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Original Article

Investigation into Mechanism of Action of Antiulcer Activity of Polyherbal Formulation in Experimentally Induced Gastric Ulcers in Rats

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Abstract

Background and Objective: The polyherbal formulation (PHF) consisted of *Piper betel* leaves and *Acacia catechu*. The leaves of *Piper betel* has major active constituents as Hydroxychavicol and eugenol which are readily extracted by water. The bark of *Acacia catechu* mainly contains catechin, and epicatechin. The extract of *Piper betel* have been tested for Antiplatelet activity against various pro aggregating substances, like PAF, TXB2, PGD2, and ADP, collagen and arachidonic acid. Anti-cancer activity was evaluated by MTT (tetrazolium) assay using human breast cancer cell line (MCF-7). Anti-arthritic activity of Hydroxychavicol was evaluated at graded doses which significantly decreased the expression of IL-1β, PGE2, LTB4, and nitric oxide levels. Antioxidant and anti-inflammatory activities of Hydroxychavicol was assessed using hyaluronidase (HYA), xanthine oxidase (XOD) and lipoxygenase (LOX) inhibition assays. The extracts of *Piper betel* and *Acacia catechu* also exhibited significant inhibition in XOD and LOX assays. Antimutagenic activity was exhibited by dose dependent suppression of dimethylbenzanthracene induced mutagenesis in *S. typhimurium* strain TA98 with metabolic activation and also showed that Hydroxychavicol was more potent than eugenol in this respect. The anticoagulant activity of the compound was assayed by the activated partial thromboplastin time (APTT), prothrombin time (PT), and these assays were compared with the anticoagulant heparin which showed that it acted on the intrinsic as well as extrinsic pathways of the blood coagulation systems. It could be a potential therapeutic agent for prevention and treatment of Atherosclerosis and other cardiovascular diseases.

Literature search reveals the traditional use of *Piper betel* leaves as an aromatic, stimulant, carminative, astringent and antiseptic (Kirtikar and Basu, 1987 and Panda, 2004). The Acacia catechu has antiviral, anti-inflammatory, hepatoprotective and spasmolytic (Dahanukar et al, 2000). To confirm the traditional claim of antiulcer activity of *Piper betel* leaves and *Acacia catechu* scientifically, the study was undertaken to investigate the antiulcer and antisecretory activity of Poly Herbal Formulation (PHF) consisting of *Piper betel* leaves and *Acacia catechu* in experimentally induced gastric ulcer models in rats. The study supported that PHF has anti-ulcerogenic activity as it decreased ulcer index, volume of gastric acid secretion, total acidity, total acid output, pepsin output, TC/PR ratio, protein content and MDA content while increasing total carbohydrates as compared to disease control group. Thus, the antisecretory and cytoprotective activity of PHF are confirmed and further studies are warranted to investigate the efficacy of the same. *Methods:* Wistar albino rats of either sex were treated with PHF in dose of 100mg/kg and 200mg/kg for 7 days p.o. once a day in Aspirin + pylorus ligation induced gastric ulcer model and 200mg/kg in ethanol induced gastric ulcer model in rats. The physical parameters, acid secretory parameters, dissolved

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mucosubstances and MDA content were analyzed. *Results and Discussion:* When compared to disease control group, PHF treated group showed significant decrease in ulcer index ($p<0.001$), Volume of gastric acid secretion ($p<0.01$), Total Acidity ($p<0.01$), Total Acid Output ($p<0.01$), Pepsin output ($p<0.01$) and Protein content ($p<0.01$) while significant increase in Total carbohydrates ($p<0.01$) and total carbohydrate: Protein (TC:PR) ratio ($p<0.01$) in Aspirin + Pylorus ligation induced gastric ulcer model. Significant decrease in ulcer index ($p<0.01$) and MDA content ($p<0.001$) was seen in ethanol induced gastric ulcer model in rats. *Conclusion:* The present study demonstrated that PHF possess significant antiulcer activity against experimentally induced gastric ulcer in rats which might be attributed to its antioxidant activity of major phytochemical constituent Hydroxychavicol and catechin.

Keywords: Hydroxychavicol; Catechin; Acacia Catechu; *Piper Betel* Leaves; Anti Ulceractivity.

Introduction

Peptic ulceration is one of the common diseases affecting nearly 10% of the world population[1]. Ulcers are identified as ulcerative colitis and peptic ulcers depending on the location in the GI tract. Peptic ulcer disease (PUD) affects substantial number of people worldwide. It develops due to imbalance between the 'aggressive factors' i.e. infection by Helicobacter pylori, gastric acid, pepsins, NSAIDs, bile acids, hypoxia, ischemia, smoking and alcohol intake; and 'protective factors' i.e. bicarbonate, mucus layer, mucosal blood flow, PGs and growth factors at the gastric epithelial lining [2].

The existing treatments involve use of antacids (aluminum hydroxide, magnesium trisilicate), acid suppressive agents (Anti-secretory drugs) like proton pump H+/K+ ATPase inhibitors (omeprazole, lanzoprazole), histamine H2 receptor antagonist (cimetidine, ranitidine) and anticholinergic (M1) (pirenzepine), cytoprotective agents (sucralfate and prostaglandin analogues (misoprostol), antimicrobials for eradication of H. pylori (amoxicillin, clarithromycin) and Triple therapy (one week triple therapy consisting of a proton pump inhibitor such as Omeprazole and the antibiotics Clarithromycin and Amoxicillin) [2,3].

A widespread search has been launched to identify new natural anti-ulcer therapies to replace currently used drugs of doubtful efficacy and safety. There is a rich heritage of plants reputed in traditional medicine known to possess antiulcer properties. These can be a valuable source of new molecules which after chemical manipulation can provide new and improved anti-ulcer treatment [1].

Piper betel is a twining plant cultivated in hotter and damper parts of India. The major active protective phyto constituents are Hydroxychavicol and eugenol which are found in its aqueous extract [4]. Hydroxyclavicol has been reported for its antioxidant, anti-inflammatory, anti-arthritic, anti-enteropathogenic, anticancer, superoxide radical and hydroxyl radical scavenging activity, COX-1/COX-2 inhibiting, antimicrobial, antimutagenic and antifungal activities [4-6].

Acacia catechu Willd .(Family: Mimosaceae) is a perennial tree and is found throughout India. It has antiviral, anti-inflammatory, hepatoprotective and spasmolytic activity (7 Dahanukar et al, 2000). Aqueous extracts of Acacia catechu are rich source of catechin and epicatechin (gallic acid derivatives), with smaller amounts of flavonoids. Potent antioxidant activity has been well established in both in vitro and in vivo studies. This antioxidant activity is believed to be responsible for the anti-inflammatory, tissue protectant, antineoplastic, and analgesic activities that have been demonstrated and clearly established in animal and cell culture systems. Furthermore, antihyperglycemic, antidiarrheal, antinociceptive, and antipyretic activities have been demonstrated in animal studies (8 Stohs and Bagchi, 2015).

Thus, it was deduced from literature survey that due to its antioxidant and free radical scavenging ability, the PHF may show anti-ulcerative effect. Thus this study was undertaken to evaluate the antiulcer activity of a PHF in experimentally induced gastric ulcer in rats.

Materials and Method

Plant Extract Preparation

The *Piper betel* leaves and heartwood of *Acacia catechu* were authenticated at Department of Botany, Urban Science College, Mehsana. The aqueous extract of the *Piper betel* dried leaves was used for the study. Accurately weighed 100 gm of fresh *Piper betel* leaves were grinded by mixing with 500ml of distilled water. The mixture was heated at 80°C for about 30 minutes and filtered using vacuum filter assembly. Then the filtrate was concentrated[9]. The dry powder was prepared by spray drying. The clumpy dry powder obtained was scraped off and fine powder

was packed in air tight plastic container [10].

The fresh heartwood of *Acacia catechu* was dried in shade, cut and crushed. The cut and crushed Acacia catechu wood was plant material (20 g) and was extracted in hot ethyl alcohol by Soxhlet extraction method. The extract was concentrated and the solvent was evaporated off. A part of the residue was used for further studies.

Experimental Animals

Wistar albino rats weighing between 250 to 300 g were housed in groups of four at temperatures (25-28°C), under a standard light/dark cycle. The rats were fed with a standard pellet diet and water. The animals were utilised after acclimatization period. The experimental procedures were performed in accordance with the Institutional Animal Ethical Committee (IAEC) constituted as per the directions of the Council for Medical Research and the Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA), New Delhi, India. (SSPC/IAEC/17/07/2013)

Treatment

Polyherbal formulation (PHF) consisted of *Piper betel* and *Acacia catechu* extracts in equal proportion. PHF (aqueous extract) was administered in two different dose regimes- 100 mg/Kg and 200 mg/Kg, orally, once daily for 6 days to study the effect of drug. The efficacious dose of PHF 200mg/Kg was further tested in Ethanol induced gastric ulcer model in rats.

Experimental Protocols

Aspirin + Pylorus Ligation Model

Rats were divided into four groups of six animals each. Group I animals were treated as Disease control animals (Aspirin + pylorus ligation and Vehicle). Group II animals received standard drug treatment of Ranitidine (50mg/kg). Group III and IV animals were treated with PHF 100 mg/kg and 200mg/kg dose respectively. From days 4 to 6, animals of all the groups received aspirin orally as an aqueous suspension at a dose of 200 mg/kg. Two hours after the administration of respective drug treatment, animals in all the groups were fasted for eighteen hours and anaesthetized with ether. Pyloric ligation was performed by ligating the pyloric end of stomach. After four hours of pyloric ligation the animals of all the groups were sacrificed and gastric contents were collected for analysis for Ulcer Index, Volume of gastric acid secretion, Total Acidity, Total Acid output, Pepsin output, Total Carbohydrates(TC), Protein content (PR) and TC/PR ratio. The stomachs were removed and opened along the greater curvature to determine the ulcer index [11,12].

Ethanol-Induced Gastric Ulcers in Rats

Animals were treated by respective treatments for 7 days. Group I animals were treated as Disease control animals and were administered 1 ml of 80% Ethanol p.o. Group II animals received standard drug treatment of Ranitidine (50mg/kg). Group III animals were treated with PHF (200mg/kg). On 7th day the animals were fasted for 36 hours before the experiment. 1 ml of 80% ethanol was administered p.o. in the fasted animals [13–15]. In treated group, drug was administered p.o., 1 h before the administration of ethanol. After 2 h of ethanol administration, animals were sacrificed and stomach was removed, and opened along the greater curvature and subjected to measurement of ulcer index. After that, the stomach was homogenised for thiobarbituric acid reactive substances assay (MDA content). The following parameters investigated:

1. Physical parameters- (Ulcer index [1] and Volume of gastric acid secretion [16]),
2. Acid secretory parameters- (Total acidity [16], Total acid output [16], Pepsin output [17]),
3. Dissolved mucosubstances- (Total carbohydrates (TC) [18], Protein content (PR) [19], TC/ PR ratio)
4. Thiobarbituric acid reactive substances assay (MDA content) [20].

Results

Gastric ulcers were induced by Aspirin + pylorus ligation model to assess the efficacy of PHF at 100 mg/Kg and 200 mg/Kg. The physical parameters i.e. ulcer index, volume of gastric acid secretion, total acidity and total acid output showed reduction by PHF treatment and by the standard treatment Ranitidine as compared to disease control animals (Table 1). The biochemical parameters involved estimation of pepsin output which was found to be decreased as compared to diseased animals. Pepsin output by PHF extract at 200 mg/Kg dose was comparable to the standard treatment ranitidine (Graph 1). Total carbohydrate content was found to

be increased in the treated animals in comparison with diseased animals. PHF extract (100 mg/Kg) treated animals showed similar increase in total carbohydrate content as Ranitidine (Graph 2). The protein content found to be decreased in all the treatment arms when compared to disease control animals (Graph 3). TC/PR ratio showed significant difference in disease treated with aqueous extract of PHF 200 mg/kg dose as compared to 100 mg/kg dose and the ranitidine treatment which had comparable effects (Graph 4).

The previous study was suggestive that the efficacy of PHF was found maximum at 200 mg/Kg and thus was further analysed for cytoprotection using Ethanol-induced gastric ulcer model. Ulcer index in treatment groups was found to be reduced as compared to disease control group. The aqueous extract showed equivalent effect in reduction of ulcer index as in ranitidine treatment arm (Graph 5). MDA content was higher in disease control group as compared to PHF treated group. Reduction in MDA content in PHF was comparable to ranitidine treated group.

Table 1: Physical parameters of aspirin + pylorus ligated gastric ulcer model

Groups	Ulcer Index	Volume of Gastric acid (ml)	Total Acidity (mEq/L)	Total Acid Output (mEq/L/100g.b.w.)
Disease control	2.25 ± 0.087	8.03 ± 0.39	61.4 ± 0.65	493.04 ± 15.52
Ranitidine (50mg/kg)	1.21±0.14***	4.96±0.11**	32.87±0.56**	163.02 ± 5.68**
PHF (100mg/kg)	2.10±0.086**	6.88 ± 0.14*	45.0± 0.63*	309.67 ± 5.71*
PHF (200mg/kg)	0.97±0.099***	5.85±0.22**	28.73±0.54**	168.07 ± 6.044**

n=6, Values are expressed as mean± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with disease control group

Table 2: Acid secretory parameters of aspirin + pylorus ligated gastric ulcer model

Groups	Pepsin Output	Total Carbohydrates	Protein Content	TC/PR Ratio
Disease control	20.5 ± 0.057	602.03 ± 0.49	325.4 ± 0.45	1.85±0.55
Ranitidine (50mg/kg)	7.1±0.17**	1004.6±0.21***	210.57±0.56**	4.77±.033**
PHF (100mg/kg)	15.20±0.076*	1002.68 ± 0.24***	245.0± 0.63**	4.09± 0.71**
PHF (200mg/kg)	0.57±0.08***	1200.43±0.12***	154.23±0.14***	7.78±0.14***

n=6, Values are expressed as mean± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared with disease control group

Table 3: Parameters for ethanol induced gastric ulcer model

Groups	Ulcer Index	MDA Content
Disease control	4.48±0.34	18.27±0.46
Ranitidine (50mg/kg)	2.98±0.11*	5.56±0.23**
PHF (200mg/kg)	3.01±0.23*	6.02±0.8**

n=6, Values are expressed as mean± SEM, * $p < 0.05$, ** $p < 0.001$ when compared with disease control group

Discussion

With the associated side effects of the modern medicine, traditional medicines are gaining importance and are now being studied to find the scientific basis of their therapeutic actions [21]. Ulcers mainly result from an imbalance between aggressive factors and the maintenance of the mucosal integrity through the endogenous defence mechanism [3]. To regain the balance, different therapeutic agents including herbal preparations are used to inhibit the gastric acid secretion or to boost the mucosal defence mechanism by increasing mucus production [22]. Keeping insight the above mentioned facts, the present investigation was designed to evaluate anti-ulcerogenic potential of aqueous extract of *Piper betel* leaves.

HPTLC analysis of aqueous extract of *Piper betel leaves* at 366 nm showed presence of Hydroxychavicol (rf value = 0.44) and Eugenol (rf value = 0.96). Our results are in concordance with the findings of Pin et al. (2010)[23]. This insight provided the basis for our study that Hydroxychavicol present in Aqueous extract of *Piper betel* leaves might be responsible for antiulcer activity by its free radical scavenging and antioxidant activity. In our study the % yield of hydroxychavicol was found to be 1.5. Hydroxychavicol and Eugenol both were detected in the aqueous extract but they differed in amounts. Aqueous extraction of Hydroxychavicol was carried out by Pin KY et al. The extraction yield of water was significant compared with other solvents. This indicated that the major phytochemical constituents in betel leaves

are mostly high in polarity and soluble in water. The chemical profile of water extract varied significantly from other extracts because it contained only two major peaks, one for hydroxychavicol and one for Eugenol [9]. Our results are in concordance with it and confirmed that Hydroxychavicol is the major active principle in aqueous extract of *Piper betel* leaves which might be responsible for antiulcer activity.The TLC of the alcoholic plant extract of *Acacia catechu* conducted using CEF (chloroform ethyl acetate formic acid, 5:4:1) as mobile phase and DPPH as spray reagent, gave yellow spots indicating presence of antioxidant constituents in the extract.

Acute toxicity test was performed by Katedeshmukh et al and Pingale for *Piper betel* leaves and *Acacia catechu* respectively. They showed that mice were free of any toxicity as per acceptable range given by the OECD guidelines up to the dose of 2000mg/kg and 6000mg/kg respectively [24,25].

NSAIDS like aspirin causes gastric mucosal damage by decreasing prost aglandins levels through inhibition of prostaglandins synthesis, increasing acid secretion, decreasing mucin activity and back diffusion of H+ ions and thus leading to breaking up of mucosal barrier. Furthermore, it results in generation of free radicals, imbalance in gastric secretion, elevated pepsin, protein content and back diffusion of H+ ions into gastric mucosa leading to necrosis and ulceration [26]. The increased protein content of the gastric juice suggests the mechanism of leakage of plasma protein into gastric juice. Pylorus ligation induced ulcers are due to auto digestion of gastric mucosa and breakdown of the gastric mucosal barrier. The PHF extract showed significant reduction in ulcer index and protein content. It also reduced volume of gastric acid secretion, total acid output and pepsin concentration indicating its anti-secretory effect. Decrease in protein content in gastric juice also signifies decrease in leakage from mucosal cells indicating mucosal resistance. Mucin secretion is a crucial factor in the protection of gastric mucosa from the gastric lesions and has been regarded as an important defensive factor in the gastric mucus barrier. Decrease in synthesis of mucus glycol proteins has been implicated in etiology of gastric ulcer[27]. The increase in carbohydrate: protein (TC:PR) ratio is the direct reflection of mucin activity.

Cytoprotection has been defined as the ability of pharmacological agents (PGs) to prevent or reduce gastric mucosal injury by mechanism other than inhibition of gastri cacid secretion. The mechanism behind cytoprotection may be mainly to maintain physico-chemical properties and integrity of the gastric mucosal barrier. Ethanol is reported to damage mucosa, alteration in permeability and free radical production. This is attributed to release of superoxide anion and hydroperoxy free radicals and have found to be involved in the mechanism of acute and chronic ulceration in gastric mucosa. Alcohol rapidly penetrates the gastric mucosa apparently causing cell and plasma membrane damage leading to increased intracellular membrane permeability to sodium and water. The massive intracellular accumulation of calcium represents a major step in pathogenesis of gastric mucosal injury. This leads to cell death and exfoliation in the surface epithelium. It was observed that the PHF extract significantly reduced ethanol induced ulcer. This may be due to cytoprotective effect of extract which was measured in terms of the inhibition of lipid peroxidation (MDA Content). The PHF extract showed protection against characteristic lesions produced by ethanol administration which might be attributed to both reduction in gastric acid secretion and increase in gastric cytoprotection.

In summary, our data suggests that the PHF extract seems to be effective against gastric ulcers induced by Aspirin together with pylorus ligation model and Ethanol model. The antiulcer effect of the drug could be attributed to its free radical scavenging property, inhibition of gastric acidity leading to prevention of H+ ion back diffusion from stomach mucosa and strengthening of the gastric mucosal barrier.

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