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# EXTRACTION AND PURIFICATION OF TANNINS FROM *PLANTAGO LANCEOLATA* L. AND ASSESSMENT THEIR ANTIBACTERIAL ACTIVITY ON PATHOGENESIS OF ENTEROPATHOGENIC *E.COLI* *IN VITRO* AND *IN VIVO*

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**ABSTRACT**
The antimicrobial activity of *Lactobacilli* has been widely exploited for prevention of food –borne pathogens e.g.: *Escherichia coli* being the major cause of diarrhoea especially in children, because of bacteriocin activity and the importance of herbal drugs, hence this study was designed to evaluate the effect of plant extract on the growth and pathogenesis of Enteropathogenic *Ecoli* . The tannins of *Plantago Lanceolata* were extracted and purified by TLC and HPLC and the antibacterial activity of tannins of *Plantago Lanceolata* with concentration 150 mg/ml was showed highly antibacterial activity *in vitro* and *in vivo.* The result showed effect of *Plantago Lanceolata* extract after experimental infection that induced by orally dosing with *Escherichia coli in vivo*. A result of histopathological study was recorded recovery of tissue.

**KEY WORDS:** Antimicrobial, *Escherichia coli, Plantago lanceolata*

## INTRODUCTION

*Plantago Lanceolata* is a perennial plant from Plantaginaceae family, *P. Lanceolata* has also been used as an anesthetic, antiviral, anti-inflammatory, astringent, anti-helmintic, analgesic, analeptic, antihistaminic, anti-rheumatic, antitumor, anti-ulcer, diuretic, expectorant and hypotensive in traditional medicine . (Kobeasy *et al*., 2011; Basri *et al*., 2011). Kobeasy *et al*., (2011) shows that the *P. major* and *C. tetragonoloba* contained important biologically active compounds and *P. major* leaves had the highest total phenol, flavonoid and tannin content. In addition, ethanol, cold and hot extracts of the same plants showed antioxidant activity, but the highest antioxidant activity was found in ethanolic extract of *P. major* leaves .Also, ethanolic extract of *P. major* leaves had the greatest effect on tumor cell growth followed by hot water extract of *P. major* leaves. The antibacterial and antifungal activity of an ethanolic extract from *Plantago lanceolata* were also investigated by AL-Ukaily, (2009) by agar diffusion and microdilution methods using *E. coli*, *Proteus mirabilis*, *Enterococcus faecalis*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Candida albicans*, *Candida kruzei* and *Candida parapsilosis*. Antibacterial or antifungal effects were not observed for *Plantago lanceolata*. *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 (Enayat *et al*., 2011). Hence, The Present study was designed to evaluate the effect of plant (*Plantago Lanceolata*, L.) extract on the growth and pathogenesis of Enteropathogenic *E. coli.*

## MATERIALS AND METHODS

### Plant Materials

*Plantago lanceolata* leaves were collected from the field of the AL –Kut city -Iraq. Later these plant (leaves) were 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.

### Extraction and purification of tannin of *Plantago Lanceolata*

According to Moosophin *et al*., (2010) with some modification. Thirty grams of *Plantago Lanceolata* was extracted in three subsequent mixtures of 95% ethanol and water (1:1 v/v) with the ratio of 1:10 at 80°C for 2 hours. Insoluble materials which contained very little tannin were removed by filtration with a nylon filter and centrifuged at 3500 rpm for 15 minutes. The ethanol in the solution was removed by a rotary evaporator at 40°C. The remaining tannin was mixed with an equal volume of 1 mM acetate buffer at pH 4, and the remaining aqueous phase was extracted twice with an equal volume of ethyl acetate. The aqueous phase was evaporated to dryness by the rotary evaporator, redissolved in a minimum volume of 80:20 ethanol water (v/v), and then chromatographed on a 2.5 cm x 30 cm column of Sephadex LH-20 (Pharmacia Fine Chemicals), which was previously equilibrated with absolute ethanol. The gel was washed repeatedly with absolute ethanol at the flow rate of 0.8 ml/min, and monitored at 280 nm. The gel was then washed with 50:50 acetone-water (v/v) at the flow rate of 0.9 ml/min and monitored the absorbance at 540 nm. The 50% acetone fraction, which contained the tannin, was evaporated by the rotary evaporator to remove acetone, and the aqueous solution was extracted three times with an equal volume of liquefied phenol. The aqueous phase was washed with a small amount of diethyl ether to remove phenol, evaporated to dryness, and redissolved in a minimum volume of absolute ethanol. This material was chromatographed on Sephadex LH-20 with an absolute ethanol mobile phase at the flow rate of 0.9 ml/min, and monitored at 280 nm. When a stable base line was reached, the mobile phase was changed

  

to 50% aqueous acetone, and monitored at 540 nm. The fractions containing tannin were pooled on the basis of the retention time.

**Analysis of tannin by Thin Layer Chromatography (**Konan *et al*., 2008 **)**

An aliquot of every extract is dissolved in 1 ml of appropriate solvent (generally $CHCl_3$). For TLC, we used silica gel sheets (silufol 60 F254, aluminum support; Merck) in appropriate solvent system: $CHCl_3$ /MeOH/AcOH (18:1:1, v/v/v), revealing - $FeCl_3$ for tannins. Rf was calculated for every constituent.

*Thin layer chromatography* - Kieselgel GF254 plates, 20 x 20 cm, 1 mm thick, were used. TLC plates were run in duplicate and one set was used as the reference chromatogram. Spots and bands were visualized by UV irradiation (254 and 366 nm) and $H_2SO_4$ spray reagent.

**Analysis of tannin by High performance liquid chromatography ( HPLC )**

HPLC fingerprints were prepared using achemito LC6600 model , equipped with isocratic pump and UV-VIS detector. Solvents were pre-filtered by using a millipore system and analysis were performed on reverse phase Lickrospher C18 column (250× 4.6 mm i.d.,5µm).For injection in HPLC system the active spots were scraped from the reference TLC plates and dissolved in methanol. Injection volume was 5µl for all the cases. All the extracts were detected at UV wavelength of 318 nm .The flow rate was 1.0 ml /min in all cases .Mobile phase used for different extracts were acetonitrate and acetic acid ( 70:30 ) (Sharma *et al*., 2008).

***In vitro* antibacterial activity of tannins of *Plantago lanceolata***

Tannins from *Plantago lanceolata* was examined for inhibitory activity against EPEC strains of bacteria using the Agar Well Diffusion (AWD) assay (Lasta *et al*., 2008).

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 tannin. 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. (Adetunji and Olaoye,2011).

**Experimental Design**

Three groups of male mice (weight 24-28 gram) including four mice/group were divided randomly into:

**A** - Mice were dosed 0.25ml of DMSO per dose 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 $1.5\times 10^8$ for 7 days, then was treated for 14 days with different way. Mice of this group were treated orally.

**Statistical analysis**: Completely randomized design (CRC) program( SAS, 2001). Was used to test the effect of the treatments on traits involved in this study. The least significant difference (LSD) test was also used to compare significance between the means (Steel and Torrie, 1980).

## RESULTS AND DISCUSSION

Thin layer chromatography using Chloroform: Methanol: Acetic acid (18: 1: 1) as the developing solvent was able to separate different chemicals having different retention factor (Rf value) present in plant extracts. TLC resulted in identification of 2 spots with the Rf Value 0.424 and 0.563. To assess the major active constituent responsible for antimicrobial activity against bacteria, TLC bioautography was performed. Clear inhibition zone were observed at $R_f$ of 0.424 , 0.563 for *P.Lanceolata* indicated the presences of tannins (Fig. 1) and this result was agreement with Békro *et al*., (2008) showed the tannin appear as gray spotlights with $R_f$ (0.47 and 0.56). TLC is a standard technique, which separates the organic compounds of lower molecular weight according to their polarity.

It is expected that more active compounds can be detected by TLC bioautography, if different solvent systems, microbial strains and more plant extracts are used. Bioautography of thin layer chromatographic plate showed clear zones containing substances that inhibited the growth of bacteria over the region containing the components with high and medium polarity, in majority of the plants tannins were observed as most active constituents. (Sharma *et al*., 2010; DE, *et al*., 2010).

The results of the HPLC analysis of *Plantago Lanceolata* showed Tannins with 3 prominent peaks with retention time of 1.732 , 2.088 , 2.451 minutes , Figure (2) and the two peaks which come with 2.088 and 2.451 its belong to tannin when compound with tannin which came at 2.144 minute .Figure (3).

One peak have been identified as tannin using standard solution under similar condition ,HPLC analysis revealed the identity of bioactive constituents present in the plant Phytochemical analysis of extracts demonstrated the presence of phytoconstituents like tannin . Antimicrobial action of tannin may be related to their ability to inactivate microbial adhesions, enzymes, cell envelops transport proteins (Sharma *et al*., 2010 ).

  

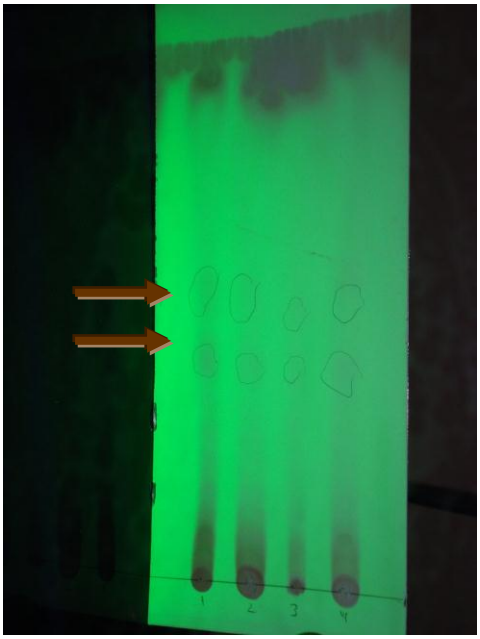

**Figure 1. Thin layer chromatography plates of *Plantago Lanceolata* extract the brown zone indicate the presence of tannin**

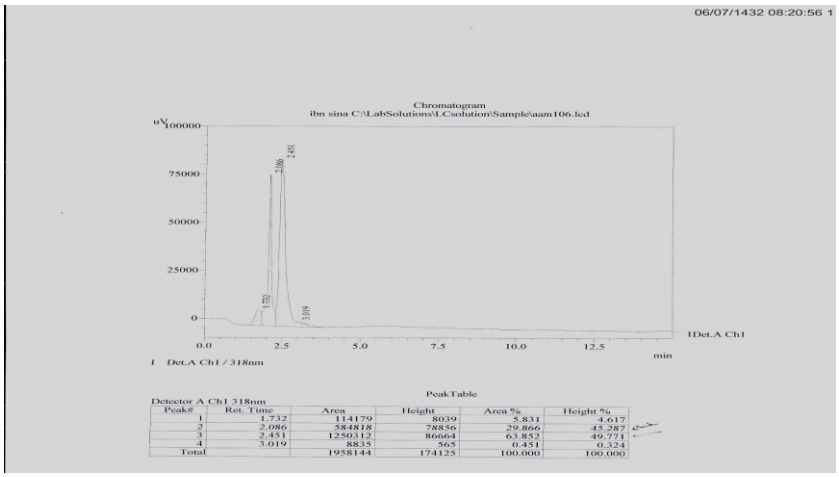


**Figure 2. HPLC chromatography for tannin purification from *Plantago Lanceolata***

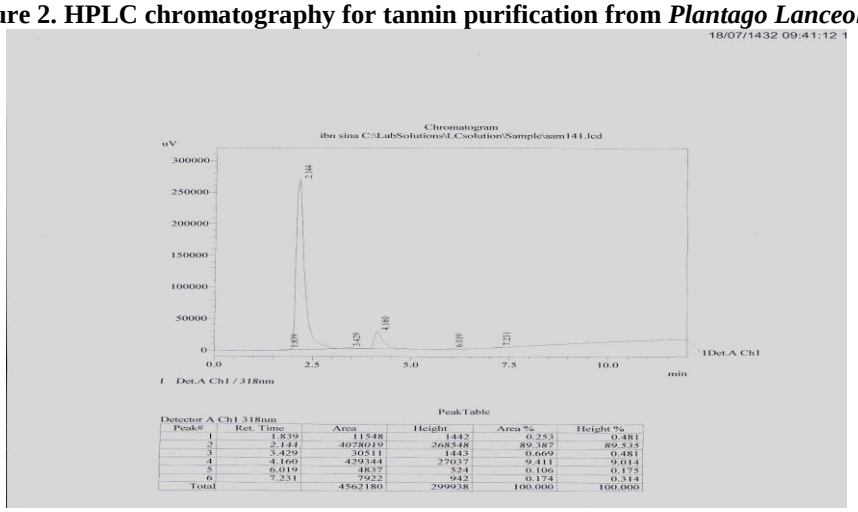


**Figure 3 . HPLC chromatography standard *In vitro* antibacterial activity of tannin.**

  

The result has showed that tannin extract 150 mg/ml affected the growth of the EPEC.The 150 mg/ml showed highly inhibition zone (29.66 mm ± 0.29) for *E.coli*. (Table 1).

**Table 1. *In vitro* antibacterial activity of *Manjakani* &Plantaricin**

| Bacteria | Tannin **150** mg/ml | DMSO |
|---|---|---|
| **E .coli** | **a 29.66 ± 0.29 A** | **0±0 D** |

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

**Effect of tannin *in vivo***

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 effect of tannin showed that no clear pathology lesion except mononuclear cell in lamina properia . Figure 5.

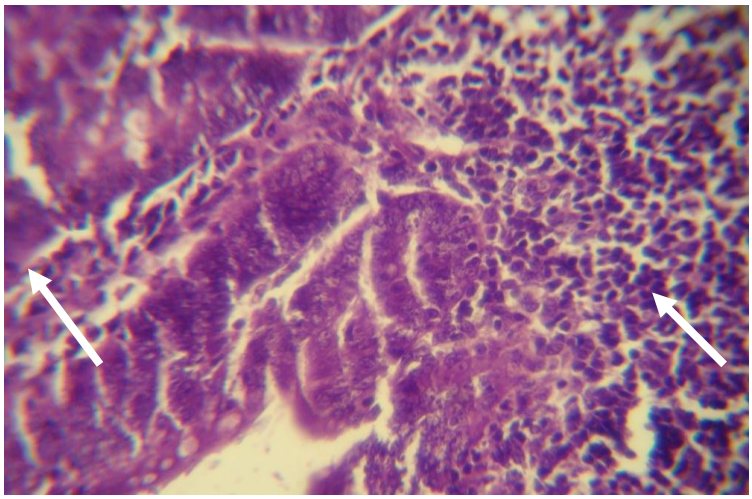

**Figure 4. Histological section in intestine of mice in control positive show mononuclear cell infiltration in sub epithelial layer and goblet cell hyperplasia and erosion of epithelial lining of villi ← (H&E 40 X).**

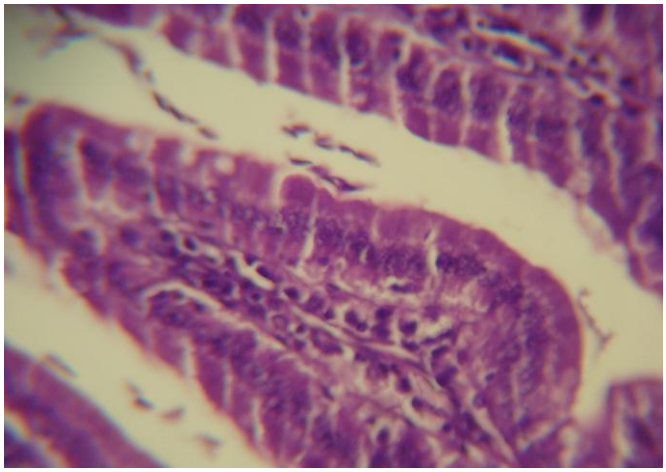

**Figure 5. Histological section of mice intestine in group treated with tannin show no clear pathology lesion ← (H and E 40 X).**

  

The *plantago* extract contain tannin has anti-inflammatory effect which helps to controls all indications of gastritis and irritating bowel disorders by its ability to bind to the proteins of the pathogenic or by precipitation the pathogenic proteins as well as by its ability to promot, alter the nature of the pathogenic proteins from normal state to shrink by hydroxyls or carboxyl group which bind to the protein and alter its nature through formation a strong complex and this was agreement with (AL-Ukaily, 2009; Effraim *et al*., 2000)The histological structure of the organ of the group treated with acidocin, showed hyperplasia of goblet cell with moderate mononuclear cell infiltration in lamina properia , the hyperplasia of goblet cell ,few mononuclear cell infiltration in lamina properia and hyperplasia of lymphoid tissue , After an inflammatory stimulus, goblet cells, present in the intestine, release mucous, The enhanced presence of goblet cells would insure an improved synthesis of mucous, which improve the intestinal barrier and functioning, and thereby increase host protection against infections (Kaur *et al*., 2011).

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