Ashwagandha Seeds Extract Supplementation Afford Comparable Therapeutic Effect to Proton Pump Inhibitors in Stress induced Gastric Ulcer in rats

Mai Adawi¹, Karima El-Sayed¹, Amaal Nabil², Wael Maher³,⁴ Bassma M. Dessouki⁵, Sally M. Abdelmonem¹

¹ Medical Physiology Department, Faculty of Medicine, Suez Canal University Ismailia, Egypt
² Medical Pharmacology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
³ Pathology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt
⁴ Department of Basic Sciences, College of Medicine, Sulaiman Al Rajhi University, Al Bukayriyah, Saudi Arabia
⁵ Department of Human Anatomy and Embryology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Abstract
Background: Gastric ulcer (GU) is the most common disease with a prevalence of 20–60 per 100,000 population and accounts for 5–10% mortality worldwide. Along term occurrence with GU has maximum risk of stomach cancer. Ashwagandha can be used in herbal medicine for treatment of stress, headache, muscle pains and convulsions. Proton pump inhibitors (PPIs) are drugs widely prescribed for many patients to treat gastrointestinal disorders, such gastroesophageal reflux, and peptic ulcer. Aim: To investigate whether ashwagandha and/or PPIs could protect against stress induced gastric ulcer and if this protection is mediated the synthesis and release of inflammatory and oxidative stress markers. Material and Methods: Thirty adult male rats were randomized, into 5 groups/6 rats each Stress gastric ulcer was induced, both Ashwagandha and PPI were administrated orally for 15 days after induction of GU. Withdrawal of blood samples for chemical and spectral assay of antioxidant markers were done. Histopathology and immunohistochemistry of TGF-β and TNF-α were done. Results: Both ashwagandha treated group and PPI treated group showed significant decrease in MDA level. The combined treated group showed much more decrease in the MDA level and There was a significant increase in GSH level in Ashwagandha treated group. Conclusion: Ashwagandha possesses promising stress-induced gastric ulcers healing activity. It improved stomach function, gastric pH, acid secretion, and reduced mucosal hemorrhagic lesions with restoration of the architecture of the mucosal layer in rats by its antioxidant activity, suppressing the inflammatory cascade, promotion of gastric barrier repair and inhibiting TGF-β/SMAD4 pathway.

Keywords
- TNF-α
- Tumor necrosis factor – α
- TGF-β
- Transforming growth factor-β
- Reactive oxygen species

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Corresponding author: Ahmed, Karima El-Sayed, assistant professor of Medical Physiology. Tel. : 01003541288
Email : karima_ahmed@med.suez.edu.eg
Introduction

Gastric ulcer (GU) is the most common disease with a prevalence of 20–60 per 100,000 population and accounts for 5–10% mortality worldwide (1). Long term occurrence with GU or duodenal ulcer has maximum risk of stomach cancer (2). It has been reported that GU is more common in patients with liver cirrhosis, type 2 diabetes mellitus and mental health disorders (3). GU occurs due to the presence of several factors such as acid, pepsin, bile acids, Helicobacter pylori, ethanol, stress and non-steroidal anti-inflammatory drugs (NSAIDs) that interfere with defensive factors such as tight junctions between epithelial cells, microvascular blood circulation, bicarbonate secretion, availability of prostaglandins and nitric oxide (4). Ashwagandha, Withania somnifera, is known as “Indian Winter cherry” or “Indian Ginseng”. Ashwagandha is found as a churns or powder which mixed with water, butter or honey. In traditional medicine and remedies its popular herb is used (5). Ashwagandha is memory enhancer and improves the brain and nervous system functions. It enhances resilience of the body to stress as it has adaptive power. Ashwagandha improves the cell-mediated immunity increasing the body's defense against disease. Moreover, its extract elicits antioxidant capacity that protect against cellular damage caused by free radicals (6). In addition, ashwagandha displays anti-inflammatory activity, and its extracts have possessed neuroregenerative, anti-stress and anticancer potentials (7). To our knowledge, Ashwagandha’s pharmacological effects have not been understood to large extent, therefore; the present study investigated the activity of Ashwagandha.

Proton pump inhibitors (PPIs) are drugs widely prescribed for many patients to treat gastrointestinal disorders, such gastro-esophageal reflux disease, peptic ulcer (particularly related to Helicobacter pylori infection), acid-related dyspepsia and gastro-duodenal lesions (8). They reduce gastric acid production by inhibiting the (H+/K+ ATPase) located on the secretory surface of gastric parietal cells (9). There are five PPIs available in the market, including omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole. It has been shown that continuous maintenance treatment with esomeprazole provides more effective acid suppression than other PPIs in patients with gastric esophageal reflux (10). Esomeprazole has slower plasma clearance, which results in higher plasma concentrations. This offers better clinical efficacy and more effective management of the hyperacidity diseases (11).

2. Materials and Methods

2.1. Experimental Animals

Eight-week-old male albino rats (weight 150 – 200 g) were purchased from animal house in faculty of Veterinary Medicine, SCU, Ismailia, Egypt; they were housed in the animal house of Faculty of Medicine, Suez Canal University and maintained in polyethylene cages in a clean animal room. Experimental conditions: temperature 20°C; normal light-dark cycle; and continuously available food and water. Animals were acclimatized a week before starting the experiment. All procedures were performed at the Physiology Department, Faculty of Medicine, Suez Canal University (Ismailia, Egypt). This study was performed in accordance with the guide for the care and use of laboratory animals.
2.2. Ethical statement:
All experimental procedures on animals that were being used for this study followed the standards of the Research Ethics Committee of the Faculty of Medicine, Suez Canal University, Ismailia, Egypt. Ethical approval for conducting the work was obtained from Suez Canal University (SCU) Animal Ethics Committee (number: 5443). All the rats (three rats per cage) were housed in polypropylene cage under standard laboratory conditions with food and water provided ad libitum. Animals were handled gently, housed with suitable environmental and nutritional conditions, assessed for general health and body weight and anesthetized prior to sample collection, by an intraperitoneal injection of ketamine/xylazine, followed by cervical dislocation to minimize pain.

2.3. Chemicals
The drugs used for the study were ashwaganda and PPIs. Esomeprazole was manufactured by AstraZeneca, (Egypt) in the form of sachet containing 10 mg. Each sachet was dissolved in 10 ml distilled water then 5mg/kg (1ml of the prepared solution), was given orally by an intragastric tube once daily for 15 days (12). Ashwagandawas purchased from Pimus pharmaceutical Inc. (Scottsdale, AZ, USA).

2.4. Experimental Model of stress gastric ulcer.
Twenty four hours before the experiment, rats were deprived of food in mesh-bottomed cages with free access to water until the last two hours before the experiment. Cold gastric stress ulcer was induced in rats by fixing the four limbs to a wooden board and they were placed in a refrigerator at 4°C for 3 hours(13).

2.5. Study Groups:
Thirty adult male rats were allocated into five groups (6 rats/group): Group 1 (control group, CTL): Rats in this group were fed with normal chow diet and received 0.2 ml of 0.5% carboxymethylcellulose (CMC) orally once daily for 15 days. Group 2 (gastric ulcer group: GU): Stress gastric ulcer was induced fixing the four limbs to a wooden board and they were placed in a refrigerator at 4oC for 3 hours; Group 3 (ashwaganda treated group): Rats in this group have stress gastric ulcer as in group2 and then were treated with ashwaganda for 15 days, ashwaganda was given orally by gavage at the dose of 50 mg/kg/day was dissolved in 0.5% carboxymethylcellulose (CMC). Group 4 (Esomeprazole treated group. PPIs): Rats in this group have stress gastric ulcer as in group2 and then were treated with Esomeprazole 5ml/kg/day by oral gavage 15 days. Group 5 (gastric ulcer, treated with both ashwaganda and Esomeprazole treated group) (GU, combined treated group): Rats in this group have stress gastric ulcer as in group2 and then were treated with both ashwaganda and Esomeprazole for 15 days. After 15 days, rats were anaesthetized and sacrificed; blood samples were collected by direct cardiac puncture for lab parameters. Gastric tissue was collected for histopathological examination.

2.6. Evaluation Parameters:
2.6. a. Spectral assay of antioxidant markers:
Serum malondialdehyde (MDA) and Reduced Glutathione (GSH) levels were measured using a colorimetric assay. Serum MDA and GSH were assessed using kits supplied by Sigma Aldrich Co., USA, according to the instructions of the
Ashwagandha Seeds Extract Supplementation Afford Comparable Therapeutic Effect to Proton Pump Inhibitors in Stress induced Gastric Ulcer in rats

manufacturer and GSP kits supplied by Sigma Aldrich Co., USA (14)

At the end of the experiment animals were sacrificed. The rats were dissected and after opening the stomach the gastric juice was collected.

2.6. b. Ulcer Index:
The stomachs were washed with cold saline and the gross mucosal lesions were scored by two investigators and expressed in terms of ulcer index (U.I.) as previously described:
0=no lesions; 1=lesions <1 mm length; 2=lesions 2-4 mm length and 3=lesions >4 mm length. Then score for each rat was calculated as the number of lesions in the rat multiplied by respective factor (15)

2.6. c. Gastric Acidity:
After dissection and removal of the stomach, gastric juice was collected. Then it was centrifuged for 10 min at 3000 x g while the supernatant was separated and used to analyze for pH, gastric juice volume, and total acidity (16)

2.6. c.1. Gastric Juice Volume
The volume of gastric juice (ml) was determined.

2.6. c.2. PH
The pH of the collected supernatant was measured with a pH meter (16)

2.6. c.3. Total Acidity
Total acid secretion in the gastric juice supernatant was determined by titration to pH 7.0, using a 0.01 N NaOH solution, and phenolphthalein indicator. The acidity obtained from the titration method was converted to total acid output (Eq) using the following equation: (17)

Total acid output (Eq) = titratable acidity (ml)×2×10

2.6. c.4. Adherent Mucus Weight
Adherent mucus weight was determined as follows: the mucus covering the stomach wall was carefully scrapped using a glass slide into a small sample tube containing 1 mL of water whose weight was predetermined. The weight of the container and mucus was taken using a digital electronic balance and the difference taken as the weight of the mucus (2)

2.7. Histopathological evaluation:
The samples were fixed with 10% formalin and embedded in paraffin. From each block, sections of 3µm thickness were submitted, mounted to glass slide, stained by hematoxylin and eosin (H&E) and examined by an independent pathologist. Slides were scanned and images were processed using ImageJ scanner and viewer software (LOCI, University of Wisconsin, US)

- **Histopathological study of stomach tissue sections:**
Gastric tissue sections were examined for ulcerative and inflammatory changes and for healing process, according to the following and scored for the following: (18)

  - Inflammation (none = 0, mild = 1, moderate = 2, and severe = 3)
  - Inflamed area/extent (none = 0, mucosa = 1, mucosa and submucosa = 2, and transmural = 3)
  - Percent involvement (none = 0, 1–25% = 1, 26–50% = 2, 51–75% = 3, and 76–100% = 4).
  - Scar tissue formation (none=0, mature scar tissue=1, granulation tissue=2)

2.8. Immunohistochemical staining:
Sections from the selected paraffin blocks were cut into 4 µm thick sections for immunohistochemical
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(IHC) staining. Slides were prepared and incubated with primary anti-transforming growth factor beta (TGF-β) antibody (ab215715, EPR21143, Abcam; Cambridge, UK) and anti-tumor necrosis factor alpha (TNF-α) (ab6671, Abcam). This was followed by incubations with the properate secondary antibody (Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP, Abcam; Cambridge, UK). All slides are lightly counterstained with hematoxylin for 30s prior to dehydration and mounting.

- **Immunohistochemical evaluation**

Cells with cytoplasmic reaction to TGF-β and/or TNF-α antibodies were considered positive. Semi-quantitative analysis of positive-stained tissue sections was performed through modified Allred scoring system guidelines **. The percentage of positive cells was estimated in 3 different high-power fields (hpf) (400x) and the average percentage (±SD) was calculated. Individual scores of the percentage of positive cells (0–5) and the staining intensity of the cytoplasm (0–3) were summed up to obtain the final grades. The percentage of positive cells was set as follows: 1-less than 10% positive cells; 2- from 10% to 20% of positive cells; 3-from 20% to 50% positive cells; 4-from 50% to 70% positive cells; and score 5-more than 70% positive cells. The staining intensity of SMA positivity in the cytoplasm was scored as: 1-weak; 2-medium; and 3-strong (19).

2.9. Statistical Analysis

Statistical tests were performed using the SPSS IBM version 25. Data were expressed as means ±SD. Statistical differences in numeric data showing a normal distribution were evaluated using one-way ANOVA followed by Bonferroni post hoc test for comparing study groups. Three high power fields (magnification 100) from five serial sections (fixed 15 _minter-sections) per animal for each group were examined. Data from scoring data were presented as medians and analyzed by a nonparametric ANOVA and a post hoc test. The differences were considered significant when p ≤ 0.05.

![Figure 1: serum malondialdehyde and reduced glutathione levels.](image)

Values are mean ± SD

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha
Figure 2: Comparison of ulcer index among rats groups. Values are mean ± SD
a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+ Ashwagandha

Figure 3: Comparison of PH among rats groups Values are mean ± SD
a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+ Ashwagandha

Figure 4: Comparison of total acidity among rats groups. Values are mean ± SD
a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+ Ashwagandha

Figure 5: Comparison of adherent mucus weight among rats groups. Values are mean ± SD
a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+ Ashwagandha
Figure 6: Comparison of gastric juice volume among rats groups. Values are mean ± SD

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU + Ashwagandha

A) Gross pictures:

<table>
<thead>
<tr>
<th>G1/Normal: Stomach shows uniform grey, white mucosa with preserved rugae</th>
<th>G2/Diseased (GU): Inner wall of the mucosa becomes reddish congested with foci of hemorrhage (Black arrows) and an area of mucosal flattening (Black rectangle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3/GU+Ashwaganda: There is slight reduction in mucosal congestion. No evidence of hemorrhagic foci. There is still an area of mucosal flattening (Black rectangle)</td>
<td>G4/GU+PPi: The mucosal congestion significantly reduced with no evidence of hemorrhagic foci. There are scattered foci of mucosal flattening (Black arrows)</td>
</tr>
<tr>
<td>G5/GU+Combined treatment: The mucosa regained its uniform grey appearance, with uniform rugae.</td>
<td></td>
</tr>
</tbody>
</table>
B) Microscopic pictures

1- Group 1 (Normal control)

Uniform gastric tissue, with regular surface epithelium (Black arrows), underlying uniform gastric glands (Red arrows) with no evidence of inflammation (H&E, 20x. Scale bar=50 µm)

Weak focal expression of TGF-β in superficial gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

Weak expression of TNF-α in scattered gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

2- Group 2 (GU)

There is surface ulceration (Black arrows) with underlying dense chronic lympho-plasmacytic inflammatory cells (Black arrowheads). There are few detected gastric glands (Red arrows) (H&E, 10x. Scale bar=100 µm)

Higher magnification of the previous figure showing surface ulceration (Black arrows), dense chronic inflammatory cells (Black arrowheads) and gastric glands (Red arrows) (H&E, 20x. Scale bar=50 µm)

Strong diffuse expression of TGF-β in gastric mucosal glands (Black arrows) next to ulcerated mucosa (IHC, 20x. Scale bar=50 µm)

Moderate focal expression of TNF-α in superficial gastric mucosal glands (Black arrows) next to ulcerated mucosa (IHC, 20x. Scale bar=50 µm)
3- Group 3 (GU+Ashwaganda treated)

There is restoration to the surface epithelium (Black arrows). Mucosal glands are regenerated (Red arrows) with areas of fibrosis present (Red arrowheads). There is significant reduction in the inflammatory cells infiltrate (Black arrowheads) with areas of edema (Green arrows) (H&E, 20x. Scale bar=50 µm)

The expression of TGF-β was noticed in lamina propria (Black arrowheads) with focal expression in few regenerating gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

The expression of TNF-α was noticed in lamina propria (Black arrowheads) with focal expression in base of few regenerating gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

4- Group 4 (GU+Ppi treated)

There is restoration to the surface epithelium (Black arrows). Mucosal glands are regenerated (Red arrows) with areas of fibrosis present (Red arrowheads). The deeper glands show regenerating epithelium with hyperchromatic nuclei (Blue arrowheads). There is significant reduction in the inflammatory cells infiltrate (Black arrowheads) (H&E, 20x. Scale bar=50 µm)

Strong diffuse expression of TGF-β in regenerating gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

Weak focal expression of TNF-α in superficial gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)
5- Group 5 (GU+ Combined treated)

There is restoration to the surface epithelium (Black arrows). Mucosal glands are regenerated (Red arrows). No areas of fibrosis detected. The deeper glands show regenerating epithelium with hyperchromatic nuclei (Blue arrowheads). There are no detected inflammatory cells infiltrate (Black arrowheads) (H&E, 20x. Scale bar=50 µm)

Moderate focal expression of TGF-β in gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

Weak focal expression of TNF-α in superficial gastric mucosal glands (Black arrows) (IHC, 20x. Scale bar=50 µm)

Results

A- Markers of oxidative stress

Table 1: Comparison of serum MDA and GSH among rats groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GU group</th>
<th>GU+Ashwagandha group</th>
<th>GU+PPI group</th>
<th>GU+combined treated group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA mean±SD</td>
<td>155.29±2.69</td>
<td>195.57±4.65</td>
<td>27.57±1.72</td>
<td>30±04.32</td>
<td>21.57±0.761.99</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>GSH mean±SD</td>
<td>6.56±0.13</td>
<td>6.13±0.18</td>
<td>15.27±0.39</td>
<td>16.54±0.35</td>
<td>17.51±0.41</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant as p<0.05 using ANOVA

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha group, d: statistically significant with GU+PPI group, e: statistically significant with GU+combined Treated group.

In this table, MDA showed statistical significant difference between groups with the lowest mean among GU+combined treated group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between GU+Ashwagandha group and GU+PPI group. GSH showed statistical significant difference between groups with the highest mean among GU+combined treatment group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between control group and GU group.

B- Results of Ulcer Index

Table 2: Comparison of ulcer index among rats groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GU group</th>
<th>GU+Ashwagandha group</th>
<th>GU+PPI group</th>
<th>GU+combined Treated group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcer index mean±SD</td>
<td>0±0</td>
<td>24.14±1.95</td>
<td>6.57±1.27</td>
<td>4.86±0.69</td>
<td>2.71±0.76</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant as p<0.05 using ANOVA

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha group d: statistically significant with GU+PPI group, e: statistically significant with GU+combined Treated group.

In this table, ulcer index showed statistical significant difference between groups with the lowest mean among control group followed by GU+combined treated group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between GU+Ashwagandha group and GU+PPI group.
C. Results of gastric acidity

1. PH Results:

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GU group</th>
<th>GU+Ashwagandha group</th>
<th>GU+PPI group</th>
<th>GU+combined treated group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td>3.59±0.39&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>2.13±0.22&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>4.51±0.32&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>5.33±0.28&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>5.61±0.20&lt;sup&gt;abcd&lt;/sup&gt;</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant as p<0.05 using ANOVA

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha group, d: statistically significant with GU+PPI group, e: statistically significant with GU+combined TTT group.

In this table, PH showed statistical significant difference between groups with the highest mean among GU+combined treated group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between GU+PPI group and GU+combined treated group.

2. Total acidity Results:

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GU group</th>
<th>GU+Ashwagandha group</th>
<th>GU+PPI group</th>
<th>GU+combined treated group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total acidity mean±SD</td>
<td>68.86±2.41&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>91.14±1.22&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>38.71±1.11&lt;sup&gt;abcde&lt;/sup&gt;</td>
<td>34.29±1.79&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>33.71±1.98&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant as p<0.05 using ANOVA

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha group, d: statistically significant with GU+PPI group, e: statistically significant with GU+combined TTT group.

In this table, total acidity showed statistical significant difference between groups with the lowest mean among GU+combined treated group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between GU+Ashwagandha group and GU+PPI group.

3. Adherent mucus weight Results:

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GU group</th>
<th>GU+Ashwagandha group</th>
<th>GU+PPI group</th>
<th>GU+combined treated group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent mucus weight mean±SD</td>
<td>0.98±0.0079&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>0.45±0.013&lt;sup&gt;bcde&lt;/sup&gt;</td>
<td>0.63±0.034&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.737±0.016&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.846±0.03&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant as p<0.05 using ANOVA

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha group, d: statistically significant with GU+PPI group, e: statistically significant with GU+combined TTT group.

In this table, adherent mucus weight showed statistical significant difference between groups with the highest mean among control group followed by GU+combined treated group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between GU+Ashwagandha group and GU+PPI group.

4. Gastric juice volume Results:

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>GU group</th>
<th>GU+Ashwagandha group</th>
<th>GU+PPI group</th>
<th>GU+combined treated group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric juice volume mean±SD</td>
<td>1.868±0.49&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>4.16±0.15&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>3.01±0.19&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.53±0.33&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.39±0.29&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant as p<0.05 using ANOVA

a: statistically significant with control group, b: statistically significant with GU group, c: statistically significant with GU+Ashwagandha group, d: statistically significant with GU+PPI group, e: statistically significant with GU+combined TTT group.

In this table, gastric juice volume showed statistical significant difference between groups with the lowest mean among control group followed by GU+combined treated group (p<0.001). Regarding comparison between groups, post hoc test showed statistical significant differences between each two groups except between GU+PPI group and GU+ combined treated group.
**D- Histopathological Results:**

**Table 7: Histopathological evaluation of gastric tissue sections**

<table>
<thead>
<tr>
<th>Gastric tissue</th>
<th>Histopathological scoring</th>
<th>Final score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Normal)</td>
<td>• Inflammation (none=0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Inflamed area/extent (none=0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Percent involvement (none=0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Scar tissue formation (none=0)</td>
<td></td>
</tr>
<tr>
<td>Group 2 (GU)</td>
<td>• Inflammation (severe=3)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>• Inflamed area/extent (mucosa and submucosa=2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Percent involvement (70%=3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Scar tissue formation (none=0)</td>
<td></td>
</tr>
<tr>
<td>Group 3 (GU+ashwaganda)</td>
<td>• Inflammation (moderate=2)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>• Inflamed area/extent (mucosa=1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Percent involvement (50%=2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Scar tissue formation (Fibrosis=1)</td>
<td></td>
</tr>
<tr>
<td>Group 4 (GU+ Ppi)</td>
<td>• Inflammation (mild=1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>• Inflamed area/extent (mucosa=1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Percent involvement (25%=1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Scar tissue formation (Fibrosis=1)</td>
<td></td>
</tr>
<tr>
<td>Group 5 (Combined treatent)</td>
<td>• Inflammation (none=0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>• Inflamed area/extent (none=0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Percent involvement (none=0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Scar tissue formation (none=0)</td>
<td></td>
</tr>
</tbody>
</table>

GU: Gastric ulcer, Ppi.; Proton pump inhibitor.

**Table 8: Immunohistochemical scoring for TGF-β expression in gastric tissue.**

<table>
<thead>
<tr>
<th>Gastric tissue</th>
<th>Percentage of positive cells (Average ± SD) (Score)</th>
<th>Staining intensity (Score)</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Normal)</td>
<td>15 ±8.7 (Score 2)</td>
<td>Weak (Score 1)</td>
<td>3</td>
</tr>
<tr>
<td>Group 2 (GU)</td>
<td>76.7 ±7.6 (Score 5)</td>
<td>Strong (Score 3)</td>
<td>8</td>
</tr>
<tr>
<td>Group 3 (GU+ashwaganda)</td>
<td>65 ±8.7 (Score 4)</td>
<td>Strong (Score 3)</td>
<td>7</td>
</tr>
<tr>
<td>Group 4 (GU+ Ppi)</td>
<td>55 ±5 (Score 4)</td>
<td>Strong (Score 3)</td>
<td>7</td>
</tr>
<tr>
<td>Group 5 (Combined treatent)</td>
<td>26.7 ±2.9 (Score 3)</td>
<td>Moderate (Score 2)</td>
<td>5</td>
</tr>
</tbody>
</table>

GU: Gastric ulcer, Ppi.; Proton pump inhibitor.

**Table 9: Immunohistochemical scoring for TNF-α expression in gastric tissue.**

<table>
<thead>
<tr>
<th>Gastric tissue</th>
<th>Percentage of positive cells (Average ± SD) (Score)</th>
<th>Staining intensity (Score)</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Normal)</td>
<td>3.3 ±2.9 (Score 1)</td>
<td>Weak (Score 1)</td>
<td>2</td>
</tr>
<tr>
<td>Group 2 (GU)</td>
<td>45 ±5 (Score 3)</td>
<td>Moderate (Score 2)</td>
<td>5</td>
</tr>
<tr>
<td>Group 3 (GU+ ashwaganda)</td>
<td>20 ±5 (Score 3)</td>
<td>Weak (Score 1)</td>
<td>4</td>
</tr>
<tr>
<td>Group 4 (GU+ Ppi)</td>
<td>15 ±5 (Score 2)</td>
<td>Weak (Score 1)</td>
<td>3</td>
</tr>
<tr>
<td>Group 5 (Combined treatent)</td>
<td>6.7 ±2.9 (Score 1)</td>
<td>Weak (Score 1)</td>
<td>2</td>
</tr>
</tbody>
</table>

GU: Gastric ulcer, Ppi.; Proton pump inhibitor.

**Discussion**

The pathogenesis of peptic ulcer in human is attributed to different factors as, stress, chronic use of NSAIDs, H. pylori infection, alcohol, smoking, and some dietary lifestyle (20). Gastric mucosal damage is stimulated, both directly and indirectly, through reactive oxygen species (ROS) and cytokines (21). Peptic ulcer can be treated through different drugs but unfortunately, the currently used medications can cause various side effects, delay the healing process, and increase the recurrence, thereby causing a financial burden on patients and healthcare systems worldwide. This encourages the interest in finding alternative natural products as a therapy (22). The present study aimed to investigate the potential
comparable therapeutic effect of ashwagandha to proton pump inhibitors in cold stress gastric ulcer in rats. The relation between the psychobiological and physiological stress and gastric damage has long been established. The animal model used in this study was cold restraint stress (CRS) gastric ulcer model. It was selected as it produces a model of gastric ulceration that is suitable for studies of healing effect of test agents. In addition, cold-restraint stress ulcers in animals are completely like human ulcer. CRS stimulates the hypothalamic-pituitary-adrenal axis which induces cortisol release. Additionally, gastric hormones are disrupted, and free radicals are generated. Increased the level of catecholamines enhance the inflammatory mediator’s expression as tumor necrosis factor-α (TNF-α) and (IL-6). The oxidative stress leads to disruption of the biological tissues imposing their injury. The damage of gastric cells includes peroxidation of the lipids of cell membrane, associated with release of intracellular components. As a result, the inflammatory process in the gastric mucosa starts.

In the current study, histopathological scoring and ulcer index confirmed the occurrence of gastric ulceration in the CRS ulcer model with the highest score & index in the GU group which was similarly reported by previous studies. The presence of acute hemorrhagic lesions in the glandular tissue is a characteristic of stress ulcer. In our study administration of Esomeprazole, (a drug commonly prescribed to ulcer patients), improved pathological scoring, ulcer index, gastric acidity and ph of the gastric juice. This observation is consistent with the results of previous researchers. Esomeprazole belongs to proton pump inhibitors (PPIs) and is used in clinical practice because of its acid inhibition effect. Ashwagandha is an essential herb in medical systems. Its biologically active components support its pharmacological importance as anti-inflammatory, immunomodulatory, anxiolytic, hypolipidemic, and antidepressant agent.

Ashwagandha treatment ameliorated the U.I and improved scoring thus produced significant healing potential compared to CRS model with less acidic gastric juice evidenced by both pH and total acidity tests. These results are in line with a previous study which reported that treatment of Withaniasomnifera extract (Ashwagandha) significantly reduced ulcer index and volume of gastric content. However, in our study only 50 mg/ kg of Ashwagandha were used daily compared to 100 mg/ kg in Bhatnagar et al. research. In order to explore the co use effect of Ashwagandha and Esomeprazole, the fifth group had received both agents. This group showed the best results regarding ulcer index, histopathological scoring, gastric acidity, pH, gastric content volume and adherent mucus weight. Thus, suggesting a potentially synergistic interaction between both agents.

On basis of present data, it can be concluded that stress induced gastric ulceration increases the gastric acid secretion evidenced by lower PH and increased total acidity in CRS induced ulcer group. This may be due to the associated increased histamine secretion by the parietal cells in CRS ulceration. Additionally, it may be related to vagal stimulation of gastrin, which stimulates acid secretion. It has been accepted that acid secretion and GIT motility alteration manifest in stressful conditions leading to multiple ulcerations.
(30). Our results reported that CRS produces oxidative stress status associated with severe inflammatory response in rats. That was evidenced by elevated oxidative stress markers as MDA. Increase in generation of Reactive oxygen species (ROS) during stress imposing oxidative damage and injury to the mucosa (20) In the current study, both ashwagandha treated group and PPI treated group showed significant decrease in MDA level. The combined treated group showed much more decrease in the MDA level.

GSH, which is a known antioxidative marker, is significantly increased in GU+Ash, GU+ PPI and combined ttt groups. Previous studies investigated the antioxidant activity of ashwagandha particularly in stressed individuals and reported significant improvements in MDA level (31). The significant increase in GSH level in Ashwagandha treated groups may be as a result of induction of nuclear factor erythroid 2-related factor 2 (Nrf2) release, a transcription factor helps in regulation of antioxidant enzyme genes expression (31)

The antioxidant properties of the bioactive compounds of Ash support its use to restore oxidative balance and prevent oxidative stress-associated diseases. The modulatory effects of Ashwagandha on levels of cortisol in individuals with stress/anxiety have been reported previously. Additionally, the ulcer healing potential of ashwagandha may be attributed to decrease TNF α as shown in the immunohistochemical results. Pro-inflammatory cytokines as (tumor necrosis factor-alpha [TNF-α], interleukin-6 [IL-6], and IL-1 beta [IL-1β]) play a major role in gastric ulceration. (32). Stress has been reported to increase the gastric mucosal level of many inflammatory markers because of increased gastric expression of nuclear factor kappa B (NF-kB), which controls the generation of pro-inflammatory cytokines (32). TNF-α stimulates caspase-3 in epithelial and endothelial cells of gastric mucosa and thus contributes to apoptosis and subsequent damage (33)Ashwagandha root was found to inhibit the NF-κB and MAPK. Transforming growth factor (TGF)-β is an important profibrotic cytokine in fibrotic diseases. TGF-β is a multifunctional mediator regulating many biological operations such as apoptosis, differentiation, adhesion, proliferation, and wound repair (34).

Immunostaining images in our study clearly demonstrated elevation in gastric TGF- β1 expression in rats with GU, and both esomeprazole and ashwagandha treatment groups reduced the percentage of positive gastric cells with TGF- β1 immune staining with marked reduction in the group received mixed treatment. In accordance with the present results, Tominaga et al, showed increased expression of TGF- β1 in gastric tissue in ulcered rats (35). However, in contrast to our results they revealed that reduced TGF-β1 expression accompanied by delayed ulcer healing. This apparent inconsistency could be explained by Hassan et al. research who investigated TGF-β in GU. They revealed that inhibition of TGF-β/Smad signaling pathway could be a potential therapeutic goal for ameliorating GU. They evaluated TGF-β1 level and SMAD-4 content of gastric tissues and found that treatment with genistein significantly suppressed TGF-β1 and SMAD4 expression thus improving ulcer healing (34). Based on Hassan et al. explanation, the improvement of gastric healing in our study with co-treatment with Ash and esomeprazole could be due to inhibition of gastric
tissue fibrosis via suppression of TGF-β/SMAD4 pathway.

**Conclusion:**
On the basis of present data, it can be concluded that ashwagandha possesses promising ulcer healing activity and is effective in the management of stress-induced gastric ulcers alone and combined with esomeprazole. It improved stomach function, gastric pH, acid secretion, and reduced mucosal hemorrhagic lesions with restoration of the architecture of the mucosal layer in rats. These effects could be explained by its antioxidant activity, suppressing the inflammatory cascade, promotion of gastric barrier repair and inhibiting TGF-β/SMAD4 pathway.

Overall data clearly showed that effects of ashwagandha can be compared with commonly prescribed drugs as esomeprazole. However, the effect of the combination of both agents was superior to that of every agent alone due to the consequent synergistic effects, which may help in reducing the drug dose and thus minimizing side effects.

**References**


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