CHAPTER II

In vitro and in vivo evaluation of the safety profile of
Grewia tiliaefolia and vitexin
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4.5. Summary
4.1. INTRODUCTION

According to the World Health Organization, about 80% of the human populations depend on plant derived herbal formulation and natural remedies for their health benefits (Campbell-Tofte et al., 2012). Traditional medicines and medicinal plants are being commonly used as a daily health care by urban population for various ailments ranging from minor to chronic due to increase in the cost of synthetic medicines and the side effects caused by them due to their prolonged usage (Leonti and Casu, 2013). Although the use of herbas and their phytochemicals are extensively practiced with the view that they are non-toxic and safe (Asiimve et al., 2014), there are reports on them to develop toxicity and cause adverse effects (Park et al., 2010; George, 2011; Haq, 2004). Of all the traditional herbal medicines practiced, only a few have been tested in clinical trials for their efficacy and safety (Cheng et al., 2009). Considering this fact, research is being carried out to provide vital information about the toxicological and pharmacological profile of herbal plants, their extracts, bioactive components and formulations over the past decades in order to identify their safety and efficacy so that they can used in indigenous system of medicine.

Generally, the toxicological profile of any compound or formulations can be tested by evaluating the cytotoxic, genotoxic and mutagenic potentials (Verschaeve and Staden, 2008; Varalakshmi et al., 2011). Mutagenicity refers to the ability of a chemical or physical agent to induce permanent transmissible changes in the genetic material of the cells, whereas genotoxicity is the ability of a compound to interact with the genetic material and also with other cellular components that maintains the fidelity of the genome (Maurici et al., 2003). According to ICH and WHO guidelines (WHO Drug Information, 2000) non-clinical safety study of herbal drugs has to be carried out before carrying out human clinical trials. Since the results of preliminary work showed methanol extract of G. tiliaefolia (GT) and its bioactive compound vitexin to be promising, safety evaluation has to be carried out before commencing intensive research on their neuroprotective effect. Therefore the present study focuses on toxicological evaluation of GT and vitexin under in vitro and in vivo conditions.
4.2. MATERIALS AND METHODS

4.2.1. Extract preparation

Methanol leaf extract of *G. tiliaefolia* (GT) was prepared as mentioned in Section 3.2.1.

4.2.2. In vitro cytotoxicity assay using PBMC

4.2.2.1. Isolation of PBMC

Fresh blood samples (5 ml) were collected from healthy volunteers (age group of 22-30; both male and female) by venipuncture in tubes containing EDTA (Institutional Ethical Committee of Alagappa University, No.IEC/AU/2014/7). The blood sample was layered onto Lymphocyte separation medium and PBMC cells were separated by density gradient centrifugation at 400 g. The cells were taken and washed twice with RPMI 1640 medium. The cells obtained were finally suspended in complete medium containing RPMI 1640, 10% FBS and 2 mM Penicillin-Streptomycin antibiotic solution.

4.2.2.2. Trypan blue exclusion assay

Cells were divided into 10 groups each containing 1x10⁶ cells and were incubated with vehicle control (RPMI 1640 alone), positive control (250 µM H₂O₂) and different concentrations (250, 500, 1000 and 2000 µg/ml) of GT and vitexin for 24 h. After the incubation period, 10 µl of trypan blue was added with the same volume of cells, kept for 5 min and the number of dead (blue) and live cells (transparent) were counted in a haemocytometer and observed under light microscope. Percentage of cell viability was calculated by using the following formula.

\[
\text{% of viability} = \frac{\text{Total number of cells} - \text{Number of dead cells}}{\text{Total number of cells}} \times 100
\]

4.2.3. Evaluation of genotoxicity by comet assay

Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>1% Normal Melting Agarose (NMA)</td>
<td>Melt 0.5 g of NMA in 50 ml of PBS</td>
</tr>
<tr>
<td>0.7% Low Melting Agarose (LMA)</td>
<td>Melt 0.21 g of LMA in 30 ml of PBS in thermostatic bath (60-70°C). Store at 4°C.</td>
</tr>
<tr>
<td>Lysis solution (50 ml)</td>
<td></td>
</tr>
<tr>
<td>Sodium lauryl sarcosinate</td>
<td>1% (0.5 g)</td>
</tr>
</tbody>
</table>
NaCl - 2.5 M (7.3 g)
Na₂EDTA - 100 mM (1.86 g)
Tris-HCl (pH 10) - (10 mM (60.57 m))
Triton X-100 - 1% (0.5 ml)
DMSO - 10% (5 ml)

Electrophoresis Buffer
SolA-Na₂EDTA - 100 mM (0.744 g/20 ml)
Sol B -NaOH - 1 M (8 g/200 ml)
Add 6 ml of A and 180 ml of B and make up to 600ml with water

Neutralization buffer (pH7.5)

Stock Tris HCl - 1 M (12.114 g/ml)
Working Conc. - 0.4 M

The alkaline comet assay was performed to assess the extent of DNA damage caused by GT and vitexin in PBMC. 1x10⁶ cells/ml were incubated with various concentrations (1000 and 2000 μg/ml) of GT and vitexin for 24 h. H₂O₂ (250 μM) served as the positive control. After exposure, cells were mixed with 0.75% low-melting point agarose in PBS and added to slides pre-coated with a layer of 1% normal melting point agarose in PBS. Agarose were allowed to set at 4°C for 5–10 min and then the slides were immersed in ice-cold freshly prepared lysis solution overnight at 4°C. Electrophoresis was carried out in freshly prepared pre-chilled electrophoresis buffer for 20 min at 300 mA. The slides were neutralized with Tris–HCl buffer (pH 7.5) and stained with ethidium-bromide (10 μg/ml). Comets were visualized and imaged using Confocal laser scanning microscope (Model: LSM 710, Carl Zeiss, and Germany) with an excitation filter of 515-560 nm under 20X magnification. The comet parameters were analysed using Autocomet™ scoring software to determine the percentage of DNA in tail, tail moment and olive tail moment (Singh et al., 1988).

4.2.4. Evaluation of mutagenicity by Ames assay

Reagents

Glucose minimal agar
1.5% agar - 1.5 g in 100 ml
40% glucose - 40 g/100 ml sterile water
50X VB salt solution (100 ml)
MgSO₄ - 1 g
Citric acid monohydrate - 10 g
K₂HPO₄ - 50 g
Sodium ammonium phosphate - 17.5 g
Molten agar
0.6% agar - 0.6 g/100 ml
0.6% NaCl - 0.6 g/100 ml

The salmonella mutagenicity assay was performed using the microsuspension protocol developed by Kado and Eisenstadt, (1983) which is a modified method of Ames test using the Salmonella typhimurium strains TA98 and TA100. An overnight culture of Salmonella typhimurium (10⁹ cells/ml) were concentrated by centrifugation at 4000 g at 4°C for 15 min and resuspended in 0.015 M sodium phosphate buffer (pH 7.4). Then 50 µl of 0.015 M sodium phosphate buffer, 1000 – 2000 µg/ml GT/vitexin and 50 µl of bacterial suspension were added to the assay tube. The mixtures were incubated at 37°C for 90 min without shaking in the presence or absence of S9 fraction. After incubation, 2.5 ml of molten surface (0.6% NaCl, 0.6% agar, 0.05 mM L-Histidine, 0.05 mM Biotin) was added to the tubes, and the mixture was poured into a petri dish containing glucose minimal agar. The plates were incubated at 37°C for 48-66 h, and the His⁺ revertant colonies were counted.

The standard mutagens such as sodium azide (1µg/plate) for TA100, 4-nitriquinoline N-oxide (0.1µg/plate) for TA98 were used as positive controls. The ratio of mutagenicity (RM) was calculated by using the following formula

\[ RM = \frac{\text{Number of revertants in the treated sample (induced spontaneous)}}{\text{Number of spontaneous revertants (Negative control)}} \]

The compound is considered as mutagenic if the ratio of mutagenicity (RM) is higher than 2.0.

4.2.5. In vivo toxicity evaluation

4.2.5.1. Animal procurement and maintenance

Adult male Wistar rats (150-180 g) and Swiss albino mice (25-30 g) were obtained from Sri Venkateshwara Enterprises, Bangalore, India. The animals were acclimatized to laboratory condition for one week and maintained with standard animal diet and water in excess. All the animal experiments were performed in accordance with the Internationally acclaimed Ethical standards and approved by the Institutional Animal Ethics Committee.
4.2.5.2. Acute toxicity studies

Acute oral toxicity was performed in accordance with the Organisation for Economical Cooperation and Development (OECD) guidelines 423. For acute toxicity studies of *G. tiliaefolia* extract, *Wistar* rats were randomly divided into 2 groups (n=5/group). Group-I (control) was treated with water and Group-II were orally administered with a single dose of GT (2000 mg/kg b.w). For vitexin, Swiss albino mice were divided into two groups of 5 animals each (Group I- Control; Group II – Vitexin 2000 mg/kg b.w.). Animals were monitored continuously for 14 days for mortality and any possible behavioral changes like tremors, altered feeding, salivation and diarrhoea post treatment. On day 14, animals were sacrificed and subjected to various haematological and biochemical analyses.

4.2.5.3. Haematological and biochemical analysis

Blood samples were collected in two different tubes, one with the anticoagulant Ethylenediaminetetraacetic acid (EDTA) and other without anticoagulant. Both samples were subjected to haematological and biochemical analyses, which were analyzed using autoanalyzer. The haematological parameters include hemoglobin, total red blood cell count (RBC), total white blood cell count (WBC), differential count, platelet count and biochemical parameters include glucose, blood urea nitrogen (BUN), creatinine, Alanine transaminase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP).

4.2.5.4. Histopathological observation

At the end of the drug treatment, the animals were quickly dissected and the organs like liver, spleen, lungs, kidney, brain and heart were excised and weighed. For histopathological observation, liver, kidney and brain specimens were fixed in 10% formalin, stained with hematoxylin and eosin and examined under microscope for any morphological changes.
4.2.6. Statistical analysis

For comparison of results among the experimental groups, statistical analysis was performed using one way ANOVA (SPSS version 17.0) by using the Duncan’s test \((p<0.05)\) comparing between the groups control vs treated.

4.3. RESULTS AND DISCUSSION

Herbal medicine, which provides natural remedy for various ailments including diabetes and cancer, has been presumed to be safe and widely used as self medication. However, certain bioactive principles present in them may have the ability to cause adverse deleterious effects (Bent and Ko, 2004). Only less attention is being paid to the unseemly effects of these herbal medical practices. It is therefore important to assess the safety profile and toxicity because of the reports about illness and mortality in the use of these plants in the recent past.

4.3.1. Assessment of cytotoxicity of GT/vitexin by Trypan Blue exclusion assay

*In vitro* cytotoxicity studies have been performed since decades for assessing the toxicity of any compound and have found profound interest by the scientific community (Bernauer et al., 2005). One of the commonly used target cells for such kind of study is human PBMC. The cytotoxic effect of GT was assessed by trypan blue exclusion assay. The viable cells having intact membrane exclude the uptake of trypan blue whereas, the dead cells stains blue. The viability of the cells by the treatment of various concentrations (250-2000 \(\mu\)g/ml) of GT/vitexin was assessed by trypan blue exclusion assay. The results of the cytotoxic evaluation in PBMC cells incubated with the various concentrations (250-2000 \(\mu\)g/ml) of GT and vitexin are represented in Fig-4.1. In \(\text{H}_2\text{O}_2\) treated group, the cell viability was significantly reduced (6.8 \(\pm\) 3.7% survival) when compared to the control, whereas no significant changes \((p<0.05)\) were observed in the viability of cells co-treated with GT/vitexin. At a concentration of 2000 \(\mu\)g/ml GT and vitexin showed 96.4 \(\pm\) 1.06% and 77.4 \(\pm\) 3.8% cell viability respectively at 24 h. Results of the assay indicates that GT/vitexin does not induce toxicity to PBMC at the tested concentrations (250 -2000 \(\mu\)g/ml).
Concentration (µg/ml)

Fig-4.1: Effect of GT and vitexin on PBMC viability evaluated by Trypan blue exclusion assay (significant level at \( p<0.05 \); # Control vs \( \text{H}_2\text{O}_2 \); * \( \text{H}_2\text{O}_2 \) vs GT/Vitexin)

4.3.2. Genotoxic assessment by Comet assay

Comet assay has been proven as a rapid and sensitive technique to detect single strand breaks and alkali labile sites occurring in individual cells and to screen chemicals for their genotoxicity (Leffa et al., 2012). Broken DNA fragments in cells under the influence of electric field migrate further from nucleus than the intact DNA and the extent of DNA damage is the measure of the length of the comet tail and the percentage of DNA in the comet tail (Tice et al., 2008). The genotoxic effect of various concentrations of GT/vitexin on the DNA of PBMC was evaluated by alkaline comet assay and the results were compared against control group as well as \( \text{H}_2\text{O}_2 \) treated (positive control) group (Fig-4.2). Several researchers have reported the genotoxic behaviour of \( \text{H}_2\text{O}_2 \) in various cells (Benhusein et al., 2010; Paino et al., 2012). As apparent with the previous studies, \( \text{H}_2\text{O}_2 \) treatment to PBMC showed high level of DNA damage and comet formation when compared to the control. Treatment of 250 µM \( \text{H}_2\text{O}_2 \) induced DNA breaks, which is evident from the comet parameters like percentage of DNA in tail (17 ±12.16%), Tail moment(26.85 ± 10.81) and Olive Tail moment (14.09 ±4.96). PBMC treated with various concentrations of GT/vitexin (1000 -2000 µg/ml) did not induce any significant levels of DNA strand breaks and all the tested parameters were
similar to that of control. The results indicate that GT/vitexin does not exhibit genotoxic effects in the tested concentration (1000, 2000 μg/ml) and can be considered safe.

**Fig-4.2:** Confocal microscopic images examining the genotoxic effect of GT/vitexin in PBMC following 24 h treatment (A) Control (B) 250 μM H₂O₂ (C) GT-1000 μg/ml (D) GT-2000μg/ml (E) Vitexin-1000 μg/ml (F) Vitexin-2000 μg/ml (G) Quantitative analysis of genotoxic parameters evaluated by AutoComet software.

### 4.3.3. Mutagenicity assessment by Ames assay

Ames assay is the most commonly used bacterial reverse mutation assay which has been effectively used to screen mutagenic and anti-mutagenic agents (Mortelmans and Zeiger, 2000). Histidine auxotrophic mutants of *Salmonella typhimurium* (TA98, TA100) were used and the results are tabulated in Table-4.1. In the absence of metabolic activation the standard mutagens 4-NQO (TA 98) and sodium azide (TA 100) causes frame shift and base pair mutation respectively thereby reversing the auxotrophs to prototrophs (Maron and Ames, 1983). The positive controls 4-NQO and sodium azide tested showed more than two fold increase in the mutagenicity index (MI) than the negative control which confirms the mutagenicity of these chemicals, whereas the various tested concentrations of GT/vitexin (1000-2000 μg/ml) showed MI less than 2 suggesting that they are non-mutagenic in nature (Santos et al., 2006). In the same way, Ames assay performed for these strains in the presence of metabolic activation to detect an indirect mutagenic effect caused by metabolites of GT/vitexin did not show any mutagenicity, but
the positive control 2-aminofluorene exhibited a significant increase in the average number of revertant colonies with the MI of 7.22 and 6.25 for TA98 and TA100 respectively. The results clearly indicate the lack of *in vitro* mutagenic activity of GT and vitexin.

**Table-4.1: Mutagenic evaluation of GT/vitexin by Ames test**

<table>
<thead>
<tr>
<th></th>
<th>TA98</th>
<th>TA100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-S9</td>
<td>+S9</td>
</tr>
<tr>
<td>Control</td>
<td>0.03±0.01</td>
<td>0.86±0.19</td>
</tr>
<tr>
<td>Positive control</td>
<td>5.21±0.84</td>
<td>7.22±0.18</td>
</tr>
<tr>
<td>GT-1000 µg/plate</td>
<td>1.35±0.14</td>
<td>1.55±0.65</td>
</tr>
<tr>
<td>GT-2000 µg/plate</td>
<td>1.45±0.24</td>
<td>1.43±0.7</td>
</tr>
<tr>
<td>Vit-1000 µg/ml</td>
<td>0.92±0.42</td>
<td>1.49±0.13</td>
</tr>
<tr>
<td>Vit-2000 µg/ml</td>
<td>0.72±0.22</td>
<td>0.62±0.13</td>
</tr>
</tbody>
</table>

*Statistical significance at *p*<0.05 in comparison with control

4.3.4. Acute toxicity studies

Acute toxicity studies provide preliminary data on the toxic effect of any compound on single exposure. Oral administration of GT and vitexin at 2000 mg/kg b.w did not develop any signs of distress or clinical signs of toxicity or mortality. No significant changes were observed in the organ weights of GT and vitexin treated animals compared to the control (Table-4.2).
Table-4.2: Relative organ weights of rats/mice treated with 2000 mg/kg b.w of GT and vitexin

<table>
<thead>
<tr>
<th>Organs</th>
<th>Control (g)</th>
<th>GT-2000 mg/kg b.w (g)</th>
<th>Control (g)</th>
<th>Vit-2000 mg/kg b.w (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>1.60±0.17</td>
<td>1.83±0.06</td>
<td>0.39±0.02</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>0.53±0.06</td>
<td>0.60±0.00</td>
<td>0.17±0.02</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.23±0.15</td>
<td>1.83±0.40</td>
<td>0.47±0.02</td>
<td>0.45±0.01</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.40±0.26</td>
<td>1.73±0.35</td>
<td>0.17±0.01</td>
<td>0.18±0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.87±0.29</td>
<td>1.60±0.20</td>
<td>0.06±0.004</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>6.10±1.15</td>
<td>7.27±0.93</td>
<td>1.47±0.12</td>
<td>1.5±0.26</td>
</tr>
</tbody>
</table>

Table-4.3: Hematological parameters of rats/mice treated with 2000 μg/ml GT and vitexin with the respective control

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>GT-2000 mg/kg b.w</th>
<th>Control</th>
<th>Vit-2000 mg/kg b.w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.73±0.93</td>
<td>12.8±1.15</td>
<td>14.47±0.42</td>
<td>14.87±0.32</td>
</tr>
<tr>
<td>RBC (x10^6/μl)</td>
<td>4.47±0.42</td>
<td>4.57±0.25</td>
<td>4.77±0.15</td>
<td>4.9±0.1</td>
</tr>
<tr>
<td>WBC (x10^3/μl)</td>
<td>1.38±0.68</td>
<td>2.52±0.34</td>
<td>1.12±0.06</td>
<td>1.27±0.1</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>52.24±2.73</td>
<td>50.32±0.22</td>
<td>43.67±3.06</td>
<td>46.33±2.31</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>41.79±3.23</td>
<td>45.04±0.12</td>
<td>52.0±2.0</td>
<td>48.67±1.53</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>5.97±1.59</td>
<td>4.63±0.30</td>
<td>4.33±1.15</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>Platelet (x10^5/μl)</td>
<td>6.81±1.59</td>
<td>7.89±1.55</td>
<td>11.26±1.37</td>
<td>11.47±0.56</td>
</tr>
</tbody>
</table>

Analysis of blood parameters is a pertinent method to assess the risk of toxicity in tested animals for application in humans. Hematopoietic system serves as one of the most sensitive targets for any toxic compounds and the alterations in them act as a higher predictive value for toxicity (Adeneye et al., 2006). Various haematological parameters like the level of haemoglobin, RBC, WBC, lymphocytes and neutrophils and the biochemical parameters like AST, ALT, ALP, BUN, creatinine were analyzed (Table-4.3 and 4.4). The results also did not show any significant alteration between the control and
tested groups indicating normal function of the liver and kidney. These results reveal that there are no extract/vitexin related changes in the hepatic and renal function. Even though there is a slight increase or decrease in few of the parameters from those of the control group, these values lie within the normal ranges and did not reach statistical significance. Hence the observed variations may be due to normal variation among the animal groups. Observations of oral acute toxicity suggest that GT and vitexin are non-toxic at single dose administration and the LD$_{50}$ is above 2000 mg/kg b.w.

Table 4.4: Biochemical parameters of rats/mice treated with 2000 µg/ml GT and vitexin with the respective control

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>GT-2000 mg/kg b.w</th>
<th>Control</th>
<th>Vit-2000 mg/kg b.w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>73.76±6.84</td>
<td>92.77±56.69</td>
<td>71±2.62</td>
<td>64.37±4.25</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>37.4±15.56</td>
<td>32.77±9.85</td>
<td>36.43±4.45</td>
<td>42.8±2.62</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.43±0.91</td>
<td>1.9±1.41</td>
<td>0.67±0.31</td>
<td>1.13±0.06</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>94.33±56.89</td>
<td>94.13±31.18</td>
<td>136.46±42.29</td>
<td>96.2±1.83</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>206.87±106.9</td>
<td>223.63±134.7</td>
<td>73.5±20.81</td>
<td>79.4±3.10</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>224.3±50.4</td>
<td>200±56.35</td>
<td>100±23.06</td>
<td>106.8±25.09</td>
</tr>
</tbody>
</table>

4.3.4.1. Histopathological observations

Histopathological observations showed that the internal organs were intact and revealed normal architecture with nodegenerative or infiltrative lesions. Brain sections showed normal white and grey matter with normal astrocytes, kidney section showed normal tubules and glomerulus and liver showed normal central vein and normal hepatocytes with nodegenerative or infiltrative lesions at the concentration (2000 mg/kg b.w) tested (Fig. 4.3), which substantiates the safety profile of GT and vitexin.
Fig-4.3: Histopathological observations of organs following acute toxicity studies (Control vs GT/vitexin treated (1000 mg/kg b.w.) (A,G) Control Brain (D,J) Treated Brain (B,H) Control Kidney (E,K) Treated Kidney (C,I) Control Liver (F,L) Treated Liver.
4.4. CONCLUSION

Safety assessment of methanol extract of *Grewia tiliacefolia* and vitexin was performed through *in vitro* and *in vivo* toxicity assays.

- The results of *in vitro* tests suggest that the GT/vitexin is devoid of cytotoxic, mutagenic and genotoxic effects.
- The *in vivo* acute toxicity tests suggest that GT/vitexin does not exhibit any significant alterations in hematological and biochemical parameters.
- Moreover, the histopathological analysis of vital organs like liver, kidney and brain revealed that GT/vitexin did not cause any adverse pathological effects.

Overall the observations indicate that the methanol extract of *Grewia tiliacefolia* and its active constituent vitexin has broad safety margin both under *in vitro* and *in vivo* conditions and thereby offer support to the use of this plant and the compound in indigenous system of medicine.

4.5. SUMMARY

- **G. tiliacefolia** and vitexin
  - **In vitro**
    - Non-cytotoxic
  - **In vivo**
    - Non-genotoxic
    - Non-mutagenic
    - No observable adverse effects