Chapter 7.

SUMMARY AND CONCLUSIONS
7.1. Summary

In this thesis, ripened berries of *Rivina humilis* L. (Phytolaccaceae), commonly called as pigeon berry, were investigated to characterise its betalain pigments, enhance pigment accumulation, assess stability and safety of the pigment extract. Betalains are vacuolar pigments with a nitrogen containing chromophore, betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid]. In plants, these pigments are categorised into red-violet betacyanins (such as betanidin, betanin, isobetanin, amaranthin, gomphrenin) and yellow betaxanthins (such as dopaxanthin, indicaxanthin, humilixanthin, tyrosine-betaxanthin). Betalains are probably synthesised in cytosol starting with hydroxylation of tyrosine in the presence of tyrosinase to produce DOPA, which undergoes ring opening reaction catalysed by DOPA dioxygenase to form betalamic acid. Various spontaneous cyclisation and condensation steps complement the short pathway. For example, DOPA is oxidised to dopaquinone in presence of a cytochrome P450, CYP76AD1, and then *cyclo-DOPA* is formed by cyclisation of dopaquinone. *cyclo-DOPA* condenses with betalamic acid to form betanidin, which is glucosylated at 5-O position catalysed by betanidin-5-O-glucosyltransferase to produce betanin. Betaxanthins are condensation products of betalamic acid and amino acids or amines. In ripened *Rivina* berries, ten betalains (two betacyanins and eight betaxanthins) were successfully identified viz. betanin, isobetanin, indicaxanthin, dopaxanthin, vulgaxanthin I, glutamine-betaxanthin, aspartic acid-betaxanthin, humilixanthin, tyrosine-betaxanthin and miraxanthin V based on their absorbance and mass spectral characteristics. Total betalains content was 0.35% fresh weight, and 1.7% dry weight of berries. The pattern of pigment accumulation during berry ontogeny hinted at the involvement of single pool of betalamic acid for the synthesis of both betacyanins and betaxanthins. Betalains biosynthesis is affected by various ecophysiological factors including elicitors, both biotic and abiotic, that can substantially increase pigment production for purported commercial scale extraction. Abiotic elicitor (salicylic acid) and biotic elicitor (chitosan) was used to enhance betalains accumulation in ripened *Rivina* berries. It was observed that salicylic acid (0.1 mM) and chitosan (0.5%, w/v) treatment in flowers resulted in significant (*P < 0.05*) increase (about 1.3-fold) in betalains content in berries, probably by inducing betalamic acid formation. The extracted betalain pigments are less stable as various physicochemical factors seem to affect them. Some chemical stabilisers such as ascorbic acid, isoascorbic acid have been known to protect betalains degradation. Encapsulation is an emerging technology for stabilisation of these pigments, whereas copigmentation, complex formation and metal chelating agents are promising alternative methods. Ripened *Rivina* berry juice betalains were comparatively more stable at pH 5, low temperature (5°C), and in the presence of ascorbic acid (0.25%, w/v). Ascorbic acid protected the pigments from metal-induced bleaching. Presence of ascorbic acid (0.25%, w/v)+selenium (40 µg/mL) enhanced...
the half–life time of betacyanins at 90°C by five–fold and also regenerated efficiently the pigments on storage at 5°C after thermal destruction. The samples containing ascorbic acid (0.25%, w/v)+Se⁴⁺ (40 μg/mL) produced an orange tinge resulting in the lowest values of colour parameters (Hunter’s Lab), probably because of betanin/isobetanin-Se complex formation. HPLC analysis further supported complex formation in ascorbic acid+selenium containing samples as there was a bathochromic shift in λ_max of betanin/isobetanin from 535 nm to 544 nm. In general, betalains are completely safe to consume, and contributes to health through various biological activities. Rivina berry juice, which is rich in betalains, has not been evaluated before for its safety. Acute, subacute and subchronic safety assessment was conducted as part of this thesis by following standard protocols of oral and dietary feeding. The juice failed to induce signs of toxicity in animal models indicating that the juice was unlikely to be toxic in human system.

### 7.2. Conclusions

1. Total betalains content in *R. humilis* berries was 0.35% fresh weight, and 1.7% dry weight.
2. *R. humilis* berries contained more betaxanthins than betacyanins.
3. Two betacyanins and eight betaxanthins were identified structurally through their absorbance and mass spectral characteristics. The pigments identified were: betanin, isobetanin, indicaxanthin, dopaxanthin, vulgaxanthin I, glutamine-betaxanthin, aspartic acid-betaxanthin, humilixanthin, tyrosine-betaxanthin and miraxanthin V.
4. Partial separation of betalains resulted in betacyanins (70% pure, 23.5 mg/100 g fresh weight) and betaxanthins (95% pure, 6.1 mg/100 g fresh weight).
5. Antiradical activity and reducing power of purified betacyanins and betaxanthins were higher than that of standard antioxidants ascorbic acid and gallic acid.
6. After 24 h exposure, betaxanthins exhibited strong cytotoxicity (IC₅₀–12.0 μg/mL) against HepG2 cells. The cytotoxicity increased on increasing the exposure time.
7. Study of berries from different stages of ontogeny revealed that red/matured berries accumulated maximum pigment and probably a single pool of betalamic acid contributes to the biosynthesis of both betacyanins and betaxanthins.
8. During berry development from green to red, expression levels of a putative *Rivina humilis* betanidin-5-O-glucosyltransferase 2 (RhBGT2) did not change significantly, whereas superoxide dismutase (SOD) and catalase (CAT) expression decreased even as betalains content increased exponentially. The reduction in antioxidant enzymes expression concomitant with betalains accumulation indicates that betalains might be involved in combating ROS in planta.
9. Nutritionally important metabolites of *Rivina* berries were extensively investigated.

10. Betalain pigments in red/matured berries were enhanced through elicitor (salicylic acid, 0.1 mM; chitosan, 0.5%, w/v) treatment to flowers, probably owing to induction of betalamic acid formation.

11. Administration of chitosan (1%, w/v) in *Rivina* flower inhibited SOD gene expression in berries.

12. Gene expression pattern of a putative RhBGT2, CAT and SOD in elicitor-treated berries did not correlate with pigment increase.

13. Betalains in *R. humilis* berry are stable at pH 5 at low temperature, in dark and after flushing with nitrogen gas.

14. Ascorbic acid protected betalains from Cu$^{2+}$ and Zn$^{2+}$-induced bleaching.

15. *Rivina* berry juice betalains can be further stabilised by ascorbic acid (AA), preferably 0.25% (w/v), and addition of selenium (Se$^{4+}$) (40 μg/mL) in presence of AA enhances pigment stability.

16. *Rivina* berry juice betalains are more stable than its purified counterpart.

17. In presence of AA (0.25%, w/v)+Se$^{4+}$ (40 μg/mL), betacyanins are regenerated efficiently on storage at 5°C after intermittent or continuous thermal treatment. This procedure could be adopted industrially for blanching of betalains containing juice to minimise pigment loss.

18. Rats tolerated *R. humilis* berry juice (RBJ) upto 5 g/kg bw in acute and subacute toxicity studies.

19. In the subacute study for 35 days in growing rats, RBJ failed to induce any significant alterations in biomarkers of toxicity such as feed intake, growth, organ weight, histology, haematological indices, serum and liver biochemical parameters.

20. Subchronic studies involving dietary feeding (upto 2.0% RBJ in diet) for 90 days among growing rats revealed that RBJ was safe as growth of rats was normal and routine toxicity evaluation after terminal autopsy did not show significant difference among control and treated rats.

21. Based on these observations, it was concluded that *R. humilis* berry juice was safe to rats indicating that the juice is unlikely to induce toxicity to human body.

22. *Rivina* berry juice coloured product (*Rivina*-banana spread) retained 68% colour after 6 months of storage at 5°C.

23. The product had good overall sensory quality and was liked by consumers indicating the possibility of its usage as food colourant.