9.1. Introduction
In the development of diseases, free radicals have emerged as the major entity causing damage to cells. These toxic metabolites are generated by aerobic metabolism in the cell, which in turn significantly increases pathological conditions, leading to free radical-mediated denaturation of protein, enzymatic deactivation, base hydroxylation of nucleic acids, cross-linking or strand scission, mutation or even cell death (Maxwell, 1995). However, the physiological system has a series of defense mechanisms including antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPX) and other free radical scavengers, β-carotene, α-tocopherol, ascorbic acid, α-lipoic acid and glutathione to protect the cell against cytotoxic ROS (reactive oxygen species) (Kazim et al., 2002). However, when the balance between the oxygen species and antioxidants is altered, a state of oxidative stress results, possibly leading to permanent cellular damage. There is evidence that antioxidants may be useful in preventing the deleterious consequences of oxidative stress, and there is an increasing interest in the protective biochemical function of natural antioxidants contained in vegetables, fruits and medicinal herbs (Rice-Evans et al., 1995). Generally, plants containing flavonoids have been reported to possess strong antioxidant properties (Raj and Shalini, 1999).

Traditionally, *Cytisus scoparius* used for hypnotic & sedative (Siegel, 1976), diabetes (Rebelo and Castro, 1998) and liver disease (Rivera and Obon, 1995). Pharmacological studies have confirmed its hypotensive activity and anti spasmodic activity (Bhakuni et al., 1969). The *Cytisus scoparius* contains the flavone such as 6′′ O acetyl scoparin (Brum-Bousquet et al., 1977), flavonals namely rutin, quercetin, quercitrin, isorhamnetin and kaempferol (Brum-Bousquet and Paris, 1974) and some isoflavones like genistein and sarothenmoside (Viscardi et al., 1984). The latex part of *Asclepias curassavica* is applied to corns, also as an emetic and bactericidal property (Zargari, 1992; Verpoorte and Dihal, 1987). The fresh (or) dried and pulverized leaf is reported to be used against cancer (Duke and Ayensu, 1985). The ethanolic fraction of *Asclepias curassavica* exhibited strong cardiotonic action (Bhagirath and Rastogi, 1969). The *Asclepias curassavica* extract stimulated central nervous system as reflected by increased level of serotonin and noradrenalline (Rastogi and Mehrotra, 1993). An inhibitory activity against carcinomatous cells of human nasopharynx has been shown by the plant extract (Neto et al., 2002). Several flavonols (Haribal et al., 1996) cardenolides (Abe et al., 1991; Seiber et al., 1982) and alkaloids (Rothschild et al., 1984) have been isolated from different parts of plant.

In this respect, flavonoids and other polyphenolic compounds have received the greatest attention (Costantino et al., 1992; Mukherjee, 2002c; Merfort et al., 1996). Based on these reports, this study was designed to investigate the *in vivo* antioxidant activities of hydroalcoholic extracts of aerial part of *Cytisus scoparius* and *Asclepias curassavica*.

9.2. Materials and Methods
9.2.1. Plant materials and Extraction
Hydroalcoholic extracts of aerial part of *Cytisus scoparius* and *Asclepias curassavica* (described in Chapter 4) were used in this experiment.
9.2.2. Test animals and Groups

Wistar albino rats (200 –250 g) of either sex were maintained under standard environmental conditions and had free access to feed and water ad libitum. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee. Wistar albino rats of either sex were divided into five groups of six animals each. Group-1 served as control and was given the vehicle alone (sodium carboxy methyl cellulose, 0.3% w/v). Groups- 2 and 3 received hydroalcoholic extract of *Cytisus scoparius* orally at 250 and 500 mg/kg body weight, respectively. Groups-4 and 5 received hydroalcoholic extract of *Asclepias curassavica* orally at 250 and 500 mg/kg body weight, respectively. The treatments were given for 14 days and on 15th day of the experiment, all the animals were sacrificed by decapitation. The heart, liver and kidney were removed, washed in cold saline and stored in liquid nitrogen for further biochemical studies. This in vivo antioxidant activity was analyzed by the method described by Rajlakshmi et al (2003).

9.2.3. Ferric reducing ability of plasma (FRAP) assay

Total plasma antioxidant capacity was measured according to the FRAP method (Benzie and Strain, 1996). The blood samples were collected from the rat retro-orbital venous plexus into heparinized glass tubes at 0, 7th and 14th days of treatment. Briefly, 3 mL of freshly prepared and warm (37°C) FRAP reagent [1 mL of 10 mM 2,4,6 tripyridyl-s- triazine (TPTZ) solution in 40 mM HCl, 1 mL 20 mM FeCl₂.6H₂O, 10 mL of 0.3 M/L acetate buffer (pH 3.6)] was mixed with 0.375 mL distilled water and 0.025 mL of plasma samples. The absorbance of developed colour in organic layer was measured at 593 nm. The temperature was maintained at 37°C. The readings at 180 sec were selected for the calculation of FRAP values. Ferrous sulphate (FeSO₄.7H₂O) was used as a standard for calibration and the data expressed as nM Fe²⁺/L.

9.2.4. Biochemical estimation of markers of oxidative stress

9.2.4.1. Preparation of rat heart, liver and kidney homogenate

Tissue homogenate was prepared in a ratio of 1 g of wet tissue to 10 times (w/v) 0.05M ice cold phosphate buffer (pH 7.4) and homogenized by using a Teflon homogenizer. 0.2 mL of sample homogenate was used for estimation of thiobarbituric acid reactive substance (TBARS). The remaining part of the homogenate was divided into two parts. One part was mixed with 10% trichloro acetic acid (1:1), centrifuged at 5000g (4°C, for 10 min) and the supernatant was used for GSH estimation. The second part of the homogenate was centrifuged at 15,000 g at 4°C for 60 minutes and the supernatant was used for superoxide dismutase, catalase and protein estimation (Bruce and Baudry, 1995).

9.2.4.2. Estimation of thiobarbituric acid reactive substance (TBARS)

Lipid peroxidation was measured by the method of Liu et al (1990). Acetic acid 1.5 mL (20%, pH 3.5), 1.5 mL thiobarbituric acid (0.8%) and 0.2 mL sodium dodecyl sulphate (8.1 %) were added to 0.1 mL of supernatant, and then heated at 100°C for 60 min. The mixture was cooled and 5 mL of n-butanol-pyridine (15:1) mixture and 1 mL of distilled water was added and vortexed vigorously. After centrifugation at 4000 rpm
for 10 min, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer and concentration of TBARS was expressed as nM/g tissue.

9.2.4.3. Estimation of superoxide dismutase (SOD)
SOD activity was analyzed by the method described by Kakkar et al (1984). Assay mixture contained 0.1 mL of supernatant, 1.2 mL of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 mL of phenazine methosulphate (186 μM), 0.3 mL of nitro blue tetrazolium (300 mM) and 0.2 mL of nicotinamide adenine dinucleotide reduced disodium salt (NADH, 750 μM). Reaction was started by addition of NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 0.1 mL of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 mL of n-butanol. Mixture was allowed to stand for 10 min, centrifuged and the butanol layer was separated. Colour intensity of the chromogen in the butanol was measured at 560 nm by spectrophotometrically and concentration of SOD was expressed as U/mg of protein.

9.2.4.4. Estimation of catalase (CAT)
Catalase activity was measured by the method of Aebi (1974). Supernatant (0.1 mL) was added to cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H$_2$O$_2$. The rate of decomposition of H$_2$O$_2$ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as U/mg of protein.

9.2.4.5. Estimation of reduced glutathione (GSH)
Reduced glutathione was measured according to the method of Ellman (1959). The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 mL of this supernatant, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of 5’5-dithio, bis (2-nitrobenzoic acid) [DTNB] and 0.4 mL double distilled water were added. Mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/g tissue.

9.2.4.6. Estimation of protein
Protein concentration was estimated according to the method of Lowry et al (1951), using bovine serum albumin as a standard.

9.2.5. Statistical analysis
The data were expressed as mean ± standard error of the mean (S.E.M). The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnet-t-test with the SPSS statistical software for comparison to the control group. P<0.05 was considered as significant.

9.3. Results
9.3.1. Ferric reducing ability of plasma (FRAP) assay
The total antioxidant capacities measured as FRAP in the plasma of rat after administration of hydroalcoholic extract of Cytisus scoparius and Asclepias
Curassavica over a period of 14 days is shown in Figure 9.1. In control group (group-1), there was no significant change in FRAP value on days 7 (1032 nM Fe²⁺/L) and 14 (1034 nM Fe²⁺/L) when compared to day zero (0) (1015 nM Fe²⁺/L. However, in groups-2, 3, 4 and 5 on days 7 (1224, 1416, 1223 and 1415 nM Fe²⁺/L, respectively) and 14 (1419, 1518, 1417 and 1517 nM Fe²⁺/L, respectively) there was a significant (p<0.001) increase in FRAP value as compared to day 0 (1032 and 1034 nM Fe²⁺/L, respectively). Maximum enhancement of FRAP level was obtained in groups 3 and 5, which correspond to animals administered with 500 mg/kg body weight of the hydroalcoholic extract of *Cytisus scoparius* and *Asclepias curassavica*, respectively.

![Figure 9.1](image)

Figure 9.1. Changes in rat total antioxidant capacity of ferric reducing ability of plasma (FRAP) measured by Fe²⁺ equivalent after administration of hydroalcoholic extract of *Cytisus scoparius & Asclepias curassavica*. values are mean ± s.d (n=6). Groups II, III, IV and V (plant extracts treated rats) compared to Group I (control rats). ***P< 0.001

9.3.2. Estimation of TBARS

The effect of various doses of hydroalcoholic extract of *Cytisus scoparius* and *Asclepias curassavica* on the lipid peroxidation and endogenous antioxidants of liver, heart and kidney of rats is shown in Figure 9.2, 9.3, and 9.4, respectively. A significant (p< 0.01 and p< 0.001) decrease in TBARS concentration in liver (287 nM/g and 299 nM/g wet wt tissues) and kidney (264 nM/g and 260 nM/g wet wt tissue tissues) for group-2 and group-3 when compared to control group (427 nM/g and 268 nM/g wet wt tissue) was observed. For groups 4 and 5, a significant (p< 0.01 and p< 0.001) decrease in TBARS concentration in liver (267 and 279 nM/g wet weight [wt] tissue, respectively) and kidney (265 and 262 nM/g wet wt tissue, respectively) was observed and compared to the control group (427 and 268 nM/g wet wt tissue in liver and kidney, respectively). There was no change in TBARS and endogenous
antioxidant levels of heart tissue in the treated groups 2 (312 nM/g), 3 (306 nM/g), 4 (314 nM/g) and 5 (304 nM/g wet wt tissue) respectively, compared to control group (300 nM/g wet wt tissue).

![Graph showing changes in TBARS, SOD, CAT, and GSH levels](image)

Figure 9.2. Changes in rat liver thiobarbituric acid reactive substance (TBARS; nM/g wet weight [wt]), superoxide dismutase (SOD; 10⁻¹ U/mg protein), catalase (CAT; 10⁻¹ U/mg protein) and reduced glutathione (GSH; 10¹ µg/g wet wt) following oral administration of hydroalcoholic extract of *Cytisus scoparius* & *Asclepias curassavica*. Values are mean ± s.d. (n=6). Groups II, III, IV and V (plant extracts treated rats) compared with Group I (control rats). ***P< 0.001, **P< 0.01, *P< 0.05

9.3.3. Estimation of SOD
The administration of hydroalcoholic extract of both plants caused no significant decrease in group 2 (39.5 U/mg of protein), group 3 (46.9 U/mg of protein), group 4 (38.5 U/mg of protein) and group 5 (46.8 U/mg of protein) in the level of SOD in heart, when compared to that of control (47.5 U/mg of protein). However, the level of SOD in the kidney and liver of the plant extracts treated rats was not dose related and found to be significantly increased (p< 0.001 and p< 0.01) in group 2 (32.3 U/mg of protein & 41.4 U/mg of protein), group 3 (31.9 U/mg of protein & 44.5 U/mg of protein), group 4 (32.1 and 40.4 U/mg of protein, respectively) and group 5 (32.3 and 42.5 U/mg of protein, respectively), when compared with control (group-1) (31.0 and 39.1 U/mg of protein). The results were shown in Figure 9.2, 9.3, and 9.4.

9.3.4. Estimation of CAT
The treatment to normal rats for 14 days with hydroalcoholic extract of both plants induced a dose dependent increase in the level of catalase in the liver and kidney however decrease in the heart. The results are significantly (p< 0.001 and p< 0.05) increased at group 3 and group 5 respectively for liver (31.4 U/mg of protein and 31.0 U/mg, respectively) and kidney (44.3 U/mg of protein and 44.2 U/mg of protein), when compared to control group (28.5 and 43.1 U/mg of protein, respectively). However, there was no significant change in the endogenous antioxidant levels in
groups-2 and 4 for liver & kidney and all groups for heart tissue. The results have been mentioned in Figure 9.2, 9.3, and 9.4.

![Graph showing changes in various biochemical parameters](image)

**Figure 9.3.** Changes in rat kidney thiobarbituric acid reactive substance (TBARS; nM/g wet weight [wt]), superoxide dismutase (SOD; 10^{-1} U/ mg protein), catalase (CAT; 10^{-1} U/mg protein) and reduced glutathione (GSH; 10^{-1} µg/g wet wt) following oral administration of hydroalcoholic extract of *Cytisus scoparius* and *Asclepias curassavica*. Values are mean ± s.d. (n=6). Groups II, III, IV and V (plant extracts treated rats) compared with Group I (control rats). ***P< 0.001, **P< 0.01

### 9.3.5. Estimation of GSH
For groups 2, 3, 4 and 5, there was no significant change in GSH level in liver (1.170, 1.130, 1.168 and 1.128 mg/g wet wt tissue, respectively), heart (373, 378, 374 and 379 µg/g wet wt tissue, respectively) and kidney (12.70, 12.55, 12.80 and 12.60 µg/g wet wt tissue, respectively) as compared to the control group of liver, heart and kidney (1.150 mg/g, 380 µg/g and 12.5 µg/g wet wt of tissues, respectively) (Figure 9.2, 9.3, and 9.4).

### 9.4. Discussion
Excessive production of reactive oxygen species plays an important role in the pathogenesis and progression of various diseases involving different organs (Visioli et al., 2000). Lipid peroxides, produced from unsaturated fatty acids via free radicals, cause toxic effects and promote the formation of additional free radicals in a chain reaction. An endogenous antioxidant enzyme such as superoxide dismutase converts the superoxide free radical anion to hydrogen peroxide. Catalase is capable of scavenging the hydrogen peroxide radical, which is formed during various biochemical and metabolic reactions. The glutathione is involved in many important cellular functions, ranging from the control of physico chemical properties of cellular proteins and peptides to the detoxification of free radicals (Meister and Anderson, 1983). If the *in vivo* activity of enzymes or scavengers is not adequate to neutralize these radicals, oxidative stress develops and leads to various diseases (Niki, 1995).
However, when the balance between these species and antioxidants is altered, a state of oxidative stress results, possible leading to permanent cellular damage.

Figure 9.4. Changes in rat heart thiobarbituric acid reactive substance (TBARS; nM/g wet weight [wt]), superoxide dismutase (SOD; $10^{-1}$ U/mg protein), catalase (CAT; $10^{-1}$ U/mg protein) and reduced glutathione (GSH; µg/g wet wt) following oral administration of hydroalcoholic extract of *Cytisus scoparius* & *Asclepias curassavica*. Values are mean ± s.d. (n=6). Groups II, III, IV and V (plant extracts treated rats) compared with Group I (control rats).

The rationale for the use of antioxidants is well established in prevention and treatment of chronic diseases where oxidative stress plays a major aetiological role. Various population studies support that consumption of natural sources of fruits and vegetables, rich in antioxidant compounds, are associated with a lower incidence of oxidative stress–induced diseases (Vayalil, 2002).

In the present study, the FRAP test measured total antioxidant capacity determined by non-enzymatic antioxidants. Several methods have been developed to assess the total antioxidant capacity of serum or plasma because of the difficulty in measuring each antioxidant component separately in the serum or plasma (Cao and Prior, 1998). One of these is the FRAP, which measures the reduction of ferric to ferrous iron in the presence of water soluble exogenous antioxidants (Benzie and Strain, 1996). The significant increase in FRAP level after oral administration of hydroalcoholic extract of both plants indicates the presence of bio-available antioxidants in both the plants. Increased total antioxidant capacity in plasma following consumption of the plant extract was also associated with the decreased lipid peroxidation in *in vivo* experiments. The superoxide anion, hydrogen peroxide and the hydroxyl radical are the major reactive oxygen species that function in concert to induce LPO (lipid peroxidation) of cell membrane lipids. The toxic peroxidative products cause widespread cellular injury (Fridovich, 1986). The present study showed the depletion in the lipid peroxidation as observed by significant decrease in the TBARS level of the liver and kidney in the plant extracts treated groups, but there was no change of TBARS level in heart as compared to control.
SOD metabolizes the superoxide radical anion. It is an effective defense of the cell against endogenous and exogenous generation of superoxide (Brawn and Fridovich, 1980). The ROS scavenging activity of SOD is effective only when it is followed by the action of CAT and GPX, because of the dismutase activity of SOD generates hydrogen peroxide from the superoxide ion, which is more toxic than oxygen derived free radicals and required to be scavenged further by CAT and GPX (Blake et al., 1987). The administration of hydroalcoholic extract of both plants at 500 mg/kg body weight significantly increased the level of SOD and catalase in liver and kidney. This shows the antioxidant nature of the extracts. Generally, results for the kidney have shown fewer changes in antioxidant activity compared to liver (Jadwiga et al., 2000). However, decrease in the level of SOD and CAT was observed in the heart, which could explain the present observation. Reduced glutathione is a protective molecule against chemical induced cytotoxicity (Orrenius and Moldeus, 1984). GSH metabolism plays a vital role in many biological processes; such as the detoxification of xenobiotics. The hydroalcoholic extract of both plants has been found to increase GSH levels in liver and kidney. However, a decrease in the level of GSH was observed in the treated groups in the heart. However, long-term administration of hydroalcoholic extract of both plants did not show significant decrease in the GSH levels in the different organs, indicating a protective antioxidant effect. It can be concluded that, the hydroalcoholic extract of Cytisus scoparius and Asclepias curassavica had significant antioxidant activity. The antioxidant action of plant extracts may be attributed to the presence of known flavonol, which provides maximum conjugation with free radical species, thus reducing the number of free radicals available as well as oxidative stress-related diseases of major organs such as liver, kidney and heart.

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