were performed using crystal violet experiment in order to evaluate plant extract inhibition ability against *E.coli* attachments at concentration MIC value in glass and plastic tube (figure 1 and 3). Results of alcoholic extract of *P.harmaline* in vitro antibiofilm activity could inhibit bacteria cell attachment Biofilm formation inhibition induced on *E.coli* Up to 95% show in figure 6 and 8 of glass and plastic tube respectively.

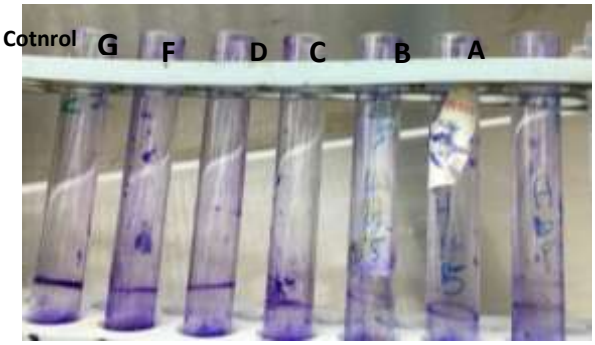

Figure 1: Antibiofilm of plant extract concentration A:20; B:10; C:5; D:2.5; E:1.25; F:0.625; G:0.312 mg/ml and H: control positive in glass tube

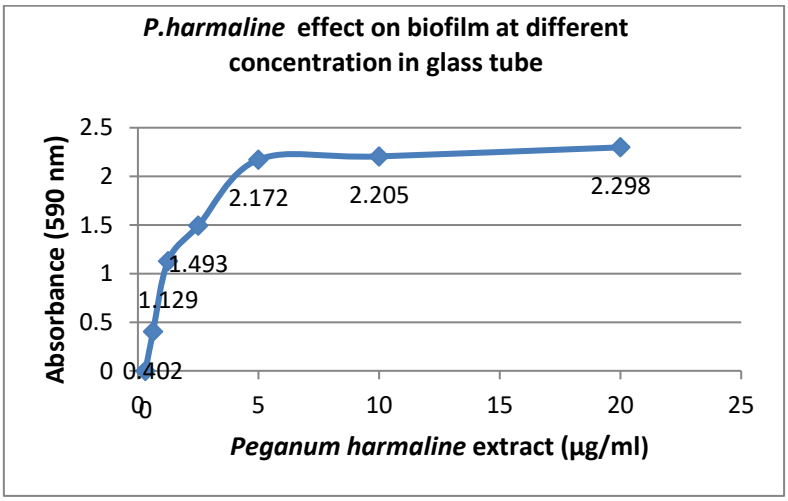

Figure 2: Antibiofilm of plant extract concentration curve revealed the higher inhibition at 20mg/ml of *P.harmaline* at 590 nm on glass tube

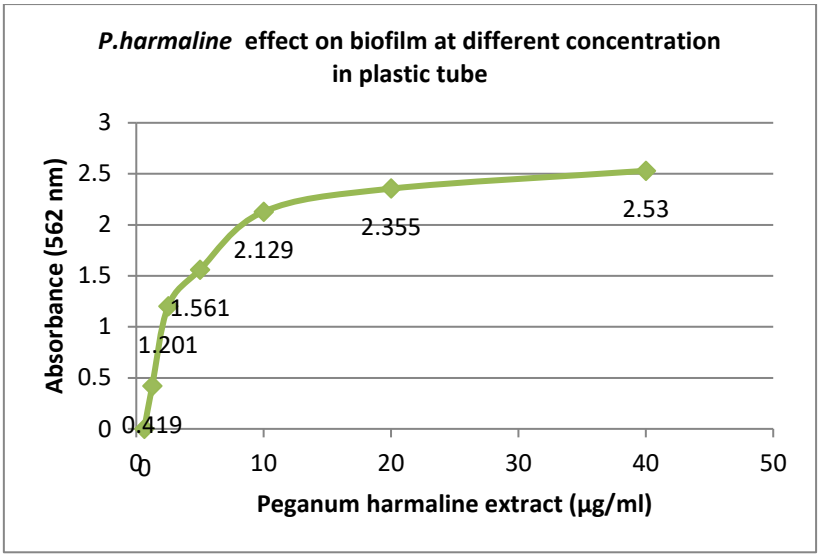

Figure 3: Antibiofilm of plant extract concentration curve revealed the higher inhibition at 40mg/ml of *P.harmaline* at 590 nm on plastic tube.

5. Histopathological study of *P.harmaline* against *E.coli* infection

The intestine was shown to invade few mononuclear cells in the intestinal mucosal lamina properia. after ten days of treatment with plant extract, likewise aggregation of mononuclear cell in the sub-mucosa and mucosal glands after twenty days of treated animals as shown in, furthermore the intestine of animal at day 30 of treatment shows marked mononuclear cells infiltration between mucosal glands(figure 4) whereas the control group showed focal enteritis (figure 5).

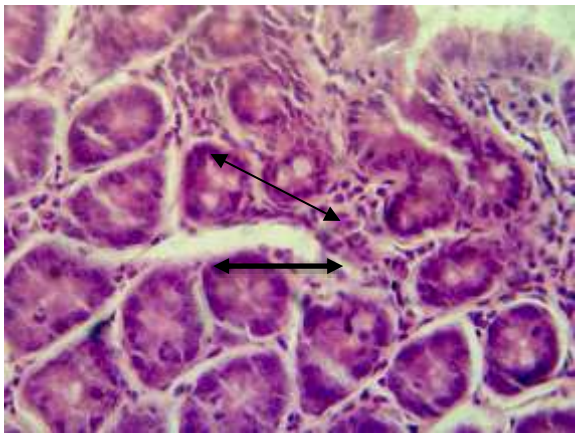

Figure 4 :Section in the intestine animal at day 30 shows marked mononuclear cells infiltration between mucosal glands (H&E stain 400X)

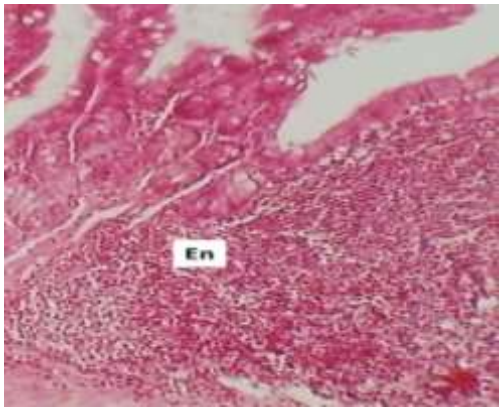

Figure 5: section of small intestine shows; focal enteritis (En). H&E stain.40x in control positive group.

Liver sections showed pathological changes in different treated group compare with control group that appeare sever changes without treatment (Figure 6 and 7).

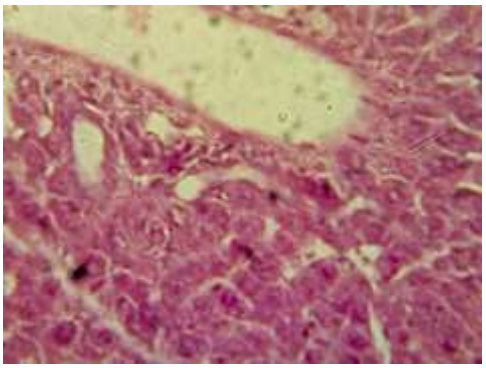

Figure 6: Section in the liver animal at day 30 shows few mononuclear cells infiltration in portal area (H&E stain 400X

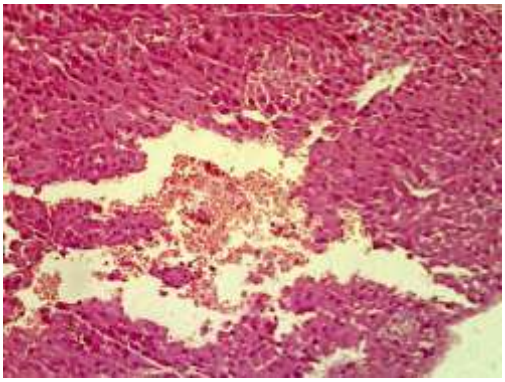

Figure 7: Section in the liver of control animal shows necrotic area replacement with RBCs (H&E stain 400X)

DISCUSSION

Escherichia coli is the gram-negative microbe most commonly isolated and recognized in laboratories of clinical microbiology [17]. Neonatal diarrhea resulting from *E.coli* is a major problem for newborns in developing countries [18] and for farm animal breeding, in particular for calves and piglets [19-21], Horses were very susceptible to *E.coli* infection, especially after administered with cefquinome alone or in combination with other antimicrobial agents which produce CTX-M *E.coli* in hospitalized horses [22]. Researchers were encouraged to identify alternatives for the treatment of infections due to the increasing complexity of most microbial infections and resistance to conventional therapy. In this regard, plant extracts and other biologically active compounds isolated from plants have attracted widespread interest as they are recognized to treat diseases. Biofilm is one of the main virulent factors of most pathogenic microorganisms *P.harmaline* displayed a qualitative improvement in biofilm morphology and decreased thicknesses in differen concenraion thus due to the active ingredian which is alkaloid that present in *P.harmaline* [23]. The antibiofilm effect obtained was indicated as being dose-dependent [24]. Although the biofilm formed weaked on glass tube and strong on plastic tube so more concentrated need to remove the biofilm on plastic surface than glass surfaces [25]. Thus, recent developments in effective and safe medicinal products, especially plant extracts with antibacterial effects, have received increasing interest from both the scholarly and manufacturing sectors. [26]. This finding suggests that *P.harmaline* can have a highly protective effect against *E.coli* infection likewise *P.harmaline* can be beneficial in preventing diarrhea in humans and farm animals, *P.harmaline* demonstrated 100% inhibitory activity towards *E.coli* at 20mg/ml with lowest MIC values furthermore Oral dosing of *P.harmaline* was stopped pathogenic *E. coli* infection in mice and increased induction of macrophage as appear in histopahological changes of intestine and liver in differen groups compare with control, although the crude extract has antibiofilm activity against bacterial biofilm in both plastic and glass tube at 10 mg/ml in glass tube otherwise 20mg/ml antibiofilm in plastic tube . We assume that this study would help humans, farmers, pharmaceutical manufacturers and researchers concerned although In this study, we found that *peganum harmaline* treatment was very effective in preventing a pathogenic *Escherichia coli* disease in mice this is the same finding in previous study which focus on the role of plant extract for treated *E.coli* [27].

CONCLUSIONS

P.harmaline have antimicrobial and antibiofilm activity towards *E.coli* infection with lowest MIC values furthermore Oral dosing of *P.harmaline* was stopped pathogenic *E. coli* infection in mice and increased induction of macrophage.

CONFLICT OF INTEREST

None

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