Objectives: (AR) or custard apple belongs to family Annonaceae. The plant is traditionally used to treat various ailments and is also known for its medicinal properties. Objectives: The aim of this research work was to evaluate the safety of usage of AR leaf extract in nutraceutical formulations through acute toxicity study. Materials and Methods: The leaves of AR were cleaned, dried and powdered; and aqueous extract (ARAQ) was prepared. The acute toxicity test was conducted using female Swiss albino mice. As per Organization for Economic Co-operation and Development (OECD) 423 guideline, a single dose of 2000 mg/kg body weight of ARAQ was administered via oral gavage for 14 days. Mortality, signs of toxicity, body weight and behavioral changes were observed during the study period. Following the 14-day treatment, the mice were sacrificed for hematochemical, biochemical and histopathology studies. Results: No mortality, signs of toxicity and changes in behavior were observed at 2000 mg/kg body weight. In addition, no significant differences (p>0.05) were noticed in body and organ weight between the control and ARAQ treated groups. Also, there were no significant elevations observed in hematological and biochemical blood parameters. Further, histopathological examination revealed normal architecture of liver, kidney and pancreas. No significant adverse effects were observed in these organs. Conclusion: Overall, ARAQ did not produce any significant toxic effect in mice and the results also indicate the safety of the oral administration of ARAQ at 2000 mg/kg body weight. Hence, ARAQ can be utilized in nutraceutical formulations.

Key words: Acute Toxicity, Annona reticulata, Nutraceutical, Animal studies, Swiss albino mice, OECD 423.

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INTRODUCTION

World Health Organization (WHO) defines herbal medicine as a plant-derived material or preparation with therapeutic values and other human health benefits, which contains either raw or processed ingredients from one or more plants. Due to lesser side effects caused by herbal medicines, many pharmaceutical companies target herbal plant sources for the synthesis of synthetic compounds. Annona reticulata (AR) is one of the traditionally important herbal medicinal plants used for the treatment of various ailments and also possess several medicinal properties such as analgesic, anti-inflammatory, anti-hyperglycemic, anthelmintic, anti-ulcer, wound healing and anti-cancer effects. It is commonly known as Bullock’s heart or Custard apple in English and Ramaphala in Kannada. It belongs to family Annonaceae. The plant is indigenous to the West Indies. It is widely cultivated in West Bengal and southern regions of India as a fruit consuming plant and deciduous tree. Traditionally, the plant is used for treating dysentery, cardiac problem, epilepsy, constipation, haemorrhage, bacterial infection, dysuria, fever and ulcer. Different parts of AR have several phytoconstituents. Stem bark contains alkaloid, tannins and phenolic compounds. Leaves contain flavonoids, amino acids, alkaloids, carbohydrates, steroids, tannins, proteins, glycosides and phenolics. Root has acetogenin, alkaloid, carbohydrates, proteins, flavonoids and tannins. The plant also found to be rich in minerals viz., K, Mg, Fe, Cu, Se, Na, Cl, S, Mn, Zn, Co, Cr, P, Ni and Ca. Also, in our preliminary screening, the aqueous extract of AR has exhibited the presence of rutin, a flavonoid compound known for its apoptosis inducing potential. A study reported cytotoxic effect of AR leaves in Caco-2, Hep G2, HEK cell lines. The roots of AR also exhibited in vitro anticaner activity against melanoma cells in mice. and in vitro cytotoxic activity on MDA-MB-435 human melanoma cells. Biological activities such as DPPH free radical scavenging activity; antibacterial and antifungal activity of leaf extract of AR has also been reported in a study. Anti-hyperglycemic effect of AR leaves extract has been reported in Streptozotocin (STZ) induced diabetic rat model, proving it to be a potent glucose-lowering agent. As there are no earlier reports on toxicity assessment of aqueous extract of Annona reticulata leaves, the present study aimed to ascertain and establish the safety profile of aqueous extract of AR leaves through acute toxicity study as per OECD guidelines.

MATERIALS AND METHODS

Chemicals and reagents
Clinical diagnostics kits were purchased from M/s. Agappe Diagnostics Ltd, Kerala, India. All other chemicals and reagents used in the study were of analytical grade.

Plant Materials
Fresh leaf samples of Annona reticulata (AR) were collected during July 2016 and the plant samples were authenticated (Reference no: Tree reg. vol.1. page no.2 annona 10) and supplied by Dr. GSK Swamy from College of Horticulture, Mysuru, Karnataka, India.

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Preparation of Aqueous Extract

The leaves of AR were cleaned, washed and dried in the oven at 40°C overnight, powdered, passed through 60 mesh sieve and stored at 4°C until further use. About 15g of powdered leaf sample was extracted with 150 ml of distilled water (1:10 w/v) on a mechanical shaker for 24 hr, at room temperature. After 24 hr, the solvent mixture was filtered and the supernatant was evaporated to dryness at -50°C under vacuum in a freeze dryer (ModulyoD, Thermo Electron Corporation, USA). The dried extract was stored in an airtight container at 4°C until further use. The total yield of dry extract obtained from 15g of powdered leaf sample was 1.708g (11.38% w/w).

Experimental Animals

Adult nulliparous, non-pregnant female swiss albino mice of 7-8 weeks old and weighing around 20-30g were procured from the animal house facility of the University of Mysore, Mysuru. The obtained animals were kept in polycrylic cages at a temperature of 25 ± 2°C, 45 to 60% RH and 12 hr light and dark cycles. The animals were fed with pellet diet (procured from Amrut feeds, Pune, India) and water ad libitum. Before the initiation of the test, the animals were acclimatized to laboratory conditions for 14 days. During acclimatization period, the animals were monitored for general conditions every day. Experiments were conducted in accordance with ethical norms and the protocols of toxicity study were reviewed and approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Institutional Animal Ethical Committee (UOM/IAEC/06/2017, dated:20.04.2017).

Acute toxicity study

The acute toxicity study was conducted using female swiss albino mice. The test was executed according to Organization for Economic Co-operation and Development (OECD) 423 guideline for testing of chemicals.12 The animals were grouped into 2 groups viz., Group I – Control; Group II – Treated with aqueous extract of Annona reticulata leaves (ARAQ) consisting of 3 animals each using Randomized Block Design. As per OECD 423 guideline, a single dose (0.5 ml) of 2000mg/kg body weight of ARAQ in distilled water was administered to mice of group II via oral gavage for 14 days. After initiation of dose, the animals were observed individually for first 30 min and at every half an h interval for 6 hr and thereafter once in 24h for 14 days. Mortality, signs of toxicity, body weight and behavioral changes were individually observed during the study period. Following the 14th day of treatment, animals were sacrificed under anesthesia by cardiac puncture for hematological, biochemical and histopathological examination. The hematological and biochemical investigations were carried out in auto analyser (Nihon Kohden, Japan) using agappe diagnostic kits. Vital organs such as liver, kidney and pancreas from both the groups were isolated, weighed, fixed with 10% buffered formalin for 24 hr, embedded in paraffin blocks and sectioned at approximately 3-5 µm thickness, stained with Hematoxylin and Eosin (H&E) and then observed under photomicroscope (Olympus U-CMAD3, Japan and camera: JENOPTIK) for histopathological examination.

Statistical analysis

Statistical analysis was performed using statistical analysis program (SPSS, 16.0, International Business Machines, USA). The results are expressed as the mean ± SD and comparisons between groups (control and treated) were performed by Independent t-test. Statistical significance was accepted at p<0.05.

RESULTS

Acute toxicity effects and behavioral studies of Annona reticulata aqueous extract

The data on toxic symptoms, behavioral pattern and mortality of mice are depicted in Table 1. No mortality or signs of toxicity and changes in behavior were observed in any animals, for a period of 14 days after administration of ARAQ at 2000 mg/kg body weight. Thus, the approximate LD₅₀ of ARAQ in female mice was found to be higher than 2000 mg/kg body weight.

Table 1: Acute oral toxicity record sheet for the control and extract treated mice.

<table>
<thead>
<tr>
<th>Parameters of Behavioral study (n = 3)</th>
<th>CNS depression</th>
<th>CNS stimulation</th>
<th>ANS stimulation</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water (5 ml/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARAQ</td>
<td>2000 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Doses  | Toxicity | Mortality | Hypoactivity | Passivity | Relaxation | Narcosis | Ataxia | Hyperactivity | Irritability | Stereotypy | Tremors | Convulsions | Straub tail | Analgesia | Posis | Exophthalmia | Diarrhoea | Skin and Fur | Eye Laxation | Salivation | Respiratory distress |
|--------|----------|-----------|--------------|-----------|------------|----------|--------|--------------|-------------|-----------|---------|-----------|------------|-----------|--------|--------|-------------|---------|-------------|--------------|-----------|------------------|
| Nil    | 0/3      | 0/3       | 0/3          | 0/3       | 0/3        | 0/3      | 0/3    | 0/3          | 0/3         | 0/3       | 0/3     | 0/3       | 0/3        | 0/3      | 0/3    | 0/3          | 0/3     | 0/3         | 0/3          | 0/3       | 0/3              |
|       | 0/3      | 0/3       | 0/3          | 0/3       | 0/3        | 0/3      | 0/3    | 0/3          | 0/3         | 0/3       | 0/3     | 0/3       | 0/3        | 0/3      | 0/3    | 0/3          | 0/3     | 0/3         | 0/3          | 0/3       | 0/3              |

Data presented as sign of toxicity/number of animals.
Effect of *Annona reticulata* aqueous extract on body and organ weights

The changes in body and organ weights of both control and ARAQ treated (2000 mg/kg) are presented in Figure 1 and Table 2 respectively. The body weight increased gradually during the treatment period in the groups viz., control and ARAQ. However, no significant difference (p<0.05) was observed. The mice treated with ARAQ at 2000 mg/kg had relative organ weights similar to the control and were not significantly different (p<0.05).

Effect of *Annona reticulata* aqueous extract on hematological parameters

Table 3 depicts the changes in hematological parameters of mice treated with ARAQ. The analysis did not show much variation between the control and ARAQ treated groups and; were not significantly different (p<0.05).

![Figure 1: Effect of *Annona reticulata* aqueous extract on body weight changes in female mice. Data provided as mean ± SD (n=3); *p>0.05 treated groups vs control.](image)

![Figure 2: (a-f): Photomicrographs of cross sections of kidney of control (a) and ARAQ treated (b) groups, liver of control (c) and ARAQ treated (d) groups and pancreas of control (e) and ARAQ treated (f) groups. Kidney, liver and pancreas of ARAQ treated groups showed normal histological architecture similar to that of control groups. 200X (H&E). AC- Acini, H- Hepatocytes, IL-islets of Langerhans, RC-Renal Corpuscle.](image)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose mg/kg (p.o.)</th>
<th>Kidney (g)</th>
<th>Liver (g)</th>
<th>Pancreas (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Distilled water (5 ml/kg)</td>
<td>0.899 ± 0.18</td>
<td>2.874 ± 0.31</td>
<td>0.337 ± 0.03</td>
</tr>
<tr>
<td>ARAQ</td>
<td>2000 mg/kg</td>
<td>0.844 ± 0.03*</td>
<td>2.838 ± 0.48*</td>
<td>0.295 ± 0.07*</td>
</tr>
</tbody>
</table>

Data provided as mean ± SD (n=3); *p>0.05 treated groups vs control.

**DISCUSSION**

Since ancient time, medicinal plants have been utilized in traditional medicine because of their therapeutic properties, low cost and also, due to their ease of availability. Recently, medicinal plants have gained importance and are used as an alternative to conventional therapy. Due to adverse reactions and side effects caused by the administration of these herbal medicines in humans, it is very important to evaluate the safety of medicinal plants and ensure their safe usage. In this study, the fixed-dose procedure was employed to evaluate the safety profile of ARAQ, as there were no earlier reports on toxicity assessment of aqueous extract of *Annona reticulata* leaves. The study revealed that there was no mortality, signs of toxicity, respiratory distress, excessive salivation and diarrhea observed in female mice at 2000 mg/kg, during the entire study period. Also, there were no significant changes in behavior pattern, central nervous system and locomotor activity suggesting the safety of ARAQ with LD$_{50}$ exceeding 2000mg/kg in female mice.

Internal organs are essential for central metabolism of the body. Exposure to toxic substances is directly related to changes in body weight and internal organs, which proves to be indicative makers of toxicity. Female mice in both the study groups had a gradual increase in body weight and also were found to be active throughout the study period corresponding to normal food and water consumption, which aided the weight gain. Also, no significant changes were observed in relative organ...
weights between control and ARAQ treated groups implying that the extract was non-toxic to organs.

The hematopoietic system is a vital marker of physiological and pathological status in human and animal since it is a sensitive target for toxic substances. In hematological analysis, there were no significant differences between the treated and control groups indicating that the ARAQ did not adversely affect the hematopoietic system and also, suggesting its safety.

Assessment of serum biochemical parameters such as lipid profile, renal and liver function tests were performed in this study. The lipid profile assessment had no significant variation in cholesterol, VLDL, HDL and triglycerides levels in ARAQ treated groups when compared with control groups. The vital markers of renal dysfunction such as urea and creatinine were assessed to verify the impact of ARAQ on kidney function. The ARAQ was found to be non-toxic and safe to kidneys since there were no significant changes observed in the levels of urea and creatinine.

The liver, also known as the metabolic hub of the body, maintains metabolic integration of all other organs. In the event of hepatic damage, the normal metabolic function of the liver will be hindered, which leads to an elevation in serum levels of biochemical markers such as SGOT, SGPT, ALP and bilirubin. No significant changes in liver serum biochemical markers were observed in ARAQ treated mice. Thus, ARAQ proved to be non-hepatotoxic. The above-mentioned biochemical analysis were in association with the histopathological examinations. No sign of degenerative changes were observed in liver as well as kidney and pancreas. Overall, administration of ARAQ did not exert any adverse effects in mice. Also, similar observation was reported for the dose ≤ 2000 mg/kg body weight, in different species of Annonaceae family.

CONCLUSION

Annona reticulata leaf powder is non-toxic and non-allergic since the extract did not produce any significant toxic effect in mice and the data provided mean ± SD (n=3); *p>0.05 treated groups vs control.

### Table 3: Evaluation of hematological parameters of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Hb (g/dL)</th>
<th>Total RBC (10^6/µL)</th>
<th>Total WBC (10^3/µL)</th>
<th>Platelet Count (10^3/µL)</th>
<th>HCT (%)</th>
<th>Differential Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.20±1.85</td>
<td>10.80±0.55</td>
<td>2.10±1.15</td>
<td>582.33±41.01</td>
<td>42.50±5.59</td>
<td></td>
</tr>
<tr>
<td>ARAQ 2000 mg/kg</td>
<td>13.46±0.35</td>
<td>10.53±0.11</td>
<td>3.10±0.30</td>
<td>662.0±35.55</td>
<td>42.40±0.52</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4: Evaluation of serum biochemical parameters of mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood Urea (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Total Cholesterol (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>VLDL (mg/dL)</th>
<th>Total Proteins (gm/dL)</th>
<th>Albumin (gm/dL)</th>
<th>SGOT (IU/L)</th>
<th>SGPT (IU/L)</th>
<th>ALP (IU/L)</th>
<th>Total Bilirubin (mg/dL)</th>
<th>Direct Bilirubin (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.66±1.52</td>
<td>0.80±0.10</td>
<td>87.33±16.25</td>
<td>93.00±7.93</td>
<td>45.33±3.05</td>
<td>17.00±2.64</td>
<td>3.33±0.15</td>
<td>25.66±6.65</td>
<td>15.33±4.61</td>
<td>20.00±5.29</td>
<td>78.66±4.59</td>
<td>0.96±0.05</td>
<td>0.43±0.15</td>
</tr>
<tr>
<td>ARAQ 2000 mg/kg</td>
<td>19.33±1.15</td>
<td>0.93±0.05</td>
<td>85.66±12.25</td>
<td>91.00±7.93</td>
<td>41.33±4.72</td>
<td>17.33±3.51</td>
<td>3.06±0.30</td>
<td>25.66±6.65</td>
<td>15.33±4.61</td>
<td>20.00±5.29</td>
<td>78.66±4.59</td>
<td>0.96±0.05</td>
<td>0.43±0.15</td>
</tr>
</tbody>
</table>

Data provided as mean ± SD (n=3); *p>0.05 treated groups vs control.
also indicate the safe usage of ARAQ orally at 2000mg/kg body weight. Hence, with no toxicity, ARAQ may be utilized in developing a low-cost disease-specific functional food formulation, which can be a boon to the field of nutraceutical.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS


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