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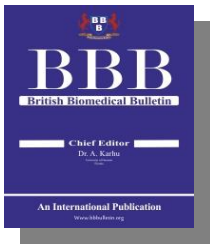

Original

Protective Effect of *Vitis vinifera* and *Cichorium intybus* on Stress Induced Ulcers in Albino Rats

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ABSTRACT

Objective: The objective of the present investigation was to assess the protective effect of ethanolic extracts of *Vitis vinifera* and C*ichorium intybus* seeds on chronic forced swimming stress induced ulcers in albino rats.
Method: The ethanolic extracts of *Vitis vinifera* seeds and C*ichorium intybus* roots were prepared and subjected to preliminary qualitative phytochemical screening. Acute toxicity of both the extracts determined in albino mice. Chronic forced swimming stress induced ulcers was evaluated by measuring the level of ulcer number, severity of gastric ulcers, pH and ulcer percentage in albino rats. Histopathological changes were observed.
Results: Both the extracts did not show any mortality or a moribound status in mice upto the doses of 5g/kg body weight. The results indicated that pretreatment with ethanolic extracts of *Vitis vinifera* seed and C*ichorium intybus* root exhibited significant protective effect and prevented induction of gastric ulcers by chronic forced swimming stress at the tested doses of 250mg/kg and 500mg/kg body weight. The protective effect of *Vitis vinifera* seed and C*ichorium intybus* root at the dose of 500mg/kg body weight was comparable to the standard drug.
Conclusion: On the basis of results it was concluded that *Vitis vinifera* and C*ichorium intybus* possess protective effect to combat stress induced gastric ulcers.

Introduction

Stress is an integral part of living organisms, which is counteracted by homeostatic mechanism of the body to keep internal environment within normal physiological limits. However if stress is extreme, unusual or chronic, the normal homeostatic mechanism will be disturbed leading to well known stress related diseases such as peptic ulcer, hypertension, depression, stroke, cancer, diabetes etc[1]. As peptic ulcer is the most common stress related disorder of digestive system[2], there is need for effective anti-stress agent to cope up with the stress.

The present study was therefore, undertaken to explore protective effect of *Vitis vinifera* and C*ichorium intybus* on stress induced ulcers in albino rats. *Vitis vinifera* belongs to vitaceae family commonly known as wine grape or common grape, which is widely grown throughout the world[3]. Complex matrix of grape seeds contains complex phenols, tannins, sugars, protein, oil, fibre, mineral and salts etc[4]. Proanthocyanidins are group of polyphenolic bioflavonoids present in *Vitis vinifera* seed, which are responsible for the potential pharmacological activities[5]. *Vitis vinifera* was reported to have antioxidant[6,7], antibacterial[8], Cardioprotective action[9,10], Antidiabetic[11], Antilisterial[12] and anti-thrombotic action[13]. C*ichorium intybus* belonging to Asteraceae family, is an erect perennial plant. Its phytochemical constituents are inulin, phytosterols, lactones, flavonoids, triterpenoids, sesquiterpene, coumarins (including cichorin), caffeic acid derivatives, tannins, vitamins, pectins and fats[14,15]. C*ichorium intybus* was reported to have antidiabetic[16] antimalarial[17], hepatoprotective[18], tumor inhibitory activity[19], antibacterial[20], antioxidant activity[21], gastroprotective[22], anti-convulsant[23], immunomodulatory activity[24] and antilithiatic activity[25].

While reviewing the multiple uses and properties of *Vitis vinifera* and C*ichorium intybus*, it was found that several diseases that are postulated to be induced by stress are treated by them. This prompted us to study the protective effect of *Vitis vinifera* and C*ichorium intybus* on a stress induced ulcer in albino rats.

Materials and Methods

Collection of plant material

The fruits of *Vitis vinifera* (common grapes) and roots of C*ichorium intybus* (chicory) were procured from local market of Bangalore, Karnataka. The collected material were identified, confirmed and authenticated by Dr. Ataulla khan, Botanist of Bangalore University. The voucher specimen (MMU/RMGM/VV-CI/2008) was maintained.

Preparation of Vitis vinifera extracts

Seeds were separated from the fruits. Then seeds were cleaned and washed with tap water to remove any remaining pulp. The washed seeds were dried under shade and powdered by using mechanical grinder. The fatty material was removed by subjecting seed powder to Soxhlet extraction with petroleum ether at 60^{o}C for 6h. Powder was re-extracted with 95% ethanol at 65^{o}C till the solvent entering the siphon tube was colorless. The extract was collected and filtered. To separate solvent, the filtrate was evaporated at 35^{0}C in a rotary evaporator. Finally viscous liquid was evaporated to a dry residue on water bath. Brown residue obtained was weighed and percentage yield was calculated. The yield of extract was 12%W/W.

Preparation of *Cichorium intybus* extract

Isolated roots of C*ichorium intybus* were chopped into small pieces and air dried under shade away from direct sunlight for

15days. The dried slices of roots of C*ichorium intybus* were powdered using mechanical grinder and passed through sieve. In Soxhlet extractor, coarse powder of C*ichorium intybus* was packed. Ethanol was used as solvent because previous investigator had used only ethanol as solvent[16,19,24]. The end point of extraction was colourless solvent leaving the siphon tube. The extract was concentrated using rotary evaporator. Finally brownish yellow residue was obtained by heating on water bath. It was stored in refrigerator. The yield of concentrated crude alcoholic extract of C*ichorium intybus* root was 14%W/W.

Preliminary phytochemical testing

Vitis vinifera and C*ichorium intybus* extracts were tested for phytoconstituents as per standard tests[26].

Approval of experimental protocols

Ethical clearance was obtained for procuring of animals and for evaluating anti-stress activity of *Vitis vinifera* and *Cichorium intybus*. (Approval No. MMUCP/ IAEC/03/2008-09).

Experimental animals

Experimental study was carried out using normal adult Albino mice (24±2 g) and Albino rats (200±20 g) of either sex of wistar strain. Animals were housed in a clean and sanitized polypropylene cages under standard environmental conditions of relative humidity (50±5%) room temperature (25±2^{o}C) and photocycle (12:12h of light/dark period with lights on 0700h). Feed of animals was dietary pellets (pellets of Amruth Lab.). Drinking water was maintained adlibitum.

Acute toxicity study

In this study a single dose (5000mg/kg body weight) of either *Vitis vinifera* extract or *Cichorium intybus* extract was administered to albino mice by oral route. It was conducted as per guidelines of Organization for Economic Co-operation and Development (OECD guideline No. 425). Individual mice were observed continuously for the first 30 m after the administration of extracts. Then mice were observed after every 30 m during the first 4 h. later on observation continued for 48 h and daily there after for a period of 14 days for delayed toxicity. Parameters of observation were mortality, moribund status of mice and gross behavior.

Selection and preparation of dose of drugs

Ethanolic extracts of *Vitis vinifera* seed and C*ichorium intybus* root have good margin of safety as both the extracts did not produce any lethal effects on albino mice upto the doses of 5000mg/kg body weight. Therefore 5% and 10% of the maximum tolerated dose i.e. 250 and 500 mg/kg body weight were selected for the study of anti-stress activity of *Vitis vinifera* seed and C*ichorium intybus* root extracts. Suspension of standard and the extract was prepared by suspending omeprazole or extracts of *Vitis vinifera* seed and C*ichorium intybus* roots in distilled water using 1%W/V acacia as a suspending agent. For control rats normal saline having 1%w/v gum acacia was selected as a vehicle.

Evaluation of anti-stress activity

Protective effect of *Vitis vinifera* and C*ichorium intybus* on stress induced ulcers in albino rats was evaluated by chronic forced swimming stress method. Seven groups of albino rats were used. Each group comprised of 6 animals of either sex.

Preparation of animals

Rats were acclimatized and habituated to laboratory conditions prior to experiment to minimize nonspecific stress condition for a period of 1week. Before

subjecting to stress, the rats were fasted overnight with free access to water *ad libitum* so as to ensure complete gastric emptying and a steady state gastric acid secretion. Care was taken to prevent coprophagy by providing perforated mesh at the bottom.

Administration of vehicle, standard and extract

Vehicle, test and standard were administered to the Albino rats 60 m before subjecting to stress for 10 consecutive days. Dose of extracts was calculated according to the body weight of the rats. Drugs were administered to rats by oral route using oral gavage. Tube was passed laterally through the inter dental space by rotating motions so that tube should advanced into the esophagus. Then drug solution was introduced slowly. Food was withheld for a further 2 h after the administration of the drugs.

Chronic forced swimming stress

Rats were forced to swim in the transparent tub (50cm deep × 27cm wide) containing water to the dept of 40 cm maintained at 25±2°C. Swimming time was 15 m daily for 10 consecutive days to get chronic stress induced ulcers.[27] Water level was maintained upto the neck region. Rats were forced to swim. Rats suffered stress as they were not allowed to stand on bottom or grasping the side of the tub. Rats were removed from the water after 15 m and dried by towel and were placed under a 60 watts bulb for drying.

Dissection of stomach

On tenth day after subjecting to stress, rats were sacrificed by cervical dislocation. Rats were secured on operating table and its abdomen was opened. The anterior abdominal wall muscle of rat was incised using a sharp scalpel. The stomach was carefully dissected out from the body of rat. Glandular portion of the stomach was opened along greater curvature and gently rinsed with water. The stomach was stretched and pinned on soft foam board in such a way that mucosal site was up. Photograph was taken.

Assessment of ulcer

Mucosa was examined under microscope with 10X magnification for the following parameters.

1. Ulcer number:- The number of ulcer into the stomach were counted.
2. Ulcer severity score:- The severity of ulcer was assessed in the range of 0 to 3scale. Severity of ulcer scale adopted as follows[28].

- Stomach without ulcer = score 0
- Stomach with superficial ulcers=score 1
- Stomach with deep Ulcers = score 2
- Stomach with perforation = score 3

3. Ulcer index:- For each rat ulcer index was determined as follows[28].

- $U_I = U_N + U_S + U_P \times 10^{-1}$

Where U_I = Mean ulcer index.

- U_N = Mean ulcer number of rat.
- U_S = Mean ulcer severity score of rat.
- U_P = Ulcer percentage of rat.

4. Percentage of ulcer protection:- It was calculated as follows.

% ulcer protection = Mean ulcer index of control - Mean ulcer index of test X 100 / Mean ulcer index of control.

5. Gastric secretion pH:- The collected gastric contents were centrifuge at 1000 rpm for 10 m.[28] Supernatant liquid of the centrifuged sample was taken and diluted with distilled water. pH was recorded using pH meter.
6. Histopathological study:- It was carried out by immersing dissected stomach in 10% formalin solution for 24h. The tissue was processed through alcohol and xylene and it was embedded in paraffin blocks. Sections were made

with the help of microtome and stained with haematoxylin-eosin stain (HE-Stain). The stained slides were examined under a research microscope with magnification of 100X and photograph were taken. The different histopatho-logical indices screened were changes in lining of mucosal epithelium, submucosal oedema, necrosis, congestion and hemorrhage in tissue.

Statistical analysis

Values were given as mean ± S.E.M (n=6). Data was analyzed by one way ANOVA. Individual comparison was done by Dunnet's test.

Results

The preliminary qualitative phytochemical screening revealed the presence of carbohydrats, proteins, phenolic compounds as major chemical constituents in *Vitis vinifera* seed extract. The C*ichorium intybus* root extracts have shown the presence of carbohydrates, proteins, phenolic compounds and phytosterols as major chemical constituents.

During 14 days of observation in acute toxicity study, mice did not show any mortality or a moribound status or gross behavioral changes upto the dose of 5g/kg body weight.

Vitis vinifera seed extract and C*ichorium intybus* root extract have shown a strong preventive effect on gastric ulcers induced by chronic forced swimming stress. As shown in table 2, the chronic forced swimming stress induced marked increase in ulcer number, severity of gastric ulcers and ulcer percentage with decrease in pH. These effects were significantly attenuated dose dependently by *Vitis vinifera* seed extract (200 and 500mg/kg body weight) and C*ichorium intybus* root extract (200 and 500mg/kg body weight). As shown in the figure 1, the stress controlled group of rats exhibited ulcer, hemorrhagic streaks and perforated ulcers to a marked degree. Gastric ulcer induction was strongly suppressed in rats that were given omeprazole with 84.22% ulcer protection. *Vitis vinifera* and C*ichorium intybus* extracts have shown statistically significant suppression of ulcer index. The protective effect was stronger in those rats administered with C*ichorium intybus* root extract (83.77%) and *Vitis vinifera* seed extract (83.86%) at the tested dose of 500mg/kg body weight and it was comparable to the standard drug.

Histopathological studies revealed following conditions of different groups. Control group has shown normal mucosa and normal columnar epithelium. The lamina propria and muscularis mucosa appeared unremarkable. The muscle layer and serosa appeared normal. Stress controlled group revealed ulceration of mucosa, erosion and hemorrhage. Submucosa appeared edematous and congested. The mucosal glands have shown intra epithelial lymphocytes. The base of the lamina and the junction of muscularis mucosa have shown dense mixed inflammatory infiltrate with mild fibrosis. The standard drug treated group, revealed similar mild features with congestion. *Vitis vinifera* and C*ichorium intybus* at 250 mg/kg body weight have not shown protection against histopathological changes. Both extracts, at a dose of 500 mg/kg body weight have prevented histopathological changes.

Discussion

A clinical endocrinologist and experimental biologist Dr. Hans selye during 1930's defined stress as the reaction of an organism with sum of all non specific responses of the body to any type of external stimuli acting upon it[29]. The fact that stress is one of the pathogenesis factor of gastric ulcers in man was published by selye, during

1936, who used restraint as stress for the first time[30]. The causes of the stress induced ulceration is not known but several factors have been evaluated. Factors involved are, increased acid concentration with reduced mucosal blood flow[31,32], reduced mucus secretion, reduced gastric epithelial cell turn over[33] and activation of hypothalamic pituitary adrenal axis[34].

Various studies and research have been done to understand the neuroendocrine component of stress ulcers. During stress, the hypothalamic-pituitary- adrenal (HPA) axis activation leads to the release of corticotrophin releasing hormone (CRH). CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) from pituitary gland. ACTH acts on adrenal cortex leading to release of glucocorticoids[35,36]. Stressors release glucocorticoids as a protective hormone which combat stress response and maintain homeostasis of the organism[37]. However peptic ulcer can be induced or aggravated by large amount of glucocorticoids by different mechanisms like impairment of tissue repair, gastric mucosa, reduction in gastric mucus production and increased gastric acid levels. Gastric ulcers in rats due to stress with decreased gastric and elevated ACTH level indicat the role of HPA axis in stress ulcers[38,39].

It has been reported that ulcers formed during swimming stress models for animals closely resemble stress ulcers of human beings and gastric injury formed during stress[40]. Porsolt *et al*. developed forced swimming stress model that has been widely used to study physical stress in animals[27]. During forced swimming, animal shows despair behavior (emotional stress) and vigorous muscular activity (physiological stress) due to its inability to escape stressful stimuli. As emotional stress and physiological stress are responsible for generation of gastric ulcers, forced swimming stress is one of the best model developed for inducing stress in rodents. Stress can be given to animal for a single short period (acute stress) or repeatedly for a long period on daily basis (chronic stress). Physiological and neuroendocrine changes have been found in chronic stress model[41], which activate HPA axis resulting in elevated level of ACTH and cortisol[42].

In the present study, both the extracts of *Vitis vinifera* seeds and C*ichorium intybus* roots on pre treatment have shown significant ($p<0.001$) protective effect. This suggests the extracts may have increased nonspecific resistance of the rats by altering hypothalamo-hypophyseal-adrenal axis (neuroendocrine component).

Conclusion

On the basis of results it was concluded that *Vitis vinifera* and C*ichorium intybus* possess protective effect to combat stress induced gastric ulcers and seemed to be appropriate for providing beneficial health effects by increasing the nonspecific resistance of the organism against various stressor.

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Table 1. Experimental design for anti-stress activity in rats

Group	Treatment	Dose/kg body wt.
I. (Control)	Vehicle (1%w/v gum acacia prepared in saline)	2 ml; *p.o*
II. (Stress control)	Vehicle (1%w/v gum acacia prepared in saline) + chronic forced swimming stress	2ml; *p.o*
III. (Standard + stress)	Omeprazole (Suspended in 1%w/v gum acacia) + chronic forced swimming stress	10mg; *p.o*
IV. (VV250 +stress)	Ethanolic extract of *Vitis vinifera* (suspended in water by 1%w/v gum acacia)+chronic forced swimming stress.	250mg; *p.o*
V. (VV500 + stress)	Ethanolic extract of *Vitis vinifera* (suspended in water by 1%w/v gum acacia)+chronic forced swimming stress	500mg; *p.o*
VI. (CI 250 + stress)	Ethanolic extract of C*ichorium intybus* (suspended in water by 1%w/v gum acacia)+chronic forced swimming stress	250mg; *p.o*
VII. (CI 500 +stress)	Ethanolic extract of C*ichorium intybus* (suspended in water by 1%w/v gum acacia)+chronic forced swimming stress	500mg; *p.o*

Table 2. Protective effect of ethanolic extracts of *Vitis vinifera* seeds and C*ichorium intybus* roots in chronic forced swimming stress in albino rats

S. No.	Group	pH	Mean ulcer number	Mean ulcer severity score	Ulcer percentage %	Mean ulcer index	% ulcer protection
I	Control	3.10±0.04	0	0	0	0	100
II	Stress control	1.85±0.09[a]	7.66±0.55[a]	2.66±0.20[a]	100	11.03	0
III	STD + stress	3.23±0.15[b]	0.66±0.66[b]	0.166±0.16[b]	16.66	1.74	84.22
IV	VV250+stress	2.79±0.07[bf]	0.83±1.04[bf]	0.66±0.49[bf]	33.33	3.48	68.44
V	VV500+stress	3.04±0.08[bf]	0.66±1.09[bf]	0.5±0.5[bf]	16.66	1.78	83.86
VI	CI 250+stress	2.46±0.06[ce]	2.83±0.94[df]	1±0.36[df]	66.66	7.04	36.17
VII	CI 500+stress	2.96±0.06[bf]	0.83±0.83[bf]	0.5±0.5[bf]	16.66	1.79	83.77

Values shown for each group of the mean ± S.E.M. obtained from six observations.
Where [a] $p<0.001$ compared to control group and [b] $p<0.001$, [c] $p<0.02$, [d] $p<0.01$ compared with stress control group. [e] $p<0.01$and [f] $p>0.05$(Non significant)compared with standard.

***Vitis vinifera* plant**

***Cichorium intybus* plant**

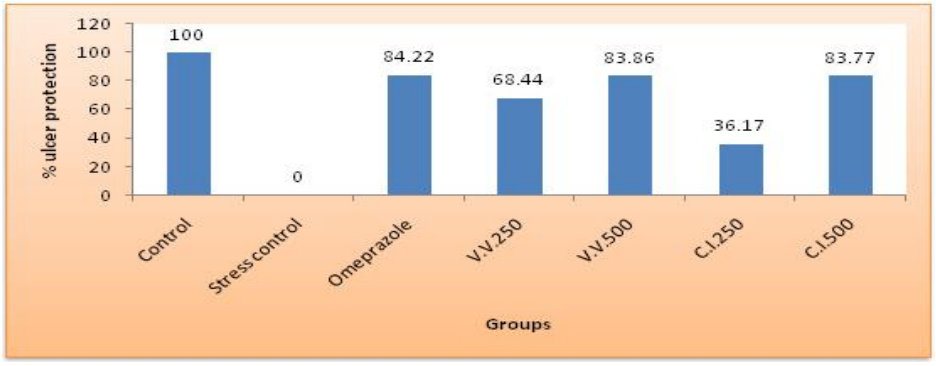

Figure 1. Protective effect of ethanolic extracts of *Vitis vinifera* seeds and *Chicorium intybus* root on chronic forced swimming stress in albino rats

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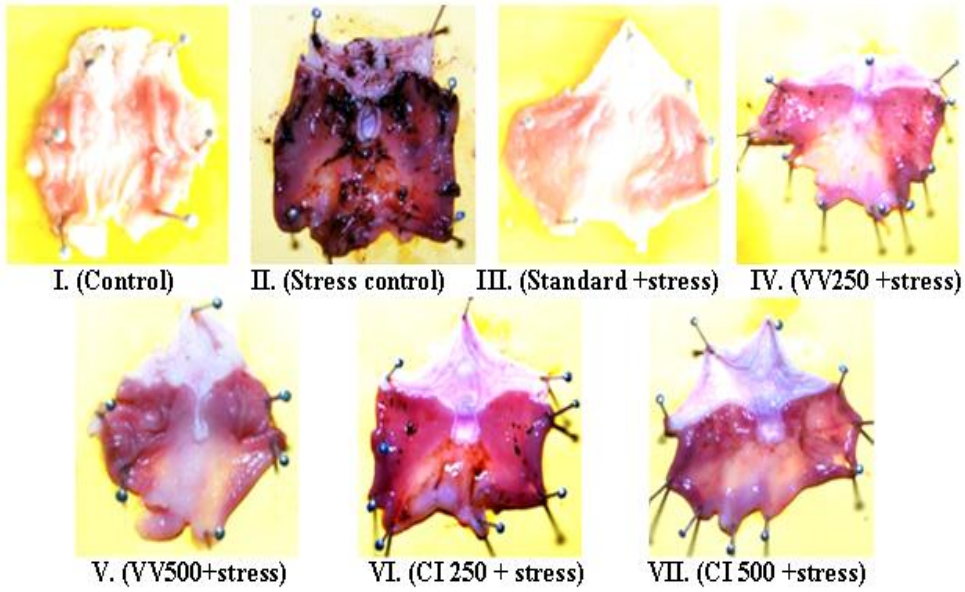

Figure 2. Representative photographs of different groups of rats stomachs showing gastric ulcers induced by chronic forced swimming stress

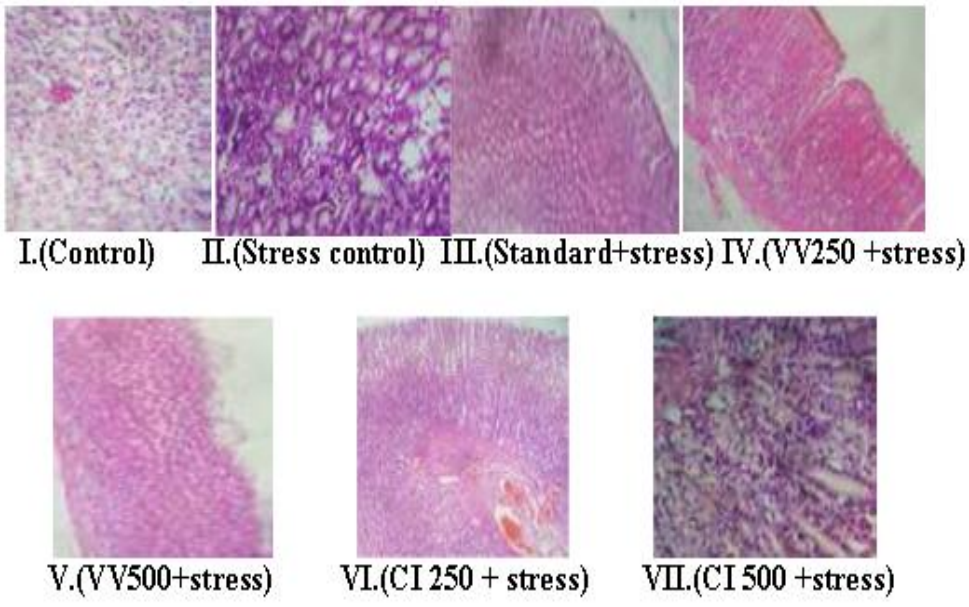

Figure 3. Representative photographs of different groups, showing histopathology of gastric ulcers induced by chronic forced swimming stress