May 2012

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Journal of Ethnopharmacology 141 (2012) 403–410

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Anti-inflammatory and cytoprotective effects of selected Pakistani medicinal plants in *Helicobacter pylori*-infected gastric epithelial cells

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ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form 1 March 2012
Accepted 4 March 2012
Available online 13 March 2012

Keywords:
Helicobacter pylori
Pakistani medicinal plants
Interleukin-8
Reactive oxygen species
Cinnamomum cassia

ABSTRACT

Ethnopharmacological relevance: Helicobacter pylori infection is associated with gastritis, peptic ulcer, and gastric cancer. Due to its high global prevalence and uprising resistance to available antibiotics, efforts are now directed to identify alternative source to treat and prevent associated disorders. In the present study, effect of selected indigenous medicinal plants of Pakistan was evaluated on the secretion of interleukin-8 (IL-8) and generation of reactive oxygen species (ROS) in a bid to rationalize their medicinal use and to examine the anti-inflammatory and cytoprotective effects in gastric epithelial cells.

Materials and methods: AGS cells and clinically isolated *Helicobacter pylori* strain (193C) were employed for co-culture experiments. Anti-*Helicobacter pylori* activity and cytotoxic effects of the selected plants were determined by serial dilution method and DNA fragmentation assay respectively. ELISA and flow cytometry were performed to evaluate the effect on IL-8 secretion and ROS generation in *Helicobacter pylori*-infected cells.

Results: At 100 μg/ml, extracts of *Alpinia galangal*, *Cinnamomum cassia*, *Cinnamomum tamala*, *Mentha arvensis*, *Myrtus communis*, *Oligochaeta ramose*, *Polygonum bistorta*, *Rosa damascena*, *Ruta graveolens*, *Syzygium aromaticum*, *Tamarix dioica*, and *Terminalia chebula* exhibited strong inhibitory activity against IL-8 secretion. Of these, four extracts of *Cinnamomum cassia*, *Myrtus communis*, *Syzygium aromaticum*, and *Terminalia chebula* markedly inhibited IL-8 secretion at both 50 and 100 μg/ml. *Cinnamomum cassia* was further assessed at different concentrations against *Helicobacter pylori* and TNF-α stimulated IL-8 secretion, which displayed significant suppression of IL-8 in a concentration-dependent-manner. Among the plants examined against ROS generation, *Achillea millefolium*, *Berberis aristata*, *Coriandrum sativum*, *Foeniculum vulgare*, *Matricaria chamomilla* and *Prunus domestica* demonstrated significant suppression of ROS from *Helicobacter pylori*-infected cells ($p < 0.01$).

Conclusion: Results of the study revealed anti-inflammatory and cytoprotective effects of selected medicinal plants which could partially validate the traditional use of these plants in GI disorders particularly associated with *Helicobacter pylori*. Furthermore, results obtained may lead to possible future candidates of chemoprevention against peptic ulcer or gastric cancer.

1. Introduction

Helicobacter pylori, a major cause of bacterial gastrointestinal infections, is prevalent in more than 50% of the world population with up to 100% infection in the residents of developing countries (Marshal and Gilman, 1999). Socio-economic status including personal hygiene and age are the two predictors of risk of infection, with higher rate of infection found in all age groups of lower socioeconomic status (Anon., 2000). Conditions like atrophic gastritis, peptic ulcer, and gastric adenocarcinoma are usually associated with *Helicobacter pylori* infection (Covacci et al., 1999). Infact, *Helicobacter pylori* has been designated as a class I carcinogen by WHO and its eradication has been reported to be beneficial in preventing gastric disorders especially ulcer and cancer (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). Eradication of *Helicobacter pylori* with different combinations of therapeutic agents such as antibiotics, proton pump inhibitors and

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doi:10.1016/j.jep.2012.03.001

H_2-blockers has been employed (Hentschel et al., 1993). However, emerging resistance to antibiotics, especially clarithromycin and metronidazole limits their use in the treatment of infections in both developed and developing countries (Sullivan et al., 1990; Lind et al., 1999; Sherif et al., 2004). In addition, adverse effects of the chemical drugs and declining patient compliance have created an exclusive need to dig out alternative approaches of eradicating and chemopreventing infections or their associated pathological disorders (O'Gara et al., 2000).

Helicobacter pylori interaction with gastric mucosa is followed by several key events linked to the pathogenesis, (1) an inflammatory response with release of various cytokines and reactive oxygen species (ROS), (2) glandular atrophy after long-term infection and interaction with host responses, and (3) cellular proliferative changes like dysplasia and metaplasia (Holian et al., 2002). Among other inflammatory cytokines, interleukin-8 (IL-8) is one of the most critical cytokines inducing *Helicobacter pylori*-induced host inflammatory response, which is also a potent neutrophil chemoattractant (Crabtree, 1996; Foryst-Ludwig et al., 2004). The aberrant secretion of IL-8 from *Helicobacter pylori*-infected gastric epithelial cells may lead to free-radical generation and release of proteolytic enzymes from activated neutrophils ultimately affecting mucosal integrity (Yoshida et al., 1993). Oxidative stress is another hallmark induced by *Helicobacter pylori* infection and amplified levels of reactive oxygen species (ROS) were detected in gastric epithelial cells which could lead to altered epithelial proliferation and oxidative DNA damage (Ding et al., 2007). Among the ROS, superoxide anion has been considered as an important factor in triggering gastric mucosal cell responses against *Helicobacter pylori* (Yoshida et al., 1993; Teshima et al., 1998). Hence, an agent that could modulate these key events might propose an effective strategy to prevent *Helicobacter pylori*-induced pathological disorders. Previous studies, including from our group, have documented such candidates that could be useful in attenuating *Helicobacter pylori*-related inflammatory processes like curcumin and resveratrol (Foryst-Ludwig et al., 2004; Zaidi et al., 2009b,c). Furthermore, gastroprotective effects of *Phyllanthus urinaria* and red ginseng extracts in *Helicobacter pylori*-infected cells have been reported earlier (Park et al., 2007; Lai et al., 2008).

Recently, we have reported for the first time, bactericidal activity of fifty indigenous Pakistani medicinal plants against *Helicobacter pylori* which are employed to treat gastrointestinal disorders like dyspepsia, ulcer, and gastritis (Zaidi et al., 2009a). However, several plants either exhibited no or weak anti-*Helicobacter pylori* activity in the above mentioned study. Since, these medicinal plants are extensively prescribed for gastrointestinal (GI) disorders in Unani (Greco-Arab) system of medicine, it was hypothesized that these herbs might possess gastroprotective or anti-inflammatory activity against *Helicobacter pylori*-induced pathological sequel. Hence, in the present study we examined whether selected Pakistani medicinal plants could modulate IL-8 secretion and ROS generation from the infected cells, which will simultaneously support in justifying their medicinal use in the above mentioned diseases.

2. Materials and methods

2.1. Selected Pakistani medicinal plants and extract preparation

Totally twenty four medicinal plants were employed in this study which is given in Table 1 with families, abbreviations, and traditional uses. These medicinal plants were selected on the basis of their (minimum bactericidal concentration) MBC values ($\geq$500 μg/ml) against *Helicobacter pylori* reported earlier (Zaidi et al., 2009a). All the plants were purchased from a local market of Karachi, Pakistan, authenticated by Dr Iqbal Ahzar, Department of Pharmacognosy, University of Karachi, Karachi, Pakistan, and authentic voucher specimens have been deposited in the Museum of Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Toyama, Japan (Zaidi et al., 2009a).

As described previously, the powdered plant material (5–50 g) was soaked twice with 50–100 ml of aqueous ethanol (30:70) for 48 h at room temperature (Zaidi et al., 2009a). Solvent was evaporated under reduced pressure and each extract was dissolved at 100 mg/ml with dimethyl sulfoxide (DMSO) and the final concentration of DMSO was <0.1% in the cell culture medium which has no effect on any of the experiment performed in this study.

Table 1
List of selected Pakistani medicinal plants used in this study.

Plant name	Abbreviation	Family	Part used	Therapeutic applications
Achillea millefolium L.	AML	Asteraceae	Aerial part	Carminative, inflammations
Alpinia galangal Willd.	AGW	Zingiberaceae	Rhizome	Expectorant, carminative
Amomum subulatum Roxb.	ASR	Zingiberaceae	Fruit	Carminative, diarrhea, flatulence
Berberis aristata DC.	BADC	Berberidaceae	Root	Fevers, inflammation, digestive
Brassica nigra (L.) Koch.	BNK	Cruciferae	Seed	Meningitis, paralysis, carminative
Cinnamomum cassia Blume	CCB	Lauraceae	Bark	Dyspepsia, flatulence, vomiting
Cinnamomum tamala (Ham.) Nees	CTN	Lauraceae	Leaf	Indigestion, stomach ache, cardiac tonic
Citrus medica L.	CML	Rutaceae	Fruit	Dyspepsia, flatulence, vomiting
Coriandrum sativum L.	CSL	Apiaceae	Seed	Dyspepsia, flatulence, brain tonic
Cuscuta reflexa Roxb.	CRR	Convolvulaceae	Seed	Ulcers, liver and spleen inflammations
Foeniculum vulgare Miller	FVM	Apiaceae	Seed	Indigestion, gastritis, flatulence
Matricaria chamomilla L.	MACL	Asteraceae	Flower	Inflammation, jaundice, epilepsy
Melilotus officinalis (L.) Desr.	MOD	Papilionaceae	Fruit	Liver, stomach and spleen inflammation
Mentha arvensis L.	MAL	Lamiaceae	Leaf	Stomach ache, fevers, dysmennorhea
Myrtus communis L.	MYCL	Myrtaceae	Fruit	Diarrhea, indigestion, palpitation
Oligochaeta ramosa (Roxb.) Wagenitz	ORW	Asteraceae	Aerial part	Fevers, cough, diarrhea
Polygonum bistorta L.	PBL	Polygonaceae	Root	Dysentry, diarrhea, hematuria
Prunus domestica L.	PDL	Rosaceae	Fruit	Dyspepsia, nausea, vomiting
Rosa damascene Miller	RDM	Rosaceae	Flower	Stomach and intestine tonic, inflammation
Ruta graveolens L.	RGL	Rutaceae	Leaf	Indigestion, flatulence, rheumatism
Syzygium aromaticum L.	SAL	Myrtaceae	Flower bud	Toothache, inflammation
Tamarix dioica Roxb.	TDR	Tamaricaceae	Aerial part	Diarrhea, dysentery, liver inflammation
Terminalia chebula Retz.	TCR	Combretaceae	Fruit	Stomach and brain tonic, diarrhea
Trigonella foenum-graecum L.	TFGL	Papilionaceae	Seed	Carminative, resolvent

2.2. Helicobacter pylori and cell culture conditions

Helicobacter pylori clinical isolate 193C was cultured in Brucella broth (BBL™, BD, Franklin Lakes, USA) medium supplemented with 10% fetal bovine serum (Marchildon et al., 2003). The bacteria was subcultured before co-culture experiments in 10 ml brucella broth liquid culture for 24–48 h under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2 at 37 °C; Sanyo-Multigas Incubator, SANYO Electric Co., Ltd., Tokyo, Japan) on a gyratory shaker at 160 rpm with 100% humidity. The bacterial concentration was estimated by using the formula as an absorbance of 0.1 = 10^8 bacteria/ml (Meyer et al., 2000).

Human gastric cancer cell line AGS (American Type Culture Collection) was grown in RPMI 1640 (Wako, Osaka, Japan) containing 2 mmol/L L-glutamine supplemented with antibiotics and 10% FBS at 37 °C in 5% CO_2. Cells were routinely passaged every 3 days. Cells were seeded into 6 cm culture dish and grown for overnight followed by washing with phosphate-buffered saline (PBS) three times. The medium RPMI 1640, without antibiotics and FBS, was added and the cells were pretreated with plant extracts for 30 min before addition of *Helicobacter pylori*.

2.3. Effect of selected extracts on Helicobacter pylori viability

The anti-*Helicobacter pylori* effects of extracts were evaluated by the method described earlier (Zaidi et al., 2009b) with minor modifications. Briefly, *Helicobacter pylori* was either left untreated or treated with plant extracts at the concentration of 100 μg/ml for 2 h at 37 °C. Bacteria were then serially diluted and inoculated onto commercial selective Pylori agar plates (Kyokuto; Tokyo, Japan) under microaerophilic conditions as presented above. After the incubation of 2–3 days, the bacterial colonies were counted and the colony forming units (CFUs) were calculated. Data is expressed as percent of survival. The results are representative of at least three independent experiments.

2.4. Determination of DNA fragmentation in AGS cells

Cytotoxic effect of the selected extracts was determined by quantitative DNA fragmentation assay as described previously (Sellins and Cohen, 1987). Briefly, cells were either pretreated with or without extracts for 4 h and were lysed in a lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) followed by centrifugation at 13,000 × *g* for 10 min. Each DNA sample in the supernatant and the resulting pellet were then precipitated in 12.5% trichloro acetic acid (TCA) at 4 °C, and quantified using a diphenylamine reagent after hydrolysis in 5% TCA at 90 °C for 20 min. The percentage of fragmented DNA for each sample was calculated as the amount of DNA in the supernatant divided by the total DNA for that sample (supernatant plus pellet).

2.5. Enzyme-linked immunosorbent assay (ELISA) for IL-8

AGS cells were co-cultured with *Helicobacter pylori* at multiplicity of infection (MOI) of 50:1 or treated with TNF-α (10 ng/ml) for 4 h in the presence or absence of selected plant extracts. IL-8 secretion in the supernatant from the treated cells was analyzed by using ELISA (R & D System). After 4 h of culture, the supernatant medium was collected and IL-8 content was determined according to the manufacturer's instructions. A standard curve of recombinant IL-8 (R & D) was employed to determine IL-8 concentrations which were expressed in pg/ml.

2.6. Assessment of intracellular reactive oxygen species

AGS cells (1×10^6) were seeded in culture dishes for overnight, washed with PBS three times and pre-incubated with or without plant extracts in antibiotic/FBS free medium followed by addition of *Helicobacter pylori* at MOI of 300:1 for 1 h. Trypsin/EDTA was used to collect the cells and intracellular ROS was measured after staining the cells with 2 μM hydroethidine (HE) (Molecular Probes, Eugene, OR) to detect intracellular superoxide (O_2^-). HE is oxidized within the cell and fluoresces when it intercalates into DNA (Cui et al., 2004). The fluorescence emission was analyzed by flow cytometry.

2.7. Statistical analysis

The results are expressed as the mean ± standard deviation. Statistical significance ($p < 0.01$, $p < 0.05$) was evaluated by one-way ANOVA followed by Bonferroni *post hoc* test for IL-8 and ROS.

3. Results

3.1. Effect of selected plants on Helicobacter pylori and AGS cells

To evaluate the effect of selected medicinal plants in *Helicobacter pylori*-infected cells, we first analyzed the viability of *Helicobacter pylori* in the presence of these extracts at 100 μg/ml which was five times lower than that of their MBC values reported earlier (Zaidi et al., 2009a). The results revealed no significant effect of any of the extract on *Helicobacter pylori* viability at this concentration. The CFUs recovered from the liquid cultures containing extracts 100 μg/ml (AML, 96% CFU; AGW, 98% CFU; ASR, 99% CFU; BADC, 93% CFU; BNK, 96% CFU; CCB, 93% CFU; CTN, 97% CFU; CML, 99% CFU; CSL, 95% CFU; CRR, 100% CFU; FVM, 92% CFU; MACL, 94% CFU; MOD, 93% CFU; MAL, 98% CFU; MYCL, 99% CFU; ORW, 95% CFU; PBL, 96% CFU; PDL, 94% CFU; RDM, 90% CFU; RGL, 99% CFU; SAL, 97% CFU; TDR, 98% CFU; TCR, 93% CFU and TFGL, 94% CFU) were comparable with that of control (100% CFU).

To examine cytotoxic effect of selected extracts against AGS cells, DNA fragmentation assay was employed. DNA fragmentation, a hallmark of apoptosis, was measured at the concentration of 100 μg/ml at 4 h of incubation with extracts. The results revealed no significant induction of DNA fragmentation by any of the evaluated extracts (AML, 4.4 ± 1.0%; AGW, 6.9 ± 1.1%; ASR, 5.3 ± 0.7%; BADC, 5.8 ± 0.3%; BNK, 5.6 ± 0.9%; CCB, 5.8 ± 1.1%; CTN, 5.4 ± 1.1%; CML, 5.1 ± 0.6%; CSL, 5.3 ± 0.8%; CRR, 4.9 ± 0.7%; FVM, 5.8 ± 0.7%; MACL, 6.5 ± 1.2%; MOD, 5.8 ± 0.8%; MAL, 5.4 ± 0.8%; MYCL, 5.9 ± 0.9%; ORW, 6.3 ± 1.2%; PBL, 7.1 ± 1.1%; PDL, 5.3 ± 0.8%; RDM, 7.4 ± 0.9%; RGL, 5.8 ± 0.7%; SAL, 5.4 ± 0.9%; TDR, 5.5 ± 1.2%; TCR, 6.1 ± 1.4% and TFGL, 6.5 ± 1.2%) when compared with untreated cells (4.5 ± 0.8%). These results suggested that the effect of these herbs in *Helicobacter pylori*/cell co-culture system will not be due to either alteration in *Helicobacter pylori* viability or toxicity to the cells.

3.2. Pre-treatment with extracts inhibited Helicobacter pylori-induced IL-8 secretion

Among the inflammatory mediators, IL-8 plays a crucial role in initiating inflammatory response by chemoattracting and activating neutrophils to the *Helicobacter pylori*-infected gastric mucosa (Crabtree, 1996). To explore the pharmacological basis for the medicinal use of these selected plants in gastrointestinal disorders, we examined their effect on *Helicobacter pylori*-stimulated IL-8 secretion in AGS cells. Supernatant from infected cells either left untreated or pretreated with extracts (50–100 μg/ml) was collected and IL-8 specific ELISAs were performed. The results revealed that addition of *Helicobacter pylori* in AGS cells resulted

in a marked elevation of IL-8 content compared to the uninfected cells (Fig. 1). Depending on the inhibitory activity on IL-8 secretion, data was divided into three categories as mild inhibition (IL-8 secretion >2000 pg/ml), moderate inhibition (IL-8 secretion between 1000 and 2000 pg/ml) and strong inhibition (IL-8 secretion <1000 pg/ml). At 50 μg/ml, twelve extracts (AML, ASR, BADC, BNK, CML, CSL, CRR, FVM, MACL, MOD, PDL, and TFGL) exhibited mild inhibitory activity, seven extracts (AGW, CTN, MAL, ORW, PBL, RDM, and RGL) demonstrated moderate inhibitory activity and four extracts (CCB, MYCL, SAL, and TCR) showed strong inhibitory activity on IL-8 secretion in *Helicobacter pylori*-infected cells. At 100 μg/ml, seven extracts (ASR, BNK, CML, CSL, CRR, MACL, and PDL) exhibited mild inhibitory activity, five extracts (AML, BADC, FVM, MOD, and TFGL) demonstrated moderate inhibitory activity and twelve extracts (AGW, CCB, CTN, MAL, MYCL, ORW, PBL, RDM, RGL, SAL, TDR, and TCR) showed strong inhibitory activity on IL-8 secretion in *Helicobacter pylori*-infected cells. Among all the evaluated herbs, four extracts namely *Cinnamomum cassia*, *Myrtus communis*, *Syzygium aromaticum*, and *Terminalia chebula* displayed strong inhibitory activity at both 50 and 100 μg/ml which reflects their potential for further evaluation as a future candidate to prevent *Helicobacter pylori*-induced inflammatory processes.

3.3. Effect of Cinnamomum cassia on Helicobacter pylori- and TNF-α-induced IL-8 secretion in a dose-dependent manner

Of the herbs employed, *Cinnamomum cassia* Blume (CCB) was found to be the strongest inhibitor of IL-8 secretion from *Helicobacter pylori*-infected epithelial cells at both 50 and 100 μg/ml (Fig. 1). We further examined dose-dependent effects on IL-8 secretion by them. The results revealed that CCB significantly ($p < 0.01$) suppressed IL-8 secretion up to the concentration of 3.12 μg/ml in the infected cells while there was no effect on IL-8 secretion in CCB treated cells only (Fig. 2). At the concentration of 50 and 100 μg/ml, CCB almost completely inhibited the secretion of IL-8 from *Helicobacter pylori*-infected epithelial cells which is comparable to positive control, curcumin (40 μM), a well-known anti-inflammatory agent from natural source (Foryst-Ludwig et al., 2004).

To analyze whether CCB can modulate non-infectious or exogenous inflammatory stimulus, AGS cells were treated with TNF-α (a potent proinflammatory cytokine) and the effect of CCB was determined on IL-8 secretion. In TNF-α stimulated cells, IL-8 content was drastically increased even higher than that of *Helicobacter pylori*-infected cells (Fig. 3). No prominent suppression of IL-8 secretion was seen at 3.12 μg/ml of CCB in TNF-α-stimulated cells while significant ($p < 0.01$) inhibition was observed at 12.5–100 μg/ml.

3.4. Suppression of Helicobacter pylori-generated ROS by plant extracts

As mentioned above, *Helicobacter pylori* infection drastically creates oxidative stress which leads to DNA damage and tissue injury. We next investigated the effect of selected herbs on *Helicobacter pylori*-induced ROS generation in AGS cells. Twelve herbs (AML, ASR, BADC, BNK, CML, CSL, CRR, FVM, MACL, MOD, PDL, and TFGL) with mild IL-8 inhibitory activity (Fig. 1) were selected for this assay in order to assess their role in *Helicobacter pylori*-associated inflammatory cascade. Addition of *Helicobacter pylori* in AGS cells prominently enhanced the generation of ROS (Fig. 4). Of twelve extracts evaluated against ROS generation, six plants namely *Achillea millefolium* (AML), *Berberis aristata* (BADC), *Coriandrum sativum* (CSL), *Foeniculum vulgare* (FVM), *Matricaria chamomilla* (MACL) and *Prunus domestica* (PDL) displayed significant ($p < 0.01$) suppression of ROS from *Helicobacter pylori*-infected cells with BADC possessing highest efficacy.

4. Discussion

The confined progress achieved by cancer therapy in the last three decades has diverted the interest of researchers to cancer chemoprevention (Parkin et al., 2005; Lopez-Lazaro, 2008). Cancer chemoprevention (the use of chemicals to prevent, stop, or reverse

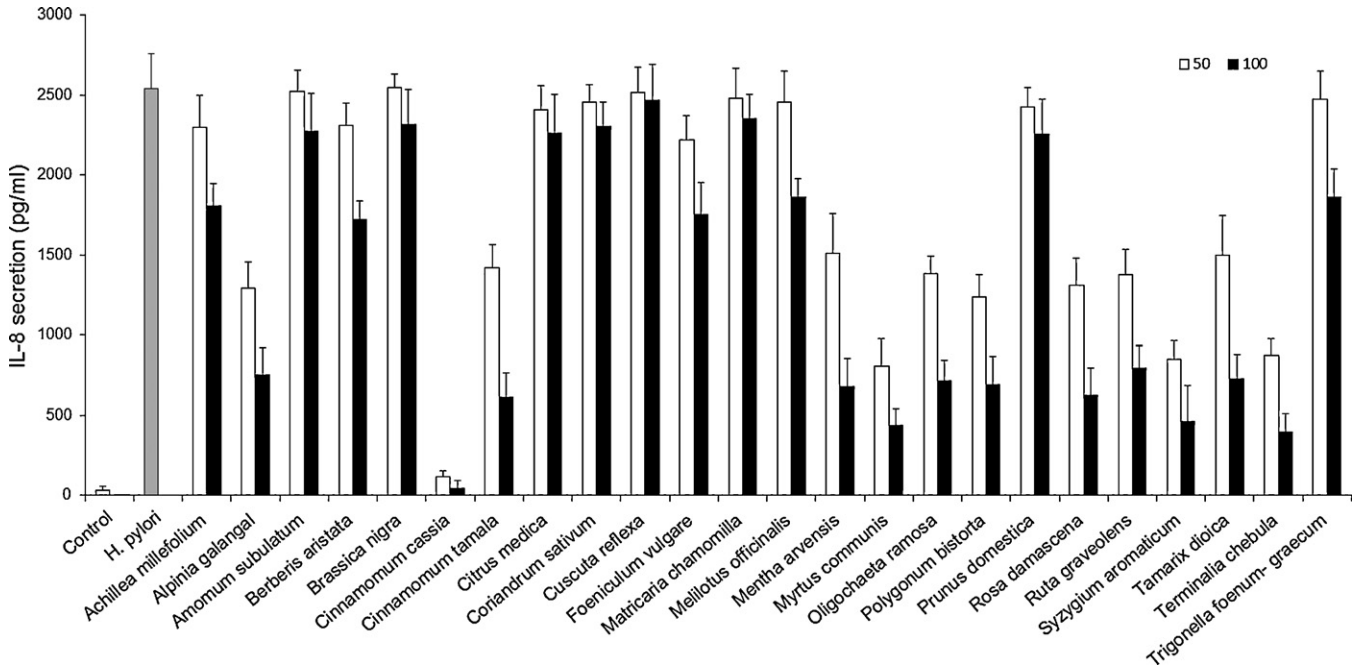

Fig. 1. Effect of selected medicinal plants on IL-8 secretion in *Helicobacter pylori*-infected cells. Cells were pretreated with different concentrations of plant extracts (50 and 100 μg/ml) and supernatants from *Helicobacter pylori*-co-cultured cells were analyzed for IL-8 content. Each value represents the average of duplicate of three independent experiments.

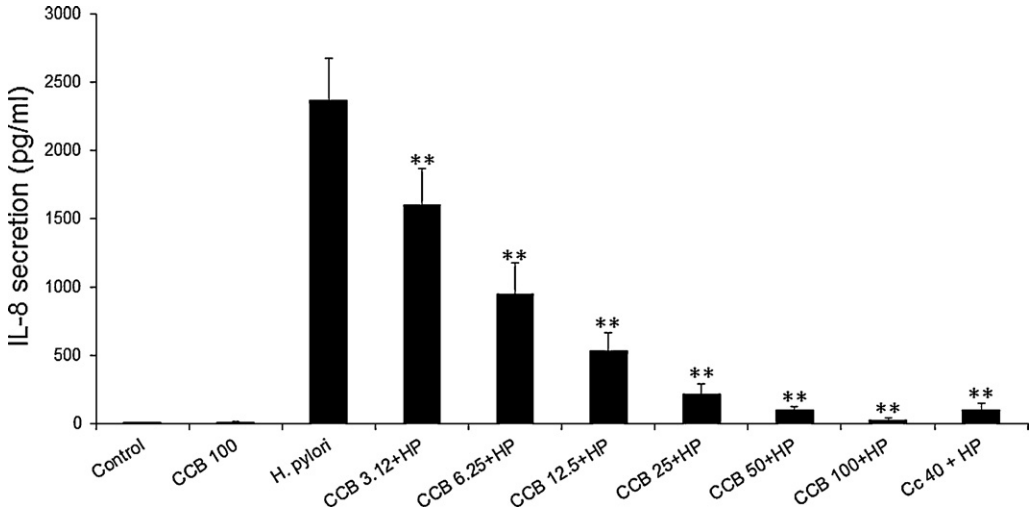

Fig. 2. Dose-dependent inhibitory effect of *Cinnamomum cassia* on *Helicobacter pylori*-induced IL-8 production from AGS cells. Cells were pretreated with various concentrations of *Cinnamomum cassia* (CCB: 3.12–100 μg/ml) and curcumin (Cc: 40 μM) and supernatants from *Helicobacter pylori*-co-cultured cells were analyzed for IL-8 content. Each value represents the mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{*}p < 0.05$ (compared to *Helicobacter pylori*-infected cells). HP stands for *Helicobacter pylori*. The experiments were conducted three times independently.

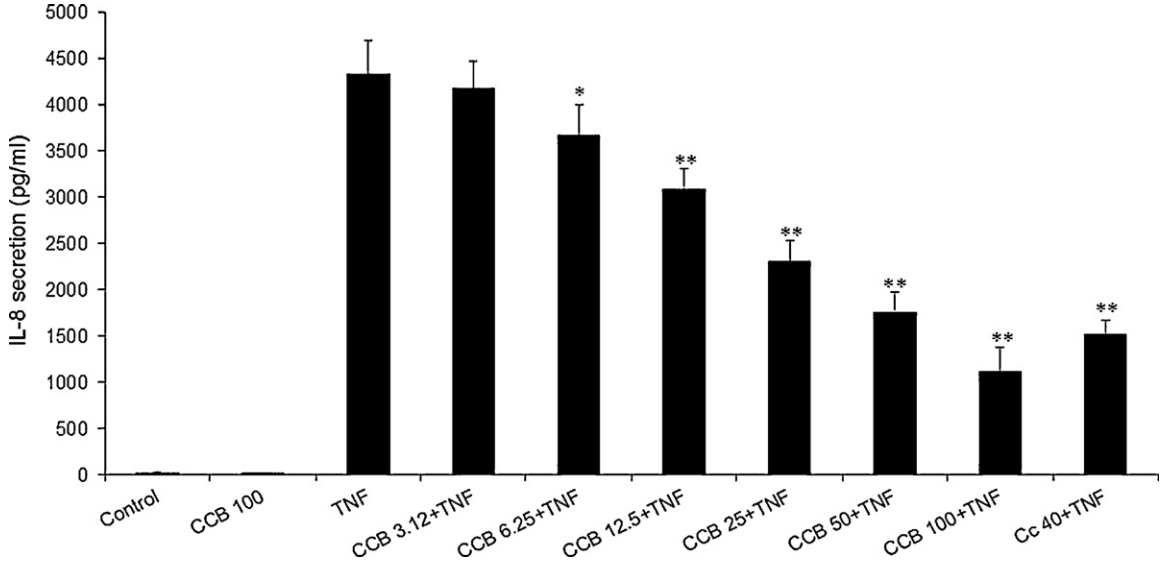

Fig. 3. Suppression of TNF-α-induced IL-8 secretion by *Cinnamomum cassia* from AGS cells. Cells were pretreated with various concentrations of *Cinnamomum cassia* (CCB: 3.12–100 μg/ml) and curcumin (Cc: 40 μM) and supernatants from TNF-α stimulated cells were analyzed for IL-8 content. Each value represents the mean ± SD ($n = 3$). $^{**}p < 0.01$, $^{*}p < 0.05$ (compared to TNF-α treated cells). TNF stands for tumor necrosis factor-alpha. The experiments were conducted three times independently.

the process of carcinogenesis) is becoming an essential approach to controlling cancer. In addition, the process of carcinogenesis is a chronic process and can take several decades to complete. Therefore, it is logical to prevent cancer rather to wait until the disease has reached its final stages, where it becomes indispensable to use more toxic chemicals (chemotherapy) with severe side effects (Lopez-Lazaro, 2008). Similar is the case with *Helicobacter pylori*-associated gastric cancer which becomes symptomatic at late ages and already has advanced to incurable disease at the time of presentation. Also it continues to stay among the top cancers causing death worldwide (Zhang and Fan, 2010). This might be attributed to high prevalence of organism, increasing antibiotic resistance, decreased patient compliance in taking antibiotics and high consumption of preserved, salty and smoked foods in daily life (Lind et al., 1999; Marshal and Gilman, 1999; Loh et al., 2007; Liu et al., 2009). Hence several studies have directed their efforts to explore new alternative strategies to prevent and control *Helicobacter pylori*-associated gastric cancer (Lee et al., 2008). Among the alternative approaches, phytomedicine has always played a leading role in identifying potential candidates both for chemotherapy and chemoprevention. Some studies have so far reported such candidates from phytomedicine like the red ginseng (at 1–100 μg/ml), *Phyllanthus urinaria* extract (at 250 μg/ml) and San-Huang-Xie-Xin-Tang (at 100 μg/ml) significantly suppressed *Helicobacter pylori*-induced inflammatory molecules like lipoxygenase and IL-8 in gastric epithelial cells (Park et al., 2007; Shih et al., 2007; Lai et al., 2008). However, still there is a surplus need to find new candidates which are easily available, cost effective, and relatively safe.

One plausible way to search such candidates is to explore the use of traditional medicinal plants in respective diseases on scientific basis. Hence, we had recently evaluated anti-*Helicobacter pylori* activity of fifty medicinal plants from Pakistan which are frequently prescribed for GI disorders in Unani system of medicine (Zaidi et al., 2009a). Although several of these herbs were found to possess bactericidal activity against *Helicobacter pylori*, still many

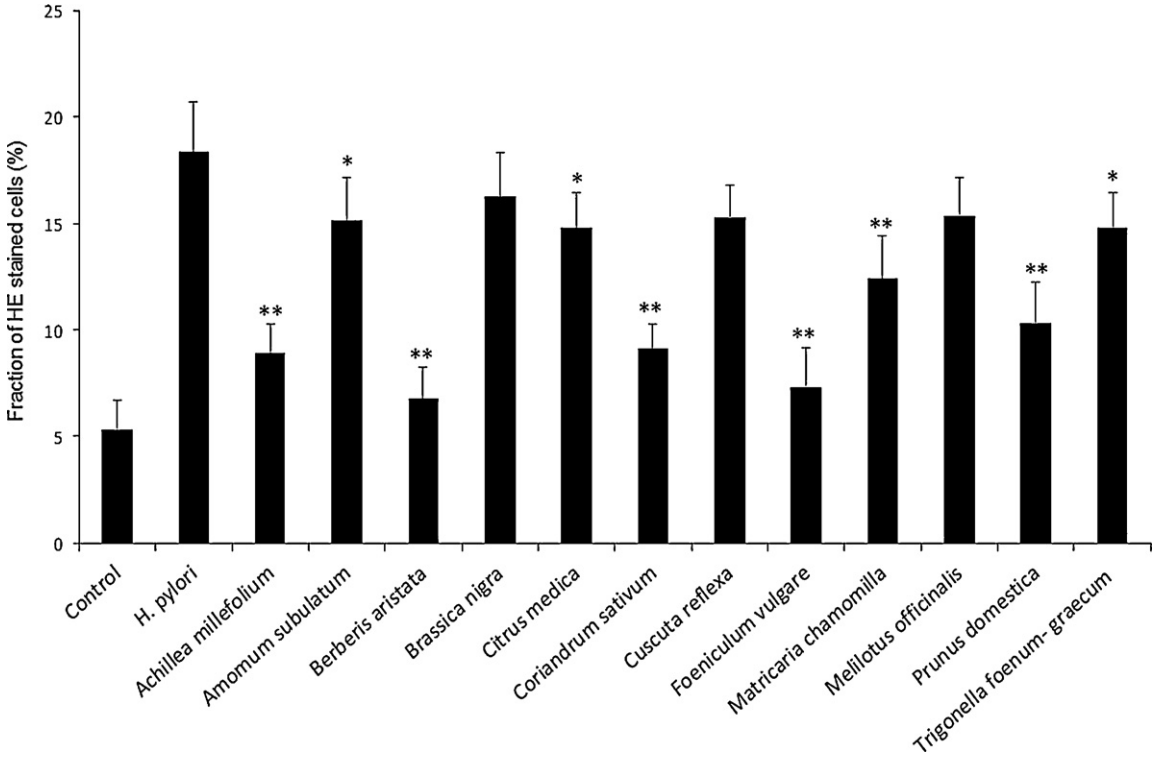

Fig. 4. Effect of selected extracts on *Helicobacter pylori*-induced ROS generation. Cells were preincubated with various extracts (100 μg/ml) for 1 h and then exposed to *Helicobacter pylori* for 1 h. ROS production was then determined by flow cytometry as described in Section 2. Data represents the mean values of three independent experiments. $^{**}p < 0.01$, $^{*}p < 0.05$ (compared to *Helicobacter pylori*-infected cells).

of them displayed weak or no bactericidal activity at 500 μg/ml. Since, these medicinal plants are frequently employed in gastrointestinal disorders, we hypothesized that they might have anti-inflammatory activity in *Helicobacter pylori*-infected gastric mucosa by some other mechanisms. The major hallmark by which *Helicobacter pylori* initiates inflammation in gastric mucosa is the production of inflammatory cytokines and oxidative stress which leads to gastritis in all infected individuals (Keates et al., 1997). Hence, it is highly desirable to search for strategies with agents having both anti-*Helicobacter pylori* and anti-inflammatory properties to kill *Helicobacter pylori* at one side and to reduce inflammation on the other. Phytomedicines can be ideal candidates for such strategies because of numerous phytochemicals with multiple pharmacological properties in single herb (Gilani and Rahman, 2005). Furthermore, in Unani system of medicine, usually compound formulations (more than one plant in formulation) are prescribed by traditional practitioners, partly with a concept of curing the disease from various angles. Keeping this in view, results from our previous study demonstrated anti-*Helicobacter pylori* activity of Pakistani medicinal plants (Zaidi et al., 2009a) and in this study we displayed anti-inflammatory activity of these plants in *Helicobacter pylori*-infected cells. To the best of our knowledge, all of the employed medicinal plants in this study are evaluated for the first time against *Helicobacter pylori*-induced IL-8 secretion, a major inflammatory cytokine involved in *Helicobacter pylori*-associated gastritis. More than half of the evaluated herbs markedly suppressed IL-8 secretion at either 50 or 100 μg/ml in *Helicobacter pylori*-infected cells which may partially signify their use in *Helicobacter pylori*-linked GI disorders. The strongest IL-8 inhibitory activity was demonstrated by four extracts namely *Cinnamomum cassia*, *Myrtus communis*, *Syzygium aromaticum*, and *Terminalia chebula* at both 50 and 100 μg/ml.

Cinnamomum cassia has been used both as traditional herbal medicine and spice for centuries (Gruenwald et al., 2010). The German Commission E and the European Scientific Cooperative on Phtyotherapy (ESCOP) approved two medicinal herbs of the genus *Cinnamomum* including *Cinnamomum cassia* (Blumenthal et al., 1998). In Unani system of medicine practiced in Pakistan, *Cinnamomum cassia* has been widely employed as carminative, antiseptic, expectorant, aphrodisiac and in gastrointestinal complaints such as dyspepsia, flatulence, diarrhea and vomiting (Usmanghani et al., 1997). Previous pharmacological studies on cinnamon have revealed its antioxidant, antimicrobial, anti-inflammatory, hypoglycemic, and antiviral activities (Friedman et al., 2002; Hong et al., 2002; Dragland et al., 2003; Gruenwald et al., 2010). Tabak et al. have reported strong anti-*Helicobacter pylori* activity of methylene chloride extract of *Cinnamomum cassia* while weak inhibitory activity of ethanolic extract (Tabak et al., 1999). This is in line with our results from pervious study demonstrating weak bactericidal activity of aqueous-ethanol extract of *Cinnamomum cassia* in seven clinical isolates (Zaidi et al., 2009a). Furthermore, a controlled clinical trial of cinnamon extract failed to eradicate *Helicobacter pylori* but the extract was well tolerated with minimal side effects (Nir et al., 2000). Our results revealed for the first time strong IL-8 inhibitory activity of *Cinnamomum cassia* not only in *Helicobacter pylori*-infected cells but also against TNF-α-stimulated IL-8 secretion revealing its potential therapeutic effect in both infectious and non-infectious inflammatory scenarios. A previous study reported cinnamaldehyde, a major active constituent of cinnamon, as effective inhibitor of nuclear factor kappa B (NF-κB) (Kim et al., 2007). As IL-8 expression is also regulated by NF-κB (Crabtree, 1996), the IL-8 inhibitory effect of CCB in *Helicobacter pylori*-infected cells might be due to cinnamaldehyde content. The exact molecular mechanism behind CCB inhibitory effect on IL-8 expression in *Helicobacter pylori*-infected cells is currently under investigation and will be reported elsewhere.

Another plant, *Myrtus communis* (MYCL), also strongly suppressed IL-8 secretion in a dose dependent manner. Fruit of MYCL is

traditionally used as antidiarrheal, antidysentric, carminative, and cardiac tonic (Usmanghani et al., 1997). Two recent reports have documented potent anti-inflammatory including interleukin-1β and tumor necrosis factor-α inhibitory activity by Myrtucommulone, an acylphloroglucinol from MYCL (Koeberle et al., 2009; Rossi et al., 2009). Therefore, the IL-8 inhibitory activity of MYCL in our study might be due to the presence of Myrtucommulone in the extract which clearly needs further investigation. The extract from flower buds of *Syzygium aromaticum* (SAL) has also shown strong inhibitory effect on *Helicobacter pylori*-induced IL-8 secretion. No other study has so far evaluated the effect of SAL on human IL-8 secretion, however, Lee et al. showed inhibitory effect of SAL on cytokine-induced neutrophil chemoattractant, a rat interleukin-8, in lipopolysaccharide-activated peritoneal macrophages (Lee et al., 1995). A recent study reported gastroprotective and antiulcerogenic activity of essential oil from *Syzygium aromaticum* (L.) Merr. Et Perry by stimulating the synthesis of mucus in animal model (Santin et al., 2010). These results together from our study open new dimensions for SAL to be a potential candidate against gastric disorders. The fruits of *Terminalia chebula* (TCR) have been extensively employed in various alternative systems of medicines like Unani, Ayurvedic, and Homeopathic medicines (Usmanghani et al., 1997; Sharma et al., 2011). It has traditionally been used as antidiarrheal, stomachic, antiulcer, and antiasthmatic (Usmanghani et al., 1997). Previous studies have demonstrated anticancer, antidiabetic, and antibacterial activities of TCR (Sabu and Kuttan, 2002; Saleem et al., 2002; Aqil et al., 2005). More recently, Sharma et al. reported potent antiulcerogenic activity of 70% hydroalcoholic extract of TCR fruit in aspirin, ethanol and cold resistant stress-induced ulcers in rats (Sharma et al., 2011). Another recent study showed potent NF-κB and IL-8 inhibitory activities of TCR in human lymphoblastic T cells (Das et al., 2011). These results support our findings of IL-8 suppression by TCR in *Helicobacter pylori*-infected cells and create a dire need to further explore the potential use of TCR in *Helicobacter pylori*-associated disorders like peptic ulcer and gastric cancer.

Next, we evaluated the antioxidant activity of those extracts which have no or mild IL-8 inhibitory activity (>2000 pg/ml) in order to clarify their use in GI disorders. As reported earlier *Helicobacter pylori* interaction with gastric epithelial cell lines was associated with a rapid increase in ROS compared to levels of ROS measured in uninfected control gastric epithelial cells and that ROS levels were increased in cells isolated from *Helicobacter pylori*-infected subjects compared to cells from uninfected individuals, pointing its importance in *Helicobacter pylori*-related pathogenesis (Ding et al., 2007). Majority of the evaluated herbs against ROS generation in this study have been reported earlier for their antioxidant activity in various experimental designs (Kayano et al., 2002; Singh and Kakkar, 2009; Cemek et al., 2010; Potrich et al., 2010; Samojlik et al., 2010; Mohamad et al., 2011) but not in *Helicobacter pylori*-infected cells. Results from our study revealed significant suppression of ROS by half of the evaluated herbs (*Achillea millefolium*, *Berberis aristata*, *Coriandrum sativum*, *Foeniculum vulgare*, *Matricaria chamomilla* and *Prunus domestica*) which may lend pharmacological evidence to the traditional use of these herbs in GI disorders specially associated with *Helicobacter pylori*. The most prominent activity was demonstrated by the root extract of *Berberis aristata* against *Helicobacter pylori*-generated ROS. An earlier study by Singh et al. reported the strong potential of *Berberis aristata* (root) extract in regulating glucose homeostasis through decreased gluconeogenesis and oxidative stress (Singh and Kakkar, 2009). Berberine, a major active constituent of berberis species has been reported to exhibit strong antioxidant properties (Sun et al., 2009) which might have imparted its effect on suppressing ROS by *Berberis aristata* extract in our study.

5. Conclusion

In summary, we reported herein anti-inflammatory and cytoprotective activities of selected Pakistani medicinal plants which are commonly prescribed for GI disorders. Selected herbs either inhibited IL-8 secretion or suppressed ROS generation in *Helicobacter pylori*-infected gastric epithelial cells validating the traditional concept of compound formulations or combination therapies while curing a disease. As both IL-8 and ROS are crucial in the inflammatory response generated by *Helicobacter pylori*, findings of our study may therefore provide molecular credence to the traditional therapeutic use of these herbs in GI disorders like gastritis or peptic ulcer. Our findings also revealed that these medicinal plants can act in multiple ways to overcome the pathogenesis of *Helicobacter pylori*-associated disorders. Furthermore, herbs like *Cinnamomum cassia* might become a trail as future chemopreventive candidates against gastric cancer, although, extensive *in vitro*, *in vivo* and clinical studies are direly required before counting them as chemopreventive agents.

Acknowledgements

We are thankful to Dr Kanwal Ahmed (Department of Biological and Biomedical Sciences, Faculty of Health Sciences, Aga Khan University, Karachi, Pakistan) and Dr Takashi Kondo (Department of Radiological Sciences, Faculty of Medicine, University of Toyama, Toyama, Japan) for their assistance in measuring ROS. This work was partially funded by a seed money grant from the FHS-Research Committee of the Aga Khan University, Karachi, Pakistan.

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