Chapter 5

PHYTOCHEMISTRY AND PHARMACOGNOSY

Materials and Methods

5.1 Collection and authentication of plant materials
   5.1.1 Plant profile
   5.1.2 Chemicals and reagents used

5.2 Pharmacognostic studies
   5.2.1 Microscopic study - transverse section
   5.2.2 Loss on drying
   5.2.3 Determination of ash values
      5.2.3.1 Determination of total ash
      5.2.3.2 Acid insoluble ash
      5.2.3.3 Water soluble ash
      5.2.3.4 Sulphated ash
   5.2.4 Extractive values
      5.2.4.1 Determination of chloroform soluble extractives
      5.2.4.2 Determination of water soluble extractives
   5.2.5 Fluorescence analysis

5.3 Preparation of the extract
   5.3.1 Procedure followed
      5.3.1.1 Test sample
      5.3.1.2 Chemicals used
      5.3.1.3 Basic operation

5.4 Phytochemical screening
   5.4.1 Detection of carbohydrates
      5.4.1.1 Molisch’s reagent
      5.4.1.2 Molisch’s test
   5.4.2 Detection of glycosides
      5.4.2.1 Keller-Killiani test
5.4.3 Detection of saponins 44
  5.4.3.1 Foam test 44
5.4.4 Detection of alkaloids 44
  5.4.4.1 Mayer’s reagent 44
  5.4.4.2 Mayer’s test 44
  5.4.4.3 Dragendorff’s reagent 44
  5.4.4.4 Dragendorff’s test 45
5.4.5 Detection of flavonoids 45
  5.4.5.1 Alkaline reagent test 45
  5.4.5.2 Shinoda test 45
5.4.6 Detection of phenolics and tannins 45
  5.4.6.1 Ferric chloride test 45
  5.4.6.2 Test for tannins 45
5.4.7 Detection of phytosterols and triterpenoids 45
  5.4.7.1 Liebermann’s test 46
  5.4.7.2 Liebermann-Burchard test 46
  5.4.7.3 Salkowski’s test 46
5.4.8 Detection of fixed oils and fats 46
  5.4.8.1 Oil spot test 46
5.5 Selection of the plant based on comparative glucose uptake profile of both the plant extracts 46
5.6 Chemical investigation of the ethanol (50%) extract of C. macrophylla roots (CMRH) 47
  5.6.1 Fractionation of the more efficaceous ethanol (50%) extract by column chromatography 47
  5.6.2 Solvents used and procedure 47
  5.6.3 Identification of the components present in the methanol eluate of ethanol (50%) extract of C. macrophylla roots 49
    5.6.3.1 UV spectral studies 49
    5.6.3.2 Shift reagent study 49
    5.6.3.3 IR spectral studies 49
    5.6.3.4 NMR spectral studies 49
5.6.3.5 Thin layer chromatography

5.6.4 Estimation of total flavonoids in the column chromatography methanol eluate of ethanol (50%) extract of *C. macrophylla* roots (ME-CMRH)

5.6.4.1 Chemicals and reagents
5.6.4.2 Preparation of the test solutions
5.6.4.3 Procedure

5.6.5 Identification and estimation of the components present in methanol eluate of ethanol (50%) extract of *C. macrophylla* root (ME-CMRH) by HPLC

5.6.5.1 Materials and Methods
5.6.5.2 Chemicals
5.6.5.3 Instrument and other details
5.6.5.4 Procedure
5.6.5.5 Standard and sample preparation

5.6.6 LC MS of *C. macrophylla*, methanol eluate of ethanol (50%) extract

5.6.6.1 Instrument-Mass spectrometer

5.7 Chemical investigations of defatted ethanol (50%) extract of *H. arifolia* leaves (HALH)

5.7.1 Separation of components present in ethanol (50%) extract of *H. arifolia* leaves
5.7.2 Paper chromatography
5.7.3 HPLC of HALH
5.7.3.1 Instrument
5.7.3.2 Standard and sample preparation

5.7.4 LC MS-MS of HALH
5.7.4.1 Standard and sample preparation

5.7.5 LCMS of defatted *H. arifolia* leaf extract (HALH)
5.7.5.1 Instrument – Mass spectrometer
Results
5.8 Pharmacognosy parameters 58
  5.8.1 TS of C. macrophylla root (milky) latex sample 58
  5.8.2 H. arifolia leaf sample 59
5.9 Results of phytochemical studies 62
5.10 Discussion 65
  5.10.1 Identification and estimation of the components present in ME-CMRH by HPLC 68
    5.10.1.1 LCMS data of ME-CMRH 80
  5.10.2 Identification of the components present in H. arifolia leaf
    5.10.2.1 HPLC results of HALH 81
    5.10.2.2 LC MS-MS estimation of HALH 94
    5.10.2.3 LCMS analysis of HALH 95

Chapter 6
IN VITRO STUDIES
6.1 Introduction 96
  6.1.1 Biochemistry of oxidative stress 98
  6.1.2 Action path of oxidants/free radicals 98
    6.1.2.1 Reactive oxygen species 98
    6.1.2.2 Reactive nitrogen species 100
  6.1.3 Response and signals during oxidative stress 101
  6.1.4 Classification of antioxidants 101
    6.1.4.1 Superoxide dismutase 102
    6.1.4.2 Catalase 102
    6.1.4.3 Glutathione peroxidase 103
  6.1.5 Antioxidant defence and repair mechanisms 104
  6.1.6 Non-enzymatic and phytochemical antioxidants 105
    6.1.6.1 Broad chemical classification of antioxidants 106
6.2 Evaluation of antioxidant activity 107
  6.2.1 Experiments which can be performed by invitro steady state free radical scavenging studies 107
6.2.2 Experiments which can be performed by \textit{in vitro} time-resolved free radical scavenging studies

6.2.3 ABTS radical scavenging assay

6.2.4 DPPH scavenging assay

6.2.5 Nitric oxide scavenging assay

6.2.6 Lipid peroxidation inhibition assay

6.3 Enzyme inhibition studies

6.3.1 Alpha amylase inhibition studies

6.3.2 Alpha glucosidase inhibition studies

6.3.3 DPP-IV inhibition studies

6.4 Cell line studies

6.4.1 Determination of cell viability by MTT assay

6.4.2 Diabetic screening – \textit{in vitro}

Materials and Methods

6.5 Materials and methods for antioxidant studies

6.5.1 Instruments used

6.5.2 Preparation of the test solutions

6.5.3 ABTS scavenging assay

6.5.3.1 Chemicals used

6.5.3.2 Reagents

6.5.3.3 Procedure

6.5.4 DPPH radical scavenging assay

6.5.4.1 Chemicals

6.5.4.2 Reagents

6.5.4.3 Procedure

6.5.5 Scavenging of nitric oxide radical

6.5.5.1 Chemicals

6.5.5.2 Reagents

6.5.5.3 Procedure

6.5.6 Lipid peroxidation inhibitory activity

6.5.6.1 Chemicals

6.5.6.2 Reagents

6.5.6.3 Procedure
6.6 Materials and methods for enzyme inhibition studies

6.6.1 Preparation of the test solutions

6.6.2 Alpha amylase inhibition studies

6.6.2.1 Chemicals
6.6.2.2 Reagents
6.6.2.3 Procedure

6.6.3 Alpha glucosidase inhibition studies

6.6.3.1 Chemicals
6.6.3.2 Reagents
6.6.3.3 Plates used
6.6.3.4 Procedure

6.6.4 DPP-IV inhibition studies

6.6.4.1 Chemicals
6.6.4.2 Reagents
6.6.4.3 Micro-titer plates
6.6.4.4 Preparation of test solutions
6.6.4.5 Procedure

6.7 Materials and methods for cell line studies

6.7.1 Chemicals and reagents

6.7.2 Culturing of L-6 cells (myoblast)

6.7.3 Preparation of test solutions

6.7.4 MTT assay - procedure

6.7.5 Glucose uptake studies (in vitro) on L-6 muscle cell lines - procedure

6.8 Materials and methods for the evaluation of mechanism of action

6.8.1 GLUT-4 Gene expression studies

6.8.1.1 RT-PCR Procedure
6.8.1.2 Amplification conditions for GLUT-4 gene
6.8.1.3 Analysis of amplified sequences

6.8.2 Gamma PPAR gene expression studies

6.8.2.1 RT-PCR Procedure
6.8.2.2 Amplification conditions for PPARγ gene
Results
6.9 Results of antioxidant studies 131
6.10 Results of enzyme inhibition studies 140
6.11 Results of cell line studies 146
   6.11.1 Cytotoxic studies by MTT assay 146
   6.11.2 Glucose uptake on L-6 muscle cells 148
6.12 Results for evaluation of mechanism of action 151
   6.12.1 GLUT-4 translocation studies on L-6 muscle cells 151
      6.12.1.1 Studies on ME-CMRH sample 151
      6.12.1.2 Inference 152
   6.12.2 Gamma PPAR studies on L-6 muscle cells 153
      6.12.2.1 Studies on ME-CMRH sample 153
      6.12.2.2 Inference 154
   6.12.3 GLUT-4 Translocation studies on L-6 muscle cells 155
      6.12.3.1 Studies on defatted HALH sample 155
      6.12.3.2 Inference 156
6.13 Discussion 156

Chapter 7
DOCKING STUDIES
7.1 Drug design 160
   7.1.1 General steps in the synthesis of a new compound 160
   7.1.2 Stereochemistry and drug design 160
   7.1.3 The type of group 160
7.2 The SAR and QSAR approaches to drug design 161
   7.2.1 Structure activity relationship (SAR) 161
   7.2.2 Quantitative structure activity relationship (QSAR) 162
   7.2.3 Molecular descriptors analysis 162
7.3 LIPINSKI’S Rule of 5 164
7.4 In silico drug design programs and resources 164
   7.4.1 Computer-aided drug design 164
7.5 Types of drug design 165
7.5.1 Receptor based approach 165
7.5.2 Ligand based approach 165
7.5.3 Combinatorial based approach 166

7.6 Docking 166

Materials and methods

7.7 Software used 167

Results

7.8 Drug molecule-receptor protein interaction-ribbon structure 171
7.9 Ligand molecule interaction diagram 172
7.10 Discussion 175

Chapter 8

IN VIVO STUDIES

8.1 Acute toxicity study in rats with methanol eluate of C. macrophylla roots ethanol (50%) extract (ME-CMRH) - (OECD guidelines 423) 176
8.1.1 Treatment 176

8.2 Glucose uptake by rat hemi diaphragm 176
8.2.1 Isolation of diaphragm 176
8.2.2 Experimental design 177
8.2.3 Statistical evaluation 178
8.2.4 Chemicals and kits 178

8.3 Studies on male Wistar albino rats 178
8.3.1 Grouping and drug treatment 178
8.3.2 Blood and organ collection 179
8.3.3 Preparation of hemolysate 179

8.4 Histopathology studies on pancreas 180

Results

8.5 Acute toxicity studies 180
8.6 Glucose uptake study in rat hemidiaphragm of test extract (ME-CMRH) 181
8.7 Inference 181
8.8 Biochemical findings 182
8.9 Graphical representation of results 184
8.10 Histopathology report 186
  8.10.1 Microscopy 186
    8.10.1.1 Normal control 186
    8.10.1.2 Positive control (Diabetic control) 187
    8.10.1.3 ME-CMRH (250 mg/kg b.wt) 188
    8.10.1.4 ME-CMRH (500 mg/kg b.wt) 188
    8.10.1.5 Standard (Gliclazide 25 mg/kg b.wt) 189
8.11 Discussion 190

Chapter 9
SUMMARY AND CONCLUSION 191

REFERENCES 196
Appendix-I  HPLC of ME-CMRH 220
Appendix-II  LCMS analysis of CMR crude 225
Appendix-III  HPLC analysis of defatted HALH 230
Appendix-IV  LC MS-MS of defatted HALH 233
Appendix-V  LCMS analysis of defatted HALH 243