8.1. Introduction
Oxidative stress has been implicated in the pathogenesis of various diseases such as atherosclerosis, ischemic heart diseases, ageing, diabetes, neurogenerative disorders (Harmann, 1988). These radicals are formed by one electron reduction process of molecular oxygen (O₂). Reactive oxygen species (ROS) can easily initiate the lipid peroxidation of membrane lipids, causing damage of the cell membrane of phospholipids, lipoprotein by propagating a chain reaction cycle (Pryor, 1973). Thus, antioxidant defense systems have coevolved with aerobic metabolism to counteract oxidative damages from ROS. Most living species have efficient defense systems to prevent themselves against oxidative stress induced by ROS (Sato et al., 1996). Natural plant extracts with proven antioxidant activity usually contain compounds with a phenolic moiety, for example coumarins, flavonoids, tocopherols and catechins (Mukherjee, 2002c). Organic acids, carotenoids, protein hydrolysates and tannins can also be present and act as antioxidants (or) have a synergistic effect with phenolic compounds (Dapkevicious et al., 1998).

In ethnomedical information, *Cytisus scoparius* used for diuretics, hypnotic & sedative (Siegel, 1976), diabetes (Rebelo and Castro, 1998) and liver disease (Rivera and Obon, 1995). Pharmacological studies have confirmed its uterine stimulant effect (Joachimovits, 1935) 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 sarothennesside (Viscardi et al., 1984). The juice of leaves of *Asclepias curassavica* has been strongly recommended as anthelmintic, antidysentric, and gonorrhoea. (Manandhar, 1995; Hirschmann et al., 1990). The latex part of plant is applied to corns, also as an emetic and bactericidal property (Zargari, 1992; Verpoorte and Dihal, 1987). In Mexico, 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). Several cardenolides (Abe et al., 1991; Seiber et al., 1982; Abe et al., 1992), alkaloids (Rothschild et al., 1984), flavonols (Haribal et al., 1996) and alicyclic compounds (Abe et al., 2000) have been isolated from different parts of plant. Most of the reported biological activities and active constituents of these plants may be related to its antioxidant nature. Therefore, this study was designed to investigate the in vitro antioxidant properties of the hydroalcoholic extracts of aerial parts of *Cytisus scoparius* and *Asclepias curassavica* and to establish their potential therapeutic value if any.

8.2. Materials and Methods
8.2.1. Plant materials
The chloroform, ethyl acetate, methanol and hydroalcoholic extracts of aerial parts of *Cytisus scoparius* and *Asclepias curassavica* (described in Chapter 4) were used in this experiment.
8.2.2. Assay of in vitro antioxidant activity
The antioxidant activity of chloroform, ethyl acetate, methanol and hydroalcoholic extracts of both plants were determined according to the thiocyanate method (Yen and Chen, 1995; Yildirim et al., 2001). Briefly, 500 μl of the individual extract of Cytisus scoparius and Asclepias curassavica separately at different concentrations (25µg, 50µg, 100µg and 200µg) were mixed with 2.5 mL of 0.02 M linoleic acid emulsion (contains equal weight of tween-20 in phosphate buffered saline, pH 7.4) and the final volume was adjusted to 5 mL with phosphate buffered saline in a test tube and incubated at 37°C in dark place. The amount of peroxide was determined by measuring absorbance at 500 nm after coloring with 0.1 mL of FeCl₂ (0.02M) and 0.1 mL thiocyanate (30%) at intervals during incubation. The solutions without the plant extracts were taken as blank for the study. α-Tocopherol was used as a reference compound.

8.2.3. DPPH radical scavenging activity
The free radical scavenging capacity of the extracts was determined using DPPH. A methanol DPPH solution (0.15%) was mixed with serial dilutions (0.5µg to 10 µg) of the individual hydroalcoholic extract of Cytisus scoparius and Asclepias curassavica separately, and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (Perkin – Elmer Lambda 20 UV – visible spectrophotometer). Vitamin C used as a standard. The inhibition curve was plotted and IC₅₀ values were obtained (Viturro et al., 1999).

8.2.4. Nitric oxide radical inhibition assay
Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (Garrat, 1964). In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and the individual hydroalcoholic extract of Cytisus scoparius and Asclepias curassavica separately (10 µg to 320 µg) or standard solution (rutin, 0.5 mL) was incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink coloured chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Rutin was used as a standard.

8.2.5. Superoxide anion scavenging activity
Measurement of superoxide anion scavenging activity of each plant extract was done based on the Nishimiki method (Nishimiki et al., 1972). About 1 mL of nitroblue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of the hydroalcoholic extracts of Cytisus scoparius and Asclepias curassavica were mixed separately (1.25 µg to 40 µg) in water. The reaction was started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the
absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Curcumin was used as a positive control.

8.2.6. Lipid peroxidation assay
The rat liver microsomal fraction was prepared by the method of Bouchet et al (1998). The reaction mixture contained 500 µL of liver microsomal fraction, 300 µL buffer containing the hydroalcoholic extracts of *Cytisus scoparius* and *Asclepias curassavica* separately (50-150 µg), 100 µL of FeCl₃ (1 mM) and 100 µL ascorbic acid (1 mM) in a final volume of 1.0 mL to start peroxidation. Samples were incubated at 37°C for 1 h and lipid peroxidation was measured using the reaction with thiobarbituric acid (TBA). Thiobarbituric acid reactive substances were determined by the methods of Houghton et al (1995) and Aruoma et al (1989). The absorbance of the organic layer was measured at 532 nm. All reactions were carried out in triplicate. Vitamin E was used as a standard.

8.2.7. Hydroxyl radical scavenging assay
The assay was performed as described by Halliwell et al (1987) with minor changes. All solutions were prepared freshly. 1.0 mL of the reaction mixture contained 100 µL of 28 mM 2-deoxy-2-ribose (dissolved in phosphate buffer pH 7.4), 500 µL solution of various concentrations of the hydroalcoholic extracts of *Cytisus scoparius* and *Asclepias curassavica* separately (10 to 100 µg), 200 µL of 200 µM FeCl₃ and 1.04 mM ethylenediamine tetracetic acid (EDTA, 1:1 v/v), 100 µL H₂O₂ (1.0 mM) and 100 µL ascorbic acid (1.0 mM). After an incubation period of 1 h at 37°C, the extent of deoxyribose degradation was measured by the TBA reaction (Nishimiki et al., 1972; Bouchet et al., 1998). The absorbance was measured at about 532 nm against the blank solution. Vitamin E was used as a positive control.

8.2.8. Reducing power
The reducing power of plant extracts was determined according to Oyaizu method (1986). Different concentration of the hydroalcoholic extracts of *Cytisus scoparius* and *Asclepias curassavica* separately (100 µg – 1000 µg) in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl₃ (0.1 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Butylated hydroxy toluene was used as a standard.

8.2.9. Determination of total phenolic compounds
Total soluble phenolics in the hydroalcoholic extract of each plant were determined with Folin–Ciocalteu reagent according to the reported method (Slinkard and Singleton, 1977) using pyrocatechol as a standard. Briefly, 0.1 mL of each of the hydroalcoholic extracts of *Cytisus scoparius* and *Asclepias curassavica* were separately taken (contains 1000 µg extracts) in a volumetric flask and diluted with
distilled water (46 mL). About 1 mL of Folin – Ciocalteu reagent was added and the contents of the flask mixed thoroughly. After 3 min, 3 mL of Na₂CO₃ (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the plant extracts was determined as microgram of pyrocatechol equivalent by using the following equation (Gulcin et al., 2002).

\[
\text{Absorbance} = 0.001 \times \text{Pyrocatechol (µg)} + 0.0033
\]

8.2.10. Statistical analysis
All the in vitro experimental results were mean ± S.D of five parallel measurements.

8.3. Results
8.3.1.1. Assay of in vitro antioxidant activity
The effects of various extracts on peroxidation of linoleic acid emulsion are shown in Figure 8.1. All extracts showed inhibition of lipid peroxidation (LPO) and the relative antioxidant potencies for different extracts of both the plants were in the order as follows, hydroalcohol extract > ethyl acetate extract > methanol extract > chloroform extract. Least antioxidant activity was observed for chloroform extract of both plants at all concentrations studied. The ethyl acetate and methanol extracts of both plants showed comparable lipid peroxidation effects with that of the standard, α-tocopherol, and the IC₅₀ values were 94.1 µg/mL and 109.2 µg/mL for ethyl acetate and methanol extracts of Cytisus scoparius, respectively. The ethyl acetate and methanol extracts of Asclepias curassavica showed the scavenging activity with IC₅₀ values of 115.2 µg/mL and 135.3 µg/mL, respectively. All concentrations of hydroalcoholic extract of both plants showed higher antioxidant activity than the other extracts and that of standard α-tocopherol. The IC₅₀ values were found to be 30.2 µg/mL and 31.2 µg/mL for hydroalcoholic extract of Cytisus scoparius and Asclepias curassavica, respectively and 66.1 µg/mL for standard, α-tocopherol.

8.3.1.2.
Maximum lipid peroxidation inhibition was found with the hydroalcoholic extract of Cytisus scoparius and Asclepias curassavica in the in vitro study. Therefore, only the hydroalcoholic extract of both plants was used for the different in vitro studies.

8.3.2. DPPH radical scavenging activity
The hydroalcoholic extract of each plant extract decreased the concentration of DPPH radical due to free radical scavenging ability. The results are shown in Figure 8.2. The extract of Asclepias curassavica had a well hydrogen donating ability with an IC₅₀ value of 8.7 µg/mL and the value was found to be higher than that of standard, vitamin C (IC₅₀ value of 3.1 µg/mL). The IC₅₀ value (1.5µg/mL) of the extract of Cytisus scoparius was found to be lesser than the standard, vitamin C.

8.3.3. Nitric oxide radical inhibition assay
The scavenging of nitric oxide by the extracts of Cytisus scoparius and Asclepias curassavica was increased in a dose-dependent manner as illustrated in Figure 8.3. At concentration of 116.0 µg/mL of Cytisus scoparius extract 50% of nitric oxide
generated by incubation was scavenged. This IC$_{50}$ value of *Cytisus scoparius* extract was found to be lesser than that of standard, rutin (IC$_{50}$ 160.0 µg/mL). The IC$_{50}$ value of extract of *Asclepias curassavica* (198.4 µg/mL) was found to be higher than rutin.

![Figure 8.1 Percentage inhibition of lipid peroxidation using the standard (α-tocopherol) and using different doses of extracts (chloroform, ethyl acetate, methanol and hydroalcohol [3:7 ratios] of *Cytisus scoparius* & *Asclepias curassavica* in linoleic acid emulsion. Results are mean ± S.D of five parallel measurements.](image)

Chlor-cs → chloroform extract–*Cytisus scoparius*; Etoac-cs → ethyl acetate extract-*Cytisus scoparius*; Meoh-cs → methanol extract-*Cytisus scoparius*; Hydr-alco-cs → hydroalcohol extract-*Cytisus scoparius*; Chlor-ac → chloroform extract–*Asclepias curassavica*; Etoac-ac → ethyl acetate extract-*Asclepias curassavica*; Meoh-ac → methanol extract-*Asclepias curassavica*; Hydr-alco-ac → hydroalcohol extract-*Asclepias curassavica*; tocopherol (std) → α-tocopherol (standard)

### 8.3.4. Superoxide anion scavenging activity
The superoxide anion derived from dissolved oxygen by phenazine methosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The decrease in the absorbance at 560 nm with the extracts of *Cytisus scoparius* and *Asclepias curassavica* indicated the consumption of superoxide anion in the reaction mixture. As mentioned in Figure 8.4, the extracts of *Cytisus scoparius, Asclepias curassavica* as well as curcumin showed the scavenging activity with the IC$_{50}$ values, 4.7 µg/mL, 21.7 µg/mL and 5.84 µg/mL, respectively.

### 8.3.5. Lipid peroxidation assay
Activity of extracts of *Cytisus scoparius* and *Asclepias curassavica* against non-enzymatic lipid peroxidation in rat liver microsomes are shown in Figure 8.5. Addition
of Fe$^{2+}$/ascorbate to the liver microsomes caused increase in lipid peroxidation. The extract of *Cytisus scoparius* showed inhibition of peroxidation effect in all concentrations, which showed 50% inhibition effect at 104.0 µg/mL. The extract inhibition value was found to be lesser than the standard, vitamin E (IC$_{50}$ 120.5 µg/mL). The extract of *Asclepias curassavica* exhibited 50% inhibition of peroxidation at 134.2 µg/mL. The effect of the extract depends on concentration in the reaction mixture and has higher inhibition value when compared to reference compound, vitamin-E.

![Graph](image-url)

Figure 8.2. Scavenging effect of hydroalcoholic extracts of *Cytisus scoparius* & *Asclepias curassavica* and standard vitamin C on 1, 1'-Diphenyl-2-picryl hydrazyl (DPPH) radical. Results are mean ± S.D of five parallel measurements.

![Graph](image-url)

Figure 8.3. Scavenging effect of hydroalcoholic extracts of *Cytisus scoparius* & *Asclepias curassavica* and standard rutin on Nitric oxide radical. Results are mean ± S.D of five parallel measurements.
8.3.6. Hydroxyl radical scavenging assay

To attack the substrate deoxyribose hydroxyl radicals were generated by reaction of Ferric-EDTA together with H₂O₂ and ascorbic acid. When the plant extracts were incubated with the above reaction mixture, it could prevent the damage against sugar. The results were shown in Figure 8.6, the concentrations of 50% inhibition were found to be 27.0 µg/mL and 32.5 µg/mL for the *Cytisus scoparius* extract and standard compound, vitamin-E, respectively. The inhibition value of *Cytisus scoparius* extract was found to be lesser than the standard. The IC₅₀ value (41.4 µg/mL) of the extract of *Asclepias curassavica* was found to be higher than the standard.

![Graph](image)

Figure 8.4. Effect of hydroalcoholic extracts of *Cytisus scoparius* & *Asclepias curassavica* and curcumin on scavenging of superoxide anion radical formation. Results are mean ± S.D of five parallel measurements.

![Graph](image)

Figure 8.5. Effect of hydroalcoholic extracts of *Cytisus scoparius* & *Asclepias curassavica* and vitamin E on lipid peroxidation of liver microsome induced by Fe²⁺/ascorbate. Results are mean ± S.D of five parallel measurements.
8.3.7. Reducing power
Figure 8.7 shows the reductive capabilities of the extracts of *Cytisus scoparius* and *Asclepias curassavica* compared to butylated hydroxy toluene. The reducing power of the extracts of both plants was very high and the power of the extracts was increased with quantity of sample. The extracts of *Cytisus scoparius* and *Asclepias curassavica* could reduce most of the Fe$^{3+}$ ions, but the reductive activity was less than that of the standard, butylated hydroxy toluene.

8.3.8. Determination of total phenolic compounds
The total phenolic contents of hydroalcoholic extracts of *Cytisus scoparius* and *Asclepias curassavica* were 0.0589 µg and 0.0495 µg pyrocatechol equivalent /mg, respectively.

8.4. Discussion
Free radicals, species with one (or) more unpaired electrons, are produced in normal (or) pathological cell metabolism from xenobiotics, (or) through ionizing radiation. Electron acceptors such as molecular oxygen react easily with free radicals to become radicals themselves ROS (Reactive oxygen species). The primary derivatives of oxygen (O$_2$, H$_2$O$_2$, OH, O$_2$) play an important role in mediating ROS related effects. Short-lived reactive species generated in situation can react with non radicals and produce chain reaction (Halliwell and Gutteridge, 1984a). Free radicals have aroused significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Rose et al., 1982). Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity (Aruoma and Cuppett, 1997). There is increasing evidences, which suggest that consumption of natural antioxidant contained in vegetables, fruits and medicinal herbs are useful in preventing the deleterious consequences of oxidative stress (Rice-Evans et al., 1995).

In the present study, *in vitro* antioxidant activities of chloroform, ethyl acetate, methanol and hydroalcoholic extracts of both the plants were determined using the thiocyanate method. From this method, the amount of peroxides formed in emulsion during incubation was determined spectrophotometrically by measuring absorbance at 500 nm. High absorption is an indication of high concentration of peroxides formed. Therefore, low absorbance indicates high antioxidant activity (Yen and Chen, 1995). All extracts of both the plants and standard of α–tocopherol were shown to inhibit the formation of peroxides at various concentrations. However, maximum inhibition of peroxidation was found with hydroalcoholic extract of both the plants over the other organic extracts.

The present investigation demonstrated that, DPPH, a free radical, stable at room temperature, which produces a purple colour solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured methanol solutions.
Figure 8.2 illustrates the decrease in the concentration of DPPH radical due to free radical scavenging ability of hydroalcoholic extracts of both plants and vitamin C, which is comparable to the reported value of Thabrew et al (1998).

![Graph showing inhibition percentage vs sample concentration](image1)

Figure 8.6. Effect of hydroalcoholic extracts of *Cytisus scoparius* & *Asclepias curassavica* and vitamin E on deoxyribose degradation assay. Results are mean ± S.D of five parallel measurements.

![Graph showing inhibition percentage vs sample concentration](image2)

Figure 8.7. Reductive ability of hydroalcoholic extracts of *Cytisus scoparius* & *Asclepias curassavica* and butylated hydroxy toluene. Results are mean ± S.D of five parallel measurements.

Nitric oxide radical inhibition study proved that aerial parts of the both plant extracts are a potent scavenger of nitric oxide. This nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract of both plants inhibits nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Lucia Marcocci et al., 1994). From the nitric oxide
test, rutin was used as a standard. The IC\textsubscript{50} value of the rutin is comparable to the reported value of Badami et al (2003). In the PMS/NADH –NBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Addition of various concentrations of both plant extracts as well as curcumin (standard) in above coupling reaction showed decrease in absorbance. The antioxidant property of curcumin is generally attributed to its phenolic nature (Toda et al., 1988). Sreejayan and Rao (1997) have earlier observed that for superoxide and DPPH scavenging, the order of activity was: curcumin > demethoxycurcumin > bisdemethoxycurcumin > diacetylcurcumin (almost inactive).

The liver microsomal fraction undergoes rapid non-enzymatic peroxidation when incubated with FeCl\textsubscript{3} and ascorbic acid. The use of Fe (III) in the presence of a reducing agent such as ascorbate produces \textbulletOH (Aruoma, 1996) and they attack the biological material. This leads to the formation of MDA (malondialdehyde) and other aldehydes, which form a pink chromogen with TBA, absorbing at 532 nm (Kosugi et al., 1987). The extract of both plants and vitamin E exhibited strong scavenging effect of hydroxyl radical, which could inhibit lipid damage at different concentrations. The scavenging effect of vitamin E is in accordance with the report of Hemanth et al (2002). The extract inhibits lipid damage caused by hydroxyl radicals and the inhibition values mentioned in Figure 8.5. The antioxidant effect of the extract may be due to its flavonol content (Haribal et al., 1996). The extracts were examined for its ability to act as \textbulletOH radical scavenging agent. Ferric EDTA was incubated with H\textsubscript{2}O\textsubscript{2} and ascorbic acid at pH -7.4; hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2- ribose into fragments that on heating with TBA at low pH form a pink chromogen (Aruoma et al., 1989; Halliwell et al., 1987). When extracts of both plants and vitamin E were added to the reaction mixture, they removed hydroxyl radicals and prevented the degradation of 2-deoxy-2- ribose as mentioned above. The observed IC\textsubscript{50} values of both plant extract and Vitamin E were analogous to the reported values of Sen et al (2002). Figure 8.7 shows the reductive capabilities of both the plant extract compared to that of butylated hydroxy toluene. For the measurements of the reductive ability, investigated the Fe\textsuperscript{3+} to Fe\textsuperscript{2+} transformation in the presence of hydroalcoholic extracts of two plants using the method of Oyaizu et al (1986). The reducing power increased with increasing the amount of extracts in both plants. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The absorbance values of the extracts at different concentrations were found to be less than that of the reference compound. The value of reference compound is in accordance with the report of Illhami et al (2002). The phenolic compounds may contribute directly to anti-oxidative action (Duh et al., 1999). This result indicates that polyphenol present in aerial part of both plants could be partly responsible for the beneficial effects.

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