5 SUMMARY AND CONCLUSION

Natural products display a wide range of structural diversity, and plants contain a large number of compounds. Plant secondary metabolite is a generic term used for more than 200,000 different substances, which play essential roles in plant survivability and many of them are used as nutrients, colorants, flavors, fragrances, and medicines. Flavonoids are polyphenolic plant secondary metabolites that possess a common phenyl benzopyrone structure (C6-C3-C6). Quercetin (3,3',4',5,7-pentahydroxyflavone) is a naturally occurring flavone found in many fruits and vegetables in the form of quercetin glucosides. Some metabolites in plant *in vitro* cultures can be accumulated with a higher content than those in parent plants, suggesting that the production of plant-specific secondary metabolites by plant cell cultures instead of whole plants cultivation possesses definite potential.

Abutilon indicum is abundantly found as a weed, showed anti-oxidant properties as well as inhibitory effect on cancer cells and used for the treatment of Bronchial asthma. The species *Albizia julibrissin*, commonly named mimosa, powder-puff tree, and has also been shown to possess the anti-inflammatory and antitumor properties. *Caesalpinia pulcherrima* is a commonly used medicinal herb in India and used in common remedies to treat a number of disorders including menoxenia, pyrexia, bronchitis, wheezing, and malarial infection. *Clitoria ternatea*, a medicinal herb native to tropical equatorial Asia, is commonly called as 'shankupushpam' and used in folk medicine to treat liver diseases and also showed memory-enhancing and anxiolytic activity. The whole plant of *Euphobia hirta* (Linn.) is a very popular herb amongst practitioners of traditional medicine and has also been reported as nephroprotectant, antidiabetic and antioxidant agent. *Psidium guajava* is grown in tropical and subtropical countries as a food and is also widely used for the treatment of inflammatory, diabetes, hypertension, wounds, pain, and fever.

The present study was undertaken to isolate and identify quercetin glycosides from six plants including *Abutilon indicum*, *Albizia julibrissin*, *Caesalpinia pulcherrima*,

Clitoria ternatea, *Euphorbia hirta* and *Psidium guajava* plant parts, culture them *in vitro* and to estimate quercetin in explants and their respective cell suspension cultures of selected plants.

The study entitled, "**Isolation, Purification and Structural Characterization of Quercetin and its Derivatives from *In Vitro* Suspension Cultures of Selected Plants and Its Comparison to *In Vivo* Plant Parts"** was carried out in five phases.

In the first phase of the study, extraction, separation and quantification of flavonoids and quercetin was carried out. Extractive value is an indicator of the solvents capacity to solubilise secondary metabolites from plant tissues. In the selected six plants, ten solvents were used to determine the extractive value, which ranged between 0.7 -10.72 g/100g DW. The highest extractive value was 10.72 g/100g DW for methanol: ethyl acetate (60:40) followed by 9.53 g/100g DW and 8.75 g/100g DW for methanol and ethanol. Among other solvents used petroleum ether showed highest extractive value, followed by benzene and chloroform.

The total content of flavonoids in selected plants was measured in terms of quercetin equivalent using colorimetric method. The amount of flavonoids in plants ranged from a minimum of 5.03 mg/g to a maximum of 92.22 mg/g. The quantity of flavonoids was organ-dependent, the concentration decreased in the order of leaves, flowers, stems and roots. Among the leaf and stem extracts of all selected plants assessed for flavonoid content, *E.hirta* showed the maximum accumulation of flavonoids in both leaf and stem extracts (92.22 mg/g and 64.23 mg/g, respectively).

Total flavonoids are comprised of flavonoid glycosides predominantly and flavonoid aglycones in all plants. Hydrolysis of all flavonoid glycosides to aglycones allows one to obtain more accurate data on total flavonoid concentration in the samples. Hence, methanol: ethyl acetate (60:40) extracts of selected plants were hydrolyzed by acid (1.5M HCl). Aglycones are measured easily and identified by spectrophotometric, TLC and HPLC methods. The contents of total flavonoids of acid treated extracts varied

from 37.71±1.15 to 109.11±1.28 mg/g, with leaf extracts of *E.hirta* recording the highest values (109.11±1.28 mg/g).

Different compositions of the mobile phase for separation of quercetin using TLC analysis were tested in order to obtain high resolution and reproducible spots. Based on the comparability of standard and sample R_F values and the clarity of spots (without tailing) of quercetin, the best chromatographic system was found to be based on solvent system n-Butanol: acetic acid: water in the ratio of 40:10:50.

HPLC analysis of acid treated methanolic ethylacetate (60:40) leaf extracts indicated the presence of the main component namely quercetin aglycone. The peak whose retention time (3.1-3.2mins) was identical to quercetin standard showed significantly higher concentration of quercetin (0.226 - 0.522 mg/g) as calculated from peak area, in *A.indicum, C.pulcherrima*, and *E.hirta* leaf extracts followed by *C.ternatea* (0.153 mg/g), *P.guajava* (0.126 mg/g), and *A.julibrissin* (0.122 mg/g).

In this study, three plants, *A.indicum, C.pulcherrima*, and *E.hirta* emerged as promising sources of quercetin, warranting the further investigation.

Based on the higher content of quercetin in the leaf extracts, in second phase, *in vitro* tissue culture studies were carried out for *A.indicum, C.pulcherrima*, and *E.hirta* using leaf explants. Influence of sucrose concentration and growth regulators on callus induction was tested. Maximum callus induction (90.77 - 99.92%) and highest callus proliferation (556.14 - 1651.75 mg FW) was observed in MS medium supplemented with 2,4-D (2.5 mg/L) and Kn (1.5- 2.5mg/L) with 5% sucrose for all the leaf explants. The harvested cell biomass was subjected to initiate cell suspension cultures.

The effect of growth regulators, BA, 2.4-D, Kn, NAA individually or in combinations on biomass and quercetin production in suspension cultures of *A.indicum, C.pulcherrima*, and *E.hirta* were studied. Fresh cell suspension cultures of selected plants were analyzed at regular intervals for 30 days. From day 6, a linear increase in biomass was observed which reached a maximum value on day 24, irrespective of plant

species, varying from 5.14 - 8.21 g FW depending upon the explant sp.. The liquid MS media supplemented with 2.5mg/L BA and 4mg/L Kn was found to be the most effective for biomass production in *A.indicum* cultures and liquid MS medium supplemented with combination of 2.5mg/l of BA along with 2.5 mg/l of 2,4-D and 1.5mg/l of Kn produced more amount of biomass in *C.pulcherrima* and *E.hirta* cultures than all the other treatments.

When cell suspension cultures were analyzed for production of quercetin, detectable amounts were recorded from the 6^{th} day onwards, increasing steadily until the 24^{th} day, after which it decreased. The cells cultured in MS medium supplemented with 2.5mg/L BA along with 1mg/L 2,4-D and 1mg/L Kn exhibited the lowest quercetin content (1.36 mg/g), where as cells cultured in MS medium supplemented with 2.5 mg/L BA along with 2.5mg/L 2,4-D and 2.5 mg/L Kn produced the highest quercetin content of 2.95mg/g DW on 24^{th} day.

The content of quercetin increased with the growth phase of the suspension culture. The quercetin contents recorded in cell suspension cultures were significantly higher compared with wild plants grown in fields, as evident from cultures of *A.indicum* which recorded a 5.84 mg/g DW in culture as compared to 0.226 mg/g from leaves.

After confirming the ability of the cell suspension cultures to produce quercetin in optimized MS medium, in third phase of the study, elicitor and precursor treatments were designed to enhance quercetin biosynthesis. UV-B rays (60mins treatment) increased the yield of quercetin. *E.hirta* cell suspension cultures showed higher quercetin content (2.51mg/g DW) on 18^{th} day compared to other plants which showed a significant accumulation on 24^{th} day under physical elicitation by UV rays.

The addition of organic elicitors like peptone water and yeast extract did not significantly accelerate quercetin production. However, among organic elicitors, MS media supplemented with coconut water (10 - 25%) showed higher quercetin biosynthesis in *A.indicum* cultures (4.68mg/g DW) compared to *E.hirta* (3.46mg/g DW) and *C.pulcherrima* (3.14mg/g DW) cultures.

The addition of phenylalanine as precursor at lower concentrations enhanced quercetin content of cells in all the three plants, which ranged from 2.97 - 5.68 mg/g DW. Chemical elicitors, which include GA_3, ABA and SA at different concentrations were successful in raising quercetin production in cell suspension cultures of *A.indicum* (5.84 mg/g), *C.pulcherrima* (4.22 mg/g DW), and *E.hirta* (4.29 mg/g DW). When the influence of heavy metals was tested, the result was conclusive as Zinc sulphate along with SA at lower concentration showed higher quercetin accumulation (5.34 mg/g DW), but content was lower compared to SA or ABA treatments.

Regardless of plant variety, the most promising effect on quercetin biosynthesis in cell suspension cultures was shown by chemical elicitors, SA (50µM) and ABA (0.1mg/l) (5.84 mg/g DW and 5.38 mg/g DW, respectively).

. In phase IV, acid treated methanolic ethyl acetate extracts of leaves, cell suspension cultures of *A.indicum*, *C.pulcherrima* and *E.hirta* were subjected to preparative thin layer chromatography to separate and identify quercetin and quercetin derivatives. Structural characterization of purified compounds using NMR analysis showed the presence of eight compounds which includes, quercetin, isoquercetin, quercetrin, rutin, quercetin 3-O-β-D-xyloside (Reinutrin), quercetin 3-O-arabinopyranoside (Guajaverin), quercetin 3-O- α-arabinopyranosyl (1→2) β-galactopyranoside, and Isorhamnetin 3-O-rutinoside (Narcissoside). Besides the expected products five new quercetin glycosides from *in vitro* cultures were identified by NMR analysis for the first time in these plants, of which the pharmacological importance remains to be unraveled.

Quercetin isolated from both leaf and cell suspension extracts were tested along with the crude extracts of plants for their antimicrobial activity. On comparison with crude plant and cell suspension extracts, purified quercetin fractions of both leaf and suspension cultures were effective even at 50µg/ml concentration, which showed highest antibacterial activity against *Bacillus cereus* (19.05±0.11mm, zone of inhibition) and

least activity against *Staphylococcus epidermis* (12.20±0.02mm) compared to standard antibiotic Gentamycin (18.20±1.54mm).

Leaves and cell suspension cultures of selected plants were tested against eleven fungal isolates for antifungal activity, partially purified quercetin fractions of *A.indicum* cell suspension cultures possessed potent antifungal activity (~14.81mm) amongst all the partially purified quercetin fractions. This quercetin fraction also showed higher zone of inhibition against *C.albicans* (23.04±0.22mm), which was also comparable to standard quercetin (25.56±0.08mm) and Ketaconazole (20.00±0.12mm).

Conclusion

The present study showed that *in vitro* approach could be an alternate for quercetin glycosides production in *A.indicum, C.pulcherrima* and *E.hirta* leaf explants. Also new quercetin glycosides were identified in cell suspension cultures. The content of quercetin is comparable to the one available in natural habitat. The application of this study could be extended to industry for the supply of product independent of the availability of plant, climate and geographical location. This would also extend the feasibility to scale up production of quercetin at industrial levels utilizing bioreactors in future.

Future prospects

- Characterization of channelling metabolites related to flavonoid biosynthesis.
- Reduce the time required for successful adaptation will better enable the increasing need for quercetin productions to be met.
- Genetic engineering, together with bioreactor design, will favour success of large scale production of metabolites.
- Intensify study on the new compounds identified.