Results
TOTAL FLAVONOID AND POLYPHENOL CONTENT IN CHLOROFORM, METHANOL AND AQUEOUS EXTRACTS OF Solanum trilobatum

Figure 1 illustrates the level of total flavonoid and polyphenol present in chloroform, methanol and aqueous extracts of Solanum trilobatum. The chloroform extract of Solanum trilobatum (CST) was observed to contain high flavonoids and polyphenols content when compared to methanolic and aqueous extracts. Henceforth, the experimental studies were carried out using chloroform extract of Solanum trilobatum.

FIXATION OF DOSAGE AND DURATION

Figure 2 depicts the trial studies carried out with different concentrations of CST (50, 100, 150, 200 and 250 mg) dissolved in alkaline saline and supplemented orally by gavage to rats at different durations (30, 60, 90 and 120 days). The dosage (150 mg/kg body weight/day) and duration (90 days) were selected on the basis of the concentration at which CST was capable of effectively inhibiting the lipid peroxidation. No significant difference in the body weight of animals at the dosage of 150-mg/kg/body weight/day for 90 days was observed during the experimental period.
Figure 1. Total Flavonoids and Total Polyphenols in chloroform, methanol and aqueous extracts of *Solanum trilobatum*

Total Flavonoids

Values are expressed as Mean ± SD
Figure 2. Level of lipid peroxidation inhibited on CST supplementation at different dosage and duration in erythrocytes of rats.
OXIDANT PRODUCTION IN ERYTHROCYTES

The non-fluorescent probe dichlorofluorescein diacetate (DCFH-DA) gets oxidized in the presence of ROS to fluorescent DCF, which helps us to determine the level of oxidants present in the cells. Figure 3 demonstrates the level of oxidant production in erythrocytes of control and experimental rats when analyzed using DCFH-DA. The DCF level was significantly increased by 1.4 fold in erythrocytes of aged rats when compared to that of young rats. CST treatment to aged rats decreased the level of DCF fluorescence by 1.3 fold. Insignificant changes were observed in erythrocytes of young rats on CST administration. Moreover, young rats supplemented with CST did not reveal any noticeable alterations in any of the forth coming parameters investigated.

FREE RADICAL LEVELS

Free radical restrains an atom with an unpaired electron in their outer orbit, which are deleterious and can damage cellular macromolecules. Table 1 depicts the levels of superoxide anions, hydroxyl radicals and hydrogen peroxides in erythrocytes of control and CST treated young and aged rats. An increased production of superoxide anion by 63%, hydroxyl radicals by 90% and hydrogen peroxide by 42% was observed in erythrocytes of aged rats when compared to young rats. CST treatment to aged rats significantly declined the levels of superoxide anion by 25%, hydroxyl radical by 31% and hydrogen peroxide by 23% respectively.
Figure 3. Level of oxidant production in erythrocytes of control and CST treated young and aged rats using fluorescence probe DCFH-DA

Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated
Values are expressed as Mean ± SD for six rats
a - Group IIa compared with Group Ia  b - Group IIb compared with Group IIa
* represents p < 0.05
Table 1: Levels of Superoxide anions, Hydroxyl Radicals and H₂O₂ in control and CST treated young and aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young rats</th>
<th>Aged rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td>Group Ib</td>
</tr>
<tr>
<td>Superoxide anions</td>
<td>32.71 ± 3.84</td>
<td>30.11 ± 3.90</td>
</tr>
<tr>
<td>Hydroxyl radicals</td>
<td>15.71 ± 1.35</td>
<td>14.20 ± 1.76</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>67.36 ± 6.45</td>
<td>64.81 ± 5.82</td>
</tr>
</tbody>
</table>

*Group Ia – Young control, Group Ib – Young CST treated, Group IIa – Aged control, Group IIb – Aged CST treated

Units: Superoxide anions: Hydroxyl radicals, H₂O₂: μmoles/hr 10⁷ cells

Values are expressed as Mean ± SD for six rats

'a' - Group IIa compared with Group Ia. 'b' - Group IIb compared with Group IIa

*represents p < 0.05
ENZYMATIC ANTIOXIDANT STATUS

SOD regulates the intracellular concentration of superoxide anion and is an important enzymatic component of erythrocyte oxidant defense system. Erythrocyte catalases primarily work to catalyze the decomposition of hydrogen peroxide to water sharing the function with GPx and GR. Figure 4 highlights the activities of enzymatic antioxidants SOD and CAT in erythrocytes of young and aged rats. The activities of SOD and CAT were decreased significantly by 33% and 47% respectively in aged rats when compared to young rats. CST supplementation to aged rats enhanced the activities of SOD by 31% and CAT by 68%.

Erythrocyte GPx is an important enzyme that scavenges hydrogen peroxide and has a critical need for glutathione and GST. These enzymes together are capable of inactivating reactive electrophilic mutagens including the aldehyde products of lipid peroxidation. Figure 5 shows the alterations in the antioxidant activities of GPx and GST in the erythrocytes of young and aged rats. The activities of GPx and GST were decreased in aged rats by 38% when compared to erythrocytes of young rats. Improvement in these antioxidant enzymes occurred purported to CST therapy, the increase being 39% for GPx and 43% for GST.

Erythrocyte G6PD is an important enzyme in pentose phosphate pathway that supplies NADPH needed for the regeneration of GSH which is catalyzed by GR. Figure 6 depicts the activities of antioxidant enzymes GR and G6PD in erythrocytes of young and aged rats. The activities of GR were
Figure 4. Activities of Superoxide Dismutase and Catalase in erythrocytes of control and CST treated young and aged rats

Superoxide Dismutase

Catalase

Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated

Values are expressed as Mean ± SD for six rats

a - Group IIa compared with Group Ia, 'b' - Group IIb compared with Group IIa

* represents $p < 0.05$
Figure 5. Activities of Glutathione Peroxidase and Glutathione-S-Transferase in erythrocytes of control and CST treated young and aged rats

Glutathione Peroxidase

1.5
1.0
0.5
0.0
Group Ia
Group Ib
Group IIa
Group IIb

Glutathione-S-Transferase

16
14
12
10
8
6
4
2
0
Group Ia
Group Ib
Group IIa
Group IIb

Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated
Values are expressed as Mean ± SD for six rats
a - Group IIa compared with Group Ia  b - Group IIb compared with Group IIa
* represents p < 0.05
Figure 6. Activities of Glutathione Reductase and Glucose-6-Phosphate Dehydrogenase in erythrocytes of control and CST treated young and aged rats

**Glutathione Reductase**

- **Group Ia**
- **Group Ib**
- **Group IIa**
- **Group IIb**

**Glucose-6-Phosphate Dehydrogenase**

- **Group Ia**
- **Group Ib**
- **Group IIa**
- **Group IIb**

*Group Ia* - Young Control, *Group Ib* - Young CST treated, *Group IIa* - Aged control, *Group IIb* - Aged CST treated

Values are expressed as Mean ± SD for six rats.

'a' - Group IIa compared with Group Ia, 'b' - Group IIb compared with Group IIa

* represents p < 0.05
found to be significantly decreased by 39% and G6PD by 45% in erythrocytes of aged rats compared to young rats. Administration of CST to aged rats increased the levels of GR (45%) and G6PD (59%). The results thus confirmed the potential antioxidant role of CST in increasing the enzymatic antioxidant status.

**GLUTATHIONE REDOX STATUS**

*Glutathione serves several essential functions within the cell including the detoxification of free radicals, metals and other electrophilic compounds.* Table 2 demonstrates the glutathione status in erythrocytes of control and CST treated young and aged rats. Remarkable decline in GSH level (38%) with increase in GSSG level (67%) was noted in aged rat erythrocytes. These alterations indicate increased oxidation of glutathione in erythrocytes with advancement of age. Further, significant decrease in both GSH/GSSG ratio and redox index by 2.7 fold was also observed in aged rat erythrocytes. CST treatment to aged rats increased the GSH level by 42% and reduced GSSG levels by 40% with subsequent increase in GSH/GSSG ratio by 2.3 fold and redox index by 1.4 fold.

**TOTAL THIOLS**

*Thiol groups play a prominent role in antioxidant reactions, catalysis, regulation and preservation of protein structure.* Figure 7 represents the levels of total thiol in erythrocytes of young and aged rats. Significant decrease in the levels of total thiols by 34% was noticed in erythrocytes of
Table 2: Levels of GSH, GSSG, GSH/GSSG and redox state in control and CST treated young and aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young rats</th>
<th>Aged rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td>Group Ib</td>
</tr>
<tr>
<td>GSH</td>
<td>3.12 ± 0.37</td>
<td>3.20 ± 0.39</td>
</tr>
<tr>
<td>GSSG</td>
<td>0.06 ± 0.006</td>
<td>0.06 ± 0.007</td>
</tr>
<tr>
<td>GSH/GSSG Ratio</td>
<td>52.00 ± 5.03</td>
<td>53.33 ± 4.18</td>
</tr>
<tr>
<td>Redox State</td>
<td>0.27 ± 0.02</td>
<td>0.27 ± 0.03</td>
</tr>
</tbody>
</table>

\(^a^\) - Group IIa compared with Group Ia, \(^b^\) - Group IIb compared with Group IIA

* represents \( p < 0.05 \)

Group Ia – Young control, Group Ib – Young CST treated, Group IIa – Aged control, Group IIb – Aged CST treated

Units: GSH, GSSG: µmoles/g Hb

Values are expressed as Mean ± SD for six rats.
Figure 7. Level of total thiols in erythrocytes of control and CST treated young and aged rats

**Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated**

Values are expressed as Mean ± SD for six rats.

a' - Group IIa compared with Group Ia  b - Group IIb compared with Group IIa

* represents p < 0.05
aged rats compared to young rats. CST supplementation to aged rats increased the total thiol levels in erythrocytes by 23%.

**NON-ENZYMATIC ANTIOXIDANTS**

Increase in oxidant formation and depletion in antioxidant levels can tip the oxidative balance causing cell damage and dysfunction. Non-enzymatic lipophilic antioxidant, α-tocopherol, exerts protective function against oxidative stress by interacting with ascorbate (a broad spectrum radical scavenger) and enhances the antioxidant capacity of the cell.

Figure 8 shows the levels of non-enzymatic antioxidants ascorbic acid and α-tocopherol in control and CST treated young and aged rats. Significant decrease in the levels ascorbic acid by 34% and α-tocopherol by 31% was observed in aged rats when compared to young rats. CST supplementation to aged rats significantly increased ascorbic acid (32%) and α-tocopherol (30%) levels, demonstrating the non-enzymatic antioxidant elevating property of CST.

**HEMATOLOGICAL INDICES**

Modification in the hematological indices may be one of the physiological changes associated with free radical attack. Table 3 depicts the levels of hematological indices in control and CST supplemented young and aged rats. Significant decrease in erythrocyte count (26%), reticulocyte count (40%), PCV (37%) Hb (41%), MCV (26%), and MCH (46%), MCHC (48%) were observed in aged rats when compared to young rats. CST administration
Figure 8. Levels of Ascorbic Acid and α-Tocopherol in control and CST treated young and aged rats

Ascorbic acid

μmoles/dl

Group Ia  Group Ib  Group Ia  Group Ib

α-Tocopherol

μg/mg protein

Group Ia  Group Ib  Group Ia  Group Ib

* Group Ia - Young Control, Group Ib - Young CST treated, Group Ia - Aged control, Group Iib - Aged CST treated
* Values are expressed as Mean ± SD for six rats
  a - Group Ila compared with Group Ia  b - Group Iib compared with Group Ila
* represents p < 0.05
Table 3: Hematological indices in control and CST treated young and aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young rats</th>
<th>Aged rats</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td>Group Ib</td>
<td>Group IIa</td>
<td>Group IIb</td>
<td></td>
</tr>
<tr>
<td>Erythrocyte count</td>
<td>5.34 ± 0.51</td>
<td>5.68 ± 0.62</td>
<td>3.94 ± 0.44(^{a*})</td>
<td>5.09 ± 0.53(^{b*})</td>
<td></td>
</tr>
<tr>
<td>Reticulocyte count</td>
<td>2.20 ± 0.28</td>
<td>2.31 ± 0.27</td>
<td>1.34 ± 0.79(^{a*})</td>
<td>2.03 ± 0.32(^{b*})</td>
<td></td>
</tr>
<tr>
<td>PCV</td>
<td>38.16 ± 4.04</td>
<td>39.51 ± 3.24</td>
<td>24.17 ± 2.40(^{a*})</td>
<td>33.34 ± 3.07(^{b*})</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>13.43 ± 1.02</td>
<td>13.72 ± 1.61</td>
<td>7.86 ± 0.83(^{a*})</td>
<td>11.31 ± 1.72(^{b*})</td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>67.33 ± 5.37</td>
<td>68.37 ± 5.89</td>
<td>49.71 ± 3.95(^{a*})</td>
<td>61.14 ± 6.23(^{a*})</td>
<td></td>
</tr>
<tr>
<td>MCH</td>
<td>20.53 ± 2.69</td>
<td>20.37 ± 2.28</td>
<td>11.07 ± 1.38(^{a*})</td>
<td>17.44 ± 1.63(^{b*})</td>
<td></td>
</tr>
<tr>
<td>MCHC</td>
<td>19.62 ± 1.93</td>
<td>20.21 ± 1.99</td>
<td>10.22 ± 1.04(^{a*})</td>
<td>16.24 ± 2.71(^{b*})</td>
<td></td>
</tr>
</tbody>
</table>

* Group Ia – Young control, Group Ib – Young CST treated, Group IIa – Aged control, Group IIb – Aged CST treated

Units: Erythrocyte count - million/mm\(^3\); Reticulocytes count, PCV - %; Hb, MCHC - g/dl; MCV – fl;

MCH – pg

Values are expressed as Mean ± SD for six rats.

'\(^{a}\)' - Group Ila compared with Group Ia. '\(^{b}\)' - Group IIb compared with Group IIa

* represents p < 0.05
to aged rats (90 days) significantly increased the level of erythrocyte count (29%), reticulocyte count (51%), PCV (38%). Hb (44%), MCV (23%), MCH (58%) and MCHC (59%). The results thus indicate the efficacy of CST in maintaining the hematological indices in aged rats.

**PROTEIN CARBONYLS**

*Oxidative damage to protein is reflected by an increase in protein carbonyl content.* Figure 9 shows the levels of protein carbonyls in erythrocytes and plasma of control and CST treated young and aged rats. Increase in protein carbonyls by 1.8 fold was observed in erythrocyte membrane and plasma of aged rats when compared with young rats. Elevation in protein carbonyl content indicates increased protein oxidation with advancement of animal age. CST treatment to aged rats showed significant decrease in protein carbonyl levels by 1.3 fold in erythrocyte membrane and 1.4 fold in plasma.

**ERYTHROCYTE MEMBRANE PROTEIN**

Damage and alterations to *membrane proteins leads to their degradation with lose of structure and catalytic function*. SDS–PAGE profile of control and experimental rats is depicted in Figure 10. Normal electrophoretic profile of membrane cytoskeleton was observed in erythrocytes of young control (lane 2). Significant alterations in the electrophoretic pattern with decrease in α-spectrin and β spectrin, ankyrin, band 3, band 4.1a and band 4.1b with an abnormal elevation of high
Figure 9. Levels of Erythrocyte Membrane and Plasma protein carbonyls in control and CST treated young and aged rats. Oxidation of proteins are expressed in terms of protein carbonyls.

**Erythrocyte Membrane**

- Group 1a
- Group 1b
- Group 11a
- Group 11b

**Plasma**

- Group 1a
- Group 1b
- Group 11a
- Group 11b

*Group 1a - Young Control, Group 1b - Young CST treated, Group 11a - Aged control, Group 11b - Aged CST treated*

Values are expressed as Mean ± SD for six rats.

'a' - Group 11a compared with Group 1a  'b' - Group 11b compared with Group 11a

* represents p < 0.05
**Figure 10.** SDS-PAGE of erythrocyte membrane protein of control and experimental rats

Lane 1: Protein molecular weight marker
Lane 2: Young control rats
Lane 3: Aged control rats
Lane 4: CST treated aged rats
molecular weight polymer (HMWP) was observed in aged rats (lane 3). CST
treatment to aged rats decreased the HMWP levels and restored α and β
spectrin, band 3, ankyrin, band 4.1a and band 4.1b levels (lane 4) thereby
illustrating the membrane protective role of CST.

SURFACE CHARGE

Erythrocyte membrane has a total negative charge that determines the
correct course of many processes like transport of metabolic substrates;
transfer of information and more importantly in preventing the aggregation of
erthrocytes from each other. Figure 11 illustrates the surface charge in
erthrocytes of young and experimental rats in terms of partition coefficient
ratio. Significant (p < 0.05) decrease in surface charge level in erythrocytes
was noticed in aged rats when compared to young rats. CST supplementation
to aged rats showed increased the surface charge (p < 0.05) level significantly.

GLYCOPROTEINS

Cell surface carbohydrate serves directly as molecular determinants
for normal cell functioning and alterations in their level profoundly have
deleterious effects in affecting cell survival. Table 4 depicts the levels of
glycoproteins like hexose, hexosamine and sialic acid in erythrocyte
membrane of control and CST treated young and aged rats. Profound decrease
in the levels of hexose, hexosamine and sialic acid by was decreased by 23%.
34% and 22% respectively in erythrocytes of aged rats was observed.
Figure 11. Level of surface charge in erythrocytes of control and CST treated young and aged rats. Surface charge was analysed using two phase aqueous system and expressed in terms of partition coefficient ratio.

*Group Ia - Young Control, Group Ib - Young CST treated, Group IIA - Aged control, Group IIb - Aged CST treated*

Values are expressed as Mean ± SD for six rats

'\text{*a*} - Group IIA compared with Group Ia  \quad \text{'b*} - Group IIb compared with Group IIA

* represents $p < 0.05$
Table 4: Levels of Glycoprotein in erythrocytes and plasma of control and CST treated young and aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young rats</th>
<th>Aged rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td>Group Ib</td>
</tr>
<tr>
<td>Hexose</td>
<td>550 08 ± 50 85</td>
<td>581 20 ± 51 20</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>415 03 ± 41 76</td>
<td>425 64 ± 40 24</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>71 72 ± 7 13</td>
<td>72 56 ± 7 35</td>
</tr>
</tbody>
</table>

*Group Ia – Young control, Group Ib – Young CST treated, Group IIa – Aged control, Group IIb – Aged CST treated*

Units µg/mg protein

Values are expressed as Mean ± SD for six rats

'a' - Group IIa compared with Group Ia. 'b' - Group IIb compared with Group IIa

* represents p < 0.05
Supplementation of CST enhanced the level of hexose by 23%, hexosamine by 41% and sialic acid by 24% in aged rat erythrocytes.

**LIPID PEROXIDATION LEVEL**

_Lipid peroxidation has been identified as the basic deteriorative reaction in cellular mechanisms to monitor the extent of oxidative cell damages._ Figure 12 shows the level of lipid peroxidation in erythrocyte membrane and plasma of control and CST treated young and aged rats. Level of MDA was profoundly increased by 1.7 fold in erythrocytes and 1.8 fold in plasma of aged rats when compared to young rats. Administration of CST significantly reduced the level of lipid peroxidation in erythrocyte membrane by 1.3 fold and in plasma by 1.7 fold respectively, thereby displaying the antioxidant capacity of CST in combating oxidative stress in aged animals.

**FLUORESCENCE ANISOTROPY - MEMBRANE FLUIDITY**

_Fluidity of membrane is important in maintaining the structure and function of erythrocytes. Erythrocyte membrane fluidity was measured in terms of fluorescence anisotropy using fluorescent lipid probe, 1,6 diphenyl hexa 3,4,5 triene (DPH)._ Figure 13 illustrates the level of fluorescence anisotropy in erythrocytes of control and CST treated young and aged rats. Fluorescence anisotropy was significantly increased by 21% in erythrocytes of aged rats when compared with young rats illustrating decrease in erythrocyte membrane fluidity with advancement of age. CST treatment to aged rats significantly decreased the level of anisotropy by 14% probably
Figure 12. Lipid peroxidation in Erythrocyte Membrane and Plasma of control and CST treated young and aged rats

Erythrocyte membrane

![Graph showing lipid peroxidation in Erythrocyte Membrane for different groups.]

Plasma

![Graph showing lipid peroxidation in Plasma for different groups.]

*Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated*

Values are expressed as Mean ± SD for six rats

'a' - Group IIa compared with Group Ia, 'b' - Group IIb compared with Group IIa

*represents p < 0.05
Figure 13. Level of fluorescence anisotropy in erythrocytes of control and CST treated young and aged rats using fluorescent probe, DPH

Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated

Values are expressed as Mean ± SD for six rats

a' - Group IIa compared with Group Ia  b - Group IIb compared with Group IIa

* represents p < 0.05
through the membrane protective role and antioxidant role of flavonoids present in CST

**LIPID PROFILE**

*Lipids are important class of molecules highly susceptible to free radical mediated modifications* The levels of cholesterol and phospholipids in erythrocyte membrane of control and CST treated young and aged rats are exhibited in Table 5. The level of cholesterol was elevated by 50% and phospholipid was declined by 28% significantly in erythrocyte membrane of aged rats when compared with young rats. CST treatment to aged rats significantly elevated the levels cholesterol by 25% and declined phospholipids by 32% in erythrocyte membrane.

The cholesterol/phospholipid ratio of young and aged rats before and after supplementation of CST is depicted in Table 6. Significant increase (p < 0.05) in the C/P ratio was observed in aged rats when compared to young control rats. CST supplementation to aged rats decreased the cholesterol levels and increased the phospholipid levels thereby restoring the C/P ratio.

Figure 14 illustrates the plasma lipid profile (total free and esterified cholesterol) in young and aged rats before and after supplementation of CST. Significant increase in total, free and esterified cholesterol by 67%, 62% and 70%, respectively, were observed in plasma of aged rats compared to young rats. However, CST therapy decreased the levels of total cholesterol (31%)
Table 5: Total Cholesterol, Total Phospholipid and Cholesterol: Phospholipid ratio in erythrocyte membrane of control and CST treated young and aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young rats</th>
<th>Aged rats</th>
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<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td>Group Ib</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>120 52 ± 12 24</td>
<td>115 12 ± 11 28</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>241 59 ± 23 72</td>
<td>246 40 ± 21 02</td>
</tr>
<tr>
<td>C/P ratio</td>
<td>0 53 ± 0 05</td>
<td>0 51 ± 0 04</td>
</tr>
</tbody>
</table>

<sup>a</sup> - Group IIa compared with Group Ia. <sup>b</sup> - Group IIb compared with Group IIa

Group Ia – Young control, Group Ib – Young CST treated, Group IIa – Aged control, Group IIb – Aged CST treated

Units: cholesterol, phospholipid - μg/mg protein

Values are expressed as Mean ± SD for six rats

* represents p < 0.05
Figure 14. Levels of total, free and ester cholesterol in plasma of control and CST treated young and aged rats

* Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated

Values are expressed as Mean ± SD for six rats
'a' - Group IIa compared with Group Ia, 'b' - Group IIb compared with Group IIa
* represents p < 0.05
free cholesterol (27%) and ester cholesterol (30%) and in erythrocyte membrane of aged rats

**MEMBRANE BOUND ATPASES**

Membrane bound enzymes play a major role in the maintenance of the ionic gradients between the intracellular and extracellular compartments of erythrocytes. Figure 15 represents the activities of ATPase in the erythrocyte membrane of control and CST treated young and aged rats. Significant decline in the activities of Na\(^+\)K\(^+\)ATPase by 36% Ca\(^2+\)ATPase by 56% and Mg\(^2+\)ATPases by 51% were observed in erythrocytes of aged rats. CSI administration to aged rats improved the activities of these ion motive ATPase by 30% for Na\(^+\)K\(^+\)ATPase 56% for Ca\(^2+\)ATPase and 60% for Mg\(^2+\)ATPase. This confirms the protective effect of CSI in maintaining the ionic gradient in erythrocytes with advancement of animal age.

**OSMOTIC FRAGILITY**

Integrity of the red blood cells can be determined by measuring the changes in erythrocyte osmotic fragility. Figure 16 and Table 6 depicts the osmotic fragility in erythrocytes of control and CST treated young and aged rats. Fifty percent hemolysis occurred at concentration 0.41% and 0.43% of isotonic saline in control and CST treated young rat erythrocytes respectively whereas aged control rats showed 50% hemolysis at 0.55% saline concentration. These results thereby illustrate the decline in erythrocyte membrane integrity with advancement of age. On treatment with CST the
Figure 15. Activities of erythrocyte membrane bound ATPases in control and CST treated young and aged rats

Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated

Values are expressed as Mean ± SD for six rats

'a' - Group IIa compared with Group Ia  'b' - Group IIb compared with Group IIa

* represents p < 0.05
Figure 16. Osmotic fragility of erythrocytes in control and CST treated young and aged rats at different saline concentrations

Figure 17. Correlation between osmotic fragility and lipid peroxidation in aged rat erythrocytes

Group Ia - Young Control, Group Ib - Young CST treated, Group IIA - Aged control, Group IIB - Aged CST treated
aged rat erythrocyte showed 50% hemolysis at 0.48% isotonic saline. Thus, CST administration was proved to be efficacious in maintaining the membrane stability of aged rat erythrocytes. Figure 17 depicts a positive correlation ($r = 0.71$) between lipid peroxidation and osmotic fragility in erythrocytes of aged rats. The correlation result signifies that increase in lipid peroxidation level consequently increases the fragility of erythrocytes in aged rats.

**MORPHOLOGICAL STUDIES**

**PHASE CONTRAST AND SCANNING ELECTRON MICROSCOPIC ANALYSIS**

Figure 18 and 19 represents the phase contrast and scanning electron microscopic pictures of erythrocytes in control and experimental rats. Erythrocytes of young rats showed normal biconcave shape with regular surface and few spiculated cells. Aged rats showed many spiculated and echinocyte type cells along with biconcave shaped cells. CST therapy to aged animals decreased the adverse morphological modifications in erythrocytes thereby reducing the number of spiculated cells.

**RED CELL PHYSICAL PROPERTIES**

Table 6 represents the physical properties of erythrocytes in young and aged rats measured under the phase contrast microscope and CCD camera. Significant decrease ($p < 0.05$) in the physical properties including radius,
Figure 18. Photographs of erythrocytes in control and CST treated young and aged rats viewed under phase contrast microscope (450 X)

Young control rats

Aged control rats

CST treated aged rats
Figure 19. Scanning Electron microscopic pictures of erythrocytes in control and CST treated young and aged rats (1000 x)

Young control rats

Aged control rats

CST treated aged rats
### Table 6: Physical properties of erythrocytes in control and CST treated young and aged rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young rats</th>
<th></th>
<th>Aged rats</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Ia</td>
<td>Group Ib</td>
<td>Group IIa</td>
<td>Group IIb</td>
</tr>
<tr>
<td>Radius</td>
<td>3.45 ± 0.31</td>
<td>3.46 ± 0.33</td>
<td>2.75 ± 0.10(^a)</td>
<td>3.24 ± 0.21(^b)</td>
</tr>
<tr>
<td>Area</td>
<td>102.13 ± 10.02</td>
<td>105.17 ± 9.13</td>
<td>73.15 ± 7.34(^a)</td>
<td>94.2 ± 8.97(^b)</td>
</tr>
<tr>
<td>Volume</td>
<td>63.26 ± 5.7</td>
<td>65.23 ± 6.1</td>
<td>46.28 ± 5.83(^a)</td>
<td>57.2 ± 5.22(^b)</td>
</tr>
<tr>
<td>Osmotic fragility</td>
<td>0.41 ± 0.04</td>
<td>0.43 ± 0.03</td>
<td>0.55 ± 0.05(^a)</td>
<td>0.48 ± 0.04(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Young control, Group Ib – Young CST treated, Group IIa – Aged control, Group IIb – Aged CST treated

Units: Radius - µm, Area - µm\(^2\), Volume - µm, Osmotic fragility – Concentration of NaCl in % at 50% hemolysis of erythrocytes

Values are expressed as Mean ± SD for six rats

'a' - Group IIa compared with Group Ia  
'b' - Group IIb compared with Group IIa

* represents p < 0.05
area and volume was observed in aged rats when compared to young rats. CST treatment to aged rats reverted to average levels with increase in radius by 1.2 fold, area by 1.3 fold and volume by 1.2 fold.

**INTRACELLULAR FREE CALCIUM IONS**

*Modulation in calcium ion level is a serious threat to calcium dependent metabolic pathways which can endanger the survival of red blood cells during oxidative stress.* Figure 20 depicts the intracellular calcium ion levels in erythrocytes of control and experimental rats. Significant (p < 0.05) elevation in intracellular calcium ion level was observed in aged rat erythrocytes. On administration of CST to aged rats the intracellular calcium ion level was declined significantly by 43%. This shows that polyphenols in CST has the ability to maintain calcium levels in erythrocytes with advancement of age.

**ELISA AND IMMUNOBLOT ANALYSIS OF CALPAIN**

*Calpain (Ca²⁺- activated protease) is believed to regulate the function of membrane enzymes and can modify the behavior of membrane structural proteins.* In the present study, the level of cytosolic calpain in cytosol of young and aged rat erythrocytes was studied using monoclonal antibody to µ-calpain (Figure 21). Erythrocyte of aged rats depicted decrease in the level of cytosolic calpain by 40% when compared to young rats. This decrease indicates the translocation of calpain from cytosol to membrane. CST treatment efficiently (p< 0.05) improved the cytosolic calpain level in aged rat
Figure 20. Intracellular calcium ions level in erythrocytes of control and CST treated young and aged rats using FURA-2AM fluorescent probe.

Group Ia - Young Control, Group Ib - Young CST treated, Group IIa - Aged control, Group IIb - Aged CST treated.
Values are expressed as Mean ± SD for six rats.
'a' - Group IIa compared with Group Ia
'b' - Group IIb compared with Group IIa
* represents p < 0.05.
Figure 21. Level of cytosolic calpain in erythrocytes of control and experimental rats

Values are expressed as Mean ± SD for six rats. 'a' - Group IIa compared with Group Ia, 'b' - Group IIb compared with Group Ila; * represents p < 0.05

Figure 22. Immunoblot analysis of cytosolic calpain expression in erythrocytes of control and experimental rats

Lane 1: Protein molecular weight marker
Lane 2: Young control rats
Lane 3: Aged control rats
Lane 4: CST treated aged rats
erythrocytes. This was further confirmed by immunoblot assay (Figure 22) illustrating the favorable effect of CST in maintaining the level of calpain level with advancement of animal age.

**PHOSPHATIDYLERINE (PS) EXPOSURE**

*Exposure of PS on the outer side of erythrocyte bilayer (membrane outer leaflet) is a strong signal for the recognition of macrophages in circulation for subsequent phagocytosis and cell death.* Figure 23 illustrates the level of Annexin V-FITC binding on erythrocytes of control and experimental rats by flow cytometric analysis. The fluorescence intensity (which is directly proportional to the cell binding Annexin V-FITC) of labeled erythrocytes was measured as FACS (Fluorescence Activated Cell Sorter) generated plots of forward light scatter versus right angle light scatter for each sample. The FACS analysis of aged rat erythrocytes showed 11% increase in erythrocytes with Annexin V - FITC binding compared to young rats. Supplementation of CST to aged rats decreased the level of PS exposure to almost 9%. This demonstrates the membrane stabilizing activity of CST in decreasing the PS externalization and thereby preventing the premature erythrocyte cell death in aged animals.
Figure 23. Flow cytometric analysis of erythrocytes with phosphatidylserine exposure using fluorescent probe FITC-Annexin V in control and experimental rats

Young control rats

Aged control rats

CST treated aged rats