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Acute, subacute and subchronic safety assessment of betalains rich *Rivina humilis* L. berry juice in rats

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ABSTRACT

Rivina humilis L. (Phytolaccaceae) accumulates vacuolar pigments betalains. These pigments are synthesized by plants of 11 families in the order caryophyllales. Red beet is the only industrial source of these hydrophilic and low acidic pigments. Betalains rich *R. humilis* berry juice (RBJ) could be used as alternative source of these pigments. However, there is no information on safety of these berries. In this research work, RBJ was fed to adult (single-dose: 1, 2 and 5 g RBJ/kg bw) and growing (repeated-dosing: 2.5 and 5 g RBJ/kg bw for 35 days; dietary feeding: 0.5%, 1% and 2% RBJ in diet, w/w for 90 days) male rats to assess acute, subacute and subchronic toxic responses. In all the three studies, RBJ was well tolerated plus the feed intake, body and organ weights of RBJ administered groups were comparable to that of untreated control rats. Data on hematology, histology of vital organs, biochemical measurements in serum and liver of RBJ treated rats were comparable to that of control in repeated-dosing and subchronic dietary study. These results suggest that intake of RBJ does not affect growth and normal biochemical homeostasis. Hence, RBJ is safe to consume without any adverse effects in the body.

1. Introduction

Rivina humilis L. (Phytolaccaceae), commonly called as pigeon berry, is a wild herbaceous bushy perennial. The plant is found in colonies that grow on various types of shaded soils. It grows up to a height of 120 cm (4 ft). This plant is native to the Caribbean and tropical America and now widely naturalized in Indo-Malaysia and Pacific regions (Swarbick, 1997; Wealth of India, 2008). The berries of the plant contain high level of betalain pigments (unpublished data).

Betalain pigments are present in plants of 11 families that belong to the order caryophyllales (Clement and Mabry, 1996) and fungi of some genera within Basidiomycetes (Strack et al., 2003). Betalains are hydrophilic *N*-heterocyclic compounds having chromophore betalamic acid, which is structurally similar to synthetic antioxidant ethoxyquin (Lin and Olcott, 1975). Betalains can be sub-divided into violet betacyanins (glucosides of immonium derivatives which may or may not be acylated) and yellow betaxanthins (amine or amino derivatives without glycosylation or acylation) (Strack et al., 2003). In past five decades, various aspects of betalains have been studied with an intention to use them as water soluble colorant for food, pharma, or cosmetics. These studies have proven the inferior stability of betalains over anthocyanins, which are also water soluble pigments (Stintzing and Carle, 2007). However, betalains have been suggested to be suitable for short shelf-life foods such as yoghurt, confectionery, ice cream, frozen dessert, fruit preparation, dry soup premixes, dry fruit drink premix, dry milk shake premixes, etc. Red beet is the only commercially exploited source of betalains, followed far behind by cactus pear fruit, leaf and grain amaranth (Stintzing and Carle, 2007).

Several evidences suggest that betalains possess various biological activities including anti-inflammatory, anti-carcinogenic, anti-malaria, neuroprotection, etc. (Stintzing and Carle, 2007). At low pH, betacyanins exhibit high DPPH radical scavenging activity (~60%), however at physiological pH, or close to it, radical scavenging capacity of betalains reduce to only ~20% (Pavlov et al., 2005). Contrary to this, free radical scavenging activity of betaxanthins increased, when pH was increased, due to deprotonation at high pH (Gandia-Herrero et al., 2009).

Biological activity, *in vivo*, of any dietary component is largely dependent on its bioavailability. Betalains from red beet juice have been reported as low (<1%) bioavailable (Kanner et al., 2001). However, better bio-absorption of betalains from cactus pear fruit has been observed (Tesoriere et al., 2004) indicating variation in

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bioavailability of betalains depending upon the dietary source. Nevertheless, there are only few sources of betalains. Hence, it is imperative to explore new sources of betalains.

In recent times, plant pigments such as lycopene, anthocyanins and betalains rich berries of *Tinospora cordifolia* (Khan et al., 2011a), *Santalum album* and *R. humilis* (unpublished data), respectively, have been studied. In addition, the levels of berberine, lycopene and other phytochemicals during ontogeny of *T. cordifolia* berries have been documented (Khan et al., 2011b). In view of high content of betalains and various *in vitro* antioxidant activities attributed to *R. humilis* berries (unpublished), there is possibility of utilizing *R. humilis* berries as industrial/dietary source of betalains. Website of Pacific Island Endangered species at Risk (PIER) (http://www.hear.org/pier/species/rivina_humilis.htm, accessed on January 22, 2011) mentions that *R. humilis* berries are eaten by numerous species of passerine birds. However, there is no report on human consumption of these berries. Thus, owing to the high betalains content, rich nutritional components and *in vitro* antioxidant properties of *R. humilis* berries, we have assessed its safety employing acute, subacute and subchronic toxicity studies in rats.

2. Materials and methods

2.1. Source of berries and extraction of berry juice

Ripened berries of *R. humilis* L. (red variety) were collected during September–November, 2010 from shady areas of the environs of CFTRI, Mysore (India) located geographically between 12° 18′ 26″ north latitude and 76° 38′ 59″ east longitude. Berries were stored in −20 °C until use. *R. humilis* berry juice (RBJ) was extracted manually from fresh berries (100 g) to give 67–70 mL. Seed weight was 12% of berry weight. Therefore, 100 g deseeded berry yielded about 76 mL juice. The juice was filtered through two layers of cheese cloth. Juice was tightly sealed in *ca.* 5 mL glass tubes and frozen at −20 °C until use. One tube at a time was withdrawn and juice was used for feeding rats. Every 15 days fresh juice was extracted. Betalains content in RBJ was quantified using molar extinction co-efficients of betanin (60000 L/mol cm) and vulgaxanthin I (48000 L/mol cm) in water in an equation reported earlier (Stintzing et al., 2003). Betaxanthins and betacyanins contents of RBJ were 209.7 ± 12.2 and 155.5 ± 7.5 mg/100 mL (total betalains content was 365.2 mg/100 mL). Density and pH of the juice was recorded as 1.08 ± 0.01 g/mL and 6.2 ± 0.05, respectively, at 25 ± 1 °C.

2.2. Animals and care

Male albino rats (CFT-Wistar strain) randomly drawn from the stock colony of CFTRI Animal House Facility (Mysore, India) were used for safety assessment employing acute, subacute and subchronic protocols. Rats were housed in polypropylene cages, with dust-free paddy husk as bedding material, or metal cages. They were maintained on rat pellets (M/s Sai Durga Feeds & Foods, Bangalore) *ad libitum* and had free access to tap water. Environmental conditions were maintained at 12-h light/dark cycles with temperature of 25 ± 2 °C and relative humidity of 60 ± 10%.

2.3. Acute toxicity study

Following safety assessment guidelines (Schilter et al., 2003), single-dose study (15 days) was conducted employing adult (8–10 weeks old) male albino rats (198 ± 8 g bw) of Wistar strain. Dosages were based on body weight of the animal (expressed as g *Rivina* deseeded berry equivalent per kg body weight of the animal). Rats were randomly divided into four groups (untreated control, treatment I – 1 g/kg bw, treatment II – 2 g/kg bw, treatment III – 5 g/kg bw) consisting of five animals ($n = 5$) in each group and rats of a group were held in same box. RBJ was administered orally (using a ball-tipped incubation steel needle fitted onto a graded disposable syringe) as a single bolus dose (150, 300 and 750 μL RBJ). Dosages for feeding rats were computed considering equivalent deseeded berry weight (i.e., 761 μL RBJ is equivalent to 1 g deseeded berry) as the juice was used without further purification. Control rats received distilled water (750 μL). Prior to dosing, rats were fasted overnight to eliminate feed from gastro-intestinal tract. All the rats were thoroughly observed for the onset of any toxic signs immediately and also during 14 days of observation period to record any delayed toxic effects. Survival, feed intake (from day 7 to 15), and body weight (day 0 and every four days) were monitored. Rats were sacrificed under mild ether anesthesia on day 15, and selected vital organs including liver, kidney, testis, adrenals, spleen, heart, brain were excised, blotted and weighed.

2.4. Subacute (35 days) toxicity study

As per recommended guidelines (Schilter et al., 2003), 3 week old weanling rats (56 ± 5 g) were used for the repeated oral dosing study. Rats were administered RBJ through oral gavage as described earlier. Three groups ($n = 6$) were randomly selected and administered RBJ (treatment I – 2.5 g/kg bw and treatment II – 5 g/kg bw) or distilled water (untreated control) every day for 35 days. Feed intake was monitored daily and body weight was recorded on day 1 and every following week. On day 36, rats were sacrificed under mild ether anesthesia, through cardiac puncture 2 mL of blood was collected in EDTA vials for hematological examination, and whole blood was collected in 15-mL falcon tubes for serum separation. Vital organs were excised, blotted and weighed. Liver, kidney and testis were processed for histopathology examination. Biochemical studies in serum were carried out for liver function (alkaline phosphatase, ALP; aspartate aminotransferase, AST; alanine aminotransferase, ALT; total bilirubin), kidney function (urea, creatinine), and other chemicals (glucose, triglyceride, cholesterol, protein) assessment using commercially available standard biochemical assay kits (Span Diagnostics Ltd., Gujarat, India).

2.5. Subchronic (90 days) toxicity study

Three week old weanling rats (56 ± 4 g) were randomly divided into four groups of six rats each and each rat was held in separate metal cage. Commercial rat pellets (M/s Sai Durga Feeds & Foods, Bangalore) were ground in a kitchen type grinder to prepare coarse powder diet. RBJ was mixed in the powdered diet at 0.5%, 1% and 2% concentration in diet after diluting with distilled water (20 mL for 100 g diet) to mix the juice homogenously in the diet. Control diet was mixed with 20 mL demineralised water per 100 g diet. Diet was prepared fresh every morning throughout the study. Feed intake and body weights of rats were monitored as described earlier. After 90 days, rats were sacrificed as described in previous section, and assessment of biomarkers of safety was carried out as described in previous section according to prescribed guidelines (Schilter et al., 2003). Activities of enzymes (AST, ALT), contents of total bilirubin and protein were also studied in liver homogenate.

2.6. Statistical analysis

All samples in each group were analysed either in duplicates or triplicates. Results are expressed in mean ± standard error of means (SEM) of 5 or 6 samples in each group. Data on weekly body weights, food intake, relative organ weights and biochemical determinations were analysed by using Two-way ANOVA test of Microsoft Excel program of Windows 7 software. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. General condition, symptoms and mortality

All animals appeared to tolerate well all RBJ doses and no mortality occurred in any of the treatment groups. No abnormal behavior or cases of diarrhea and soft feces were observed except for one rat receiving 2% RBJ in diet in the initial days of subchronic study. No ophthalmological abnormalities were recorded in any of the treatment groups. Hence, the eyes were excluded from histological examinations. In general, dosing of RBJ in animals did not induce any clinical signs of toxicity in both acute and subacute regimens. In case of subchronic dietary study, mean body weights of treated rats were marginally lower than the mean body weights of control rats but the differences were not significant at $P < 0.05$.

3.2. Acute study

The single-dose study in rats revealed no untoward physiological events that may arise out of an acute exposure of a test material in target species. Both feed intake and body weight gain (data not shown) of treated groups were not significantly different from that of untreated control rats. Feed conversion efficiency of untreated control and RBJ administered groups were 10.2%, 9.4%, 9.9% and 9.9%, respectively. Relative weight of organs (Table 1) in rats of control and treatment groups did not show significant difference. No mortality, no alteration in growth and normal state of vital organs of adult rats in this study showed that they can tolerate maximum recommended dose (Schilter et al., 2003) of RBJ. Similar observations during safety study of betalains rich garambullo fruit