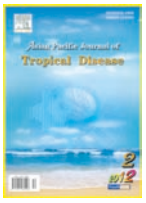
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage:www.elsevier.com/locate/apjtd



Document heading

Therapeutic effect of *H. pylori* nosode, a homeopathic preparation in healing of chronic *H. pylori* infected ulcers in laboratory animals

Tejas Prabhakar Gosavi[1], Pinaki Ghosh[2], Amit Dattatraya Kandhare[2], V. Shiva Kumar[2], Mohammad Adil[2], Anuchandra Ramchandra Rajmane[2], Subhash L. Bodhankar[2*]

[1] *Department of Homeopathic Pharmacy, Bharati Vidyapeeth Deemed University, Dhankawadi, Katraj, Pune, Maharashtra, 411043, India*
[2] *Department of Pharmacology, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune, Maharashtra, 411038, India*

ARTICLE INFO

Article history:
Received 9 August 2012
Received in revised from 11 September 2012
Accepted 9 December 2012
Available online 28 December 2012

Keywords:
Epidemiology
H. pylori
Homeopathy
Nosode
Prevention

ABSTRACT

Objective: *H. pylori* is classified as class I carcinogen by World Health Organization and is a global as it is the primary cause of gastric carcinoma. The objective of present investigation was made to investigate the protective effect of homeopathically potentized *H. pylori* nosode in rats infected with *H. pylori*. **Methods:** The infection was induced in the rats using a bacterial suspension of 5 x 10^8 CFU per ml. The various animals were treated with three potencies of *H. pylori* nosode (3X, 6X and 12X) and standard (Clarithromycin 25mg/kg + amoxicillin 50 mg/kg + omeprazole 20 mg/kg). A period of ten weeks followed and then the rats were sacrificed. **Results:** *H. pylori* nosode was able to stop the initiation of infection in the pretreatment group of animals. This defines and provides us with the data regarding its sphere of action. A plethora of parameters such as ulcer area, infection status, biomarkers of oxidative stress, total intracellular ROS, degree of apoptosis, TNF level and load of bacteria in the gastric tissue served as the hallmarks to prove the preventive role of *H. pylori* nosode in the amelioration of the infection. It is evident that homeopathic drug *H. pylori* nosode was able to transform the susceptibility of the animals to the induction of infection. It provides a novel avenue in the field of research in homeopathy as the results suggest that *H. pylori* nosode was able to initiate the infection resistive forces in the animals before being subjected to the infection of *H. pylori*. **Conclusion:** *H. pylori* nosode possesses potent prophylactic anti *H. pylori* activity *in vivo*.

1. Introduction

Helicobacter pylori is a gram negative panmictic microarerophilic bacteria thriving in the acidic environment of stomach and transforms pre-existing ulcer into gastric lymphomas[1]. Ever since the dawn of gastroenterology *H. pylori* has intrigued physicians and scientists. It has the unique property of recrudescence and reinfection[2]. It has ability to cause severe gastritis accompanied by malena, hematemesis, early satiety, post prandial fullness and excruciating epigastric pain[3].

There are various treatment strategies available for treatment of *H. pylori* in allopathy and ayurveda[4, 5]. Homeopathy has always been a part of community medicine proving effective in management of epidemics[6]. Homeopathy is based upon the principles of Hahnemann[7]. Nosode and isode are closely related associated therapeutic dosage form used in homeopathy. In the light of Hahnemann philosophy nosode have the ability to wane off epidemics.

History also showed similar evidences like prevention and cure of cholera epidemic by camphor and scarlet fever by Belladonna[8]. But this never satisfied the demographic immunological statements in terms of vaccination, prevention as well as specific therapeutic interventions to reduce mortality and morbidity[9]. Probability of preventive action of homeopathically potentized medicines could be very similar to that of the vaccine especially when they are prepared from miasmatic agents[10].

Western medicine has an array of medicaments and therapeutic strategies to treat active *H. pylori* infection. However, it has been seen that there is rampant resistance of the bacteria to the well known triple drug regimen[5]. *H. pylori* nosode (HPN) was selected on the basis of homeopathic principles and due to the fact that it is clinically proven in case of *H. pylori* infection[11].

*Corresponding author: Dr. S. L. Bodhankar, Dept. of Pharmacology, Poona college of Pharmacy,Bharati Vidyapeeth Deemed University, Erandwane. Pune-411038 Maharashtra.
E-mail: sbodh@yahoo.com

Hence, the objective of present investigation was to elucidate the therapeutic role of *H. pylori* nosode in an animal model of *H. pylori* infected unhealed ulcer in laboratory rats.

2. Material and methods

2.1. Animals

Healthy adult male wistar rats (230-250 g) were obtained from the National Toxicological Centre, Pune (India). The animals were housed separately in groups of 4 in solid bottom polypropylene cages. Rats were maintained at 24 ℃ ± 1 ℃, 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 housed under pathogen free conditions. The animals had free access to standard pellet chow (Pranav Agro industries Ltd., Sangli, India). The animals had access to filtered water. The research protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Pune (CPCSEA/12/2011) and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.

2.2. Drugs and Chemicals

Clarithromycin was 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 rRNA gene were synthesized at Ocimum Biosolutions, Hyderabad, India. *H. pylori* was provided in cryopreserved state by Centre for liver Research and diagnostics, Owaisi hospital, Hyderabad, India.

2.3. Preparation of homeopathic medicines

On decimal scale *H. pylori* nosode was prepared from preformed mother tincture according to HPI[12] guidelines in the laboratory itself. Briefly, *H. pylori* was killed at 300 ℃ and mixed in dispensing alcohol i.e. mother solution. Medicines were potentized (3X, 6X and 12X) manually with strokes on pad in glass vial by 1:9 succession and serial dilution. Culture of *H. pylori* was obtained from Centre for liver Research and diagnostics, Owaisi hospital, Hyderabad, India.

2.4. Experimental set up

The animals were divided into six groups consisting of 8 rats in each group as follows:

[A] Non-infected and non-ulcered:

Group 1: Normal animals (received 2 ml/kg/day of distilled water)

[B] Infected and ulcered:

Group 2: Vehicle control animals (received 2 ml/kg/day of distilled water)

Group 3: CAO treated animals (received Clarithromycin 25 mg/kg + Amoxicillin 50 mg/kg + Omeprazole 20 mg/kg)

Group 4: HPN3XP (received *H. pylori* nosode 3XPOTENCY 2 mg/kg/day, for 4 weeks)

Group 5: HPN6XP (received *H. pylori* nosode 6XPOTENCY 2 mg/kg/day, for 4 weeks)

Group 6:HPN12XP (received *H. pylori* nosode 12XPOTENCY 2 mg/kg/day, for 4 weeks)

2.5. Induction of ulcers and H. pylori infection

Ulcers were induced in the rats using the method mentioned by Takagi et al[13]. The rats were anesthetized with ketamine (60 mg/kg i.p). An epigastric incision was made through midline and stomach was exposed. 0.3 ml of a 20% solution of acetic acid was injected into the sub serosal layer of the glandular portion of the stomach with the aid of a tuberculin syringe. Subsequently stomach was re-internalized; the abdomen was closed and sutured. The animals were maintained in individual cages with meshed bottom to prevent coprophagy. The size of the mesh (4 X 4 mm) allowed feces to fall to the floor of the cage below the mesh. After the induction of ulcers, five days are required for the ulcers to develop fully. During this period, *H. pylori* inoculums in mid log phase was administered to the rats for three alternate days at a concentration of 5×10^8 CFU / mL *H. pylori* using 0.5 McFarland's turbidity standard (NCCS).

On the seventh day after surgery, infection status was determined by randomly sacrificing two animals from each group and determining their ulcer and infection status. Then treatment was initiated and continued for four weeks. At the end of the treatments, after 24 h fasting, the rats were sacrificed and the stomachs were dissected and opened out along the greater curvature. It was washed with normal saline and each stomach was photographed using a CCD scanner at a magnification of 2400 DPI to determine ulcer area[14]. Thereafter the fundic portion was excised off and corpus antrum along with pylorus portion of the stomach was divided into four strips of 200 mg each. They were separately homogenised in 2 ml of PBS using sterile homogeniser.

The first portion was used for the determination of infectious status by quantitative culture and DNA isolation which was further used for polymerase chain reaction (PCR) amplification. The second portion was used for the determination of oxidative stress parameter. The third portion was used for the flowcytometric determination of apoptosis and ROS whereas fourth portion was used for determination of cytokine i.e. TNF-α.

2.6. Determination of Ulcer area

The animals were sacrificed by cervical dislocation and the stomach isolated out. It was dissected along the greater curvature. It was washed with normal saline and the image scanned at 2400 DPI. This image was stored and then the ulcer area was calculated using Adobe Photoshop and Image J software. The ulcer area was calculated for each animal

using the scanned image of the stomach according to method reported elsewhere[14].

2.7. Determination of H. pylori infection status

The infection was determined by the amplification of 16s rRNA gene and the virulent genes such as cag E, cag T in the gastric mucosa of all the animals according to method mentioned by Tiwari et al[15]. The infection was determined at 10th week using polymerase chain reaction (PCR). A small portion of the pylorus of the isolated stomach was used for extraction of DNA to be amplified by PCR.

2.8. Quantitative Culture

The Quantitative Culture was determined according to method reported elsewhere[16]. At the end of the study, a strip from the greater curvature of the stomach glandular stomach was cultured quantitatively. Bacterial load was expressed as log CFU per gram of stomach tissue. Challenged rats that were culture negative were assigned a value of 1 CFU/g in order to calculate the log CFU per gram and geometric means.

2.9. DNA Isolation

DNA isolation from gastric tissue was performed according to standard acetyl tri-methyl ammonium bromide method mentioned by Tiwari et al[15]. Briefly, the tissue samples were suspended in 250 μL of digestion buffer II. {0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0), 1% SDS} containing 100 μg/ml of proteinase K (Vivantis, India). To this, 250 μl 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 56 ℃ overnight. DNA was extracted with an equal volume of phenol chloroform and precipitated with 0.6 volume isopropanol. The DNA pellets were washed thrice with 80%, 75% and 70% ethanol, respectively, and finally resuspended in 50 μl - 100 μl of sterile water for injection. All the steps were performed in aseptic conditions to minimize contamination.

2.10. PCR amplification of 16S rRNA, cag E, cag T gene

PCR amplifications were performed according to protocol mentioned by Tiwari et al[16]. All the primers were designed as per the specific genes and adequately diluted. Briefly, 2 μl of the template DNA isolated from gastric tissue was added to 18 μl 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 MgCl2, 200uM concentrations of each dNTPs, 10 pMol of each primer & 1U of Taq polymerase. The following thermal cycle steps were used in the PCR amplification: initial denaturization at 96 ℃ for 5 minutes, 40 cycles with 1 cycle consisting of 94 ℃ for 1 minute, 56 ℃ for 1 second and 72 ℃ 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 (Eppendorff). Thermal cycles were altered according to the melting point (Tm) of each primer. 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 rRNA F and 16s rRNA R. The PCR-amplified products were analyzed by agarose gel electrophoresis. 10 μl of each amplified product was added to 3 μl 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.

2.11. Determination of gastric superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA) and myeloperoxidase (MPO) contents

The mucosal pathological alteration occurs due to the overproduction of ROS. Gastric SOD, GSH, MDA and MPO assay were determined as previously reported elsewhere[14]. SOD activity was expressed as U/mg protein.

2.12. Analysis of CD4 and CD8 cells

Analysis of CD4 and CD8 cells according to the method described by Santhosh Kumar et al[17]. Prior to dissection, 3 mL of blood was collected from each rats in EDTA coated vacutainers (Becton & Dickinson India Pvt. Ltd., Gurgaon, India). Quantification of CD4 and CD8 cells was carried out in three-color flow cytometer FACS Calibur (Becton & Dickinson, San Diego CA, USA). Briefly, 20 μL of the antibody and 50 μL of the anti coagulated blood were incubated in 5 mL polystyrene round-bottomed tube (Cat. No. 352003, Becton & Dickinson, San Diego, USA) for 15-20 min. Then the RBCs were lysed using 1×FACS lysing solution (Cat. No. 349202, Becton & Dickinson, San Diego CA, USA). The samples were incubated in dark for 10-12 min and subsequently washed twice with 2 mL sheath solution (BD FACS Flow Cat. No. 342003, Becton & Dickinson, San Diego CA, USA) to remove the debris. Finally the precipitate was re suspended in 450 μL of the sheath solution for flow cytometric analysis. Acquisition and analysis of the processed samples was performed using CELL Quest software (Becton & Dickinson, San Diego, USA).

2.13. Analysis of total intracellular ROS

A single cell suspension was prepared and the oxidative damage to gastric tissue was evaluated by estimating ROS levels according to the method described by Kobayashi et al and Lawler et al[18, 19]. ROS production was quantified by the dichlorofluorescin diacetate (DCFH-DA) method based on the ROS-dependent oxidation of DCFH-DA to DCF according to the method described Kobayashi et al[18].

2.14. Determination of TNF alpha

TNF alpha was quantified in blood using ELISA kit (Thermo Fischer Ltd., USA) according to the manufacturer's instructions. The assay employed the sandwich enzyme

immunoassay technique. The values were expressed as pg/ml[20].

2.15. Determination of Apoptosis

Apoptosis was measured using Apo Alert (Anexin-FITC PI staining kit) Takara Ltd., USA using FACS Caliber flow cytometer. Samples were processed according to the manufacturer's instructions. Whole rat stomachs were harvested and processed. As a first step in the analysis, gastric epithelial cells were disrupted and released by incubating rats stomachs in HBSS (Hank's buffered salt solution) containing 10% FBS (fetal bovine serum), 15 mM HEPES, 5 mM EDTA, and 0.014% DTT (Di Thio Threitol) for 1 h at 37 ℃. The released epithelial cells were discarded, and stomachs were then digested for 30 min at 37 ℃ in RPMI (Roswell park memorial institute medium) 1640 containing 10% FBS and 1 mg/ml collagenase A. The suspension was passed through a 70 μm cell strainer (BD Biosciences. Cells were harvested by centrifugation. Cells were washed, resuspended in FACS buffer. The cell suspension was diluted to obtain 5 x 106 cells/ml and analyzed on a BD LSR II flow cytometer (BD Biosciences). The number of each cell type in a sample was calculated as previously described by Algood et al[21]. Acquisition and analysis of the processed samples was performed using CELL Quest software (Becton & Dickinson, San Diego, USA).

2.16. Histopathological analysis

Freshly excised stomach of one animal from each group was washed with saline and preserved in 10% formaldehyde solution for histopathological studies. It processed for 12 hr using isopropyl alcohol, xylene and paraffin embedded for light microscopic study (Nikon E200). Paraffin-embedded tissue section cut in 5 μm thickness were prepared and stained after deparaffination using haematoxylin and eosin stain (H and E) to verify morphological assessment of stomach damage. Photomicrographs were captured at a magnification of 100 X.

All cases were examined by a pathologist and the following parameters were analyzed:

Inflammation – presence of lymphocytes and plasmocytes in the lamina propria;

Activity – characterized by the presence of neutrophil inside the superficial and glandular epithelial layers;

Atrophy – reduction of glandular structures;

Metaplasia – presence of caliciform cells with an intestinal morphology.

2.17. Statistical analysis

Data were expressed as mean ± standard error mean (SEM). The data was analyzed using one-way analysis of variance (ANOVA), Dunnett's multiple range test was applied for post hoc analysis. Analysis of all the statistical data was performed using GraphPad Prism 5.0 (GraphPad, San Diego, USA). $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of HPN on ulcer Area

The mean ulcer area in vehicle control animals was significantly increased as compared to normal animals.

Table 1.
Effect of chronic treatment of HPN on ulcer area, log CFU per gram and PCR amplification of DNA

Treatment	Ulcer area (mm^2)	Log CFU per gram	Infection status i.e. PCR amplification of DNA (16s rRNA, cag E, cag T)
Normal	0.00 ± 0.00	0.00 ± 0.00	---
Vehicle control	20.51 ± 0.73###	8.80 ± 0.48###	6/6
CAO	6.55 ± 0.90***	2.26 ± 0.29***	1/6
HPN3XP	17.53 ± 0.68*	7.20 ± 0.50*	5/6
HPN6XP	13.14 ± 0.75**	5.34 ± 0.44**	3/6
HPN12XP	9.45 ± 0.78***	3.18 ± 0.45***	2/6

Data are expressed as mean ± S.E.M. and analyze by one way ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to vehicle control animals and ###$P < 0.001$ as compared to normal animals.

Table 2.
Effect of chronic treatment of HPN on antioxidant enzyme, malondialdehyde and myeloperoxidase levels

Treatment	SOD (U/mg protein)	GSH (U/mg protein)	MDA (nM /mg protein)	MPO (U/gm)
Normal	6.44 ± 0.40	3.38 ± 0.21	3.78 ± 0.44	1.68 ± 0.23
Vehicle control	2.64 ± 0.32###	0.95 ± 0.15###	12.55 ± 0.69###	6.07 ± 0.41###
CAO	5.73 ± 0.37***	2.98 ± 0.14***	4.74 ± 0.48***	2.39 ± 0.28***
HPN3XP	3.09 ± 0.32	1.40 ± 0.16	11.91 ± 0.44	5.66 ± 0.33*
HPN6XP	4.51 ± 0.44**	1.94 ± 0.15**	9.59 ± 0.34**	4.37 ± 0.42**
HPN12XP	5.30 ±0.35***	2.63 ± 0.18***	7.04 ± 0.34***	3.01 ± 0.26***

Data are expressed as mean ± S.E.M. and analyze by one way ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to vehicle control animals and ###$P < 0.001$ as compared to normal animals.

Chronic treatment with HPN (3X, 6X and 12X) for 4 weeks significantly ($P < 0.05$, $P < 0.01$ and $P < 0.001$ resp.) and dose dependently decreased mean ulcer area as compared to vehicle control animals. Whereas treatment with CAO also showed significant attenuation of increased in mean ulcer area ($P < 0.001$) when compared with vehicle control animals. (Table 1).

Table 3.
Effect of chronic treatment of HPN on CD4:CD8 ratio and TNF-alpha level

Treatment	CD4:CD8 ratio	TNF-α (pg/ml)
Normal	4.35 ± 0.19	11.87 ± 1.14
Vehicle control	2.11 ± 0.21###	37.26 ± 2.02###
CAO	3.83 ± 0.25***	17.08 ± 1.50***
HPN3XP	2.55 ± 0.24	32.91 ±1.93*
HPN6XP	2.97 ± 0.14**	27.78 ± 2.51**
HPN12XP	3.45 ± 0.22***	23.26 ± 2.46***

Data are expressed as mean ± S.E.M. and analyze by one way ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to vehicle control animals and ###$P < 0.001$ as compared to normal animals.

3.2. Effect of HPN on H. pylori infection status

DNA isolated from all the sample was amplified to get a 534 base pair fragment amplicon corresponding to 16s rRNA gene as well as 329 base pair fragment amplicon of cag E gene and 842 base pair fragment amplicon of cag T gene. Oral administration of *H. pylori* resulted in significant induction of infection in the vehicle control animals assessed by the PCR amplification of various genes included 16s rRNA, cag E, cag T. There was 100% of animals were get infected with the *H. pylori* oral administration. Treatment with HPN (3X, 6X and 12X) significantly ($P < 0.05$, $P < 0.01$ and $P < 0.001$ resp.) and dose dependently decreased PCR amplification of various genes as compared to vehicle control animals. The infection was ameliorated to 16.66%, 50.00% and 66.66% along the entire period of investigation in HPN (3X, 6X and 12X) treated animals. Treatment with CAO also significantly ($P < 0.001$) ameliorated infection (100%) as compared to vehicle control animals. (Table 1 and Figure 1)

Table 4.
Effect of HPN on Histopathological alteration

Treatment	Inflammation	Neutrophil infiltration	Atrophy	Metaplasia
Normal	0	+	0	0
Vehicle control	+++	++++	++++	+++
CAO	+	++	0	+
HPN12XP	+	+	+	+

Pathological grade: 0: no abnormality detected; +: damage/ active changes up to less than 25%;++: damage/ active changes up to less than 50%; +++: damage/ active changes up to less than 75%;++++: damage/ active changes more than 75%.

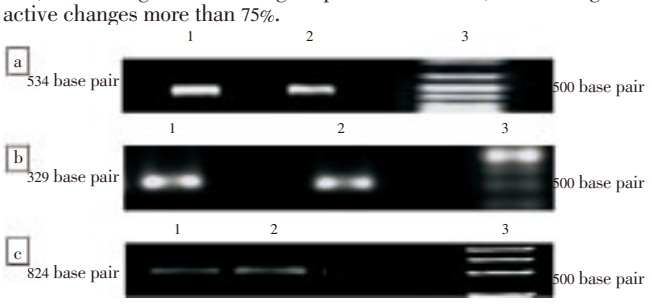


Figure 1. Representative gel image of successful amplification of *H. pylori* specific genes (a) 16s rRNA (534 base pair) (b) cag E gene (329 base pair) (c) cag T gene (842 base pair) to confirm the presence of *H. pylori* in the various treatment groups of animals.

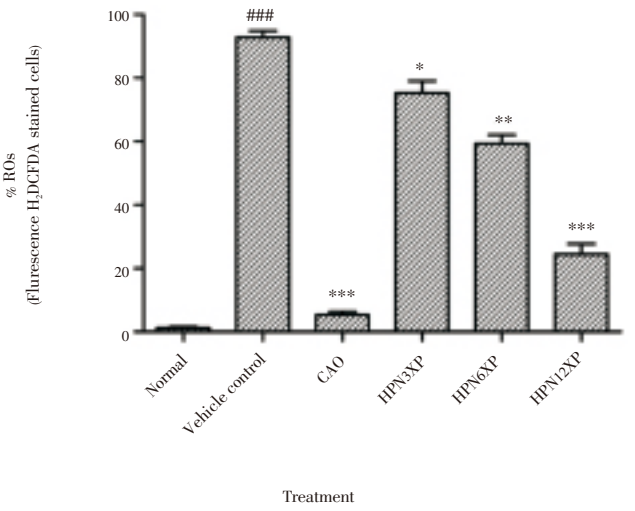


Figure 2. Effect of chronic treatment of HPN and CAO on% of ROS observed after flow cytometric analysis using H_2DCFDA method.
Data are expressed as mean ± S.E.M. and analyze by one way ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to vehicle control animals and ###$P < 0.001$ as compared to normal animals.

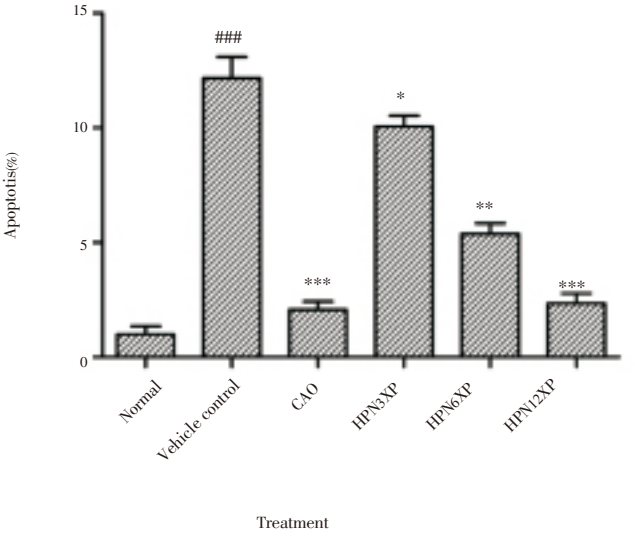


Figure 3. Effect of chronic treatment of HPN and CAO on% of apoptosis cell populations observed after FACS analysis using Annexin V/FITCPI stain.
Data are expressed as mean ± S.E.M. and analyze by one way ANOVA followed by Dunnett's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to vehicle control animals and ###$P < 0.001$ as compared to normal animals.

3.3. Effect of HPN on quantitative culture

The bacterial load was assessed by determining log CFU per gram in all animals after oral administration of *H. pylori* and in vehicle control animals it was found to be significantly elevated ($P < 0.001$) as compared to normal animals. Chronic treatment with HPN (3X, 6X and 12X) for 4 weeks significantly and dose dependently ($P < 0.05$, $P < 0.01$ and $P < 0.001$ resp.) decreased bacterial load as compared to

vehicle control animals. (Table 1)

3.4. Effect of HPN on antioxidant enzymes

Oral administration of *H. pylori* results significant decreased ($P < 0.001$) in level of antioxidant enzyme i.e. SOD and GSH in the vehicle control animals as compared to normal animals. Treatment with HPN (6X and 12X) for 4 weeks significantly and dose dependently ($P < 0.01$ and $P < 0.001$ resp.) increased levels of antioxidant enzyme as compared to vehicle control animals. Treatment with CAO also significantly attenuated ($P < 0.001$) these decreased level of SOD and GSH as compared to vehicle control animals. (Table 2)

3.5. Effect of HPN on malondialdehyde activity

The level of malondialdehyde was significantly increased ($P < 0.001$) in vehicle control animals after oral administration of *H. pylori* when compared with the normal animals. Treatment with HPN (6X and 12X) for 4 weeks significantly ($P < 0.01$ and $P < 0.001$ resp.) and dose dependently decreased activity of MDA as compared to vehicle control animals. Treatment with CAO also significantly ($P < 0.001$) decreased level of malondialdehyde as compared to vehicle control animals. (Table 2)

3.6. Effect of HPN on myeloperoxidase activity

The myeloperoxidase activity was significantly increased ($P < 0.001$) in vehicle control animals after oral administration of *H. pylori* when compared with the normal animals. Chronic treatment with HPN (3X, 6X and 12X) for 4 weeks significantly ($P < 0.05$, $P < 0.01$ and $P < 0.001$ resp.) and dose dependently decreased activity of MPO as compared to vehicle control animals. Myeloperoxidase activity in the CAO treated group was significantly decreased ($P < 0.001$) when compared with vehicle control animals. (Table 2)

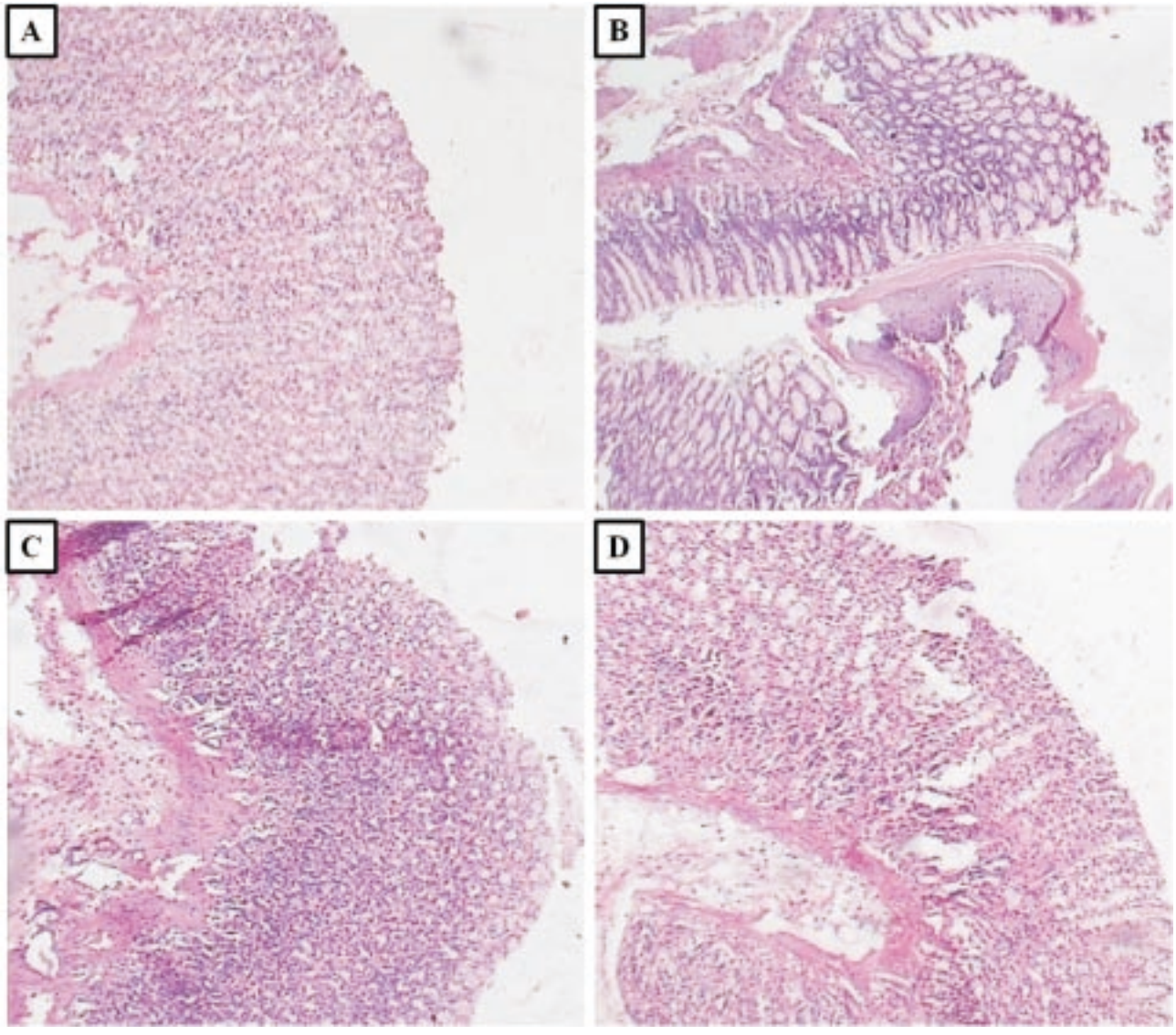


Figure 4. Photomicrography of the gastric wall stained with H&E. Stomach microscopic image of (A) Normal animal (B) Vehicle control animal (C) CAO treated animal (D) HPN (12X) treated animal. Images (X 100 magnification) are typical and representative of each study group.

3.7. Effect of HPN on CD4 and CD8 cells

Oral administration of *H. pylori* results significant decreased ($P < 0.001$) in CD4:CD8 ratio in vehicle control animals as compared to normal animals. Treatment with HPN (6X and 12X) for 4 weeks raised ratio of CD4:CD8 significantly ($P < 0.01$ and $P < 0.001$ resp.) and in a dose dependent pattern when compared with vehicle control animals. Chronic treatment with CAO also significantly raised the ratio of CD4:CD8 ($P < 0.001$) when compared with vehicle control animals. (Table 3)

3.8. Effect of HPN on TNF alpha concentration

TNF alpha levels were significantly increased ($P < 0.001$) in vehicle control animals after oral administration of *H. pylori* as compared to normal animals. Chronic treatment with HPN (3X, 6X and 12X) for 4 weeks significantly ($P < 0.05$, P < 0.01 and $P < 0.001$ resp.) and dose dependently decreased level of TNF alpha as compared to vehicle control animals. Increased in TNF alpha level was significantly attenuated ($P < 0.001$) in CAO treated group when compared with vehicle control animals. (Table 3)

3.9. Effect of HPN on total intracellular ROS

Vehicle control animals showed significantly enhanced ($P < 0.001$) intracellular levels of ROS in the form of H2DCFDA fluorescence intensity after oral administration of *H. pylori* as compared to normal animals. Total intracellular ROS levels were decreased significantly and in a dose dependent pattern in HPN3XP ($P < 0.05$), HPN6XP ($P < 0.01$), HPN12XP ($P < 0.001$) when compared with vehicle control animals. Treatment with CAO showed significant restoration of H2DCFDA fluorescence intensity ($P < 0.001$) when compared with the vehicle control group of animals. (Figure 2)

3.10. Effect of HPN on apoptosis

Percent apoptosis was significantly increased ($P < 0.001$) in vehicle control animals after oral administration of *H. pylori* when compared with normal animals. Chronic treatment with HPN (3X, 6X and 12X) for 4 weeks significantly ($P < 0.05$, P < 0.01 and $P < 0.001$ resp.) and dose dependently attenuated this elevated level of apoptosis as compared to vehicle control animals. Treatment with CAO showed significant decreased in level of apoptosis ($P < 0.001$) when compared with the vehicle control group of animals (Figure 3)

3.11. Effect of HPN on histopathological alteration

In the normal animals groups presented intact gastric mucosa and did not exhibit any alterations in their morphologies. Histopathological changes in vehicle control animals showed degeneration, hemorrhage, edematous appearance of the gastric tissue. Where as in CAO and HPN (12 X) treated animals showed regeneration and prevents the formation of hemorrhage and edema. (Table 4 and sFigure 4)

4. Discussion

Ulcer healing is a programmed replicative process including many interdependent processes. *H. pylori* aggravates the expression of malefic factors and down regulates the protective one[22, 23]. Intracellular and intercellular cross talk result in disregulation of the normal gastric epithelization process and milieu leading to carcinoma[24]. The present investigation was designed to evaluate the role of *H. pylori* nosode in modulation of various intermediate of ulcer healing and other biochemical factors leading to gastric ulcers healing.

Ulcer area is a quantitative parameter depicting ulcer healing. HPN at potencies of 3X, 6X and 12X showed healing of ulcer and reduction in ulcer area. *H. pylori* residue in gastric pits of pyloric antrum and exerts its pathological effect[25]. In the light of these fact log CFU of *H. pylori*/gm was determine. HPN 3X, 6X and 12X showed reduction of log CFU/gm of stomach tissue.

Free radicals are universally accepted intermediates involved in cellular and molecular damage of cells. They are the markers that help us in determining the state of gastric mucosa in stressful condition[14]. Various intermediates involved in these processes are SOD, GPx, GSH and MDA. These are indispensible biomarkers leading to down regulation of mucosal integrity[26]. HPN increase the concentration of SOD depicting its role in restoration of mucosal damage. *H. pylori* is a known potentiater of oxidative stress. HPN demonstrate its ability to reduce the oxidative damage.

MPO is a biomarker corresponding to the elevated neutrophil infiltration[27]. In the ulcerated tissue, the elevated level of the neutrophil infiltration may be associated with the increase in MPO enzyme activity as well as H_2O_2[28]. Neutrophils exhibit an important role in the pathogenesis of ulceration by dent of increase in an array of reactive oxygen species and superoxide anions which in turn up regulate hydroxyradical and peroxide exacerbation leading to mucosal dysfunction and tissue necrosis[29]. HPN attenuated elevated levels of neutrophil infiltration.

It has been documented that that presence of lymphocytes and especially the predominance of CD8 cells may delay certain stages of the healing process[30]. It has been reported that CD4 cells are sensitized in vivo and migrate to gastric mucosa where they induce gastritis in response to *H. pylori* antigens, suggesting that CD4-dependent *H. pylori* induced gastritis could lead to epithelial damage with proliferative and metaplastic responses. Hence, *H. pylori* modulate the immune system of the host and decrease the CD4: CD8 ratio[31]. HPN was able to elevate the diminished CD4: CD8 ratio representing its ability to modulate the immune system.

Cytokines have been implicated to play an indispensible role in vicious cycle of oxidative stress[32]. TNF-α is a potent stimulator of neutrophil infiltration[20]. TNF-α elevation results due to chronic infection and disruption of the gastric mucosa. HPN attenuated elevated levels of TNF-α dose dependently.

Apoptosis is characterized by programmed cell death

culminating to synergistic interplay of oxido-nitrosative cytokine and other intracellular aberrations[33]. It is measured by flow cytometry using FITC-Annexin V- PI staining depicting the degree of differential apoptosis in discrete samples. Phosphotidyl serine is over expressed on the cell membrane of a normal cell under stressful condition[34]. Over expression of phosphotidyl serine leads to increased binding of FITC to phosphotidyl serine molecule resulting in to changes in fluorescence intensity registered by flow cytometer. It is observed that over expression of phosphotidyl serine is a representative cellular event which can be traced by flow cytometer. The population of cell in late apoptotic stage was reduced in dose dependant manner mirroring the antiapoptotic effect of HPN. H2DFDA is a dye having an inherent ability to couple with intracellular reactive oxygen intermediates. Enhanced intensity corresponded to excess ROS in the sample hence this dye was employed to measure the intracellular status of oxidative stress[35]. HPN was able to down regulate the elevated amount of oxidative stress precipitated by the *H. pylori* infection in dose dependant manner.

Infection status was determined by polymerase chain reaction[36]. DNA of *H. pylori* was isolated from excised gastric mucosa from the infected animals. 16 s rRNA gene is present in the highly conserved region of *H. pylori* genome[15]. It is the gold standard for detection of the *H. pylori* in clinical and preclinical setting. Cag A, Cag E and Cag T are the three important genes responsible for the injection of cytotoxine into the gastrointestinal tract[37]. It is a part of the Cag pathogenecity inland responsible for the elucidating the detrimental effect of *H. pylori* on the gastrointestinal tract[15]. It was evident from the present investigation as 1/6 animals were infected at the end of study.

The present antibiotics treatment regimen for *H. pylori* includes clarithromycin, metronidazole, omeprazole, etc. However the side effects of these drugs are well documented in literature[38, 39]. Homeopathy is proven in many medical areas[40, 41]. A glint of hope in dilapidated treatment armory is visible from the present investigation. HPN has been an unexploited medicament for *H. pylori* treatment. However, further studies are needed to elucidate its mechanism of action.

The present investigation shows that *H. pylori* nosode can be looked upon as a potential therapeutic strategy to provide prophylactic treatment to patients who have a history of ulcers and are constantly under the threat of being infected with *H. pylori*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors would like acknowledge Dr. S. S. Kadam, Vice-Chancellor and Dr. K. R. Mahadik, Principal, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune, India, for providing necessary facilities to carry out the study. The authors are thankful to the Dr. Aleem. A. Khan, Dr. Santosh. K. Tiwari, Dr. Manoj Gopi, Dr. G. S. Shivaram and Dr. A. Bardia of Centre for Liver Research and Diagnostics, Owaisi hospital, Hyderabad for valuable guidance in molecular biology and flow cytometric techniques in the investigation.

References

[1] Kamangar F, Qiao YL, Blaser MJ, Sun XD, Katki H, Fan JH, et al. *H. pylori* and oesophageal and gastric cancers in a prospective study in China. *Br J Cancer* 2007;**96**(1): 172-176.

[2] Ghosh P, Bodhankar SL. Application of polymerase chain reaction for determination of prevalence of *helicobacter pylori* in salivary samples of asymptomatic subjects. *Asian J Pharm Clinical Res* 2011; **4**(4):21-24.

[3] Akhter Y, Ahmed I, Devi SM, Ahmed N. The co-evolved *H. pylori* and gastric cancer: trinity of bacterial virulence, host susceptibility and lifestyle. *Infect Agent Cancer* 2007;**2**:2.

[4] Kim YS, Kim SJ, Yoon JH, Suk KT, Kim JB, Kim DJ, et al. Randomised clinical trial: the efficacy of a 10-day sequential therapy vs. a 14-day standard proton pump inhibitor-based triple therapy for *Helicobacter pylori* in Korea. *Aliment Pharmacol Ther* 2011; **34**(9): 1098-105.

[5] Ghosh P, Kandhare AD, Gauba D, Raygude KS, Bodhankar SL. Determination of efficacy, adverse drug reactions and cost effectiveness of three triple drug regimens for the treatment of *H. pylori* infected acid peptic disease patients. Asian Pac J Trop Biomed 2012a. In press

[6] Gosavi TP, Ghosh P, Kandhare AD, Bodhankar SL. Unwrapping Homeopathic Principles in the Wake of Research: Serendipity, Placebo or True Therapeutic Milestones. *Pharmacologyonline* 2011a;**1**:894-906.

[7] Hahnemann CFS. edn. Organon of Medicine. 6th edn. New Delhi: B. Jain Publishers pp 1991;105-45.

[8] Bellavite P, Contorti A, Dontarllo F, Ortolani R. Immunology and Homeopathy: 2. Cells of the immune system and inflammation. *Evid Based Complement Alternat Med* 2006;**3**:13-24.

[9] Gosavi TP, Kandhare AD, Raygude KS, Ghosh P, Bodhankar SL. A comparative study on the efficacy, safety and cost effectiveness of Viscum album and Rauwolfia serpentina mother tincture in hypertensive patients. Deccan *J. Natural Products* 2011b;**2**(3):29-35.

[10] Heine H, Schmolz M. Immunoregulation via 'bystander suppression' needs minute amounts of substances-a basis for homeopathic therapy? *Med Hypotheses* 2000;**54**:392-393.

[11] Hofbauer R, Pasching E, Moser D, Frass M. Heparin-binding epidermal growth factor expression in KATO-III cells after *Helicobacter pylori* stimulation under the influence of wstrychnos Nux vomica and Calendula officinalis. *Homeopathy* 2010;**99**(3):177-82.

[12] Homoeopathic Pharmacopoeia of India. Government of India ministry of Health & Family Welfare. 1974;Vol-I p. 178-188, 211, 219.

[13] Takagi K, Okabe S, Saziki R. A new method for the production of chronic gastric ulcer in rats and the effect of several drugs on its healing. *Japanese J Pharmacol* 1969;**19**:418-426.

[14] Kandhare AD, Raygude KS, Ghosh P, Bodhankar SL. The ameliorative effect of fisetin, a bioflavonoid, on ethanol-induced and pylorus ligation-induced gastric ulcer in rats. *Int J Green Pharm* 2011;**5**:236-43.

[15] Tiwari SK, Khan AA, Manoj G, et al. A simple multiplex PCR assay for diagnosing virulent *H. pylori* infection in human gastric biopsy specimens from subjects with gastric carcinoma and other gastro-duodenal diseases. *J Appl Microbiol* 2007;**103**(6):2353-2360.

[16] Ghosh P, Badgujar L, Ajmera A, Garg H, Purohit A, Bodhankar S. Effect of ethanolic extract of Psoralea corylifolia on *H. pylori* infection in laboratory rats. *Pharmacologyonline* 2009;**2**: 557-569.

[17] Santhosh Kumar B, Tiwari SK, Saikant R, et al. Antibacterial and ulcer healing effects of organoselenium compounds in naproxen induced and *H. pylori* infected Wistar rat model. *J Trace Elem Med Bio* 2010;**24**:263-270.

[18] Kobayashi D, Kondo K, Uehara N, Otokozawa S, Tsuji N, Yagihashi A. Endogenous reactive oxygen species is an important endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. *Phytomed* 2008;**15**:391-399.

[19] Lawler JM, Kwak HB, Kim JH, Suk MH. Exercise training inducibility of MnSOD protein expression and activity is retained while reducing prooxidant signaling in the heart of senescent rats. *Am J Physiol Regul Integr Comp Physio*l 2009;**296**(5):1496-1502.

[20] Kandhare AD, Raygude KS, Ghosh P, Ghule AE, Gosavi TP, Badole SL, Bodhankar SL. Effect of hydroalcoholic extract of Hibiscus rosa sinensis Linn. leaves in experimental colitis in rats. *Asian Pac J Trop Biomed* 2012a;**5**:337-344.

[21] Algood HM, Gallo-Romero J, Wilson KT, Peek RM Jr., Cover TL. Host response to *H. pylori* infection before initiation of the adaptive immune response. *FEMS Immunol Med Microbiol* 2007;**51**:577-586.

[22] Raygude KS, Kandhare AD, Ghosh P, Gosavi TP, Bodhankar SL. Consumption of Alcohol and *H. pylori* infection: A Cumulative Meta-Analysis of Literature. *Asian J Biochem Pharm Res* 2011;**3**(1):338-345.

[23] Ghosh P, Kandhare AD, Raygude KS, Gauba D, Gosavi TP, Bodhankar SL. Cigarette smoking and *H. pylori* infection: A meta-analysis of literature. *Der Pharmacia Lettre* 2012b;**4**(1):128-134.

[24] Ghoshal UC, Chaturvedi R, Correa P. The enigma of *H. pylori* and gastric cancer. *Indian J Gastroenterol* 2010;**29**(3):95-100.

[25] Ghosh P, Bodhankar SL. Determination of risk factors and transmission pathways of *Helicobacter pylori* in asymptomatic subjects in Western India using polymerase chain reaction. *Asian Pac J Trop Dis* 2012a;**2**(1):12-17.

[26] Sahin E, Gumuslu S. Immobilization stress in rat tissues: Alterations in protein oxidation, lipid peroxidation and antioxidant defense system. *Comp Biochem Physiol C Toxicol Pharmacol* 2007;**144**:342-347.

[27] Patil MVK, Kandhare AD, Bhise SD. Anti-arthritic and anti-inflammatory activity of Xanthium srtumarium L. ethanolic extract in Freund's complete adjuvant induced arthritis. *Biomed Aging Pathol* 2012;**2**(1):6-15.

[28] Tariq M, Khan HA, Elfaki I, et al. Gastric antisecretory and antiulcer effects of simvastatin in rats. *J Gastroenterol Hepatol* 2007;**22**:2316-2323.

[29] Bayir Y, Odabasoglu F, Cakir A, et al. The inhibition of gastric mucosal lesion, oxidative stress and neutrophil-infiltration in rats by the lichen constituent diffractaic acid. *Phytomed* 2006;**13**:584-590.

[30] Takimoto H, Nakamura T, Takeuchi M, Sumi Y, Tanaka T, Nomoto K, et al. Age-associated increase in number of CD4+CD8+ intestinal intraepitelial lymphocytes in rats. *Eur J Immunol* 1992;**22**:159-64.

[31] Peterson RA, Hoepf T, Eaton KA. Adoptive transfer of splenocytes in SCID mice implicates CD4+ T cells in apoptosis and epithelial proliferation associated with *H. pylori* induced gastritis. *Comp Med* 2003;**53**:498-509.

[32] Ding SZ, Lam SK, Yuen ST, et al. Prostaglandin, tumor necrosis factor alpha and neutrophils: causative relationship in indomethacin induced stomach injuries. *Eur J Pharmacol* 1998;**348**(2-3):257-263.

[33] Kandhare AD, Raygude KS, Ghosh P, Ghule AE, Bodhankar SL. Therapeutic role of curcumin in prevention of biochemical and behavioral aberration induced by alcoholic neuropathy in laboratory animals. *Neurosci Lett* 2012b;**511**:18-22.

[34] Kandhare AD, Raygude KS, Ghosh P, Ghule AE, Bodhankar SL. Neuroprotective effect of naringin by modulation of endogenous biomarkers in streptozotocin induced painful diabetic neuropathy. *Fitoterapia* 2012c;**83**:650-9.

[35] Jones NL, Day AS, Jennings HA, Sherman PM. *H. pylori* induces gastric epithelial cell apoptosis in association with increased Fas receptor expression. *Infect Immun* 1999;**67**:4237-4242.

[36] Ghosh P, Pandit VA, Karandikar Y, Diwan AG, Bodhankar SL. A cross sectional study to determine risk factors associated with prevalence of *H. pylori* infection in salivary samples of acid peptic disease patients in western India. *Pharmacologia* 2012c;**3**(11):554-564.

[37] Romo-Gonzalez C, Salama NR, Burgeno-Ferreira J, et al. Differences in genome content among *H. pylori* isolates from patients with gastritis, duodenal ulcer or gastric cancer reveal novel disease-associated genes. *Infect Immun* 2009;**77**(5):2201-2211.

[38] Ghosh P, Bodhankar SL. Association of smoking, alcohol and NSAIDs use with expression of cagA and cagT genes of *Helicobacter pylori* in salivary samples of asymptomatic subjects. *Asian Pac J Trop Biomed* 2012b;**2**(6):479-484.

[39] Ghosh P, Bodhankar SL. A Cross Sectional Study to Determine Association of the Socio Demographic Risk Factors with Prevalence and Virulence of *H. pylori* Infection in Salivary Samples of Asymptomatic Subjects by Application of Polymerase Chain Reaction Technique. *Pharmacologia* 2012c;**3**:481-489.

[40] Camerlink I, Ellinger L, Bakker EJ, Lantinga EA. Homeopathy as replacement to antibiotics in the case of Escherichia coli diarrhoea in neonatal piglets. *Homeopathy* 2010;**99**(1):57-62.

[41] Mathie RT. Clinical outcomes research: contributions to the evidence base for homeopathy. *Homeopathy* 2003;**92**:56-57.