Discussion
Ageing, a major risk factor for diseases and death is characterized by progressive decline in the biochemical and physiological functions of various tissues and organs in an organism. Oxidative stress, an unavoidable consequence in metabolism of oxygen in aerobic cells is a major factor in ageing and age associated diseases. Erythrocytes, devoid of repair and synthesizing machinery are valuable model system for observing age associated changes in cellular macromolecules. In recent years, various anti ageing agents are being ushered into the field of medicine for successful ageing. Herbal remedies as antioxidant supplement is globally growing up owing to broad spectrum of beneficial biological properties. Flavonoids, the potential dietary antioxidant found ubiquitously in plants, attracted global interest in combating the devastating effects of oxidation in cells and tissues of an organism. Ageing formerly thought to be one of the most immutable and inescapable facts of life are now becoming more amenable due to the intervention of antioxidant supplementation as effective medicinal therapy.

ANTIOXIDANT ACTIVITY OF CHLOROFORM EXTRACT OF SOLANUM TRILOBATUM (CST)

Epidemiological data have strongly suggested that “herbal health diets” contains phytochemicals, which are rich in antioxidant potential and have protective effect against oxidative damages (Elliot, 1999). In the present investigation, CST was shown to possess high levels of flavonoids and polyphenols (Figure 1). Polyphenols and flavonoids are thus the
phytochemical compounds present in CST that play a central role in protecting the macromolecular structure and functions of cells and tissues during oxidative stress (Amir and Kumar. 2004: Sini and Devi. 2004; vanHaroon et al., 2006).

**REACTIVE OXYGEN SPECIES**

Oxidative stress resulting from increased production of reactive oxygen species and a decrease in antioxidant defense, leads to damage of biological macromolecules and disruption of normal metabolism and physiology (Trevisan et al., 2001). Structural and functional integrity of erythrocytes is conditioned by normal energy production and maintenance of the reductive state of the cell, which are challenged by highly activated forms of oxygen such as superoxide anions (O$_2^-$), hydroxyl radicals (·OH) and hydrogen peroxide (H$_2$O$_2$). Erythrocytes being rich in oxygen are the likely sites for free radical formation. Hemoglobin undergoes a slow auto-oxidation to physiologically inactive ferric hemoglobin, further enhancing free radical production (Winterbourne and Carrell, 1974). Moreover, during oxidative stress redox active metal (iron redox couple) involved in the Fenton reaction generating highly reactive radicals could oxidatively modify cellular components during ageing (Rice Evans and Baysal, 1987; Floyd and Carney, 1993).

Enhanced levels of oxidant production (Figure 3) and significant increase in the levels of superoxide anions, hydroxyl radicals and hydrogen peroxide observed in erythrocytes of aged rats (Table 1) thus signifies the
prevalence of oxidative stress. Another possibility for the enhanced production of oxidants and free radicals may be due to the impaired antioxidant defense system with advancement of age. The accumulation of these oxygen free radicals result in oxidative damage to critical biological molecules particularly in erythrocytes and contributes to the detrimental effects of ageing (Harman, 1992; Ames et al., 1993).

The protective effect of CST against age related increase in oxidant production is connected with the ability of its free radical scavenging activity (Laranjinha et al., 1996). In addition, the phenolic compounds present in CST may also be attributed to the hydrogen donating abilities that would possibly nullify the effects of free radicals in CST supplemented rats (Sini and Devi, 2004; Mohanan and Devi, 1998a). Studies established that the orthohydroxy structure in B-ring and 3-hydroxy group in unsaturated C-ring of flavonoids could have effectively interacted with free radicals and decreased their levels (Rice-Evans et al., 1996).

**ANTIOXIDANT ENZYMES**

The biological effects of ROS are tightly controlled by a wide spectrum of enzymatic antioxidant defense systems (Halliwell, 1985). Erythrocytes being regularly exposed to free radicals are however equipped with antioxidants far in excess of normal requirement and also function as an effective oxidative sink in the organism (Davis and Goldgerg, 1987). Cu-Zn SOD, a major antioxidant enzyme in erythrocytes, protects against oxygen free radicals by catalyzing the removal of superoxide radical (O$_2^-$) and
catalase (CAT) a haemoprotein primarily works to catalyze the decomposition of hydrogen peroxide to water (Anderson et al. 1997). In particular, the oxidation or autoxidation of hemoglobin (Hb-Fe⁺ into Hb-Fe³⁺) in the erythrocytes results in the continuous formation of superoxide radicals (Hebbel and Eaton 1989) which is reflected as declined activities of SOD and CAT in erythrocytes of aged rats (Figure 4).

Significant increase in SOD and CAT activity on supplementation of CST to near normalcy in aged rat erythrocyte may be due to the potential quenching of free radicals (Sri and Devi 2004) by phenolic acids present in it (Table 1). Further reports have suggested that polyphenols in CST are effectual scavenger of superoxide and hydroxyl radicals (Sri and Devi 2004) thereby sparing the antioxidant enzymes valuable in protecting erythrocytes from oxidative damages.

**GLUTATHIONE REDOX CYCLE**

Glutathione, a tripeptide containing γ-glutamic acid cysteine and glycine provides the first line of defense against ROS and protects erythrocytes from oxidative damage that acts as a radical quencher. The glutathione redox enzymes glutathione peroxidase, glutathione-S-transferase, glutathione reductase, and glucose-6-phosphate dehydrogenase provide the second line of defense through detoxification of noxious by-products and alleviation of free radicals mediated macromolecular damages in erythrocytes (Pastore et al. 2003).
Significant increase in the level of GSSG with a concomitant depletion in the concentration of GSH, the redox status (Table 2) and the glutathione metabolizing enzymes may represent an imbalance in prooxidant and antioxidant status in aged rats (Figure 5 and 6). Significant decrease in the activity of glutathione metabolizing enzymes is well correlated with the declined availability of its substrate, glutathione with advancement of age (Hazelton and Lang, 1991). Reports show that elevated exposure of erythrocytes to free radicals may be the possible reason behind GSH depletion (John et al., 2001). Further, studies suggested that enhanced utilisation of glutathione by enzymes such as GPx, GST and reduced activity of glutathione regenerating enzyme G6PD, due to the upsurge of ROS production may decrease the GSH status in erythrocytes of aged animals (Sastre et al., 1992). On the other hand, fall in the activity of G6PD essential for an adequate supply of NADPH (enzymes involved in GSH regeneration) creates an imbalance in GSH/GSSG ratio and thereby a shift in the redox state of cells with advancement of age (Chiu et al., 1982).

Glutathione along with total thiols play an important role in a variety of detoxification processes, including nullification of peroxide damage. A direct link between the thiol status of the membrane and cellular glutathione has reported by Kosower and Zipser (1982). An age-dependent reduction in total thiol (Figure 7) concentration was observed in the erythrocytes of rats. This is consistent with the study of Hazelton and Lang (1991) that showed decreased erythrocyte total thiols with increasing age. Efficiency of S-thiolation as a mechanism of antioxidant defense, decreases with age, which
creates an increased risk of irreversible oxidation of –SH groups of proteins (Malgorzata et al., 2004) may be quoted as a possible reason for the reduction in the levels of total thiols.

The flavonoids and polyphenols in CST protected the sulphydryl groups and thiols from oxidative damage thereby elevating their levels in erythrocytes of aged rats (Ishige et al., 2001). Also the metal chelating action of CST improved total thiol levels, which in turn would have spared the activities of glutathione metabolizing enzymes (GPx, GST, GR, and G6PD) in aged rats (Myhrstad et al., 2002). Moreover, the decrease in free radical levels by polyphenols in CST might also have contributed to the improvement of glutathione metabolizing enzymes (Nakagawa and Miyazawa 1997).

**NONENZYMATIC ANTIOXIDANTS**

Non enzymatic antioxidants play an important role in preventing free radical damages associated with ageing by directly interfering with the generation of radicals and scavenging them. Ascorbic acid, the hydrophilic antioxidant, plays an important role with the lipophilic antioxidant alpha tocopherol in preventing oxidative damage to cell membrane induced by free radicals. The reduction in levels of nonenzymatic antioxidants in aged rat (Figure 8) may be attributed to the elevated oxidative damages on erythrocyte membrane. Further, decrease in ascorbic acid contents might contribute to the decreased availability of glutathione, as glutathione functions in the reduction of dehydroascorbate to ascorbate (Halliwell and Gutteridge, 1999). Moreover,
ascorbate is essential for the recycling of tocopherol radical to tocopherol (Packer et al., 1997), which is consistent to the observed decrease in alpha tocopherol levels.

Therapeutic supplementation of CST enhanced significantly the levels of ascorbic acid and α-tocopherol in erythrocyte of aged rats. The effects of flavonoids to metabolically spare ascorbic acid, stabilize ascorbic acid, reduce dehydroascorbate to ascorbate and increase ascorbic acid absorption might have improved the levels of ascorbic acid (Hughes and Wilson, 1977) in erythrocytes of aged rats. Earlier reports suggest that polyphenols regenerate α-tocopherol from tocopherol radical through an H-transferring mechanism, thereby behaving as a sacrificial antioxidant (Hirano et al., 2001). This property might have contributed to the improved α-tocopherol levels on CST therapy.

HEMATOLOGICAL INDICES

Hematological indices portray the overall picture of alterations in blood cells in circulation that helps in the diagnosis of many diseases such as infection and anemia. Hematological alterations have great impact on blood rheological properties and are associated with various risk factors of cardiovascular disorders (Seki et al., 2006). In the present study, reports confirm that the increased formation of free radicals has profound effect in disturbing the hematological indices in aged rats (el-Demerdash et al., 2004). Earlier our laboratory study has showed profound decrease in hematological
parameters as a consequence of antioxidant reduction (Jayachandran and Panneerselvam, 1997).

Increased oxidative damage to erythrocyte membrane and decrease in blood viscosity at higher shear rates from aged animals may contribute to alterations in the hematological parameters (Glass and Gershon 1984; Coppola et al., 2000). Significant reduction in hematological indices was observed in aged rats (Table 3) compared to young rats. This may be due to the peroxidative damage to haemoglobin, brought about by diminished cellular protection resulting in partial denaturation of the globin molecules and subsequent loss of the haemoglobin moiety (Jacob, 1974). In addition, erythrocyte hemoglobin contains metallic iron, which is considered as a salient target for free radical attack. Also the decrease MCHC and MCH levels observed in aged rat erythrocytes might be due to declined GSH levels, as GSH play an important role in protecting the thiol groups of hemoglobin and thereby preventing its loss.

The antioxidant augmentating action of CST might account for the improvement of hematological parameters in aged rats. The GSH elevating action of CST (Table 2) might have contributed to the improvement of MCHC, MCV and MCH levels. Report by Mesbah et al. (2004) suggests that the maintenance of haematocrit and hemoglobin levels to normal values by flavonoids are owing to the increased GSH concentration supports the present findings.
PROTEIN CARBONYL

There is large body of evidence implicating oxidative damage to proteins in normal ageing (Beal, 2002). The gradual increase in cellular content of oxidized proteins is involved in age related loss of biochemical functions (Stadtman, 1992). Aged rat erythrocyte membrane is associated with protein oxidative damage as evidenced by the increase in the protein carbonyls (Figure 9). The present findings displayed an enhanced level of carbonyl formation in erythrocytes of aged rats that may be due to age dependent increase in the rate of oxidized protein degradation (Stadtman and Levine, 2000).

Modification of proteins can take two forms: (a) addition reactions by highly reactive intermediate products of lipid peroxidation or and glycosidation, and (b) direct oxidative modification of macromolecules (Hyun et al., 2003). Thus the increased accumulation of protein carbonyls in aged rats is due to excessive oxidation of proteins or decreased capacity to clear up oxidatively damaged proteins. Though several antioxidant defense systems have evolved to prevent ROS damage, oxidized proteins appear to accumulate with age (Carney et al., 1991).

Administration of CST decreased the levels cf protein carbonyls, which may probably be due to the presence of polyphenolic compounds functioning as an in vivo antioxidant that prevents protein oxidation (Sato et al., 2002) with advancement of age. The bioactivity of CST to directly react
with ROS holds an effective reason for the decrease in the protein carbonyls in erythrocyte membrane of aged rats

**ERYTHROCYTE MEMBRANE CYTOSKELETAL PROTEINS**

Protein oxidation generally causes the loss of structure or catalytic function and contributes to serious deleterious effects on cellular function (Levin and Stadman, 2001) The SDS-PAGE analysis of aged rat erythrocytes membrane indicates the elevated degradation of functional proteins like spectrin actin ankyrin band 4.1 and generation of HMWP aggregates (Figure 10) The HMWP are non-dissociable aggregates formed due to sulphydryl group oxidation of membrane proteins during oxidative stress (Lin and Hung 1997) Peroxidised lipids in the membrane can effectively favor the polymerization reactions with spectrins forming HMWP (Caprari et al 1995) thus affecting the membrane functions and integrity. In accordance enhancement of cytoskeletal proteins in erythrocyte membrane of aged rats was accompanied by the generation of high molecular weight proteins

Age-dependent degradation of cytoskeletal protein spectrin in erythrocytes might be due to oxidative reduction of ankyrin and band 3 proteins, as ankyrin bind with beta-chain of spectrin on one side and band 3 on the other side (Weaver and Marchesi, 1984, Schwartz et al 1991) Further, decrease in band 4.1b was probably due to protein degradation and partial oxidation of band 4.1 (a globular protein is responsible for the connection between spectrin and actin in the membrane skeleton), which makes them
unable to participate in the formation of the spectrin-protein 4.1-actin complex leading to destabilization of cytoskeletal structure (Advani et al., 1992). It has been suggested that a fraction of band 3 protein may, via limited oxidation and/or proteolysis, undergo conformational modifications and serve as a source for senescence signal(s), marking the erythrocyte for removal from the circulation (Bosman and Kay, 1988).

Enhanced protein degradation has been shown to play a role in the events leading to the shortened life of the erythrocyte in old individuals (Gershon and Gershon, 1988; Danon and Marikovsky, 1988). Further, studies by Ferrali et al. (1997) indicate that the membrane protein alteration, resulting in the formation of SCA, appears to be related to the release of iron. The results of the present report seem to be, in agreement to previous reports (Signorini et al., 1995; Ferrali et al., 1997) that when an effective iron chelator is present in the cell in sufficient concentration, the iron released under stress conditions is chelated and thus rendered inactive for redox-cycling reactions allegedly involving several membrane components. In addition, earlier reports have proposed that vitamin E deficiency would lead to increased oxidation of band 3 proteins during ageing (Kay, 1991). Therefore decrease in the level of iron chelator and vitamin E observed in the present study. (Figure 8) could be attributed to the increased oxidation of proteins in aged rats.

Supplementation of CST to aged rats showed an improved pattern of the altered erythrocytes membrane protein profiles. The ability of CST in protecting erythrocyte membrane sulfhydryl groups in GSH from oxidative damage (el-Alfy et al., 2005) would have prevented the cytoskeletal proteins
in aged rats from degradation. The possible mechanism by which GSH functions as a stabilizer in erythrocytes is with the cooperation of GPx in transporting reducing equivalents into erythrocyte cytosol via -SH group of membrane proteins that would have eventually protected the erythrocyte membrane and thus the cytoskeletal proteins from oxidative damages (Flohe and Gunzler, 1976). Moreover, flavonoids have the capacity to enter the cells and chelate iron, thus affording a protection against erythrocyte membrane damage (l lipid peroxidation and hemolysis) and erythrocyte SCA formation (Ferrali et al., 1997; Korkina and Afanas’ev, 1997). Therefore, destructive side chain reactions causing damage to proteins and the metabolic machinery are alleviated upon CST therapy in the aged rats possibly, due to free radical scavenging effect (Hirano et al., 2001) and chain breaking activity (Laranjinha et al., 1996) of flavonoids present in them.

**SURFACE CHARGE AND GLYCOPROTEINS**

Aggregation of red blood cells is a reversible process that occurs when the bridging force due to the adsorption of macromolecules on adjacent cell surfaces exceeds the disaggregating forces caused by electrostatic repulsion (Stolz and Donner, 1987). Erythrocyte aggregation is mainly dependent on net negative surface charge and is one of the main determinants influencing blood circulation at low shear rates by increasing blood viscosity and inducing ‘sludging’ in the capillary (Chien et al., 1979). In the present study, significant reduction in erythrocyte surface charge was observed in aged rats when compared with young rats (Figure 11). The shedding of microvesicles probably containing sialoglycoproteins such as glycophorin A and Band 3, is
the main reason behind surface charge decrease in aged rats as the negative surface charge is mainly attributed by sialic acid residues located on glycoproteins in erythrocyte membrane surface (Cook et al., 1961; Ejlar et al., 1962).

In accordance, a profound decrease in the levels of glycoproteins (Hexose, hexosamine and sialic acid) was evidenced in erythrocyte of aged rats in the present study (Table 4). The observed decrease in surface charge and glycoproteins level may also be attributed to the increased protein carbonyl levels with advancement of age (Sangeetha et al., 2005). Oxidative stress or other damaging effects with advancement of age would have decreased the surface sialosaccharides (Wauiter et al., 1981). Reports reveal that removal of sialic acid residues of the saccharide chains of glycophorin and decrease in surface charge levels in erythrocytes results in exposure of new antigen recognized by immunoglobulin G, causing premature elimination of erythrocytes from circulation (Kay, 1981; Beppu et al., 1995).

CST supplementation lead to an increase in glycoproteins especially sialic acid level that ultimately increased the membrane surface charge level. Significant increase in surface charge noted in erythrocytes of CST treated rats would have been possibly due to the preservation of protein carbonyl levels by flavonoids (Figure 9). Moreover, the ability to maintain redox state of sulphydryl groups in membrane proteins by flavonoids (van Duin et al., 1998) would have contributed to the maintenance of glycoprotein levels and thus the membrane surface charge in erythrocytes of aged rats.
LIPID PEROXIDATION

Lipid peroxidation is both a free radical-mediated process and a source of secondary free radicals. The uncontrolled peroxidation of biomembranes can lead to profound effects on the membrane structure and function thereby leading to cell death (Spiteller, 2002). The high polyunsaturated fatty acid content of erythrocyte membrane and the continuous exposure to high concentration of oxygen and iron in hemoglobin are the factors that make erythrocytes very sensitive to the lipid peroxidative injury (Clemens and Waller, 1987). The end products of lipid peroxidation (MDA) are usually more stable than the free radicals and their longer life allow them to be toxic, indicative of the extent of LPO, since MDA is known to be one of the most abundant aldehydes formed as a byproduct of lipid peroxidation (Gurer et al., 1998).

Tremendous increase in the levels of MDA in erythrocyte membrane and plasma of aged rats indicated the possibility of increased radical production, and higher rate of lipid peroxidation in these rats (Figure 12). Data demonstrated that the age related membrane rigidity and impaired membrane-related functions diminished RBC survival, which may probably be due to the increase in lipid peroxide levels formed upon oxidation of lipids through iron released from hemoglobin (Choe et al., 1995). In addition, decrease in vitamin E. in erythrocytes could enhance the lipid peroxidation process, as it is effective in preventing erythrocyte membrane lipid from oxidative damages (Koyu et al., 2005). Since the ageing process is characterised as time dependent, the age-related modification of lipid
structures should be considered as one of the major contributors for the age-dependent diseases (Pepe et al., 1999).

CST supplementation to aged rats significantly decreased the level of lipid peroxidation. Flavonoids present in CST can scavenge lipid peroxides by binding metal ions and by inhibiting of enzymatic systems responsible for free radical generation in a way declining lipid peroxidation levels (Laughton et al., 1991; Cotelle et al., 1996). Thus CST has the capacity to modify membrane dependent processes, such as free-radical-induced membrane lipoperoxidation and is related not only to structural characteristics but also to its ability to interact with and/or penetrate cell membrane. In addition, polyphenols in CST would have inhibited the formation of phospholipid hydroperoxides and spared the lipophilic antioxidant vitamin E to protect phospholipids from free radical damage (Carini et al., 2000).

**ERYTHROCYTE MEMBRANE FLUIDITY AND LIPID PROFILES**

Maintenance of fluidity of cell membrane at an optimum state is necessary for the proper functioning of the membrane. The fluidity of membrane influences cellular deformability and membrane microviscosity that contribute to various pathophysiological implications such as cardiovascular diseases, hyper tension and stroke (Zicha et al., 1980). In the present study, DPH probe was used to monitor the fluidity changes in erythrocyte membranes. Their fluorescence anisotropy values respond to lipid arrangement in various regions of membranes. Significant increase in fluorescence anisotropy therefore indicated the increased membrane rigidity
in aged rat erythrocytes in the present study (Figure 13). Various gerontological studies have illustrated altered membrane fluidity with advancement of animal age (Yu et al., 1992).

Cholesterol plays a key role in erythrocyte membrane fluidity as it appears to regulate the mobility of phospholipid fatty acyl chains by condensing hydrophobic interaction leading to increased rigidity to membrane lipids (Peddada et al., 1997). Reports by Marino et al. (2002) also showed that an increase in cholesterol with the advancement of age to be the underlying cause for the membrane rigidity. Significant increase in the total cholesterol content and decrease in total phospholipids of the erythrocyte membrane with increased C/P ratio was observed in aged rats when compared with the young rats (Table 5). An implication behind the increased cholesterol level in erythrocyte membrane of aged rats might be due to the increased presence of plasma cholesterol. Reports demonstrate that exchange of cholesterol between plasma and erythrocytes can take place effectively in aged rats leading to increased cholesterol incorporation into erythrocyte membrane (Malhotra and Kritchevsky, 1975). In accordance, significant increase in the level of total, free and ester cholesterol were observed in plasma of aged rats compared to young rats (Figure 14).

Supplementation of CST to the aged rats increased the membrane fluidity to near normaley indicating the protection afforded by the flavonoids present in the CST. Flavonoids can potentially reduce oxidative modifications of membrane by restraining the access of oxidants to the bilayer and propagation of lipid oxidation in the hydrophobic membrane matrix thereby
improving their fluidity (Saija et al., 1995; Halder and Bhaduri, 1998). Further, the decrease in erythrocyte membrane rigidity on CST was possibly due to hypolipidemic role of flavonoids present in CST which would have altered the lipid profiles (Sudheesh et al., 1997). Supplementation of CST would have also positively increased the phospholipid levels due to the property of flavonoids, which are known to anchor the polar head of main phospholipids through hydrogen bonds forming reversible physiochemical complexes (Roychowdhury et al., 2001). Moreover, polyphenols can lower the total cholesterol and l.DL oxidability level by inhibiting cholesteryl ester hydroperoxides formation (Preuss et al., 2000; Vinson et al., 2002) thereby altering plasma and erythrocyte lipid profiles in CST treated aged rats. Thus the aforementioned properties prove CST as an efficient membrane stabilising antioxidant in improving the fluidity of the erythrocyte membrane with advancement of animal age.

**MEMBRANE-BOUND ATPASES**

Erythrocyte membrane bound ATPases play an important role in the maintenance of the ionic gradients between the intracellular and extracellular compartment of the cell. Changes in the ionic concentration can bring about diverse ripples of cell injury and ultimately cell death (Trump et al., 1980). Significant decrease in the activities of Na⁺-K⁺ ATPase, Ca²⁺ ATPases and Mg²⁺ ATPases was observed in the erythrocytes of aged rats compared to young rats (Figure 15).
Modifications in the fatty acid composition of red cell phospholipids can change the allosteric behavior of membrane-bound enzymes (Yu et al., 1992). Reports showed that the most possible mechanism for the alterations in ATPases activities through changes in lipid-protein interactions due to alterations of the lipid-bilayer environment caused by free radicals and lipid peroxidation (Srivatsava, 1994). As thiol status also contributes in maintaining the structure and function of ATPases (Iiu and Wei, 1999), the decrease in thiol levels in erythrocytes of aged rats (Figure 7) as evidenced from the present study could also have contributed the decrease in ATPases activities in aged rat erythrocytes. Another mechanism underlying these alterations is increased oxidative stress occurring in erythrocyte membrane, possibly due to decreased antioxidant defenses within the cell (Figure 4, 5 and 6).

Significant loss of Na⁺-K⁺ ATPase activity might well be related to the loss of phospholipids following peroxidant injury due to localization of the enzyme inside the red cell membrane. Furthermore, the carbonyl-containing substances derived from peroxidised phospholipids are potent inhibitors of erythrocyte membrane Na⁺-K⁺ ATPase. The activity of Ca²⁺ ATPases strongly inhibited by high concentration of calcium (Shao and McCarthy, 1995) and increase in calcium concentration means a serious threat to calcium-dependent metabolic pathways, which can endanger the survival of erythrocytes. Significant reduction in the activity of Mg²⁺ ATPase in this study may be due to an increase in the level of lipid peroxidation as well as due to decrease in the level of ATP upon hydrolysis during the transport of
ions. Moreover, Mg\(^{2+}\) enzyme inhibition may produce abnormalities related to the modulation of magnesium ion cellular environment as well as to the Mg\(^{2+}\) dependent enzyme activities (Tsakiris et al., 2002).

The phenolic compounds present in CST, with different functional properties such as scavenging of ROS, lipid peroxidation chain breaking activity and thiol group replenishing properties would have accounted for the improvement of ATPases in aged rats (Laranjinha et al., 1996; Zainol et al., 2003). Further, thiol group elevating property (Figure 7) and membrane-stabilizing effects of CST could also have contributed for the enhanced activity of ATPases in aged rats.

**OSMOTIC FRAGILITY**

Osmotic fragility determines the functions and integrity of the erythrocytes. Alteration in osmotic fragility is an important indicator for various pathological conditions in erythrocytes (Jain and Shohet, 1981). Increased fragility of erythrocytes was observed in aged rats when compared to young rat erythrocytes in the present study (Figure 16 and Table 6). ATP depletion with alterations in membrane cytoskeletal proteins and lipids has been implicated to increased fragility of erythrocytes (Tesoriere et al., 1999). Further, decrease in the activity of Na\(^+\), K\(^+\) ATPase (Figure 15), upon ageing is critically important in altering the osmotic fragility, owing to its importance in the maintenance of osmotic balance and cell volume (Sweedner, 1991). Additionally, peroxidative damage of phospholipids was reported to determine the fragility of erythrocytes (Lopez-Revuelta et al., 2005). In
accordance, a positive correlation was observed between lipid peroxidation and osmotic fragility in erythrocytes of aged rats (Figure 18).

CST supplementation has shown to decrease osmotic fragility of erythrocytes in aged rats. This was possibly due to free radical scavenging and antioxidant property of flavonoid present in CST that would have prevented macromolecular damages and maintained the erythrocyte membrane integrity in aged rats (Rizvi et al., 1995; Pawlikowska-Pawlega et al., 2003). In addition, decrease in protein and lipid peroxidation (Figure 9 and 12) through antioxidant property of CST would have ultimately increased the erythrocyte bilayer integrity and decreased the fragility of erythrocyte membrane in aged rats.

**MORPHOLOGICAL CHANGES**

Erythrocytes are prone to be converted from normal biconcave to speculated, echinocyte and spherocytic forms during adverse conditions (Strauss et al., 1992). Maintenance of biconcave discocyte shape of erythrocytes is immensely important for its proper functioning and survival of cells. Photographic studies using phase contrast (Figure 18) and scanning electron microscope (Figure 19) showed many cells with serrated boundaries and dark centres with loss of biconcave shape when compared with young rat erythrocytes. Oxidative protein modifications causing denaturation and cross linking of cytoskeletal components in erythrocytes results in significant alterations of its shape, represented by cell swelling, blebbing or shrinkage (Mohanndas and Evans, 1983). Further, abnormalities of spectrin-ankyrin
binding site due to oxidative damage would be expected to lead an abnormal shape of erythrocytes with unstable membrane (Zail and Coetzter, 1984). Reports have shown that oxidative product of hemoglobin, hemin, extensively bind to spectrin to cause oxidative damage leading to shape change (Chiu et al., 1982). Additionally, depletion of membrane bound enzymes can also cause ionic imbalances resulting in cell shape changes, i.e., forming echinocytes (Waugh et al., 1992). Thus increased protein oxidation (Figure 9) and decreased ATPases activities (Figure 15) in the present study would have been the reason behind shape changes in aged rat erythrocytes.

The pliability and elasticity are the salient features of erythrocyte membrane, which facilitates their continual passage through the circulation for a longer time. Cell volume, viscosity of the intracellular milieu and the viscoelastic and viscoplastic properties of the membrane are the major cellular factors that regulate cells ability to maintain shape (Strauss et al., 1992). In accordance, aged rat erythrocytes illustrated decrease in surface area, radius and volume (Table 6) as viewed through phase contrast microscope. Alterations in cell shape with subsequent decrease in physical properties including surface area, radius and volume was believed to be due to the pinching off of membrane fragments from the cells as a result of oxidative stress mediated membrane integrity (Waugh et al., 1992). Additionally cholesterol enrichment in erythrocytes of aged rats as shown in the present study, (Table 5) may alter the red cell surface area and cause distortion in the shape of erythrocytes (Nash and Wyard, 1981). Glass et al. (1983) also evidenced that decrease in hemoglobin content with increasing donor age
could also be the reason behind the decrease in cell volume and shape of erythrocytes. Further, studies by Cruz Silva et al. (2000) demonstrated decrease in alpha tocopherol levels with increase in free radical attack on phospholipids lead to deformation of erythrocytes to echinocyte like structure accompanied with microvesicle formation.

CST supplementation to aged rats improved the morphological characteristics of erythrocytes that could possibly be due to the interaction of flavonoid in CST with membrane proteins of aged rats leading to changes in shape and physical properties like surface area, radius and volume of erythrocytes. Nakagawa and Miyazawa (1997) claimed that plant phenolics have a plannar structure favourable to enter into the cell membrane and get localized effectively in the interface of the membranes thereby exhibiting its antioxidative nature and contributing to the increase in the number of biconcave shaped erythrocytes.

**INTRACELLULAR CALCIUM**

Modulation of Ca\(^{2+}\) level in the intracellular compartment plays a significant role in aging and cell death (Orrenius et al., 1991). The level of intracellular free calcium (Ca\(^{2+}\)) was appreciably increased in erythrocytes of aged rats than young rat erythrocytes (Figure 20). Age related elevation in intra cellular calcium level may be due to the decreased ATP level that would have decreased the activity of Ca\(^{2+}\)-ATPase, which plays an important role and maintaining their normal levels (Larsen et al., 1981). An elevated calcium ion stimulates the activation of proteins, which induce an oxidative burst
(Jabs et al., 1994) by generation of ROS. Moreover, increase in calcium ions concentration can cause peroxidation of erythrocyte phospholipids leading to membrane lipid asymmetry (Jain and Shohet, 1981).

The phenolic compounds of CSTI declined intracellular calcium levels by maintaining Ca\(^{2+}\) ATPase activity probably through their membrane stabilizing and free radical scavenging properties. Further, Ca\(^{2+}\) chelating property of phenolic compounds would have decreased Ca\(^{2+}\) ions level in erythrocyte of aged rats (Pawlikowska-Pawlega et al., 2003).

**CALPAIN**

Eryptosis is considered as a special form of apoptosis typical for the clearance of anucleated RBC, characterized by externalization of PS, cellular shrinkage, ceramide formation, opening of cation channels, and increase of intracellular calcium ions. An important effect caused by raised cell calcium concerns the activation of Calpain – a neutral Ca\(^{2+}\)-dependent thiol protease. It has been suggested that, in the presence of Ca\(^{2+}\), calpain is activated by autolysis a process that is enhanced by the translocation of calpain from cytosol to the cell membrane causing degradation of proteins and enzymes present there (Croall and Demartino 1991). In the present study, decrease in the level of cytosolic calpain level was observed in aged rat erythrocytes compared to young rats demonstrated that increased calcium level would have elevated calpain autolysis leading to its loss in cytosol (Figure 21 and 22). The calpain decrease was possibly due to increased oxidative stress in erythrocytes of aged rats, since reports have shown irreversible loss of calpain
from erythrocyte cytosol during oxidative insults (Mortensen and Novak, 1991). Further, thiol depletion in erythrocytes especially in the presence of calcium ions leads to decrease in cytosolic calpain in aged individuals (Glaser et al., 1994). Thus elevated oxidative stress, raised intracellular calcium ions and decreased thiol levels would have decreased the cytosolic calpain in aged rat erythrocytes.

CST treatment diminished the calpain autolysis and thereby increased the level of cytosolic calpain in erythrocytes of aged rats. This may also be due to the antioxidant properties and Ca$^{2+}$ chelating properties of polyphenols and flavonoids present in CST (Stevens et al., 1997; Chen et al., 2003). Further, polyphenols can decrease oxidative stress that can protect protein thiols from degradation (Balu et al., 2005) in a way, decreased oxidative stress and thereby decreased calpain autolysis and loss of calpain from cytosol.

**PHOSPHATIDYLSERINE EXPOSURE - ERYPTOSIS**

Activation of apoptotic signals in the absence of hemolysis follow phagocytic recognition of exposed PS by a scavenger receptor on the macrophage (Schwartz et al., 1985 and McEvoy et al., 1986). Increased production of ROS may promote the process of apoptosis. Oxidants can indirectly induce apoptosis by depleting GSH, reducing ATP levels, and decreasing reducing equivalents, such as NADH and NADPH (Bernadi, 1996; Bernadi and Petronili, 1996; Costantini et al., 1996). Elevated externalization of PS in erythrocyte membrane of aged rats was evident by flow cytometric
analysis using fluorescent probe, FITC-AnV (Figure 23). Choudhury et al. (1999) suggested that the oxidation of phospholipids in erythrocyte membrane bilayer exposes aminophospholipids that lead them to premature cell death.

PS externalization might possibly be due to the high calcium concentrations that activate lipid scramblase and block the co-operative action of ATP dependent aminophospholipid translocases and flocases leading to randomization of phospholipids across the membrane bilayer (Schroit 1985; Callahan et al., 2000). Daleke and Huestis (1985) confirmed the aforesaid statement by the studies using ATP-depleted erythrocytes, which showed rapid translocation of both PE and PS in favor of inner monolayer, only when the ghosts were resealed in the presence of Mg\(^{2+}\) ATP. Elevated intracellular calcium ions (Figure 21) were reported to increase the PS exposure in aged rats, as the aminophospholipid translocase activity can be inhibited with increased cytosolic calcium ions levels (Bitbol et al., 1987). Reports also suggest that lipid peroxides (Figure 12) within the membrane has devastating effect on the functional state of the membrane because they allow Ca\(^{2+}\) upon oxidation causing cell death. It was also proposed that decline in the intracellular level of ATP in erythrocytes during aging would affect the translocation of phospholipids from inner bilayer of erythrocyte membrane (Beleznay et al., 1993).

Further, cytoskeletal proteins assist in the maintenance of membrane phospholipid asymmetry by selectively interacting with aminophospholipids (Franck et al., 1985). Thus the degradation of cytoskeleton proteins observed
in the present study, (Figure 10) can cause the interaction between
eytoskeletal proteins and PS that becomes weak during lipid symmery
leading to externalization of PS in aged rats. Collectively, these data suggest
that the expression of PS on the outer leaflet of cells were possibly, due to
stress mediated alterations in normal membrane lipid asymmetry that plays a
pivotal role in the recognition and subsequent removal of RBC by the
macrophages (Allen et al., 1988) in aged rats.

Administration of CST to aged rats decreased the levels of intracellular
calcium that might have prevented PS exposure in erythrocyte of aged rats.
This might be due to the presence of phenolic acids and flavonoids present in
CST that have inhibitory properties on calcium elevation and PS exposure
(Summanen et al., 2001). Further, the antioxidant property of polyphenols in
CST can facilitate the augmentation of GSH and ATP levels that would
improve the aminophospholipid asymmetry and thereby prevent PS
exposure and early recognition of erythrocyte by macrophages with
advancement of animal age (Sini and Devi. 2004; Valls-Belles et al., 2006).