CHAPTER 4

EXPERIMENTAL INVESTIGATIONS

Plant material

- Fruits of *Vitis vinifera*
- Roots of *Cichorium intybus*

Both the plant materials were procured from local market of Bangalore district, Karnataka.

Animals

- Albino mice in the range of 22-26g
- Albino rats in the range of 180-220g

Experimental study was carried out using adult animals of either sex of wistar strain. Animals were procured from Srivenkateshwara enterprises, Bangalore.

Chemicals and Drugs

All the chemicals used in this investigation were of analytical grade, and obtained from S.D. Fine chemicals, Sigma, Qualigens, Merck, Ranbaxy and Himalaya drugs.

4.1 Collection of plant material and its authentication

The selected plants namely, fruits of *Vitis vinifera* (common grapes) and roots of *Cichorium intybus* (chicory) were procured from Bangalore, Karnataka. The collected material were identified, confirmed and authenticated by Dr. Athaula khan, Botanist. The voucher specimen (MMU/RMGM/VV-CI/2008-09) was maintained.

4.2 Extraction of selected plant material.

The extraction method was based on isolation of active constituents, using 95% ethanol as a solvent. Alcohol was selected as a solvent for an extraction, because alcohol is moderate in polarity, which helps in extracting most of the active constituents. In previous reported studies of *Vitis vinifera and Cichorium intybus*, investigators have used ethanol as solvent. In this investigation, Soxhlet’s apparatus was used for extraction. According to the Soxhlet’s procedure, active phytoconstituents are extracted by continuous washing or percolation with solvent.

4.2.1 Extraction of *Vitis vinifera*.

The collected fruits of *Vitis vinifera* (grapes) were pressed to isolate seeds. Skin and pulp of the fruit were removed by washing the seeds. The cleaned seeds were then left to dry openly under shade, for a week. The seeds were powdered using coffee
grinder (mechanical grinder), and it was passed through sieve number 40. Extraction of *Vitis vinifera* was carried out in batches with 300 g of powdered material in each batch. 300 g of powdered material was packed in the thimble, which was made from thick filter paper. After plugging the siphon tube with cotton, the thimble was placed in an extraction chamber or Soxhlet’s extractor. Cotton bed was placed on the top of sample to prevent its loss. Petroleum ether was taken in the round bottomed flask to remove fatty material from seed powder. Few porcelain chips were added into the flask to avoid bumping of the solvent. The Soxhlet’s assembly was inserted into the flask and the reflex condenser was fixed to the assembly with continuous water supply. The flask was heated at sixty degree Celsius, by setting temperature of heating mantel. Vaporized petroleum ether solvent was condensed back into liquid and allowed to trickles into the packed powder in the assembly. When the solvent surrounding the sample was exceeded to a certain level, it was overflowed and emptied by a siphon tube into the flask. The solvent percolated the powder continuously, resulting in separation of fatty material. During each cycle, the fatty material was collected into the round bottomed flask. The extraction of fatty material was done for six hours. After cooling the assembly, the petroleum ether extract was replaced with 2000 ml of 95% ethanol as a solvent. The packed powdered material was re-extracted with 95% ethanol at sixty five degree Celsius till the solvent entering into the siphon tube was colorless. After cooling, the extract was filtered with the help of filter paper. Solvent was recovered from the extract by evaporating under reduced pressure using rotary evaporator. Finally the viscous liquid was evaporated on water bath to a dry residue and kept in amber colored air tight bottle in the refrigerator.

**Determination of yield and physical characterization of extract:** The dried residue was weighed and its physical characters such a color, consistency and % yield were determined. The results of this determination were compiled in the table no 5.1.

**4.2.2 Extraction of *Cichorium intybus***

*Cichorium intybus* roots were isolated and chopped into small pieces and dried under shade for 2 weeks. The dried slices of roots were grounded to powder using mechanical grinder. The coarse powder of roots was obtained by passing through sieve. Extraction of *Cichorium intybus* was carried out in batches with 250 g of powdered material in each batch. 250gm of powder was charged in the thimble of the Soxhlet’s assembly, after plugging siphon tube with cotton. Top of the sample was packed with cotton. About 1.5 liter of 95% ethanol as a solvent was taken in the flask,
with few porcelain chips. Flask, extraction chamber and reflux condenser were fixed and solvent was heated at seventy degree Celsius using heating mantle. Entry of colorless solvent into the siphon tube was considered as the end of extraction. After cooling, the extract was evaporated using rotary evaporator to collect solvent. The viscous liquid was converted into dry residue on water bath and kept in amber colored air tight bottle in the refrigerator.

**Determination of yield and physical characterization of extract:** The dried residue was weighed and its physical characters such a color, consistency and % yield were determined. The results of this determination were compiled in the table no 5.1.

**4.3 Preliminary qualitative studies by phytochemical investigation**

*Vitis vinifera* seeds and *Cichorium intybus* root extracts were subjected to systematic chemical test analysis. The presence of different phytoconstituent was investigated, as described by the well established methods [167-168].

**Carbohydrates**

About 300 mg of solvent free extracts of *Vitis vinifera* and *Cichorium intybus* were dissolved in 6ml of distilled water and filtered. The filtrates were used in the following tests.

1. Molisch’s test: 2 ml of filtrate was mixed with 3 drops of ethanolic solution of \( \alpha \)-naphthol. Concentrated Sulphuric acid was added slowly, down the sides of the test tube without mixing to observe purple to violet ring between acid and test layer.

2. Barfoed’s test: 1 ml filtrate was mixed with Barfoed’s reagent, and heated for 5 min to get brick red precipitate.

3. Fehling’s test: 1 ml of filtrate was mixed with 1ml of Fehling’s solutions A (copper sulphate) and 1ml of Fehling’s solutions B (sodium potassium tartarate). Mixed solution was heated to observe red precipitate of cuprous oxide.

4. Benedict’s test: 1 ml of filtrate was mixed with Benedict’s reagent and heated to get red precipitate of cuprous oxide.
Proteins and free amino acids

About 200 mg of solvent free extracts of *Vitis vinifera* and *Cichorium intybus* were dissolved in 10ml of distilled water and filtered. The filtrates were used in the following tests.

1. Biuret test: 2 ml of filtrate was mixed with few drops of sodium hydroxide and 0.7 % copper sulphate solutions, to get violet or pink colour.
2. Millon’s test: 2 ml of filtrate was mixed with 3 drops of Millon’s reagent to get white precipitate. The solution was heated to get red colour.
3. Ninhydrin test: 2 ml of filtrate was mixed with 3 drops of 5% Ninhydrin solution, and boiled for few min to get purple or blue colour.

Alkaloids

About 100 mg of solvent free extracts of *Vitis vinifera* and *Cichorium intybus* were dissolved in small quantity of dilute hydrochloric acid and filtered. The filtrates were used in the following tests.

1. Mayer’s test: 2 ml of filtrate was mixed with 2 drops of Mayer’s reagent (potassium mercuric iodide), to get creamy precipitate.
2. Dragendroff’s test: 2 ml of filtrate was mixed with 3 drops of Dragendroff’s reagent (potassium bismuth iodide solution), to get orange brown precipitate.
3. Hager’s test: 2 ml of filtrate was mixed with Hager’s reagent (saturated picric acid solution), to get yellow precipitate.
4. Wagner’s test: 2 ml of filtrate was mixed with few drops of Wagner’s reagent (iodine reagent), to get reddish brown precipitate.

Glycosides

About 100 mg of solvent free extracts of *Vitis vinifera* and *Cichorium intybus* were hydrolyzed with dilute hydrochloric acid for few hours on a water bath. The hydrolysate was used in the following tests.

1. Borntrager’s test: 2 ml of hydrolysate was mixed with chloroform solvent and shaken well. The organic (chloroform layer) layer was mixed with ammonia solution to get pink color.
2. Legal test: 2 ml of hydrolysate was mixed with 1ml of pyridine and few drops of Sodium nitropruside. It was made alkaline to get pink or red color.

Phytosterols or Triterpenoids and their glycosides

1. Liebermann-Burchard’s test: 2 ml of extract was mixed with 1ml of chloroform, and then few drops of acetic anhydride were added and boiled.
After cooling, 1ml of concentrated Sulphuric acid was added along the sides of the test tube, to get brown or red ring at the junction of two layers or green color in the upper layer.

2. Salkowski’s test: 2 ml of extract was mixed with 2ml of chloroform, and then few drops of concentrated Sulphuric acid was added and shaken well. The mixture was allowed to stand, to get red or violet color at the junction or greenish fluorescence from acid layer.

Phenolic compounds and tannins
About 200 mg of solvent free extracts of *Vitis vinifera* and *Cichorium intybus* were dissolved in 10ml of distilled water and filtered. The filtrates were used in the following tests.

1. Ferric chloride test: 2 ml of extract was mixed with few drops of 5% ferric chloride solution to develop blue or green or violet color.
2. Lead acetate test: 2 ml of extract was mixed with 3ml of 10% lead acetate solution to develop white precipitate.
3. Gelatin test: 2 ml of extract was mixed with 2ml of 1% Gelatin solution, containing 10% sodium chloride. The mixture was shaken to develop white precipitate.

Flavonoids
1. Shinoda Test: 2 ml of extract was dissolved in alcohol and then few fragments of magnesium ribbon were added into it. Concentrated Hydrochloric acid was added drop wise to develop pink or magenta or green blue color.
2. Sodium hydroxide test: Solution of extract was mixed with few ml of Sodium hydroxide solution to develop yellow color. Colorless with the addition of Hydrochloric acid.
3. Lead acetate test: Extract solution was mixed with 10% lead acetate solution to develop yellow precipitate.

Saponins
Foam test: Extract was mixed in 20 ml of water and shaken vigorously in a cylinder for 15 min to get foam.

Fixed oils and fats
Filter paper stain test: Drop of extract was pressed between filter paper to get oil stain on the paper.

The results of this investigation were compiled in the table no 5.2.
4.4 Experimental animals
The pharmacological investigations were conducted on albino mice and albino rats of either sex.

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

- Animal maintenance (Housing and feeding condition)
  Experimental studies were 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 clean and sanitized polypropylene cages under standard environmental conditions of relative humidity (50±5%), room temperature (25±2°C) and photocycle (12:12h of light/dark period with lights on 0700h). Feed of animals was dietary pellets (pellets of Amruth Lab. Bangalore). Drinking water was maintained *ad libitum*.

  All animals were acclimatized and habituated to laboratory conditions, for seven days prior to experiment, to minimize nonspecific stress conditions.

4.5 Acute toxicity study
In this study, a single dose of the drug was administered orally to each animal for the determination of gross behavior and median lethal dose (LD$_{50}$), which can be expected to cause death in fifty percent of animals.

**Principle:** An acute toxicity study was conducted for *Vitis vinifera* and *Cichorium intybus* extracts, as per guidelines set by Organization for Economic Co-operation and Development (OECD guideline No. 425) received from CPCSEA.

Limit test at 5000 mg/kg body weight was considered to determine acute toxicity study of both the extracts. As per guidelines of OECD, this limit test would have a direct relevance for protecting human and animal health. As per this principle, animal was administered with the extract at the dose of 5000 mg per kg body weight. After the survival of the first animal, then two additional animals were administered with the extract at the dose of 5000 mg per kg body weight. Test was terminated after the survival of additional animals. Observation of animals was carried for 14 days.
Method: The following steps were followed.

1. Selection of animal: Six adult albino mice in the age group of 90±10 days were used for the study. All the six female mice were nulliparous and nonpregnant.

2. Conditioning of animals: The female mice were randomly selected and marked for identification. Mice were acclimatized for a period of one week to the laboratory conditions and then mice were fasted for four hours prior to the administration of the extract. But mice were allowed free access to water as and when required. To avoid coprophagy mice were fasted in wire bottomed cages. Body weights of all six mice were recorded.

3. Selection of dose: Dose of *Vitis vinifera* seeds and *Cichorium intybus* roots extracts were fixed at 5000mg per kg body weight of mice (Limit test dose as per guidelines no.425).

4. Preparation of stock solutions of extracts: Fresh suspension of extracts was prepared by suspending 50g of *Vitis vinifera* seeds extract or *Cichorium intybus* roots extract in 100 ml of distilled water using 1% w/v gum acacia.

5. Administration of extracts: Dose of extracts was calculated according to body wt of mice. Extracts were administered in a single dose, taking care so that volume of dose should not exceed 0.25ml for a 25gm of albino mice.

   Intra gastric administration of extract into the mice was done by using polythene tube of 3 cm long sleeved on 18 gauge blunted hypodermic needle. Mice were grasped by the nape of neck, holding the whole animal with the left hand. Tube was passed laterally thorough the interdental space by rotating motions, so that tube should advance into the esophagus. Then extracts fluid was introduced slowly. Food was withheld for further two hours, after administration of extract.

6. Observation: 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 and gross behavior. Gross behavior observations were behavior, neurological and autonomic responses [169].
I. Behavioral observation

1. Awareness
   - Alertness: Stimulation or depression of the CNS was recorded by observing alertness or loss of consciousness (stupor).
   - Stereotypy: Another parameter for central stimulation or depression was recorded by observing stereotypy. It was noted by observing frequent repetitive circling or searching movements of the head.
   - Visual placing: Motor incoordination was recorded by observing visual placing. In this, animal was observed for orientation itself without fall, when placed in different position.
   - Passivity: Central depression was also recorded by observing passivity. Here mouse was placed in unaccustomed positions to observe struggle behaviors.

2. Mood
   - Vocalization: Sound produced by speech organs and uttered by the mouth of mouse was observed to rule out noxious stimulus.
   - Grooming: Excess of grooming was recorded as central stimulation.
   - Restlessness: Central stimulation was also recorded by observing restlessness in mouse.
   - Irritability: Aggressiveness was observed for any CNS stimulation.
   - Fearfulness: it was recognized from gentle manipulation of the mice to any condition.

3. Motor activity
   - Spontaneous activity: it was observed by placing the mouse in a bell jar to find CNS stimulation or depression.
   - Reactivity: Observed by placing mice on a table to find CNS effect.
   - Touch response: it was observed by touching the neck or abdomen of the mouse with forceps, to find anesthetic activity.
   - Pain response: it was noted by pressing tail base with artery clamp

II. Neurological observation

1. Central excitation
   - Straub response: Elevation of tail of mouse was observed.
   - Tremor: shaking of mouse limbs was observed.
   - Convulsion: Epilepsy was observed as a central excitation.
2. Motor incoordination
   - Body position: Change in normal position was observed to find neuromuscular blockade.
   - Limb position: Deviation in normal limb position was observed to find neuromuscular blockade or central disturbance.
   - Somersault test (Righting reflex): Mouse was picked up by the tail and tossed in the air, so that a somersault of 2 or 3 turns was made before the mouse rest upon rubber pad. This was repeated 5 times.

3. Muscle tone
   - Limb tone: Resistance to extension of the forepaw of the mouse was observed when it was grasped.
   - Grip strength: it was measured by allowing the animal to grasp a pencil.
   - Body tone: it was observed by comparing muscle tone with control animals.
   - Abdominal tone: it was observed by comparing tone of muscles with control mouse.

4. Reflexes
   - Pinna reflex: Ability of mouse to withdraw from irritating hair was observed, when pinna was touched with the hair.
   - Corneal reflex: Ability of mouse to withdraw from irritating hair was observed, when Cornea was touched with the hair.

III. Autonomic profile.
   1. Optical signs
      - Pupil size: Constriction or dilatation of the pupil size was measured.
   2. Secretory signs
      - Urination and salivation of mouse was observed.
   3. General signs
      - Writhing: It was observed for possible irritation of tissue.
      - Piloerection: Erection of hair of mouse was observed as a possible compensation for lower temperature.
      - Skin color: Change in color of ear was observed.

The results of this investigation were compiled in the table no 5.3 to 5.4.
4.6.1 Effect of extracts of *Vitis vinifera* seeds and *Cichorium intybus* roots on swimming endurance of mice.

**Purpose and rationale:** In this method, the antistress effect of extracts was evaluated by determining the improvement in swimming endurance period and overall performance of the animals, when subjected to swim in restricted space like water vessel [145, 179].

**Animals:** Experimental investigation was carried out using albino mice of either sex, weighing between 22 to 26 g. Experiment was conducted in compliance with the guidelines provided by CPCSEA.

**Drugs:** Testing drugs were *Vitis vinifera* seeds and *Cichorium intybus* roots extracts suspended in distilled water using 1% w/v gum acacia. Geriforte was used as standard drug suspended in distilled water using 1% w/v gum acacia. Vehicle was 1% w/v gum acacia prepared in normal saline.

**Dose selection:** The dose of plant extracts selected in this investigation was 200 and 400 mg/kg body weight of animals. The dose of Geriforte was 100 mg/kg body weight of animal, selected form previous reported studies of Geriforte as antistress agent [38]. The dose of vehicle was 10 ml/kg body weight of animal.

**Treatment protocol:** Albino mice of either sex were randomly assigned into six groups, and each group consisted of six mice as follows:

- **Group 1:** Animals of this stress control group were administered with vehicle and subjected for swimming stress.

- **Group 2:** Albino mice of this group were administered with *Vitis vinifera* seeds extract at the dose of 200 mg/kg body weight and subjected for swimming stress.

- **Group 3:** This group of animals were administered with *Vitis vinifera* seeds extract at the dose of 400 mg/kg body weight and subjected for swimming stress.

- **Group 4:** Albino mice of this group were administered with *Cichorium intybus* roots extract at the dose of 200 mg/kg body weight and subjected for swimming stress.

- **Group 5:** Albino mice of this group were administered with *Cichorium intybus* roots extract at the dose of 400 mg/kg body weight and subjected for swimming stress.

- **Group 6:** In this standard group, animals were administered with Geriforte at the dose of 100 mg/kg body weight and then subjected for swimming stress.
Method: The swimming endurance was accomplished as described by previous investigators [170]. Albino mice of all groups were treated with vehicle, extracts and Geriforte by oral route in the above order. The treatment was given for a period of 10 consecutive days. On tenth day, swimming stress was given to all the mice, by allowing for swimming in the transparent tub containing water at room temperature. The height and diameter of the transparent tub were 30 cm and 25 cm respectively. Water level was maintained to the height of 25 cm in the transparent tub, so that mice were unable to touch the bottom or escape from the top. The swimming of these mice was not restrained and they allowed for swimming till they got exhausted. The end point of this swimming endurance was drowning and death of the mice. The swimming endurance of each mouse was recorded by observing duration of survival time, during swimming stress. The obtained results were expressed as mean survival time for each group with standard error of mean. The results of this investigation were compiled in the table 5.5 and graphically represented in figure no.5.1

Statistical analysis: The date of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with p<0.05 was considered as significant.

4.6.2 Effect of extracts of Vitis vinifera seeds and Cichorium intybus roots on drug induced narcosis in mice.

Purpose and rationale: In this method, the antistress effect of extracts was evaluated by determining their (Adaptogen) ability to synergize with CNS depressant such as barbiturates, and it could be the mechanism of antistress activity [146].

Animals: Experimental investigation was carried out using albino mice of either sex, weighing between 22 to 26 g. Experiment was conducted in compliance to the guidelines provided by CPCSEA.

Drugs: Testing drugs were Vitis vinifera seeds and Cichorium intybus roots extracts suspended in distilled water using 1% w/v gum acacia. Pentobarbitone sodium was used as CNS depressant, whereas Chlorpromazine was used as standard synergizing CNS depressant.

Dose selection: The dose of plant extracts selected in this investigation was 200 and 400 mg/kg body weight of animals. The dose of Pentobarbitone sodium was 50 mg/kg body weight of animal, whereas dose of Chlorpromazine was 3 mg/kg body weight of animal, based on previous reported studies [146,171]. The dose of vehicle was 10 ml/kg body weight of animal.
**Treatment protocol:** Albino mice of either sex were randomly assigned into seven groups, and each group consisted of six mice as follows:

Group 1: Albino mice of this group were administered with vehicle, thirty minutes prior to Pentobarbitone sodium administration.

Group 2: Animals of this group were administered with *Vitis vinifera* seeds extract at the dose of 200 mg/kg body weight, thirty minutes prior to Pentobarbitone sodium administration.

Group 3: This group of animals were administered with *Vitis vinifera* seeds extract at the dose of 400 mg/kg body weight, thirty minutes prior to Pentobarbitone sodium administration.

Group 4: Albino mice of this group were administered with *Cichorium intybus* roots extract at the dose of 200 mg/kg body weight, thirty minutes prior to Pentobarbitone sodium administration.

Group 5: Albino mice of this group were administered with *Cichorium intybus* roots extract at the dose of 400 mg/kg body weight, thirty minutes prior to Pentobarbitone sodium administration.

Group 6: In this standard group, animals were administered with were given Chlorpromazine at the dose of 3 mg/kg body weight, thirty minutes prior to Pentobarbitone sodium administration.

Group 7: Albino mice of this group were given Chlorpromazine at the dose of 3 mg/kg body weight.

**Method:** Albino mice of group 1, group 2, group 3, group 4 and group 5 were treated orally with vehicle, *Vitis vinifera* seeds extract at 200 mg per kg body wt., *Vitis vinifera* seeds extract at 400 mg per kg body wt., *Cichorium intybus* roots extract at the dose of 200 mg/kg body weight and *Cichorium intybus* roots extract at the dose of 400 mg/kg body weight respectively. Albino mice of group 6 and group 7 were treated with Chlorpromazine at the dose of 3 mg per kg body wt. by intraperitoneal route. After 30 minutes Pentobarbitone sodium was administered by intraperitoneal route to all the groups except group 7. In each mouse narcosis time was recorded, by observing the loss of righting reflex and regain of the righting reflex due to CNS depression. This narcosis time was expressed in minute.

The obtained results were expressed as mean narcosis time for each group with standard error of mean. The results of this investigation were compiled in the table no 5.6 and graphically represented in figure no. 5.2
Statistical analysis: The data of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with p<0.05 was considered as significant.

4.6.3 Effect of extracts of *Vitis vinifera* seeds and *Cichorium intybus* roots on cold and restraint stress induced brain lipid peroxidation.

**Purpose and rationale:** In this method, the antistress effect of extracts was evaluated by determining their adaptogenic ability of protection against stress induced brain lipid peroxidation and oxidative damage of tissue [149]. This could be the mechanism of antistress activity.

**Animals:** Experimental investigation was carried out using albino rats of either sex, weighing between 180 to 210g. Animals were handled and subjected for investigation in compliance to the guidelines provided by animal ethics committee.

**Drugs:** *Vitis vinifera* seeds and *Cichorium intybus* roots extracts were testing drugs, whereas Geriforte was used as standard antistress agent. All drugs were suspended in distilled water using 1% w/v gum acacia. Vehicle was 1% w/v gum acacia prepared in normal saline.

**Dose selection:** The dose of plant extracts selected in this investigation was 250 and 500 mg/kg body weight of animals. That is 5% and 10% of the maximum tolerated dose was selected. The dose of Geriforte was 100 mg/kg body weight of animal, based on previous reported studies of Geriforte as antistress agent [38]. The dose of vehicle was 10 ml/kg body weight of animal.

**Treatment protocol:** Albino rats of either sex were randomly assigned into seven groups, and each group consisted of six animals as follows:

- **Group 1:** Albino rats of this control group received vehicle.
- **Group 2:** Animals of this stress control group were administered with vehicle, and then subjected for cold and restraint stress.
- **Group 3:** In this standard group, animals were administered with Geriforte at the dose of 100 mg/kg body weight, and then subjected for cold and restraint stress.
- **Group 4:** Albino rats of this group were administered with *Vitis vinifera* seeds extract at the dose of 250 mg/kg body weight, and then subjected for cold and restraint stress.
Group 5: This group of animals were administered with *Vitis vinifera* seeds extract at the dose of 500 mg/kg body weight, and then subjected for cold and restraint stress.

Group 6: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 250 mg/kg body weight, and then subjected for cold and restraint stress.

Group 7: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 500 mg/kg body weight, and then subjected for cold and restraint stress.

**Method:** Rats of all groups were treated with vehicle, extracts and Geriforte by oral route in the above order. The treatment was given one hour prior to stress procedure for a period of 21 consecutive days. Animals of all groups were subjected to stress except group-1. It was accomplished by immobilizing the animal at cold temperature. Rats were immobilized on wooden plank and kept at 4–6 °C for 15 min. To avoid habituation, rats were subjected to stress at random time, once a day. On 21st day, rats of all groups were sacrificed by decapitation. Skull was dissected to excise brain. Isolated brain was rinsed in ice cold saline solution to remove blood. Then it was blotted, weighed and its homogenate was prepared for the analysis of malondialdehyde.

**Estimation of Malondialdehyde (MDA):** It was carried by the well-established method of Ohkawa H et. al., as follows [172]. 1g of wet tissue and 9ml of 1.15% of potassium chloride were homogenized to get 10% w/v of brain homogenate. 0.2ml of homogenate was taken in a test tube and mixed with 0.2ml of 8.1% sodium dodecyl sulphate. Then 1.5 ml of 20% acetic acid solution was added to the above test tube. The pH of acetic acid solution was 3.5, and it was adjusted with sodium hydroxide. Finally 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to the above test tube. The volume of mixture in the test tube was made up to 4 ml with distilled water. The mixture of solution was heated at 95 °C for one hour to obtain maximum color intensity. The solution was cooled and 1ml of distilled water was added to it. To extract reaction product, 5ml of mixture of n-butanol and pyridine (15:1v/v) was added and centrifuged. The upper organic layer was separated and its absorbance was measured in UV Spectrophotometer at 532nm against the reagent blank. Standard solution of Malondialdehyde was prepared using 1, 1, 3, 3-tetra methoxy propane, and
its absorbance was measured at different concentration to prepare calibration curve. Finally the level of lipid peroxidation in different samples of isolated brain tissue was calculated using calibration curve and it was expressed as nmol MDA/g of wet tissue. The obtained results were expressed as mean nmol MDA/g of wet tissue for each group with standard error of mean. The results of this investigation were compiled in the table no 5.7 and graphically represented in figure no.5.3.

**Statistical analysis:** The data of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with p<0.05 was considered as significant.

### 4.6.4 Effect of extracts of *Vitis vinifera* seeds and *Cichorium intybus* roots on swimming stress induced gastric ulceration in albino rats.

**Purpose and rationale:** In this method, the antistress effect of extracts was evaluated by determining their adaptogenic ability of protection against gastric ulceration in albino rats, induced by Chronic forced swimming stress.

**Animals:** Experimental investigation was carried out using normal adult albino rats of Wister strain, weighing between 180 to 220g. Animal experiments were carried out under the spirit of CPCSEA guidelines provided by animal ethics committee.

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.

**Drugs:** *Vitis vinifera* seeds and *Cichorium intybus* roots extracts were testing drugs, whereas Omeprazole was used as standard drug. Drugs were suspended in distilled water using 1% w/v gum acacia. Vehicle was 1% w/v gum acacia prepared in normal saline.

**Dose selection:** The dose of plant extracts selected in this investigation was 250 and 500 mg/kg body weight of animals. The dose of Omeprazole was 10 mg/kg body weight of animal, selected form previous reported studies [173]. The dose of vehicle was 10 ml/kg b. w. of animal.
**Treatment protocol:** Albino rats of either sex were randomly assigned into seven groups, and each group consisted of six animals as follows:

Group 1: Albino rats of this control group received vehicle only.

Group 2: Albino rats of this stress control group received vehicle and then subjected for chronic forced swimming stress.

Group 3: Albino rats of this standard group were administered with Omeprazole at the dose of 10mg/kg body weight, and then subjected for chronic forced swimming stress.

Group 4: Albino rats of this group were administered with *Vitis vinifera* seeds extract at the dose of 250 mg/kg body weight, and then subjected for chronic forced swimming stress.

Group 5: Albino rats of this group were administered with *Vitis vinifera* seeds extract at the dose of 500 mg/kg body weight, and then subjected for chronic forced swimming stress.

Group 6: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 250 mg/kg body weight, and then subjected for chronic forced swimming stress.

Group 7: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 500 mg/kg body weight, and then subjected for chronic forced swimming stress.

Rats of all groups were treated with vehicle, extracts and omeprazole by oral route in the above order. The treatment was given one hour prior to stress procedure for a period of 10 consecutive days. Drugs were administered to rats by oral route using oral gavage. Tube was passed lateral space through the interdentally by rotating motions so that tube should advance into the esophagus. Then drug solution was introduced slowly. Food was withheld for a further 2 h after the administration of the drugs. Animals of all groups were subjected to forced swimming stress except group-1. Forced swimming stress was accomplished as described by previous investigators [19, 174]. In this model rats were forced to swim in the transparent tub (50cm deep × 27cm wide) containing water to the depth of 40 cm maintained at 25±2°C. Swimming time was 15 min daily for 10 consecutive days to get chronic stress induced ulcers. Water level was maintained up to their neck region and they were forced to swim. Albino rats suffered stress as they were not allowed to stand on bottom or grasping the side of the tub. Even it was ensured that the animals could not climb out of tub.
Rats were removed from the water after 15 minute and dried by towel and were placed under a 60 watts bulb for drying. 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 numbers of ulcer and severity of ulcer.

1. **Ulcer number:** The numbers of ulcer into the stomach were counted.
2. **Ulcer severity score:** The severity of ulcer was assessed in the range of 0 to 3 scale. Severity of ulcer scale adopted was as follows [175].
   
   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 [175].

\[ U_I = U_N + U_S + U_P \times 10^{-1}, \text{ Where} \]
\[ U_I = \text{Mean ulcer index.} \]
\[ U_N = \text{Mean ulcer number of rat.} \]
\[ U_S = \text{Mean ulcer severity score of rat.} \]
\[ U_P = \text{Ulcer percentage of rat.} \]

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

\[ \% \text{ ulcer protection} = \frac{\text{Mean ulcer index of control} - \text{Mean ulcer index of test}}{\text{Mean ulcer index of control}} \times 100 \]

5. **Gastric secretion pH:** The collected gastric contents were centrifuge at 1000 rpm for 10 min. Supernatant liquid of the centrifuged sample was taken and diluted with distilled water. pH was recorded.

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 haematoxyline-eosin stain (HE-Stain). The stained
slides were examined under a research microscope with magnification of 100X and photographs were taken. The different histopathological indices screened were changes in lining of mucosal epithelium, sub mucosal edema, necrosis, congestion and hemorrhage in tissue.

The obtained results were expressed as mean Ulcer Number, mean Ulcer Severity score, Ulcer Percentage, mean Ulcer index, % ulcer protection and pH for each group with standard error of mean. The results of this investigation were compiled in the table no 5.8 and graphically represented in figure no.5.4.

**Statistical analysis:** The data of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with p<0.05 was considered as significant.

### 4.6.5 Effects of adaptogenic plant extract on adrenocortical activity in stress induced rats.

**Purpose and rationale:** In this method, the antistress effect of extracts was evaluated by determining their adaptogenic effect on adrenocortical activity in albino rats, during cold water swimming stress. This could be the mechanism of antistress activity.

**Animals:** Experimental investigation was carried out using albino rats of Wister strain, weighing between 190 to 220g. Animal experiments were carried out under the spirit of CPCSEA guidelines provided by animal ethics committee.

**Drugs:** *Vitis vinifera* seeds and *Cichorium intybus* roots extracts were testing drugs, whereas Diazepam was used as standard drug. Drugs were suspended in distilled water using 1% w/v gum acacia. Vehicle was 1% w/v gum acacia prepared in normal saline.

**Dose selection:** The dose of plant extracts selected in this investigation was 250 and 500 mg/kg body weight of animals. The dose of Diazepam was 5 mg/kg body weight of animal, based on previous reported studies [176]. The dose of vehicle was 10 ml/kg body weight of animal.

**Treatment protocol:** Albino rats of either sex were randomly assigned into nine groups, and each group consisted of six animals as follows:

- **Group 1:** Albino rats of this control group received vehicle.
Group 2: Animals of this stress control group were administered with Vehicle, and then subjected for cold water swimming stress.

Group 3: In this standard group, animals were administered with diazepam at the dose of 5 mg/kg body weight, and then subjected for cold water swimming stress.

Group 4: Albino rats of this group were administered with *Vitis vinifera* seeds extract at the dose of 250 mg/kg body weight, and then subjected for cold water swimming stress.

Group 5: Albino rats of this group were administered with *Vitis vinifera* seeds extract at the dose of 500 mg/kg body weight, and then subjected for cold water swimming stress.

Group 6: This group of animals were administered with *Cichorium intybus* root extract at the dose of 250 mg/kg body weight, and then subjected for cold water swimming stress.

Group 7: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 500 mg/kg body weight, and then subjected for cold water swimming stress.

Group 8: This group of animals were administered with *Vitis vinifera* seeds extract at the dose of 500 mg/kg body weight. Animals were not subjected for cold water swimming stress.

Group 9: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 500 mg/kg body weight. Animals were not subjected for cold water swimming stress.

Rats of all groups were treated with vehicle, extracts and diazepam by oral route in the above order. The treatment was given daily for a period of 10 consecutive days. Drugs were administered to rats by oral route using oral gavages. Food was withheld for a further 1 h after the administration of the drugs. On tenth day, animals of group 2 to group 7 were subjected to cold water swimming stress for an acute period, after one hour of drug administration. Cold water swimming stress was accomplished as described by previous investigators [19, 157]. In this model rats were allowed to swim in the cold water of transparent tub (60cm depth × 40cm width) containing water to the depth of 50 cm maintained at 15±5°C. Water level was maintained up to the neck region. 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 minutes. To avoid
further stress, rats were anaesthetized with diethyl ether to stage of surgical anesthesia. Blood samples were withdrawn from retro orbital sinus of the eye (inner canthus of the eye) using microhaematocrit capillary tube. Uncotted blood for separation of plasma was collected in ice cold tubes containing heparin (100 unit heparin/ ml of blood). Blood was centrifuged at 3000 rpm for 10 minute and supernatant was separated as plasma and stored in cold condition (±4°C) until further processing for estimation of plasma corticosterone. Rats were euthanized by cervical dislocation method. Adrenal glands were dissected out, washed with alcohol and their weights were recorded with respect to their body weights (expressed as weight of adrenal gland / 100 g of body weight of rats).

**Estimation of plasma corticosterone formed during activation of adrenocortical activity in albino rats:** It was carried by the well-established method of Mattingly, as follows [177].

**Materials:** Fluorescence reagent: it was prepared by mixing concentrated sulphuric acid and absolute alcohol in the ratio of 7:3. Then it was cooled .The reagent was colorless and stable at room temperature.

- Standard stock solution of corticosterone: 50 mg of corticosterone was dissolved in 50 ml of absolute alcohol. 1 ml of above solution was diluted up to 100 ml with distilled water to produce 10µg/ml stock solution. Both these solutions were kept at cold condition to remain stable for long period. Working standard solution was prepared by diluting above stock solution to produce 1µg/ml.
- Sample: It was obtained by extraction of corticosterone from plasma. Two ml of above separated plasma was taken into a 250 ml of conical flask and 15 ml of methylene chloride (organic solvent) was added into it, to extract the corticosterone. The conical flask was shaken well using mechanical shaker for 10 minute. Two layers were formed. The upper layer was aspirated and discarded. 10 ml of lower layer of methylene chloride extract was collected into a 20 ml of glass stoppered extraction tube.
- Reagent blank: Two ml of distilled water was taken into a conical flask and remaining procedure was carried as per that of sample treatment.
Standard: Two ml of corticosterone solution of known strength (1µg/ml) was taken into a conical flask and remaining procedure was carried as per that of sample treatment.

Fluorimetric analysis method: Either 10 ml of plasma extractor or reagent blank or standard was taken into a 20 ml of glass stoppered extraction tube. 5ml of fluorescence reagent was added into it. The extraction tube was shaken vigorously for 20 second. Again supernatant methylene chloride layer was sucked off. The lower acid extract layer was taken into a glass cell and reading was recorded using Systronic photofluorimeter. Excitation was done at 470 nm and readings were taken at peak transmission at 530nm. The concentration of plasma corticosterone was determined as follows: Concentration of plasma corticosterone= \text{Reading of unknown} \times 100 \over \text{Reading of known}

The concentration of plasma corticosterone was expressed as µg/100 ml plasma. The obtained results were expressed as mean µg/100 ml plasma for each group with standard error of mean. The results of this investigation were compiled in the table no 5.9 and graphically represented in figure no.5.6 and 5.7.

Statistical analysis: The data of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with p<0.05 was considered as significant.

4.6.6 Effects of adaptogenic plant extract on liver glycogen of albino rats during weight loaded forced swimming stress.

Purpose and rationale: In this method, the antistress effect of extracts was evaluated by determining their adaptogenic effect or their protective effect on depletion of liver glycogen and behavioral despair in albino rats, during weight loaded forced swimming stress.

Animals: Experimental investigation was carried out using albino rats of Wister strain, weighing 200±25g. Animal experiments were carried out under the spirit of CPCSEA guidelines provided by animal ethics committee.

Drugs: Vitis vinifera seeds and Cichorium intybus roots extracts were testing drugs, whereas Ashwagandha was used as standard antistress agent. Drugs were suspended in distilled water using 1% w/v gum acacia. Vehicle was 1% w/v gum acacia prepared in normal saline.
**Dose selection:** The dose of plant extracts selected in this investigation was 250 and 500 mg/kg body weight of animals. The dose of Ashwagandha was 100mg/kg body weight of animal, based on previous reported studies [157]. The dose of vehicle was 10 ml/kg body weight of animal.

**Treatment protocol:** After acclimatization period, the albino rats of either sex were randomly assigned into nine groups, and each group consisted of six animals as follows:

Group 1: Albino rats of this control group received vehicle.

Group 2: Animals of this stress control group were administered with vehicle, and then subjected for weight loaded forced swimming stress.

Group 3: In this standard group, animals were administered with Ashwagandha at the dose of 100 mg/kg body weight, and then subjected for weight loaded forced swimming stress.

Group 4: Albino rats of this group were administered with *Vitis vinifera* seeds extract at the dose of 250 mg/kg body weight, and then subjected for weight loaded forced swimming stress.

Group 5: This group of animals were administered with *Vitis vinifera* seeds extract at the dose of 500 mg/kg body weight, and then subjected for weight loaded forced swimming stress.

Group 6: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 250 mg/kg body weight, and then subjected for weight loaded forced swimming stress.

Group 7: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 500 mg/kg body weight, and then subjected for weight loaded forced swimming stress.

Group 8: This group of animals were administered with *Vitis vinifera* seeds extract at the dose of 500 mg/kg body weight. Animals were not subjected for stress.

Group 9: Albino rats of this group were administered with *Cichorium intybus* root extract at the dose of 500 mg/kg body weight. Animals were not subjected for stress.

Rats of all groups were treated with vehicle, extracts and Ashwagandha by oral route in the above order. The treatment was given one hour prior to stress procedure for a period of 10 consecutive days. Drugs were administered to rats by oral route.
using oral gavage. Food was withheld for a further 1 h after the administration of the drugs. Animals of group 2 to group 7 were subjected to weight loaded forced swimming stress. Weight loaded forced swimming stress was accomplished as described by previous investigators [158]. In this model rats were forced to swim in the transparent tub (60cm depth × 40cm width) containing water to the depth of 50 cm at room temperature. Each albino rat was loaded with steel weight (approximately 4% of their body weight), and it was fixed to their tail. Swimming time was 15 m daily for 10 consecutive days to get chronic stress. Water level was maintained up to their neck region and they were forced to swim. Albino rats suffered stress as they were not allowed to stand on bottom or grasping the side of the tub. Even it was ensured that the animals could not climb out of tub. Rats were removed from the water after 15 minutes and dried by towel and were placed under a 60 watts bulb for drying.

Assessment of swim stress induced behavioral despair.

The animal immobility period was used to assess behavioral despair. Duration of immobility of albino rats were observed on the first and last day (10th day) of weight loaded forced swimming stress treatment. After initial 10 minutes of swimming with attempts to escape, animals were observed for duration of immobility with in the next 5 minute period. Albino rats were considered immobile, when they stopped swimming and head was floating above the surface of water level [145]. Stress induced behavioral despair (duration of immobility) of albino rats were observed in the group 2 to 7.

Dissection of liver: On tenth day after subjecting to stress session for 15 minutes, albino rats were first anaesthetized with ether to avoid further stress. Then animals were euthanized by cervical dislocation. Albino rats were secured on operating table and its abdomen was opened. The anterior abdominal wall muscle of rat was incised using a sharp scalpel. Livers were immediately excised from the albino rats, cleaned of extraneous tissue and blotted on filter paper. The livers were kept at cold condition.

Estimation ofLiver glycogen in albino rats: It was done by well established method explained by Montgomery, as follows [178]. This method is based on reaction of phenol sulfuric acid with alkali soluble polysaccharide (glycogen) of tissue and subsequently the developed color intensity is measured by colorimeter. The color intensity indicates the amount of glycogen in liver.
50 mg of liver sample was taken randomly, as glycogen appeared to be distributed evenly throughout liver [178]. It was digested for 20 minute on a boiling water bath with 2ml of 30% potassium hydroxide solution, which stopped all enzymatic action and prevented rapid glycogenolysis. Glycogen was extracted from liver by precipitation with alcohol, as glycogen is insoluble in ethanol. So 2.8 ml of absolute alcohol was added to above digested mixture. After centrifugation at 2000 rpm for 5 minute, the supernatant was discarded and the precipitate was dissolved in 2 ml of water. Then 0.2 ml of above mixture was mixed with 1.7 ml of distilled water and 0.1mol of 80 % phenol. Finally 5 ml of concentrated sulphuric acid was added within 5 second. Good mixing was ensured by directing the stream of acid against the liquid surface and shaking the tube. After 30 minute of standing the reaction mixture, the yellow orange color absorbance was measured at 490nm using spectrophotometer. The yellow orange color was stable for several hours. Reagent blank and standard were run simultaneously. Calibration curve was prepared by using standard glycogen. Reagent blank value or sample blank value is substracted from sample value. The amount of liver glycogen was expressed as mg/gm for each group with standard error of mean. The results of this investigation were compiled in the table no 5.10 to 5.11 and graphically represented in figure no 5.8.

Statistical analysis: The data of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with p<0.05 was considered as significant.

4.6.7. Effect of extracts of Vitis vinifera and Cichorium intybus as Immunomodulatory during stress and drug induced myelosuppression in albino rats.

Purpose and rationale: In this method, the antistress effect of extracts was evaluated by determining their adaptogenic protective effect on immunosuppression.

Animals: Experimental investigation was carried out using albino rats of Wister strain, weighing 175 to 210 g. Animal experiments were carried out as per CPCSEA guidelines provided by IAEC.

Drugs: Vitis vinifera seeds and Cichorium intybus roots extracts were testing drugs, whereas Diazepam was used as standard drug. Drugs were suspended in distilled water using 1% w/v gum acacia. Vehicle was 1% w/v gum acacia prepared in normal saline.
Dose selection: The dose of plant extracts selected in this investigation was 250 and 500 mg/kg body weight of animals. The dose of Diazepam was 5 mg/kg body weight of animal, based on previous reported studies [176]. The dose of Cyclophosphamide was 3 mg/kg body weight of animal, based on previous reported studies [163]. The dose of vehicle was 10 ml/kg body weight of animal. The dose of Ashwagandha was 100mg/kg body weight selected from the previous reported studies [163].

Treatment protocol: After acclimatization period, the albino rats of either sex were randomly assigned into eight groups, and each group consisted of six animals as follows:

Group 1: Albino rats of this control group received vehicle.
Group 2: Animals of this stress control group were administered with vehicle, and then subjected for stress.
Group 3: In this standard group, animals were administered with Diazepam at the dose of 5 mg/kg b.w., and then subjected for Restraint stress.
Group 4: Albino rats of this group were administered with Vitis vinifera seeds extract at the dose of 500 mg/kg b.w., and then subjected for Restraint stress.
Group 5: This group of animals were administered with Cichorium intybus root extract at the dose of 500 mg/kg b.w., and then subjected for Restraint stress.

Group 6: Albino rats of this group were administered with Cyclophosphamide alone at the dose of 3 mg/kg b.w.

Group 7: In this standard group, animals were pretreated with Ashwagandha at the dose of 100mg/kg b.w. then cyclophosphamide was administered at the dose of 3 mg/kg b.w.

Group 8: Animals of this group were administered with Vitis vinifera seeds extract at the dose of 500 mg/kg b.w. After 30 minute, Cyclophosphamide was administered at the dose of 3 mg/kg b.w.

Group 9: Albino rats of this group were administered with Cichorium intybus root extract at the dose of 500 mg/kg b. w. After 30 minute, Cyclophosphamide was administered at the dose of 3 mg/kg b.w.

Group 10: Albino rats of this group were administered with Vitis vinifera seeds extract at the dose of 500 mg/kg b.w.

Group 11: This group of animals were administered with Cichorium intybus root extract at the dose of 500 mg/kg b.w.
Animals of all groups were treated with vehicle, extracts, diazepam, Ashwagandha and Cyclophosphamide by oral route in the above order. Animals of group 2, 3, 4 & 5 were treated with vehicle, diazepam, Vitis vinifera & Cichorium intybus respectively, for a period of 10 consecutive days. On 10\textsuperscript{th} day animals of group 2 to 5 were subjected to restraint stress as described by previous investigators [176]. Animal were restrained on wooden board for a period of 5 hrs. Animals of group 6 to 9 were treated with Cyclophosphamide. Groups 7, 8 and 9 were pretreated with Ashwagandha, Vitis vinifera and Cichorium intybus respectively. Treatment was given for a period of 10 consecutive days as described by previous investigators [163,176].

Animals of group 1, 10 & 11 were received vehicle, Vitis vinifera & Cichorium intybus respectively for a period of 10 consecutive days. On 10\textsuperscript{th} day blood samples were collected from all animals. Blood samples were withdrawn from retro orbital sinus of the eye (inner canthus of the eye) using microhaematocrit capillary tube. Blood was collected in a vial containing anticoagulant for evaluating haematological parameter viz; total leucocytes count, differential leucocytes count, RBC count, haemoglobin content and platelet count. The results of this investigation were compiled in the table no: 5.12 & 5.13.where as it was represented graphically in figure 5.9.

\textbf{Statistical analysis}: The data of this investigation was subjected to one way analysis of variance, followed by Dunnet’s test for comparison between the groups of treatment. Difference with \( p<0.05 \) was considered as significant.