Evaluation of Naringin and Rutin to Reverse Doxorubicin-Induced Chemobrain Associated Memory Deficits in Healthy Rats

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Evaluation of Naringin and Rutin to Reverse Doxorubicin-Induced Chemobrain Associated Memory Deficits in Healthy Rats

7.1. Introduction
Incidence of chemobrain complications are reported clinically, however no FDA approved interventions are available till date to manage or mitigate the cognitive problems associated with chemotherapy. To date, few anti-inflammatory agents, modafinil, acetylcholinesterase inhibitors, methylphenidate, ginkgobiloba, resveratrol, N-acetyl cysteine are tried clinically to manage the chemofog, but they are found to be not effective in relieving the memory deficits associated with chemobrain (Davis et al., 2013; Fardell et al., 2011). Animal studies support the fact that, flavonoids can improve various components of cognitive processes through neuronal differentiation, long-term potentiation and also by enhancing the synaptic plasticity (Spencer, 2009; Wang et al., 2006; Williams et al., 2004). Hence we designed studies to evaluate the protective role of flavonoids, NAR and RUT against DOX-induced cognitive dysfunction.

7.2. Materials and Methods
7.2.1. Animals
Healthy female Wistar rats of body weight ranging from 170-180 g were obtained from central animal research facility of Manipal University, Manipal. All the experimental procedures were approved by IAEC with approval number, IAEC/KMC/17/2013.

7.2.2. Chemicals
The chemicals used were already described in previous chapters.

7.2.3. Apparatus
The apparatus used for ORT and MWM were same as described in chapter 4, page 24 and chapter 6, page 46.

7.2.4. Experimental design
Total of six groups of animals were used to assess the protective effect of NAR and RUT flavonoids. Group 1 is a healthy control treated with normal saline, whereas the other three groups, (i.e. Group 2, 3 & 4) were treated with DOX at a dose of 2.5 mg/kg, once in every 5 days intraperitoneally with a dose volume of 2 ml/kg. All the groups were continued to receive the treatments up to 50 days (i.e. 10 treatment cycles).
In addition to the above treatment, flavonoid treatments were given daily, one week before start of the DOX or saline administration as follows. Group 1 & 2 were treated with sodium CMC, \textit{p.o.} whereas group 3 & 4 were treated with NAR and RUT at a fixed dose of 50 mg/kg, \textit{p.o.} and continued throughout the study period including the duration of behavioural assessment.

To know the individual \textit{per se} effects of test flavonoids, animals of Group 5 & 6 were treated with NAR and RUT respectively on a daily basis orally and normal saline was given instead of DOX once in 5 days by \textit{i.p.} Detailed experimental protocol was illustrated in Fig.7.1

![Chemobrain Protocol Diagram](image)

**Fig. 7.1** Illustration represents the developed chemobrain protocol with the administration of test flavonoids and DOX

**7.2.5. Formulations and treatments**

NAR and RUT were formulated as suspensions in 0.25% w/v sodium CMC in distilled water using a dose volume of 2 ml/kg. DOX was formulated as clear solution in normal saline at a dose volume of 2 ml/kg. All the treatments were given as mentioned in the experimental design section either orally or intraperitoneally for the prescribed period as per the protocol mentioned above.

**7.2.6. In-vivo chemobrain studies**

**7.2.6.1. Object recognition task (ORT)**

The basic procedure involved was similar to that of protocols used in chapter 4, page 25. We used 2 h trial delay to assess DOX-induced cognitive deficits and the accompanying chemobrain complications without the confounding influence of time-induced cognitive deficits. Exploration time, discriminative and recognition indices were calculated and compared among the various groups.
7.2.6.2. Morris water maze (MWM) - Spatial memory model

The procedure involved was similar to the protocol mentioned in chapter 6, page 47. Escape latency, swim speed and path length were assessed during the acquisition trials whereas, time spent in the target quadrant (Q4 i.e. quadrant having platform during acquisition phase), latency to reach target quadrant were noted during retention trials and compared.

7.2.7. Open field test for locomotor activity

Animals were assessed for locomotion using an open field paradigm. OF test was done using square arenas used for object recognition task (Abel, 1995; Prut and Belzung, 2003).

7.2.8. Change in body weight

Body weight was recorded once in three days and monitored throughout the study. The average body weight was calculated and compared among different treatment groups.

7.2.9. Haematological profiling

Blood sampling was made by retro-orbital puncture. Complete blood profile was assessed using automated veterinary blood cell counter (ERMA-PCE-210VET, Japan).

7.2.10. Biochemical analysis

Complete biochemical profiling was carried out in serum using standard kits. Biochemical analysis was carried out for AST, ALT, ALP, Cholesterol, total protein, urea, CKL, creatinine etc., by fully automated autoanalyser (Cobas C111; Roche Diagnostics Limited, Switzerland) using the company’s standard kits following the manufacturer’s protocol.

Animals were deeply anaesthetized with ketamine and whole body perfusion was carried out with ice cold saline. Organ collection was made for kidney, liver, brain and heart. The weight of each organ per 100 g body weight of the animal was calculated as organ index and compared.

7.2.11. Acetylcholinesterase activity

Estimating the AChE activity provides valuable information on cholinergic function. Following hippocampal and frontal cortex isolation, samples were homogenized with phosphate buffer (pH 7.4) and supernatants were collected and stored in aliquots. The method for acetyl cholinesterase estimation was as per the earlier method (Ellman et al., 1961).

Methodology

**Reagents**

0.1 M Phosphate buffer (PB) was prepared as follows. 5.22 g of K$_2$HPO$_4$ and 4.68 g of NaH$_2$PO$_4$ were dissolved in 150 ml of distilled water which is Solution-I. 6.2 g NaOH was dissolved in 150 ml of distilled water (Solution-II). Solution I and II were added to get
desired pH (pH 8.0). DTNB reagent was prepared by dissolving 39.6 mg of DTNB and 5 mg NaHCO₃ in 10 ml of 0.1 M phosphate buffer (pH 7.0)

**Procedure**

21.67 mg of acetylthiocholine iodide was dissolved in 1 ml distilled water and 0.4 ml aliquot of the homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1 M, pH 8) and 100 µl of DTNB. The contents of the cuvette thoroughly mixed and absorbance was measured at 412 nm in UV spectrophotometer. 20 µl of substrate i.e., acetylthiocholine iodide was added and change in absorbance was recorded for a period of 3 min. Change in the absorbance per minute was determined.

The enzyme activity was calculated using the following formula,

\[ R = 5.74 \times 10^{-4} \times \frac{A}{C} \]

Where, \( R \) = Rate in moles of substrate hydrolyzed/minute/mg protein  
\( A \) = Change in absorbance / min  
\( C \) = Original concentration of the tissue protein (mg/ml)

**7.2.12. TNF-alpha levels**

Using the rat ELISA kit (Invitrogen, Novex, Catalogue -KRC3011), the levels of TNF-alpha were assessed in homogenates of hippocampal and frontal cortex regions and expressed as pg per mg of protein.

**Methodology**

- 100 µl of the standard diluent buffer was added to the zero standard wells
- 100 µl of standards were added to the to the appropriate wells
- 100 µl of prepared samples and/or controls was added to the appropriate wells
- Covered plate with plate cover and incubated for 2 h at RT
- Aspirated the contents and washed the wells 4 times
- 100 µl of biotinylated rat TNF-alpha biotin conjugate solution was then added into each well except the chromogen blank and incubated for 1 h at RT
- Washed the wells for 4 times and 100 µl streptavidin-HRP working solution was added to each well except chromogen blank and incubated for 30 minutes at RT
- Washed for 4 times and added 100 µl of stabilized chromogen to each well. The liquid in the wells began to turn blue, incubate for 30 minutes at RT in the dark
- 100 µl stop solution was added and color was then changed from blue to yellow
- Read the absorbance of each well at 450 nm against a chromogen blank composed of 100 µl each of stabilized chromogen and stop solution
- Read the concentrations for unknown samples and controls from the standard curve
Chapter 7  Evaluation of NAR and RUT to Alleviate Chemobrain in Healthy Rats

7.2.13. Oxidative stress markers
The antioxidant defense systems like SOD, catalase, GSH, total thiols were estimated in hippocampus, frontal cortex according to the procedures described elsewhere (Aebi, 1974; Buege and Aust, 1978; Misra and Fridovich, 1972; Moron et al., 1979; Sedlak and Lindsay, 1968).

Methodology
Preparation of Tissue Homogenate
Animals were deeply anesthetized by excess ketamine and were perfused transcardially with an ice-cold saline. The whole body was perfused with ice-cold saline, brain was dissected out for isolation of hippocampus and frontal cortex. A 10% w/v tissue homogenate was prepared with ice-cold KCl (150 mM) using Teflon – glass homogenizer (Yamato LSG LH-21, Japan). The homogenate was centrifuged at 10,000 rpm for 10 min and the pellet discarded. The supernatant obtained was used for further estimations.

7.2.13.1. Glutathione (GSH)
5, 5’-Di thiobis (2-Nitrobenzoic acid) (DTNB) is reduced by sulphhydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration.

Reagents
10% w/v trichloroacetic acid (TCA), Phosphate buffer (0.2 M, pH-8.0) 0.218 g sodium dihydrogen phosphate and 2.641 g disodium hydrogen phosphate dissolved in 100 ml distilled water. DTNB (0.6 mM) – 11.9 mg in 50 ml Phosphate buffer (pH 8)

Procedure
From the tissue homogenate, proteins were precipitated by 10% TCA, centrifuged and supernatant was collected. 1 ml supernatant was mixed with 6 ml 0.2 M phosphate buffer and 1 ml 0.6 mM DTNB and incubated for 10 min at RT. The absorbance of the samples was recorded against the blank at 412 nm and the GSH concentration was calculated from the standard curve.

7.2.13.2. Catalase
In the ultraviolet range, \( \text{H}_2\text{O}_2 \) shows a continual increase in absorption with decreasing wavelength. The decomposition of \( \text{H}_2\text{O}_2 \) can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of the catalase activity.

Methodology
Reagents
Chapter 7: Evaluation of NAR and RUT to Alleviate Chemobrain in Healthy Rats

Phosphate buffer (PB): KH₂PO₄ - 1.703 g dissolved in 250 ml distilled water, Na₂HPO₄ - 1.773 g dissolved in 250 ml. 100 ml of KH₂PO₄ solution and 150 ml of Na₂HPO₄ were mixed and pH was adjusted to 7.0; PBS-H₂O₂ solution: 50 ml of PB and 75 µl of H₂O₂ were mixed. The absorbance of solution was adjusted to 0.3-0.5.

**Procedure**

3 ml of H₂O₂-PBS solution was added to 50 µl of tissue homogenate. Placed in cuvette and took absorbance at 240 nm at zero time. The difference in absorbance per unit time is a measure of the catalytic activity.

7.2.13.3. Superoxide dismutase (SOD)

Superoxide dismutase plays major role in protection of cells against oxidative damage. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide.

**Reagents**

Sodium carbonate buffer (0.1 M, pH 10.2): 1.05 g of Na₂CO₃ in 100 ml of distilled water; Adrenaline bitartarate (250 µM).

**Procedure**

The activity of superoxide dismutase was determined based upon activity that SOD inhibits the autoxidation of epinephrine to adrenochrome at alkaline pH. 50 µl supernatant was taken in 900 µl carbonate buffer (0.1 M, pH-11). After adjusting the epinephrine concentration to 250 µM, followed by addition, the increase in absorbance was measured at 480 nm using kinetic method (from 0-60 s).

7.2.13.4. Total thiols

**Reagents**

Tris buffer (0.25 M): Dissolve 1.5514 g of tris buffer in 50 ml distilled water (pH-8.2); EDTA solution (20 mM): 404 mg of EDTA dissolved in 50 ml of Tris buffer; DTNB solution: 20 mg dissolved in 5 ml of methanol

**Procedure**

100 µl tissue homogenate was mixed with 100 µl tris EDTA and 40 µl DTNB. Then 3.16 ml of methanol was added and centrifuged at 2000 rpm. Absorbance of the supernatant was taken at 412 nm and subtracted from DTNB blank and a blank containing homogenate without DTNB. The -SH groups was calculated using molar extinction coefficient 13,600 cm⁻¹ M⁻¹.

7.2.14. Histopathological analysis

Histopathological analysis was carried out for major organs like liver, heart, kidney and brain. Gross structural changes as a result of toxicant, i.e. DOX and protective ability of test
flavonoids to reverse these changes present were reported. Conventional paraffin tissue embedding and the slides of tissue sections were made by using microtome.

**Methodology**

- Deparaffinizing and rehydrating sections: Carried out by using serial rinsing and washing with xylene, 100% ethanol, 95% ethanol, 80% ethanol and finally with deionized H2O
- Hematoxylin staining: Hematoxylin followed by rinsing with deionized water and then by tap water, followed by dipping 8-12 times in acid ethanol. Then rinsed with tap water and later by deionized water
- Eosin staining and dehydration: Placed the slides for 30 sec in Eosin followed by rinsing in 95% ethanol and 100% ethanol, then finally with xylene
- Slides were air dried and DPX mount media was applied along with cover slip and left over night in hood

**7.2.15. Statistical analysis**

Statistical analysis was carried out using GraphPad prism software as mentioned earlier. Locomotor activity, acetyl cholinesterase, biochemical and haematological data are analyzed by one-way ANOVA followed by Tukey’s posthoc test.

**7.3. Results**

**7.3.1. In-vivo chemobrain studies**

**7.3.1.1. Object recognition task (ORT)**

Rats treated with vehicle and normal saline have spent significantly more time exploring the novel object compared to familiar one which indicates that, the healthy control group remembered the familiar object and discriminated the novel object. Group treated with sodium CMC along with DOX has spent almost equal time in exploring both novel and familiar objects which indicates that, these animals could not remember the familiar object, therefore did not show any discrimination between novel and familiar objects.

This shows that long-term treatment with DOX resulted in episodic memory deficits to recognize the objects which may be correlated or taken as a neurocognitive complication accompanying chemobrain or chemofog. However, rats treated with NAR and RUT at 50 mg/kg, p.o. along with DOX have discriminated the novel object from the familiar one and they spent significantly more time exploring the novel object compared to the familiar object, the most effective being the RUT treatment. This infers that, flavonoids treatment protected against DOX-induced cognitive deterioration (Fig.7.2 & Fig. 7.3).
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Fig. 7.2. Protective effect of NAR and RUT against DOX-induced cognitive deficits in ORT. Data represents mean ± SEM of exploration time during choice trial, *p<0.05, **p<0.01 & ***p<0.001 vs. familiar object (n=7-9).

Fig. 7.3. Protective effect of NAR and RUT against DOX-induced cognitive deficits in ORT. Data represents mean ± SEM of (a) Recognition, and (b) Discriminative indices during choice trial, *p<0.05, **p<0.01 & ***p<0.001 vs. DOX control (n=7-9).

7.3.1.2. Morris Water Maze (MWM)
In case of MWM task for assessing spatial memory, long-term DOX treatment did not result in significant deficits as there was no difference noted in escape latency, path length and swim speed among the treatment groups during the acquisition trials (Fig.7.4). Further, we did not observe any changes in the Q4 latency and Q4 time during the retention trial among the treatment groups (Fig.7.5). This indicates that, long-term treatment with DOX did not affect the spatial memory though it negatively affected the episodic memory. This could be attributed to the stress involved in swimming and struggle for survival. Furthermore, complete hippocampus deterioration is essential for loss of spatial memories.
This might be the reason why the spatial memory is not impacted even after chronic DOX administration. One more possible reason could be that, the animals were healthy and chemotherapy may have more pronounced deleterious effects in cancer bearing animals, i.e. mammary carcinoma bearing female rats.

Fig. 7.4. Effect of DOX and test flavonoids on spatial acquisition memory in MWM. Data represents mean ± SEM of (a) Escape latency or latency to reach target, (b) Path length, (c) Swim speed from day 1 to day 4, *p<0.05, **p<0.01 compared to DOX (n=9).

Fig. 7.5. Effect of DOX and test flavonoids on spatial retention memory in MWM. Data represents mean ± SEM of (a) Q4 Time and (b) Q4 Latency during retention or probe trial on day 5 (n=9).
7.3.2. Open field test for locomotor activity

It was found that neither DOX nor the flavonoids (NAR & RUT) have influence on locomotor activity (Fig. 7.6). This confirms that cognitive impairment induced by DOX had no confounding influence by locomotor activity of rats.

![Chart showing distance travelled (m) by different treatments](image)

**Fig. 7.6. Effect of DOX and test flavonoids on locomotor activity. Data represents mean ± SEM of distance travelled, n=9.**

7.3.3. Change in body weight

We observed gradual increase in body weight throughout groups. It was noted that, increase in body weight was significantly less in DOX control group as compared to vehicle from day 42 to day 63, however no significant differences were noted among treatment groups at the end of the study period (Fig. 7.7). Highest mean body weight was observed in groups treated with only vehicle or flavonoids, NAR and RUT. Treatment with test flavonoids at a tested dose of 50 mg/kg, p.o. for 60 days has protected from loss of body weight observed with DOX control.

![Chart showing % increase in body weight over days](image)

**Fig. 7.7. Effect of DOX and test flavonoids on % increase in body weight. Data represents mean ± SEM of %IBW, p*<0.05, p**<0.01, p***<0.001 vs. DOX control, n=9.**
7.3.4. Haematological profiling

Treatment with DOX for 50 days significantly \((p<0.001)\) reduced RBC, hemoglobin compared to vehicle control which was completely reversed by NAR and RUT co-administration at a dose of 50 mg/kg, \(p.o.\) WBC were reduced in DOX group compared to vehicle, however the differences were not statistically significant. This supports NAR and RUT improved blood forming potential. Platelet count was unaffected by DOX treatment (Table 7.1).

**Table 7.1. Effect of DOX and test flavonoids on haematological profile.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RBC ((\times10^6) cells/µl)</th>
<th>Hb % (g/dl)</th>
<th>WBC ((\times10^3) cells/µl)</th>
<th>Granulocytes ((\times10^3) cells/µl)</th>
<th>Lymphocytes ((\times10^3) cells/µl)</th>
<th>Monocytes ((\times10^3) cells/µl)</th>
<th>Platelets ((\times10^3) cells/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>7.94 ± 0.15</td>
<td>12.86 ± 0.17</td>
<td>8.68 ± 0.68</td>
<td>1.18 ± 0.10</td>
<td>8.04 ± 0.55</td>
<td>1.11 ± 0.10</td>
<td>538.11 ± 23.5</td>
</tr>
<tr>
<td>DOX control</td>
<td>5.93 ± 0.22*</td>
<td>10.81 ± 0.16</td>
<td>7.01 ± 0.48</td>
<td>0.94 ± 0.07</td>
<td>5.62 ± 0.46</td>
<td>1.05 ± 0.05</td>
<td>521.11 ± 22.5</td>
</tr>
<tr>
<td>NAR + DOX</td>
<td>8.75 ± 0.51#</td>
<td>11.82 ± 0.20</td>
<td>6.89 ± 0.71</td>
<td>1.02 ± 0.14</td>
<td>7.55 ± 0.81</td>
<td>1.11 ± 0.09</td>
<td>533.88 ± 41.8</td>
</tr>
<tr>
<td>Rutin + DOX</td>
<td>9.07 ± 0.42#</td>
<td>13.82 ± 0.39</td>
<td>7.32 ± 0.68</td>
<td>1.22 ± 0.15</td>
<td>7.33 ± 0.27</td>
<td>1.11 ± 0.12</td>
<td>523.88 ± 31.0</td>
</tr>
<tr>
<td>NAR</td>
<td>8.28 ± 0.76#</td>
<td>13.14 ± 0.48</td>
<td>7.77 ± 0.71</td>
<td>1.11 ± 0.27</td>
<td>6.91 ± 0.66</td>
<td>0.99 ± 0.06</td>
<td>566.12 ± 25.3</td>
</tr>
<tr>
<td>RUT</td>
<td>9.28 ± 0.12#</td>
<td>14.14 ± 0.52</td>
<td>8.17 ± 1.3 ± 0.28</td>
<td>6.85 ± 0.29</td>
<td>0.88 ± 0.07</td>
<td>581.55 ± 17.8</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:** Data represents mean ± SEM of all the major haematological parameters, *\(p<0.01\) compared to vehicle control, #\(p<0.01\) compared to DOX control (n=6).

7.3.5. Organ index

No significant difference was observed among the treatment groups for the organ index of heart, liver, brain, kidney etc. (data not shown).

7.3.6. Biochemical analysis

It was found that the creatine kinase (CKL), urea, triglyceride and total cholesterols were significantly elevated in DOX control group compared to control. However NAR and RUT co-administration has prevented the above changes observed with DOX treatment. Other parameters, viz., glucose, AST, ALT, ALP, total bilirubin, total protein, albumin, creatinine levels were found to be unchanged among different groups (Table 7.2).
Table 7.2. Effect of DOX and test flavonoids on clinical biochemistry parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle control</th>
<th>DOX control</th>
<th>NAR + DOX</th>
<th>RUT + DOX</th>
<th>NAR</th>
<th>RUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>128.72 ± 8.44</td>
<td>122.53 ± 4.85</td>
<td>116.50 ± 8.26</td>
<td>127.06 ± 11.05</td>
<td>126.40 ± 7.55</td>
<td>117.68 ± 8.15</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>43.60 ± 3.03</td>
<td>45.47 ± 3.19</td>
<td>44.75 ± 3.53</td>
<td>40.71 ± 3.61</td>
<td>42.87 ± 4.06</td>
<td>43.98 ± 2.38</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>103.53 ± 5.80</td>
<td>107.98 ± 7.55</td>
<td>95.44 ± 6.16</td>
<td>105.44 ± 7.17</td>
<td>87.94 ± 7.86</td>
<td>98.13 ± 8.29</td>
</tr>
<tr>
<td>CKL (U/L)</td>
<td>376.05 ± 38.37</td>
<td>801.83 ± 167.91*</td>
<td>398.73 ± 52.92#</td>
<td>391.66 ± 33.91#</td>
<td>410.98 ± 48.76#</td>
<td>401.18 ± 37.80#</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>73.46 ± 6.92</td>
<td>81.65 ± 2.92</td>
<td>73.67 ± 5.22</td>
<td>76.54 ± 5.64</td>
<td>73.67 ± 5.22</td>
<td>80.28 ± 4.20</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>36.66 ± 5.06</td>
<td>96.96 ± 8.87***</td>
<td>41.21 ± 7.82###</td>
<td>36.25 ± 6.19###</td>
<td>39.15 ± 5.66###</td>
<td>33.10 ± 4.32###</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>55.12 ± 3.18</td>
<td>86.40 ± 3.77***</td>
<td>61.54 ± 2.17###</td>
<td>49.52 ± 3.56###</td>
<td>58.23 ± 4.59###</td>
<td>58.44 ± 3.29###</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>61.98 ± 4.67</td>
<td>147.58 ± 7.53***</td>
<td>79.13 ± 5.46##</td>
<td>51.36 ± 4.61##</td>
<td>56.81 ± 4.32##</td>
<td>49.733 ± 4.41##</td>
</tr>
<tr>
<td>Total Bilirubin (mg/dL)</td>
<td>0.10 ± 0.00</td>
<td>0.10 ± 0.000</td>
<td>0.08 ± 0.02</td>
<td>0.1 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>31.39 ± 2.17</td>
<td>31.22 ± 1.88</td>
<td>30.06 ± 0.55</td>
<td>29.87 ± 3.09</td>
<td>27.18 ± 0.27</td>
<td>30.53 ± 2.01</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>7.02 ± 0.41</td>
<td>7.87 ± 0.17</td>
<td>6.55 ± 0.26</td>
<td>6.89 ± 0.54</td>
<td>7.18 ± 0.55</td>
<td>6.43 ± 0.24</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>4.33 ± 0.30</td>
<td>4.52 ± 0.23</td>
<td>3.98 ± 0.37</td>
<td>3.97 ± 0.21</td>
<td>4.21 ± 0.31</td>
<td>4.01 ± 0.15</td>
</tr>
</tbody>
</table>

**Notes:** Data represents mean ± SEM of various biochemical parameters (n=6). *p<0.05, ***p<0.001 compared to vehicle control and #p<0.05, ###p<0.01, ####p<0.001 compared to DOX control.

### 7.3.7. Acetylcholinesterase activity

Acetylcholinesterase activity was not differing significantly among various treatment groups in either hippocampus or frontal cortex regions (Fig. 7.8). This indicates that, the pathology associated with chemobrain complications are different from Alzheimer’s disease (AD) in which acetylcholinesterase inhibitors are proved to be efficacious although they are not disease modifying.
7.3.8 TNF-alpha levels
A significant increase in TNF-alpha levels was noticed in hippocampus and frontal cortex of DOX control group as compared to healthy controls. However treatment with NAR and RUT resulted in significant reduction of DOX-induced increase in TNF-alpha cytokine levels (Fig.7.9).
Fig. 7.9. Effect of DOX and test flavonoids on neuro-inflammatory marker, TNF-α levels in Hippocampus and Frontal cortex. Data represents mean ± SEM of inflammatory marker, TNF-α per mg of protein in (a) Hippocampus and (b) Frontal cortex, ***p<0.001 compared to vehicle control and #p<0.05, ##p<0.01 compared to DOX control, n=6.

7.3.9. Oxidative stress markers
The antioxidant defense systems like, GSH, catalase. SOD etc. were significantly reduced in hippocampus and frontal cortex regions in DOX control group as compared to healthy rats. NAR and RUT treatment has significantly protected the depletion of these antioxidant defense systems which may possibly underlie their potential to improve the chemobrain complications observed with DOX (Table 7.3).
### Table 7.3. Effect of flavonoids and DOX on oxidative stress markers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Catalase (Units/mg of protein)</th>
<th>SOD (Units/mg of protein)</th>
<th>GSH (µg/mg of protein)</th>
<th>Total thiols (µg/mg of protein)</th>
<th>Catalase (Units/mg of protein)</th>
<th>SOD (Units/mg of protein)</th>
<th>GSH (µg/mg of protein)</th>
<th>Total thiols (µg/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>1.90 ± 0.11</td>
<td>12.45 ± 1.04</td>
<td>4.77 ± 0.71</td>
<td>11.01 ± 0.87</td>
<td>2.04 ± 0.11</td>
<td>12.94 ± 1.44</td>
<td>6.68 ± 0.66</td>
<td>12.98 ± 1.01</td>
</tr>
<tr>
<td>DOX control</td>
<td>0.94 ± 0.13*</td>
<td>7.35 ± 0.54*</td>
<td>2.11 ± 0.31*</td>
<td>5.60 ± 0.71</td>
<td>1.21 ± 0.12*</td>
<td>5.44 ± 0.83*</td>
<td>3.40 ± 0.33*</td>
<td>6.45 ± 0.89*</td>
</tr>
<tr>
<td>NAR + DOX</td>
<td>1.85 ± 0.42*</td>
<td>11.08 ± 1.23*</td>
<td>4.12 ± 0.62*</td>
<td>10.82 ± 1.15*</td>
<td>1.95 ± 0.22*</td>
<td>13.08 ± 1.23*</td>
<td>5.92 ± 0.96*</td>
<td>12.95 ± 2.65*</td>
</tr>
<tr>
<td>RUT + DOX</td>
<td>1.89 ± 0.32*</td>
<td>12.18 ± 1.04*</td>
<td>4.52 ± 0.52*</td>
<td>11.02 ± 1.22*</td>
<td>1.92 ± 0.12*</td>
<td>12.08 ± 1.13*</td>
<td>5.95 ± 0.69*</td>
<td>12.05 ± 1.46*</td>
</tr>
<tr>
<td>NAR</td>
<td>1.91 ± 0.12*</td>
<td>13.58 ± 1.18*</td>
<td>5.07 ± 0.52*</td>
<td>12.31 ± 2.28*</td>
<td>1.99 ± 0.32</td>
<td>13.58 ± 1.18*</td>
<td>6.05 ± 0.51*</td>
<td>12.31 ± 1.22*</td>
</tr>
<tr>
<td>RUT</td>
<td>2.01 ± 0.11*</td>
<td>13.08 ± 1.12*</td>
<td>4.90 ± 0.42*</td>
<td>11.31 ± 2.08*</td>
<td>2.19 ± 0.13</td>
<td>13.56 ± 1.10*</td>
<td>6.55 ± 0.42*</td>
<td>12.51 ± 1.31*</td>
</tr>
</tbody>
</table>

**Notes:** Data represents mean ± SEM of oxidative stress markers in hippocampal and frontal cortex regions in brain, *p<0.01 compared to vehicle control, #p<0.01 compared to DOX control (n=6).

### 7.3.10. Histopathological analysis

DOX chronic treatment produced pathological changes in major organ systems, i.e. heart, liver, kidney and brain. These changes observed in histopathological analysis were not observed when we co-administer the flavonoids, especially rutin along with DOX which indicates that, NAR and RUT were able to protect the major organ systems by preventing the organ level toxicity as a result of chemotherapy (Fig. 7.10.). The histology of various organs are described below.

**Brain:** Healthy vehicle showing normal architecture whereas DOX control showing irregular and degenerated morphological features along with marked gliosis. Rats treated with DOX and naringin (NAR; 50 mg/kg, *p.o.*) reflecting the minor morphological changes with reduced gliosis as compared to DOX control while treatment with DOX and rutin (RUT; 50 mg/kg, *p.o.*) showing the histological features comparable to that of healthy control (Fig. 7.10.1).

**Heart:** Normal rats displayed normal histological architecture of healthy myocardium, while DOX showed numerous vacuoles and degeneration of myocardial structures. Rats treated with DOX and naringin (NAR; 50 mg/kg, *p.o.*) reflecting slight histological changes as
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compared to normal control. Rats treated with DOX and RUT (50 mg/kg, *p.o.*) displayed healthy myocardial architecture as that of normal healthy control (Fig. 7.10.2).

**Liver:** Normal rats exhibited histological architecture of healthy hepatocyte parenchyma and central vein with no abnormalities while DOX was showing disruption of hepatocyte parenchyma with central vein dilation and necrosis. Rats treated with DOX and NAR (50 mg/kg, *p.o.*) displayed minor changes of hepatocyte parenchymal and central vein histological architecture whereas treatment with DOX and RUT (50 mg/kg, *p.o.*) exhibited liver histology as that of normal healthy control (Fig. 7.10.3).

**Kidney:** Healthy control rats showing normal histological morphology of kidney with complete renal corpuscle and proximal tubules and DOX control rats showed abnormal architecture with necrosis of renal corpuscles, and proximal tubules with medullary congestion. Rats treated with DOX and NAR (50 mg/kg, *p.o.*) displayed healthy histological features as that of normal control and the rats treated with DOX and RUT (50 mg/kg, *p.o.*) exhibited kidney morphology comparable to healthy control (Fig. 7.10.4).

Fig. 7.10. Histopathological analysis of major organ systems by H & E staining method. 7.10.1. Cerebral cortex, 7.10.2. Heart, 7.10.3. Liver and 7.10.4. Kidney. A-Healthy vehicle control, B- DOX control, C- DOX + NAR (50 mg/kg, *p.o.*), D- DOX + RUT (50 mg/kg, *p.o.*) (All the images were captured under 40X magnification lens).
7.4. Discussion

Long-term treatment with DOX resulted in episodic memory deficits as we noticed in object recognition task, however these deficits were significantly prevented by chronic co-administration with NAR and RUT daily at a dose of 50 mg/kg, *p.o.* The most effective treatment in alleviating DOX-induced episodic memory deficits was RUT at a dose of 50 mg/kg, *p.o.* as compared to NAR at 50 mg/kg, *p.o.* However, we did not notice any spatial acquisition or retention memory deficits with 10 cycles of DOX as we observed in MWM task. This confirms that, DOX long-term treatment did not produce any impairment of spatial memory. Also one of the earlier studies reported that, certain chemotherapeutic agents in fact, improved the spatial memory instead of deterioration of cognitive function (Lee et al., 2006). Further, chronic DOX treatment did not affect locomotor activity which would otherwise become a confounding influence in assessing the cognitive function.

AChE found to play key role in determining the levels of acetylcholine that ultimately affects the cholinergic neurotransmission which underlies cognitive processing. In our present study, we did not find any significant changes among the various groups which indicate that the pathology of chemobrain is entirely different from normal AD phenomenon where inhibition of AChE is found to be an effective therapy.

Myelosuppression was noticed especially for RBC count with DOX treatment, however this was significantly reversed by co-treatment with NAR and RUT at tested doses, i.e. 50 mg/kg, *p.o.* respectively. Earlier reports proved that, myelosuppressive effect along with reduced blood flow and oxygen supply as a result of cytotoxic drugs was one of the underlying cause for memory complications observed in cancer survivors (Mizusawa et al., 1988; Silverman et al., 2007). Improved blood profile may be one of the mechanism underlying memory enhancing effect of flavonoids against DOX chemotherapy.

Most of the biochemical parameters were found to be unchanged except few. DOX was found to induce cardiac toxicity as we noticed significantly elevated levels of creatinine kinase (CKL). However treatment with either NAR or RUT protected from this cardiac toxicity as a result of DOX. Also cholesterol, triglyceride and urea levels were significantly increased in DOX control group as compared to vehicle. However these changes were significantly reversed by co-administration with NAR or RUT at 50 mg/kg, *p.o.*

Cytokines plays an important role in regulation of immune response and also are important in the inflammatory process. Cytokine dysregulation known to affect the cognitive processing (Banks et al., 2002). In our present study, TNF-alpha levels were found to be elevated.
significantly in DOX control group as compared to saline control which indicates that, DOX through an indirect peripheral mechanism has produced enhanced cytokine levels which can easily cross BBB and result in glial activation and neuro-inflammation. Flavonoids, NAR and RUT were able to prevent the production of TNF-alpha and thereby neuro-inflammation which could be the possible mechanism underlying their efficacy to protect from DOX-induced cognitive deterioration.

Oxidative stress is found to be elevated following the treatment with most chemotherapeutic drugs. DOX is also known for its oxidative free radical generation and is one of the major mechanisms of anticancer effects. Earlier report proved that DOX can produce chemobrain through generation of free radicals (Joshi et al., 2005). In this present study also, we have noted elevated oxidative stress as a result of exhausted antioxidant defense systems. However treatment with test flavonoids protected from this elevated oxidative stress by preserving the catalase, glutathione, total thiols and SOD antioxidant defense systems.

Ten cycles of DOX treatment has resulted in organ level toxicity as noticed with the structural abnormalities to the major organ systems, i.e. brain, heart, liver and kidney. However these histological abnormalities were found to be prevented by the protective effect of flavonoids, NAR and RUT, most effective being RUT treatment.

7.5. Conclusion

The present findings supported our hypothesis that, NAR and RUT protects from DOX-induced cognitive dysfunction. Further, RUT was found to be more effective than NAR.
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Bibliography


