CHAPTER 6

DISCUSSION

Acute toxicity studies are performed to determine the adverse effects of the drugs, which also provide insight into the drugs activity and degree of lethality. Thus, in the present investigation, acute toxicity study of plant extracts of *Vitis vinifera* and *Cichorium intybus* was performed by determining the quantitative aspect like LD$_{50}$ and observing gross behavior. Both the plant extracts did not show any mortality or a moribund status in mice.

Testing of swimming endurance of mice is widely used method to evaluate antistress activity of drugs. When animals are subjected to swim in a water vessel, which is not their natural habitat, they initially show struggling and swimming activity to escape from the water. Later on they become immobile due to depression, which is a common response during stress [145]. As a result of this immobility, drowning and death of mice occurs, and this parameter was used as an evaluation parameter in this investigation. Extracts of *Vitis vinifera* and *Cichorium intybus* have significantly increased the survival time or swimming endurance of mice at the tested doses of 200 and 400 mg per kg body weight when compared to the untreated mice. In a dose response study, when the doses of *Vitis vinifera* and *Cichorium intybus* extracts were raised, the swimming survival response of mice was also found to be increased. Even the effect of *Vitis vinifera* extracts at 400 mg per kg body weight was found to be highest, but non significant compared to the Geriforte as standard drug. Geriforte, a combination of several plant ingredients, exhibited antistress effect in experiment by previous investigators [38].

Effect of adaptogenic plant extract on drug induced narcosis in mice as a method, was designed by Ahumada et. al. [146]. They also reported that, mechanism of antistress activity could be the capacity of adaptogen or antistress agent to depress the CNS [146]. Whenever CNS of animal is depressed, they are unable to maintain posture, is which called as loss of righting reflex. This parameter was used as an evaluation parameter in this investigation. Extracts of *Vitis vinifera* and *Cichorium intybus* have significantly increased the narcosis time or prolonged the duration of loss of righting reflex in mice. This effect of extracts was observed at the tested doses of 200 and 400 mg per kg body weight, when compared to the control group of mice. In a dose response study, when the doses of *Vitis vinifera* and *Cichorium intybus*
extracts were raised, the synergism between extracts and Pentobarbitone sodium was also found to be increased, with enhanced narcosis time of mice. Even the effect of *Vitis vinifera* seed extracts at 400 mg per kg body weight was found to be approximately equally effective with that of standard drug.

Recently evaluation of lipid peroxidation in tissue due to stress is employed as a method to study antistress effects of drugs [149, 180-181]. During this method, the effects of *Vitis vinifera and Cichorium intybus* extracts on lipid peroxidation in brain of rats was investigated. The target for different stressors is the brain, as it is more sensitive for degeneration due to stress [149]. During this investigation, cold and restraint stress was used to induce stress as described by previous investigators with some modification [149]. Both cold temperature and restraint of animal produces synergism leading to intense stress. It results in HPA axis activation, leading to release of adrenocortical hormone responsible for stressful response [19]. In this model chronic or repeated exposure of rats for 21 days to stressful condition was carried, as it causes a wide range of physiological and neuroendocrine changes [148]. During this physical and emotional stress, lipid peroxidation in the brain of rats occurs [150], as brain tissue contains large amount of polyunsaturated fatty acids. There are several investigations that indicate stress leads to lipid peroxidation in brain, on exposure to various stress models [149].

In this study the results showed that cold and restraint stress enhanced lipid peroxidation significantly. Pretreatment with standard Geriforte prevented stress induced rise in brain lipid peroxidation level significantly. Whereas *Vitis vinifera and Cichorium intybus* extracts at the tested doses were found to be effective significantly, for preventing rise in brain lipid peroxidation. The effect of extracts against stress induced brain lipid peroxidation was found to be dose dependent. *Vitis vinifera* at the dose of 500 mg per kg body weight of animal exhibited similar effect to that of standard Geriforte drug. But Geriforte exhibited maximum prevention of stress induced rise in brain lipid peroxidation level significantly.

In another method, antistress activity of *Vitis vinifera and Cichorium intybus* extracts was evaluated, by finding their protective effect on stress induced ulcers in albino rats. During 1930’s, Dr. Hans Selye reported that the stress is one of the pathogenesis factors of gastric ulcers. He used restraint as stress factor to induce ulcer in animal for the first time [175]. During stress both autonomic and neuroendocrine
system are activated leading to change in both the offensive and defensive mechanism involved in the pathogenesis of peptic ulcer [145]. Among the most likely mechanism are increased gastric acid secretion, reduced gastric mucosal blood flow, inhibition of gastric mucosal prostaglandin synthesis, reduced mucous secretion, disruption of gastric mucosal barrier, reduced gastric epithelial cell proliferation or growth [182-184]. Bhargava et. al. have demonstrated both adrenergic and cholinergic nervous systems are involved in the CNS and at the periphery in the pathogenesis of gastric ulcer during stress [185]. The pathological changes in the gastro intestinal system during stress may be modulated (positively or negatively) by the amygdala and hippocampal formation (parts of limbic system) [148]. Swimming of rats or mice in water has been widely used to investigate stress induced physiological changes [174]. It has been reported that swimming stress induced ulcers is similar to stress induced ulcer in human, and gastric injury may be due to the involvement of the CNS [186].

In this investigation forced swimming stress model was used to induce gastric ulcers as described by previous investigators. Forcing albino rats to swim in water within the restricted space has been widely used and accepted model for studying stress in animals [174, 182, and 187]. Forced swimming stress model provides both emotional and physiological stress. In this model behavioral despair (emotional stress) and vigorous muscular activity (physiological stress) of animals are responsible for the pathogenesis of gastric ulcers [187]. In this model rats were exposed to stressful condition repeatedly, as chronic stress is a potent inducer of stress ulcers [148].

In this study the results showed that forcing albino rats to swim exhibited ulcer formation significantly. Pre-treatment with standard omeprazole reduced the ulcer formation in albino rats, showing highest protection against stress induced ulceration significantly. Both the extracts of *Vitis vinifera* and *Chichorium intybus* on pretreatment have shown significant protective effect for stress induced ulcers in albino rats at the tested doses. The effect of extracts on stress induced ulceration was found to be dose dependent. *Vitis vinifera* at the dose of 500 mg/kg body weight of animal exhibited maximum protection. Flavonoids of *Vitis vinifera* are responsible for protection against stress induced ulcers, as previous studies have revealed the ulcer protective effect of flavonoids [182-183, 188]. This study demonstrates the presence of antistress activity in *Vitis vinifera* and *Chichorium intybus*, which may have increased nonspecific resistance of the rats by altering neuroendocrine components.
During stress, hypothalamic-pituitary-adrenocortical axis activation leads to increase in plasma corticosterone and alteration in weights of adrenal gland [145, 156 and 174]. This has been utilized as evaluation parameter of stress. It has been found that rats in lukewarm water of temperature 36°C could survive for 80 hour of long period. But water temperature above or below than 36°C, influences the animal behavior with the involvement of glucocorticoids [174]. In this investigation cold water swimming stress model was used to induce effect on adrenocortical activity in rats, as described by previous investigators [19, 157 and 174]. It has been well established phenomenon that cold swimming stress increases plasma corticosterone level [157].

In this model stress is induced by vigorous muscular activity, behavioral despair and temperature fluctuation [19, 187]. Due to cold stress temperature regulatory centre in the hypothalamus is activated, resulting in activation of HPA axis with increase in plasma corticosterone level [19]. During stress, adrenal gland stimulation leads to hyperplasia and hypertrophy of cells and it is a well established observation. When adrenal cortex is stimulated for prolonged period by ACTH, it leads to hypertrophy and proliferation of adrenocortical cells [26]. Even forced swimming stress has shown adrenaline release with rise of adrenal gland weight [174]. It has been well established that acute stress, like forced swimming stress for a brief period of one day induces profound alteration in the physiology of animal [148,174]. When animals are exposed to acute stress, plasma ACTH level and corticosterone level increases within 5 minute and 30-45 minute respectively [148].

In this study the results reveal that cold water swimming stress of rats exhibited elevation in the plasma corticosterone and adrenal gland weight, which is in accordance with the previous studies of other antistress agent [157, 174 and 187]. Pretreatment with diazepam prevented elevation of plasma corticosterone and adrenal gland weight in albino rats due to stress. This is in agreement with previous reported studies [187]. Pretreatment with the extracts of Vitis vinifera seeds and Cichorium intybus roots extracts have shown significant protective affect against cold water swimming stress, by preventing rise in plasma corticosterone and adrenal gland weight. So Vitis vinifera and Cichorium intybus extracts have exerted antistress effect by regulating homeostasis mechanism through hypothalamic pituitary adrenal axis of the animal.
During swimming induced stress, the energy demand occurs [159], and this energy is provided from breakdown of stored glycogen in liver [161]. Severe depletion of liver glycogen was observed in guinea pigs, when subjected to swimming [158]. So assessment of liver glycogen depletion is a biomarker of stress. Even depletion of glycogen in liver is related to immobility of animal, when subjected to prolonged physical exercise, such as swimming of rodents in water [157, 158]. Behavioral despair like immobility of albino rats after a period of vigorous activity occurs, when they are subjected for forced swimming [145, 162].

In this investigation weight loaded forced swimming stress model was used to induce effect on liver glycogen and to assess swim stress induced behavioral despair in albino rats as described by previous investigators [145, 158]. This model fulfills basic criteria responsible to induce Selye’s stress syndrome, such as chronicity, excessiveness of stressor and inability of the organism to escape from the stressor. During stress, depletion of liver glycogen has been well established [158]. Liver contains glycogen in highest concentration compared to other polysaccharides [178]. Glycogen was isolated from liver by precipitation with alcohol, and then it was estimated by the well established method explained by Montgomery [178]. This method was selected, as it is simple, sensitive (up to 5µg of glycogen), rapid, reproducible and reagents are inexpensive with stability on storage [178].

In this study the results showed that weight loaded forced swimming stress on albino rats exhibited depletion in liver glycogen and behavioral despair like immobility which is in accordance with previous studies [145, 158]. In this model the animal was forced to swimming exercise against downward pressure of applied weight which leads to rapid utilization of energy. As a result the liver glycogen level might have got depleted in stress control animal. Energy for swimming exercise is derived from glucose due to glycogenolysis in liver, on prolonged period of exercise. During resistance phase of Selye’s general adaptation syndrome, the additional glucose resources of the body are mobilized from glycogenolysis through growth hormone, whereas thyroid hormone stimulates the production of ATP (energy) from glucose [18]. The overall action of these two hormones is to provide additional resources of energy in the form of ATP required for metabolically hyperactive cells of the brain, heart and muscles [18]. When animals are subjected to weight loaded forced swimming stress, initially they try to escape from water. But after repetitive failure of efforts, they show immobility as a behavioral despair. Behavioral despair represents
an experimental model of endogenous depression in animal [145]. Depression occurs if stress is continued for prolonged period of time [145, 152].

In this study weight loaded forced swimming stress induced significant increase in duration of immobility, when compared to control group. Pretreatment with *Vitis vinifera* seeds and *Cichorium intybus* roots extracts exhibited significant decrease in duration of immobility of albino rats and prevented swimming stress induced behavioral despair. It is in accordance with the previous studies of other antistress agent [145]. Pretreatment with Ashwagandha as a standard antistress agent prevented depletion of liver glycogen. Ashwagandha as an antistress agent is well known for attenuating ATP-depletion and other energy related indices during forced swimming stress [189]. Ashwagandha was found to be safest antistress agent in human being [190]. Even immobility was found to be less, and it could be due to reduced rate of hepatic glycogen depletion responsible for enhancement of exercise capacity in albino rats [158].

As shown in the results, the liver glycogen level in *Vitis vinifera* seeds and *Cichorium intybus* roots extracts treated groups were significantly higher than that of stress control group, indicating prevention of depletion of glycogen store in liver. Alternatively energy might have been provided by prevention of stress induced increase in nitric oxide and the associated decline in ATP production. Consequently rise of energy (ATP) might have increased performance and endurance of animals [45]. The antistress activity of *Vitis vinifera* and *Cichorium intybus* extracts seems to be mediated by attenuating energy depletion. It might have resulted in augmentation of the endurance capacity of the animal. This is also confirmed with the reduced behavioral despair observation of animal during investigation.

In another method, effect of *Vitis vinifera* and *Cichorium intybus* extracts on restraint stress and cyclophosphamide-induced immunosuppression was evaluated. Influence of stress on immunity is a well known phenomenon. Physical or psychological stress influences function and efficiency of the immune system, resulting in immunosuppression [163]. Restraint stress is a combination of psychological and physical (immobilization) stress, which produces intense stress on the animal leading to activation of HPA axis with subsequent increase in plasma corticosterone [176]. During chronic stress, corticosterone level remains high, resulting in reduction of white blood cell numbers in circulating blood [166]. It has been found that stress in rodents has induced decrease in leucocyte number of blood
and it was due to glucocorticoid hormone released during stress [165]. Cyclophosphamide is immunosuppressive drug and it is relatively selective for lymphoid tissue [163]. Cyclophosphamide induced immunosuppression in animal is a reliable, convenient and reproducible method [163]. In this investigation, immune response to stress and cyclophosphamide was evaluated by quantitative measurement of number of cells and percentage of each type of cells in blood as described by previous investigators with some modifications [163, 165 and 176].

In this study, the results reveal that restraint stress has significantly decreased total leucocyte count. Differential count of leucocyte in stress control animals exhibited a significant relative lowering of lymphocyte and monocyte percentage whereas increase in neutrophil percentage was observed. There was significant decrease in RBC count, haemoglobin content and platelet count. These changes in haematological parameters are in agreement with previous reported studies [165]. Pretreatment with diazepam *Vitis vinifera* and *Cichorium intybus* extracts have prevented immunosuppression in albino rats due to stress, thereby showing significant protective effect against restraint stress induced immunosupression. Protection of immunosuppression due to stress was highest by *Cichorium intybus*. In cyclophosphamide treated animals there was significant lowering of total WBC, RBC, Haemoglobin content, Platelet count and Lymphocyte percentage with elevation of Neutrophil percentage. This is in accordance with previous reported studies [163, 176].

Pretreatment with Ashwagandha, *Vitis vinifera* and *Cichorium intybus* extracts have prevented myelosuppression in albino rats due to Cyclophosphamide (myelosuppressive drug). There was significant protective effect against myelosuppression. This is in accordance with previous reported studies [163]. *Cichorium intybus* extract possesses maximum potential for immunomodulation.

The results of all the above studies performed by different methods, clearly indicates that *Vitis vinifera* and *Cichorium intybus* extracts were found to possess a significant antistress activity at the tested doses. *Vitis vinifera* at the tested dose of 500mg/kg body weight of animal exhibited maximum antistress activity.