Chapter 9
SUMMARY AND CONCLUSION

The fact that *Diabetes mellitus* is a global disease which is mostly the result of a high calorie diet and lack of physical activity coupled with genetic factors. Biostatistical trends predict that it will soon become the seventh leading cause for death in the world. A total death from diabetes is projected to rise more than 50% in the next ten years. Uncontrolled diabetes increases the rate of death by cardiovascular involvement. Though the current methods of treatment have considerably improved over the last two decades, much needs to be done to arrive at a satisfactory therapeutic regimen.

Several plant extracts and pure plant products have been tried through Ayurvedic and herbal methods for diabetes. In the wake of the large plant biodiversity in our country and a rich documented system of TIM, there is enough scope for further improvement in the control of diabetes. The efforts made by earlier researchers in isolating API from plant extracts have been found to be very effective and encouraging. These are highlighted in the introductory chapter.

Certain medicinal plants with acclaimed antidiabetic activity in the TIM were considered for detailed study with respect to their chemical constituents and their efficacy and mechanism of their action. Two plants were then selected for this purpose. 1) *C. macrophylla* (*Apocyanaceae*) and 2) *H. arifolia* (*Hemionitidaceae)*.

Objectives planned in the work covered the following cardinal points.

- Evaluation of antidiabetic activity in the crude extract
- Bioactivity guided fractionation of the most potent plant fraction and dose optimization
- Investigations related to mechanism of action such as *in vitro* radical scavenging antioxidant activity, *in vitro* α-amylase, α-glucosidase and DPP-IV inhibition activity, cell line glucose uptake studies and characterization of the isolated chemical constituents by spectral analytical data followed by docking studies of the isolated compounds.
Available literature on the title plants focusing on their chemical investigations as well as antidiabetic related bioactivity studies have been collected covering the last two decades. Emphasis has been given to their recent literature covering the last decade. Modern investigative tools employed in the investigation of antidiabetic principles from plant sources have been given utmost importance while preparing the review of the literature.

In chapter 4, a plan of work followed to arrive at a reasonable conclusion regarding the chemical constituents, their antidiabetic activity, related mechanisms, histopathological studies and docking studies is presented.

In chapter 5 is included the materials and methods used in the chemical investigations, pharmacognostical studies, results obtained therefrom as well as logical conclusions derived.

The extractive values of *C. macrophylla* root and *H. arifolia* leaves in 50% ethanol using soxhlet were 14 and 15.6% w/w respectively. Chemical tests indicated the presence of phenolics, flavonoids and tannins in the above extracts.

The extract prepared from the roots of *C. macrophylla* was further fractionated using a silica gel column into petroleum ether soluble eluate (2.2), chloroform soluble eluate (7.0), ethyl acetate soluble eluate (1.5) and methanol soluble eluate (9.1). The extractive values in % w/w are given in brackets.

In the TLC experiments performed using silica gel, rutin, quercetin and gallic acid were identified. It was further confirmed by HPLC method of analysis. The total phenolic content was determined by the aluminium chloride colorimetric method and was 24.45 µg/ml of rutin. The UV spectra of both quercetin and rutin were recorded and shift reagent studies using sodium hydroxide was also performed. IR and NMR spectra were also recorded using the separated compounds. In the LCMS studies performed by using the extract, mass peak m/z value corresponding to quercetin was detected.

Chapter 6 focuses on the *in vitro* studies which were needed to establish the antioxidant and antidiabetic nature of the extract and the various fractions. Methods used for antioxidant studies include ABTS assay, DPPH assay, Nitric oxide
scavenging assay and Lipid peroxidation assays. IC\textsubscript{50} values of CMRE, CMRH, HALE and HALH in ABTS assay methods were 2.30±0.20, 5.63±0.74, 2.33±0.15 and 3.87±7.51 µg/ml respectively. IC\textsubscript{50} values of CMRE, CMRH, HALE and HALH in DPPH assay methods were 72.33±2.89, 71.10±10.58, 90.33±7.51 and 61.33±2.08 µg/ml respectively. IC\textsubscript{50} values of CMRE, CMRH, HALE and HALH in Nitric oxide scavenging assay methods were 923.33±7.5, >1000, 360±20.0 and 953.33±15.33 µg/ml respectively. IC\textsubscript{50} values of CMRE, CMRH, HALE and HALH in Lipid peroxidation assay methods were 210±10.00, >1000, >1000 and >1000 µg/ml respectively.

These antioxidant results provided the impetus to continue the antidiabetic studies. It was then decided to do the following enzyme inhibition and cell line studies. They include 1) \(\alpha\)-amylase inhibition 2) \(\alpha\)-glucosidase inhibition 3) MTT assay 4) L-6 muscle cell line glucose uptake studies 5) DPP-IV inhibition 6) Glut-4 translocation and 7) PPAR\(\gamma\) up regulation studies.

The \(\alpha\)-amylase inhibitory activity of CMRE and CMRH were evaluated and the IC\textsubscript{50} values were found to be greater than 1000µg/ml. Simultaneously the IC\textsubscript{50} values of quercetin and rutin were determined and were 931±9.28µg/ml and > 2000µg/ml respectively.

In the \(\alpha\)-glucosidase inhibition assay performed with ME-CMRH fraction, the IC\textsubscript{50} value was found to be 249.50±2.35µg/ml. Simultaneously the IC\textsubscript{50} values of quercetin , rutin and acarbose were also determined and were 200.30±3.65, 85.65±2.45 and 13.5±1.09 µg/ml respectively.

In the MTT assay, CMRH was found to be toxic only at a concentration of 353.33µg/ml on exposure for 72 hours.

The glucose uptake values demonstrated by ME-CMRH(500µg/ml) and standard drug metformin(100µg/ml) were 68.5±7.33 and 115.8±3.49 percentage uptake respectively.

DPP-IV enzyme was inhibited (20.80%) by ME-CMRH fraction at a concentration of 500µg/ml compared to the standard Ile-Pro-Ile.
In the Glut-4 translocation studies performed, ME-CMRH at a concentration of 500 µg/ml showed 0.3 fold upregulation compared to the normal control.

In the PPARγ upregulation studies, ME-CMRH at a concentration of 500 µg/ml showed 0.31 fold upregulation compared to the standard metformin.

Chapter 7 focuses on docking studies. In the docking studies, the glide score of all the five molecules studied (quercetin, rutin and gallic acid from C. macrophylla and quercetin, rutin, apigenin and kaempferol from H. arifolia) were found to be in the range of -6 to -11. Out of the five molecules studied only rutin violated one among the five rules of Lipinski’s rule of five. Thus it was concluded that the results are promising.

Upregulation of PPARγ happens through the 3SZ1 receptor selected for docking studies. PPARγ upregulation is one among the different mechanisms through which antidiabetic action is produced without insulin (Panda S, Kar A, 2007), (Mansor F et al, 2013). ME-CMRH fraction demonstrated PPARγ upregulation in the antidiabetic mechanism identification studies.

Chapter 8, comprises in vivo studies carried out on the most promising fraction obtained from C. macrophylla. Dose selection was carried out after a systematic acute toxicity study. LD-50 was found to be 2000 mg/kg body weight. Two doses selected were 250 and 500 mg/kg body weight respectively. Significant reduction in blood glucose level was observed from the 14th day onwards in a schedule of a 28 days study as compared to the standard drug gliclazide 25 mg/kg body weight. Out of the two selected doses, 250 and 500 mg/kg body weight, only the latter could give an effective response.

Similar results were obtained in the glucose uptake experiments performed by rat hemidiaphragm method. Only the larger dose (500 μg/ml) could give a glucose uptake of (11.14±1.94) as compared to that obtained (17.43±2.93) with insulin (1 IU/ml).

Histopathological data obtained were very much congruent with the in vivo findings reported above. e.g. At a dose of 500 mg/kg body weight, the destruction of β-cells induced by streptozotocin could be prevented to an extend of 70%. The
antioxidant property identified earlier may be protecting the cells of pancreas from further destruction. This is very much comparable with that obtained with Gliclazide (65%) at 25 mg/kg body weight dose.

In the extract of *H. arifolia* only phytochemical and *in vitro* studies were performed. Earlier, antidiabetic studies in rats were performed and reported (Ajikumaran Nair S et al., 2006). In the present study, the presence of quercetin, kaempferol, apigenin and rutin were detected by paper chromatography experiment.

Using RPHPLC, the presence of rutin, quercetin and kaempferol were confirmed and were also quantified. Further LC MS-MS studies were performed and the presence of rutin (0.04% w/w) apigenin (0.010% w/w) and kaempferol (0.013% w/w) were confirmed and quantified. In the next stage, spectral studies of all the above compounds were performed. LCMS studies were performed on the defatted extract and the presence of nine more different m/z values of molecules other than the above identified four components were detected.

In the MTT assay, the cytotoxic concentration was found to be above 1000 µg/ml. In the α-amylase inhibition assay, the IC$_{50}$ value was found to be greater than 1000 µg/ml. In the DPP-IV inhibition studies, defatted HALH extract at a concentration of 500 µg/ml demonstrated only 15.83% inhibition compared to the standard Ile-Pro-Ille. In the Glut-4 translocation studies performed defatted HALH showed 0.15 fold up regulation compared to the standard metformin.

Thus it can be concluded that, further detailed investigations and evaluations of these active plant extracts using more animals and sophisticated techniques followed by clinical investigations need to be carried out to bring out their actual potential as a therapeutic component in the armamentarium of antidiabetic therapy.

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