EFFECT OF ETHANOLIC EXTRACT OF *PSORALEA CORYLIFOLIA* ON *H. PYLORI* INFECTION IN LABORATORY RATS

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Summary

The objective of the present study was to evaluate in vivo anti *H. pylori* activity ethanolic extract of *Psoralea corylifolia* (called as PCE)in laboratory rats. Naproxen was administered orally to produce ulcers and then rats were orally inoculated with *H. pylori.* The animals were divided into following five treatment groups: Clarithromycin 30 mg/kg, PCE at 100, 200 and 400 mg/kg/day, p.o and vehicle (0.25 % Sodium CMC) for 10 weeks. On 4th, 7th and 10th week infection status was determined by polymerase chain reaction, rapid urease test and histopathology. The extent of neutrophil infiltration was estimated by myeloperoxidase assay. Clarithromycin treated animals demonstrated absence of infection at 4th, 7th and 10th week whereas the control group of animals showed the presence of infection throughout the treatment regimen. Ethanolic extract of Psoralea eradicated the *H. pylori* infection in a dose and time dependent manner. The level of myeloperoxidase was elevated in the control group whereas significantly reduced in the clarithromycin and PCE treated groups when compared with the vehicle treated group of animals but decreased in the clarithromycin and PCE treated groups. It is concluded that PCE showed anti *H. pylori* activity *in vivo*.

Keywords: PCE, polymerase chain reaction, *Helicobacter pylori.*

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Introduction

Helicobacter pylori has colonized in the gastric mucosa of humans since times immemorial. However, it has taken the shape of almost an epidemic and more than half the population worldwide is infected with *H. pylori*[1]. From numerous studies it has become evident that *H. pylori* infection has cost humans, chiefly in the form of gastric carcinoma, adenocarcinoma, GERD (gastroesophagal reflux disease) and MALT (mucosa associated lymphoid tissue) lymphoma[2-6]. However, after acquiring the infection most patients remain asymptomatic until ulceration is induced by an agent. In most cases, chronic treatment with NSAIDs stimulates ulcer formation[7].

In the recent past, various genes of *H. pylori* including *vacA* (Vacuolating associated cytotoxin), *iceA* (Induced by contact with the epithelium), *hrgA* (*H. pylori* Restriction Endonuclease replacing gene), *babA* (blood group antigen binding adhesion gene), *fla A&B* (flagellin genes), *napA* (neutrophil activating protein) etc. have been identified. However, *16s r RNA* gene is considered to be marker of *H. pylori* as it is present in the highly conserved region of *H. pylori* [8]. Advances in molecular biology has enabled the sequencing of these genes and designing the respective primers to amplify these genes using polymerase chain reaction[9,10].

The current anti *H. pylori* therapy is punctuated with adverse drug reactions such as hepatotoxicty and renal failure[11]. It has been found that the infection persists even after completion of the therapy due to resistance to the antibiotic drug regimens leading to concomitant administration of three to four drugs. It is worth noting that the herbal drugs are devoid of the side effects. Hence, in the present investigation, an attempt was made to elucidate the anti *H. pylori* potential of *Psoralia corylifolia* (Family Fabaceae). It has been used to cure gynecological bleeding, vitiligo and psoriasis. It has been claimed in the traditional Indian and Chinese system of medicine to possess antiplatelet[12] anti depressant[13,14] and DNA polymerase and topoisomerase II inhibitory activity[15]. It has been reported to possess potent broad spectrum antibacterial activity against gram positive and gram negative bacteria**.** However, there is paucity of reports of this plant on eradication of ulcers induced by NSAIDs (Non steroidal anti inflammatory drugs) and infected by *H. pylori*. A wide range of animal models replicating the human pathological state have been reported[16]. However, the most relevant model is NSAID induced *H. pylori* infected model which closely resembles the human pathological condition[17]. The objective of the present study was to elucidate the *in vivo* anti *H. pylori* activity of ethanolic extract of *Psoralea corylifolia* (called as PCE) in laboratory rats.

Materials and methods

Collection of plant material: The seeds of *Psoralea corylifolia* were collected from Pune region of Maharashtra. They were authenticated from Botanical survey of India, Pune. A voucher specimen was deposited at the herbarium in the institute (BSI/WC/identi/tech/2008/1159).

Preparation of extract: The seeds were dried under shade and powdered.1000 grams of dried powder were extracted with 2 liters of 70% ethanol in soxhlet apparatus. The hydro alcoholic extract was concentrated in rotary vacuum evaporator under reduced pressure, at less than 40 °C and then further concentrated in vacuo at room temperature. The yield was found to be equal to yield 215 grams.

Animals Used: Male wistar rats (230-250 grams) and swiss albino mice (18-22) grams were procured from National Toxicological Centre, Pune. The animals were housed in groups of 4 in solid bottom polypropylene cages. They were maintained at 24 ^{o}C ± 1 ^{o}C, with relative humidity of 45-55% and 12:12 h dark/light cycle. The animals were acclimatized for a period of two weeks and were kept under pathogen free conditions. The animals had free access to standard pellet chow (Chakan Oil Mills, Sangli) throughout the experimental protocol, with the exception of overnight fasting before induction of the ulcer. The animals were provided with filtered water. The pharmacological and acute toxicity protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/8/2008).

Drugs and Chemicals: Naproxen sodium and clarithromycin were a gift samples from Symed laboratories, Hyderabad. Brucella agar and broth were purchased from Becton–Dickinson (USA). All the chemicals for DNA extraction were procured from S.D. Fine chemicals, Mumbai, India. The reagents for polymerase chain reaction were purchased from Vivantis,Thane, India. The forward and reverse primers for *16s r RNA* gene were synthesized at Ocimum Biosolutions, Hyderabad, India. *H. pylori* was kindly provided in cryopreserved state by Centre for liver research and diagnostics, Owaisi hospital, Hyderabad, India.

Acute Toxicity Testing: The acute oral toxicity study was carried out in swiss albino mice as per the guideline Acute Oral Toxicity 425 set by the organization for economic co-operation and development. Acute oral toxicity study was performed in female swiss albino mice.

Experimental set up: The animals were divided into 5 treatment groups having 18 animals in each group. PCE suspended in 0.5% w/w Sodium C.M.C. was administered at a dose of 50,100 and 200 mg/kg/day.p.o. Clarithromycin suspended in 0.5% w/w Sodium C.M.C. was administered at a dose of 30 mg/kg/day.p.o. The control group of animals was administered 1ml of 0.5% w/w Sodium C.M.C. At the end of 4th, 7th and 10th week, six animals were sacrificed to determine the infection status.

Induction of ulcers and *H. pylori* infection: Ulcers were induced using a method modified from that described by Kim et al., [18]. Briefly, Naproxen sodium was administered (30 mg/kg.p.o) for three consecutive days. Once the ulcers were induced, the animals were infected with *H. pylori* as suspension in brucella broth media. The number of bacteria/ml was adjusted to 10^8 cfu/ml using McFarland's turbidity standards[19]. 1 ml of *H.pylori* suspension was administered for 3 consecutive days to the ulcerated animals to induce infection. The treatment with drugs was initiated 1 week after *H. pylori* infection.

Determination infection status and myeloperoxidase activity: Six animals from each group were sacrificed by cervical dislocation on 4th, 7th and 10th for the assessment of the infection status and myeloperoxidase activity in the gastric mucosa. The stomach of each animal was isolated and cut along the greater curvature. The inner mucosal surface of stomach was washed with normal saline.

Determination of *H. pylori* infection status: A small portion of the pylorus of the isolated stomach was used for rapid urease test and extraction of DNA to be amplified by polymerase chain reaction. The rest of the stomach was used to determine the histopathological changes.

Rapid urease test: The rapid urease test solution was prepared by dissolving 10 grams of urea in 100ml millipore water followed by autoclaving. 0.002 grams of phenol red was added into the solution as indicator. The pH of the solution was adjusted to 6. The isolated pyloric tissue was immediately immersed in RUT solution[20]. The change of color from yellow to red within 10 minutes indicated the presence of *H. pylori.*

Molecular diagnostic techniques

DNA Isolation: DNA isolation from gastric tissue was performed according to standard cetyl tri methyl ammonium bromide method mentioned by Tiwari et al., 2005. Briefly**,** the tissue samples were suspended in 250µL of digestion buffer II. {0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0), 1% SDS} containing 100µg/ml of proteinase k (Vivantis, India). To this, 250µl of digestion buffer I {0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0)} was added and incubated at 56ºC overnight. DNA was extracted with an equal volume of phenol chloroform and precipitated with 0.6 volume iso propanol. The DNA pellets were washed thrice with 80%, 75% and 70% ethanol, respectively, and finally resuspended in 50µl-100µl of sterile water for injection. All the steps were performed in aseptic conditions to minimize contamination.

PCR amplification of *16S rRNA* gene: PCR amplification was performed according to protocol mentioned by Tiwari et al., 2005. Briefly, 2µl of the template DNA isolated from gastric tissue was added to 18µl of the reaction mixture containing 1X PCR buffer {50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5% (vol/vol) Triton X-100}, 1.5mM $MgCl_2$, 200uM concentrations of each dNTPs, 10pMol of each primer, & 1U of Taq polymerase. The following thermal cycle steps were used in the PCR amplification: initial denaturation at 96ºC for 5 minutes, 40 cycles with 1 cycle consisting of 94ºC for 1 minute, 56ºC for 1 second, 72ºC for 2 minutes. The final cycle comprised of a 6-minute extension step to ensure full extension of the PCR products. PCR amplification was performed in a thermal cycler (Eppendorf). DNA of the ATCC 26695 type strain was used as a positive control in each batch of PCR assays while negative control consisted of all the reagents of the master mix excluding the template DNA. The primers were 16s r RNA F and 16s r RNA R (Tiwari et al., 2005). The PCR-amplified products were analyzed by agarose gel electrophoresis. 10µl of each amplified product was added to 3µl of loading buffer (20 ml of glycerol 50%, 25 mg of bromophenol blue, 3 drops of 1N NaOH) and subjected to electrophoresis in a 2% agarose gel. The gel was examined in gel documentation instrument (Alpha innotech) and image was captured.

Histopathological studies: The pylorus portion of the stomach was fixed in formalin and 3µm sections were stained with eosin haematoxylin stain to determine the presence or absence of *H. pylori*. The images were captured at 40X magnification.

Determination of myeloperoxidase activity: The myeloperoxidase assay was performed according to Krawisz et al., [21]. Briefly, The mucosa was scrapped to remove the mucus layer using a glass slide and the mucosal scrapings were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH 6), before sonication in an ice bath for 10 seconds. The homogenates were freeze-thawed three times, repeating the sonication after which they were centrifuged for 15 min at 20,000 × g. The level of MPO activity was measured spectrophotometrically. 0.1 ml of the supernatant was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide.

The change in absorbance at 460 nm was then measured for 5 min using JASCO V -530 UV/VIS spectrophotometer. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 25^0 C and was expressed in units per gram (mU/mg) of wet scrapings.

Results

Infection status

The infection was determined at the end of 4^{th},7^{th} and 10^{th} week in the animals using all the three above mentioned techniques (figure 2 to 4).The infection of *H. pylori* was ameliorated up to varying extents in the different treatment groups of animals (Table1). Clarithromycin treated animals demonstrated absence of infection at 4^{th}, 7^{th} and 10^{th} week whereas the control group of animals showed the presence of infection throughout the treatment regimen. Hydro alcoholic extract of psoralea eradicated the *H. pylori* infection in a dose and time dependent manner. It was unable to eradicate the infection at a dose of 100 mg/kg where 100 % (6/6) animals were infected throughout the dosage regimen. At a dose of 200 mg/kg/day of PCE, 100 % (6/6) animals were found *H. pylori* positive after 4 weeks of treatment and 83.33% (5/6) animals were found *H. pylori* positive after seventh and tenth week of treatment. At a dose of 400 mg/kg/day of PCE, 66.67%(5/6), 50%(4/6), and 0%(0/6) of the animals were found to be infected at the end of 4^{th}, 7^{th} and 10^{th} week of treatment.

Table 1: Status of *H. pylori* infection

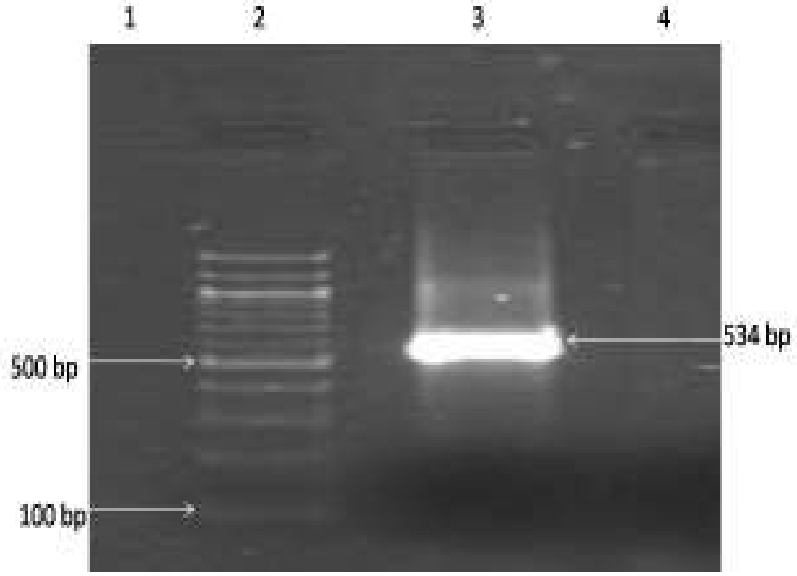
Weeks	**4**	**7**	**10**
No. of infected rats in treatment groups			
Control(vehicle)	6/6	6/6	6/6
Clarithromycin	0/6	0/6	0/6
PCE 100 mg/kg	6/6	6/6	6/6
PCE 200 mg/kg	6/6	5/6	5/6
PCE 400 mg/kg	5/6	4/6	0/6

Note: Evaluation of *H. pylori* infection by polymerase chain reaction and rapid urease test during the treatment of *H. pylori* infected rats with ethanolic extract of psoralea throughout the treatment regimen of 10 weeks. The number of infected rats out of six rats sacrificed after 4^{th}, 7^{th} and 10^{th} weeks of treatment. RUT: rapid urease test.

Myeloperoxidase activity

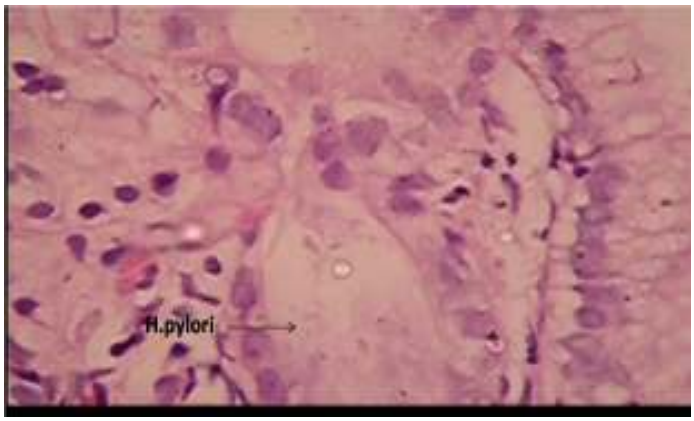
The level of myeloperoxidase in the clarithromycin treated group was found to be 1.307, 1.238 and 1.007 mU/mg at 4^{th}, 7^{th} and 10 weeks of treatment. The level of myeloperoxidase in the control group was found to be 1.552, 1.603 and 1.618 mU/mg at 4^{th}, 7^{th} and 10 weeks of treatment. The level of myeloperoxidase in the PCE 100mg/kg.p.o. treated group was found to be 1.535, 1.512 and 1.452 mU/mg at 4^{th}, 7th and 10 weeks of treatment. The level of myeloperoxidase in the PCE 200 mg/kg.p.o. treated group was found to be 1.477, 1.455 and 1.358 mU/mg at 4^{th}, 7th and 10 weeks of treatment. The level of myeloperoxidase in the PCE 400mg/kg.p.o. treated group was found to be 1.407, 1.372 and 1.145 mU/mg at 4^{th},7^{th} and 10 weeks of treatment. The elevated myeloperoxidase activity was reduced significantly in the clarithromycin treated group ($p<0.001$) and at a dose of 200 and 400 mg/kg of PCE ($p< 0.01$) on 4^{th},7^{th} and 10^{th} week when compared with the control group of animals (Figure 4).

Figure 1: Gel image of *16S rRNA* gene



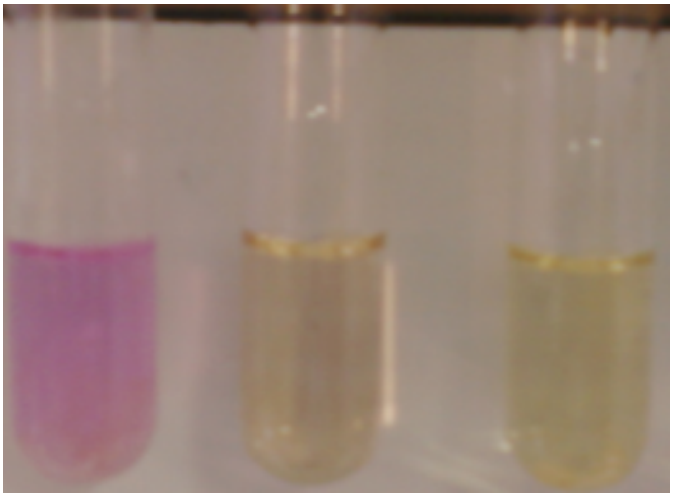
Note: Determination of infection status by isolation of *H. pylori* DNA and amplification of *16s rRNA* gene by PCR (product size 534 base pairs). Gel image showing successful amplification of *16srRNA* gene by polymerase chain reaction using DNA isolated from the harvested stomach of infected rats at the end of 10 weeks of treatment. Lane1and 4 show PCR product of clarithromycin and 400mg/kg PCE treated group. Lane 3 shows pcr product of control group of animals (product size 534 base pairs). Lane 2 shows 100 base pair DNA ladder.

Figure 2: Histopathology of the gastric mucosa of infected rat



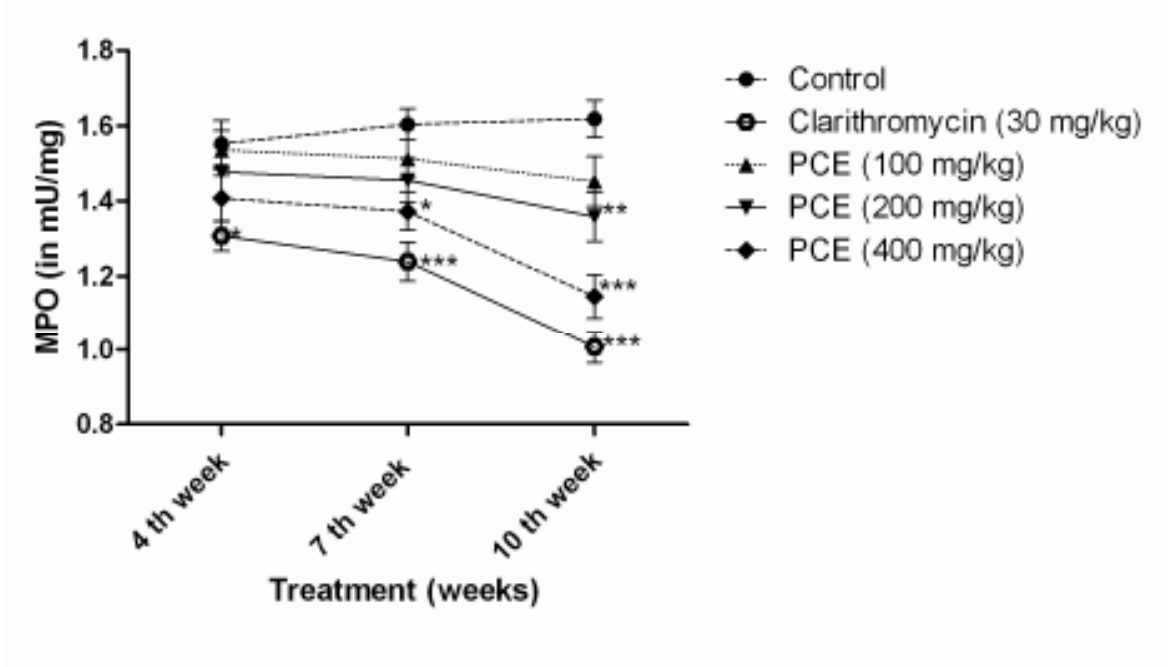
Note: Histopathological study of the gastric mucosa of the control group of animals showing presence of *H. pylori* (haematoxyline & eosin staining) under 40X magnification.

Figure 3 Rapid urease test



Note: Determination of infection status by rapid urease test. The conversion of yellow to red color shows presence of *Helicobacter pylori*.

Figure 4: Myeloperoxidase activity



Note: Changes in the myeloperoxidase activity in the vehicle treated and clarithromycin and PCE (100,200,400 mg/kg/day.p.o) treated animals for 10 weeks after induction of ulcers and *H. pylori* inoculation. Data analyzed by two way ANOVA followed by Bonferroni post test.

(* $p<0.05$,**p,0.01,***$p<0.001$).

Discussion

A plethora of treatment regimens have been proposed to ameliorate *H. pylori* infection without much success and effective control of infection is not achieved by the triple drug regimen. Hence, herbal antimicrobials seem to be the key to this rising menace.

The rapid urease test (RUT) is a rapid and widely used test to diagnose the *H. pylori* infection[22]. When the gastric tissue is immersed in the solution containing urea it leads to formation of ammonia and CO_2 due to the urease enzyme present in *H. pylori*. The liberated ammonia binds with water leading to formation of ammonium hydroxide which raises the pH of the solution. The RUT solution contains phenol red indicator which immediately turns from yellow to red indicating the presence of ammonia and *H. pylori*[23]. RUT is routinely used by the physicians for the diagnosis of the infection and it provides reproducible results[24-25]. Among the wide range of strategies used for the detection of *H. pylori* polymerase chain reaction technique has carved out a special niche for itself. It has the advantage of being sensitive, reproducible, reliable and specific[26-27]. The infection can be determined by the isolation of DNA of *H. pylori* from the gastric tissue and amplifying the specific genes using DNA as the template and suitable primers along with appropriate thermal cycles[28].

Cetyl tri methyl ammonium bromide (CTAB) method is a reproducible method to isolate bacterial DNA from the gastric tissue[10,19,] The isolated DNA when subjected to PCR with suitable primers shows the presence of the *16S rRNA* gene which serves as a marker for the presence of *H. pylori*. *16S rRNA* and *hrgA* genes serve as markers of *H. pylori* and hence serve as reproducible diagnostic tool[29] . In the present investigation, *16s r RNA* gene was successfully amplified in animals in which the infection persisted but PCR negative results confirmed the amelioration of the infection among the clarithromycin and test drug treated animals.

It has been reported that various extracts of Psoralia possess potent broad spectrum antimicrobial activity against gram positive and gram negative bacteria[30-35]. On the other hand, Psoralia has been reported to possess *in vitro* activity to inhibit the growth of *H. pylori*[36,37]. The antibacterial activity could be attributed to the psoracarylifols A to E which were isolated from the ethanolic extract of *corylifolia* and were found to possess *in vitro* activity equivalent to metronidazole in inhibition of the *H. pylori* growth *in vitro*[12]. In the present investigation, the ulcerated animals were infected with *H. pylori* and treated with either clarithromycin or increasing doses of PCE. The amelioration of infection was assessed using histopathological as well as molecular biology techniques. The results potent inhibition of *H. pylori* infection in the clarithromycin treated animals. PCE was ineffective in arresting the *H. pylori* infection at lower doses of 100 and 200 mg/kg. At a dose of 400 mg/kg it showed a tardy inhibition of *H. pylori* infection.

Myeloperoxidase (MPO) is an enzyme existing in the gastric tissue whose expression is enhanced in the inflammatory conditions of the gastric mucosa and is responsible for elevation of oxidative stress[38]. MPO seems to act on macrophage as deduced by the increased production of cytokines and oxidative burst when macrophages are exposed to MPO or other peroxidases[39]. MPO is a key weapon of the innate immune system, providing a first line defense. At the cellular level MPO occurs in the macrophages. In the inflammatory conditions, monocyte infiltration leads to elevation of MPO and hypochlorous acid within the macrophage[40,41]. MPO has been attributed to its unique capacity to produce hypochlorous acid and other toxic agents that create an environment within the phagolysosome of neutrophills that inhibits or kills ingested microbes. MPO utilizes H_2O_2 to increase posttranslational modifications of target molecules, following a paradigm utilized by all members of the animal peroxidase family, although the capacity to oxidize Cl^- to Cl^+ at physiologic pH is a property unique to MPO. Concomitant with release of MPO into the phagosome, the NADPH-dependent oxidase of phagocytes is activated to generate the required H_2O_2 for MPO to mediate HOCl (hypochlorous acid) generation[42]. Usually at inflammatory sites neutrophills precede macrophage infiltration. It appears that macrophages can acquire MPO by engulfing neutrophills or MPO released by these cells at the inflammatory site[43-44]. Macrophages and neutrophills vary from a resting to a fully activated state. The activation includes the expression of several types of proteins, such as membrane receptors, soluble factors and enzymes. Reactive oxygen intermediates appear to be important in some signaling pathways involved in phagocyte activation[45,46]. Hence, the cellular activity of enzymes that utilize or generate reactive oxygen species could reveal the steady state concentration of these species. These mediators lead to necrosis and dysfunction of the gastric mucosa[47-49]. The endogenous cellular antioxidants are exhausted which promotes further ulceration of the gastric mucosa[50,51]. Macrophages enriched in MPO content are found in atherosclerotic lesions[52] or in brain lesions of patients with multiple sclerosis[53]. The levels of myeloperoxidase in the gastric mucosa represent the extent of gastric mucosal damage. PCE was also found to significantly reduce the myeloperoxidase activity in a dose dependent manner.

MPO is regarded as a measure of neutrophil infiltration and oxidative stress[54,55]. Hydro alcoholic extract of psoralea has been reported to possess potent *in vitro* anti oxidant and free radical scavenging activity[35]. Hence, our investigation provides support to the previous *in vitro* studies. The anti oxidant profile may also play a role in the protection of the gastric mucosa from the *H. pylori* cytotoxins and the neutrophil infiltration due to naproxen administration. The present investigation clearly demonstrates that significant in vivo inhibition of myeloperoxidase requires continued treatment for ten weeks.

The present study reveals a new facet in the therapeutic potential of Psoralea. However, the antimicrobial activity is tardy and the antioxidant activity was found to be significant on long term administration. This could be explained by the possibility of delayed formation of active ingredients and building up of their sufficient antimicrobial concentration. Further investigations should be carried out to determine the isolated phytochemical responsible for the *in vivo* anti *H. pylori* activity of ethanolic extract of Psoralea.

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