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Research Article

PROTECTIVE EFFECTS OF TINOSPORA CORDIFOLIA AGAINST RESERPINE INDUCED ULCER MODEL
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ABSTRACT
We investigated the mechanism underlying the protective effects of Tinospora cordifolia (50 mg/kg and 100 mg/kg) against gastric damage induced by reserpine in adult Sprague Dawley rats. Ulceration was induced in rats by administering 10mg/kg reserpine intraperitoneally (i.p.). The plant was evaluated for its anti-oxidative profile by estimating MDA, GSH, nitrite/nitrate and SOD in rat’s stomach. Pre-treatment with aqueous extract of AETC significantly increased level of endogenous antioxidants (GSH, SOD) and attenuated MDA and nitrite/nitrate levels in the reserpine-treated rats. Thus, it may be concluded that AETC reduced the oxidative stress and consequently improved the integrity of stomach in reserpine-induced ulcers in rats.

Keywords: Tinospora cordifolia, Gastric ulcers, Oxidative stress, Ulcer index.

INTRODUCTION
Reserpine is derived from the roots of the Rawolfia serpentina. It is an indole alkaloid having antipsychotic and antihypertensive property. It has been reported to have pivotal role in the progression of ulcer since it has been showed how reserpine caused gastric ulceration is not clear. Various reports indicated that reserpine causes degranulation of mast cells with increase in the gastric acid secretion by sympathetic activation. Reserpine is documented to generate free radicals and inhibit the prostaglandin synthesis. It has been demonstrated that reserpine produced gastric ulceration by depleting catecholamine, serotonin and histamine stores at both peripheral level and central nervous system. Moreover reserpine also increases the acidity and gastric motility and alters the blood vessels of the mucosa. Reports indicated that administration of reserpine cause a marked increase in oxidative stress leading to the increase generation of reactive oxygen species (ROS). ROS is reported in the pathophysiology of gastric ulcer. A number of synthetic therapeutic interventions are available in the market for the management of ulcers but they possess severe adverse drug reactions (ADRs). So, this research has been designed to explore such herbal drugs in the management of ulcers that possess fewer side effects but more therapeutic efficacy. T. cordifolia belongs to the family Menispermaceae. Clinically, the plant has been found to possess immunomodulatory, antidiabetic, anti-inflammatory, hepatoprotective. However, the mechanism underlying the protective effects of T. cordifolia against gastric damage is unclear. Here, we investigated the antiulcer effects of T. cordifolia in reserpine-induced gastric ulcer model rats.

MATERIALS AND METHODS
Healthy adult Sprague Dawley rats weighing 180-220 g were used in the present study. The rats were housed in colony cages at an ambient temperature of 25±2°C and 45%-55% relative humidity with 12h light-dark cycle. They were fasted in individual cages with measures taken to avoid coprophagy for 24 h prior to the experiment with free access to water. All procedures were approved and carried out as per the guidelines of committee for the purpose of control and supervision of experimental (CPCSEA).

PLANT MATERIAL
Procurement of extract
Standardized dried aqueous extract of T. cordifolia (AETC) roots was obtained from Amsar Pvt. Ltd., Indore (M.P.).

Preliminary phytochemical screening
The preliminary phytochemical screening of the above AETC was carried out according to the methods described by Kokate et al., (2005). Phytochemical analysis of the extract was performed for the identification of phytochemicals like alkaloids, terpenoids, tannins, glycosides, steroids, phenolics, and polysaccharides etc.

Induction of Gastric Ulcers in rats
In order to produce gastric lesions, 10 mg/kg dose of reserpine (dissolved in few drops of glacial acetic acid and then diluted with distilled water) was administered intraperitoneally (i.p.) according to the method of Gupta et al. (1974). AETC and ranitidine treatment was given orally 30 min before reserpine treatment. After 20 hrs, animals were sacrificed and their stomach were removed for the evaluation of microscopic and biochemical parameters.

Experimental Protocol
Five groups were employed in the present study and each group comprised of 6 rats. The AETC and Ranitidine were dissolved in 0.5% w/v carboxymethyl cellulose (CMC). Reserpine was dissolved in few drops of glacial acetic acid and final volume made up with distilled water.

Group I (Normal Control): Rats were maintained on standard food and water and no treatment was given. Group II (Reserpine Control): Rats were administered single dose of reserpine (10 mg/kg, i.p). Group III (Ranitidine treated reserpine): Rats were treated with ranitidine (50 mg/kg, p.o.) dissolved in 0.5% w/v of CMC, 30 mins prior to reserpine administration. Group IV [AETC (50 mg/kg) treated reserpine]: Rats administered reserpine (10 mg/kg, i.p.) were treated with AETC (50 mg/kg, p.o.) and the treatment was administered 30 min prior to reserpine administration. Group V [AETC (100 mg/kg) treated reserpine]: Rats administered reserpine (10 mg/kg, i.p.) were treated with AETC (100 mg/kg, p.o.) and the treatment was administered 30 min prior to reserpine administration.

Assessment of integrity of stomach using histopathological studies
Stomach was excised and immersed in 10% buffered formalin. They were then dehydrated in the graded concentrations of ethanol, immersed in xylene, and then embedded in paraffin. From the paraffin blocks, 4 mm thin sections were cut, and stained with haematoxylin (0.6% w/v) for 15 min followed by counterstaining with eosin (1% w/v) for 2 min. They were then examined using light microscopy.
to analyze integrity of stomach, using an image analysis program (NIH Scion image analyzer).

Assessment of stomach damage

Macroscopic scoring of stomach in reserpine-induced ulcers

On completion of experimental protocol, all the rats in each group were sacrificed. The entire stomach was excised and opened along the greater curvature, rinsed with cold saline. The stomachs were stretched on a corkboard and the ulcer index was obtained according to scoring method of Suzuki et al. (1974)\(^1\) as follows:

- Score 1: maximal diameter of 1 mm
- Score 2: maximal diameter of 1-2 mm
- Score 3: maximal diameter of 2-3 mm
- Score 4: maximal diameter of 3-4 mm
- Score 5: maximal diameter of 4-5 mm
- Score 10: an ulcer over 5 mm in diameter
- Score 25: a perforated ulcer

Ulcer Protection

The percent protection with each test drug dose was calculated by the following formula:

\[
\% \text{ Protection} = \left( \frac{\text{UI control} - \text{UI treated}}{\text{UI control}} \right) \times 100
\]

where UI stands for ulcer index.

ASSESSMENT OF OXIDATIVE STRESS PARAMETERS

Preparation of Homogenate

For the determination of tissue (TBARS), superoxide dismutase (SOD), reduced glutathione (GSH), nitrite/nitrate level. Stomach was homogenized in ice-cold PBS (pH 7.4) and centrifuged at 3000 rpm for 10 min at 4°C. Estimation of tissue TBARS level

The quantitative measurement of thiobarbituric acid reactive substances (TBARS) is an index of lipid peroxidation in the gastrointestinal tract. This estimation was performed according to the method of Ohkawa et al. (1979)\(^1\). 0.2 ml of tissue supernatant was pipetted out in a test tube, followed by addition of 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 30% acetic acid (pH 3.5) and 1.5 ml of thiobarbituric acid. The volume was made up to 4 ml with distilled water. The test tubes were incubated at 95°C for 1 hr. The test tubes were then cooled and 1 ml of distilled water was added followed by the addition of 5 ml of n-butanol-pyridine mixture (15:1 v/v). The tubes were centrifuged at 4000 g for 10 min. The absorbance of the developed pink colour was measured spectrophotometrically. The concentration of TBARS was expressed as nmol per mg protein.

Estimation of tissue Reduced Glutathione (GSH)

GSH content in colonic tissue was estimated using method of Beutler et al., (1963)\(^1\). The supernatant was mixed with trichloroacetic acid (10% w/v) in 1:1 ratio. The tubes were centrifuged at 1000 g for 10 min at 4°C. The tissue supernatant obtained (0.5 ml) was mixed with 2 ml of 0.3 M disodium hydrogen phosphate. Then 0.25 ml of 0.001 M freshly prepared DTNB was added and absorbance was noted spectrophotometrically at 412 nm. Results were expressed as micromoles of reduced glutathione per mg of protein.

Estimation of tissue nitrite/nitrate content

Levels of nitrite/nitrate were determined with the Griess reagent according to the method described by Green et al. (1982)\(^1\). Tissue supernatant was mixed with an equal volume of fresh Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulphanilamide in 2.5% phosphoric acid], incubated for 10 min at room temperature, and then the absorbance of the mixture was determined spectrophotometrically at 540 nm. Concentration of tissue nitrite/nitrate was calculated in micromoles per mg of protein.

Estimation of Superoxide Dismutase (SOD)

SOD activity was estimated by the method of Misra and Fridovich (1972)\(^1\). 0.5 ml of tissue homogenate was mixed with 0.5 ml of cold distilled water. Then 0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform were added and mixed well using cyclomixer for 5 min and centrifuged at 2500 rpm for 15 min at 4°C. To 0.5 ml of supernatant, 1.5 ml of carbonate buffer (pH 10.2) and 0.5 ml of 0.4 M ethylenediaminetetraacetate (EDTA) solutions were added. The reaction was initiated by the addition of 0.4 ml of epinephrine bitartrate solution (3 mM) and the change in optical density/minute was measured at 480 nm against reaction blank. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. SOD activity was expressed as units/mg of protein.

Estimation of tissue total protein

The quantitative measurement of the total protein was determined by Lowry’s method\(^5\).

STATISTICAL ANALYSIS

The results are expressed as mean±S.D. The microscopic and biochemical values were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using statistical Graph Pad Prism software. P < 0.05 was set to be statistically significant.

RESULTS

The treatment with AETC (50 mg/kg and 100 mg/kg) per se to normal rats did not produce any significant effects on various parameters (UI, TBARS, GSH, SOD and nitrite/nitrate) performed in the present study. However, when results of single dose of reserpine administration were compared with normal control, reserpine was found to produce significant injury to the gastric mucosa and altered the gastric mucosal indicators.

Effects of AETC on gastric mucosal ulcer index

UI is shown in Table: 2. Intraperitoneally reserpine administered rats (10 mg/kg) showed gross mucosal lesion in their stomach. These lesions were hemorrhagic and were linear or dotted in shape. Pre-treatment with AETC (50 mg/kg and 100 mg/kg) were found to inhibit reserpine-induced gastric mucosal injury in a dose dependent manner.

Histopathological study

Histological study revealed that rats receiving reserpine showed extensive damage to the gastric mucosa along with edema and lymphocytes infiltration of the mucosal layer when compared to normal control rats. However, rats pre-treated 30 min prior to reserpine with the AETC (100 mg/kg and 50 mg/kg) showed comparatively better protection of the gastric mucosa indicated by reduction in ulcer area, caused reduction of mucosal edema and lymphocytes infiltration in a dose dependent manner. Dose of 100 mg/kg was found to be more effective than 50 mg/kg (Figure 1).

Effect on Oxidative Stress Parameters

Effect of AETC on tissue TBARS level in reserpine-induced ulcer

Table: 1 depicts reserpine administration caused a significant increase in the level of TBARS in rats (p < 0.001). However treatment with AETC (50 and 100 mg/kg) and ranitidine (50 mg/kg) markedly attenuated the level of TBARS. A dose dependent response was obtained with 100 mg/kg dose of AETC (p < 0.001) and 50 mg/kg dose of AETC (p < 0.01).
Effect of AETC on GSH level in reserpine-induced ulcer

Induction of gastric ulcers in reserpine control group produced a significant decrease in colonic GSH level (p < 0.001) as compared to the normal group. However, AETC (50 mg/kg and 100 mg/kg) and ranitidine (50 mg/kg) pre-treatment significantly increased GSH content as compared to the reserpine control group (Table: 1). A dose dependent response was obtained with 100 mg/kg dose of AETC (p < 0.001) and 50 mg/kg dose of AETC (p < 0.05).

Effect of AETC on SOD level in reserpine-induced UC

Reserpine administration produced a significant decrease in SOD content (p < 0.001) as compared to the normal control group. AETC (50 and 100 mg/kg) pre-treatment 30 min prior to reserpine administration significantly increased SOD content as compared with reserpine control group (p < 0.01 and p < 0.01; respectively) dose dependently. Ranitidine also protects significantly against SOD depletion induced by reserpine level (p < 0.001) (Table: 1).

Effect of AETC on nitrite/ nitrate level in reserpine-induced ulcers

Administration of reserpine resulted in significant increase in nitrite/ nitrate level compared to the normal control. Pre-treatment with AETC (50 and 100 mg/kg) dose dependently inhibited the elevation of nitrite/ nitrate in stomach compared to the reserpine control group. The effect of AETC (100 mg/kg, p<0.001) was found to be more potent than AETC (50 mg/kg, p<0.01) (Table: 1).

DISCUSSION

Gastric ulcers are the most wide state disease and are a very common global problem today. While in most of the cases the etiology of ulcer is unknown, but it is usually accepted that it ensues from an imbalance between aggressive factors and mucosal integrity through the endogenous mechanism. Among the aggressive factors, it is generally accepted that increased gastric secretion, reduced gastric mucosal blood flow, increased gut motility, degranulation of mast cells and inhibition of prostaglandin biosynthesis are crucial factors which induce gastric erosions. The peculiar biological mechanisms involved in mucosal defence include a mucus coat provided by surface epithelial cells that secrete mucus and bicarbonate, maintenance of adequate blood flow and production of prostaglandins by mucosa. To retrieve the balance, different therapeutic agents including herbal preparations are used to inhibit the gastric acid secretion or to elevate the mucosal defence mechanism by increasing mucus production.

The antiulcer drugs like proton pump inhibitors, antimuscaranics, H2-receptors blockers, produce adverse effects such as hypersensitivity, arrhythmia, impotence and heamopoietic changes. As a substitute to other approaches scientific research on plants used in traditional medicine is receiving more concern as a way of identifying new agents. There is marked evidence that oxygen derived free radicals plays an essential role in the pathogenesis of the injury of gastrointestinal tract. Literature review indicated that ROS generated by metabolism of arachidonic acid, platelets, macrophages and smooth muscle may lead to gastric mucosal damage. Hence by scavenging free radicals, the reactive oxygen metabolites will defend the gastric mucosa from oxidative damage or yet heal the gastric ulcers. A number of studies have supported the antioxidant, anti-inflammatory, immunomodulator, properties of T.cordifolia. The anti-ulcerative effects of T.cordifolia have previously been investigated in experimental gastric ulcer models. However, the mechanism underlying the protective effects of T. cordifolia against gastric damage is unclear. In the present study, the anti-ulcerative effects of T.cordifolia were investigated in reserpine-induced gastric.

Phytochemical screening of T.cordifolia depicts the presence of alkaloids, terpenoids, tannins, glycosides, steroids, phenolics, and polysaccharides. The root extract has been known to recognized that it contain choline, isocolcumin, palmatine, tetrahydropalmatine, magnoflorine and sitosterol. There are data in the literature indicating that gastroprotective activity is due to the presence of alkaloids, palmatine, a new type of protoberberine alkaloid accelerated ulcer-healing and increased the gastric mucus production. Thus the gastroprotective and antioxidant property of T.cordifolia could be due to the presence palmatine and terpenes which can reduce free radical by scavenging them. Various reports indicated the antioxidant activity of terpenes in various stress-induced ulcer models.

In the present study, we assessed the effect of T.cordifolia in animal model employed in an attempt to radiate the animal model gastric ulcers, namely reserpine-induced ulcers in rats. Reserpine-induced ulcer model is an easily inducible model of gastric ulcer in rats and share many of the characteristics of human ulcers in terms of histopathological appearances. Reserpine causes intracellular acidification resulting in massive epithelial damage. In our study, an increased number of inflammatory cells infiltrations, massive necrosis and hemorrhages were detected in the stomach by microscopic evaluation. Compared to the reserpine control group, orally administered AETC in the present study, dose dependently reduced the severity and extent of necrosis and hemorrhages in the involved tissue, which was associated with a significant reduced microscopic stomach ulcer index. On the other hand, treatment with AETC was able to reduce inflammatory cells infiltration in the stomach tissue. This inhibitory effect of AETC at the doses of 50 and 100 mg/kg against the infiltration of inflammatory cells into the gastric mucosa may account for its beneficial effect against tissue injury, most likely through a combination of several antiinflammatory mechanisms.

However, the mechanism by which reserpine produces ulceration is not completely understood but various mechanisms have been suggested to be responsible for this action. Reserpine-induced gastric ulceration has been attributed to vagotonic hypermotility, degranulation of gastric mast cells and an increase in gastric acid secretion which is thought to be mediated cholinergically. Mobilization of superoxide (O2-) and hydroxyl radicals (OH) are also known to be responsible for inhibition of mucus release and stimulation of surface mucus breakdown via β-adrenoceptor stimulation that have been assigned to the ulcerative potentials of reserpine. In the present study, we investigated that reserpine administration increased lipid peroxidation and nitrite content; decrease SOD and GSH, thus leading to oxidative stress. So, alterations in the antioxidant status have been manifested after reserpine administration in rats.

NO is thought to be mediator not only of gastointestinal mucosal defence, but also of its damage. In general, the mucosal nitric oxide synthase (mNOS) and endothelial nitric oxide synthase (eNOS) isoforms raise low amounts of NO. However, the high quantity of NO produced by inhibitory nitric oxide synthase NO (iNOS) damages the epithelium. In our study, we firstly discovered and authentically exposed that AETC significantly inhibit NO overproduction and...
decreased TBARS levels in reserpine-induced gastric ulcers in a dose dependent manner. In healthy individuals, antioxidants e.g., SOD, GSH protect components of the body against free radical damage. SOD restrains the lipid peroxidation in stomach by eliminating free radicals, converting superoxide into peroxide (H₂O₂). Glutathione is found almost solely in its reduced forms, since its oxidized form (GSH) is constitutively active and inductive only upon oxidative stress and inhibits free radical mediated lipid peroxidation. The results of the present study depicted significant increased activity of SOD and GSH in AETC-treated in a dose dependant manner.

This study suggests a possible link between marked healing effects of AETC and its antioxidant. Further studies for investigating the tissue damage in the pathological progress of gastric ulcers need to be carried out in order to elucidate underlying molecular mechanisms responsible for the therapeutic effects of AETC.

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REFERENCES


Table 1: Effect of AETC on various biochemical parameters in reserpine-induced gastric ulcers

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS(nm/mg protein)</th>
<th>GSH(µm/mg protein)</th>
<th>Nitrite(µm/mg protein)</th>
<th>SOD(U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>0.540±0.140</td>
<td>1.966±0.287</td>
<td>0.322±0.171</td>
<td>38.010±3.082</td>
</tr>
<tr>
<td>Reserpine Control</td>
<td>1.640±0.205**</td>
<td>0.620±0.164*</td>
<td>2.174±0.355**</td>
<td>6.335±1.846**</td>
</tr>
<tr>
<td>Ranitidine + Reserpine</td>
<td>0.791±0.186**</td>
<td>1.360±0.332</td>
<td>0.858±0.202**</td>
<td>26.750±2.378**</td>
</tr>
<tr>
<td>AETC (50 mg/kg) + Reserpine</td>
<td>1.220±0.167**</td>
<td>1.755±0.384</td>
<td>1.437±0.366*</td>
<td>13.330±1.818</td>
</tr>
<tr>
<td>AETC (100 mg/kg) + Reserpine</td>
<td>0.843±0.211**</td>
<td>1.121±0.176</td>
<td>0.542±0.264**</td>
<td>28.790±172**</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± S.D. (n=6) where * represents p<0.001 as compared to normal control group; ** represents p<0.01, *** represent <0.05 as compared to reserpine group; **** represents p<0.001 and ***** represents p< 0.01 as compared to AETC (50 mg/kg) +reserpine group; administered with AETC at the dose of 100 mg/kg, p.o. in normal control rats.

Table 2: Effect of AETC on ulcer index in reserpine-induced gastric ulcers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of animals Used</th>
<th>Ulcer index (mean ± S.D.)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6</td>
<td>29.51±4.899</td>
<td>-</td>
</tr>
<tr>
<td>Reserpine control</td>
<td>6</td>
<td>12.76±2.806</td>
<td>56.76</td>
</tr>
<tr>
<td>Ranitidine + Reserpine</td>
<td>6</td>
<td>21.18±3.478</td>
<td>28.22</td>
</tr>
<tr>
<td>AETC (50 mg/kg) + Reserpine</td>
<td>6</td>
<td>5.859±2.870</td>
<td>80.14</td>
</tr>
<tr>
<td>AETC (100 mg/kg) + Reserpine</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source of support: Nil, Conflict of interest: None Declared

Figure 1. Histopathology of stomach of rats administered with reserpine (10 mg/kg, i.p.)
A. Normal Control: a. Intact mucosal layer with no lymphocytes infiltration in between the epithelial site b. Intact sub mucosa.
C. AETC (50 mg/kg) treatment: a. Mucosal layer shows small erosions. b. Lyphocytes infiltration in the mucosal layer.
D. AETC (100 mg/kg) treatment: a. Mild erosions on the surface of mucosal layer. b. Submucosa showing mild edema and lymphocytes infiltration.
E. Ranitidine (50 mg/kg) treatment: a. Small erosions in the mucosal layer b. No lymphocytes infiltration in-between the cells of mucosa

Source of support: Nil, Conflict of interest: None Declared