ANALYTICAL, BIOLOGICAL, PHARMACOKINETIC AND STABILITY STUDIES OF Piper sarmentosum Roxb. EXTRACTS AND SELECTED STUDIES OF Orthosiphon stamineus Benth. EXTRACTS

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By

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Dedicated to my parents, wife and children

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TABLE OF CONTENT

Page

DEDICATION ii

ACKNOWLEDGEMENTS iii

TABLE OF CONTENT v

LIST OF TABLES xvii

LIST OF FIGURES xix

LIST OF PLATES xxiii

LIST OF ABBREVIATIONS xxiv

ABSTRAK xxx

ABSTRACT xxxiii

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW 01

Therapeutic potential of flora 01

1.2 Ethnobotany of Malaysia 03

1.3 Phytochemical analysis 04

- 1.3.1 Standardisation 05
- 1.3.2 Development and validation of analytical methods 08
    - 1.3.2.1 Validation 09
        - 1.3.2.1(a) Linearity and calibration 09
        - 1.3.2.1(b) Precision 10
        - 1.3.2.1(c) Capacity factor 10
        - 1.3.2.1(d) Resolution 11
        - 1.3.2.1(e) Accuracy 11
        - 1.3.2.1(f) Sensitivity 11
        - 1.3.2.1(g) Specificity and system suitability 12
        - 1.3.2.1(h) Peak purity 12
        - 1.3.2.1(i) Robustness 12

1.4 The discovery of herbal drugs 13

1.4.1 Phytochemical and taxonomical screening 13

1.4.2 *In vitro* biological studies 13

1.4.3 *In vivo* biological studies 14

1.4.4 Pharmacokinetic studies 14

1.5 Stability studies 15

1.5.2 Physical factors effecting stability 15

1.5.1.1 Temperature 15

1.5.1.2 Moisture 16

1.5.1.3 Light 16

1.5.2 Chemical factors effecting stability 16

1.5.2.1 Hydrolysis 16

1.5.2.2 Oxidation 16

1.5.2.3 Isomerisation and polymerisation 17

1.6 Accelerated stability 17

1.7 Literature review of *Piper sarmentosum* 18

1.7.1 Botanical description 18

1.7.2 Ethnopharmacology 19

1.7.3 Review of chemical constituents 19

1.7.4 Review of biological activities 26

1.8 Literature review of *Orthosiphon stamineus* 29

1.8.1 Botanical description 30

1.8.2 Ethnopharmacology 30

1.8.3 Review of chemical constituents 30

1.8.4 Review of biological activities 36

1.9 Objectives of the study 40

CHAPTER 2: ANALYTICAL STUDIES 43

2.1 Introduction 43

2.2 Materials and methods 44

2.2.1 Plant materials 44

2.2.2 Chemicals and solvents 45

2.2.3 Instruments 45

2.2.4 Physicochemical analysis of *Piper sarmentosum* and *Orthosiphon stamineus* crude powders 47

  2.2.4.1 Moisture content 47

  2.2.4.2 Total ash 47

  2.2.4.3 Acid insoluble ash 47

  2.2.4.4 Sulphated ash 48

  2.2.4.5 Extractive values 48

    2.2.4.5a Alcohol soluble extractives 48

    2.2.4.5b Water soluble extractive 48

  2.2.5 Qualitative analysis of crude powders by FTIR spectroscopy 49

    2.2.5.1 *Piper sarmentosum* 49

    2.2.5.2 *Orthosiphon stamineus* 49

2.2.6 Preparation of extracts 50

  2.2.6.1 *Piper sarmentosum* 50

  2.2.6.2 *Orthosiphon stamineus* 50

2.2.7 Estimation of total content of primary and secondary metabolites of *Piper sarmentosum* and *Orthosiphon stamineus* extracts 50

  2.2.7.1 Estimation of total proteins 51

  2.2.7.2 Estimation of total polysaccharides 51

  2.2.7.3 Estimation of total glycosaponins 52

  2.2.7.4 Estimation of total phenolics 52

    2.2.7.4a Total polyphenolics 52

    2.2.7.4b Total flavonoids 53

  2.2.7.5 Estimation of total amides 54

2.2.8 Analysis of *Piper sarmentosum* and *Orthosiphon stamineus* extracts by Ultraviolet/Visible spectroscopy 54

2.2.9 Qualitative analysis of *Piper sarmentosum* and *Orthosiphon stamineus* extracts of by HPTLC 55

2.2.9.1 *Piper sarmentosum* 55

2.2.9.2 *Orthosiphon stamineus* 55

2.2.10 Semi quantitative analysis of *Piper sarmentosum* and *Orthosiphon stamineus* extracts by HPTLC 56

2.2.10.1 Quantification of rutin and naringenin in *Piper sarmentosum* extracts 56

2.2.10.1a Preparation of sample and standard solutions 56

2.2.10.1b Sample application 56

2.2.10.1bi For quantification of rutin 56

2.2.10.1bii For quantification of naringenin 56

2.2.10.1c Chromatographic conditions 57

2.2.10.1d Densitometry 57

2.2.10.1e Documentation 57

2.2.10.2 Quantification of betulinic acid and sinensitin in *Orthosiphon stamineus* extracts 57

2.2.10.2a Preparation of sample and standard solutions 57

2.2.10.2b Sample application 58

2.2.10.2bi For quantification of betulinic acid 58

2.2.10.2bii For quantification of sinensitin 59

2.2.10.2c Chromatographic conditions and documentation 59

2.2.11 Isolation of marker compounds from fruit ethanol extract of *Piper sarmentosum* 60

2.2.11.1 Column chromatography 60

2.2.11.2 Purification and characterization of the markers 61

2.2.12 Development and application of HPLC methods for quantitative analysis of *Piper sarmentosum* and *Orthosiphon stamineus* extracts 62

2.2.12.1 Analysis of *Piper sarmentosum* extracts 62

2.2.12.1a Chromatographic conditions 62

2.2.12.1ai For rutin and flavonone 62

2.2.12.1aii For pellitorine, sarmentine and sarmentosine 63

2.2.12.1b Preparation of standard solutions 63

2.2.12.1c Limit of detection, limit of quantification and linearity 63

2.2.12.1d Validation of HPLC method 64

2.2.12.1e Preparation of sample solutions and analysis 65

2.2.12.2 Analysis of *Orthosiphon stamineus* extracts 66

2.2.12.2a Chromatographic conditions 66

2.2.12.2b Preparation of standard solutions 66

2.2.12.2c Limit of detection, limit of quantification and linearity 67

2.2.12.2d Validation of HPLC method 67

2.2.12.2e Preparation of sample solutions and analysis 68

2.2.13 Statistical analysis 68

2.3 Results and discussion 69

2.3.1 Physicochemical analysis of *Piper sarmentosum* and *Orthosiphon stamineus* crude powders 69

2.3.2 Analysis of *Piper sarmentosum* and *Orthosiphon stamineus* crude powders by FTIR spectroscopy 70

2.3.3 Analysis of FTIR spectra of *Piper sarmentosum* and *Orthosiphon stamineus* crude powders by principal component analysis 72

2.3.4 Qualitative analysis of *Piper sarmentosum* and *Orthosiphon stamineus* extracts by UV and HPTLC 74

2.3.5 Estimation of total content of primary and secondary metabolites in *Piper sarmentosum* and *Orthosiphon stamineus* extracts 77

2.3.4.1 *Piper sarmentosum* 77

2.3.4.2 *Orthosiphon stamineus* 78

2.3.5 Validation of HPTLC method 79

2.3.6 Quantitative analysis of *Piper sarmentosum* and *Orthosiphon stamineus* extracts by HPTLC 82

2.3.7 Isolation and characterization of markers from fruit ethanol extract of *Piper sarmentosum* 92

2.3.8 Development and validation of HPLC method 100

2.3.9 Analysis of *Piper sarmentosum* extracts by HPLC 105

2.3.10 Analysis of *Orthosiphon stamineus* extracts by HPLC 107

2.4 Conclusion 107

CHAPTER 3: BIOLOGICAL ACTIVITIES 114

3.1 Antioxidant and hepatoprotective activities 114

3.1.1 Introduction 114

3.1.2 *In vitro* antioxidant activity of *Piper sarmentosum* extracts 116

3.1.2.1 Chemicals and solvents 116

3.1.2.2 Instruments 116

3.1.2.3 Plant samples 117

3.1.2.4 Free radical scavenging activity 117

3.1.2.5 Antiradical activities of the potent extract by DPPH model 117

3.1.2.6 Antioxidant activity by β-carotene linoleate model 118

3.1.3 Acute oral toxicity 119

3.1.3.1 Introduction 119

3.1.3.2 Material and methods 120

3.1.3.2a Preparation of doses of extracts 120

3.1.3.2b Animals, housing and feeding 121

3.1.3.2c Preparation of animals and dose administration 121

3.1.3.2d Acute oral toxicity of extracts 121

3.1.3.2e Observations 122

3.1.3.2f Body weight 122

3.1.3.2g Data or animal outcome and calculations of LD_{50} 122

3.1.4 *In vivo* antioxidant studies 122

3.1.4.1 Selection of dose 122

3.1.4.2 Preparation and grouping of animals 123

3.1.4.3 Preparation and administration of dose 123

3.1.4.4 Collection of samples 123

3.1.4.5 Determination of total protein content 124

3.1.4.6 Determination of total plasma antioxidant activity 124

3.1.4.7 Determination of superoxide dismutase 125

3.1.4.8 Determination of catalase levels 125

3.1.4.9 Determination TBARS levels 126

3.1.5 *In vivo* hepatoprotective studies 126

3.1.5.1 Determination of markers of hepatic toxicity 126

3.1.5.2 Hepatic histopathology 126

3.1.5.2a Preservation of the specimens and tissue processing 126

3.1.5.2b Embedding and microtomy 127

3.1.5.2c Staining by haematoxylin and eosin 127

3.1.6 Statistical analysis 128

3.1.7 Results and discussion 128

3.1.7.1 *In vitro* antioxidant activity of *Piper sarmentosum* 128

3.1.7.2 Acute oral toxicity 134

3.1.7.3 *In vivo* antioxidant studies of *Piper sarmentosum* extracts 135

3.1.7.4 *In vivo* hepatoprotective studies of *Piper sarmentosum* extracts 141

3.1.8 Conclusion 147

3.2 Antiangiogenic and cytotoxicity studies 150

3.2.1 Introduction 150

3.2.2 Materials and methods 152

3.2.2.1 Chemicals and solvents 152

3.2.2.2 Instruments 152

3.2.2.3 Animals and tissue preparation 152

3.2.2.4 Preparation of media 153

3.2.2.5 Antiangiogenic assay 153

3.2.2.6 Evaluation of angiogenesis inhibition 154

3.2.2.7 Cytotoxicity (MTT cell viability assay) 154

3.2.2.8 Evaluation of *Piper sarmentosum* extracts for antiangiogenesis 155

3.2.2.8a Plant material, extraction and fractionation 155

3.2.2.8b Preparation of samples and antiangiogenic studies 155

3.2.2.8c Dose response relationship studies 155

3.2.2.8d Preparation of samples and cytotoxicity studies 155

3.2.2.9 Characterization of the chloroform extract 156

3.2.2.10 Evaluation of *Orthosiphon stamineus* extract (*NHSIDE06*) for antiangiogenesis 156

3.2.2.10a Plant materials, sample preparation and antiangiogenic evaluation 156

3.2.2.10b Fractionation of (*NHSIDE06*) and antiangiogenic evaluation 156

3.2.2.10(c) Bioassay-guided isolation 157

3.2.2.10d Characterization of compounds 1-3 158

3.2.2.10e Evaluation of compounds 1-3 for antiangiogenesis 158

3.2.2.9f Dose response relationship of *n*-hexane fraction and compounds 1-3 158

3.2.2.11 Statistical analysis 158

3.2.3 Results and discussion 158

3.2.3.1 *Piper sarmentosum* 158

3.2.3.2 *Orthosiphon stamineus* 162

3.2.4. Conclusion 174

3.3 Evaluation of interaction between isoniazid and extracts of *Piper sarmentosum* and *Orthosiphon stamineus* 175

3.3.1 Introduction 175

3.3.2 Materials and methods 178

3.3.2.1 Chemicals 178

3.3.2.2 Microorganism and media 178

3.3.2.3 Preparation of inoculums 179

3.3.2.4 Determination of MIC 179

3.3.2.5 Interaction calculations 180

3.3.2.6 Interaction studies on *Piper sarmentosum* extracts 180

3.3.2.6a Plant materials 180

3.3.2.6b Sample preparation for anti-TB and Interaction evaluations 180

3.3.2.6c Determination of MIC 181

3.3.2.7 Determination of total amides 181

3.3.2.8 Interaction studies on *Orthosiphon stamineus* extracts 181

3.3.2.8a Plant materials 181

3.3.2.8b Sample preparation for anti-TB and interaction evaluations 181

3.3.2.8c Determination of MIC 182

3.3.2.9 Characterization of the extract and its fraction 182

3.3.2.10 Statistical analysis 182

3.3.3 Results and discussion 182

3.3.3.1 Interaction between INH and *Piper sarmentosum* extracts/fractions 182

3.3.3.2 Interaction between INH and *Orthosiphon stamineus* extracts/fractions 184

3.3.4 Conclusion 186

CHAPTER FOUR: PHARMACOKINETIC STUDIES OF *Piper sarmentosum* EXTRACTS 189

4.1 Introduction 189

4.2 Materials and methods 191

4.2.1 Chemicals and solvents 191

4.2.2 Instruments and chromatographic conditions 191

4.2.3 Limit of detection, limit of quantification and linearity 191

4.2.4 Extraction of markers from plasma, tissues, urine and fecal matter 191

4.2.5 Validation of HPLC method 193

4.2.6 Pharmacokinetic studies 193

4.2.6.1 Preparation of extracts and quantification of the markers 193

4.2.6.2 Animals and dosage 194

4.2.6.3 Collection of blood samples for absorption studies 194

4.2.6.4 Sampling for tissue distribution studies 195

4.2.6.5 Collection of urine and excreta 195

4.2.7 Analysis of samples of plasma, tissues, urine and fecal matter 196

4.2.8 Determination of pharmacokinetic parameters 196

4.2.9 Statistical analysis 197

4.3 Results and discussion 197

4.3.1 Development and validation of HPLC method 197

4.3.2 Pharmacokinetics parameters 198

4.4 Conclusion 201

CHAPTER 5: STABILITY STUDIES 209

5.1 Introduction 209

5.2 Stability studies on fruit ethanol extract of *Piper sarmentosum* 211

5.2.1 Materials 211

5.2.2 Instruments 212

5.2.3 Stability study protocol 212

5.2.4 Preparation of samples for analysis 212

5.2.5 Analysis of extracts using different methods 213

5.2.5a FTIR spectroscopy 213

5.2.5b HPTLC method 213

5.2.5c HPLC method 214

5.2.6 Calculations of chemical kinetics 214

5.2.6a Order of the reaction 214

5.2.6b Activation energy (Ea) 215

5.2.6c Shelf life (t_{90}) 215

5.2.7 Data analysis 216

5.3 Results and discussion 216

5.3.1 Stability by FTIR spectroscopy and principle component analysis 216

5.3.2 Stability study by high performance thin layer chromatography 218

5.3.3 Stability studies by HPLC and chemical kinetic parameters 219

5.3.3a Percentage remaining of the marker compounds 219

5.3.3b Determination of order of the reaction 222

5.3.3c Determination of rate constant (K) of the marker compounds and Arrhenius plot 222

5.3.3d Estimation of activation energy and pre exponential factor 223

5.3.3e Estimation of shelf life (t_{90}) 223

5.4 Conclusion 224

CHAPTER: 6 SUMMARY AND SUGGESTIONS FOR FUTURE WORKS 231

6.1 Summary 231

6.2 Suggestions for future work 234

REFERENCES 236

APPENDICES

A 1 Approval letter from the Animal Ethical Committee for *in vivo* activities, acute oral toxicity and pharmacokinetics 251

A 2 Approval letter from the Animal Ethical Committee for *in vitro/ex vivo* antiangiogenic studies 252

A 3 Antioxidant activity of different extracts of *Piper sarmentosum* by DPPH model and analysis by one way ANOVA 253

A 4 Concentration dependent response (FRSA) of the ethanol extract of the leaf of *Piper sarmentosum* by DPPH Method 254

A 5 Antioxidant activity of different extracts of *Piper sarmentosum* by β-carotene linoleate assay 255

A 6 Correlations between antioxidant activity and total content of metabolites 256

A 7a Acute oral toxicity of leaf ethanol extract of *Piper sarmentosum* at dose of 2000 mg/kg 257

A 7b Acute oral toxicity of fruit ethanol extract of *Piper sarmentosum* at dose of 2000 mg/kg 257

A 8 Total protein content in the liver homogenate and total plasma antioxidant activity in CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 258

A 9 Content of superoxide dismutase (SOD) and catalase (CAT) in liver homogenate of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 259

A 10 Content of thiobarbituric acid reactive species (TBARS) in liver homogenate and alanine aminotransferase (ALT) in rat plasma of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 260

A 11 Content of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in rat plasma of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 261

A 12 A 12 Content of lactate dehydrgenase (LDH) and total protein in rat plasma of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 262

A 13 Correlation of markers of antioxidant and hepatoprotective activities and total content of total flavonoids and total amides 263

B 1 Calibration curves of pellitorine, sarmentine and sarmentosine for Pharmacokinetic studies 264

B 2 HPLC chromatograms of mix standard solution of pellitorine, sarmentine and sarmentosine, plasma, tissues and fruit ethanol extracts of *Piper sarmentosum* At 260 nm 265

B 3 Pharmacokinetic data of pellitorine and sarmentine after oral dose of fruit ethanol extract *Piper sarmentosum* 266

B 4 Calibration curves for excretion studies and HPLC chromatograms of fecal matter and urine at different time intervals 267

C.1 Standard curves of markers used for stability studies of extract of *Piper sarmentosum* by high performance liquid chromatography 268

C 2 Order of the chemical reaction of pellitorine in ethanol extract of *Piper sarmentosum* 269

C 3 Order of the chemical reaction of sarmentosine in fruit ethanol extract of *Piper sarmentosum* 270

C.4 Order of the chemical reaction of sarmentine in fruit ethanol extract of *Piper sarmentosum* 271

LIST OF PUBLICATIONS, AWARDS AND SEMINARS 272

LIST OF TABLES

Page

Table 1.1 Chemical constituents of *Piper sarmentosum* 20

Table 1.2 Chemical constituents of *Orthosiphon stamineus* 31

Table 2.1 Physciochemical properties of different parts of *Piper sarmentosum* and leaves of *Orthosiphon stamineus* 70

Table 2.2 Recovery, intraday and inter day accuracy and precision values of rutin, naringenin, sinensitin and betulinic acid by HPTLC 81

Table 2.3 The content of rutin and naringenin in ethanol and aqueous extracts of different parts of *Piper sarmentosum* by HPTLC 85

Table 2.4 The content of betulinic acid and sinensitin in methanol and aqueous extracts of *Orthosiphon stamineus* by HPTLC 91

Table 2.5 Results of calibration, LOD and LOQ of rutin and flavonone by HPLC at 340 nm 104

Table 2.6 Recovery, intraday and inter day accuracy and precision values of rutin and flavonone by HPLC at 340 nm 104

Table 2.7 Results of calibration, LOD and LOQ of amides FK1 (pellitorine), FK2 (sarmentine) and FK3 (sarmentosine) by HPLC with UV detection at 260 nm 104

Table 2.8 Recovery, intraday and inter day accuracy and precision values of pellitorine (FK1), sarmentine (FK2) and sarmentosine (FK3) by HPLC, detection at 260 nm 106

Table 2.9 Recovery, intraday and inter day accuracy and precision values of betulinic acid by HPLC, detection at 210 nm 107

Table 2.10 The content of markers, rutin and flavonone, in extracts of different parts of *Piper sarmentosum* by HPLC 110

Table 2.11 Content of betulinic acid in different extracts of *Orthosiphon stamineus* by HPLC, detection at 210 nm 110

Table 2.12 Content of amides FK1 (pellitorine), FK2 (sarmentine) and FK3 (sarmentosine) in different parts of *Piper sarmentosum* on the basis of ethanol extraction and comparison ethanol and supercritical fluid extraction 111

Table 3.1 Total amide content of sequential extracts of leaf of *Pipre sarmentosum* and fractions of methanol extract (n=3) 162

Table 3.2 The values of MICs, FICs and FICIs of sequential leaf methanol extract of *Piper sarmentosum* and its fractions, isoniazid and combinations of isoniazid and the extracts/fractions by microplate tetrazolium assay 185

Table 3.3 The values of MICs, FICs and FICIs of extract of *Orthosiphon stamineus* and its fractions, isoniazid and combinations of isoniazid and the extract/fractions by microplate tetrazolium assay 186

Table 3.4 Content of marker compounds (mg/g) in extract and fractions of aqueous extract of *Orthosiphon stamineus* by HPLC at 210 nm (n=3) 186

Table 4.1 Recovery from plasma, intraday and inter day accuracy and precision values of pellitorine, sarmentine and sarmentosine detection at 260 nm 202

Table 4.2 Recovery of pellitorine, sarmentine and sarmentosine from urine, hepatic tissue and feces; detection at 260 nm 203

Table 4.3 Pharmacokinetic parameters of pellitorine after oral dose of fruit ethanol extract of *Piper sarmntosum* 204

Table 4.4 Pharmacokinetic parameter of sarmentine after oral dose of ethanol extract of fruit of *Piper sarmntosum* 205

Table 4.5 Cumulative excretion of pellitorine, sarmentine and sarmentosine in fecal matter and urine volume in experimental and control groups after oral dose of fruit ethanol extract of *Piper sarmentosum* 206

Table 5.1 Storage conditions for stability studies of drug substances by ICH and FDA 210

Table 5.2 Climatic conditions for stability testing 211

Table 5.3 Storage conditions for stability studies 213

Table 5.4 Percentage remaining of pellitorine in fruit ethanol extract of *Piper sarmentosum* stored for six months under different storage conditions 226

Table 5.5 Percentage remaining of sarmentine in fruit ethanol extract of *Piper sarmentosum* stored for six months under different storage conditions 226

Table 5.6 Percentage remaining of sarmentosine in fruit ethanol extract of *Piper sarmentosum* stored for six months under different storage conditions 226

Table 5.7 Rate constant, activation energy and pre exponential factor of the markers in fruit ethanol extract of *Piper saremntosum* at different temperatures 227

Table 5.8 Shelf life of the markers in ethanol extract of *Piper sarmentosum* at different storage conditions 227

LIST OF FIGURES

Fig. 1.1 Structures of some chemical constituents of *Piper sarmentosum* 25

Fig. 1.2 Structures of some chemical constituents of *Orthosiphon stamineus* 35

Fig. 2.1 Comparison of FTIR spectra of different parts of *Piper sarmentosum* in mid-IR range (4000- 400 cm^{-1}) 71

Fig. 2.2 Comparison of FTIR spectra of *Orthosiphon stamineus* leaves from four different batches in mid-IR range (4000- 400 cm^{-1}) 71

Fig. 2.3 Report of principle component analysis of FTIR fingerprints of *Piper sarmentosum* fruit from different batches collected from April, 2006 to August, 2007 72

Fig. 2.4 Comparison of FTIR spectra of *Piper sarmentosum* fruit from different batches 73

Fig. 2.5 Report of principle component analysis of FTIR fingerprints of *Orthosiphon stamineus* leaves from four different batches 74

Fig. 2.6a UV spectra of aqueous and ethanol extracts of different parts of *Piper sarmentosum* in a range (400-200 nm) 76

Fig. 2.6b UV spectra of methanol extracts of *Orthosiphon stamineus* leaves from four different batches (400-200 nm) 76

Fig. 2.7 Percentage total primary and secondary metabolic content in ethanol and aqueous extracts of various parts of *Piper sarmentosum* 78

Fig. 2.8 Percentage total primary and secondary metabolic content in aqueous and methanol extracts of *Orthosiphon stamineus* (NHSIDE06) 79

Fig. 2.9 HPTLC densitograms and spectra of rutin (standard) and ethanol extracts of different parts of *Piper sarmentosum* 83

Fig. 2.10 Images of HPTLC plates of rutin (standard) and ethanol extracts of of different parts of *Piper sarmentosum* at 366 and 254 nm 83

Fig. 2.11 HPTLC densitograms and spectra of rutin (standard) and aqueous extracts of different parts of *Piper sarmentosum* 86

Fig. 2.12 Images of HPTLC plates of rutin (standard) and aqueous extracts of of different parts of *Piper sarmentosum* at 366 and 254 nm 86

Fig. 2.13 HPTLC densitograms and spectra of naringenin (standard) and ethanol extracts of stem and leaf of *Piper sarmentosum* 87

Fig. 2.14 Images of HPTLC plates of naringenin (standard) and ethanol extracts of different parts of *Piper sarmentosum* at 254 and 366 nm 87

Fig. 2.15 HPTLC densitograms and images of HPTLC plates (254 and 366 nm) of naringenin (standard) and aqueous extracts of *Piper sarmentosum* 88

Fig. 2.16 HPTLC densitograms, spectra (400-200 nm) and image of plate (366 nm) of sinensitin (standard) and methanol extracts of *Ortosiphon stamineus* 90

Fig. 2.17 HPTLC desitograms and spectra (400-200 nm) of sinensitin (standard) and aqueous extract of *Orthosiphon stamineus* (NHSIDE06) 90

Fig. 2.18 HPTLC densitograms, spectra and image of the plate of betulinic acid (standard) and aqueous extract of *Orthosiphon stamineus* (NHSIDE06) 91

Fig. 2.19 FTIR spectrum, UV scan and structure of compound FK1 (pellitorine) 94

Fig. 2.20 Mass spectra (LC-MS and GC-TOFMS) and HPLC chromatogram of compound FK1 (pellitorine) 95

Fig. 2.21 FTIR spectrum, UV scan and structure of compound FK2 (sarmentine) 96

Fig. 2.22 Mass spectra (LC-MS and GC-TOFMS), HPLC chromatogram and ^{1}H NMR spectrum of compound FK2 (sarmentine) 97

Fig. 2.23 FTIR spectrum, UV scan and structure of compound FK3 (sarmentosine) 98

Fig. 2.24 Mass spectra (LC-MS and GC-TOFMS), HPLC chromatogram and ^{1}H NMR spectrum of compound FK3 (sarmentosine) 99

Fig. 2.25 HPLC chromatograms of mix standards (Rutin and flavonone) and extracts of *Piper sarmentosum* at 340 nm 109

Fig. 2.26 HPLC chromatograms of mix standards (pellitorine (1), sarmentine (2) and sarmentosine (3) and extracts of *Piper sarmentosum* at 260 nm 112

Fig. 2.27 HPLC chromatograms of different extracts of *Piper sarmentosum* at 260 nm 112

Fig. 2.28 HPLC chromatograms of betulinic acid (standard) and different extracts of *Orthosiphon stamineus* at 210 nm 113

Fig. 3.1 Antioxidant activity of ethanol and aqueous extracts of different parts of *Piper sarmentosum* by DPPH model 131

Fig. 3.2a Plot of different concentrations of leaf ethanol extracts of *Piper sarmentosum* versus % remaining of DPPH 131

Fig. 3.2b Dose response relationship of leaf ethanol extract of *Piper sarmentosum* 132

Fig. 3.3a Antioxidant activity of ethanol and aqueous extracts of different parts of *Piper sarmentosum* by β-carotene linoleate model 132

Fig. 3.3b Kinetics of ethanol and aqueous extracts of various parts of *Piper sarmentosum*, control and butylated hydroxy anisole (BHA) 133

Fig. 3.4a Aantioxidant activity (β- carotene linoleate and DPPH models) of ethanol and aqueous extracts of different parts of *Piper sarmentosum* and their correlation with total polyphenols and flavonoids 133

Fig. 3.4b Antioxidant activity (β- carotene linoleate and DPPH models) of ethanol and aqueous extracts of different parts of *Piper sarmentosum* and their correlation with total amides 134

Fig. 3.5 Total protein content (µg/mL) in liver of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 136

Fig. 3.6 Percent total plasma antioxidant activity of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 137

Fig. 3.7 Superoxide dismutase levels in hepatic tissue of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 139

Fig. 3.8 Catalase levels in hepatic tissue of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 139

Fig. 3.9 Values of thiobarbituric acid reactive species (TBARS) in hepatic tissue of CCl_4, control and treated groups with *Piper sarmentosum* extracts and vitamin-E 141

Fig. 3.10 Serum alanine transaminase content in CCl_4, control and treated groups with extracts of *Piper sarmentosum* and vitamin-E 142

Fig. 3.11 Serum aspartate transaminase content in CCl4, control and treated groups with extracts f *Piper sarmentosum* and vitamin-E 143

Fig. 3.12 Serum lactate alkaline phosphatase content in CCl_4, control and treated groups with extracts of *Piper sarmentosum* and vitamin-E 144

Fig. 3.13 Serum lactate dehydrogenase content in CCl_4, control and treated groups with extracts of *Piper sarmentosum* and vitamin-E 145

Fig. 3.14 Serum protein content in CCl_4, control and treated groups with extracts of *Piper sarmentosum* and vitamin-E 146

Fig. 3.15 Comparison of antiangiogenic activity of different extracts of leaf of *Piper sarmentosum*, fractions of methanol extract and compounds A1-3 160

Fig. 3.16 Dose response curve of leaf chloroform extract of *Piper sarmentosum* and cytotoxicity on HEP G2 cell line 161

Fig. 3.17 Percentage inhibition of angiogenesis by extract of *Orthosiphon stamineus* and its fractions and compounds 1-3 using rat aorta model 163

Fig. 3.18 Image of TLC plate and spectra of compound 1 (betulinic acid) by HPTLC, HPLC and LC-MS 165

Fig. 3.19 FTIR spectra of compound 1 (betulinic acid) and betulinic acid (standard) and its chemical structure 166

Fig. 3.20a Image of TLC plate and spectra of compound 2 (oleanolic acid) by HPTLC, HPLC, LC-MS and FTIR 168

Fig. 3.20b ^1H NMR, ^{13}C NMR and DEPT 90 spectra and chemical structure of compound 2 (oleanolic acid) 169

Fig. 3.21a Image of TLC plate and spectra of compound 3 (ursolic acid) by HPTLC, HPLC, LC-MS and FTIR 170

Fig. 3.21b ^{1}H NMR, ^{13}C NMR and DEPT 90 spectra and chemical structure of compound 3 (ursolic acid) 171

Fig. 3.22 Dose dependent response of HF and compounds 1-3 using rat aorta antiangiogenic assay 172

Fig. 3.23 Cytotoxicity of *n*-hexane fraction of aqueous extract of *Orthosiphon stamineus* (NHSIDE 06) using human hepatocarcinoma cells (Hep G2) by MTT assay 174

Fig. 4.1 Comparison of urine out put in 24 h between control and treated rats with *Piper sarmentosum* extracts 203

Fig. 4.2 Excretion profile of pellitorine, sarmentine and sarmentosine in feces after oral dose of *Piper sarmentosum* extracts 207

Fig. 4.3 Concentration of pellitorine and sarmentine in different tissues 6 h after oral dose of *Piper sarmentosum* fruit extracts 207

Fig. 4.4 Pharmacokinetic profile of sarmentine after oral dose of fruit ethanol extract of *Piper sarmntosum* 208

Fig. 4.5 Pharmacokinetic profile of pellitorine after oral dose of fruit ethanol extract of *Piper sarmentosum* extracts 209

Fig. 5.1 FTIR spectra of fruit ethanol extract of *Piper sarmentosum* stored at 60 ^{o}C/85% RH 217

Fig. 5.2 Analysis of FTIR fingerprints of fruit ethanol extracts of *Piper sarmentosum* stored at different conditions by principle component analysis 218

Fig. 5.3 HPTLC fingerprints of fruit ethanol extracts of *Piper sarmentosum* stored at 60^{0}C/85%RH at different intervals 220

Fig. 5.4 HPTLC profile (3D) of fruit ethanol extracts of *Piper sarmentosum* kept at different storage conditions for six months 221

Fig. 5.5a HPLC chromatograms of standards of pellitorine (1), sarmentine (2) and sarmentosine (3) at 260 nm 225

Fig. 5.5b HPLC chromatograms of fruit ethanol extract of *Piper sarmentosum* stored at 30 ^{o}C/45% RH 225

Fig. 5.6 Percentage remaining of pellitorine, sarmentine and sarmentosine in fruit ethanol extract of *Piper sarmentosum* at different storage conditions 228

Fig. 5.7 Plots of concentration (C %) versus time of pellitorine, sarmentine and sarmentine for the zero order reaction in fruit ethanol extract of *Piper sarmentosum* 229

Fig. 5.8 Plots of ln(k) versus 1/T (Kelvin^{-1}) of pellitorine, sarmentine and sarmentosine at various temperatures 230

LIST OF PLATES

Plate 1.1 Pictures of the plant of *Piper sarmentosum* Roxb. 44

Plate 1.2 Pictures of the plant of *Orthosiphon stamineus* Benth. 44

Plate 3.1 Pictures of hepatic tissue of a rat of CCl_4 group (negative control), control (untreated), vitamin-E treated (positive control), leaf extract treated at 500 mg/kg and fruit treated at 500mg/kg 148

Plate 3.2 Pictures of aorta ring of control and the treated with leaf extracts of *Piper sarmentosum* and its fractions 159

Plate 3.3 Pictures of aorta ring of the control and the treated with *n*-hexane fractions of *Orthosiphon stamineus* extract (NHSIDE06) and isolated compounds 162

Plate 3.4 Pictures of the 96 well plates of extracts, fractions and combinations of INH with extracts and fractions 188

LIST OF ABBREVIATIONS

AA	antioxidant activity
ABTS	2, 2- azino-di[3-ethylbenzthiazoline-6-sulfonate
ACN	acetonitrile
ALP	alkaline phosphatase
ALT	alanine amino tranferase
ARP	antiradical power
AST	aspartate transaminase
ASTM	American Society for testing and materials
ATCC	American type culture collection
AUC	area under the curve
AUFS	Absorbance Units Full Scale
BA	betulinic acid
BAW	*n*-butanol acetic acid water
BHA	butylated hydroxyl anisole
BSA	bovine serum albumin
BuOH	*n*-butanol
CCl_4	carbon tetrachloride
DC	de Candolle
CF	chloroform fraction
Cl	clearance
C_{max}	maximum plasma concentration
COX I	cycloxygenase 1

COX II	cycloxygenase II
CV	coefficient of variance
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
EDTA	ethylendiamine tetracetic acid
EF	ethyl acetate fraction
EMEA	European Agency for the Evaluation of Medicinal Products
EtOAc	ethyl acetate
FDA	food and Drug Administration
FIC	fractional inhibitory concentration
FICI	fractional inhibitory concentration index
FRSA	Free radical scavenging activity
FTIR	Fourier Transform Infrared Spectroscopy
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GIT	gastrointestinal tract
h	hour
HF	*n*-hexane fraction
HIFBS	heat inactivated fetal bovine serum
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
IACUC	Institutional Animal Care and Use Committee
IC	Inhibitory concentration

ICD	identification classification and differentiation
ICH	International Council of Harmonization
KCl	potassium chloride
K_{el}	elimination rate constant
LC-MS	liquid chromatography mass spectrometry
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
m/z	mass charge ratio
mAU	milli absorbance unit
MDA	malonaldehyde
MIC	minimum inhibitory concentration
Miq	Miquel
MMP	matrix metalloproteinase
MPa	megapascal
MRSA	multi resistant *Staphylococcus aureus*
MTT	[(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]
NBT	nitroblue tetrazolium
NMR	nuclear magnetic resonance
NP/PEG	natural product reagent/polyethylene glycol
OADC	oleic acid, albumin, dextrose and catalase
OECD	organization for economic co operation and development
PBS	phosphate buffer saline

PCA	principal component analysis
PDA	photodiode array
PKG	protein kinase G
PMS	phenazine methosulfate
psi	pounds per square inch
PTFE	polytetrafluoroethylene
QTN	quercetin
R_f	retardation factor
RH	relative humidity
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SC	supercritical
SD	standard deviation
SFDA	State Food and Drug Administration
SFE	supercritical fluid extraction
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
SNS	sinensitin
SOASA	superoxide anion scavenging activity
SOD	superoxide dismutase
SRB	sulforhodamine B
$T_{1/2}$	half life
TB	tuberculosis
TBA	thiobarbituric acid

TBARS	thiobarbituric acid reactive species
TLC	thin layer chromatography
T_{max}	time of maximum plasma concentration
TMF	3-hydroxy-2, 6, 7, 4 tetramethoxyflavone
TPPA	total plasma antioxidant activity
UK	United Kingdom
UV/VIS	ultraviolet and visible spectroscopy
VD	volume of distribution
WHO	World Health Organization
WPHMP	Working party of the Herbal Medicinal Products

KAJIAN ANALISIS, BIOLOGIKAL, FARMAKOKINETIK DAN KESTABILAN EKSTRAK *Piper sarmentosum* Roxb. DAN BEBERAPA KAJIAN TERPILIH EKSTRAK *Orthosiphon stamineus* Benth.

ABSTRAK

Tujuan kajian adalah untuk menjalankan kajian analisis dan biologi ke atas akar, batang, daun dan buah *Piper sarmentosum* dan daun *Orthosiphon stamineus*, farmakokinetik dan kajian kestabilan ke atas ekstrak etanol buah *Piper sarmentosum*. Bahan serbuk daripada bahagian yang berlainan *Piper sarmentosum* diekstrak menggunakan etanol dan air manakala daun diekstrak berjujukan menggunakan petroleum eter, kloroform dan metanol. Daun *Orthosiphon stamineus* diekstrak dengan metanol dan beberapa ekstrak lain diperolehi daripada sumber berlainan. Ekstrak kedua-dua tumbuhan dianalisa secara kualitatif dan kuantitatif menggunakan spektroskopi Inframerah Terubah Fourier (FTIR) dan spektroskopi Ultralembayung/Tampak (UV/Vis), kromatografi lapisan nipis prestasi tinggi (HPTLC) dan kromatografi cecair prestasi tinggi (HPLC). Ekstrak akueous dan etanol daripada perbagai bahagian *Piper sarmentosum* dikaji untuk aktiviti antioksida *in vitro* dan ekstrak yang mempunyai aktiviti baik, iaitu ekstrak etanol buah dan daun, dinilai secara *in vivo* bagi aktiviti antioksida dan perlindungan hepatik menggunakan model stress oksidatif teraruh CCl_4. Ekstrak berjujukan daun *Piper sarmentosum,* ekstrak methanol berfraksi, ekstrak akueous *Orthosiphon stamineus* dan fraksinya dikaji untuk aktiviti antiangiogenik dan interaksi. Ekstrak etanol buah *Piper sarmentosum* dikaji untuk farmakokinetik dan kestabilan dipercepat dengan menggunakan pelitorin, sarmentin dan sarmentosin sebagai penanda.

Analisis kualitatif menunjukkan kehadiran flavonoid dan alkaloid di dalam ekstrak etanol bahagian berbeza *Piper sarmentosum* manakala flavonoid dan

triterpena terdapat di dalam ekstrak *Orthosiphon stamineus*. Analisis kuantitatif berbagai ekstrak *Piper sarmentosum* menggunakan HPTLC menunjukkan jumlah berbeza-beza rutin (0.0004-0.0109 mg/g) dan narigenin (0.010-0.659 mg/g). Analisis HPTLC ekstrak berbagai *Orthosiphon stamineus* menunjukkan jumlah berbeza-beza asid betulinik (0.013-0.124 mg/g) dan sinensitin (0.470-1.335 mg/g).

Kaedah HPLC baru dibangunkan, divalidasi dan digunakan untuk penentuan kuantitatif serentak rutin dan flavonon dalam ekstrak *Piper sarmentosum* yang menunjukkan amaun rutin antara 0.20-5.02 mg/g dan flavonon 0.32-15.32 mg/g. Kaedah HPLC lain dibangunkan, divalidasi dan digunakan untuk penentuan kuantitatif serentak pellitorin, sarmentin dan sarmentosin dalam ekstrak *Piper sarmentosum* yang menunjukkan amaun pellitorin antara 0.043-6.820 mg/g, sarmentin 0.006-0.420 mg/g dan sarmentosin 0.005-0.120 mg/g. Kaedah HPLC yang terdahulu untuk penentuan kuantitatif asid betulinik diperbaiki, divalidasi dan digunakan untuk analisis pelbagai jenis ekstrak *Orthosiphon stamineus* yang mempamerkan amaun asid betulinik antara 2.76-9.50 mg/g.

Ekstrak etanol buah dan daun *Piper sarmentosum* mempamerkan aktiviti antioksida *in vitro* yang baik di dalam model DPPH dengan IC_{50} pada 25.87 dan 23.66 ug/mL, masing-masing. Kedua-dua ekstrak menunjukkan aktiviti yang baik dalam model β-karotena linoleat. Aktiviti antioksida ekstrak didapati mempunyai korelasi dengan jumlah kandungan polifenol, flavonoid dan amida ($P<0.05$)

Kajian ketoksikan akut oral ekstrak etanol buah dan daun *Piper sarmentosum* menunjukkan median dos kematian (LD_{50}) melebihi 2000 mg/kg dalam tikus.

Ekstrak kedua-dua bahagian dikaji *in vivo* untuk aktiviti antioksida dan perlindungan hepatik pada dua aras dos 250 dan 500 mg/kg. Kumpulan-kumpulan prarawat mempamerkan pengekalan signifikan aktiviti antioksida dan penanda fungsi hati berbanding dengan kumpulan kontrol negatif (CCl_4) ($P<0.05$).

Ekstrak kloroform daun *Piper sarmentosum* dan fraksi n-heksana ekstrak akueous *Orthosiphon stamineus* mempamerkan 100 dan 80% aktiviti antiangiogenik, masing-masing dengan IC_{50} pada 45 ug/mL dan IC_{50} pada 45 ug/mL. Amida yang diasingkan daripada buah *Piper sarmentosum*, pelitorin, sarmentin dan sarmentosin mempamerkan 30% aktiviti antiangiogenik manakala sebatian terasing daripada fraksi n- heksana *Orthosiphon stamineus*, asid betulik, oleanolik dan ursolik mempamerkan 100% aktiviti antiangiogenik. Dalam kajian sitotoksisiti, ekstrak kloroform dan fraksi n- heksana masing-masing menunjukkan IC_{50} pada 76.24 dan 80 ug/mL, adalah lebih tinggi berbanding IC_{50} antiangiogenesis.

Fraksi kloroform dan etil asetik ekstrak methanol daun *Piper sarmentosum* menunjukkan aktiviti antimikobakteria dengan kepekatan perencatan minimum (MIC) 3.12 ug/mL manakala fraksi n- heksana ekstrak akueous *Orthosiphon stamineus* menunjukkan aktiviti MIC 3.12 ug/mL. Dalam kajian interaksi, ekstrak dan fraksi kedua-dua tumbuhan tidak menunjukkan interaksi dengan isoniazid kerana indeks kepekatan perencatan fraksi (FICI) adalah >0.5.

Kaedah HPLC baru telah dibangunkan, divalidasi dan digunakan untuk menentukan serentak pelitorin, sarmentin dan sarmentosin daripada plasma, urin, jirim najis dan tisu. Dalam kajian farmakokinetik ekstrak etanol buah *Piper* *sarmentosum* dalam tikus, pelitorin dan sarmentin menunjukkan bioperolehan oral yang baik manakala sarmentosin tidak diserap secara oral dan dikumuhkan tidak berubah dalam najis. Pelitorin dan sarmentin mempamerkan activiti tisu berbeza dan dikumuhkan di dalam urin sebagai metabolit.

Dalam kajian kestabilan dipercepat, anggaran hayat simpanan (t_{90}) pelitorin, sarmentin dan sarmentosin adalah kira-kira 16 bulan pada 25°C. Penanda menuruti tindak balas tertib sifar dan kestabilannya didepati menurun pada suhu tinggi dan kelembapan relatif (RH).

Kesimpulannya, kaedah HPLC yang dibangunkan adalah mudah dan senang dilakukan dan boleh digunakan untuk pempiawaian. Ekstrak *Piper sarmentosum* menjanjikan aktiviti antioksida dan perlindungan hepatik. Ekstrak kloroform *Piper sarmentosum* dan fraksi n-heksana ekstrak akueous *Orthosiphon stamineus* menjanjikan aktiviti antiangiogenik. Ekstrak kedua-dua tumbuhan tidak berinteraksi dengan isoniazid.

ANALYTICAL, BIOLOGICAL, PHARMACOKINETIC AND STABILITY STUDIES OF *Piper sarmentosum* Roxb. EXTRACTS AND SELECTED STUDIES OF *Orthosiphon stamineus* Benth. EXTRACTS

ABSTRACT

The study aimed to perform analytical and biological activity studies on root, stem, leaf and fruit of *Piper sarmentosum* and leaves of *Orthosiphom stamineus,* pharmacokinetic and stability studies on ethanol extract of fruit of *Piper sarmentosum.* Powdered material of different parts of *Piper sarmentosum* was extracted using ethanol and water while leaves were also extracted sequentially using petroleum ether, chloroform and methanol. Leaves of *Orthosiphom stamineus* were extracted with methanol and few prepared extracts were obtained from different sources. The extracts of both the plants were analyzed qualitatively and quantitatively using Fourier Transform Infrared (FTIR) and ultraviolet/visible (UV/Vis) spectroscopy, high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC). Aqueous and ethanol extracts of different parts of *Piper sarmentosum* were investigated for *in vitro* antioxidant activity and the extracts having good activity namely fruit and leaf ethanol extracts were evaluated for *in vivo* antioxidant and hepatoprotective activity using CCl_4 induced oxidative stress model. Sequential extracts of *Piper sarmentosum* leaf and fractions of the methanol extract, aqueous extract of *Orthosiphom stamineus* and its fractions were investigated for antiangiogenic and interaction studies. Ethanol extract of the fruit of *Piper sarmentosum* was investigated for pharmacokinetic and accelerated stability studies using pellitorine, sarmentine and sarmentosine as markers.

Qualitative analysis indicated the presence of flavonoids and alkaloids in ethanol extracts of different parts of *Piper sarmentosum* while flavonoids and triterpenes in extracts of *Orthosiphom stamineus*. Quantitative analysis of different extracts of *Piper sarmentosum* by HPTLC indicated varying amaunts of rutin (0.0004-0.0109 mg/g) and naringenin (0.010-0.659 mg/g). The HPTLC analysis of different extracts of *Orthosiphon stamineus* indicated varying amaunts of betulinic acid (0.013-0.124 mg/g) and sinensitin (0.470-1.335 mg/g).

A new HPLC method was developed, validated and applied for the simultaneous quantification of rutin and flavonone in *Piper sarmentosum* extracts which indicated varying amaunt of rutin ranging 0.20-5.02 mg/g and flavonone 0.32-15.32 mg/g. Another HPLC method was developed, validated and applied for the simultaneous quantification of pellitorine, sarmentine and sarmentosine in *Piper sarmentosum* extracts which indicated varying amaunts of pellitorine ranging 0.043-6.820 mg/g, sarmentine 0.006-0.420 mg/g and sarmentosine 0.005-0.120 mg/g. A previously used HPLC method for the quantification of betulinic acid was improved, validated and applied to analyse different extracts of *Orthosiphon stamineus* which exhibited varying amaunts of betulinic acid ranging 2.76-9.50 mg/g.

Ethanol extracts of the fruit and leaf of *Piper sarmentosum* exhibited good *in vitro* antioxidant activities in DPPH model with IC_{50} at 25.87 and 23.66 µg/mL, respectively. Both the extracts have also shown good activity in β-carotene linoleate model. The antioxidant activity of the extracts was found to be correlated with total content of polyphenolics, flavonoids and amides ($P < 0.05$).

Acute oral toxicity studies of the fruit and leaf ethanol extracts of *Piper sarmentosum* indicated median lethal dose (LD_{50}) above 2000 mg/kg in rats. The extracts of both the parts were investigated for *in vivo* antioxidant and hepatoprotective activities in two dose levels 250 and 500 mg/kg. The pretreated groups exhibited significant preservation of antioxidant activity and liver function markers as compared to negative control (CCl_4) group ($P < 0.05$).

Chloroform extract of the leaf of *Piper sarmentosum* and *n*-hexane fraction of aqueous extract of *Orthosiphon stamineus* exhibited 100 and 80% antiangiogenic activity with IC_{50} at 45 µg/mL and IC_{50} at 45 µg/mL, respectively. Amides isolated from the fruit of *Piper sarmentosum,* pellitorine, sarmentine and sarmentosine, exhibited 30 % antiangiogenic activity while the isolated compounds from the *n*-hexane fraction of *Orthosiphon stamineus*, betulinic, oleanolic and ursolic acids, exhibited 100% antiangiogenic activity. In cytotoxicity studies, the chloroform extract and the *n*-hexane fraction have shown IC_{50} at 76.24 and 80 µg/mL, respectively, which are higher as compared to IC_{50} of antiangiogenesis.

Chloroform and ethyl acetate fractions of the leaf methanol extract of *Piper sarmentosum* have shown antimycobacterial activity with minimum inhibitory concentration (MIC) 3.12 µg/mL while *n*-hexane fraction of aqueous extract of *Orthosiphon stamineus* has shown the activity MIC 3.12 µg/mL. In interaction studies, the extracts and the fraction of both the plants have not shown any interaction with isoniazid because fractional inhibitory concentration index (FICI) was found to be > 0.5.

A new HPLC method was developed, validated and applied for the simultaneous determination of pellitorine, sarmentine and sarmentosine from plasma, urine, fecal matter and tissues. In pharmacokinetic studies of the fruit ethanol extract of *Piper sarmentosum* in rats, pellitorine and sarmentine showed good oral bioavailability while sarmentosine was not absorbed orally and excreted unchanged in feces. Pellitorine and sarmentine exhibited different tissue affinities and were excreted in urine as metabolites.

In accelerated stability studies, estimated shelf life (t_{90}) of pellitorine, sarmentine and sarmentosine was approximately 16 months at 25 ^{o}C. The markers followed the zero order reaction and their stability was found to be decreasing at high temperature and relative humidity (RH).

The results of the study indicates that the developed HPLC methods are simple, easy to perform and can be applied for standardization. Extracts of *Piper sarmentosum* have promising antioxidant and hepatoprotective activity. Chloroform extracts of *Piper sarmentosum* and *n*-hexane fraction of aqueous extract of *Orthosiphon stamineus* have promising antiangiogenic activity. The extracts of both the plants have no interaction with isoniazid.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 THERAPEUTIC POTENTIAL OF FLORA

The use of natural flora in the treatment of different ailments is centuries old. The evidence of the use of natural products in therapeutics can be traced back for at least 5000 years (Goldman, 2001). The development of modern drugs is owed to the traditional use of natural substances. About 40% of the new drugs approved in North America in a period of 1983-1994 were derived from natural compounds (Simmonds, 2003). Approximately 70% of the new chemical entities reported from 1981-2006 resulted from the study of natural products (Newman and Cragg, 2007). Documentation of more than 85000 plant species globally for medical use indicates the interest of scientists and healthcare professionals in natural products (Balunas and Kinghorn, 2005).

The plants have played an important role in maintaining health by providing molecules and products to combat diseases. Many plants and plant based products are being used as folk remedies in the form of fresh or dried plant materials and in the form of extracts. According to the WHO report, 75% population of the world has therapeutic experience with herbal products (Dubey *et al.,* 2004).

In spite of many developments in synthetic chemistry, cultivation and utilization of medicinal plants is increasing due to their use as traditional medicine. Moreover, despite the development of various approaches for drug development, plants still are one of the best reservoirs of new structural entities. A single plant contains a large number of compounds which can be used as a starting material for the synthesis of novel drugs. Among 250,000 plant species, only a small percentage has been investigated for bioactive compounds (Prance, 1977). It means that the potential of plants as a source of new drugs has not explored completely.

Traditional medicine represents a vast array of therapies with proven benefits for the prevention and cure of different ailments. Nowadays, these are being used along with modern medicines in many countries. The combination of traditional and modern medicines is playing an important role in healthcare. Due to this fact herbal medicines are making a strong comeback in many countries (WHO, 2001). The increase in the popularity of herbal remedies is due to increase in the cost of treatment with modern medicines, fear of their side effects and new appreciation of natural remedies, which represents alternative healthcare movement. Due to this reason, the demand of herbal products has increased tremendously in the world market especially among ageing young generation, eagerly seeking preventive treatment of various diseases.

The actual global market size of herbal products is difficult to assess because sale of these products is not regulated and accurate statistics are not available. Till 1988, only 14 of the WHO member countries regulated the sales of herbal products and since then, 53 countries were added in the list with another 42 in the process of developing relevant regulations by 2003 (Voigt, 2006). According to a survey conducted in 2004, more than one third of the American adults use alternative medicine (Voigt, 2006). This consumer driven movement has expanded the market of natural products significantly. The worldwide sale of the medicinal plants, crude extracts and finished products was 15 billion US dollars in 1999 (Raskin *et al.,* 2002), which increased up to 32 billion US dollars in 2002 (Dubey *et al.,* 2004). According to the World Bank projection, with annual increase of 5-15%, the current market size of herbal products is about 60 billion US dollars per year (Voigt, 2006).

The use of herbs varies from country to country and every country tends to have its own particular herbs. For example, products containing Echinacea or ginkgo are popular in Germany. Feverfew, garlic, ginseng, passiflora and valerian are used more in the United Kingdom. The use of these remedies is primarily for the management or prevention of chronic age related diseases and to improve the quality and longevity of life. The pathophysiology of these conditions is completely unknown and for which presently available synthetic drugs are rarely preventive or curative (Mitscher *et al.,* 2000).

The development of spectroscopic methods for the elucidation of chemical structure of naturally occurring compounds together with development of biological sciences have opened a new era to study structure activity relationship. These developments have made possible to prepare derivatives or synthetic analogues using natural compounds as models.

1.2 ETHNOBOTANY OF MALAYSIA

Malaysia is situated in the tropics, between 1 and 6 degree north and consists of East Malaysia (Sabah and Sarawak) in the northern part of the Borneo, bordering Indonesia and South China Sea and West Malaysia, peninsula south of Thailand. The region is mountainous with peaks rising up to 2000 m. The climate has characteristic high temperature and rainfall around the year. Temperature does not vary much from month to month and there is no significant variation in the daily temperature.

The total area under the Malaysian rainforest is around 19.12 million hectares and it covers 58.1% of the land. This area is a habitat of more than 20000 plant species and out of which 2000 species are reported to have medicinal properties (Jaganath and Ng, 2000).

Many Malaysians have broad traditional knowledge about folklore uses of plants and plant derived preparations. Malaysian population is a multi-ethnic and multi-cultural comprising of three main ethnic cultures, the Malays, the Chinese and the Indian. These ethnic sects have intermixed for the past centuries but each ethnic sect has its own distinct healthcare system. The plants, which were brought by the Chinese and the Indians have adopted, naturalized and hybridized very well in the local conditions. A similar diversity of traditional medicine systems also exists, including the Malay, the Indian and the Chinese folk medicine. The three main races have their own traditions of treatment, traditional knowledge of medicinal plants and collection of herbs. A report on the utilization of plants in traditional medicine in peninsular Malaysia was published, describing the use of 543 remedies to cure various disorders (Burkhill, 1935). Another version was published on the Malay village medicine describing 650 plant species of 116 families (Burkhill, 1966). In this publication, 20 species of *Annonmaceae*, 20 species of *Zingiberaceae*, 40 species of *Rubiaceae*, 54 species of *Legunminosae* and 932 species of *Euphorbiaceae* were reported as useful medicinal plants.

The Malaysian flora is a huge reservoir of genetic diversity and a source of wealth in terms of chemicals that can be used in healthcare. There has been a great interest in the investigation of medicinal plants. The research is being carried out to develop modern drugs from local plants.

1.3 PHYTOCHEMICAL ANALYSIS

Phytochemical analysis is concerned with chemical characterization of plant materials and includes qualitative and quantitative analysis. The former deals with the identification of constituents present in crude drugs while the later deals with the determination of how much of one or more substances are present in the crude drug. Furthermore, it also includes the development and validation of analytical techniques and isolation and characterization of characteristic compounds, which can be used as markers for standardization. Therefore, analytical studies are of prime importance to produce standardised formulations for preclinical and clinical studies. Unlike synthetic compounds, analytical methods and standards are not adequate for herbal drugs, which is the main constraint of natural product industry.

1.3.1 Standardisation

More than 80% population of the developing countries relies on the use of herbal/traditional medicines in their primary healthcare because these are economical and time tested. The WHO (World Health Organization) encourages, recommends and promotes the use of traditional and herbal remedies in National Healthcare Program because these drugs are easily available at low cost, comparatively safer and people have faith in such remedies (Wani, 2007). Plant materials and herbal remedies have a substantial proportion in the global drug market. Therefore, an internationally recognized quality assessment of herbal products is necessary. The WHO in a number of resolutions has emphasized the need to ensure quality control of herbal products using modern analytical techniques because herbal products of well defined constituents are required for clinical trials. Therefore, to make herbal preparation a reliable and affordable medicine, the product must be standardized applying all the standards of quality control like any synthetic drug. Advances in chemical and biological techniques during the past 50 years have resulted in scientific evidence to substantiate the use of herbal products and have enabled manufacturers to produce evidence based standardized products (Phillipson, 2003).

Standardisation is a process to maintain consistency in claimed efficacy of a product and its batch-batch reproducibility. Standardisation of herbal preparations is a difficult task due to unavailability or inadequacy of standards and methods of analysis. Lack of standardisation is a biggest hindrance in the wider acceptance of herbal products. Moreover, it also prevents modernization or modification of production methods because there is no way to establish the equivalence between a product made by modified and the original method. Standardised herbal products have several advantages as compared to crude extracts because it assures the identification that "the herb is what it is claimed to be".

In contrast to single entity based pharmaceuticals, most of the herbal remedies lack in scientific evidence and are only based on realm and myth. In order to bring botanical products in mainstream of pharmaceutical market, solid scientific evidence is needed to support the functionality claims of the products. The major challenges in terms of scientific standardisation to adhere to industry norms are variation in the source, unknown active ingredients, lack of safety evaluations, difficulty in quality control and unclear mechanism of actions (Wang and Ren, 2002). Therefore, it is imperative to analyze plant materials to prepare standardised herbal products.

Unlike synthetic drugs, precise standardisation of herbal medicine is not easy due to unknown nature of the constituents. Synthetic drugs have well known constituents while the active components in many herbs are unknown. The use of markers helps in standardisation of herbal products. Markers were first classified into three main categories as active principles, active markers and analytical markers. Active principles are the constituents of the extract with proven activity. Active markers are the constituents which have known pharmacological activity and contribute to some extent to efficacy. However, their clinical efficacy may not be proven. Analytical markers are the constituents which are chosen when neither active principles nor active markers are available. Recently, markers have been classified into eight categories such as therapeutic components, bioactive components, synergistic components, characteristic components, main components, correlative components, toxic components and general components (Li *et al.*, 2008). The markers help in positive identification or standardisation of extracts (United States Pharmacopoeia, 2005). The identification of active principles/active markers of herbal products would be the best solution but when these are not possible, standardisation can be based on markers of different chemical classes to ensure that the extract is more representative of the original plant material.

Standardisation of herbal products can be divided into two categories. First, an active constituent extract, where biochemical principles are known and have therapeutic values, and second, a marker extract, where the active principle is not known and a characteristic compound is used as a marker to assess the presence of other biochemical compounds that make the extract active (Tierra, 2008). In active constituent extracts, the known biochemical compound(s) is isolated. But this type of standardisation has limitations that only isolated compound(s) is considered, ignoring the whole constituents of the herb which may have synergistic or buffering activities to reduce the side effects. In marker extract standardisation, where active principle is not known, partly known or mix preparation containing many crude drugs or extracts, the whole formulation is considered active in the presence of all plant constituents. In this type of products, a single isolated compound would not be used as a marker because it is not unique enough to any one plant. The standardisation of herbal drug(s) is not mere an analytical operation which ends with the identification and assay of the active principle(s) rather it embodies total information and controls which are necessary to insure consistency in composition. Therefore, it is necessary to use all possible means of standardisation like TLC, HPLC and other related techniques to quantify active constituents and other components of the extract such as total content of primary and secondary metabolites.

Metabolomic fingerprint profiling is an alternative way to standardize herbal products. Metabolomics, a system of cell biology, comprise of all the compounds other than proteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Metabolomics, detect, quantify and catalogue the time related metabolic processes of an integrated biological system under specified conditions (Zhou *et al.,* 2006). Metabolomic fingerprints are unique patterns, which indicate the presence of particular molecules based on specialized analytical techniques and are extremely useful to identify active components, contaminants and other chemicals present in herbal products (Fan *et al.,* 2006).

Many international authorities and agencies including the World Health Organization, the European Agency for Evaluation of Medicinal Products and the European Scientific Cooperation of Phytomedicine, the US Agency for Healthcare Policy and Research and the European Pharmacopoeia Commission have started creating a new mechanism for quality control and standardisation of botanical medicines. A botanical formulation is now regarded as active substance in its entity whether or not the constituents with therapeutic activity are known. This is a major step in the development of new generation of standardized herbal medicines. Several spectroscopic and chromatographic techniques like UV, FTIR, TLC, HPLC, LC/GC-MS and NMR are used to obtain fingerprints. The fingerprints in combination with chemometrics provide effective tool of identification, classification and discrimination (Sherma and Fried, 2003). The characterization of metabolic profiles not only helps in identification of active constituents but also improve the knowledge about efficacy, safety and complexity of a given therapeutic combination (Liu and Yang, 2006). Therefore, analytical studies on herbal drugs are very important to fill the big gap between indigenous herbal practices and contemporary medical sciences.

1.3.2 Development and validation of analytical methods

Great care must be taken that accurate results are obtained in an analytical procedure. Two types of error, random and systemic, may occur. Every measurement has some imprecision associated with it which results in random distribution of results. The method should be designed in such a way to narrow the range of random errors. Systemic error is an error that biases a result in one direction. New developed analytical method must be validated to get accurate results.

1.3.2.1 Validation

According to FDA guidelines on process validation (FDA, 1987), validation is the assessment of a process or instrument to assure that the process and instrument is suitable for its intended use. Validation enables an efficient and productive use of the process and instrumental variables. The objective of validation of HPTLC/HPLC method is to demonstrate that the method is suitable for quantification of analyte under prevailing experimental conditions. A new method development, change in the operators, laboratory and equipments than the one in previous method require validation. A typical validation of the method is given as follows:

1.3.2.1a Linearity and calibration curves

Linearity is the ability of the method to obtain test results that are directly proportional, without or after mathematical transformation to the analyte's concentration within a given range (United States Pharmacopoeia, 1999). The range is established by confirming that the analytical procedure provides an acceptable degree of linearity when applied to samples containing amounts of analyte within or at the extremes of the specified range of analytical procedure.

Linearity of the method is evaluated by plotting concentration versus peak area/height. Each standard is analyzed in triplicate. Calibration curves are constructed on five data points and linearity is evaluated by correlation coefficient and standard deviation. Standard curves are used to get linear regression equation, which is obtained in the form given as follows:

$$Y = mx + b \ldots\ldots\ldots\ldots\ldots\ldots 1.1$$

Where Y is the peak area/height, m is the slope of the standard curve, x is the concentration of analyte and b is the y-intercept of the line of standard curve.

1.3.2.1b Precision

Precision is a measure of the degree of repeatability of an analytical method under normal operation and is expressed in relative standard deviation (Ott and Longnecker, 2001). Precision can also be defined as the degree of agreement among individual results when analytical method is applied repeatedly to a homogenous sample (United States Pharmacopoeia, 2005). The precision in different official books has recently been reviewed (Walter *et al.*, 2008). Precision can be subdivided into repeatability, intermediate precision, and reproducibility.

Repeatability is the results of the method operating over a short time interval under the same conditions (inter-assay precision). It is determined from 3-5 replicate at 3 different concentrations i.e. low, medium and high covering the expected concentration range of analyte (United States Pharmacopoeia, 1999; ICH, 2005). Intermediate precision arises from within laboratory variations due to random events such as different days, equipment, columns, reagents, etc. The experiment is design carefully in determining intermediate precision to monitor the possible effects of the individual variables. Reproducibility is assessed by means of an inter-laboratory trial and measured as relative standard deviation (RSD) or confidence interval (CI).

1.3.2.1c Capacity factor

Capacity factor is an important parameter used widely to describe migration rate of a solute in a column. It is denoted by (k') and is calculated by using following equation.

$$k' = (t_R - t_M) / t_M \ldots\ldots\ldots\ldots\ldots\ldots 1.2$$

Where t_R is the peak retention time and t_M is the solvent retention time and is also called dead time.

1.3.2.1d Resolution

Resolution is a quantitative measure of the ability of a column to separate two analytes. It is denoted by “Rs” and given by the following expression.

$$R = 2\,(t_{R2} - t_{R1}) / w_1 + w_2 \ldots\ldots\ldots\ldots\ldots 1.3$$

Where t_{R1} is the retention time of first peak, t_{R2} is the retention time of the second peak, w_1 is peak width of peak 1 and w_2 is peak width of peak 2.

1.3.2.1e Accuracy

Accuracy is degree of agreement between true values of analyte in sample and the observed value by the method (ICH, 2005). FDA recommends establishment of accuracy by minimum of 5 determinations for at least across the 3 levels (low, medium and high) in the range of expected concentrations. The accuracy is acceptable if the agreement of true and observed values is less than 15%. It is determined as the percent of analyte recovered by analytical method after spiking samples in a blind study.

1.3.2.1f Sensitivity

Sensitivity of the method is evaluated by the limit of detection (LOD) and limit of quantification (LOQ). The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected, but can not inevitably be quantified. The limit of quantification (LOQ) is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The LOQ is verified by analyzing the standard 6 times at the LOQ concentration. Various approaches are available to determine the LOD. Usually used equations for the determination of LOD and LOQ (Stewart and Ebel, 2000) are given as follows:

$$\text{LOD} = 3.3\sigma / S \quad \ldots\ldots\ldots\ldots\ldots\ldots 1.4$$

$$\text{LOQ} = 10\,\sigma / S \quad \ldots\ldots\ldots\ldots\ldots 1.5$$

Where σ is the standard deviation of intercept of six standard curves and S is the mean slope of the standard curves.

1.3.2.1g Specificity and system suitability

Specificity is a measure of the degree of interference from such entities as blank plasma, other active ingredients, excipients, impurities and degradation products. Specificity ensures that peak response of an analyte is due to its own and there is no co-elution (Vessman, 1996). System suitability is a test used to verify that the precision and accuracy are adequate for analysis to be done (United States Pharmacopoeia, 2005).

1.3.2.1h Peak purity

Peak purity is determined by spiking a solution of the extract with standard solution of the marker. The spiked solution and untreated solution are analyzed to determine the peak overlap of the obtained chromatograms. Peak purity is further established by using Photodiode Array (PDA), LC/GC-MS. The molecular ion of the mass spectrum of the peak of standard is compared with corresponding peak of the sample. In HPLTC, peak purity is determined by scanning the spots in a range of 700-200 nm and spectra of the standards and sample are compared.

1.3.2.1i Robustness

Robustness, a measure of method reliability, is the capacity of a method to remain unaffected after small deliberate variations in method parameters. The robustness of the method is evaluated by varying method parameters such as percent mobile phase proportions, pH, ionic strength and temperature etc., and determining the effect (if any) on the results. If the results or other measurements are susceptible to variations in method parameters, then these parameters should be controlled adequately.

1.4 THE DISCOVERY OF HERBAL DRUGS

Two approaches are being used to verify the claims of traditional medicine scientifically and to develop new drugs, which would be acceptable to modern system of medicine (Patil, 2003). The first approach involves direct clinical trials of commonly used traditional remedies followed by chemical and pharmacological studies. The second approach involves broad spectrum screening of individual plants followed by chemical, pharmacological, preclinical and clinical drug development studies.

1.4.1 Phytochemical and taxonomical screening

There are many reports in the literature on the screening of randomly collected plants from various geographical localities for a group of compounds. Data derived from such screening reports show little value in the discovery of active natural products. Another approach to search biologically active compounds in plants is to consider the taxa in which known useful drugs or derivatives occur. This is not much exciting path to follow in drug development, if novel agents are desired. However, it leads to identify new indigenous sources of known useful drugs which may lower the cost of drugs.

1.4.2 *In vitro* biological studies

Extracts of the plants are subjected to preliminary screening for biological activity using *in vitro* or *ex vivo* models. After preliminary screening, extracts having significant activity are further investigated for pharmacological activities using suitable models. The active extracts are also subjected to assay guided isolation for pure compounds, which may serve as potent drug(s) or drug prototype(s).

1.4.3 *In vivo* biological studies

The extracts or pure compounds having significant *in vitro* activity are further investigated using suitable animal models. Before *in vivo* studies, plant materials are evaluated for toxicity to calculate suitable dose. The action of the drug in the living system is not only dependent on pharmacodynamics but also on pharmacokinetics. Animal studies are performed using suitable controls and positive controls.

1.4.4 Pharmacokinetic studies

Pharmacokinetics is defined as the action of body on the drug and it includes absorption, distribution, metabolism and excretion. It offers means to measure the amount of drug in blood stream, at the site of action and extent of the release of drug substance from a dosage form. Study of pharmacokinetic parameters has applications in toxicology (drug exposure) and biopharmaceutics (bioavailability and bioequivalence). The literature on pharmacokinetic studies of herbs is not sufficient due to a number of factors including lack of studies and scarce reporting**.**

Pharmacokinetics data of herbs is also useful to understand herb-drug interactions. Furthermore, it may provide valuable information which may be helpful in prescribing herbal products safely and effectively. It may also enable us to make useful predictions regarding possible interactions between herbal remedies and conventional pharmaceuticals.

Since longtime, natural product scientists have been studying the pharmacodynamics, actions of the herbs on the body, but less attention has been paid to study the effects of the body on herbal products. The action of the drug depends on the rate and extent of active components at the site of action which are monitored by pharmacokinetic parameters.

1.5 STABILITY STUDIES

Stability is defined as the time during which a drug retains its chemical integrity and labeled potency within the specified limits. The stability of a pharmaceutical preparation is its degree of resistance to chemical and physical changes. A product should be consistent in efficacy and claimed potency or may change only within the limits specified by legal provisions until the date of expiry (Racz, 1989). Stability is one of the most important factors which determine whether a compound or a mixture of compounds can be developed into a pharmaceutical product. Stability studies provide evidences on how the quality of a drug substance varies with the passage of time under the influence of environmental factors (WHO, 1996). Stability studies before developing a dosage form are the first quantitative assessment of chemical stability of a new product and its possible formulation. Stability studies are used to recommend storage conditions and predict shelf life of medicinal products.

The stability studies are essential for three main reasons. First, patient safety by assuring that the patient receives a uniform dose of drug throughout the shelf life of a product. Second, legal requirements concerning about identity, strength, purity and quality of a drug. Third is to prevent economic repercussions in developing and marketing an unstable product. The stability of a product is influenced by a number of factors, which may be classified into two main categories, physical and chemical factors.

1.5.1 Physical factors effecting stability

1.5.1.1 Temperature

Temperature increases the rate of degradation of active ingredients due to the increase in kinetic energy, which increases the number of colliding molecules. The decomposition usually increases by two to three times for every 10 °C rise in temperature. Therefore, it is important to be aware about it while storing raw material and in production of a product especially when heating is required. It is particularly important in case of thermolabile and volatile constituents.

1.5.1.2 Moisture

Moisture content increases the rate of decomposition and makes the product susceptible to hydrolysis. In case of herbal crude powders/extracts, it allows the growth of microbes which not only deteriorate the constituents but may produce toxic substances.

1.5.1.3 Light

There are many reports about the effect of sunlight on the stability of pharmaceutical products (Tonnesen, 2004). Sunlight being a form of energy can initiate and accelerates decomposition. Photolabile constituents of extracts deteriorate on exposure to sunlight (Rawlins, 1977; Pugh, 2002).

1.5.2 Chemical factors effecting stability

The constituents of extracts like other pharmaceuticals also undergo chemical degradation with the passage of time. The chemical degradation is further classified as follows:

1.5.2.1 Hydrolysis

Hydrolysis, reaction of the compound with water, is the most common cause of drug degradation (Waterman *et al.,* 2002). It is considered to be a major cause of deterioration of drugs especially for aqueous based preparations.

1.5.2.2 Oxidation

Oxidation of a compound is defined as the removal of an electropositive atom, radical or electron, or addition of an electronegative atom, radical or electron.

Decomposition of pharmaceutical preparation due to oxidation is nearly as probable as that of hydrolysis. The rate of oxidation is temperature dependent. For example peroxidation of fatty acids, break down of fatty acids into aldehydes and ketones, accelerates as the temperatures exceeds 50 °C (Rawlins, 1977).

1.5.2.3 Isomerisation and polymerisation

Polymerization is the addition of similar molecules whereas isomerisation is the change in isomeric forms. These phenomena change the biological activity of compounds.

The stability study of pharmaceuticals performed at exaggerated conditions is called accelerated stability. Accelerated stability study is performed for fast prediction of stability.

1.6 Accelerated stability

The test for examining the quality and potency of a product at suitable time intervals is conducted for a period corresponding to the normal time that the product is likely to remain in stock or in use. Degradation is usually slow at room temperature and shelf life may go up to several years. Since the period can be as long as two years, stability testing for this type will be time consuming and expensive. Therefore, accelerated stability testing is devised for rapid prediction of long term stability of a product. Though stability of drugs at room temperature is of primary interest, stability study at room temperature would take too long to be useful as a screening procedure for new formulations. Therefore, stability studies are conducted at elevated temperatures and the data is evaluated by applying Arrhenius equation (Sungthongjeen, 2004).

A prediction of shelf life (t_{90}), a time in which a product retains 90% of its original potency, may be made by accelerating the decomposition process and extrapolating the results to normal storage conditions. For this reason stability tests are often performed under exaggerated conditions (elevated temperatures) to accelerate degradation process in order to get a fast stability prediction. Application of the principles of chemical kinetics on the results of accelerated stability tests enables us to make prediction about the shelf life of a product at normal temperature.

Keeping in view the therapeutic importance of natural products, two local plants having significant potential of commercialization were selected to perform analytical work to standardize their extracts. The standardised extracts were then used to perform biological, pharmacokinetic and stability studies.

1.7 LITERATURE REVIEW OF *Piper sarmentosum*

Scientific name: *Piper sarmentosum* Roxburgh

(*Piper* Latin name of Pepper and *sarmentosum* means twiggy/ with long slender runners)

Local Name: Kadok

Common name: *Daun Kadok, Chau pulu, Jia ju*

Family:*Piperaceae*

Synonyms: *Chavica hainana* DC.; *Chavica sarmentosa* (Roxburgh**)** Miq.; *Piper alispicum* DC.; *Piper brevicaule* DC.; *Piper gymnostachyum* DC.; *Piper lolot* DC.; *Piper pierrei* DC.; *Piper saigonese* DC.

1.7.1 Botanical description

The plant is herbaceous shrub, may be creeping along the ground or erect. The plant is usually found as a weed in villages under shady places (Hsuan, 1990) and the leaves are heart shaped (Wee, 1992). It grows up to 50-60 cm and is found in Cambodia, Laos, Philippines, Burma, Thailand, Malaysia, Vietnam and China. The leaves are simple, alternate and estipulate. The blade is lanceolate-ellipitical 10 cm X 5 -14 cm X 6 -9.9 cm X 3.4 -12 cm X 2 cm, acuminate at the apex, round at the base, with two pairs of secondary nerves. The florescences are 5 mm long nerves. The fruit is green and red with 4 mm X 3 mm drupes (Wiart, 2006). The plant has a characteristic pungent odor (Hsuan, 1990; Wee, 1992).

1.7.2 Ethnopharmacology

The plant is well known in South East Asian countries due to its medicinal properties. In the Chinese traditional treatment system, leaves of the plant are used to treat fever and indigestion (Wee, 1992) and roots are used to relieve toothaches (Duke and Ayensu, 1985; Toong and Wong, 1989; Wee, 1992). Roots are also used to treat dermatomycoses, coughing and pleurisy (Perry, 1981; Duke and Ayensu, 1985; Toong and Wong, 1989). In Malaysia and southern parts of Thailand, leaves are used externally to sooth headaches. In Indonesia, roots are chewed for cough, asthma and toothaches and leaves are also used to mitigate chest pain (Wiart, 2006). The fruit is used as an expectorant (Pongboonrod, 1976; Toong and Wong, 1989).

1.7.3 Review of chemical constituents

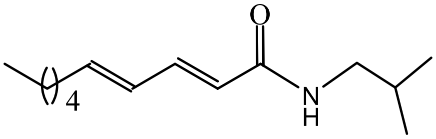
Main constituents of the plant are amide alkaloids, phenylpropanoids, pyrones, flavonoids, sterols and neolignans etc. More than 67 compounds have been separated and identified from the essential oil of *Piper sarmentosum*. The main compounds were 2, 4, 5-trimethoxy-1-propenylbenzene 23.20%, cis-caryophyllene 13.33%, 1, 2-dimethoxy-4-(1-propenyl)-benzene 12.63%, 1, 3-benzodioxole-4-methoxy-6-(2-propenyl) 5.71% and delta-cadinene 3.03% (Song et al., 2006). In an other study, the main components of the essential oil obtained from leaves of the plant were 10-Epi-γ-eudesmol 21.0%, α-cadenine 18.8%, seychellene 12.6% and (E, E)-farnesol 10.5% (Chieng *et al.*, 2005). The chemical constituents identified in this plant are given in Table-1 and structures of some chemical constituents are given in Fig. 1.1.

Table 1.1 Chemical constituents of *Piper sarmentosum*

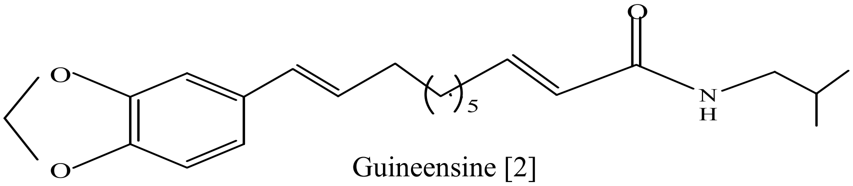
Constituents	**Reference**
Pellitorine [1]	Thitima *et al.,* 2004 Likhitwitayawuid *et al.,* 1988 Tutiwachwuttikul *et al.,* 2006
Guineensine [2]	Thitima *et al.,* 2004 Tutiwachwuttikul *et al.,* 2006
Brachystamide B [3]	Thitima *et al.,* 2004 Tutiwachwuttikul *et al.,* 2006
Sarmentine [4]	Thitima *et al.,* 2004 Tutiwachwuttikul *et al.,* 2006 Likhitwitayawuid *et al.,* 1988
Brachyamide B [5]	Thitima *et al.,* 2004
1-piperettyl pyrrolidine [6]	Thitima *et al.,* 2004
3,4,5-trimethoxycinnamoyl pyrrolidine [7]	Thitima *et al.,* 2004
Sarmentosine [8]	Thitima *et al.,* 2004 Tutiwachwuttikul *et al.,* 2006 Likhitwitayawuid *et al.,* 1988
(+) asarinin [9]	Thitima *et al.,* 2004
Sesamin [10]	Thitima *et al.,* 2004 Tutiwachwuttikul *et al.,* 2006
N-[9-(3,4-methylenedioxyphenyl)-2E,4E,8E-nonatrienoyl] pyrrolidine [11]	Tutiwachwuttikul *et al.,* 2006
Methyl piperate [12]	Thitima *et al.,* 2004
β- sitosterol [13]	Thitima *et al.,* 2004 Tutiwachwuttikul *et al.,* 2006 Likhitwitayawuid *et al.,* 1988 Niamsa *et al.,* 1983
Stigmasterol [14]	Thitima *et al.,* 2004
Aromatic alkene [15]	Tutiwachwuttikul *et al.,* 2006
1-allyl-2-methoxy-4,5-metylenedioxybenzene [16]	Tutiwachwuttikul *et al.,* 2006 Masuda *et al.,* 1991
Sarmentide C [17]	Tutiwachwuttikul *et al.,* 2006
Pyrrole amide [18]	Tutiwachwuttikul *et al.,* 2006
Sarmentamide A [19]	Tutiwachwuttikul *et al.,* 2006
Sarmentamide B [20]	Tutiwachwuttikul *et al.,* 2006
1-(3,4-methyllenedioxyphenyl)-1E-tetradecene	Thitima *et al.,* 2004
Horsfieldin [21]	Tutiwachwuttikul *et al.,* 2006
N-(2-methylbutyl)-2E,4E-decadienamide	Stoehr *et al.,* 1999
Naringenin [22]	Vimala *et al.,* 2003
Cis- caryophyllene	Song *et al.,* 2006
Hydrocinnamic acid [23]	Niamsa *et al.,* 1983
α-asarone [24]	Song *et al.,* 2006 Likhitwitayawuid *et al.,* 1988
Asaraldehyde [25]	Likhitwitayawuid *et al.,* 1988
1-(3,4-methylenedioxyphenyl)-1E-tetradecene	Likhitwitayawuid *et al.,* 1988

2,4,5-trimethoxy- 1-propenylbenzene [26]	Song *et al.,* 2006
1- allyl- 2,4,5-trimethoxybenzene [27]	Masuda *et al.,* 1991
1-(1E-propenyl)-2,4,5- trimethoxybenzene	Masuda *et al.,* 1991
1,2-dimethoxy-4-(1-propenyl)-benzene [28]	Song *et al.,* 2006
1-allyl-2-methoxy-3,4-methylenedioxybenzene [29]	Masuda *et al.,* 1991
1,3-benzodioxole-4-methoxy-6-(2-propenyl)	Song *et al.,* 2006
10-Epi-γ-eudesmol [30]	Chieng *et al.,* 2005
α-cadinene [31]	Chieng *et al.,* 2005
Seychellene [32]	Chieng *et al.,* 2005
Delta- cadinene [33]	Song *et al.,* 2006
(E, E)-farnesol [34]	Chieng *et al.,* 2005

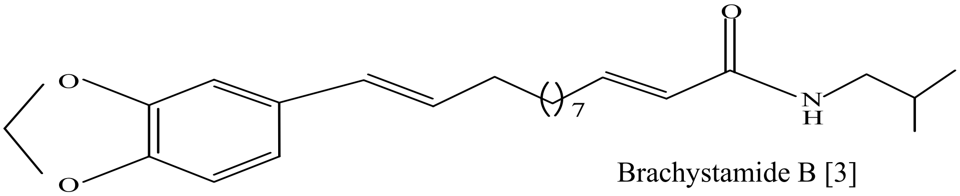
Numbers in square brackets indicate the structure



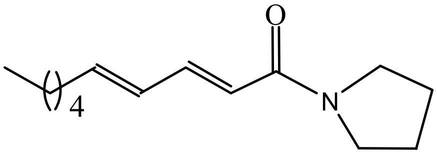
Pellitorine [1]



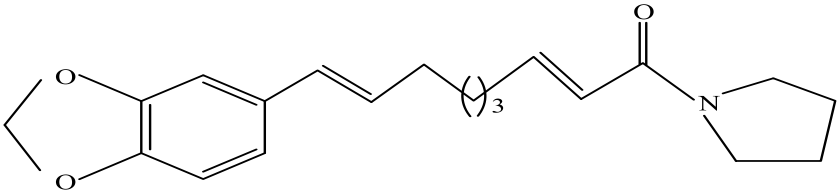
Guineensine [2]



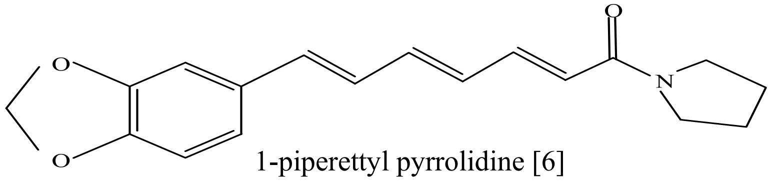
Brachystamide B [3]



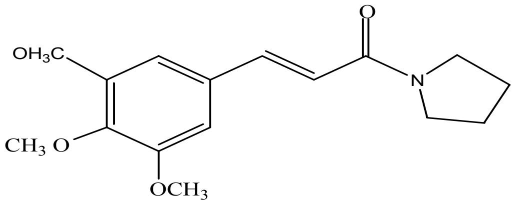
Sarmentine [4]



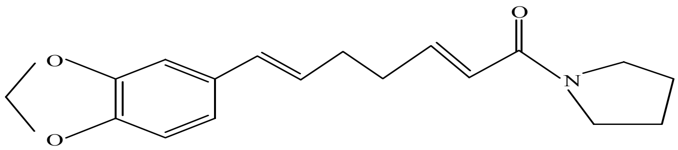
Brachyamide B [5]



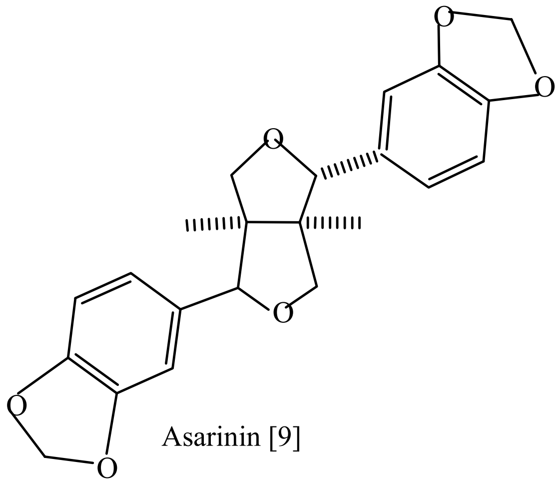
1-piperettyl pyrrolidine [6]



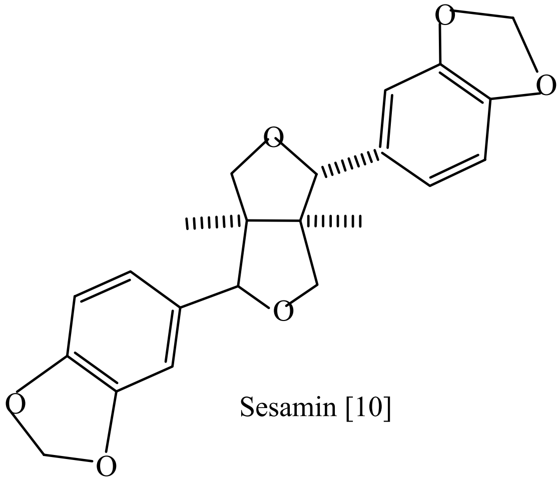
3,4,5 trimethoxy cinnamoyl pyrrolidine [7]



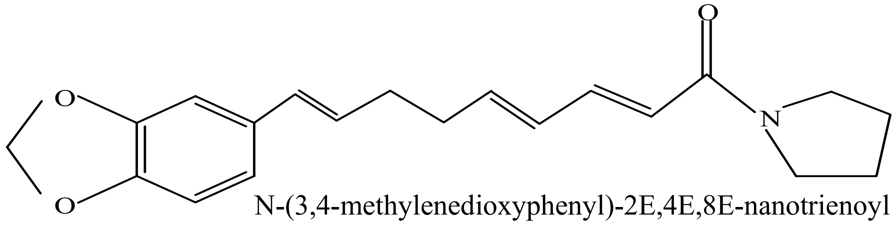
Sarmentosine [8]



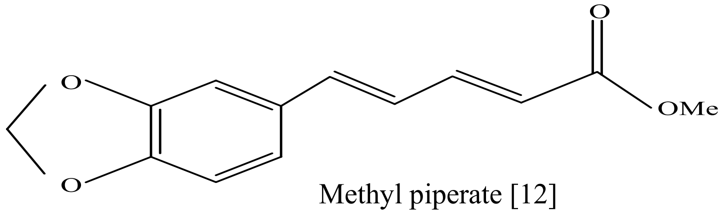
Asarinin [9]



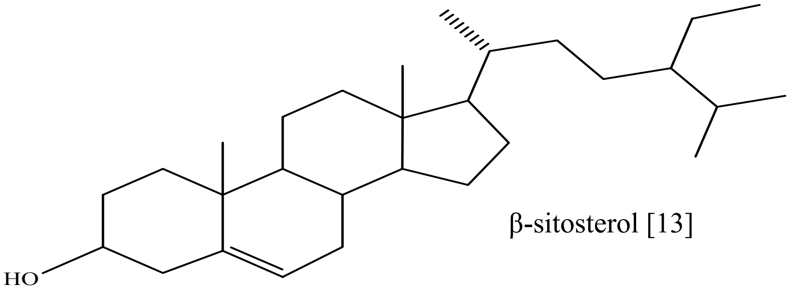
Sesamin [10]



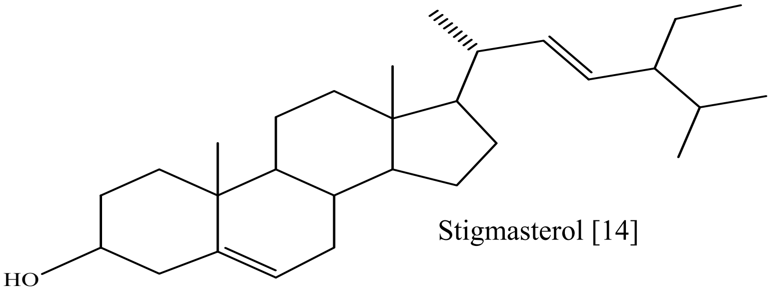
N-(3,4-methylenedioxyphenyl)-2E,4E,8E-nanotrienoyl [11]



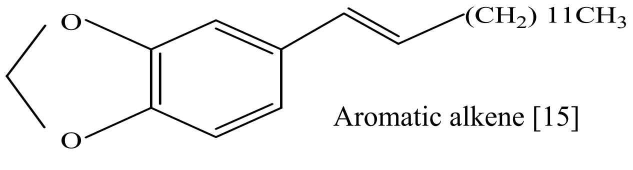
Methyl piperate [12]



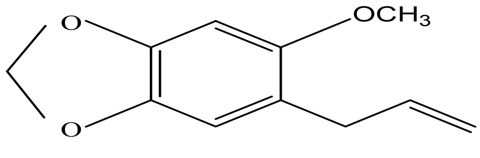
β-sitosterol [13]



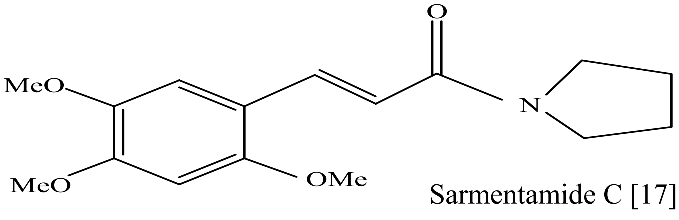
Stigmasterol [14]



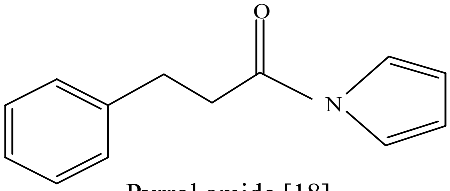
Aromatic alkene [15]



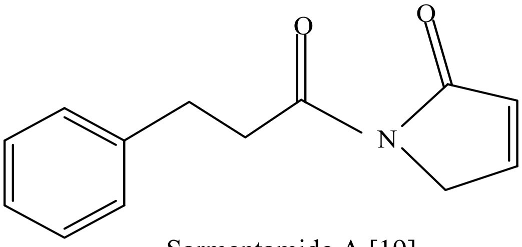
1-allyl 2-methoxy-4, 5 methylene dioxybenzene [16]



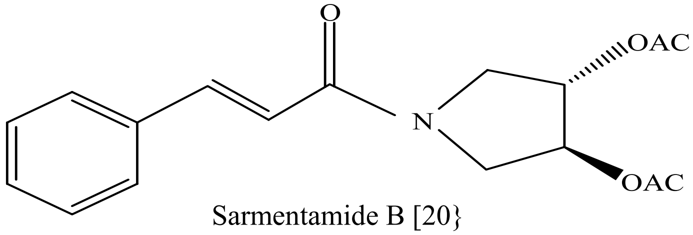
Sarmentamide C [17]



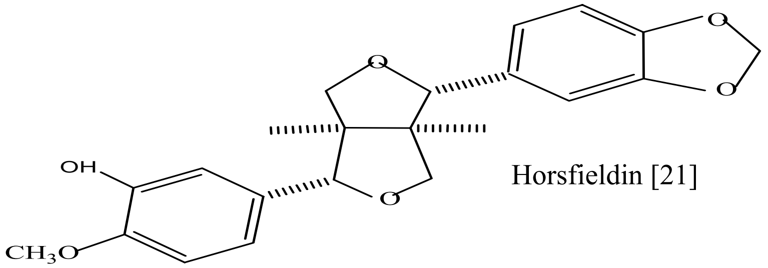
Pyrrol amide [18]



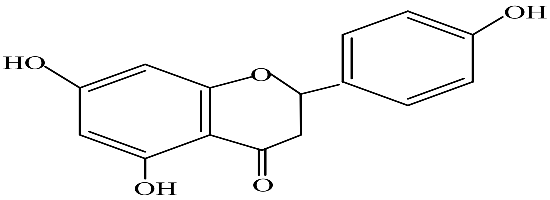
Sarmentamide A [19]



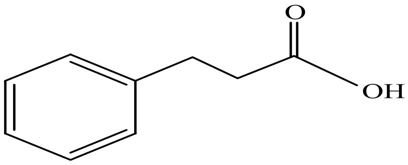
Sarmentamide B [20}



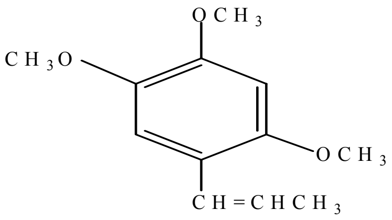
Horsfieldin [21]



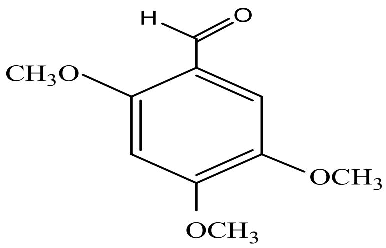
Naringenin [22]



Hydrocinnamic acid [23]



α asarone [24]



Asaraldehyde [25]