THERAPEUTIC ALTERNATIVES AGAINST HELICOBACTER PYLORI ERADICATION RATE IMPROVED WITH HERBAL THERAPY REGIMEN

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Abstract: ***Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine. Treatment of Helicobacter pylori infection is one of the effective ways to prevent gastric cancer. Present work focused for the combination of β -carotene and Plumbagin administrated to Helicobacter pylori infected mice and that eradication of this organism greatly reduces the recurrence rate of ulcers. Purification of β -carotene from carrot and isolation of Plumbagin from Plumbago zeylanica were simportant bioactive material being used to treat H. pylori infection which caused gastric cancer and peptic ulcer disease in the traditional system of medicines. β- carotene is an antioxidant isolated from carrot and quantified exactly in different steps of purification, the yield of pure β-Carotene was high when it prepared with ethanol extraction proved the recovery of HPLC separation methods. 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, β- carotene alone and combination of both plumbagin & β- carotene .At the end of the experimental period, analysis carried out with gastric tissues and the repair responses to this injury were assessed with standard omeprazole. Preliminary screening was made by the disk diffusion test and then minimum inhibitory concentration was determined by the agar dilution method. Alternative eradication therapies, which consist of two plant based purified bioactive compound are highly effective, based on western blot expression of IL-6 confirmed eradicating H. pylori also capable of inhibiting the in vitro growth of H. pylori has investigated.***

Keywords: β- carotene, Helicobacter pylori, Plumbago zeylanica, Plumbagin, eradication .

I.INTRODUCTION

Herbal drugs are use to prevent and treat diseases, Herbal drugs are the oldest form of health care known to mankind. There are many herbal products offered that assert to treat the symptoms of a broad range of problems, World Health Organization has set precise guidelines for the evaluation of the safety, efficacy, and quality of herbal medicines[1]. In every country traditional medicines find foundation in magical or religious beliefs, or popular experience and the World Health Organization is engaged to establish definitive guidelines for methodology of clinical research and the appraisal of effectiveness of traditional medicine.

Carrot (*Daucus carota* linn; Umbelliferae) is widely used as a vegetable, the different part of this plant are known to possess multifarious medicinal properties. High vegetable consumption including carrots reduces the risk of breast cancer. The protective effects of carrots in case of the colon, rectum, and lungs have also been reported. The extract of the seeds of carrot showed antitumor activity, inhibiting the growth of *Ehrlich ascites* tumor in mice. In recent years, researchers have also discovered that beta-carotene not only functions as a precursor to vitamin A but also powerful antioxidant, beta-carotene has proven to be protective against many types of cancer, but especially cancer of the lungs. Studies also indicate that it may help to protect the eyes from the damage that can lead to cataracts. Beta-carotene (β-carotene) is a type of carotenoid, an important precursor to vitamin A. Vitamin A is essential for biochemical and physiological processes in the body including vision, reproduction, cellular differentiation and immunity. β-carotene can be obtained from dark-green leafy vegetables and some (not all) yellow and orange coloured vegetables and fruits, as well as dietary supplements. At present, it is unclear whether some beneficial effects of beta-carotene and other bioactive material in humans are a result of their antioxidant activity or other non-antioxidant mechanisms [2].

Plumbago zeylanica is one such important medicinal plant which is being used the world over in the traditional system of medicines [3]. *P. zeylanica* contains a variety of important chemical compounds specifically plant possess naphthaquinones, alkaloids, glycosides, steroids, triterpenoids, tannins, phenolic compounds, flavanoids, saponins, coumarins, carbohydrates, fixed oil and fats and proteins, Of all the chemical constituents' plumbagin is the principle active compound. Plumbagin (5-hydroxy-2-methyl-1, 4- naphthoquinone- $C_{11}H_8O_3$) is primarily present in roots in higher amounts with only about 1% in the whole plants [4].

Gastric ulcer (GC) resulted from persistent erosions and damage of the stomach wall that might become perforated and developed into peritonitis and massive haemorrhage as a result of inhibition in the synthesis of mucus, bicarbonate and prostaglandins. Various factors can contribute to the formation of gastric ulcer such as the infection of stomach by *Helicobacter pylori* [5], the frequent use of non steroidal anti-inflammatory drugs (NSAIDs) [6] and consumption of alcohol. The success of commercially available antiulcer drugs in the treatment of gastric ulcer is usually overshadowed by various side effects. *H.pylori* is a helix-shaped bacterium which can inhabit the stomach mucosa. The infection route is thought to be the oral infection. The infection rate of *H.pylori* tends to be higher in developing countries and lower in developed countries. *H.pylori* causes of chronic gastritis, gastric ulcer, duodenal ulcer and deeply related to gastric cancer. Therefore, *H.pylori* eradication is strongly recommended to people infected with *H.pylori*.

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. One medicine for reducing the production gastric acid and two classes of antibiotics are used for the combination therapy with three medicines. The first *H.pylori* eradication rate is about only 70% and also the strong side effects by using potent antibiotics are caused [7].

In this study planned to design combination drug of plumbagin and beta-carotene in a single formulation of drug for that to be prepared the plumbagin bioactive compound from *Plumbago zeylanica* plant root extracts and beta-carotene from Carrot (*Daucus carota*), experimental investigation was also carried out to identify the inhibitor effect on *Helicobacter pylori* in animal model and also present study aimed at evaluating the eradication activity of human *H.pylori* infection for that an attempt was taken to infect *H.pylori* on mice model and subjected to various analyzes in the presence of *Plumbago zeylanica* and β carotene in order to confirm molecular events to inhibit or eradicate pathogenic *H.pylori* infection.

II. 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 Samples:

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].

Carrot (*Daucus carota*) vegetables were collected and extracted as follows: 20g of sample (orange pulp) was homogenized in 10mg calcium bicarbonate and 70-80% warm ethanol and filtered using Whatmann no.1 and no.42 filter paper. Again the residue was homogenised with 95% warm ethanol and filtered using whatmann no.42 filter paper. The ethanol solution was recovered and centrifuged at 5000 rpm for 10 minutes and the supernatant was discarded and small amount of double distilled water was added to the residue again centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the residue was dried in room temperature to obtain the final product β-Carotene crystals.

β- Carotene assay:

The antioxidant activity of the extracts was estimated using ß-carotene system according to the modified procedure [8]. Briefly, a stock solution of ß-carotene mixture was prepared as follows: 1 mL of ß-carotene solution in chloroform (0.2 mg/mL) was pipetted into a round-bottom flask. To the solution, 20 mg of linoleic acid and 200 mg of Tween 40 were added. After removing chloroform in a rotary evaporator at room temperature, 50mL of aerated distilled water was added to the oily residue with vigorous stirring. Aliquots (5mL) of thus obtained emulsion were transferred to a series of tubes containing 2 mg of extract or 0.5 mg of BHT (positive control) dissolved in 1 mL of 0.4% (w/v) Tween 40 solution. A tube with 1 mL of 0.4% Tween 40 solution to which no antioxidant was added, served as water control (negative control). Solution of 2 mg of an extract in 6 mL of Tween 40 solution served as blank for the corresponding extract. After addition of the emulsion to the tubes, they were placed in a water bath at 50 ˚C for 2 h. During that period, the absorbance of each sample was measured at 470 nm at 15 min intervals, starting immediately after sample preparation (t = 0 min) until the end of the experiment (t = 120 min). The antioxidant activity was calculated from the absolute changes in absorbance at t = 60 min and t = 120 min (AA-60 and AA-120, respectively). The results were normalized using both controls: the water control and the positive control. The first should offer no protection against oxidation of ß-carotene in emulsion, while the other should offer maximum protection over the time course of the assay. The percentage ß-carotene bleaching activity was calculated as: $AA\% = 1 - [(A_E^{t=0} - A_E^{t=t}) / (A_w^{t=0} - A_w^{t=t}) + (A_{BHT}^{t=0} - A_{BHT}^{t=t})] \times 100$. where $A_E^{t=0}$ is the absorbance of the extract at t = 0 min, $A_E^{t=t}$ is the absorbance of the extract at t = 60 or t = 120 min, $A_W^{t=0}$ is the absorbance of the water control at t = 0 min, $A_W^{t=t}$ is the absorbance of the water control at t = 60 min or t = 120 min, $A_{BHT}^{t=0}$ is the absorbance of BHT at t = 0 min and $A_{BHT}^{t=t}$ is the absorbance of BHT sample at t = 60 or t = 120 min.

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 (10 mg/kg/b.w/d) and β carotene (10 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.

Western blotting:

Cells were harvested in ice-cold Ham's medium and washed with ice cold phosphate-buffered saline (PBS). The cell pellets were lysed in Proteo JET Mammalian Cell lysis reagent crude extract (40µgprotein per lane) were analyzed by 15% SDS-PAGE [10]. Proteins were transferred electrophoretically to nitrocellulose filters (for 3 h at1A) using an immunoblot transfer apparatus. After transfer, the nitrocellulose was incubated for 1 h at room temperature in 3% (w/v) BSA in Tris-buffered saline (TBS; 500mM NaCl and 20mM Tris-HCl pH 7.5) to block non specific binding. The blot was incubated overnight at 4°C with 3% (w/v) BSA in TBS containing antiserum at a dilution of 1:500. After three 15 min washes with TBS containing 0.1% BSA and 0.2% Nonidet P40, the blot was incubated for 1 h at room temperature with peroxidase-conjugated goat anti (mouse immunoglobulin) diluted at 1:1000 in 3% BSA in TBS. The blot was again washed three times with TBS containing 0.1% BSA and 0.2%Nonidet P40. Antibodies were visualized using a chem.-illuminescence detection system.

III. RESULTS AND DISCUSSION:

Plumbago zeylancia root ethanolic extract principally contain a yellow naphthaquinone compound namely Plumbagin , which possesses significant activities against peptic ulcers caused by *H. pylori* are treated with plumbagin drugs that kill the bacteria, reduce stomach acid, and protect the stomach and duodenal lining.

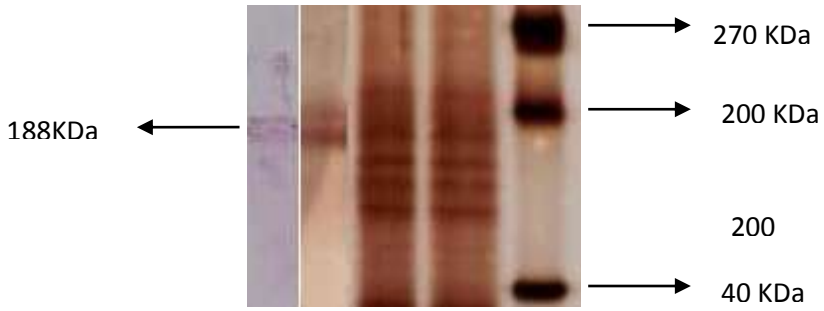
Table 1: Thin layer chromatography (TLC) of varies extracts of ***Plumbago Zeylanica*** roots for the presence of naphthoquinone.

S.NO	Name of the Sample	R_F Values
1	Quinine standard	0.63
2	Petroleum ether extract	0.56
3	Ethanol extract	0.70
4	Methanolic extract	0.60
5	Aqueous extract	0.53
6	n-butanol	0.58
7	Chloroform	0.54

Table 1 showed various solvent used to extract Plumbagin, specifically ethanolic extract confirm purity related with standard good yield which is a naphthoquinone have been separated by TLC used as folk medicine in the treatment of ulcers and inflammation. Antibiotics are used to kill *H. pylori* but antibiotic regimens may differ throughout the world because some strains of *H. pylori* have become resistant to certain antibiotics that once destroyed the bacterium is no longer effective. *Helicobacter pylori* a corkscrew shaped bacteria that reside in the stomach and intestine of more than half of the world's population, most people have no symptoms [8]. Plant based combination therapy would be suggested for *H.pylori* eradication method.

The Agar diffusion method was used to identify the efficiency of the root extract against pathogen *H.pylori*.(data not given) .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, Different fraction were collected and separated on SDS PAGE which confirmed the size of the molecules with standard Quinine. The efficiency of the molecules analyzed to test against *H. pylori* strains have the same ability to cause gastric diseases, with host genetic background, environment, diet, hygiene, and cross talk between bacterial gene products and host cells as additional determining factors. *Helicobacter pylori* colonization and bacterial load of mice stomach was assessed on day 1 and 5 post-treatment. Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections [9]. Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections. The active components were usually interfere with growth and metabolism of microorganisms in a negative manner and has quantified by determining the minimum inhibitory concentration and minimum bactericidal activity.

Figure 1: SDS PAGE analyses

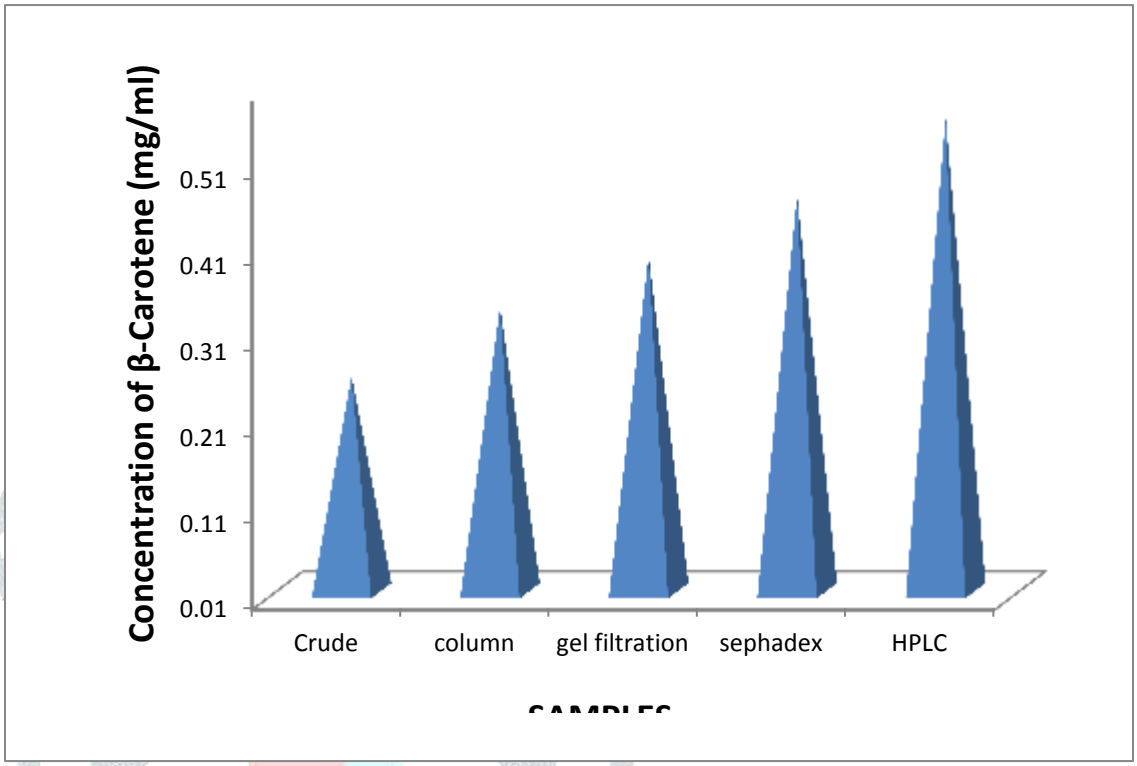


Legends: SDS PAGE analyses of the *Plumbago zeylancia* extracts eluted fraction from size exclusion chromatography. Lane1: Peak 1 from the size exclusion chromatography. Lane 2-3: The total protein loaded to size exclusion chromatography. Lane 4: Protein size marker.

Crude extracts of *plumbago zeylancia* were used to separate individual molecule according to size by SDS PAGE. Purification of molecules by adsorption chromatography and ultra filtration, followed by size exclusion chromatography was also verified through SDS PAGE. The molecular

weight of the separated molecule has confirmed on SDS PAGE as compared with standard marker and plumbagin molecular weight was confirmed as 188 and eluted plumbagin considered purified bioactive component could be used for treatment of *H. pylori* infection.

Figure 2: Estimation of β-Carotene

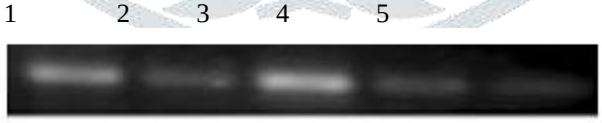


Legends:Figure showed preparation of β-carotene through classical biochemical techniques to get high yield. X – indicates varies methods of extraction such as crude extract, then it passed through column chromatography, gel filtration, sephadex -50 elution, HPLC separated fraction.

For HPLC method, excellent recoveries of β-carotene were obtained at each concentration. Quantification of β-carotene in this study was of good reproducibility, based on the precision.

Beta-carotene is an important functional ingredient among the carotenoids family, providing vitamin A activity from vegetable sources in the human food supply [10]. It is a well-known active phytochemical with many health-promoting properties. HPLC used to evaluate four methods for the quantitative determination of β-carotene (Figure 2). The five methods used to extraction of β-carotene included classical biochemical techniques to obtained pure high yield by using HPLC (High Performance Liquid Chromatography) separation and extraction-HPLC with C18 column. The results confirmed that all of these methods were sufficient for β-carotene quantification. The extraction with ethanol confirmed the concentration of β-carotene was high in carrot. Western blot resulted increased IL-6 immuno reactivity due to IL-6 mRNA expression[11] in *H. pylori* infected animal treated with single as well as combination of two bioactive component have been demonstrated to inhibit /suppress the acid effects on cells. This inhibiting activity against *H. pylori* was also confirmed by the *H pylori* count in gastric biopsies culture, where the decrease in bacterial population was observed with both extract of Plumbagin and β-carotene. The role of IL-6 in the host response to *H. pylori* has also been confirmed by acid suppressant upregulated genes IL-6 showed the greatest increase (40-fold) in the Plumbagin and β-carotene treated cells.

Figure: 3 Western Blot analysis



Legend: Figure 3 showed the expression IL-6 in the experimental tissue analyzed by western blot techniques. **Lane1.** Control shows IL-6 expression without infection; **Lane 2**. *H. pylori* infected treated with drug omeprazole expression of interleukin-6. **Lane 3**.*H. pylori* infection treated with Plumbagin and β-carotene showed IL-6 expression. **Lane *4***. *H. pylori* infected tissues with Plumbagin has less expressed compared to combination of treatment.**Lane 5**. *H. pylori* infected tissues with β-carotene showed IL-6 less expression.

To investigate the efficiency of plant based component Plumbagin and β-carotene has administrated to *H. pylori* infected mice as mention in the methods. Figure 3 revealed the efficiency of treatment effect in terms of IL-6 expression confirmed by western blot analysis. *Helicobacter pylori* infection plays an important role in upper gastrointestinal disease [12], *H. pylori* can reduce the incidence of both peptic ulcer disease and gastric cancer confirmation of *H. pylori* eradication should be performed alternative plant based bioactive combinational therapy.

IV. CONCLUSION:

Gastric cancer is the third most common cause of cancer-related death globally, thus it has been considered as an important public health burden. Infectious diseases are one of the major problems in developing as well as developed countries. Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine. Many medicinal plants have been found effective in the cure of bacterial diseases. Due to increasing antibiotic resistance in microorganisms and side effects of synthetic antibiotics medicinal plants are now

gaining popularity in the treatment of bacterial infections. Medicinal plants are considered as clinically effective and safer alternatives to the synthetic antibiotics. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs for treating bacterial infections can be developed.

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