Chapter IX

SUMMARY

AND

CONCLUSION
Oxidative stress is implicated in the pathogenesis of numerous disorders for example cardiovascular diseases such as atherosclerosis, and ischemia/reperfusion injury; cancer; inflammatory diseases; metabolic disease such as diabetes; and diseases of the central nervous system (CNS) such as Alzheimer’s, Parkinson’s, and stroke. Among the many health predictions the most alarming is that of cardiovascular diseases (CVD). Ischemic heart disease is a leading cause of death in India, with an estimated 3 million deaths per year accounting for 25% of all mortality (Mukherjee, 1995). Epidemiological studies support a positive association between plasma homocysteine concentration and risk for cardiovascular disorders (Arnesen et al., 1995; Graham et al., 1997). Homocysteine is toxic to neuronal cells (Lipton et al., 1997) and animals exposed to homocysteine accumulate this compound in the brain (Algaidi et al., 2005). Concentrations of homocysteine in the brain and cerebrospinal fluid (CSF) are elevated in several neurological diseases (Yanai et al., 1983; Regland et al., 1997). One of the complications of MI is cerebral artery embolism, leading to cerebral ischemia and stroke. Stroke is an acute and progressive neurodegenerative disorder and is the third leading cause of death throughout the world resulting from the interruption of blood flow (Feigin et al., 2003).

Recent awareness of therapeutic potential of several traditionally used plants has opened a new dimension for the study and research of medicinal plants. The partial replacement of industrial drugs by medicinal plants is advocated in recent times, with the aim of preventing the excessive consumption of pharmaceuticals, which has now reached a disturbing level. These increasing trends in the use of plants as medicines locally and globally necessitate scientific investigations especially where information regarding toxicity is lacking on such plants or their extracts (Kayang et al., 2005). Indeed, many antioxidative plants and their isolated active components have been reported to be cardioprotective in ischemia reperfusion-induced myocardial infarction (Banerjee et al., 2002; Mohanty et al., 2004; Rao et al., 2005).

It has been suggested that some herbal medicines, or their products, may improve microcirculation in the brain (Gong and Sucher, 2002; Wang et al., 2005), protect against ischemic reperfusion injury (Lee et al., 2005; Wang et al., 2005), possess neuroprotective properties (Gong and Sucher, 2002; Kim, 2005) and inhibit apoptosis (Bei et al., 2004), thus, justifying their use in ischemic stroke patients.
Embelia ribes Burn (family, Myrsinaceae), commonly known as Vidanga, a woody shrub, which is sparsely distributed in India, Sri Lanka, Malaysia and South China (Guhabakshi et al., 2001). Bhandari et al (2002) have reported diabetic dyslipidemic activity of ethanolic Embelia ribes extract (200 mg/kg body weight, p.o.) in streptozotocin-induced diabetes in rats. Further, Bhandari et al (2007) have reported antioxidant effect of Embelia ribes Burn in streptozotocin- induced diabetes in rats, using gliclazide as positive control drug. Recently, Bhandari et al (2008) have reported the cardioprotective activity of aqueous extract of Embelia ribes in isoproterenol- induced myocardial infarction in albino rats.

The present research work was designed to study the effect of the standardized Embelia ribes extracts (aqueous and ethanolic) against oxidative stress induced by left coronary artery ligation (LAD) model (Birnbaum et al., 2005), L-methionine- induced hyperhomocysteinemia model (Senaratne, 2000) and middle cerebral artery occlusion (MCAO)- induced cerebral ischemia model (Seisjo, 2000) in albino rats.

Chemically, E. ribes is reported to contain embelin, quercitol (polyphenol), tannins and alkaloids (Krishna and Verma, 1941), which may contribute to its antioxidant activity. In the present study, on phytochemical analysis of ethanolic extract of E. ribes, it was found to contain embelin, a flavonoid, alkaloid, carbohydrates, saponins and acidic compounds. Further, on standardization of aqueous extract of Embelia ribes, it was found to contain alkaloids, carbohydrates, flavonoids, phenolic compounds, proteins and saponins. It can, thus, be concluded that the antioxidant effect of Embelia ribes can be due to the presence of alkaloids, flavonoids, phenolic compounds and saponins.

The data obtained from the study demonstrated that left anterior descending coronary artery (LAD) ligation- induced ischemia reperfusion in albino rats caused significant myocardial injury, which was revealed by the decreased heart rate, Na-K-ATPase activity, and myocardial endogenous antioxidant levels and increased of serum lactate dehydrogenase (LDH) activities and myocardial LPO levels, as compared to sham operated rats (i.e. Group I).
Baseline values of heart rate were similar among all the groups. Heart rate was steadily decreased over the entire period of reperfusion (P<0.01) in the sham operated group (i.e. group I). In IR group (i.e. group VI), a significant (P<0.01) fall in heart rate was observed after left coronary artery ligation and remained decreased throughout the reperfusion period. The decrease in heart rate, during IR injury corroborates the findings of Rao et al (2005).

The decreased heart rate observed throughout the ischemia-reperfusion duration in the IR group as compared to sham control, clearly depicting the injured state of myocardium following ischemia-reperfusion induced injury.

In the present study, ischemic reperfusion injury (IRI) was associated with increased oxidative stress, as evidenced by increase in myocardial TBARS levels and depletion of Na-K-ATPase activity as well as myocardial endogenous antioxidants such as GSH, GPx, GR and GST levels. Further, a significant increase in myocardial infarct size in IR group (i.e. group VI) was observed, as compared to sham operated group (i.e. group I).

The groups treated with Embelia ribes extracts in two doses of 100 and 200 mg/kg body weight produced slight decrease in heart rate during 30 min coronary artery ligation and, thereafter, gradually increased throughout the reperfusion period and restored to normal value at the end of the 2 h. These observations were similar in sham and IR groups of albino rats.

Increase in serum LDH activity suggested the occurrence of considerable myocardial membrane damage, as reported by Mueller et al (1977). In the present study, Embelia ribes treatment was found to decrease LDH enzyme release, thereby, demonstrating its protective action on the cell membranes.

Pretreatment with Embelia ribes extracts (100 and 200 mg/kg body weight) resulted in significant increase in Na-K-ATPase activity. It increased the Na⁺ efflux, decreased concentration of Ca⁺ that resulted in decreased cardiac injury and improved functional recovery. Other investigators have also shown that increased Na-K-ATPase activity
during low-flow ischemia limited the rise in sodium and improved functional recovery (Cross et al., 1995).

It is interesting to note that different plants and plant extracts can also stimulate the synthesis of cellular antioxidants (Pathania et al., 1998; Bhattacharya et al., 1999; Banerjee et al., 2001; Gauthaman et al., 2001). Protection against ischemia reperfusion-induced oxidative stress in Embelia ribes treated rat hearts was evidenced by preservation of endogenous antioxidants and prevention in rise of TBARS.

As compared to treatment with aqueous extract of Embelia ribes, ethanolic Embelia ribes extract treatment in a dose of 200 mg/kg body weight offered the best protection against IR-induced myocardial infarction. A significant % decrease in myocardial infarct size in Embelia ribes extracts treated rats was observed as compared to IR group (Group VI) in a dose dependent manner.

The present findings suggest that the aqueous and ethanolic extracts of Embelia ribes possess a dose dependent cardioprotection against ischemia-reperfusion-induced myocardial injury and the cardioprotection may be due to its free radical scavenging activity or indirectly by enhancing the endogenous antioxidant levels or by antagonizing free radical mediated inhibition of sarcolemmal Na-K-ATPase activity.

Some of the β-blockers (atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol, timolol and carvedilol) are useful in preventing oxidative damage reported in hypertension and other cardiovascular diseases (Gomes et al., 2006). Similarly, enalapril, an angiotensin converting enzyme inhibitor (ACEIs) increases antioxidant defenses in chronic hemodialysis (HD) patients (de Cavanagh et al., 1999). The tolerance to the vascular effects of nitrates may be prevented by high doses of antioxidants (Giugliano et al., 1995). Since Embelia ribes also has antioxidant property (Bhandari et al., 2007; Joshi et al., 2007), it may be suggested that Embelia ribes could be useful clinically in the treatment of cardiovascular disorders associated with oxidative stress. Therefore, it could be having long term effects similar to β-blockers and ACEIs in reducing cardiovascular morbidity and mortality.
Chapter IX Summary and Conclusion

The mechanisms associated with homocysteine-induced endothelial dysfunction are mediated by increased oxidative stress (Kanani et al., 1999), leading to increased levels of oxidized LDL (Ventura et al., 2000). Hyperhomocysteinemia may promote the generation of reactive oxygen species (ROS) such as H$_2$O$_2$ and hydroxyl radicals via the autooxidation of sulfhydryl (-SH) group (Heinecke et al., 1987) or by decreasing the intracellular levels of GSH that is involved in the elimination of free radicals.

Homocysteine, a thiol containing amino acid derived from demethylation of dietary methionine, may generate partially reduced ROS that are able to stimulate the lipid peroxidation involved in atherosclerotic process. Thus, an imbalance in dietary methionine may contribute to the development of atherosclerosis by increasing homocysteine levels (Toborek et al., 1995). The data in our present study showed that L-methionine (1 g/kg body weight, p.o.) treatment for a period of 30 days in pathogenic control group rats significantly ($P < 0.01$) elevated the levels of homocysteine, LDH, total cholesterol, LDL-C, triglycerides in serum and LPO in heart and brain homogenates with a concomitant decrease in serum HDL-C levels and GSH content in heart and brain homogenates.

Antioxidant treatment restores several toxic effects of homocysteine (Jara-Prado et al., 2003). In the present study, elevated levels of homocysteine, LDH, total cholesterol, LDL-C and triglycerides in serum and LPO in heart and brain homogenates were reduced significantly ($P<0.01$) after treatment with *Embelia ribes* extracts (aqueous and ethanolic) in two doses of 100 and 200 mg/kg body weight, suggesting lipid lowering, cardio- and neuro-protective potential of *Embelia ribes*. Further, the levels of HDL-C in serum and GSH in heart and brain homogenates were increased significantly ($P<0.01$), thereby, enhancing the endogenous myocardial antioxidant levels. Furthermore, the results of test drug were comparable to folic acid, a standard positive control.

The results of biochemical observations in serum and heart tissues were supplemented by histopathological examination of rat’s heart and brain sections to confirm the methionine toxicity and deleterious effects of *Embelia ribes* Burn extracts. Photomicrograph of heart section of vehicle control group showed normal architecture.
with regular morphology of myocardial cell membrane. L-methionine-induced hyperhomocysteinemia resulted in vascular congestion. *Embelia ribes* extracts (aqueous and ethanolic) treated groups showed absence of necrosis, edema or congestion with regular morphology of cardiac muscles. Further, folic acid treatment in rats showed normal cardiac muscles morphology.

Furthermore, the light microscopic observations of the brain tissues of methionine-treated rats showed focal necrosis and vacuolar changes while *Embelia ribes* extracts (aqueous and ethanolic) treated rats showed normal fibrillar background and neuronal cell morphology exhibiting near normal pattern of neuronal cells, thereby, further supporting the role of *Embelia ribes* as a promising neuroprotective agent in L-methionine-induced hyperhomocysteinemia.

The present study support the inferences from the previous studies (as discussed above) that *Embelia ribes* decrease the levels of homocysteine and also reduce the necrotic / apoptotic changes in cerebral and heart tissues. Therefore, the role of *Embelia ribes* in management of hyperhomocysteinemia is suggested.

Free radical formation is an essential component of pathological mechanisms responsible for ischemic stroke, as well as an exacerbator of ischemic brain injury (Kent *et al.*, 2001; Kontos, 2001; Sugawara and Chan, 2003). During ischemia and especially reperfusion, free radicals expected to attack proteins and lipids of the cell membrane and DNA.

Many neuroprotective therapies and other interventions have been shown to limit experimental cerebral ischemic injury (Dirnagl *et al.*, 1999; Ginsberg, 2003). Beneficial effects of various antioxidants and herbal formulations in ischemic stroke have been demonstrated in a number of studies (Yousuf *et al.*, 2005; Saleem *et al.*, 2006; Zhan and Yang, 2006). The present investigation showed the neuroprotective potential of *Embelia ribes* extracts (aqueous and ethanolic) against ischemia / reperfusion-induced oxidative stress.

Free radicals are thought to cause behavioral deficits in experimental animals (Fukui *et al.*, 2001, 2002). ‘Grip Strength test’ is used as a measure of total body strength and
in stroke patients, grip strength declines due to motor function impairment as being reported earlier (Cramer et al., 1997; Kamimura and Ikuta, 2002). In the present study, MCAO-induced cerebral ischemia in albino rats produced significant \( P<0.01 \) reduction in the grip strength activity as compared to sham operated group (Group I).

After 24 h of ischemia reperfusion injury, significant \( P<0.01 \) rises in LDH was observed in the ischemic rats (group VI) relative to sham-operated animals. LDH was used as a marker of tissue breakdown as LDH is abundant in red blood cells (RBCs) and can function as a marker for hemolysis. Free radicals generated during cerebral ischemia reperfusion causes LDH leakage from RBCs and their concentration in serum increases (Callow et al., 1977).

Lipids are the most susceptible macromolecules to oxidative stress, which play a major role in the production of MDA (Chan, 2001). Due to high content of lipids, the brains neurons become high vulnerable to oxidative attack (Cui et al., 2004; Halliwell, 2001), which caused a significant elevation in the content of MDA in frontal cortex and hippocampus of the rats after 24 h of MCAO.

In present study, depletion in endogenous antioxidant (GSH, GPx, GR and GST) levels was observed in frontal cortex and hippocampus of ischemic rats. It has been shown that depletion of GSH in ischemia reperfusion injury can be attributed to several factors such as cleavage of GSH to cysteine, decrease in synthesis of GSH and the formation of mixed disulfides, causing their cellular stores depleted (Slivka and Cohen, 1993; Shivakumar et al., 1995; Rao et al., 2001).

Stadtman (1992) put forth that balanced antioxidants are required to control the cognitive and motor functions of the frontal cortex and the hippocampus. In the present study, it is suggested that Embelia ribes extracts (aqueous and ethanolic) which is a potent antioxidant, has protected neurobehavioral deficits of animals by scavenging free radicals. The exact mechanism of this hypothesis is to be explored yet.

In the present study, it has been observed that pretreatment with Embelia ribes extracts (aqueous and ethanolic) for 30 days significantly reduced the serum LDH
levels and lipid peroxidation level in frontal cortex and hippocampus, as compared to MCAO group i.e. Group VI rats.

The activity of *Emhelia ribes* extracts (aqueous and ethanolic) appears to work by restoring the altered antioxidants enzymes activity in frontal cortex and hippocampus regions of brain induced by MCAO. Our results are in agreement with Prasanthi *et al* (2005), as they have reported increased antioxidant enzyme activity in oxidative damage in rat tissue by using dietary sesame oil.

This study suggest that *Emhelia ribes* extracts (aqueous and ethanolic) administration to the normal rats did not show any effect on the activity of endogenous antioxidant enzymes and oxidative stress markers in various brain regions of normal rat. Further, there was no effect of *Emhelia ribes* per se on grip strength. Interestingly, *Emhelia ribes* extracts (aqueous and ethanolic) exerts its antioxidant effect, by decreasing LDH, total cholesterol, triglycerides LDL-C and TBARS levels, which implicated in injury. Therefore, on the basis of present observations of the study, *Emhelia ribes* could be an important herbal drug for neuroprotection.

In conclusion, we have used all standard, documented and established animal models of oxidative stress. In these models (LAD- induced myocardial infraction methionine-induced hyperhomocysteinemia and MCAO- induced cerebral ischemia), there was significant depletion of antioxidant molecules. The pre-treatment with *Embelia ribes* extract have shown restoration of levels of antioxidant molecules. Some of the clinically used drugs like β-blockers and ACEIs have long term protective effect on morbidity and mortality on cardiovascular diseases. This long term protective effect of β-blockers and ACEIs could be due to their antioxidant effect, apart from hemodynamic effect. The observations with *Emhelia ribes* pre-treatment suggest its antioxidant effect without any toxicity in the doses studied. Therefore, the biochemical and histological observations due to *Embelia ribes* treatment may also have long term antioxidant activity. Being plant origin medicine without any apparent toxicity, *Embelia ribes* may have an important role in reducing the oxidative stress in cardiovascular and cerebrovascular disorders.