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Antioxidant and antiulcer activity of aqueous extract of a polyherbal formulation

Annie Shirwaikar*, H N Aswatha Ram, P Mohapatra
Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal 576 104, India

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The aqueous extract of Hingwashtak churna was evaluated for gastroprotection in rats using the ibuprofen and ethanol induced ulcer models. Efficacy was assessed by determination of mean ulcer size, ulcer number and ulcer index. Oral administration of the aqueous extract (750 mg/kg) significantly protected against gastric lesions by 84.96% and 91.12% as compared to ranititidine (95.54 and 95.2%) in the ibuprofen and alcohol induced ulcer models respectively. The findings suggest that the significant gastroprotective activity could be mediated by its antioxidant activity which was evaluated by using different antioxidant models of screening.

Keywords*:* Alcohol, Antioxidant, Anti-ulcer, Hingwashtak churna, Ibuprofen

"Hingwashtak churna" is a polyherbal Ayurvedic medicine used as a digestive, carminative, astringent and as an antacid[1]. According to Ayurvedic physicians ulcer formation is due to the improper digestion of food. Though Hingwashtak churna is claimed to cure peptic ulcers by way of improving digestion[2,3], there is no scientific proof to support this claim. Recent studies have implicated the role played by free radicals and lipid peroxidation in the development of ulcers[4]. The present study reports the anti-ulcer activity of the aqueous extract of Hingwashtak churna on alcohol and ibuprofen induced peptic ulcer models in rats by comparison with reference drug rantitidine. Free radical scavenging activity was determined by using the DPPH, lipid peroxidation, nitric oxide and ABTS assays.

Materials and Methods

The chemicals and solvents used were 2,2-azinobis-(-3ethylbenzothiazoline-6-sulphonate) (ABTS), sodium nitroprusside, sulphanilamide, potassium superoxide, O-phosphoric acid, naphthyl ethylene diamine dihydrochloride, potassium chloride (KCl), ferrous sulphate ($FeSO_4$), thio barbituric acid (TBA), trichloro acetic acid (TCA), butyrated hydroxy toluene (BHT) and sodium hydroxide (NaOH) of analytical grade, 1,1-diphenyl, 2-picryl hydrazyl (DPPH) were obtained from Sigma Chemicals, USA. Ibuprofen and ranitidine were from Knoll Pharmaceutical Ltd., Goa, and J.B. Chemicals and Pharmaceuticals Ltd., Ankleshwar respectively.

Plant material — Hingwashtak churna consists of eight ingredients viz., *Piper nigrum, Piper longum, Zingiber officinale, Nigella sativa, Cuminum cyminum, Trachyspermum ammi, Ferula foetida*, and Rock salt (Saindhava Lavana)[5]. All these ingredients were procured locally and were authenticated by botanist Dr. K. Gopal Krishna Bhat, Professor of the Department of Botany, Poorna Prajna College, Udupi, Karnataka. A voucher specimen (PP 1-7) of the same has been deposited in the museum of the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal for future reference.

Preparation of Hingwashtak churna — The churna was prepared as per the procedure given in Ayurvedic Formulary of India. *Piper nigrum, Piper longum, Zingiber officinale, Nigella sativa, Cuminum cyminum* and *Trachyspermum ammi* (100g each) were fried in equal quantities. The fried ingredients were then powdered separately, passed through 80 # sieve and mixed together in an equal proportion with *Ferula foetida* fried in ghee and rock salt to get uniformly blended churna.

Preparation of the extract — Hingwashtak churna powder (100 g) was extracted with 2 l of chloroform water (1:1000) by maceration. The extract evaporated under vacuum gave a dry yield of extractive value

*Correspondent author
Phone : 0820 – 2922482
Fax : 0820 – 2571998
E-mail : annieshirwaikar@yahoo.com

27.45% (w/w) and was stored in a desiccator until further use. The aqueous extract of the churna obtained hereafter is referred as AQEHC.

Animals — Healthy Wistar adult albino rats of either sex, 2 to 3 months old and weighing between 150-200g were used. Animals were housed individually in polypropylene cages, maintained under standard conditions (12:12 L:D cycle; 25°±3°C and 35-60% humidity); the animals were fed with standard rat pellet diet (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance (IAEC/KMC/04/2003-2004).

In vitro antioxidant studies — *In vitro* free radical scavenging activity was determined by using DPPH, nitric oxide, lipid peroxidation assay and ABTS radical cation decolorization assay methods.

Preparation of rat brain homogenate[6] — Randomly selected rats were fasted overnight. They were sacrificed by cervical dislocation, dissected and the whole brain except cerebellum was removed quickly. It was further processed to get 10% homogenate in 0.15 *M* KCl[7] using a teflon homogenator. The homogenate was filtered to get a clear solution and used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation.

In vitro antilipid peroxidation assay — The lipid peroxidation in rat brain homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS). Different concentrations of the extract (10-1000 µg/ml) were made up with ethanol. The aqueous extract was expressed in terms of dry weight (mg/ml) in ethanol (95%). These samples were individually added to the brain homogenate (0.5 ml) and the mixture was incubated with 0.15 *M* KCl (100 µl). Lipid peroxidation was initiated by adding 100 µl of 15 mM $FeSO_4$ solution and the reaction mixture was incubated at 37°C for 30 min. An equal volume of TBA : TCA (1:1; 1ml) was added to the above solution followed by the addition of 1ml BHT. This final mixture was heated on a water bath for 20 min at 80°C, cooled, centrifuged and the absorbance read at 532 nm[8] using a spectrophotometer (Shimadzu 160 IPC). The percentage inhibition of lipid peroxidation was calculated[8] by comparing the results of the test with those controls not treated with the extract as per the formula:

$$\text{Inhibition (\%)} = \frac{(\text{control} - \text{test}) \times 100}{\text{Control}}$$

ABTS radical cation decolorisation assay[9] — ABTS radical cations ($ABTS^+$) were produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulfate. The mixture was allowed to stand in the dark at room temperature for 12-16 hr before use. Different concentrations (10-1000 µg/ml) of the aqueous extract (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up to 1 ml with ethanol. Absorbance was read at 745 nm[6] and the percentage inhibition was calculated by using the above formula.

DPPH radical scavenging activity — DPPH scavenging activity was measured by spectrophotometric method[10]. To an ethanolic solution of DPPH (200 µ*M*), 0.05 ml of aqueous extract dissolved in ethanol was added at different concentrations (10-1000 µg/ml). An equal amount of ethanol was added to the control. After 20 min the decrease in absorbance of test mixtures (due to quenching of DPPH free radicals) was read at 517 nm and the percentage inhibition was calculated[8].

Scavenging of nitric oxide radical[11,12] — Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction as described previously[13,14]. Sodium nitroprusside (5 mM) in standard phosphate buffer solution was incubated with different concentrations (10-1000 µg/ml) of the aqueous extract dissolved in phosphate buffer (0.025 M; *p*H : 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess' reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm.

Acute toxicity — Inbred male Swiss albino mice weighing 40-50g were starved overnight and divided into 6 groups of 6 each. They were orally fed with AQEHC suspended in 2% gum acacia, in increasing dose levels of 100, 500, 1000, 3000, 6000 and 10,000

mg/kg body weight[15]. The mice were observed continuously for 2 hr for behavioral, neurological and autonomic profiles and after a period of 24 and 72hr for any lethality[16].

Gastroprotective studies — Ibuprofen and alcohol induced gastric ulcer models were chosen for the experiment. Each model consisted of three groups of six rats each. The aqueous extract suspended in 2% gum acacia was administered orally at a dose level of 750 mg/kg. Ranitidine (2.5 mg/kg) suspension in acacia was used as the reference standard drug in both ibuprofen and alcohol induced gastric ulcers.

Ibuprofen induced gastric ulcers — Ibuprofen in the dose of 300 mg/kg was administered orally at 15 hr intervals to fasted rats to produce gastric ulcers[17]. The animals were sacrificed 6 hr after the second dose of ibuprofen. Stomachs were incised along the greater curvature and ulceration was scored. The stomachs were isolated and opened along the lesser curvature and the contents were washed off. The stomachs were then stretched over a frog-board and with the help of magnifying glass and millimeter scale, the number of ulcers and the length of each ulcer were measured[18]. Test drug and gum acacia were administered 1hr before the dose of ibuprofen.

Alcohol induced gastric ulcers — Rats fasted for 14-16 hr were orally administered with absolute alcohol (1ml/animal) 1hr after the administration of test drug and gum acacia alone to the vehicle control group animals[19]. Animals were sacrificed 3 hr after alcohol administration. Stomachs were incised along the greater curvature and ulceration was scored.

Histopathological evaluation — Animals of all the groups were sacrificed and the stomachs were immediately isolated, washed with saline and preserved in 10% formaldehyde solution for histopathological studies. The study was carried out within two days after the storage in formalin. The central part of the damaged or ulcerated tissue was cut into half along the long diameter and 2-5 tissue samples were taken. When there were no visible lesions in gastric mucosa, several sections were taken from different parts of antrum and corpus. After standard processing[20], the cut tissue (Eliot Inc.) embedded in paraffin and 4 μm thick were cut using a rotary microtome (Reichert Inc.), stained with haematoxylin–eosin and then examined under microscope (Olympus FHY).

Statistical analysis — The results were analysed by using one way ANOVA followed by post hoc Sheff's test using 7.5 version of SPSS computer software.

Results

In *in vitro* antioxidant studies, the AQEHC exhibited ABTS, DPPH scavenging nitric oxide generation and lipid peroxidation activity in a concentration dependent manner (Table 1). Acute toxicity studies were carried out and the extract was found to be safe up to a dose of 10,000 mg/kg. Treatment with 1ml of absolute ethanol and 300mg/kg dose of ibuprofen (Figs 2a and b; Table 2) showed formation of ulcers with an increase in histological and ulcer depth index as compared to the normal control (Figs 1, 2a and b). Treatment with 750 mg/kg AQEHC showed significant reduction in mean ulcer size, ulcer number and ulcer index as compared to ranitidine in both models tested (Figs 2c-f; Table 3). The gastroprotection due to AQEHC in ibuprofen model and alcohol model was 84.96 and 91.12% as compared to 95.54 and 95.2% due to ranitidine. Histopathological studies further confirmed that pre-treatment with AQEHC inhibited the ethanol and ibuprofen induced congestion, oedema, haemorrhage and necrosis in gastric mucosa (Table 4).

Discussion

Table 1— Effect of AQEHC on different antioxidants
[Values showing % inhibition of free radical scavenging activity are mean ± SE]

Conc. (μg/ml)	DPPH	ABTTS	Nitric oxide	Lipid peroxidation
1000	88.30±0.03	98.79±0.09	88.76±0.79	89.34±0.04
750	81.17±0.62	91.46±0.06	81.63±0.12	85.79±0.26
500	75.77±0.16	87.57±0.04	75.25±0.56	80.56±0.04
400	71.24±0.03	82.14±0.16	71.63±0.23	75.26±0.79
300	55.50±0.09	76.38±0.24	68.31±0.03	67.34±0.66
200	47.07±0.16	65.36±0.29	51.24±0.31	60.41±0.72
100	31.12±0.12	49.61±0.20	44.67±0.55	58.67±0.37
80	22.13±0.03	40.73±0.51	41.24±0.65	48.63±0.36
60	18.1±0.04	33.14±0.33	39.13±0.36	41.14±0.25
40	15.11±0.03	19.43±0.36	36.45±0.30	35.65±0.12
20	12.13±0.12	14.92±0.36	33.71±0.79	34.40±0.25
10	10.13±0.15	12.19±0.13	20.78±0.04	22.71±0.90

NSAIDS are known to induce peptic ulcer not only by denaturing mucous glyco-proteins but also by free radical formation[21,22]. Similarly, alcohol is also known to produce free radicals and induce peptic ulcers[23]. The free radicals produced cause lipid peroxidation, leading to membrane fluidity which in turn increases the influx of Ca^{2+} ions and results in the reduced membrane integrity of surface epithelial cells, thereby generating gastric ulcers[24,25]. Free radicals have been demonstrated to be a contributing factor in tissue injury and in the modulation of pain[26]. The incidence of ethanol induced ulcers, predominant in the glandular part of the stomach has been reported to stimulate the formation of leukotriene C4 (LTC4), mast cell secretory products[27] and reactive oxygen species[28] resulting in the damage of rat gastric mucosa[29].

Table 2 — Histopathological examination of gastric lesions in alcohol and ibuprofen induced gastric ulcer models

Model	Group	Grade	Histological index	Size of the ulcer in 3mm of gastric mucosa (%)	Ulcer depth index
Alcohol	Control	4	1.56	68.42	1.37
	Ranitidine	2	2.34[a]	35.63[a]	3.67
	AQEHC (750 mg/kg)	3	1.84[a]	41.27[a,b]	2.51[a]
Ibuprofen	Control	4	1.47	69.23	1.72
	Ranitidine	2	2.13[a]	36.54[a]	3.73[a]
	AQEHC (750 mg/kg)	3	1.76[a]	43.23[a,b]	2.62[a]

Grade 1: Normal appearance without any damage; 2 : Shallow damage not exceeding 25% of the mucosal depth; 3: moderate damage reaching beyond 25% of the mucosal depth but not exceeding 75%, 4: deep damage reaching beyond 75% of mucosal depth.

P values: <0.05; [a] vs control, [b] vs ranitidine

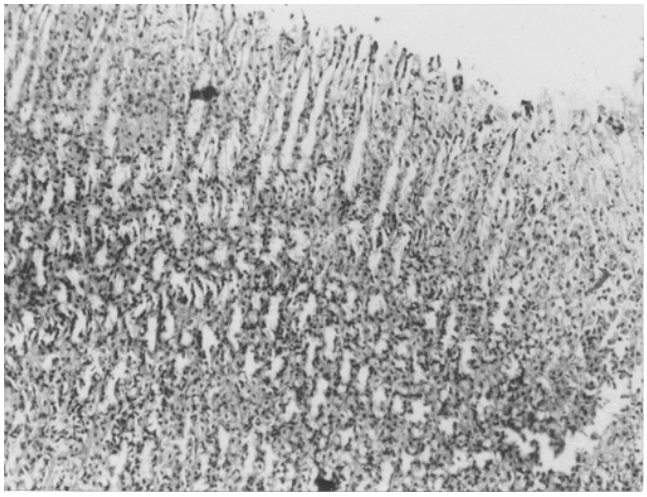

Fig. 1— The stomach wall of a control rat showing its normal appearance (H&E, 50×).

Shirwaikar *et al.*[30] reported gastroprotective activity of ethanolic extract of *Ageratum conyzoides* based on Ca^{2+} channel blocking activity by regulating gastric acid secretion and mediating free radical generation in peptic ulcers. Aswatha Ram *et al.*[31] reported that the anti-ulcer activity of the aqueous extract of *Murraya koenigii* could be due to a non-specific inhibitory effect on the parietal cell function which antagonises the ulcerogenic and gastric secretory effect in rats. In the present study, AQEHC was found to possess free radical scavenging effect in DPPH, ABTS, nitric oxide and lipid peroxidation assays, thereby showing its antioxidant property. Administration of AQEHC (750 mg/kg) exhibited marked gastro-protection in both the ibuprofen and alcohol model with the alcohol model showing more significant protection. The tissue protection by AQEHC was further confirmed by histopathological studies.

The ingredients present in Hingwashtak churna have been shown in different studies to possess biological properties related to antioxidant mechanisms eg. *Nigella sativa* has been observed to decrease the lipid peroxidation and liver enzymes and thus increases the antioxidant defence system[32]. The five phenolic amides of *Piper nigrum* are documented to possess antioxidant activity, more significant than α-tocopherol[33]. Zerumbone, a sesquiterpene and gingerol, a plant phenol both active constituents of another ingredient of Hingwashtak churna, *Zingiber officinale* are reported antioxidants[34]. while *Ferula foetida* is a known potent antioxidant documented to afford protection against free-radical mediated diseases like carcinogenesis[35]. Agarwal *et al.*[36] have reported the ability of the aqueous extract of *Piper*

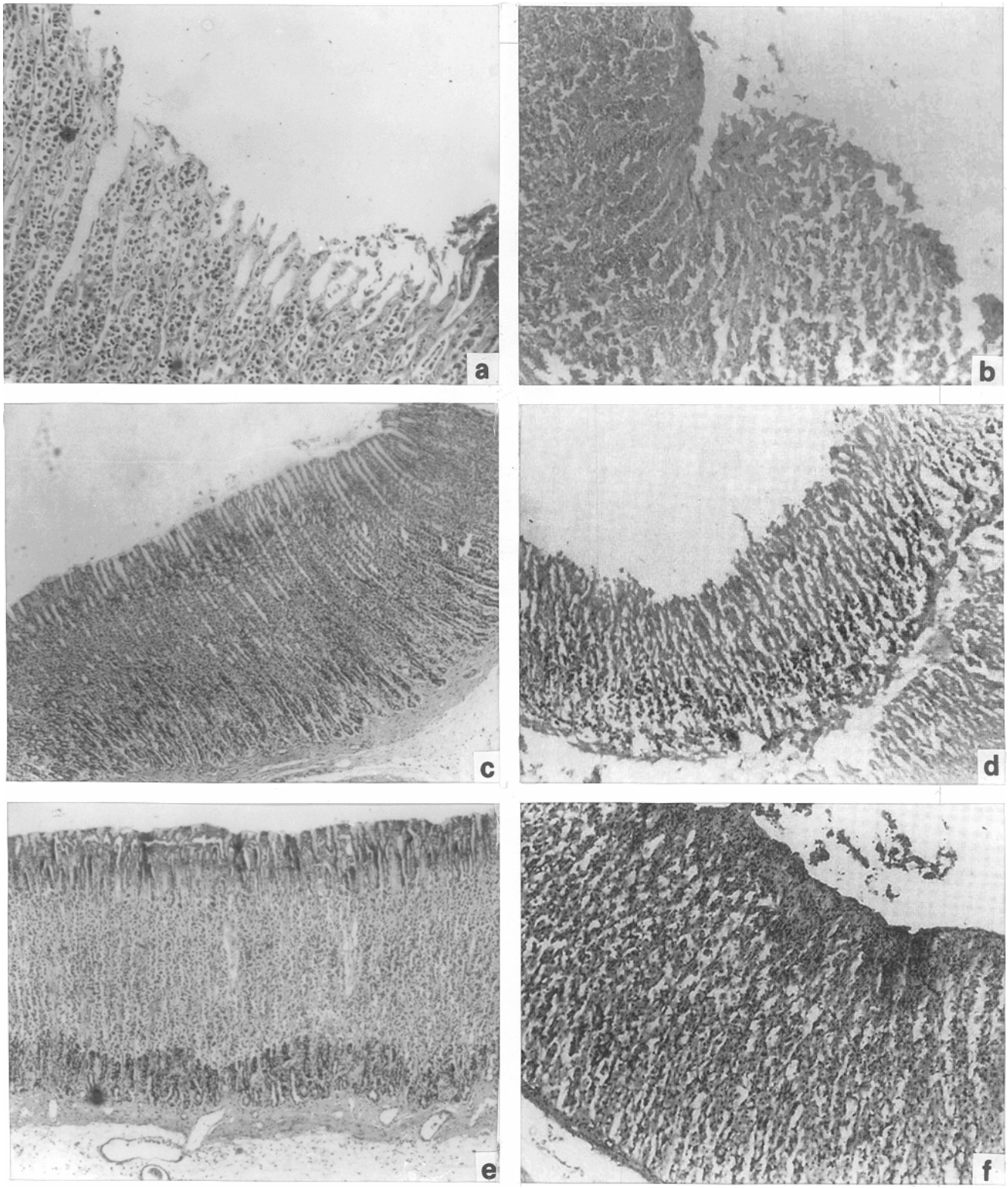

Fig. 2 — The stomach wall of a control rat after treatment by gavage (H & E, 50×) with (a) 1ml of absolute ethanol. (b) 300mg/kg dose of Ibuprofen (c) Ranitidine (2.5mg/kg) and 1ml of absolute ethanol (d) Ranitidine (2.5 mg/kg) and 300mg/kg dose of Ibuprofen (e) Hingwashtak churna (750 mg/kg) and 1ml of absolute ethanol and (f) Hingwashtak churna (750 mg/kg) and 300mg/kg dose of Ibuprofen.

Table 3— Effect of AQEHC on ibuprofen and alcohol induced gastric ulcers
Values are mean ± SE of 6 animals in each group

Groups	Parameter	Vehicle	AQEHC (750 mg/kg)	Ranitidine (2.5 mg/kg)
Ibuprofen	Ulcer number	12.64 ± 0.19	6.29 ± 0.08[a]	4.57 ± 0.11[a]
	Ulcer size (mm)	28.64 ± 0.43	8.49 ± 0.11[a,b]	3.52 ± 0.14[a]
	Ulcer index	362.12 ± 8.63	54.43 ± 0.54[a]	16.14 ± 0.76[a]
	Percentage protection	-	84.96	95.54
Alcohol	Ulcer number	23.69 ± 0.16	7.37 ± 0.14[a]	4.28 ± 0.10[a]
	Ulcer size (mm)	12.78 ± 0.36	3.6 ± 0.10[a,b]	3.36 ± 0.17[a]
	Ulcer index	299.11 ± 11.31[a]	26.54 ± 0.74[a]	14.34 ± 0.54[a]
	Percentage protection	-	91.12	95.2

One way ANOVA followed by post hoc Sheffe's test
P values: < 0.05; [a] vs control, [b] AQEHC vs ranitidine

Table 4 — Effect of AQEHC on induction of histopathological lesions in alcohol and ibuprofen induced gastric ulcer model

Treatment	Congestion	Oedema	Haemo-rrhage	Necrosis
Normal control	-	-	-	-
Alcohol control	++	++++	++	+++
Ranitidine	+	+	+	+
AQEHC (750 mg/kg)	+	++	+	++
Ibuprofen control	++	++++	+++	+++
Ranitidine	+	+	++	+
AQEHC (750 mg/kg)	+	++	++	+

Normal; +: little effect; ++: appreciable effect; +++: severe effect; ++++: very severe effect.

longum, Zingiber officinalis and Ferula species to augment mucin secretion and to decrease cell shedding in the stomach of the rat.

Hence, the significant antioxidant activity of AQEHC in the present study may be attributed to these aforementioned potent antioxidant ingredients of Hingwashtak churna. It is also probable that AQEHC may offer gastro protection by increasing prostaglandins, thus protecting the stomach from ethanol injury[37]. The effects of AQEHC were found to be comparable with that of ranitidine in reducing the ulcer number and ulcer index.

To conclude, AQEHC holds promise as an adjunct to existing drugs used in peptic ulcer therapy. Further work is necessary to elucidate the actual mechanism involved in the anti-ulcer activity of this polyherbal formulation.

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