Advances in Biochemistry & Applications in Medicine

Chapter 3

Anti-Gastric Ulcer Activity of Aqueous Extract of Terminalia Arjuna Against Helicobacter Pylori Lipopolysaccharide

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Abstract

Gastric ulcer is a common disease in humans. Every human might undergo the episode of gastric ulcer at least once in their life time. Among the various causes of gastric ulcer, *Helicobacter pylori* are one among them. The lipopolysaccharide is one of the virulence factors of *H. pylori.* The natural /holistic approach for the treatment/prevention is needed for all diseases including gastric ulcer due to its lack of adverse effects. *Terminalia arjuna* bark is one of the natural drug, which underwent clinical trials for cardio vascular diseases was chosen to study the antiulcer effect of Terminalia arjuna aqueous extract in *H.pylori* LPS induced gastric ulcer in rats. Gastric ulcer was induced in Sprague dawley rats with *H. pylori* LPS and *Terminalia arjuna* aqueous extract was administered to study the anti-ulcer activity. The assessment of anti-gastric ulcer was performed by testing the acid secretory and mucosal defensive factors. As a result of this study we could conclude that the aqueous extract of *Terminalia arjuna* offered anti-gastric ulcer effect.

1. Introduction

Gastric ulcer is a very frequent disease in the clinical practice and a challenge in the gastroenterology research [1]. Helicobacter pylori after being first isolated in human biopsies by Warren and Marshall in 1983 are now considered to be the major cause of gastric ulcers, duodenal ulcers and gastritis. *H. pylori* infection is also reported to be one of the important causes for relapse of ulcers [2]. Approximately 40 and 80% of individuals in developed and

developing countries are infected respectively, making Helicobacter pylori as one of the most common bacterial infections in humans [3].

The factors implicated in the virulent action of *H. pylori* towards mucosal integrity include CagA and VacA cytotoxins capable of inducing the release of pro-inflammatory cytokines, excessive production of ammonia known for its strong toxic effect on the gastric epithelium, and the impairment of feedback inhibition of gastrin release by somatostatin [4]. Another product of significance to the virulent action of *H. pylori* is its cell wall lipopolysaccharide [5].

H. pylori lipopolysaccharide elicited within 2 days the pattern of acute mucosal inflammatory responses accompanied by a massive epithelial cell apoptosis, increase in mucosal expression of endothelin- 1, enhancement in TNF-alpha, increase in NOS-2, decrease on cNOS activity [5], excessive nitric oxide generation, apoptotic caspase activation and a marked enhancement in gastric epithelial cell apoptosis [6]. Other pathogenic effects of *H. pylori* LPS involve progression of the mucosal inflammatory process, stimulation of NFκB nuclear translocation, disturbances in mitogen activated protein kinase (MAPK) cascades and a marked up-regulation in gastric mucosal level of endothelin-1 [7].

Eradication of *Helicobacter pylori* seems to be curative of both infection and ulcer disease. Hence successful treatment leads to the resolution of gastritis and diminished ulcer recurrence [8]. The combined treatment of proton pump inhibitors (i.e. omeprazole) with antibiotics (i.e ampicillin, amoxicillin, ofloxacin or tetracycline) have shown to be successful in some of the patients suffering from this complaint, with cure rates up to 90% [9]. Currently, the antiulcer treatment can be performed with antacid drugs, such as proton pump inhibitors (PPIs) or antagonists of the type 2 histamine receptors. However, this therapy produces serious adverse effects, including osteoporotic fracture; renal damage; infection (pneumonia and Clostridium difficile infection); rhabdomyolysis; deficiencies of vitamin B_{12}, magnesium, iron; anemia; thrombocytopenia [10], and is being associated with poor ulcer healing quality and in turn ulcer recurrence [11].

Several plants are used for the treatment of gastric ailments, including stomach ache and ulcers [12]. *Terminalia arjuna* Wight and Arnot known locally as Kumbuk, have a long history of medicinal uses in India [13], including cancer treatment [14]. Prior attempts to isolate medicinal agents from this tree yielded a variety of relatively simple compounds [15] such as flavonoids [16].

The bark is astringent, sweet, acrid, cooling, aphrodisiac, demulcent, cardiotonic, styptic, antidysenteric, urinary astringent, expectorant, alexiteric, lithontriptic and tonic. It is useful in fractures, ulcers, urethrorrhea, leucorrhea, diabetes, vitiated conditions of pitta, anemia, cardiopathy, hyperhydrosis, fatigue, asthma, bronchitis, tumours, otalgia, dysentery, inflam-

mations, internal and external haemorrhages, cirrhosis of the liver and hypertension [17]. It is reputed as cardiotonic and also possesses hypotensive and hypolipidemic activity [18]. Its use in wound healing has been mentioned by Sushruta in Sushruta Samhita [19]. The bark powder is reported to exert hypocholesterolaemic and antioxidant effect in humans [20]. The chemical constituents isolated from the plant are mainly tannins and various oleanane triterpenoids. Tannins of the leaves had been reported to have anticancer activity [21] and that from the bark possessed antimutagenic effect [22].

Terminalia arjuna bark constitutes high amounts of fibre, sugar, tannin, beta-sitosterol, carbonate of calcium, sodium, aglycones-arjunine, arjunolic acid, arjunoids I, II, III and IV [23]; and also having antioxidant polyphenolics, flavonoids-quercetin, kaempferol, pelargonidin and luteolin [24]. *Terminalia arjuna* exhibited antibacterial activity against *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris,* and *Pseudomonas aerogenes* (gram-negative bacteria) [25]. An active principle from *Terminalia arjuna* bark, Arjunaphthanoloside- a glycoside, showed potent antioxidant activity and inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated rat peritoneal macrophages [26]. Terminoside A, a new oleanane type triterpene, potently inhibited nitric oxide (NO) production and decreased inducible nitric oxide synthase (iNOS) levels in lipopolysaccharide-stimulated macrophages [27]. Hence, the present study was aimed to assess the gastroprotective effect of water extract of *Terminalia arjuna* against *Helicobacter pylori* lipopolysaccharide induced gastric ulcer.

2. Materials and Methods

2.1. Gastric ulcer induced by *Helicobacter pylori* lipopolysaccharide (Hp-LPS)

2.1.1. Preparation of Hp-LPS 26695

Helicobacter pylori lipopolysaccharide was prepared from 26695 strain of *Helicobacter pylori* by the conventional method used for the preparation of lipopolysaccharide from gram-negatives. *Helicobacter pylori* 26695 grown in Brucella broth with 5% FCS was pelleted by centrifugation, washed twice with 0.9% NaCl, heat inactivated for 2 h in steam, washed twice with 0.9% NaCl. Finally the pellet was suspended in 1 mL of 0.9% NaCl and stored at 4˚C. Then it was lyophilized and used.

2.1.2. Procurement and maintenance of animals

Male Sprague-Dawley rats weighing 150-200g were obtained from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India, was used for the study and given standard pellet diet and water *ad libitum* and deprived of food 24 h before sacrifice. All experiments were performed with 6 animals in each group. This study was conducted according to the ethical norms approved by Animal Ethics Committee of our

institution (IAEC No. 01/038/07).

2.1.3. Experimental set up for effective dose fixation of TA against Hp-LPS induced gastric ulcers

Group 1 : Control rats

Group 2 : Ulcerated rats - Ulcer was induced by *Helicobacter pylori* lipopolysaccharide (Hp-LPS) (50µg/animal, orally) for 3 days

Group 3-6 : Treatment group - Pretreatment with different doses of *Terminalia arjuna* for 7 days (100, 200, 300 and 400mg/kg bw) and subjected to *Helicobacter pylori* lipopolysaccharide induction for 3 days and then maintained on *Terminalia arjuna* extract for 4 days

Group 7 : Reference group - Pretreatment with Sucralfate for 7 days (100mg/kg bw, orally, [28]) and ulcer was induced by *Helicobacter pylori* lipopolysaccharide for 3 days and then maintained on sucralfate for 4 days.

Animals were sacrificed after 24h fasting by cervical decapitation following anesthetization. For studies requiring gastric juice, pyloric ligation was done 4 hours prior to sacrifice.

Gastric juice was collected by a 4 h pyloric ligation [29]. After 4h, the animals were sacrificed. Stomach was dissected out after tying the oesophageal end. The stomach was cut open along the greater curvature and the contents were collected into tubes, centrifuged at 1000 rpm for 10 minutes and were used for the estimation of various biochemical parameters such as volume of gastric juice, acid output, free acidity, total acidity, mucin content etc., Pyloric ligation was done only for the collection of gastric juice. For the collection of gastric mucosal tissue, no pyloric ligation was done; the rats were killed by cervical dislocation under ether anesthesia. After sacrificing the rats, the stomach was excised and cut along the greater curvature, washed carefully with 0.9% NaCl. The mucosa of the stomach was scrapped and used for gastric mucosal parameters. Blood was collected from the jugular vein and plasma was used for estimations.

Ulcer index was calculated according to the method of Okabe et al., [30]. After the collection of gastric juice, the volume was noted and p^H was measured. Free acidity and total acidity [31] were determined by titrating with 0.01 M NaOH using Toepfer's reagent and phenolphthalein as indicator. The basal and maximum acid output [32] was determined to assess the anti-secretory effect of TA.

2.1.4. Experimental setup for gastro protective evaluation of TA's efficacy against Hp-LPS induced gastric ulcers

Group 1 : Normal rats - serve as control (Control)

Group 2 : Ulcerated rats – induced for gastric ulcers with Helicobacter pylori li popolysaccharide-26695 (50 µg/ animal, orally) for 3 days (Hp-LPS)

Group 3 : Treated rats - pretreatment with water extract of Terminalia arjuna for 7 days (300 mg/kg bw, orally) and subjected to Helicobacter pylori lipopoly saccharide induction for 3 days and then maintained on water extract of Terminalia arjuna extract for 4 days (Hp-LPS+TA)

Group 4 : Reference group - Pretreatment with Sucralfate for 7 days (100mg/kg, orally, [28]) and ulcer was induced by Helicobacter pylori lipopolysac charide for 3 days and then maintained on sucralfate for 4 days (Hp-LPS+SFT)

After the experimental period, the rats were killed under anaesthesia. Gastric juice, blood and gastric mucosal tissues were collected as that of the above said methods and used for the biochemical analysis.

- Gastric mucosal barrier [33], protein [34], hexose [35], hexoseamine [36], sialic acid [37], fucose [38] were estimated in gastric mucosa and gastric juice to assess the mucoprotective efficacy of TA.

- Gastrin hormone (Gastrin hormone was estimated using RIA kit (Diagnostic products corporation, Los Angeles, USA), pepsin and pepsinogen [39] assays were done.

- Cyclooxygenase 2 expression - Stomach was cut and tissue was utilized for COX-2 analysis so as to assess the prostaglandin levels and the cytoprotective nature of Terminalia arjuna. Immunohistochemical analysis of COX-2 was done using Primary Antibody: Goat Anti-COX-2; Secondary Antibody: Rabbit Anti-Goat IgG HRP conjugate. Photomicrographs were obtained using a LABOMED CX RIII microscope (20X /0.454; 10X/0.254; 40X/0.654) connected to a SANYO digital color CCD camera.

- Nitric oxide levels [40] were estimated since it changes during Helicobacter pylori lipopolysaccharide pathogenesis.

- Serum tumor necrosis factor-alpha level was determined as it is a marker of inflammation. The level of TNF - α in plasma was measured using ELISA – kit provided by PAN-Biotech, GmBH. The protocol was a modified version of Sharma and Singh [41]. Primary antibody - Rabbit monoclonal anti TNF-α(1: 5000 dilution), Secondary antibody - Goat antirabbit antibody IgG - HRP conjugated.

- The level of IL- β was measured by using Enzyme Linked Immunosorbent assay (ELISA). The protocol was a modified version of Sharma and Singh [41]. Primary antibodies - Rabbit M-Ab Anti - IL- β (1: 5000 dilution), Secondary antibody - Goat antirabbit antibody IgG-HRP conjugated

- Proliferating cell nuclear antigen expression was examined for cell proliferation by immunohistochemistry. The gastric tissues were immunostained for PCNA using Monoclonal Mouse Anti-PCNA antibody and detected with Mouse anti- IgG HRP conjugate.

2.2. Statistical Analysis

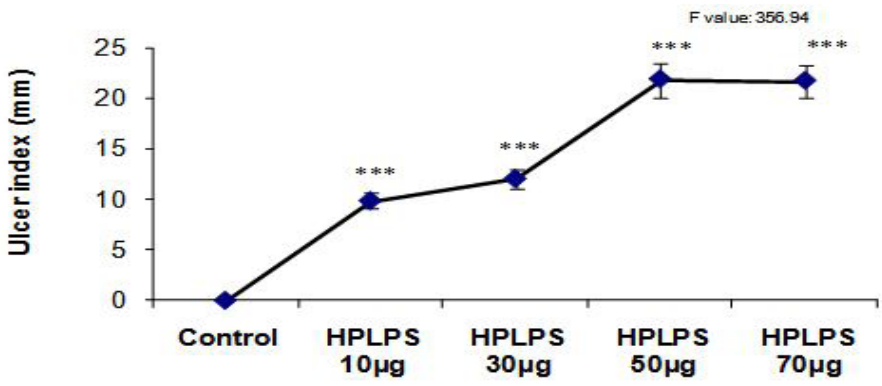
Values are represented in mean ± SD for six rats in each group and the differences between mean values was determined by one-way analysis of variance (ANOVA) followed by the Dunnett's T3 multiple comparison test by utilizing the SPSS (Statistical Package for Social Science) 10.0 software package. The values are considered significant when $p < 0.001$, $p < 0.01$ and $p < 0.05$.

3. Results and Discussion

3.1. Effective dose fixation for Hp-LPS-26695 for the induction of gastric ulcers

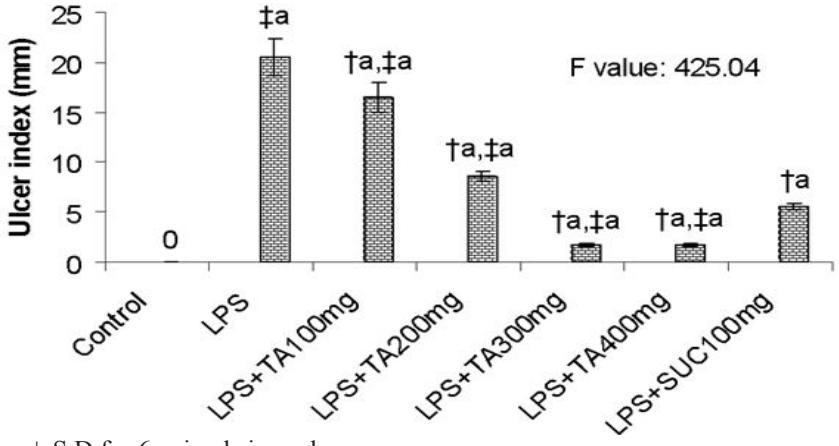
Figure 1a depicts the results of dose fixation for Hp-LPS-26695 for the induction of gastric ulcers. The effective dose of *Helicobacter pylori* lipopolysaccharide (Hp-LPS) - 26695 in inducing gastric ulceration was assessed by orally administering Hp-LPS at the doses of 10, 30, 50 and 70µg/animal per day for 3 consecutive days by dissolving it in saline. At a dose of 10 and 30µg/animal for 3 days, mild lesions were observed. Severe gastric lesions were produced at the doses of 50 and 70µg/animal for 3 days in experimental rats. From these, 50µg/animal for 3 days was fixed for inducing gastric lesions using Hp-LPS-26695, as it elicits damage at a minimum dose.

Figure1 a: Dose fixation for HP-LPS for induction of gastric lesions in rats



Values are expressed as mean ± S.D for 6 animals in each group.
Groups are compared as follows: Control vs. All groups
Significance represented as ***– $p<0.001$

Figure 2a: Effect of TA at various concentration to fix the dose against HP-LPS



Values are expressed as mean ± S.D for 6 animals in each group.
Groups are compared as: †-Hp-LPS vs. All groups; ‡-Hp-LPS+SFT vs.
All groups. Significance represented as a-p<0.001 using One way ANOVA.
Dunnett's T3 multiple comparision test.

3.2. Anti-secretory effect of TA against Hp-LPS induced gastric ulcers

Table 1a: Effect of TA on gastric secretory parameters in control and Hp-LPS induced experimental animals Values are expressed as Mean ± SD for 6 animals in each group

Parameters	Control	Hp-LPS	Hp-LPS +TA 100mg	Hp-LPS + TA 200mg	Hp-LPS+ TA 300mg	Hp-LPS+ TA 400mg	Hp-LPS+ SFT 100mg	F Value
Volume of gastric juice	1.88± 0.15 ‡NS	3.15± 0.24[a,‡a]	3.10± 0.17 †NS,‡a	2.5± 0.21†b, ‡a	1.90± 0.17†a, ‡NS	1.95± 0.14 †a,‡c	1.58± 0.15†a	75.02
pH	4.2± 0.14 ‡NS	1.87± 0.15[a,‡a]	3.47± 0.18 †a,‡c	3.97± 0.08†a, ‡NS	4.18± 0.15†a, ‡NS	4.28± 0.15 †a,‡NS	3.95± 0.19†a	193.33
Free acidity	34.67± 2.8‡NS	53.67± 2.73 [a,‡a]	52.33± 2.42† NS,‡a	42.33± 3.01 †a, ‡b	33.0± 1.79†a, ‡NS	35.17± 1.47 †a,‡NS	32.5± 3.02†a	78.16
Total acidity	64.83± 2.48 ‡NS	88.5± 3.78 [a,‡a]	81.33± 4.55† NS,‡a	75.67± 2.8†b, ‡a	65.83± 4.71 †a, ‡NS	66.67±3.01 †a,‡c	59.83± 2.93†a	50.33
Basal Acid output	38.75± 4.41 ‡a	90.63±5.5[a,‡a]	23.37± 1.03 †a,‡a	17.32± 0.81†a, ‡a	12.18± 0.86†a, ‡NS	11.65±0.87 †a,‡NS	29.42± 1.01†a	641.27
Maximum Acid Output	37.88± 112.0 ‡a	112.0±7.64 [a,‡a]	30.68± 1.29†a,‡a	18.72±0.35 †a,‡c	17.48± 0.92†a, ‡NS	16.17± 1.47 †a,‡NS	15.83± 1.47†a	726.17

Units: Volume of juice (ml/100g/4h), pH, Free Acidity (mEq/L/100g), Total Acidity(mEq/L/100g), Basal acid output (mEq/100g/4h); Maximum acid output (mEq/100g/4h).Groups are compared as follows: Control vs. Hp-LPS, † - Hp-LPS vs. All groups, ‡ - Hp-LPS+SFT vs. All groups.
Significance represented as [a]– p<0.001, [b]– p<0.01, [c]– p<0.05, [NS]– Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test

Figure 2a shows the effect of TA at various concentrations to fix the dose against Hp-LPS. Hp-LPS 26695 induced a significant increase ($p<0.001$) in the ulcer index compared to control rats. The ulcer index was significantly decreased ($p<0.001$) on treatment with TA at a dose of 100 mg/kg body weight compared to Hp-LPS induced rats, whereas a significant decrease ($p<0.001$) was noted with TA at doses of 200, 300 and 400 mg/kg bw compared to Hp-LPS ulcerated rats.

Table 1a indicates the effect of TA on gastric secretory parameters in control and Hp-LPS induced experimental animals. Hp- LPS 26695 induced a significant increase ($p<0.001$) in the gastric volume, free acidity, total acidity, maximal and basal acid output and pepsin concentration compared to control rats. In rats treated with different doses of TA a significant decrease was evident in all the secretory parameters in a dose dependent manner. Since reduction in ulcer index along with decrease in secretory parameters were noted at 300 mg/kg body weight, further studies on the assessment of gastroprotective effect of TA against Hp-LPS induced ulceration were performed at this dose.

LPS is a family of glycolipids found in the cell envelope of gram-negative bacteria, including *H.pylori* [42]. LPS from *H. pylori* can stimulate acid secretion, which possibly might contribute to mucosal damage of the stomach. The second possible mechanism by which *H. pylori* LPS can stimulate acid secretion at the gland level is by enhancing histamine release from rat ECL cells [43]. The LPS purified from the known gastric pathogen *H. pylori* has this secretory property greatly impaired and, depending on the strain of the bacterium is able to stimulate directly both pepsinogen [44] and acid secretion, potentially contributing to gastric ulcer.

T*erminalia arjuna* was reported to have antibacterial effect [45]. The reduction in the ulcer index clearly point towards the antibacterial effect of TA against the toxic effects elicited by Hp-LPS.

Maximal acid output has been indicated in the pathogenesis of mucosal ulceration where low gastric pH resulted in enhancement of H. pylori-induced NF-κB nuclear binding [46]. *H. pylori* increases basal gastrin levels, basal acid output, meal-stimulated maximal acid output and 24-h intragastric acidity. The effects on gastric acid production depend on the distribution of gastritis in the stomach [47]. *H. pylori* LPS can stimulate acid secretion at the gland level by increasing histamine release from rat ECL cells [43]. Sucralfate markedly suppresses *H. pylori* infection and the accompanying hypersecretion of acid. These effects are likely to be important mechanisms by which the drug promotes ulcer healing [48].

The Sydney strain of H. pylori in mouse model stimulated acid secretion [49] and LPS from *H. pylori* SS1 strain stimulates acid secretion, whereas other LPS preparations did not increase acid secretion. This is probably related to the differences in the molecular structure

of the tested LPS preparations [50]. However, in this study the LPS of 26695 strain stimulated acid secretion.

Table 2a: Effect of TA on pepsin, pepsinogen and gastrin in control and Hp-LPS induced experimental animals Values are expressed as mean ± S.D for 6 animals in each group

Parameters	Control	Hp-LPS	Hp-LPS+TA	Hp-LPS+SFT	F value
Pepsin (Gastric Juice-micromole tyrosine liberated/ml)	170.83±14.29	243.83±19.33a	155.33±11.47†a,‡NS	160.0±13.04†a	46.78
Pepsinogen (Gastric Mucosa-micromoles of tyrosine liberated/min/mg protein)	802.12±43.58	1044.17±55.35a	836.67±48.44†a,‡NS	851.67±40.21†a	31.94
Gastrin (plasma-pmol/L)	74.17±7.36	135.5±7.18a	75.5±6.16†a,‡NS	84.17±6.4†a	110.27

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– $p<0.001$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Table 2a shows the effect of TA on pepsin, pepsinogen and gastrin in control and Hp-LPS induced experimental animals. Pepsin concentration and Pepsinogen activity was significantly increased ($p<0.001$) in Hp-LPS induced ulcer rats compared to control rats while Hp-LPS+TA and Hp-LPS+SFT rats showed a significant decrease ($p<0.001$) in Pepsin concentration and pepsinogen activity compared to Hp-LPS induced rats. There was no significant alteration in Hp-LPS+TA treated animals compared with Hp-LPS+SFT rats.

Pepsin appears to play a crucial role in ulceration of the stomach and in the absence of pepsin; gastric acid does not cause ulceration. Hence, major benefits of antacid therapy in the treatment of ulcer disease may be inhibition of the conversion of pepsinogen to pepsin and the maintenance of a gastric luminal p^H greater than the optimum for the enzyme. Pepsin, a protease present in the gastric lumen, is secreted by the chief cells of the gastric mucosa as an inactive precursor, pepsinogen; pepsinogen is activated by acid present in the gastric lumen, which initiates digestion of protein [51].

Lipopolysaccharide of *H. pylori* (Hp-LPS) affects pepsinogen release by a nontoxic mechanism. This effect was characteristic of the organism and related to the clinical status of the strain. Physical and chemical disruption of LPS suggested that both the structure and the carbohydrate composition of LPS may play a critical role in pepsinogen release. Pepsinogen release is an innate property of all cagA+ *H. pylori* LPS. The structure of the molecule and composition of side-chains are important in this response which appears to be partially lipid A driven [52]. Stimulation of pepsinogen secretion is the important mechanism of Hp-LPS induced mucosal damage [42]. Luminal addition of *H. pylori* lipopolysaccharide resulted in a fifty-fold stimulation of pepsinogen [53].

An increase in pepsinogen activity by Hp-LPS 26695 strongly indicates the ulcerogenic potency of this LPS. Further, a decrease in the pepsinogen activity on TA administration is probably due to the efficacy of TA in inhibiting the hypersecretion of pepsinogen and protecting the mucosa from mucosal damage.

In Hp-LPS induced ulcer rats, the level of plasma gastrin was significantly increased ($p<0.001$) compared to control rats. Hp-LPS+TA and Hp-LPS+SFT rats showed a significant decrease ($p<0.001$) in the level of gastrin compared to Hp-LPS ulcer rats. No significant changes were noted between Hp-LPS+TA and Hp-LPS+SFT rats.

Gastrin is a peptide hormone that stimulates gastric acid secretion and the growth of fundic mucosa in the stomach [54]. *H. pylori* infection is associated with hypergastrinemia [55]. Proinflammatory cytokines including IL-1β, TNF-α, and IL-8 are able to stimulate gastrin release from G cells [56]. In addition, IL-1β, which can also act as a potent inhibitor of acid production, may cause hypochlorhydria resulting in hypergastrinemia [57]. The presence of *H. pylori* colonization was shown in several studies and associated with hypergastrinaemia and hyperpepsinogenaemia [58].

In the present study, Hp-LPS 26695 also elicited the plasma gastrin levels suggesting onset of inflammatory processes following Hp-LPS administration inducing the release of gastrin from G cells by proinflammatory cytokines like TNF-α and IL-1β. However in TA treated animals reduction in the inflammatory events could have resulted in the maintenance of plasma gastrin levels.

Gastric mucosal protection

Table 3a: Effect of TA on the levels of protein and protein bound carbohydrate complexes in gastric juice of control and Hp –LPS induced experimental animals
Values are expressed as mean ± S.D for 6 animals in each group

Parameters (µg/ ml)	Control	Hp-LPS	Hp-LPS+ TA	Hp-LPS+ SFT	F value
Hexose	405.14±25.64	277.29±28.23a	400.49±21.85	395.68±23.26†a	36.95
Hexoseamine	174.58±15.58	118.03±11.85a	36.95	164.5±14.03 †a	22.48
Sialic acid	40.38±3.17	27.94±1.98a	182.11±17.42	36.38±3.72 †b	17.95
Fucose	45.02±2.22	31.34±2.33a		42.64±2.23 †a	32.45
Total carbohydrate (TC)	665.12±16.01	454.59±29.95a	182.11±17.42	639.21±27.61†a	89.67
Protein (P)	272.46±12.65	392.11±25.25a	41.67±4.85	273.86±22.57 †a	42.68
TC: P ratio	2.45±0.12	1.16±0.13a		2.26±0.1†a	78.18

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

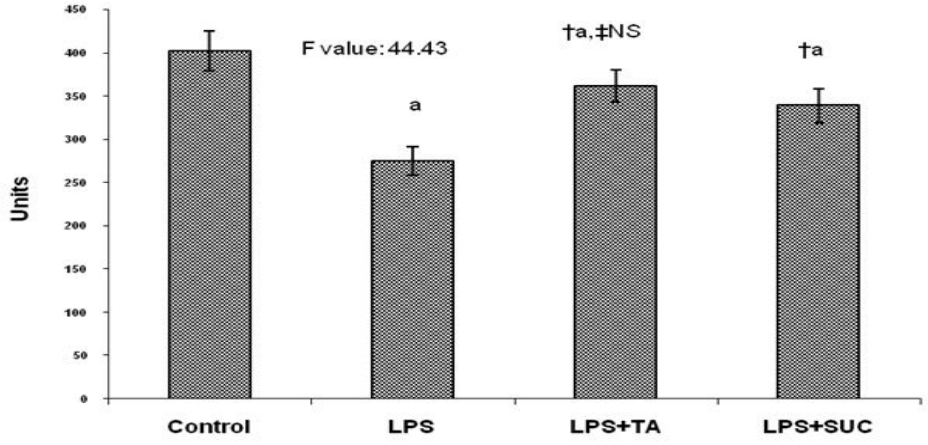
Significance represented as a– $p<0.001$, b– $p<0.01$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Table 3a shows the effect of TA on the levels of protein and protein bound carbohydrate complexes in gastric juice of control and Hp-LPS induced experimental animals. A significant decrease ($p<0.001$) in hexose, hexosamine, sialic acid, fucose, total carbohydrate and Total carbohydrate: protein ratio (TC: P) resulting from a significant increase in protein levels were noted in Hp-LPS ulcerated rats compared to control rats. On the contrary, Hp-LPS+TA treated rats registered a significant increase in hexose ($p<0.001$), hexoseamine ($p<0.001$), sialic acid ($p<0.01$), fucose ($p<0.001$), total carbohydrate and total carbohydrate: protein ratio (TC: P) ($p<0.001$) with a concomitant decrease in protein ($p<0.001$) levels compared to Hp-LPS ulcer rats. Hp-LPS+SFT rats registered a significant increase in hexose ($p<0.001$), hexoseamine ($p<0.001$), sialic acid ($p<0.01$), fucose ($p<0.001$), total carbohydrate and Total carbohydrate: protein ratio (TC: P) ($p<0.001$) with a concomitant decrease in protein levels ($p<0.001$) compared to Hp-LPS rats. There was no significant alteration in all these parameters in Hp-LPS+TA when compared with Hp-LPS+SFT rats.

Exposure of gastric mucosal cells to the LPS led to a dose-dependent decrease in mucin synthesis, accompanied by a marked increase in caspase-3 activity and apoptosis. A decrease in mucin synthesis following induction with LPS, accompanied by cells proceeding to apoptosis has been reported [59]. Also *H. pylori* LPS cause inhibition of mucin binding to the receptor [60].

H. pylori LPS has been shown to exert an inhibitory effect on the synthesis and secretion of gastric mucin, the glycoprotein that maintains the strength and mucus coat integrity [61]. In the present study, the influence of Hp-LPS 26695 on mucin content was evident from decreases in the hexose, hexoseamine, fucose and sialic acid contents. However the mucoprotective role of TA observed against other ulcer models [62] was also evident with Hp- LPS gastric ulcers and comparable to the effect of SFT.

Figure 3a: Effect of TA on the levels of Adherentmucus in control and Hp-LPs induced experimental animals



Units- Micro gram alcian blue/ g tissue.

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– $p<0.001$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Figure 3a shows the effect of TA on the levels of Adherent mucus in control and Hp-LPS induced experimental animals. The levels of adherent mucus were decreased significantly ($p<0.001$) in Hp-LPS ulcer rats compared with control rats. In Hp-LPS+TA and Hp-LPS+SFT group, there was a significant increase ($p<0.001$) in adherent mucus compared to Hp-LPS ulcer rats. No significant difference was observed in Hp-LPS+TA animals compared to Hp-LPS+SFT rats.

Table 4a: Effect of TA on the levels of protein and protein bound carbohydrate complexes in the gastric mucosa of control and Hp-LPS induced experimental animals

Values are expressed as mean ± S.D for 6 animals in each group

Parameters (mg/ g)	Control	Hp-LPS	Hp-LPS+TA	Hp-LPS+SFT	F value
Hexose	14.35±0.95	7.9±0.47a	15.58±1.23†a,‡c	13.05±1.09†a	71.18
Hexoseamine	8.78±0.42	4.53±0.22a	9.13±0.46 †a,‡NS	8.62±0.4†a	190.03
Sialic acid	1.78±0.05	0.81±0.07a	1.93±0.29 †a,‡NS	1.71±0.11†a	58.99
Fucose	3.45±0.3	2.22±0.15a	3.53±0.34 †a,‡NS	3.42±0.33†a	27.99
Total carbohydrate (TC)	28.37±1.11	15.46±0.6a	30.18±1.13 †a,‡C	26.79±1.54†a	202.43
Protein (P)	22.37±1.28	17.0±0.96a	24.61±2.04 †a,‡NS	23.25±1.53†a	29.47
TC: P ratio	1.27±0.08	0.91±0.07a	1.23±0.09 †a,‡NS	1.12±0.11†c	19.86

Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA. Significance represented as a– $p<0.001$, c– $p<0.05$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Table 4a shows the effect of TA on the levels of protein and protein bound carbohydrate complexes in gastric mucosa of control and Hp-LPS induced experimental animals. The levels of hexose, hexosamine, sialic acid, fucose, total carbohydrate, total carbohydrate: protein ratio (TC: P) and protein were decreased significantly ($p<0.001$) in Hp-LPS ulcer group compared with control rats whereas, these levels were significantly increased ($p<0.001$) in Hp-LPS+TA and Hp-LPS+SFT rats compared with Hp-LPS ulcerated rats. A non significant change was observed in Hp-LPS+TA compared with Hp-LPS+SFT rats in all these parameters except a significant increase ($p<0.05$) in hexose and total carbohydrate levels in Hp-LPS+TA animals.

Although the causative factors for ulcerogenesis may vary, the net imbalances in offensive and defensive factors are involved in ulcerogenesis [63]. The luminal surface of the gastrointestinal tract is covered by a viscoelastic mucous gel layer that acts as a protective barrier against the harsh luminal environment. The structural characteristics of this barrier are primary

indicators of its physiological function and changes to its composition have long been identified in gastrointestinal pathologies. The high molecular weight mucins are responsible for the viscoelastic properties of the mucous barrier. Mucins are implicated in the aetiology and may assist in the diagnosis of gastric intestinal metaplasia associated with gastric ulceration, *H. pylori* infection, and the risk of gastric cancer [64].

Inhibition of sulphated mucin synthesis and stimulation of pepsinogen secretion by LPS in vitro suggest the mechanisms for *H. pylori*-induced mucosal damage [42]. LPS, primarily through the lipid A component, stimulates the release of cytokines and possesses endotoxic properties including interference with the gastric epithelial cell-laminin interaction, which may lead to loss of mucosal integrity; inhibition of mucin synthesis; and stimulation of pepsinogen secretion [42]. Microvascular dysfunction was provoked by Hp-LPS [65]. Hp-LPS also exhibited alterations in the vascular permeability and the protective effect of TA may be attributed to the cytoprotective activity by increasing the integrity of mucus status.

Mucus serves as first line of defense against ulcerogens. Mucus is secreted by the mucus neck cells and covers the gastric mucosa thereby preventing physical damage and back diffusion of hydrogen ions [66]. TA significantly increased mucus secretion as observed from the increase in TC: P ratio, which is taken as reliable marker for mucin secretion [67]. This was primarily due to increase in the individual mucopolysaccharides. Further, strengthening of the gastric mucosa is evident from the decrease in the leakage of protein into the gastric juice [68]. Increase in glycoprotein content of gastric mucosa is evidenced from increase in TC: P ratio of the mucosal cells, which is taken as marker for cellular mucus [69]. This increase was due to increase in mucopolysaccharides, the major constituent of mucus and also which are responsible for viscous nature and gel-forming properties of the mucus. The gel is reported to be resistant to a number of ulcerogens including acid, ethanol and NSAIDs, i.e. indomethacin [70]. Hence an increase in the synthesis of mucus may be one of the important contributing factors for ulcer protective role of TA as against other models of gastric ulcers [62,71].

Plate 1a: Effect of TA on gastric mucosal Cyclooxygenase 2 expression

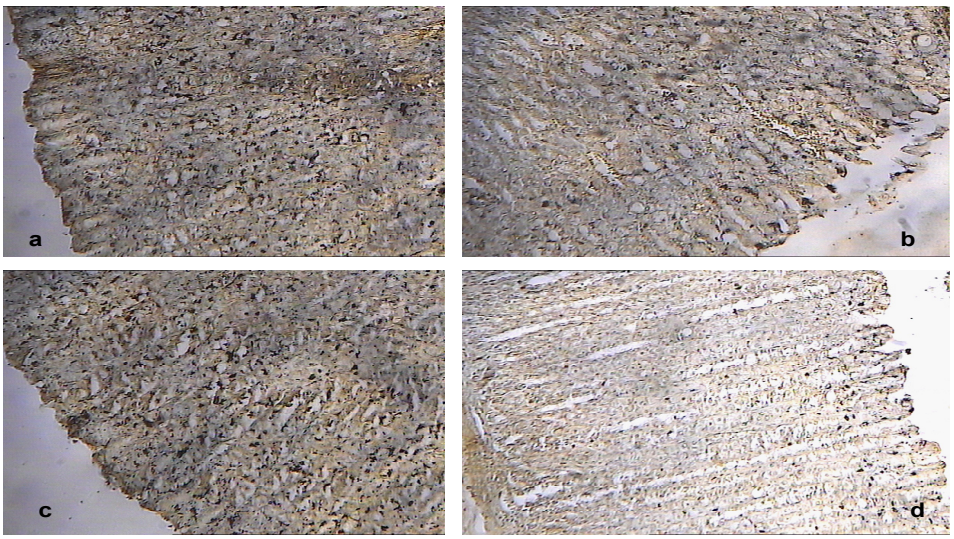
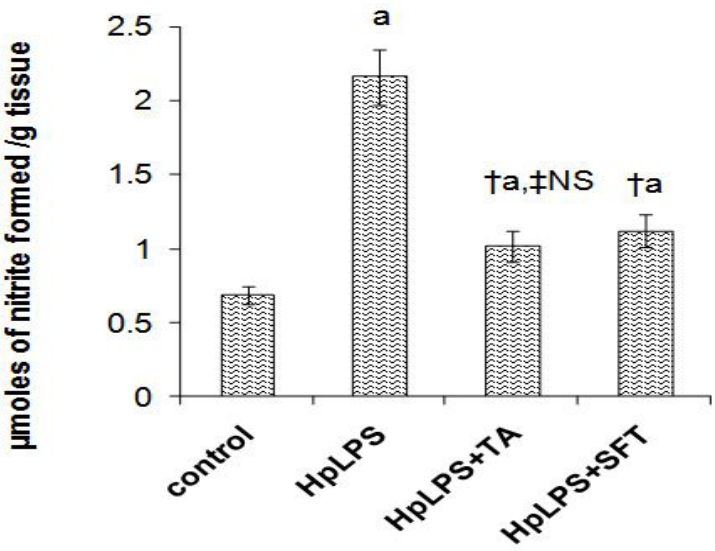


Plate 1a shows the effect of TA on gastric mucosal COX-2 expression in control and Hp-LPS induced experimental animals. Control rats showed normal expression of COX- 2 in gastric mucosa, Hp-LPS induced ulcerated rats showed an abrupt decrease in COX-2 expression in gastric mucosa, Hp-LPS+TA treated rats showed regeneration of mucosal cells and increased expression of COX-2 and Hp-LPS+SFT rats also showed regeneration of mucosal cells with reduced expression of COX-2.

Prostaglandins are known to protect the gastric mucosa against a wide variety of insults [72] and contribute to the maintenance of gastric mucosal integrity by influencing gastric mucus, bicarbonate and acid secretion as well as mucosal blood flow and epithelial cell proliferation rate [73]. *H. pylori* infection failed to induce COX-2 in gastric mucosa [74].

Likewise Hp-LPS 26695 inhibited the expression of COX-2 that could have reduced the levels of prostaglandins and resulted in the mucosal damage. The diterpene derivative ecabet sodium improves the wound repair in intestinal epithelial cells elicited by hydrogen peroxide by inducing the expression of COX-2 [75]. Terpenes present in TA might have induced COX-2 expression in Hp-LPS+TA rats.

Figure 4a: Effect of TA on nitric oxide levels



Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– $p<0.001$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Figure 4a illustrates the effect of TA on the levels of nitric oxide in gastric mucosa of control and Hp-LPS induced experimental animals. There was a significant increase ($p<0.001$) in nitric oxide level in Hp-LPS induced animals compared to control rats. Hp-LPS+TA and Hp-LPS+SFT rats showed a significant decrease ($p<0.001$) in nitric oxide level compared to Hp-LPS ulcer group. No significant differences were observed between Hp-LPS+TA and Hp-LPS+SFT rats.

Nitric oxide (NO) is also recognized as an important mediator of gastrointestinal mucosal defense, exerting many of the same actions as prostaglandins (PGs) in these tissues [76].

Both the mediators (NO and PG) are capable of modulating mucosal blood flow, mucus and bicarbonate secretions as well as the repair of gastric injury [77]. Three isoforms of NO synthase (NOS) exist: endothelial NOS, neuronal NOS, and inducible NOS (iNOS) [78].

NO plays a biphasic role in the ulcerogenic response in the gastrointestinal mucosa, as a protective effect of cNOS/NO and a proulcerogenic effect of iNOS/NO have been reported [78]. *H. pylori* LPS can initiate the expression of iNOS in the stomach following a systemic challenge, which can evoke microvascular dysfunction [65].

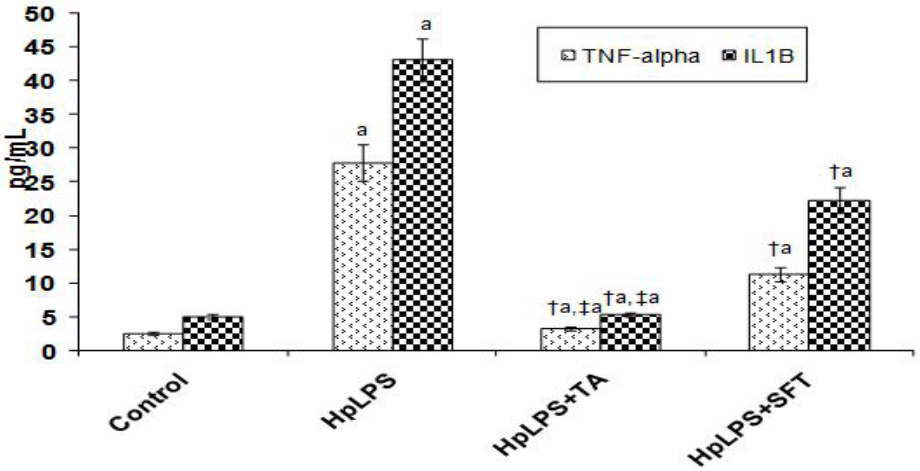
*H. pylor*i LPS-induced gastric mucosal damage is manifested by the increase in pro-inflammatory cytokine production, excessive NO and prostaglandin generation, massive rise in epithelial cell apoptosis, and a marked up-regulation in gastric mucosal ET-1 level [79]. Induction of NOS-2 leads to pro-apoptotic caspase-3 activation and the excessive formation of NO-related species that evoke transcriptional disturbances, cause alterations in prostaglandin formation, and leads to the up-regulation of pro-inflammatory cytokine production [80].

H. pylori infection may provoke damage in the stomach and duodenum by releasing soluble factors that activate inflammatory cells such as neutrophils, to produce cytotoxic mediators such as superoxide [81] and nitric oxide (NO) [82]. High concentrations of NO are known to be cytotoxic, and in combination with the superoxide radical, leads to the subsequent formation of the moieties, peroxynitrite and hydroxyl radicals, which are highly injurious to cells [83].

However, NO/iNOS also contributed to the gastric mucosal protection as induced by a mild irritant at a later time period or observed in arthritic rats [84]. The dual action of NO is not determined by the source enzyme, cNOS or iNOS, but depends more on the circumstance where NO is acting. On the other hand, Konturek et al. [85] reported that the healing of acetic acid-induced gastric ulcers was delayed and promoted by administration of NOS inhibitors and L-arginine, respectively. However, the inhibition of NO production by NG-nitro-L-arginine methyl ester (L-NAME) impaired gastric mucosal blood flow and delayed healing of acute gastric injury [86].

An increase in the levels of NO following Hp-LPS 26695 induction supports the theories of pro-ulcerogenic role of NO in Hp-LPS 26695 induced gastric ulcer. The decrease in NO levels with reduction in the ulcer size on TA administration suggests attenuation of cellular damage, mucosal injury and apoptosis. Hence the phytoconstituents present in TA may offer gastroprotection by decreasing NO production.

Figure 5a: Effect of TA on the levels of TNF-alpha and IL-1beta



Groups are compared as follows: Control vs. Hp-LPS, †- Hp-LPS vs. Hp-LPS+TA and Hp-LPS+SFT, ‡- Hp-LPS+SFT vs. Hp-LPS+TA

Significance represented as a– $p<0.001$, NS- Non-significant using One way ANOVA- Dunnett's T3 multiple comparison test.

Plate 2a: Effect of TA on PCNA expression

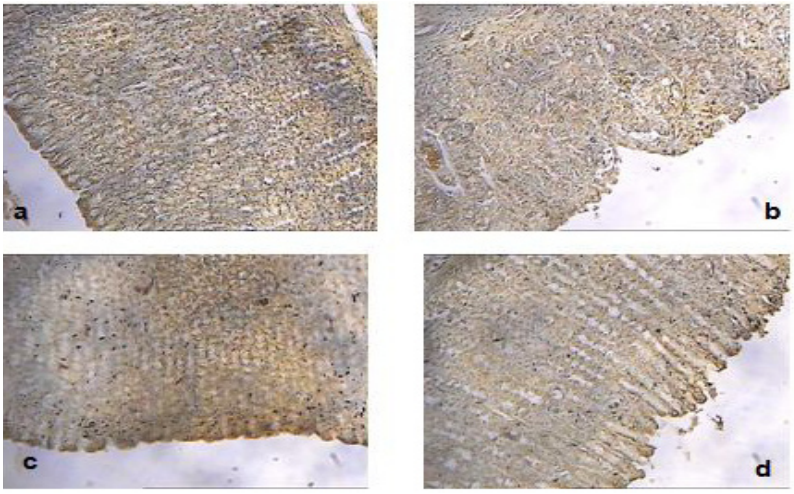


Plate 2a shows the effect of TA on PCNA expression in control and Hp-LPS induced experimental animals. PCNA positive cells were observed in gastric mucosa of control rats, Hp-LPS ulcerated rats showed lesser number of PCNA positive cells, whereas in Hp-LPS+TA rats and Hp-LPS+SFT rats abundant number of PCNA positive cells were observed.

Re-epithelialization is a key process in the ulcer-healing after mucosal injury. To restore the mucosal integrity the filling of the mucosal defect with proliferating and migrating epithelial and connective tissue cells is necessary [87]. The capacity of re-epithelialization is crucial in the recovery of the gastric mucosa after ulceration. Ulcer healing is a complex and tightly regulated process of filling the mucosal defect with proliferating and migrating epithelial and connective tissue cells [87]. Proliferating cell nuclear antigen (PCNA) plays an important role during DNA synthesis and cell proliferation. PCNA increases with *H pylori* infection [88]. Sun *et al*. [89] found PCNA was increased during healing of gastric mucosal injury. There are several evidences that PCNA assessment is a useful tool to evaluate cell proliferation [88].

Acute gastric mucosal injury is often accompanied with decreased cell proliferation and increased cell apoptosis, while cell apoptosis decreases during the healing of gastric ulcers

[90]. There is a balance between cell apoptosis and proliferation in normal gastric mucosa [91]. A few apoptotic cells exist in epithelial cells of normal gastric mucosa, but concentrated necrosis and apoptotic cells are found on the surface of ulcers [92]. In the present study, Hp-LPS induced gastric mucosal damage witnessed a significant decrease in the expression of PCNA. However, regeneration was marked in TA treated rats from a significant increase in the number of PCNA positive cells.

Figure 5a shows the effect of TA on the levels of plasma TNF-α and IL-1β in Hp-LPS induced experimental animals. Hp-LPS induced rats showed a significant increase ($p<0.001$) in TNF-α and IL-1 β levels compared to control rats, whereas Hp-LPS+TA and Hp-LPS+SFT animals showed a significant decrease ($p<0.001$) compared to Hp-LPS induced ulcer rats. A significant decrease ($p<0.001$) was observed in Hp-LPS+TA rats in both TNF α and IL-1 β levels compared with Hp-LPS+SFT.

Enhancement in gastric mucosal TNF-α production, excessive NO and prostaglandin generation, and alteration in the extent of epithelial cell apoptosis are associated with mucosal inflammatory responses in the animal model of *H. pylori* LPS-induced gastritis [93].

Acute mucosal inflammatory responses are accompanied by a massive epithelial cell apoptosis, and a marked increase in the expression of membrane-bound and soluble forms of TNF-α [94].

IL-1β is a potent inflammatory cytokine that is released as a component of the host response against bacterial infection. It is primarily expressed by activated monocytes/macrophages. IL-1β is produced as a precursor molecule, pro-IL-1β, in the cytosol of macrophages. Pro-IL-1β is a 31–34-kDa inactive form of the cytokine, which is later cleaved by caspase-1 to active 17-kDa IL-1β [95].

Soluble mediators of *H. pylori* are known to induce IL-1β. Of particular significance is the finding that IL-1β gene cluster polymorphisms suspected of enhancing production of IL-1β are associated with an increased risk of gastric cancer [96]. This makes it worthwhile to explore the mechanism of induction of IL-1β by *H. pylori*, and in particular, the role of LPS. The expression of IL-1β is regulated at the level of transcription [97], mRNA stabilization, and post-translational proteolytic processing [98].

Hp-LPS 26695 increased the levels of TNF-α and IL-1β probably in consequence to inflammatory response. As in other models of ulcers, TA also reduced the levels of TNF-α and IL-1β. Mucosal Sucralfate administration produced a reduction in the mucosal expression of TNF-α [5] which is in agreement with the present study.

4. Conclusion

The aqueous extract of *Terminalia arjuna* showed beneficial role in treating gastric ulcer induced by *Helicobacter pylori* lipopolysaccharide (one of the virulence factor for *Helicobacter pylori*). From the results observed in this study, it can be concluded that the evaluated anti-ulcer effect of aqueous extract of *Terminalia arjuna* had significant impact on inhibiting the aggressive factors such as acid and pepsin. The cytoprotective effect was evident from mucosal integrity and ulcer healing effect was mediated by maintenance of proinflammatory processes, gastric mucosal regeneration property.

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