ISSN: 2321-3760

Research Article

International Journal of Current Trends in Pharmaceutical Research

IJCTPR, 2014, Vol. 2(2): 400-404

www.pharmaresearchlibrary.com/ijctpr

Cellular mechanisms involved in *Helicobacter pylori* induced infection and its eradication strategies by potential root extracts of *Plumbago zeylanica* L

G. Renuga*[1] and A. Kennath[2]

[1]*Department of Biotechnology, Ultra College of Pharmacy, Madurai, Tamil Nadu, India.*

[2]*Department of Oral Pathology, Ultra Best Dental science college, Madurai, TN, India.*

Abstract

The research was designed to investigate *H. pylori* infection induced in mouse can be used as a model to design a drug for the treatment of peptic ulcer. A novel compound purified from root of *Plumbago zeylanica* L showed Plumbagin has the potential action of plasma membrane repair it can be considered as drug for experimental groups. The mouse were divided into four groups comprising of five animals in each group and designated as follows: Control, *H. pylori* infected animals, *H. pylori* infected animals treated with plumbagin and omeprazole. At the end of the experimental period, analysis carried out with gastric tissues and the repair responses to this injury were assessed. (FDx- green stain showed presence of membrane injury in infected but not treated animals, FDx , Red showed in the *H. pylori* infected plus treated with plumbagin and mixed spots of red & green in the case of standard drug treated mice, control has showed no *H. pylori* in the mucus. Plumbagin entry has been shown to activate a classic membrane repair response. From a biological point of view, the relationship in the outcome of eradication therapy has been analyzed by western blot results could be confirmed the mucosal expression of Interleukin-8 (IL-8) upregulated in the Plumbagin treated gastric cell than the *H. pylori* infected cells which attributed to the eradication in severity of gastritis due to the cytotoxin gene. Plumbagin might be diffused through the gastric mucosa triggered members cell surface expression cytokine thereby repair epithelial cell damage.

Keywords: *Helicobacter pylori,* Plumbagin, Western blot, Interleukin-8, Membrane repair.

Contents

1. Introduction 400
2. Experimental 401
3. Results and discussion 402
4. Conclusion 404
5. Acknowledgement. 404
6. References 404

***Corresponding author**

Dr. G. Renuga

E-mail: renugabiog@gmail.com

MS.ID: PRL2014-IJCTPR1988

PAPER-QR CODE

© 2013, IJCTPR All Rights Reserved

1. Introduction

Helicobacter pylori infection has great attention today because of its crucial role in the pathogenesis of chronic gastritis, peptic ulcer diseases and in the multi-step carcinogenic process of gastric cancer. *H. pylori* infection causes gastric mucosal inflammation, which could not only lead to chronic gastritis and peptic ulcer disease, but also to gastric adenocarcinoma or low-grade mucosa-associated lymphoid tissue (MALT) lymphoma. Prospective studies

revealed that the risk of development of gastric carcinoma was much greater in *H. pylori*-infected populations than in uninfected populations [1]. The clinical outcome of *H. pylori* infection was determined by multiple factors, including host genetic predisposition (cytokine polymorphisms) [2] bacterial strain heterogeneity and environmental factors, such as dietary salt intake [3]. *H. pylori* has heterogeneous both genotypically and phenotypically, and was adapted for survival in the gastric niche. The present knowledge of *H.pylori* infection and its etiologic importance in peptic ulcer and in gastric malignancy appears to be a major risk factor for gastric Non-Hodgkin's lymphoma and MALT lymphoma. MALT lymphoma regresses in about half of the cases when *H.pylori* infection was eradicated with antimicrobial agents [4].

There was evidence that some *H. pylori* virulence factors significantly affect bacterial susceptibility towards different antibiotics. Previous studies reported that CagA-positive *H. pylori* strains has been more susceptible to antibiotics when compared to CagA-negative. Two important bacterial factors *CagA* and *VacA* associated with *H.pylori* infection are of public health importance, would have implication for strategies of control and therapy [5]. *H. pylori* infection in animal considered as an excellent model system to study bacterial-induced epithelial cell signaling cascades which were a key feature of the increased risk of developing gastric cancer [6]. The present work focused that *H. pylori* caused plasma membrane disruption injury and also a cell proliferation responses were activated against physical and biological damage [7]. Medicinal plant has helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings. Many plants have therefore become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicine. This project was undertaken that to develop a drug with Plumbagin which was extracted from *Plumbago zeylanica* L, the novel compound already characterized in our previous work [8]. Present concept was focused to analyze the efficiency of plumbagin on the repair response to *H. pylori* activation of epithelial cell damage and monitored cellular repair mechanism behind it for *H. pylori* eradication. Strategies were designed might provide a new insight into potential eradication of *H. pylori*-induced inflammation under Plumbagin administration as drug in animal model.

2. Materials and Methods

Collection of Bacterial Strain:

The *H. pylori* (ATCC 43504) strain was obtained from the Post Graduate Institute of Medical Education and Research, Kolkata, India, which were isolated from antral mucosal biopsy specimens of patients with chronic gastritis or duodenal ulcers, and kept as reference in American Type Culture Collection strain (ATCC 43504) were used for this study.

Preparation of plant extracts:

The plant namely *Plumbago zeylancia* (Root) was taken and washed the root section cut into small pieces weighed for about 1gm plus add ethanol to prepare extract with the help of mortar and pestle grind then transfer the extract into eppendroff and keep it for centrifugation at 5000rpm for15mins at 4°C collect the supernatant and store it for further experimental use. High performance liquid chromatography (HPLC) was used to purify the plumbagin from crude root extract of *Plumbago zeylancia*. High resolution HPLC was performed using shimadzu LC –10AT up chromatograph provided with isocratic pump and UV visible detector. Column of C18 ODS, Gemini 5 µ, 110A of dimensions 250 x 4.5 mm with mobile phase 70:30:1 (methanol :water : acetic acid), was used at flow rate of 0.5 ml / min. The detection wavelength was 339 nm and injection volume was 20 m and flow rate 0.9 ml / min, range 0.0100 AUFS [Data not given].

Experimental Design:

Animals [C57BL/6] mice of both control and experimental groups were kept separately in standard conditions and were fasted for 6 h with free access to water before each inoculation. Groups of mice (5 mice per group) were inoculated with *H. pylori* cultures harvested in PBS twice in a period of 3 days, with about 10^8 CFU/mouse/inoculation [9]. Mouse groups inoculated with PBS (control group) were kept separately, two weeks after the final inoculation, a group of mice were orally fed with plumbagin (25 mg/kg) once daily for 5 days consecutively, while untreated infected ones received sterile water. All mouse groups were sacrificed 3 weeks post infection, and the gastric tissues were assessed for *H. pylori* colonization and histology. All protocols were performed in accordance with the Institutional Animal Ethical Committee (IAEC) as per the directions of the CPCSEA approved number UCP/IAEC/2009/037 (Committee for the purpose of Control and Supervision of Experiments on Animals). The mouse were divided into four groups comprising of five animals in each group and designated as follows: Group I:Control animals receiving PBS, Group II: *H. pylori* infected animals received sterile water, Group III: *H. pylori* infected animals treated with plumbagin (25 mg/kg/b.w/d) in aqueous solution orally for 5 d, Group IV: *H. pylori* infected animals given omeprazole (20mg /kg/ b.w/d) in aqueous solution orally for 5 d. At the end of the experimental period, animals were anesthetized by ketamine (12 mg/kg of body weight), followed by

cervical dislocation for killing and further analysis carried out in the *H. pylori* induces cell disruption in gastric tissues and that a novel aspect of the repair response to this injury were assessed. Gastric tissues removed from control and experimental animals were grown in 90% RPMI 1640 medium (Biological Industries) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Biological Industries). Cells were cultured at 37°C in a controlled humidified atmosphere in an incubator containing 5% CO_2.

Cell Proliferation Assay

Cells (1 × 104 cells/well for glass bead stimulation; 1.5 × 104 cells/well for electroporation) were loaded in each well of a 16-well microtiter E-plate. Each well contained microelectronic sensor arrays at the base to detect the cell index (CI) and electroporated twice at 200 V and 100 μF at 5-minute intervals. After electroporation, cells were incubated with 5 mg/mL of fluorescein isothiocyanate-dextran (FDx) for 5 minutes to allow for analysis of cell membrane damage by fluorescence microscopy. Glass beads (Sigma,St. Louis, MO, USA) were used to create membrane injury as previously described [10],with slight modifications. Briefly, glass beads (bead size, 450–600 μm; bead weight, 0.03 g/well in E-plate) were carefully placed onto a plate and gently rocked for 1 minute. The E-plate was placed in the Real-Time Cell Analyzer (RTCA) system and incubated in an incubator containing 5% CO_2 at 37 °C. The level of cell proliferation was represented as CI, which was based on the electrical impedance measured using the xCELLigence system (Roche,Penzberg, Germany).

Preparation of Genomic DNA by CTAB Method:

The frozen intestinal tissue samples were taken and homogenized with Tris (pH 7.0) buffer then samples were spin at -4°C with 10000rpm, until a compact pellet was formed. The pellets were treated with 500μl TE buffer. To this 30μl of 10%SDS and 100μl of proteinase K (concentration is 100μg/ml) was added and incubated for one hour at 37°C 100μl of 5M sodium chloride and 10μl by Cetyl Tri methyl Ammonium Bromide. CTAB solution was added and incubated for 10 minutes at 65°C.Equal volume of chloroform: isoamyl alcohol was added and spin at -4°C with 10000rpm for 5 minutes. This extraction was removed the CTAB and exopolysaccharide complexes. The aqueous layer was transferred in a sterile tube, to add phenol: chloroform: isoamyl alcohol and then spin it at -4°C with 10000rpm for 5 minutes. The supernatant solution was transferred into a fresh centrifuge tube.0.6 volume of isopropanol was added to precipitate the genomic DNA .The DNA pellets were washed with 80%,75% and 70% of ethanol respectively. Finally it was re suspended in 200μl of TE buffer [11]. Genomic DNA extracted checked by 1.2% agarose gel electrophoresis. PCR was carried out in 50μl volumes containing 10mM Tris HCL (pH 8.3), 50mM KCL ,1.5mM MgCl2, 200mM of dNTPs, 50ng of each primers, 2.5 units of Taq polymerase and 5μl DNA template extracted from animal tissue. Specific primers for 'CagA' gene was16s rRNA- F (5'-TAA GAG ATC AGC CTA TGT CC-3') and R (5'-TCC CAC GCT TTA AGC GCA AT-3') used to amplification reaction. The amplification reaction normally carried out with a programme consisting of an initial denaturation step at 94ºC for 4 minutes, annealing at 59ºC for 1 minute and an extension step for 1 minute at 72ºC. At the 40th cycle included 6 minutes to ensure full extension of the PCR products. Amplification was performed in a minicycler (M J Research Inc). DNA of the *H.pylori* was used as a positive 'Cag A' control in each batch of PCR assays. The negative control consisted of all the reagents expect the template DNA.16S rDNA amplification by PCR from template genomic DNA confirmation of amplicon size by agarose gel electrophoresis. The standard *H.pylori* DNA was used as a positive 'Cag A' control.

Western blot:

Gastric cells were harvested in ice-cold Ham's medium and washed with ice cold phosphate-buffered saline (PBS). The cell pellets were lysed supplemented with 5 mM ethylene diamine tetraacetic acid (EDTA) and 1 × ProteoBlock Protease inhibitor (Fermentas,). Proteins were separated by SDS-PAGE and western blot was performed using Immobilon- P transfer membrane (Millipore), according to standard procedures. Anti-LATS2 (clone ST-3D10, and anti-a-tubulin (clone B-5-1-2, Sigma-Aldrich, St. Louis, MO, USA) antibodies were used at a 1:500 and 1:30,000 dilutions, respectively.

3. Results and Discussion

Plasma membrane integrity was monitored in cells labeled with fluorescein isothiocyanate-dextran (FDx; green) Figure1 FDx was detected in *H. pylori*-infected untreated animal cells and *H. pylori* infected treated with plumbagin results were monitored and compared with standard drug treated cells. FDx was observed to label the cytoplasm of cells infected with *H. pylori* Hp-infected/FDx), but not the cytoplasm of non-infected control cells.

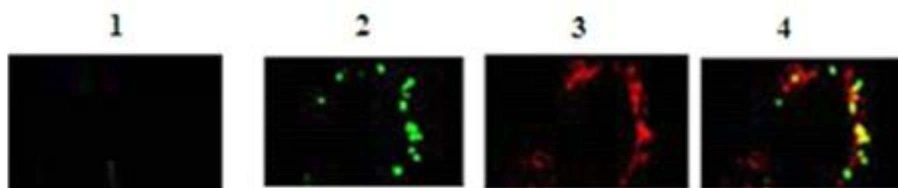

Figure 1. Membrane disruption in *H. pylori* infection of mice

Legend: Figure 1 indicates four section of observation as follows: 1.Control view without infection; 2. *H. pylori* infected /FDx green); 3.*H. pylori* infected /FDx red treated with Plumbagin .*4. H. pylori* infected/ FDx mixed spot green & red treated with drug omeprazole.

The control and *H. pylori* infected animals were kept for 2 weeks and treated with plumbagin homogenized gastric tissues were cultured in medium used membrane-impermeant fluorescein isothiocyanate-dextran (FDx; green) to detect membrane disruption in gastric tissue observed under Real-Time Cell Analyzer. (FDx- green stain for gastric tissue showed presence of membrane injury in *H. pylori* infected but not treated animals, FDx , Red showed in the *H. pylori* infected plus treated with plumbagin and mixed spots of red & green in the case of standard drug treated mice, control has showed no *H. pylori* in the mucus. Plumbagin entry has been shown to activate a classic membrane repair response. FDx was present at membrane disruption sites in cells infected *H. pylori*, which was consistent with the observations in cells infected with *H. pylori* strain. In the presence of Plumbagin FDx was present diffusely in the cytoplasm of the infected cells (Figure 2) drug omeprazole treatment had moderate effect on the patterns of FDx labeling in as mixed spot in infected cells.

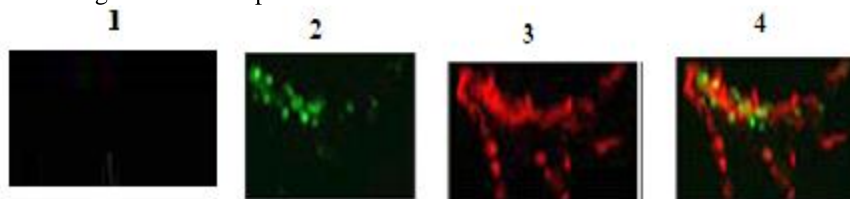

Figure 2. Role of VacA and CagA Plasma membrane injury caused by *H. pylori*

Legend: Figure 2 indicates four section of observation as follows:1. Control view without infection; 2. *H. pylori* infected/FDx green); 3. *H. pylori* infected /FDx red treated with Plumbagin. *4. H. pylori* infected/ FDx mixed spot green & red treated with drug omeprazole. After treatment with plumbagin FDx was detected in *H. pylori*-(red) infected cells, which were cultured in Ca2+ containing medium and in the absence of Ca2+, FDx was diffused throughout the infected cells with or without treatment. In present studies reveal that *H. pylori* injected into mice induced infection that could be treated with Plumbagin administrated orally to the experimental animals entered into the cytosol of the gastric host cell regulates the intracellular signal transduction in the host cell. *H. pylori* infection disrupts host plasma membrane integrity, membrane-impermeant fluorescein isothiocyanate-dextran (FDx) [12] used as a marker to detect membrane disruption *H. pylori* infection causes plasma membrane microinjury. Observation provides that *H. pylori* induces epithelial cell plasma membrane disruption could be repaired due to increased Interleukin-8 in gastric cancer cells in Plumbagin treated groups and its presence has been associated with the increase of Interleukin-8. [13] in infected cells was mediated by CagA in gastric epithelial cells induces IL-8 expression. Through this mechanism CagA may associated with increased mucosal expression of IL-8 and **i**ncrease IL-1beta production also inhibit gastric acid secretion. Western blot analysis confirmed (Figure 3) that increased expression of IL- 8 which was potent acid suppressant [14], cytokine modulation of endocrine and exocrine function in the gastric mucosa impacts on the colonization patterns of *H. pylori*, the distribution of gastritis and the pathogenesis of *H. pylori* induced atrophy.

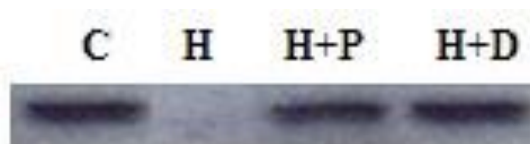

Figure 3. Analysis of *IL-8* expression by western blot

Legend: Figure 3 showed the expression IL-8 in the experimental tissue analyzed by western blot techniques. **Lane1.** Control shows IL-8 expression without infection; **Lane 2**. *H. pylori* infected tissues has not expressed **Lane 3**.*H. pylori* infected plus treated with Plumbagin showed IL-8 expression. **Lane *4*.** *H. pylori* infected treated with drug omeprazole also shows expression of interleukin-8. Western blot resulted increased IL-8 immuno reactivity due to IL-8 mRNA expression in *H. pylori* infected mucosa have been demonstrated to suppress the acid effects on cells, The importance of IL-8 in the host response to *H. pylori* has also been confirmed by acid suppressant upregulated genes IL-8 showed the greatest increase (25-fold) in the Plumbagin treated cells. *CagA* is commonly used as a marker for the entire *cag* locus. *H. pylori* adherence to epithelial cells, the secretion system translocates the CagA protein from *H. pylori* into the epithelial cell, where it undergoes tyrosine phosphorylation which was associated with dephosphorylation of host cell proteins and host cell morphological changes.

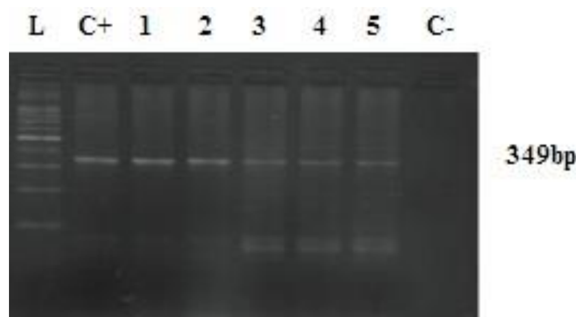

Figure 4. Amplification of cagA gene of *H. pylori*

Legend: Figure 4: 1% Agarose gel showing 349bp amplicon obtained from *H. pylori* infected animal samples: L, ladder 100 bp; C+ *H. pylori* positive**;** Infected animal sample at different period lane marked as 1 to 5 shows positive samples; C-, negative control.

H. pylori genes are differentially expressed in response to a shift in pH from 7 to 4.5. *CagA* gene downregulated at pH 5, in particular, encode outer membrane proteins, including Hop family members that were predicted to be outer membrane porins and adhesins. Loss of acid-producing parietal cells increases bacterial adhesin and increases the proportion of cells with adhesin receptors [15]. Eradication of *H. pylori* infection has the potential to reduce the risk of gastric cancer development. Furthermore, the optimal time to eradicate the bacterium was before the development of preneoplastic lesions such as atrophic gastritis and intestinal metaplasia.

4. Conclusion

In conclusion that eradication of *H. pylori* infection has the potential to reduce the risk of gastric cancer development. In plumbagin treated mice that suppress gastrin production as a result of altered epithelial cell proliferation thereby confirm plumbagin ability to reduce gastrin production might be an important aspect associated with therapeutic efficacy and the potential of anti- ulcer drugs. Furthermore, the optimal time to eradicate the bacterium was before the development of preneoplastic lesions such as atrophic gastritis and intestinal metaplasia. Plumbagin could be used as drug along with new therapeutic combinations, there was also a need to identify subjects most at risk for cancer from their genetic susceptibility and their infection with *H. pylori* genotypes of greater carcinogenic potential. PCR based diagnosis was essential to ensure the best outcome of treatment, preventing the development and the worsening of the gastric cancer.

5. Acknowledgements

This work was financial supported by CSIR, New Delhi, India. The authors are grateful to G.Madhangi Priyadharshini for her excellent technical assistance.

6. References

1. N Uemura; S Okamoto; S Yamamoto; N Matsumura; S Yamaguchi; M Yamakido; K Taniyama; N Sasaki;RJ Schlemper, J Med., 2001, 13 (11), 784-90.
2. M R Amieva; El-Omar, Gastroenterology, 2008, 134(1), 306-23.
3. D Forman; VJ Burley, Best Pract Res Clin Gastroenterol., 2006, 20(4), 633-49.
4. L Fuccio; LH Eusebi; Bazzoli F, World J Gastrointest Oncol., 2010, 2, 342-347.
5. A Alakkari; A Zullo; HJ Connor, Helicobacter. 2011, 16 (1), 33-37.
6. S Suerbaum ; C Josenhans , Nat Rev Microbiol., 2007, 5(6), 441-52.
7. MJ Blaser , EMBO Rep., 2006,7, 956–960.
8. G Renuga; A Babu thandapani, Int. J. Pharm.Biol. Res., 2013, 4 (4), 146-158.
9. H Suzuki; BJ Marshall; Hibi T, Int J Hematol., 2006, 84, 291–300.
10. CC Tzeng; CL Men ; L Jin L; HF Hsieh, Cytogenet., 1991, 55, 67–71.
11. J Sambrook; EF Frittsch; T H Maniatis, A laboratory manual 2nd eds, Cold Spring Harbor., 1989.
12. H Lu; P Hsu; DY Graham; Y Yamaoka,Gastroenterology., 2005, 128(4), 844-848.
13. SM Everett; ATR Axon, Lancet. 1998, 351 (112), 1350– 1352.
14. A Zullo; C Hassan; F Cristofari; F Perri; S Morini, World J Gastrointest Oncol., 2010, 2, 181-186.
15. J Houghton; C Stoicov; S Nomura; AB Rogers; J Carlson; H Li,Science., 2004, 306, 568–71.