

Pharmaceutical Analysis and Drug Interaction Studies:

African Potato (*Hypoxis hemerocallidea*)

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Abstract

In order for a medicinal product to produce a consistent and reliable therapeutic response, it is essential that the final composition of the product is invariable and that the active ingredient/s is/are present in appropriate, non-toxic amounts. However, due to the complexity involved in the standardization of natural products, quality control (QC) criteria and procedures for the registration and market approval of such products are conspicuously absent in most countries around the world.

African Potato (AP) is of great medical interest and this particular plant has gained tremendous popularity following the endorsement by the South African Minister of Health as a remedy for HIV/ AIDS patients. Very little information has appeared in the literature to describe methods for the quantitative analysis of hypoxoside, an important component in AP. It has also been claimed that sterols and sterolins present in AP are responsible for its medicinal property but is yet to be proven scientifically. To-date, no QC methods have been reported for the simultaneous quantitative analysis of the combination, β - sitosterol (BSS)/ stigmasterol (STG)/ stigmastanol (STN), purported to be present in preparations containing AP.

The effect of concomitant administration of AP and other herbal medicines on the safety and efficacy of conventional medicines has not yet been fully determined. Amongst the objectives of this study was to develop and validate quantitative analytical methods that are suitable for the assay and quality control of plant material, extracts and commercial formulations containing AP.

Hypoxoside was isolated from AP and characterized for use as a reference standard for the quality control of AP products and a stability-indicating HPLC/ UV assay method for the quantitative determination of hypoxoside was developed. In addition, a quantitative capillary zone electrophoretic (CZE) method was developed to determine hypoxoside, specifically for its advantages over HPLC. A HPLC method was also developed and validated for the quantitative analysis of BSS, STG and STN in commercially available oral dosage forms containing AP material or extracts thereof.

The antioxidant activity of an aqueous extract of lyophilized corms of AP along with hypoxoside and rooperol were investigated. In comparison with the AP extracts and also with hypoxoside, rooperol showed significant antioxidant activity.

The capacity of AP, (extracts, formulations, hypoxoside and rooperol as well as sterols to inhibit *in vitro* metabolism of drug substrates by human cytochrome P450 (CYP) enzymes such as CYP 3A4, 3A5 and CYP19 were investigated. Samples were also assessed for their effect on drug transport proteins such as P-glycoprotein (P-gp). Various extracts of AP, AP formulations, stigmasterol and the norlignans, in particular the aglycone rooperol, exhibited inhibitory effects on CYP 3A4, 3A5 and CYP19 mediated metabolism. These results suggest that concurrent therapy with AP and other medicines, in particular antiretroviral drugs, can have important implications for safety and efficacy.

Large discrepancies in marker content between AP products were found. Dissolution testing of AP products was investigated as a QC tool and the results also revealed inconsistencies between different AP products.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	vi
LIST OF FIGURES	xiv
LIST OF TABLES	xviii
LIST OF ABBREVIATIONS	xix
LIST OF UNITS	xxv

CHAPTER 1

Introduction	1
1.1 Quality, Safety and Efficacy of Natural Products	3
<i>1.1.1 Quality of Natural Products</i>	3
<i>1.1.2 Safety of Natural Products</i>	5
1.1.2.1 Adverse Effects	6
1.1.2.2 Adulteration and Contamination of Natural Products	7
1.1.2.3 Interactions with Orthodox Medicines	8
<i>1.1.3 Efficacy of Natural Products</i>	9
1.2 Regulatory Aspects and Alternative Medicines	10
<i>1.2.1 Definitions</i>	10
<i>1.2.2 Regulatory Situation of Complementary and Alternative Medicine (CAM) in Several Countries</i>	17
1.2.2.1 The United States of America (USA)	17
1.2.2.2 Canada	18
1.2.2.3 Europe	20
1.2.2.4 United Kingdom (UK)	22
1.2.2.5 Asia	23
1.2.2.6 Australia	25
1.2.2.6.1 Pre-market Assessment	26
1.2.2.6.2 Licensing of Manufacturers	26
1.2.2.6.3 Post-market Vigilance	26
1.2.2.7 South Africa	27
1.3 Conclusions	29

CHAPTER 2	
African Potato (<i>Hypoxis hemerocallidea</i>)	32
2.1 Background	32
2.1.1 Vernacular Names	32
2.2 Introduction	33
2.2.2 Prominent Species of the Genus <i>Hypoxis</i>	34
2.2.2.1 List of Prominent Species	35
2.2.3 Traditional Use of the Plants under the Genus <i>Hypoxis</i>	38
2.2.4 Extracts of <i>Hypoxis</i> Genus and Biological Activity	39
2.2.4.1 The Extracts of <i>Hypoxis</i>	40
2.3 Phytochemical Constituents in <i>Hypoxis</i> Species	43
2.3.1 Chemical Properties of Hypoxoside, Rooperol, Sterols, Stanols and Sterolins	49
2.4 Pharmacological Properties of Hypoxoside, Rooperol, Sterols, Stanols and Sterolins	53
2.4.1 Sterols, Stanols and Sterolins	53
2.4.2 Hypoxoside and Rooperol	56
2.4.2.1 Inhibitory Activity on Cell Growth (Cytotoxicity)	56
2.4.2.1.1 Mechanism of Cytotoxic Activity	57
2.4.2.2 Antioxidant Activity	58
2.4.2.3 Anti-HIV Activity	59
2.4.2.4 Analgesic Activity	60
2.4.2.5 Cardiovascular Effects	60
2.4.2.6 Miscellaneous Activities and Effects	61
2.5 Pharmacokinetics	61
2.5.1 Animal Studies	61
2.6 Clinical Studies	63
2.7 Patents	65
2.8 Formulations and Marketing of AP Products	68

CHAPTER 3	
Extraction, Isolation and Characterization of Hypoxoside	90
3.1 Introduction	90
3.2 Material and Methods	91
3.2.1 Reagents	91
3.2.2 Instrumentation	92
3.3 Experimental	92

3.3.1 Raw Material Collection	92
3.3.2 Extraction	93
3.3.3 Isolation	94
3.4 Characterization	95
3.4.1 Appearance	95
3.4.2 Determination of Melting Point	96
3.4.3 Fourier Transform Infrared (FTIR) Spectroscopy	97
3.4.4 UV Absorption Spectroscopy	100
3.4.5 Chromatographic Purity	101
3.4.6 LC-MS Analysis	101
3.4.7 Nuclear Magnetic Resonance (^1H and ^{13}C NMR)	105
3.5 Conclusions	109

CHAPTER 4

High-Performance Liquid Chromatography (HPLC)	110
4.1 Introduction	110
4.2 Background and Objectives	112
4.3 Stability Studies	113
4.4 Materials and Methods	114
4.4.1 Instrumentation	114
4.4.2 Reagents	114
4.5 Experimental	115
4.5.1 Method Development	115
4.5.2 Method Validation	119
4.5.2.1 Sample Treatment	119
4.5.2.2 Hypoxoside Reference Standard	120
4.5.2.3 Preparation and Standard Solutions	120
4.5.2.4 Sample Extraction	120
4.5.2.5 Stability of Hypoxoside Solutions	122
4.5.3 Forced Degradation Studies	122
4.5.3.1 Stress Testing of Hypoxoside	122
4.5.3.2 Stress Conditions	125
4.6 Results and Discussion	126
4.6.1 Method Validation	126
4.6.1.1 Linearity	126
4.6.1.2 Limits of Detection (LOD) and Quantification (LOQ)	126
4.6.1.3 Accuracy and Precision	126
4.6.1.4 Recovery	128

4.6.2 <i>Analysis of Samples</i>	129
4.6.2.1 <i>Extraction Efficiency</i>	129
4.6.2.2 <i>Commercial Formulations</i>	131
4.6.2.3 <i>Crude AP Corms</i>	134
4.6.2.4 <i>Traditional Extract of AP</i>	136
4.6.2.5 <i>Stability Studies</i>	136
4.6.3 <i>Forced Degradation</i>	136
4.7 Discussion and Conclusions	146

CHAPTER 5

High-Performance Liquid chromatography with Evaporative Light Scattering Detection	148
5.1 Introduction	148
5.2 Background and Objectives	149
5.3 Experimental	151
5.3.1 <i>Instrumentation</i>	151
5.3.2 <i>Reagents and Chemicals</i>	152
5.3.3 <i>Method Development</i>	152
5.3.4 <i>Optimization of ELSD Conditions</i>	155
5.3.5 <i>Optimization of MS Conditions</i>	157
5.3.6 <i>Preparation of Standard Solutions</i>	157
5.3.7 <i>Sample Preparation and Extraction</i>	157
5.3.7.1 <i>Hard Gelatin Capsules</i>	157
5.3.7.2 <i>Solid Oral Dosage Forms (Tablets)</i>	158
5.4 Results and Discussion	158
5.4.1 <i>Sample Extraction Efficiency</i>	158
5.4.2 <i>Method Validation</i>	159
5.4.2.1 <i>Linearity</i>	159
5.4.2.2 <i>Limits of Detection (LOD) and Quantification (LOQ)</i>	160
5.4.2.3 <i>Accuracy and Precision</i>	160
5.4.2.4 <i>Sample Analysis</i>	162
5.4.2.5 <i>Recovery</i>	163
5.4.3 <i>LC-MS Analysis</i>	166
5.5 Conclusions	168

CHAPTER 6	
Capillary Electrophoresis	169
6.1 Introduction	169
6.2 Background and Objectives	173
6.3 Stability Studies	174
6.4 Experimental	174
<i>6.4.1 Instrumentation</i>	174
<i>6.4.2 Reagents and Chemicals</i>	175
<i>6.4.3 Method Development</i>	175
6.4.3.1 Capillary Conditioning	175
6.4.3.2 Initial Conditions	176
6.4.3.3 Optimization of Running Electrolyte	176
6.4.3.4 Optimization of pH	176
6.4.3.5 Optimization of Ionic Strength	177
6.4.3.6 Optimization of Applied Voltage	179
6.4.3.7 Selection of Internal Standard	179
6.4.3.8 Summary	181
<i>6.4.4 Method Validation</i>	182
6.4.4.1 Preparation of Reference Standards	182
<i>6.4.5 Working Standards</i>	182
<i>6.4.6 Preparation of Sample Solutions</i>	182
<i>6.4.7 Stress Testing of Hypoxoside- Forced Degradation Studies</i>	183
6.5 Results and Discussion	183
<i>6.5.1 Method Validation</i>	183
6.5.1.1 Linearity	183
6.5.1.2 Limits of Detection (LOD) and Quantification (LOQ)	184
6.5.1.3 Accuracy and Precision	184
6.5.1.4 Recovery	184
<i>6.5.2 Analysis of Samples</i>	186
6.5.2.1 Extraction Efficiency	186
6.5.2.2 Analysis of Commercial Products	186
6.5.2.3 Selectivity	187
6.6 Conclusions	189

CHAPTER 7

Antioxidant Activity of Hypoxoside, Rooperol and Extracts of African Potato	190
7.1 Introduction	190
7.2 Background	194
7.3 Experimental	195
7.3.1 <i>Instrumentation</i>	195
7.3.2 <i>Reagents and Chemicals</i>	195
7.3.3 <i>Plant Material and Phytochemicals</i>	196
7.4 Experimental	196
7.4.1 <i>HPLC Method Development</i>	196
7.4.2 <i>Sample Extraction</i>	198
7.4.3 <i>Preparation of Solutions for Antioxidant Investigations</i>	199
7.4.4 <i>Antioxidant Investigations</i>	199
7.4.4.1 <i>Free Radical Scavenging Ability</i>	199
7.4.4.2 <i>Ferric Reducing Activity of Plasma (FRAP)</i>	199
7.4.4.3 <i>Animal Care and Homogenate Preparation</i>	200
7.4.4.4 <i>Lipid Peroxidation Assay</i>	200
7.4.4.5 <i>Superoxide Anion Assay (Nitroblue Tetrazolium Assay)</i>	201
7.4.5 <i>Statistical Analysis</i>	202
7.5 Results and Discussion	202
7.5.1 <i>HPLC Analysis</i>	202
7.5.2 <i>Antioxidant Investigations</i>	202
7.5.2.1 <i>Free Radical Scavenging</i>	202
7.5.2.2 <i>Ferric Reducing Activity of Plasma (FRAP)</i>	203
7.5.2.3 <i>Lipid Peroxidation Assay</i>	204
7.5.2.4 <i>Superoxide Anion Assay (Nitroblue Tetrazolium Assay)</i>	205
7.6 Conclusions	206

CHAPTER 8

Drug Interaction Studies: In vitro Evaluation of Human Cytochrome P450 Enzymes and P-glycoprotein Interactions	208
8.1 Introduction	208
8.2 Background and Objectives	210
8.3 Materials and Methods	212
8.3.1 <i>Instrumentation</i>	212

8.3.2 <i>Reagents and Chemicals</i>	212
8.4 Experimental	213
8.4.1 <i>Sample Preparation</i>	213
8.4.1.1 AP Raw Material	213
8.4.1.2 Commercial Products	214
8.4.1.3 Phytochemicals	214
8.4.2 <i>Cytochrome P450 Assay</i>	215
8.4.3 <i>P-Glycoprotein Assay</i>	216
8.4.4 <i>Phytochemical Analysis</i>	217
8.4.5 <i>Statistical Analysis</i>	218
8.5 Results	218
8.5.1 <i>Cytochrome P450 Assay</i>	219
8.5.2 <i>P-Glycoprotein Assay</i>	221
8.6 Discussion	222
8.7 Conclusion	224
 CHAPTER 9	
<i>In Vitro</i> Dissolution Studies	225
9.1 Introduction	225
9.2 Background and Objectives	230
9.3 Materials and Methods	231
9.3.1 <i>Instrumentation</i>	231
9.3.2 <i>Reagents and Chemicals</i>	231
9.4 Experimental	232
9.4.1 <i>Dissolution Conditions</i>	232
9.4.2 <i>Analyses of the Dissolution Samples</i>	232
9.4.3 <i>In Vitro Dissolution Method Development</i>	233
9.4.3.1 Products Containing Hypoxoside	233
9.4.3.2 Products Containing β -Sitosterol	234
9.4.4 <i>Dissolution Profiles of Products Containing Hypoxoside</i>	235
9.4.5 <i>Dissolution Profiles of Products Containing β-Sitosterol</i>	235
9.5 Results and Discussion	236
9.5.1 <i>Dissolution Profiles of Products containing Hypoxoside</i>	236
9.5.2 <i>Dissolution Profiles of Products containing β-Sitosterol</i>	237
9.6 Conclusions	237

CHAPTER 10	
Sterols and Sterolins in African Potato; Fact or Fiction?	239
10.1 Introduction	239
10.2 Experimental	241
<i>10.2.1 Instrumentation and Analytical Method</i>	241
<i>10.2.2 Reagents and Chemicals</i>	241
<i>10.2.3 Preparation of Standard Solutions</i>	241
<i>10.2.4 Sample Preparation</i>	241
10.2.4.1 Aqueous Extract	241
10.3 Results	242
10.4 Conclusions	243
 GENERAL CONCLUSIONS	 244
 REFERENCES	 245

LIST OF FIGURES

Figure: 2.1.	Photograph of a Young Corm of <i>Hypoxis hemerocallidea</i>	33
Figure: 2.2.	Photograph of the Trans-section of a Corm of <i>Hypoxis hemerocallidea</i>	34
Figures: 2.3a-2.3g.	The Prominent Species of <i>Hypoxis</i> in Southern Africa	36
<i>Figure: 2.3a.</i>	<i>Hypoxis obtusa</i>	36
<i>Figure: 2.3b.</i>	<i>Hypoxis colchifolia</i>	36
<i>Figure: 2.3c.</i>	<i>Hypoxis rigidula</i>	36
<i>Figure: 2.3d.</i>	<i>Hypoxis parvula</i>	36
<i>Figure: 2.3e.</i>	<i>Hypoxis membernaceae</i>	37
<i>Figure: 2.3f.</i>	<i>Hypoxis hirtusa</i>	37
<i>Figure: 2.3g.</i>	<i>Hypoxis angustifolia</i>	37
Figure: 2.4.	Honey Bees Foraging on the Flowers of <i>Hypoxis hemerocallidea</i>	42
Figure: 2.5	Bio-Synthetic Scheme of Norlignans	44
Figure: 2.6.	Structures of Norlignan Glycosides of Hypoxidaceae	45
Figure: 2.7.	Hypoxoside	49
Figure: 2.8.	Rooperol	50
Figure: 2.9.	Daucosterol (β - Sitosterol glycoside)	50
Figure: 2.10.	Stigmasterol	51
Figure: 2.11.	β - Sitosterol	51
Figure: 2.12.	Stigmastanol	52
Figure: 2.13.	Zeatin glycoside	52
Figure: 2.14.	Rooperol as a Radical Scavenger (Antioxidant)	59
Figures: 2.15a-2.15h.	Advertisements of AP Products	70-75
Figures: 2.16a-2.16h.	Media Propaganda on traditional Medicines and the Use of AP in South Africa (RSA)	76-81
<i>Figure: 2.16a.</i>	British Broadcasting Centre (BBC) News- November 07-1999	76
<i>Figure: 2.16b.</i>	Sunday Times- September 07-2000	76
<i>Figure: 2.16c.</i>	South African Broadcasting Centre- July 24-2003	77
<i>Figure: 2.16d.</i>	The Herald-2004	78
<i>Figure: 2.16e.</i>	Sunday Times-February 23-2004	78
<i>Figure: 2.16f.</i>	The Herald-November 08-2005	79
<i>Figure: 2.16g.</i>	Readers Digest- October-1997	80
<i>Figure: 2.16h.</i>	Readers Digest- November-1999.	81
Figures: 2.17a-2.17c.	Media Criticisms on the Use of AP as an Immune Booster for HIV Positive Patients	82-83
<i>Figure: 2.17a.</i>	UN Integrated Regional Information Network-February 4-2005	82

Figure: 2.17b.	Sunday Times- February 15-2004	83
Figure: 2.17c.	Sunday Times- 2004	83
Figures: 2.18a-2.18q.	Products and Raw Materials of African Potato (AP)	84-86
Figure: 2.18a.	<i>Sterols and sterolins, Hypoxis-African potato</i> -Product A	84
Figure: 2.18b.	<i>Natural remedy</i> -Product B	84
Figure: 2.18c.	<i>Hi-Vite High impact vitamin</i> -Product C	84
Figure: 2.18d.	<i>Suprimune forte</i> -Product D	84
Figure: 2.18e.	<i>Spiraforce</i> -Product E	84
Figure: 2.18f.	<i>Imu-Wize Forte</i> -Product F	84
Figure: 2.18g.	<i>Imu-wize</i> -Product G	85
Figure: 2.18h.	<i>Imu/Wize(different package)</i> -Product H	85
Figure: 2.18i.	<i>Mixture of life</i> -Product I	85
Figure: 2.18j.	<i>Phyto Force</i> -Product J	85
Figure: 2.18k.	<i>Africa's solution forte</i> -Product K	85
Figure: 2.18l.	<i>Africa's solution</i> -Product L	85
Figure: 2.18m.	<i>Viral Choice</i> -Product M	86
Figure: 2.18n.	<i>Moducare</i> -Product N	86
Figure: 2.18o.	<i>Herbology</i> -Product O	86
Figure: 2.18p.	Raw Materials of AP	86
Figure: 2.18q.	Traditional Aqueous Extract of AP	86
Figure: 3.1.	Collection of African Potato Corms	93
Figure: 3.2.	Scanning Electron Microscope Image of Hypoxoside Evaporated From an Ethanollic Solution (12 KV, 10 μ m x 400)	96
Figure: 3.3.	Scanning Electron Microscope Image of Freeze Dried Hypoxoside (12 KV, 10 μ m x 400)	96
Figure: 3.4.	Differential Scanning Calorimetry (DSC) Thermogram of Hypoxoside	98
Figure: 3.5.	Infrared spectrum of Hypoxoside	99
Figure: 3.6.	UV Absorption Spectrum of Hypoxoside	100
Figure: 3.7.	LC-MS Chromatogram of Hypoxoside	103
Figure: 3.8.	LC-MS-MS Chromatogram of Hypoxoside	104
Figure: 3.9.	LC-MS-MS Chromatogram of Rooperol	105
Figure: 3.10.	HMBC Spectrum of Hypoxoside	108
Figure: 4.1.	Traditional Aqueous Extract of African Potato	117
Figure: 4.2.	Chemical Structure of Sulphamerazine	118
Figure: 4.3.	UV Absorption Spectrum of Sulphamerazine	118
Figure: 4.4.	UV Spectra and Chromatogram of Hypoxoside and Sulphamerazine (I.S.).	119
Figures: 4.5a-4.5d.	Flow chart for Stress Studies	123-124
Figure: 4.5a.	Oxidative Degradation	123
Figure: 4.5b.	Acid/Base Hydrolysis	123
Figure: 4.5c.	Neutral Hydrolysis	124

<i>Figure: 4.5d.</i>	Photolysis	124
Figure: 4.6.	Chromatogram of Product-D spiked with Low, Medium and High Concentrations of Hypoxoside	128
Figures: 4.7a-4.7c.	HPLC Chromatograms	133-134
<i>Figure: 4.7a.</i>	Chromatogram of Product-D II	133
<i>Figure: 4.7b.</i>	Chromatogram of Product-H	133
<i>Figure: 4.7c.</i>	Chromatogram of Product-J	134
Figure: 4.8.	Chromatogram of Freeze Dried African Potato (FDAP)	135
Figure: 4.9.	UV Spectra and Chromatogram of H ₂ O ₂ (3%, v/v) after 24 hr	138
Figure: 4.10.	UV Spectra and Chromatogram of H ₂ O ₂ (3%, v/v) Stress Solution of Hypoxoside after 24 hr	138
Figure: 4.11.	UV Spectrum and Chromatogram of Acid Hydrolysis Sample of Hypoxoside Solution in 0.1 M HCl at 40° C after 8 hr	139
Figure: 4.12.	UV Spectra and Chromatogram of Alkaline Hydrolysis Sample of Hypoxoside Solution 0.1 M NaOH at 40° C after 4 hr	140
Figure: 4.13.	UV Spectra and Chromatogram of Neutral Hydrolysis (reflux) Sample of Hypoxoside Solution after 8 hr	141
Figure: 4.14.	UV Spectra and Chromatogram of Solid (dry) Photo-hydrolysis Sample of Hypoxoside after 30 hr Exposure ($\geq 1.2 \times 10^6$ lux hr at 35° C)	142
Figure: 4.15.	UV Spectra and Chromatogram of Wet (solution) Photo-hydrolysis Sample of Hypoxoside After 30 hr Exposure ($\geq 1.2 \times 10^6$ lux hr at 35° C)	142
Figure: 4.16.	UV Spectra and Chromatogram of Degradation Mixture (0.1 M HCl, 0.1 M NaOH, Neutral Hydrolysis, Dry and Wet Photo Degradation)	143
Figure: 4.17.	LC-MS Chromatogram of Neutral Hydrolysis Sample of Hypoxoside Solution Subjected to Reflux for 6 hr	145
Figure: 4.18.	Structural Re-arrangement of Pentenyne Skeleton	146
Figure: 5.1.	Principle of Evaporative Light Scattering Detector (ELSD)	149
Figure: 5.2.	Structure of Cholesterol	154
Figure: 5.3.	UV Spectra and Chromatogram of Standard Solutions	155
Figure: 5.4.	ELSD Chromatogram of Standard Solution	156
Figure: 5.5.	HPLC-ELSD Chromatogram of the Commercial Products	163
Figure: 5.6.	HPLC-UV Chromatograms of Product N Spiked with BSS, STG and STN	164
Figure: 5.7.	HPLC-ELSD Chromatograms of Product N Spiked with BSS, STG and STN	165
Figure: 5.8.	LC-MS Chromatogram of CHOL, STG, BSS and STN	166
Figure: 5.9.	LC-MS-MS Fragmentation Pattern of CHOL, STG, BSS and STN	167
Figure: 6.1.	Schematic Diagram of a CE System	170
Figure: 6.2.	Capillary Zone Electrophoresis (CZE) Separation Process	171
Figure: 6.3.	Micellar Electrokinetic Chromatography (MEKC)	172
Figure: 6.4.	Effect of pH on the Migration Time of Hypoxoside	177
Figure: 6.5.	Effect of Ionic Strength on the Migration Time of Hypoxoside	178

Figure: 6.6.	Electropherogram of Hypoxoside using 15 mM Borate Buffer	178
Figure: 6.7.	Effect of Applied Voltage on the Migration Time of Hypoxoside	179
Figure: 6.8.	Chemical Structure of Sulphafurazole	180
Figure: 6.9.	Electropherogram of Hypoxoside and Sulphafurazole (SF)	181
Figure: 6.10.	Recovery Studies on Product-D I	186
Figure: 6.11.	Electropherogram of Blank H ₂ O ₂ Solution (10%, v/v)	188
Figure: 6.12.	Electropherogram of a Mixture of Stress Treated Blank Solutions Used for Acid, Alkali and Neutral Hydrolysis	188
Figure: 6.13.	Electropherogram of a Mixture of Stress Treated Sample Solutions after Acid, Alkali and Neutral Hydrolysis	189
Figure: 7.1.	Schematic Diagram of Lipid Peroxidation	192
Figure: 7.2.	HPLC Chromatogram of Hypoxoside and Rooperol	198
Figure: 7.3.	Effect of Increasing Concentration of Rooperol, Hypoxoside and AP on QA-(1 mM) Induced Lipid Peroxidation in Rat Liver Homogenates	205
Figure: 7.4.	Effect of Increasing Concentrations of Rooperol, Hypoxoside and Aqueous Extract AP on 1 mM KCN Induced Super Oxide Radical in Rat Liver Homogenate	206
Figure: 9.1.	Dissolution of Product-D II at pH 1.2 and 4.5	233
Figure: 9.2.	Dissolution of Product-A	235
Figure: 9.3.	Dissolution Profiles of Different Products containing Hypoxoside in Phosphate Buffer (pH 1.2)	236
Figure: 9.4.	Dissolution Profiles of Different Products containing β -Sitosterol in FeSSIF (pH 5.0)	237
Figure: 10.1.	HPLC-ELSD Chromatogram of Extract of AP	242

LIST OF TABLES

Table: 2.1.	Traditional Uses of the Plants under the Genus Hypoxis	38
Table: 2.2.	Major Phytochemicals in Hypoxidaceae	47
Table: 2.3.	Pharmacokinetic Model Parameters	62
Table: 2.4.	Products of African Potato (AP)	87-88
Table: 3.1.	The IR Spectrum Absorption Bands	100
Table: 3.2.	¹³ C NMR Chemical shifts	107
Table: 4.1.	Influence of Mobile Phase on the Retention of Hypoxoside	116
Table: 4.2.	Accuracy and Precision of Hypoxoside	127
Table: 4.3.	Recovery of Hypoxoside	130
Table: 4.4.	Assay of AP Commercial Products	132
Table: 4.5.	Assay of AP Raw Materials	135
Table: 5.1.	Linearity of STG, BSS and STN	159
Table: 5.2.	Accuracy and Precision of STG, BSS and STN	161
Table: 5.3.	Content of Commercial Products (HPLC-ELSD)	162
Table: 5.4.	Recovery Studies of STG, BSS and STN	165
Table: 5.5.	Fragment Ions of CHOL, STG, BSS and STN	167
Table: 6.1.	Linearity of Hypoxoside	183
Table: 6.2.	Accuracy and Precision of Hypoxoside	185
Table: 6.3.	Recovery of Hypoxoside	185
Table: 6.4.	Assay of Formulations	187
Table: 7.1.	Comparison of Antioxidant Activities Using Quercetin as Control (DPPH assay)	203
Table: 7.2.	Comparison of Antioxidant Activities Using Ascorbic Acid as Control (FRAP assay)	204
Table: 8.1.	Contents of Commercial Products	215
Table: 8.2.	Phytochemical Content	218
Table: 8.3.	The % Inhibition of Human Cytochrome Enzymes ($n \geq 3 \pm SD$)	220
Table: 8.4.	The % Induction and % Inhibition of P-glycoprotein ($n \geq 6$)	221
Table: 9.1.	Fasted State Simulating Fluid (FaSSIF)	229
Table: 9.2.	Fed State Simulating Fluid (FeSSIF)	229

LIST OF ABBREVIATIONS

¹ H	proton
ABC	ATP binding cassette
ACD	advanced chemistry development
ACE	angiotensin converting enzyme
ADME	absorption, distribution, metabolism and excretion
ADR's	adverse drug reactions
AIDS	acquired immune deficiency syndrome
ALT	alanine transaminase
ANOVA	analysis of variance
AP	african potato
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
Ar	aromatic
ARTG	Australian register of therapeutic goods
AST	aspartate transaminase
ATCC	American type culture collection
ATM	African traditional medicine
ATP	adenosine triphosphate
ATP	lowered adenosine triphosphate
AUC	area under the curve
AUFS	absorbance units full scale
BBC	British broadcasting centre
BCS	biological classification system
BGE	background electrolyte
BHT	butylated hydroxytoluene
BP	British pharmacopoeia
BPH	benign prostatic cancer
BSS	beta sitosterol
BSSG	beta sitosterol glycoside
C	carbon
C ₈	octyl silane
C ₁₈	octadecyl silane
CA	California
CaCo	colon cancer
CAMs	complementary and alternative medicines
CANSA	cancer association of South Africa
CCD	counter current distribution

CD4	cluster of differentiation 4
CDCl ₃	deuterated chloroform
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
cGMP	current good manufacturing practice
CHMP	committee for herbal medicinal products
CHOL	cholesterol
CID	collision induced dissociation
CIEF	capillary isoelectric focusing
CITP	capillary isotachopheresis
Cl	clearance
CM	complementary medicines
C _{max}	maximum plasma concentration
CMC	complementary medicines committee
CMEC	complementary medicines evaluation committee
CMWG	complementary medicines working group
CNS	central nervous system
COX	cyclooxygenase
CPMP	committee for proprietary medicinal products
CT	computed tomography
CVD	cardio vascular diseases
CVS	cardiovascular system
CYP	cytochrome
CZE	capillary zone electrophoresis
D ₂ O	deuterium
DBF	dibenzyl fluorescein
DoH	department of health
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSC	differential scanning calorimetry
DSHEA	dietary supplement health and education act
DSVP	dietary supplement verification program
ECCMHS	expert committee on complementary medicines in the health system
ED	electrochemical detection
ED ₅₀	effective dose 50
ELSD	evaporative light scattering detection
EMA	European agency for the evaluation of medicinal products
EOF	electro-osmotic flow
ERP	expedited registration procedure
ESCOP	European scientific cooperative on phytotherapy

ESI	electro-spray ionization
EtOAc	ethyl acetate
EU	European union
FAB	fast atom bombardment
FA SI	field amplified sample injection
FaSSIF	fast state simulated intestinal fluid
FDA	food and drug administration
FDAP	freeze dried African potato
FeSSIF	fed state simulated intestinal fluid
FIP	international pharmaceutical federation
FIV	feline immuno virus
FL	Florida
FRAP	ferric reducing activity of plasma
FSDU	foods for special dietary uses
FTIR	Fourier transform infra red spectroscopy
GABA _A	γ -aminobutyric acid
GCMS	gas chromatography mass spectroscopy
GIT	gastrointestinal tract
gr	gravity
GRAS	generally recognized as safe
H ₂ O ₂	hydrogen peroxide
HAART	highly active anti-retroviral therapy
HCl	hydrochloric acid
HIV	human immune virus
HMBC	heteronuclear multiple bond correlation
HMP	herbal medicinal products
HO ₂ [•]	hydroperoxyl radical
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum correlation
I.S.	internal standard
IC ₅₀	inhibitory concentration 50
ICH	international committee for harmonization
ISO	international organization for standardization
IT	ion trap
IV	intravenous
IVIVC	<i>in vitro- in vivo</i> correlation
KCN	potassium cyanide
LC	liquid chromatographic/liquid chromatography
LC-APCI-MS	liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry

LC-MS	liquid chromatography-mass spectrometry
LC-MS-MS	liquid chromatography-tandem mass spectrometry
LD	lactate dehydrogenase
LDL	low density lipoproteins
LOD	limit of detection
LP	lipid peroxidation
MP	mobile phase
MW	molecular weight
MA	Massachusetts
MALDI	matrix-assisted laser desorption ionization
MCC	medicine control council
MDA	malondialdehyde
MeCN	acetonitrile
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MO	Missouri
MRM	multiple reaction monitoring
MRP	multidrug resistance-associated protein
MS	mass spectrometry
MWD	microwave dried african potato
N	normal
Na ₂ B ₄ O ₇ ·10H ₂ O	sodium tetraborate deca hydrate
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NBD	nitroblue diformazan
NBT	nitroblue tetrazolium
NDGA	nordihydroguaiaretic acid
NGO	non-government organisations
NHP	natural health product
NHPD	natural health products directorate
NHPR	natural health products regulations
NHPs	natural health products
NJ	New Jersey
NLEA	nutrition labelling and education act
NMR	nuclear magnetic resonance
NP	normal phase
NSAIDS	non-steroidal anti-inflammatory drugs
NY	New York
O ₂ ⁻	superoxide
OH	hydroxyl

ON	Ontario
OTC	over the counter
p.s.i.	pounds per square inch
PA	peak area
PBS	phosphate buffered saline
PC	prostate cancer
PD	plasma desorption
PDA	photodiode array
P-gp	p-glycoprotein
pI	isoelectric point
PMS	premenstrual syndrome
PUFAs	poly unsaturated fatty acids
PVDF	polyvinylidene difluoride hydrophilic filters
PVDP	low protein binding durapore
QAC	quinolic acid
QA	quality assurance
QC	quality control
QSE	quality, safety and efficacy
R	Registered
RCT	randomized clinical trials
RDA	recommended daily allowance
RE	relative error
RF-MEKC	reduced flow micellar electrokinetic chromatography
Rh123	rhodamine 123
RI	refractive index
RM	raw material
ROS	reactive oxygen species
RP	reversed phase
rpm	revolutions per minute
RSA	Republic of South Africa
RSD	relative standard deviation
S/N	signal-to-noise
SABC	South African broadcasting corporation
SC	subcutaneous
SD	standard deviation
SEM	scanning electron microscope
SF	sulphafurazole
SGF	simulated gastric fluid
SHD	shade dried African potato
SIF	simulated intestinal fluid

SIM	single ion monitoring
SOD	superoxide dismutase
SPC	summary of product characteristics
SRM	single reaction monitoring
STG	stigmasterol
STN	stigmasterol
SUN	sun dried African potato
$t_{1/2}$	elimination half life
TBA	thiobarbituric acid
TDAP	traditionally brewed African potato
TGA	therapeutic goods administration
TIC	total ion chromatogram
TM	trademark
TMD	traditional medicine
T_{max}	time at highest concentration
TMEC	traditional medicines evaluation committee
TPA	12-o-tetradecanoylphorbol-13-acetate
TPTZ	2,4,6-tripyridyl-1,3,5-triazine
TRAMED	traditional medicines project
TRM	traditional medicine programme
U.K	United Kingdom
UN	United Nations
US	United States
USA	United States of America
US FDA	United States federal drug act
USP	United States pharmacopoeia
UTI	urinary tract infection
UV-Vis	ultraviolet-visible
vs.	versus
WBC	white blood corpuscles
WHO	world health organization

LIST OF UNITS

β	beta
λ	lambda
γ	gamma
d	capillary diameter
cm	centimeter
r^2	coefficient of determination
$^{\circ}\text{C}$	degrees Celsius
g	grams
hr	hour
i.d.	inner diameter
C	ionic strength
kV	kilovolt
L	liter
m/e	mass-to-charge ratio
M	molar
μA	microampere
μg	microgram
μm	micrometer
μM	micromolar
μl	microlitre
mbar	millibar
mEq	milliequivalent
mg	milligram
ml	millilitre
mm	millimeter
min	minute
MF	molecular formula
mM	millimolar
MM	molecular mass
mOsm	milliosmolarity
ηl	nanolitre
o.d.	outer diameter
pH	negative logarithm of the hydrogen ion concentration to the base 10
pK_a	negative logarithm of the acid dissociation constant, K_a
sec	second
t	migration time of solute
V	applied voltage

Chapter 1

Introduction

Medicinal herbs and plants have been used to treat and prevent various ailments from time immemorial. In the recent past, there has been a renaissance in the use of traditional medicines whose tremendous gain in popularity has led to global expansion of the use of many traditionally used indigenous herbal drugs. Nowadays traditional medicines have not only continued to be used for primary health care of the poor in developing countries, but also found its way into countries where conventional medicine is used predominantly in the various national health care systems. Fact sheets of the World Health Organization (WHO) indicate that more than 80% of the world population relies on herbal medicines as part of their primary health care needs [1].

- In China, traditional herbal preparations account for 30-50% of the total medicinal consumption.
- In Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of children with high fever resulting from malaria is the use of herbal medicines at home.
- In Europe, North America and other industrialized regions, over 50% of the population have used complementary or alternative medicine at least once.
- In San Francisco, London and South Africa, 75% of people living with HIV/AIDS use traditional medicines (TMDs)/complementary and alternative medicines (CAMs).
- 70% of the population in Canada has used CAMs at least once.
- In Germany, 90% of the population has used a natural remedy at some point in their life. Between 1995 and 2000, the number of doctors who had undergone special training in the use of natural remedies had almost doubled.

- In the United States, 158 million of the adult population use complementary medicines and according to the USA Commission for Alternative and Complementary medicines, US\$ 17 billion was spent on traditional remedies in 2000.
- In the United Kingdom, annual expenditure on alternative medicine is about US\$ 230 million.
- The global market for herbal medicines currently stands at over US\$ 60 billion annually and is growing steadily.

Medicinal plants are considered to be responsible for more than 25% of conventional medicines on the market. It should be noted that plant constituents are not only used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. In the last decade, there has been an upsurge in the international market for herbal medicines in tune with the pharmaceutical and clinical research being carried out, although it varies between countries [1, 2].

This emerging market of CAMs has been considered to have not yet reached its full potential and many view this as the timely start of a so-called “herbal renaissance” [3]. There has been a growing trend to include natural products in the retail inventory of pharmacies. The sales of herbal medicines often have been reported to have increased over that of allopathic medicines [4]. This has led to the re-evaluation of conventional therapies by medical practitioners and recognition of the importance of a more holistic patient care approach [5]. More importantly, the impact of natural product use on the efficacy and safety of orthodox medicines has received a great deal of attention [4].

Furthermore, due to its rising popularity, some tertiary educational institutes have included certain fields of CAM as certifiable for degrees and diplomas [5]. These developments in the health care system make it evident that CAM is a ubiquitous phenomenon that requires specific recognition and attention. In fact, with its impact on other health sectors, CAM is predicted to become a permanent facet of health care systems [6].

Interestingly, many herbal drugs in the past have resulted in the production of toxic effects when administered concomitantly with allopathic drugs. Also, there have been reports of plant misidentification, mislabeling, adulteration, contamination, and

lack of standardization of various products such as, for example, dietary supplements. Past experience has left a lot to be desired with respect to quality, safety and efficacy (QSE) of such products [7].

Currently, as a prerequisite for marketing a conventional drug, proof of both safety and efficacy has to be submitted to the relevant country's drug regulatory authorities such as the Food and Drug Administration (FDA) in the USA. Herbal drugs are generally treated as dietary supplements in many countries. Many traditional/alternative medicine products are sold over the counter (OTC) and unlike conventional drugs, are mostly not regulated. It was evident from a survey conducted by WHO in 142 countries in which 99 responded that most of these products could be bought without prescription. In 39 countries, many traditional remedies were used for self-medication often through unskilled health professionals. This has led to rise of various undesirable consequences such as poor quality products, increased adverse drug interactions, overexploitation of natural resources etc. Thus, QSE of herbal medicines and traditional medicines have become important concerns for both health authorities and the general public globally, including South Africa.

1.1 Quality, Safety and Efficacy (QSE) of Natural Products

1.1.1 Quality of Natural Products

The WHO suggests that pharmaceutical assessment should cover all important aspects of quality of herbal medicines. In order for a medicinal product to produce a consistent therapeutic response, it is essential that the final composition of the product is invariable and the active ingredient/s is/are present in appropriate, nontoxic amounts. To attain consistent quality, the active constituent/s have to be unequivocally identified and isolated and the constitutional matrix should be readily controllable, as in the case of manufactured orthodox medicines [8]. Preferably, an official monograph should be developed if it doesn't already exist and all the procedures should be in accordance with current good manufacturing processes (cGMP).

Natural products, are more often than not, complex mixtures of compounds and often their therapeutic activities are reported to rely on the synergistic action of multiple and sometimes unknown components [8, 9]. Moreover, various other factors

such as, geographical location, soil type, cultivation methods, natural biological variation, season and time of harvesting, drying and processing techniques as well as storage conditions influence the levels of purported active constituents present in botanicals [10, 11]. Skilled, experienced personnel are required for correct herbal identification or confirmation of authenticity [12, 13]. During the dehydration processes, characteristic macroscopic structures may be altered, which adds to the difficulty of authenticating dried botanical products. There have been reports of plant materials being erroneously identified, processed and sold under the pretext of another product, often leading to disastrous ramifications [12]. Also, quite often, the quality of the initial plant material used in botanical products has also been reported to be dramatically compromised by adulteration by traders. Substitutions of costly herbs with more economical ones for financial benefit or attempts to dilute botanical material to comply with the increasing demands of manufacturers are typical of such practices.

In the early nineties, a significant number of end-stage renal failures and urothelial carcinomas were reported in Belgium. Investigations concluded that the concurrent administration of the nephrotoxin, aristocholic acid and the orthodox drug acetazolamide was responsible for the potentially lethal effects [12]. The aristocholic acid was reportedly derived from *Aristolochia frangchi* which was substituted for the herb *Stephania tetrandra* in a slimming regime which also contained orthodox medicines.

Information on botanical taxonomy, plant identification, definition and description of the part of the plant used as medicine should be provided for all crude plant material. In addition, an indication of the processing of the plant material should be provided. The active and characteristic constituents should be specified and the contents should be defined if possible. The limits of foreign matter, impurities and microbial content should be defined. The plant material used should be authenticated by a qualified botanist and a voucher specimen of each lot should be safely stored under appropriate storage conditions for at least a 10 year period. The material should be properly and clearly labeled and assigned a lot number [2].

Often, the content of active ingredients in a product may be altered to enhance or dilute the level of concentration by addition of pure compound/s or excipients, respectively. If the pharmacological activity is linked to a constituent or groups of constituents such as in St. John's Wort and *Ginkgo biloba*, which synergistically

contribute to the desired effect of which the mechanism is largely unknown, then standardization can be achieved by blending batches of the raw botanical material to yield higher or lower concentrations of such components provided the active markers have been identified [14].

The manufacturing procedure should be detailed and should describe whether the plant material was comminuted or powdered, whether extracts, tinctures, fatty or essential oils or expressed juices and preparations were included and whose production involved fractionation, purification and concentration. During the preparation, if any other substance has been added for any purpose or to adjust any of the characteristic constituents, a method for identification and assay of the plant preparation should be mentioned and described. It should be sufficient to identify a characteristic substance or mixture of substances (e.g. chromatographic finger print) to ensure consistent quality of the preparation, especially if the identification of the active principle is not possible [2].

For finished products, specifications should be defined along with information on the manufacturing procedure, formula and the amount of excipients included. The identification and quantitative information of the plant material in the finished product should be provided. If this is not possible, it should be sufficient to identify a characteristic substance or mixture of substances (e.g. by chromatographic finger print) to ensure consistent quality of the product. The finished product should also comply with the general requirements for any particular dosage form. Confirmation of the regulatory status of the product and raw material in the country of origin should be required for imported finished products. Also the WHO certification scheme relating to the quality of pharmaceutical products moving in international trade should be applied [9]. The shelf-life of the finished product should be established and the physical and chemical stability of the product in the container in which it is to be marketed should be tested under defined storage conditions.

1.1.2 Safety of Natural Products

There is a general misconception that herbal products are intrinsically safe and effective [10, 15]. This ominous belief is attributed to its natural origin. Often it has been noted that patients do not regard natural products as a form of medication and are unlikely to document natural product use when questioned by health care

professionals regarding their medication history [4, 10]. Natural products are indeed associated with various side effects as illustrated by the WHO which documented 9854 cases of adverse events in China during 2002, in contrast to 4000 cases which marked the entire period from 1990 to 1999 [16]. Less common but severe effects are related to central nervous system (CNS) disorders, liver toxicity, renal failure, heart palpitations and respiratory complications [17] whereas the most frequently reported adverse events include gastrointestinal symptoms, headaches, allergic skin reactions and menstrual disturbances. Some level of safety is required even when traditional herbal products have an inveterate history of use. However, it has to be understood that the determination and documentation of cumulative toxic effects or infrequent side effects in a traditional environment [8, 18] is not easy. In addition, issues and concerns of safety, when herbal preparations are produced and administered in Western arenas, may become more prominent since there might be significant differences in their traditional use [8].

1.1.2.1 Adverse Effects

According to the WHO's *Research Guidelines For Evaluating The Safety And Efficacy Of Herbal Medicines*, the reported and documented adverse effects, side-effects (recorded according to established principles of pharmacovigilance) of a herb or herb mixture, its closely related species, constituents of the herb and its preparations/finished herbal products should be taken into account when new pharmacological or toxicological studies are to be undertaken. A recent investigation in the US predicted that 99% of all adverse events due to natural product use were not detected by the implemented surveillance systems [19]. For example the anxiolytic, kava kava (*Piper methysticum*), which is a known hepatotoxin, could result in liver cancer. In 2002, in a follow-up study of 39 reported hepatotoxic effects related to the ingestion of various commercial kava preparations in Germany, nine patients developed fulminant hepatic failure and three deaths were recorded. The remaining patients spontaneously recovered after discontinuation of the kava products [20]. Furthermore, natural products may also indirectly affect patients' health and present safety risks when pertinent conventional therapy is delayed or replaced with herbal preparations that have not demonstrated reasonable efficacy [21]. In general, an

accurate reflection of the incidence of side effects caused by natural preparations is difficult to ascertain due to a lack of mandatory surveillance/pharmacovigilance systems that are generally available for orthodox drugs in most countries.

The absence of any reported or documented side effects is not an absolute assurance of safety for herbal medicines. However, a full range of toxicological tests may not be necessary. Tests that examine effects that are difficult or even impossible to detect clinically, should be encouraged. Suggested tests include immunotoxicity (e.g. tests for allergic reactions), genotoxicity, carcinogenicity and reproductive toxicity. Only when there is no documentation of long historical use of an herbal medicine, or when doubts exist about its safety, should additional toxicity studies be performed. Where possible, such studies should be carried out *in vitro*. Using *in vitro* tests can reduce the number of *in vivo* experiments. If *in vivo* studies are needed, they are to be conducted humanely, with respect for the animals' welfare and rights. Toxicity studies should be conducted in accordance with generally accepted principles, such as those described in WHO's *Research Guidelines For Evaluating The Safety And Efficacy Of Herbal Medicines* [2].

1.1.2.2 Adulteration and Contamination of Natural Products

Adulteration by heavy metals such as lead, mercury, cadmium and arsenic [12] and also the intentional inclusion of regulated proprietary drugs has often plagued botanical products. There have been incidents where prescription drugs such as alprazolam, colchicine, fenfluramine, sildenafil [11] and steroids [21], amongst others, have been identified in so-called "natural" products [11]. Furthermore, undeclared drugs commonly include ephedrine, chlorpheniramine, methyl testosterone and phenacetin. It has been reported that chemical analysis of the "herbal" remedy, PC-SPES, used in the treatment of prostate cancer, confirmed that it had been adulterated with various combinations of warfarin, indomethacin and diethylstilboestrol [11, 22]. In fact, among the traditional Chinese medicines in Taiwan, up to 25% of have been found to contain unspecified chemicals unrelated to the products' originally prescribed indications [21].

Products containing abnormal levels of bioactive components that could potentially cause toxic reactions are also considered to be adulterated [10]. In addition

to adulteration, often plant material may be contaminated with insects, residual insecticides, aflatoxins and fungal and bacterial growth [9, 13]. Incidents such as these jeopardize the health of consumers and cast doubt on the integrity of the natural product industry in its entirety.

1.1.2.3 Interactions with Orthodox Medicines

Herb-herb interactions and herb-drug interactions are a rising concern [15]. In 1998, a study revealed that 20% of US adults on prescription drugs admitted co-medicating with herbal preparations with an overall estimation of 15 million people that were at risk for serious adverse complications or therapeutic failure due to herb-drug interactions [23]. There are numerous examples of herb-drug interactions that are documented, most of which can be detrimental and even lethal [15] with few references suggesting possible beneficial effects [12]. Garlic (*Allium sativum*) is extremely popular as a spice as well as a phytomedicine used as an antihypertensive and cholesterol lowering medicine [24-28]. It has been shown that garlic affects the cytochrome P450 isoenzyme CYP3A4 as well as the intestinal transporter protein, P-glycoprotein (P-gp). Similar properties have also been observed with another prominent anti-depressant herbal medicine, St. John's wort (*Hypericum perforatum*). This herbal medicine exhibited interaction through its effect on cytochrome P450 isoenzyme CYP3A4 as well as on intestinal P-glycoprotein, thereby catalyzing the oxidative degradation of many therapeutic drugs, resulting in a fall to below their minimum effective plasma concentrations [11]. Allopathic medicines such as antiretrovirals, amitriptyline, warfarin, cyclosporine, theophylline and digoxin that are metabolised by the same metabolic pathway are likewise affected by this process [19]. Grapefruit juice, on the contrary, is an inhibitor of cytochrome P450 isoenzymes that could potentially result in elevated plasma levels of co-ingested orthodox medicines. Such interactions are of concern, especially when conventional drugs exhibit narrow therapeutic indices [18].

A review on the impact of herbal remedies on surgical patients suggested that natural products could be responsible for physiological changes in post-operative patients and may interact with prescribed medications causing severe complications such as excessive bleeding, strokes, cardiac arrests, organ transplant rejections and

alterations in anesthetic effects. It is of great concern that many patients are unaware of potential herb-drug interactions and/or adverse effects, posing questionable doubt on the circulating knowledge available to under-informed consumers [21].

1.1.3 Efficacy of Natural Products

To date, only relatively few herbal products have been subjected to rigorous randomized controlled clinical studies that are routinely used to prove the efficacy of conventional medicines. It is important for herbal medicines, and particularly for those made from mixtures of herbal products, that the requirements for proof of efficacy, including the documentation required to support the indicated claims, should depend on the nature and level of the indications. For the treatment of minor disorders, for non-specific indications, or for prophylactic uses, less stringent requirements (e.g. observational studies) may be adequate to prove efficacy, especially when the extent of traditional use and the experience with a particular herbal medicine and supportive pharmacological data are taken into account. The level of evidence and the grading of recommendations must correspond to the nature of the illness to be treated or the nature of the physical or mental function to be influenced and regulated. Definitions of levels of evidence and the grading of recommendations from the USA Agency for Health Care Policy and Research may be used as a guide. Many other national documents, such as the Australian *Guidelines For Levels And Kinds Of Evidence To Support Claims For Therapeutic Goods Administration* [29], can also be used as a reference. In most countries, natural products are not required to undergo the same testing procedures as conventional medicines and efficacy is usually credited to the fact that such medicines have well-established use or have been used traditionally over extended periods of time, sometimes even centuries [8].

Historical use certainly does give natural products a degree of efficacious merit, and it can be argued that more definitive data need to be accumulated to vindicate appropriate levels of efficacy. For example, Mistletoe has a history of anti-carcinogenic activity but a randomized controlled clinical trial has shown that it did not significantly decrease morbidity or increase the quality of life of patients suffering from head and neck cancer [8]. The therapeutic alternatives available within the

community and the risks of the herbal medicine have to be taken into account. It should be noted that in the case of herbal medicines made from mixtures, a therapeutic or scientific reason must exist for the presence of each herb in the mixture. Numerous clinical trials using herbal preparations have indeed been performed in recent years but multiple concerns have been highlighted regarding the applied methodology and overall quality of the presented data [8].

Research needs to be carried out on the possible therapeutic effects of herbal medicines made from herb mixtures or specific combinations of herbs. The regulatory situation of many world countries has been reviewed in the WHO/TRM/98 document [30]. The legal situation regarding herbal preparations varies from country to country. In some developed nations, the regulations of phytomedicines are well-established whereas in developing countries, hardly any legislative criteria exist to establish their richly used traditional herbal medicines. Some countries have tried to draw a distinction between "officially approved" products and "officially recognized" products, by which the latter products can be marketed without scientific assessment by the authority [31].

It is obvious that there are currently limitations, especially relating to the evaluation of efficacy, with regard to performing recognized clinical trials for herbal medicines and protocols need to be carefully developed and standardized. Many of these limitations and variables could be eradicated by addressing problems directly relating to the quality of herbal preparations.

1.2 Regulatory Aspects of Complementary and Alternative Medicines

1.2.1 Definitions

Some definitions for concepts involving non-conventional or natural substances used to improve health or treat diseases are used interchangeably, often incorrectly [32]. Guidelines For The Assessment Of Herbal Medicines [33] and Research Guidelines For Evaluating The Safety And Efficacy Of Herbal Medicines [2] have redefined certain terms in order to make them acceptable, consistent and internationally standard so that they could be used in the evaluation and research of herbal medicines. The following definitions are intended to differentiate terminology and are kept as neutral as possible. However, it is important to note that CAMs is

currently dynamic in nature and evolves as perspectives are altered, hence the descriptive terminology may need to be modified. Furthermore, whilst these definitions conform to those generally used, they may differ from country to country [34-38].

Active Ingredients: Active ingredients refer to ingredients of herbal medicines with therapeutic activity. In herbal medicines where the active ingredients have been identified, the preparation of these medicines should be standardized to contain a defined amount of the active ingredients, if adequate analytical methods are available. In cases where it is not possible to identify the active ingredients, the whole herbal medicine may be considered as one active ingredient.

Active Marker: A constituent of a herbal product with pharmacological activity that contributes to the efficacy of the product.

Active Principle: A constituent that has a proven clinical activity.

Analytical Marker: A constituent of a herbal product without any pharmacological activity yet can be used as a relevant marker for quality control [39].

Alternative Medicines: All health care practices that can be adopted instead of mainstream treatment regimes and are therefore mutually exclusive [40].

Aromatherapy: Therapy involving the use of aromatic oils extracted from plants primarily by steam distillation or physical compression [41].

Ayurveda: Ancient Indian treatment regime which focuses on the psycho-metabolic requirements of individuals and encompasses mineral, herbal and animal products as well as exercise and meditation in order to harmonize the inner self and promote a healthy, self-healing environment [41].

Botanicals/Botanical Health Product: A preparation of plant origin that is not ingested as a nutritive source for metabolism but contains chemical components which are claimed to maintain or promote health [42, 43].

Chinese Medicine: Studies the human body's energy patterns and aims to correct energy flow imbalances characterized by disease. Practitioners may employ various therapies to rectify the imbalance including herbal medicines, acupuncture, meditation, massage etc. [44].

Conventional and Orthodox Medicines: “Any substance or mixtures of substances used or purporting to be suitable for use of manufacture or sold for use in:-

- a) Diagnosis, treatment, mitigation, modification, or prevention of a disease, or abnormal physical or mental state, or the symptoms thereof in man, or
- b) Restoring, correcting, or modifying any somatic or psychic or organic function in man, and includes any veterinary medicine” [29, 45].

Complementary Medicines: Unconventional drug treatment regimes to be used in conjunction with mainstream health care in order to achieve an appropriate therapeutic response with minimal or reduced side effects [40].

Complementary and Alternative Medicines (CAMs): “Broad domain of healing resources that encompass all health systems, modalities, and practices and their accompanying theories and beliefs, other than those intrinsic to the politically dominant health system of a particular society or culture in a given historical period” [40].

Dietary/Nutritional Supplement: Nutrients such as essential vitamins and minerals which are usually administered independently from food stuffs in a liquid or oral dosage form and contribute to a balanced diet. However, the US has included botanical ingredients, amino acids and glandular extracts in this definition [3].

Food: Animal and/or botanical source of essential nutrients/substrates necessary for physiological metabolic processes which maintain vitality and enable growth.

Foodstuffs may include substances with little or no nutritional value such as flavourants and colourants [42, 43].

Foods for Special Dietary Uses (FSDU)/Medicinal Foods: Functional foods that cater for specific dietetic needs e.g. sugar-free foodstuffs for diabetics and products that contain cardio-protective antioxidants [3].

Functional Foods: Foodstuffs which are generally associated with a habitual diet but which also contain nutraceuticals i.e. they contribute to general health and may prevent disease states. In some instances, foods are modified in order to claim this effect [3].

Finished Herbal Products: Finished herbal products consist of herbal preparations made from one or more herbs. If more than one herb is used, the term “herbal product mixture” can also be used. Finished herbal products and herbal product mixtures may contain excipients in addition to the active ingredients. However, finished products or herbal product mixtures to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal. [46].

Flower Remedies/Essences: Uses spring water infused with flower essences to influence patients’ emotional state and restore “happiness”, thereby assisting the body to achieve wellness [44].

Gemmotherapy/Embryophytotherapy: A gentle, noninvasive treatment which effectively drains the body from toxins by using embryonic plant tissue harvested from seedlings [44].

Herbal Materials: Herbal materials include, in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir-baking with honey, alcoholic beverages or other materials [37].

Herbal Medicinal Products (HMP): Phytopharmaceuticals whose levels of QSE have been justified by rigorous scientifically approved pharmacological and clinical studies

which are usually applicable to allopathic medicines i.e. are included in rational phytotherapy [32, 42, 43]

Herbal Preparations: Manufactured herbal products containing singular or combinations of complementary herbs in various forms of powdered materials, tinctures or extracts [19, 32, 40].

Herbs: Crude botanical materials which encompass any plant matter in whole, fragmented or pulverized forms and may include stems, leaves, flowers, seeds, fruit, roots, bark, rhizomes etc. [40].

Homeopathy: A curative based approach involving administration of minute doses of substances that induce or potentiate diseased states at higher concentrations and which then stimulates the body's own physiological healing mechanisms [3].

Homotoxicology: This is based on the premise that disease is the body's defense mechanism to combat the effect of accumulated toxins and uses a collaboration of medical science as well as homeopathy in the design of anti-homotoxic medication [44].

Nutrients: Constituents inherently present in food that are essential for life and growth such as vitamins, minerals, trace elements and amino acids. Some nutrients have demonstrated reasonable evidence to be classified as nutraceuticals [3].

Nutraceutical: Any compound present in a food or dosage form that provides health benefits to the consumer beyond contributing to basic physiological nutrition [3].

Natural Health Product (NHP): Virtually synonymous with herbal preparations but may also contain mineral and animal extracts [21].

Naturopathy: The use of nontoxic treatment methods adopted from various traditional healthcare systems for the prevention and cure of diseased states with accentuation on the individuals' health in its entirety [40].

Negative Marker: A constituent that has a negative impact on the product such as a toxin [39].

Phytomedicines/Herbal Medicines: Botanical material that contains one/more inherent active constituent and are therefore theoretically capable of inducing a therapeutic response [42, 43].

Phytopharmaceuticals: Solid oral or liquid formulations originally derived from plant material and which are intended for therapeutic use [9, 32].

Phytotherapy: Treatment that involves the medicinal use of plants or extracts from plants [3].

Rational Phytotherapy: Rational allopathic treatment regime using botanical dosage forms that have been subject to the same rigorous evidence-based procedures used to prove the QSE of orthodox medicines and are therefore considered to be of the same scientific stature as conventional treatment regimes. Rational phytotherapy is intended to cure ailments or diseases [32].

Standardized Extract: A standardized extract is defined as the herbal extract that is assured to contain a specific amount of active ingredient (s) or marker compound (s), which are found in the whole herb. The quality of a standardized extract therefore, should be based on assays of selected key compounds, either those of which pharmacologic activity is known and/or those that serve as markers for the source material and biologically active compounds [37].

Therapeutic Activity: Therapeutic activity refers to the successful prevention, diagnosis and treatment of physical and mental illnesses; improvement of symptoms of illnesses as well as beneficial alteration or regulation of the physical and mental status of the body.

Tibetan (Sowa Rigpa): Based on the tenet of so-called “humoural imbalances” and adopts ayurveda, bon shamanic, chinese medicine and unani tibb practices in order to

treat the physical and psychic “being”. It is believed that certain disorders are caused by afflictive emotions and/or evil spirits and relies on karma to address or influence these diseases [47].

Traditional African Medicine: Similar to traditional phytotherapy however mineral and animal products may be included in the formulation and the treatment is of African origin [44].

Traditional Medicine: Traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness. It also refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being.

Traditional Phytotherapy: Phytopharmaceuticals whose evidence of QSE is drawn from traditional/historical use rather than scientific evidence and claim to aid in the prevention of diseases [32].

Traditional Herbal Medicinal Products: Phytopharmaceuticals which demonstrate appropriate standards of quality and safety, while efficacy is based on longstanding or historical use [44].

Unani Tibb (Unani Sidda): This is a holistic treatment regime encompassing Greek, Arabic, Egyptian and Indian medicine. It is based on the theory that all matter comprises of 4 basic elements symbolized by water, earth, air and fire in different ratios which gives the object its own unique “Temperament”. This “Temperament” is maintained by the bodily fluids which, in turn is influenced by food and liquids that are ingested. Disease is as a result of imbalance of the bodily fluids which are then corrected by natural medicines, diet, regimental and psychological therapies [44].

In order to avoid semantic problems with some terms used, terms such as herbal, CAMs and dietary supplements are used interchangeably throughout this thesis.

1.2.2 Regulatory Situation of Complementary and Alternative Medicine (CAM) in Several Countries

1.2.2.1 The United States of America (USA)

In the late 1930s, the Food, Drug and Cosmetic Act was passed and, from that time on, the Food and Drug Administration (FDA) has regulated herbal products or nutraceuticals as dietary supplements, under the Federal Food, Drug and Cosmetic Act (FD and C Act). Natural products theoretically have "generally recognized as safe" (GRAS) status, as long as qualified experts confirm this and are not contradicted by other experts. Since 1976, following the "Proxmire Bill", the FDA desisted from developing monographs on dietary supplements, vitamins, minerals and herbs, as has been done for several kinds of medicines. In 1990, the US Congress passed the Nutrition Labelling and Education Act (NLEA) which required that all food products must have nutritional labelling, and required the FDA to establish criteria for approving health benefit labelling for foods. In November 1992, the FDA established a new advisory committee of outside experts for OTC medicines [48]. With the passage of the Dietary Supplement Health and Education Act (DSHEA) of 1994, Congress amended the FD and C Act to include several provisions that apply to dietary supplements. It also established an office to coordinate research on dietary supplements, and it required an independent dietary commission to be initiated to report on the use of claims in dietary supplement labelling.

Dietary supplements may comprise of essential vitamins, minerals, glandular extracts, amino acids and herbal preparations and currently fall under the blanket cover of food [49]. Under the assumption that natural products are safe and that the consumer is entitled to affordable autonomous healthcare, the regulation of herbal preparations differs drastically from orthodox medicines in that they are not required to undergo pre-market QSE testing.

The FDA recognises that its requirement for pre-market review of dietary supplements is less than those for other products which it regulates, implies that

responsibility for checking the safety of dietary supplements and determining the truthfulness of the label claims falls in the hands of manufactureres and consumers [34]

This has left a margin for ambiguous interpretation [50] and it has been reported that up to 55% of advertising over the Internet make illegal health claims [19]. In addition, manufacturers that receive reports relating to adverse events are not obliged to relay that information to the FDA. As a result, the FDA's jurisdiction is limited to restriction measures or, in extreme cases, warranted recall of marketed products that have been already proven to jeopardize the health or safety of consumers.

The US market, in the last decade, has been riddled with reports on substandard levels of QSE of natural products. In order to save this industry, the US government was compelled to address these pertinent issues. This lead to discussions relating to the future introduction of appropriate standardized cGMP procedures that will involve validation and stability testing. Furthermore, verification of the actual content with product label information and manufacturing procedures in compliance with USP standards is to be pursued by a Dietary Supplement Verification Program (DSVP) which has been launched by the United States Pharmacopoeia (USP) [50]. DSVP logo seals will need to be displayed on products by the subscribing companies as a guarantor of quality. Frequent revision of official monographs on dietary supplements has also been committed to be undertaken by USP. Online analytical methods for routine product testing of popular dietary supplements are intended to be made available on the USP website.

FDA guidelines on the scientific evidence that is required to ratify health-related claims have been issued and the FDA has threatened legal action against companies that disseminate inept and misleading information [50].

1.2.2.2 Canada

In Canada, the Natural Health Products Directorate (NHPD) oversees natural health products (NHP) under official Natural Health Products Regulations (NHPR). It has been planned to effectively implement the regulations in increments over the next 6 years starting from January 2004 [51]. Primarily, NHPs comprise a diverse array of

compounds that may include “any product derived from a plant, animal or microorganism, vitamins, minerals, and homoeopathics that are used to diagnose, treat, prevent disease; restore or correct function, maintain or promote health”. However, items such as biological blood products, marijuana, tobacco and intravenous (IV) or subcutaneous formulations (SC) [41], which are stipulated in the exclusion list are not permitted to be incorporated into NHP formulations

The NHPRs state that all products must be licensed and issued with identification numbers. NHP licenses are attainable if suitable evidence of safety and health benefits have been put forward to the directorate [51] or if a monograph exists for the product in question [52]. Legitimate site licenses as well as individual importing, manufacturing, packaging and product labeling licenses are mandatory and the safety of NHP presides with the license holder of the product. In addition, a site license holder is considered responsible for adverse events that may occur with NHP use and quality control reports. In such cases, information must be available for inspection by the NHPD upon request. GMP procedures are required for processing, manufacturing, packaging, labeling and distribution of NHPs and include suitable standards of proficient personnel, premises, equipment, quality assurance, sanitation, stability and the maintenance of audited records, standard operating procedures etc. The same quality control (QC) standards are required for imported goods [52].

Applicable benefits associated with product according to definition could be suggested for licensed NHPs. Package labels should contain information that ensures cognitive, responsible decision-making thereby bestowing autonomy of healthcare on the consumers’ part. The information on product labels/inserts must contain indications for appropriate use, quantity, daily dosage, administration route, the recommended duration of the treatment, suitable storage conditions and also include applicable warnings, precautions, side effects and possible drug interactions [51].

It should be noted that in Canada, all CAM products and NHPs fall under the national NHPD legislation and are regulated by the Federal government, while health care practices are regulated provincially and in some cases, not at all. For example, naturopathic practitioners are regulated in four traditional areas, Chinese medicine in only one province; whilst homoeopathy is regulated in none. This could possibly have implications on the public safety, especially through different standards of training and competency of such practitioners [41].

1.2.2.3 Europe

Due to reasons attributed to historical and cultural heritage, countries within the EU differ substantially when it comes to the regulation of natural products. Natural health products are categorized in between the realm of supplements and medicines, with diverse procurements required for subsequent pre-market authorization. Hence, most natural products are regarded as either botanical or herbal medicinal products that are regulated by food or pharmaceutical legislations, respectively. As a result, natural health products are limited to the state in which authorization was granted with minimal interstate circulation especially of traditional herbal products [49].

In order to promote free movement of medicines within the common market of the European Union, a general rule called "Summary of Product Characteristics (SPC)" came into effect in 1995. Under this procedure, approval by the first authority must be taken into account and if any differences in evaluation occur between national authorities, a decision will be reached by a European Community (EC) procedure [53].

In 1989, the European Scientific Cooperative on Phytotherapy (ESCOP) was founded to establish harmonized criteria for the assessment of phytomedicines, to support scientific research and to contribute to the acceptance of phytotherapy at a European level [54].

Herbal medicinal products may obtain pre-market authorization under the European Council Directive 2001/83/EC. Registration requires adherence to official positive botanical monographs as compiled by the WHO or ESCOP or by subscribing to the prerequisites required for traditional herbal medicinal products. Official monographs are established by the Committee for Herbal Medicinal Products (CHMP) which is involved in processing requisitions for registration and pre-market approval [55] and contains information pertaining to the definition (including the plant part used in preparation), identification (fingerprints), purity, radioactivity, adulterations and content of active constituents or chemical marker compounds of the product [9]. The herbal drugs, especially those with long history of use for a minimum of 30 years globally and 15 years within the EU, are fairly easier to market in Europe with the current regulatory frame.

There are two reasons for this:

- a) Substances are approved under the ‘doctrine of reasonable charity’.
- b) The time to regulatory approval is relatively short.

This facilitates the marketing of a herbal drug with a long history of use even though it does not have any scientific evidence to validate its safety and efficacy [3]. Products containing vitamins and minerals in conjunction with traditional herbal medicines may seek licenses under historical use if the pharmaceutical action of the herbal actives supercedes that of the supplementary ingredients [55].

In Europe, Germany is considered to be the country with the highest turnover in herbal medicines and phytomedicines have shared equal status with orthodox medicines since 1978, especially regarding the requirement to submit full evidence of QSE prior to marketing. In 1978, an expert committee called Commission E was established and has now published more than 400 monographs on natural products that provide essential information on pharmacological-toxicological studies as well as the safety and clinical efficacy of popular herbal products [56]. Much of this was based on information derived from historic use of herbal drugs and preparations from medicinal plants [39, 57].

Even though, this system is highly efficacious, it has evolved over a considerable period of time and linked to exorbitant costs. In view of the significant cost implications, the natural products industry might be crippled if this system is implemented in other countries, which could lead to a decrease in availability of herbal products to consumers [12]. However with more simplified provisions these regulations could serve as a model framework for the current European Union (EU) legislation for traditional herbal medicines [49].

The EU has no laws pertaining to the regulation of botanical products as food and some EU states have developed their own regulatory approach and legislation, mostly using positive and/or negative lists [49, 58]. For example, in some EU countries, such as the Netherlands and Belgium, information directly relating to the usual health claims permitted for herbal medicinal products when present in food and food supplements is prevented by the EU food law and hence consumers remain unaware of potential health benefits and/or hazards which may be associated with those herbals.

1.2.2.4 United Kingdom (UK)

In contrast to many other countries, the regulation of CAM in the UK is focused on CAM disciplines rather than the actual products used in phytotherapies. Accordingly, three categories have been formed under CAM disciplines. They are professionally organized alternative therapies (acupuncture, chiropractic, herbal medicine, homoeopathy and osteopathy), complementary therapies (for example aromatherapy, hypnotherapy, shiatsu, nutritional medicine and yoga) and finally, alternative disciplines which includes established and traditional healthcare systems (e.g. ayurvedic, naturopathy, chinese herbal medicine and traditional chinese medicine) as well as other alternative disciplines (crystal therapy, dowsing, iridology, kinesiology and radionics). Under an Act of parliament, osteopaths and chiropractors alone are the two CAM professions that are regulated. Accordingly, legal permission should be obtained through registration with their respective Councils [58]. Hence, one should be duly qualified in either of these two professions in order to practice under their professional titles.

With the increase in popularity, it has been recognized that CAM industry needs to be effectively regulated to ensure public safety and confidence. In this respect, the Health Act (1999), has now facilitated statutory self-regulation or the registration of united, single bodied organizations within all CAM professions. Each profession pertaining to a discipline will be monitored for suitable conduct directly based on their regulatory framework [58]. For those organizations wishing to follow the recommended routes, funding in the form of grant awards have also been made available. Adequate training and competency are the additional responsibilities of the professional bodies within their field of practice [59]. Statutory bodies enforce disciplinary action in cases of confirmed malpractice [58].

In the UK, herbal preparations that enter the market are mostly imported as nutritional foods or are formulated using imported dry herbal extracts. Like elsewhere, the quality and safety of herbal products is a contentious issue and is of concern. A number of potentially serious quality control issues could arise as a direct result of accidental substitution of botanicals due to incorrect plant identification since many plants are known to have similar nomenclature but differing pharmacology/toxicology [12].

Consequently, suppliers and manufacturers of these products are required to guarantee product quality by ensuring absence of adulteration and contamination. This is accomplished by providing information regarding the authenticity of raw materials, details of product ingredients, harvesting dates and even adopted processing techniques. As mentioned earlier, the assurance to the customers of the highest possible quality and safety of herbal products is the professional obligation of the skilled CAM practitioner [60]. Appropriate and necessary education and training in the respective CAM disciplines covers these aspects [59].

Although the efficacy of CAM is considered secondary to issues of product quality and safety products which make claims to treat specific diseases are mainly directed to professionally organized alternative therapies in the first category and should have appropriate scientific evidence to prove efficacy “above and beyond the placebo effect”. Practices that fall into the second CAM category and which are generally used in conjunction with orthodox medicines are not required to provide substantial evidence of efficacy. The last group of CAM disciplines that include the traditional healthcare systems and other miscellaneous alternative therapies are accepted with little or no scientific proof of efficacy.

The general lack of CAM evidence in all disciplines has however prompted authorities to devote funding and attention to research, including the performance of appropriate Randomized Clinical Trials (RCT). This has been done with an intention to compile a database comparable to that expected of conventional medicines [59, 61].

1.2.2.5 Asia

Most of the Asian countries including China, Japan, Pakistan and India have a long history of herbal medicinal use. In many countries of this region, it is still being used at par with conventional medicine. In Japan, the Nutrition Improvement Act regulates the definition, licensing and approval of functional foods, which in turn is administered by the Japan Food and Nutrition Association. But in most countries such as Pakistan, no herbal drug law has been put into force to restrict the aggressive advertisement and free availability of the herbal products.

In India, with its rich heritage of traditional medical systems such as ayurveda, about one-third of the government’s medical posts are occupied by physicians who

belong to the traditional systems [62]. In India, traditional medicines are governed by the Drugs and Cosmetics Act of 1940 and the Drugs and Cosmetics Rules of 1945. From 1959, no products derived from traditional systems were allowed to be manufactured without a licence from the State Drug Control Authorities. The government has set up a separate expert committee and a pharmacopoeial committee for ayurvedic, naturopathy, yoga, siddha and unani drugs. The first step in granting this recognition was the creation of the Central Council of Indian Medicine Act of 1970. The mandates were to establish education and recognition of professionals, prescribe standards of professional conduct and etiquette, and develop a code of ethics to be observed by practitioners of traditional medicine in India.

The Central Council of Homeopathy, constituted in 1973, has the same mandates. The Indian Government created the Department of Indian Systems of Medicine and Homeopathy in March 1995. Through the establishment of such bodies, the Indian Government seeks the active and positive use of traditional medicine and homeopathy in national health programmes, family welfare programmes, and primary health care [63].

In 1993, an expert committee appointed by the Indian government developed guidelines for the safety and efficacy of herbal medicines and it proposed that no new herbal medicines other than those authorized by the licensing authorities be allowed to be manufactured or marketed, except for those mentioned in and manufactured in compliance with the formulae given in the "authoritative" books for ayurveda, siddha and unani herbal medicines where classification is made on the basis of process and constitution of potentially poisonous plants.

The following categories have been established:

- Category 1: medicines already in use for more than 5 years
- Category 2: medicines in use for less than 5 years
- Category 3: new medicines.

The requirements for safety and efficacy vary according to the various categories, classification and market availability of the product [64].

1.2.2.6 Australia

In Australia, the Therapeutic Goods Act 1989 has laid a framework for the regulation of therapeutic goods in Australia, and ensures their quality, safety and efficacy.

According to the Therapeutic Goods Act 1989, therapeutic goods for human use that are imported or manufactured in Australia must be included in the Australian Register of Therapeutic Goods (ARTG). The Traditional Medicines Evaluation Committee (TMEC) was established to provide expertise for the evaluation of non-prescription traditional medicines and to give advice to the authority on their registration [29, 65, 66].

Therapeutic Goods Administration in its edition "TGA Approved Terminology for Drugs", dated January 1993, has included herbs in its list of Australian Approved Names for Pharmaceutical Substances [66]. The TGA is mandated to guarantee appropriate levels of product QSE. A therapeutic product is either classified as a low or high risk substance depending on the assessed safety of the product and is then referred to as either a Listed or Registered product, respectively.

The overall safety of a product is evaluated in terms of:

- potential toxicity;
- indications for use i.e. whether the product claims overall health benefits and prevention of diseased states or intends to cure diseases or disorders;
- possibility of the onset of significant side effects associated with its use and finally;
- likelihood of adverse reactions after extended periods of administration [67].

Complementary medicines classified as listed goods are required to comply with Australian legislation but the formulations/dosage forms are not evaluated in terms of product safety, quality and efficacy. The TGA is also concerned with the processing procedures and traditional use of herbal products since, if a product has been historically used for a particular ailment and prepared accordingly, the safety of that product can be presumed and appropriate for Listing [67].

Manufacturers are not required to obtain the approval of the TGA to make structure/function claims on their product labels. However, the TGA has the

jurisdiction to request and evaluate evidence if the provided information is found to be deliberately misleading to the public or the product poses serious safety concerns after marketing. The TGA, together with the Complementary Medicines Evaluation Committee (CMEC) have drafted Guidelines for suppliers on appropriate interpretation of evidence used to substantiate therapeutic claims.

Control of the supply of therapeutic goods is performed by the TGA, who administers the provisions of the Therapeutic Goods Act 1989, and also exerts control over therapeutic drugs through three avenues:

1.2.2.6.1 Pre-market Assessment

Non-prescription items such as complementary medicines are considered as being low risk items, and as such are assessed solely for quality and safety (not efficacy). Registered complementary medicines which profess therapeutic benefits beyond structure/function claims and may pose health risks to consumers through inappropriate self-medication or long term administration, thereby requiring additional data to be provided to substantiate use. These products are required to undergo pre-market evaluation of QSE and may include both OTC and prescription medicines. Both Listed and Registered products must bear the characteristic AUST L or AUST R numbers associated with their respective classification system [67].

1.2.2.6.2 Licensing of Manufacturers

All manufacturers of therapeutic goods must be licensed, and their processes must comply with principles of Good Manufacturing Practice (GMP). Also, imported phytomedicines are subject to the same GMP requirements before they can be marketed. The TGA employs a GMP task team that inspects industrial sites and evaluates adopted manufacturing procedures before issuing licenses. Thereafter, follow-up inspections are performed at regular intervals [67].

1.2.2.6.3 Post-market Vigilance

This includes activities, such as problem reporting, monitoring activities and laboratory testing to ensure compliance to legislation. This is mostly characterized by

targeted, random audits for listed products, chemical testing of commercial products or formulary ingredients and advertising and marketing surveillance. Australia also has an integrated system in place for reporting adverse reactions associated with complementary medicine use and has expedient recall procedures in place for warranted removal of products if they are found to compromise the health and safety of the Australian consumers [67, 68].

The government has elected a new statutory committee, the Expert Committee on Complementary Medicines in the Health System (ECCMHS) to address the shortcomings in the CAM legislation [8]. This was in response to the reports of substandard quality control and complaints of severe adverse effects that caused major damage to the Australian public's confidence in CAM. Consequently, the committee has proposed that the quality of complementary medicine ingredients should be enforced legally and the evidence used by manufacturers to make claims for therapeutic use and indications be subject to thorough scrutiny. The government has also agreed to disseminate accurate, interpretable information to the public to ensure that consumers make informed decisions regarding their choice of healthcare. Improvements to the efficiency of the adverse events surveillance system are also underway and the government has committed to support and promote CAM research in Australia [45, 67].

1.2.2.7 South Africa

Various approaches to regulate complementary and alternative medicines in South Africa have been attempted over recent years and although appropriate legislation is imminent, it is not without serious contention. Also, the trade in crude indigenous herbal products is not completely regulated. However, any finished product with a health regulated claim has to go through the full medicines evaluation procedure by the Medicines Control Council (MCC) before marketing. The MCC recently appointed a Complementary Medicines Committee (CMC) as well as a separate Committee for African Traditional Medicines (ATMs) primarily to attend to issues relating to the QSE of as such [69]. However, specific regulations for the registration and control of CAMs and ATMs are yet to be implemented [70].

Since 1981, minerals and from 1985, vitamins were required to be registered with the MCC if the content in a product was higher than twice the recommended daily allowance (RDA) while exoneration of vitamin registration was granted if the concentration fell below the specified recommended level. Vitamins and minerals are now however classified as nutritional supplements in SA and will therefore be subject to the new regulations governing pre-market safety [69].

It has been estimated that up to 70% of the South African population consult traditional healers and is therefore imperative to develop an optimal QSE framework to ensure preservation of this cultural heritage [71].

The present regulations of the MCC with respect to traditional herbal medicines are comparable to those of the FDA prior to the DSHEA of 1994 [71]. In order to implement the National Drug Policy with respect to traditional medicines, the National Department of Health established the National Reference Centre for Traditional Medicines. Traditional medicines are included in the drug policy section of the government's Reconstruction and Development Programme.

In 1994, The Traditional Medicines Programme (TRAMED) at the Department of Pharmacology, University of Cape Town, was established to participate in formulating an outline proposal for the registration and control of traditional medicines. The objectives of TRAMED include, promotion of the use of safe, effective and high quality "essential" traditional medicines; promotion of the documentation of traditional medicines, and their scientific validation [72].

In August 1998, the South African Parliament decided to enlist the help of traditional healers in achieving major goals in primary health care. However, the affiliation of such healers is often being conversed [73].

In 1997, A Complementary Medicines Working Group (CMWG) was appointed to develop an appropriate registration system for complementary medicines. This paved the way for the development of a unique system for the registration of CAMs.

a) A full registration application, comprising “toxicity issues, safety and efficacy through preclinical and clinical evidence as well as quality”.

b) Registration through a listing procedure known as the Expedited Registration Procedure (ERP) or Registration through Listing Procedure.

The ERP allows many different disciplines and categories of complementary medicine and is based on the Australian system of complementary medicines. This is in contrast to the legislation in US, where the health system deals only with herbal and/or nutritional supplements; or to that of Germany where it caters only for anthroposophy, homeopathy and herbals.

In the case of traditional medicines, issues of safety and quality take precedence over demonstrations of efficacy. The aim is to regulate and not to prevent access to what many people use in preference to allopathic medicines.

In February 2002, a call up notice for all medicines frequently referred to as complementary medicines in terms of the Medicines And Related Substances Control Act, 1965 (Act 101 of 1965). Accordingly, manufacturers were required to submit information relating to their products and the “call-up” was seen to be a primary step towards registration of such medicines. The compiled data would be used to establish a data base of CAMs on the South African market [74].

Incorporation of ATMs into the current health system is to be facilitated by the implementation of the proposed Traditional Health Practitioner’s Bill. This could result in the possibility of reimbursement by medical aid schemes, and makes provision for the compulsory registration of the estimated 200,000 practicing traditional healers. Traditional healers will need to register and will be issued with numbers to help to identify approved traditional practitioners. Illegally operating healers will be prosecuted to twelve months imprisonment or a hefty fine. Through the above process a more integrated health care system has been envisioned between traditional and Western practitioners. However, the set criteria to include education/training and standards of practice, including the certification of accomplished traditional healers are yet to be specified [71].

1.3 Conclusions

According to the literature around 80% of the world’s population depend on botanical products for their health, either directly or indirectly [32]. Recent studies indicate that in many cultures the use of NHP is deep rooted from early days. Also it has been found that in western countries, NHP is rapidly gaining unprecedented popularity. As a result, the surging and lucrative NHP market has been riddled with

substandard products and practices. This has considerably jeopardized the safety of consumers through untoward incidents thereby sabotaging the integrity of the NHP industry in its entirety. Authorities in most countries have consequently recognized these implications and the need for appropriate regulatory intervention. NHPs are therefore required to yield to long overdue legislations that are proposed to ensure product QSE.

The regulation of natural products has always been a contentious issue that has provoked a worldwide debate. Public misconceptions on the inherent safety of natural preparations have often resulted in disastrous ramifications. Most regulatory frameworks, until recent times, seemed to be constructed on a trial and error basis to the ultimate detriment of the consumer. The challenge upfront is thus to maintain a delicate balance between making natural health products available to consumers at an affordable price, at the same time guaranteeing highest possible levels of QSE. Germany's phytopharmaceutical legislation is exemplary and has been in place for almost three decades. Even though inextricable from insurmountable costs, Germany's legislation is frequently used by other Western countries as a guideline to construct appropriate regulatory frameworks.

Especially in poorer countries, rigorous quality control testing, including RCTs, demand high cost and time which impede availability. In order to accommodate this, the current approach is to attribute some level of safety to products that have been used historically over extended periods of time provided the product is appropriately manufactured and administered. Accordingly, the priorities of quality and safety supercede that of efficacy associated with such products.

The intention to professionalize certain CAM practices by countries such as South Africa and the UK gives a degree of credibility to such modalities. The practitioners guilty of malpractice may ultimately face disbarment or prosecution. Even though such an approach does have its merits, it could be extremely burdensome when all the facets of QSE are the sole responsibility of the professional practitioner such as is the current situation in the UK. It would be more sensible and practical if the responsibility is delegated to the various contributing sectors, such as manufacturers and marketers of CAMs and that the professionalization of CAM practices adopts an ancillary role in terms of ensuring appropriate product quality.

In conclusion, it could be stated that more stringent criteria are required to ensure product quality and safety. Also, accountability needs to be enforced with legal

action if necessary. The importance of appropriate QSE criteria and its associated priority should be balanced with sound post-marketing surveillance. Furthermore, regulatory authorities should promulgate accurate, scientific information to the public such as special warnings, contraindications and possible interactions with orthodox medicines. This is to avoid the often inept and dangerous self-medication by consumers through misguided/ill-informed labeling and advertising claims.

Chapter 2

African Potato (*Hypoxis hemerocallidea*)

2.1 Background

The name African Potato (AP) refers to *Hypoxis hemerocallidea* (*syn. Hypoxis rooperi*). The epithet *Hypoxis* L was coined by Linnaeus in 1759 [75] from the Greek words hypo ('below') and oxy ('sharp'), which refer to the corm that is pointed at the base. In 1992, Nicoletti *et al* [76] published a review article on Hypoxidaceae. The classification of this genus has changed from time to time. Initially, the genus *Hypoxis*, along with *Curculigo* were raised to the family Hypoxideae as distinct from Amaryllidaceae by Brown (1814) [77], and is mentioned in the book by Flinders, '*A Voyage To Terra Australensis*'. *Hypoxis* and *Curculigo* were referred to the tribe, Hypoxideae, of the family Amaryllidaceae by Lindley in 1836 [78]. Hypoxideae was then reconsidered by Pax in 1889 [79] as a tribe within the sub-family Hypoxidoideae of the Amaryllidaceae. This tribe was later raised to the rank of a separate family, Hypoxidaceae, by Lotsy in 1911 [80]. Within two decades it was again brought back to Amaryllidaceae, in the second edition of 'Pflanzenfamilien' [81]. Hypoxidaceae was placed in the order of Haemodiales [82] until 1964, when the tribe Hypoxideae was moved from the family Hypoxidaceae to the order Lilliflorae, suborder Lillineae [83]. The last changes to this classification was made when *Hypoxis*, *Curculigo*, *Molineria*, *Rhodohypoxis*, *Empodium*, *Spiloxene* and *Pauridia*. were placed within the family Hypoxidaceae (order Asparagales) [84, 85]. The classification of the genus *Hypoxis*, however, cannot be considered fully settled because of the different taxonomic descriptions given by different workers to the same morphological characteristics [86, 87].

2.1.1 Vernacular Names

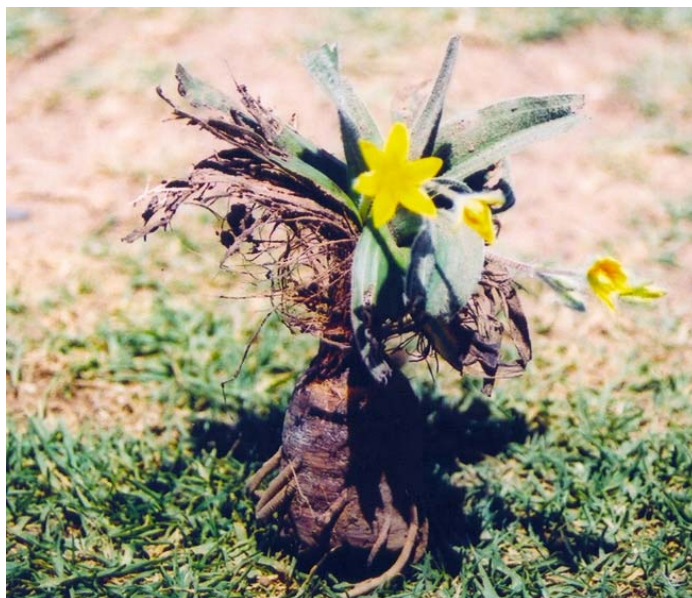
Yellow stars, Star lily, African potato (English); Sterretjie, Afrika-patat (Afrikaans); Inkomfe, Ilabatheka (isiZulu).

2.2 Introduction

Hypoxis hemerocallidea (Figure: 2.1.) plants are geophytic and they overcome winter conditions in the form of an underground rootstock called the corm. They also have adventitious roots attached to the corms that are thick and fleshy and which arise from the base of young corms. When the base of the older corms dies, they are inhabited with termites and other pests such as American bollworm, spotted maize beetle, stink bug and grasshopper. A resinous substance oozes out when the corm is cut and seals the wound in order to prevent any infection. Leaves range from linear to broadly lance-shaped and are often mistaken for nutgrass. The hairy appearance of the leaves is a major distinguishing factor. The flowering stems are unbranched, with 2-12 flowers per stalk. Flowers are symmetrical with 6 petals, which are bright yellow, giving the plant its common name "yellow stars". *Hypoxis* is predominantly a grassland genus, preferring full sunlight.

Figure: 2.1.

Photograph of a Young Corm of *Hypoxis hemerocallidea*



The plant grows well in warm and cold subtropical areas and the planting of the young corms should be done during the spring season. The one year old corms

need less space to plant (10 cms apart in rows and 20 cms between the rows), but if the intention is to leave it to grow for more than three years, then the spacing should be increased to accommodate the large size. Open fields with high organic matter and 25 mm irrigation per week for the first three months of plantation are prescribed.

The leaves of this plant die back over the winter months. Flowering stems appear with the leaves after the first rains in spring and generally a new set of leaves grow from the apex of the corm. The corms of these plants are graded on size. The small (200 g) corms are generally from one year old seedlings and the medium are at least three years old (450 g) whereas the large corms (800 g) are older. The fleshy corms of AP are yellow in color in the inside, but soon turn to dark brown due to oxidation, when exposed to air (**Figure: 2.2.**).

Figure: 2.2.

Photograph of the Tran-section of a Corm of *Hypoxis hemerocallidea*



2.2.2 Prominent Species of the Genus Hypoxis

The development of the anthers, ovules and embryos (floral morphology) indicates that *Hypoxis* species belong to a primitive family. The aerial parts of the plant are covered with soft hair and this feature differentiates them from Amaryllidaceae.

A notable feature of *Hypoxis* is that these herbaceous perennials can survive high stress climatic conditions due to the presence of abundant adventitious roots on their rhizomes or corm.

The plants of Hypoxidaceae have a geographic distribution in the southern hemisphere (South America, Southern Africa, Australia and coastal regions of Asia) and are seen growing in meadows, grasslands, low scrubs, and mountainous terrain [86, 88]. Fifty species have been recorded in the southern hemisphere whereas forty six have been recorded in the tropical areas. Of these, sixteen from America, seven from Asia and Australia and ninety six from Africa have been recorded (**Figure: 2.3**). Even though it is prevalent in sub-Saharan Africa, 41 species have been reported in Southern Africa alone. It is primarily a summer rainfall genus with a large number of species in the eastern region of South Africa where our research facility is located. Seven species occur in the Cape winter rainfall region but none of them are endemic to the region. Eleven species of these 41 are found inland at altitudes higher than 1000 m whereas the remaining species extend from the coastal region to the interior of South Africa.

2.2.2.1 List of Prominent Species

Hypoxis rooperi, *Hypoxis obtusa*, *Hypoxis obliqua*, *Hypoxis nyasica*, *Hypoxis latifolia*, *Hypoxis hirsute*, *Hypoxis decumbens*, *Hypoxis aurea*, *Hypoxis acuminata*, *Hypoxis multiceps*, *Hypoxis angustifolia*, *Hypoxis argentea*, *Hypoxis interjecta*, *Hypoxis multiceps*, *Hypoxis rigidula*, *Hypoxis scorzoneraefolia*, *Hypoxis scuroneria*, *Hypoxis brasiliensis*, *Hypoxis nitida*, *Hypoxis parvula*, *Hypoxis membranacea*, *Hypoxis pusilla*, *Hypoxis carolinensis*, *Hypoxis erecta* , *Hypoxis graminea*, *Hypoxis grandis* , *Hypoxis micrantha*, *Hypoxis pallida*, *Hypoxis villosa*, etc. Few of these species are been shown in **Figures: 2.3a-2.3g**.

Figures: 2.3a-2.3g.

The Prominent Species of *Hypoxis* in Southern Africa



Hypoxis obtusa (2.3a)



Hypoxis colchifolia (2.3b)



Hypoxis rigidula (2.3c)



Hypoxis parvula (2.3d)



Hypoxis membernaceae (2.3e)



Hypoxis hirtusa (2.3f)



Hypoxis angustifolia (2.3g)

2.2.3 Traditional Use of the Plants under the Genus *Hypoxis*

The various species under the genus *Hypoxis* have been used traditionally for a wide range of purposes by different tribes of Southern Africa (**Table: 2.1.**). They have been used by the Zulu tribe as an internal parasiticide, purgative and to treat delirium. The Maniyaka tribe applied the ash to treat wounds and help children to stop breastfeeding whereas the Karanga tribe used it as a remedy for bilious vomiting, anorexia, abdominal pain and fever. It is also used to induce vomiting [89]. *Hypoxis* species is also traditionally used in South Africa to treat benign prostatic hyperplasia [90].

Table: 2.1.
Traditional Uses of the Plants under the Genus *Hypoxis*

Species	Traditional Use/Region
<i>Hypoxis rooperi</i> <i>Hypoxis hemerocallidea</i>	Health tonic, urinary diseases, purgative, treatment of burns, headache, African medicine, fibre for rope, charm against thunder/Southern Africa, Congo [89], Prostate problems/South Africa [91], Benign prostatic hyperplasia/South Africa [92, 93], Uterine cancer/Swaziland [94], Sexual complaints/Mozambique [95].
<i>Hypoxis obtusa</i>	Urinary diseases [96], prostate hypertrophy/Mozambique, Zimbabwe [97].
<i>Hypoxis obliqua</i>	Antiseptic lotion, glue, African medicine/Southern Africa, Congo [89], Cuts and wounds/South Africa [98].
<i>Hypoxis nyasica</i>	Prostate hypertrophy, internal cancer/Malawi [99, 100], Urinary problems/Mozambique [100] , Female Infertility/Malawi [90], uterine cancer, bilharzias/Malawi [101].

<i>Hypoxis latifolia</i>	Emetic, heart weakness, purgative, ascarifuge/Southern Africa, Congo [89], Female infertility/South Africa [102], Heart problems, impotency, female infertility, insanity, vermin killer/South Africa [98].
<i>Hypoxis decumbens</i>	Testicular tumour/West Indies [103], Emmenagogue/French Guiana [104].
<i>Hypoxis aurea</i>	Tonic, rejuvenator/Himalayas [105], Philippines [106], Aphrodisiac, wound healing/China [107], Aphrodisiac/India [105].
<i>Hypoxis multiceps</i>	Charm against lightning, African medicine/Southern Africa, Congo [89].
<i>Hypoxis argentea</i>	Skin cracks, African medicine, food/Southern Africa, Congo [89]
<i>Hypoxis rigidula</i>	Gall sickness, making rope, African medicine/Southern Africa, Congo [89].
<i>Hypoxis villosa</i>	Witch craft, magic, charm against thunder, induce diarrhoea in live stock, fiber for ropes, medicine/Southern Africa [89].
<i>Hypoxis scorzoneraefolia</i>	Emmenagogue/Antilles [108].
<i>Hypoxis scuroneria</i>	Testicular tumour/West Indies [103].
<i>Hypoxis brasiliensis</i>	Forage/Brazil [109].

2.2.4 Extracts of *Hypoxis* Genus and Biological Activity

Many species under the genus *Hypoxis* have been exhaustively investigated for their phytoconstituents but none have been investigated for biological activity as much as *Hypoxis hemerocallidea*.

2.2.4.1 The Extracts of *Hypoxis*

The pharmacological activities of extracts of *Hypoxis rooperi* have been studied more extensively than any other species under this genus. Other species investigated for biological activity are: *Hypoxis obtusa*, *Hypoxis colchifolia*, *Hypoxis nyasica*, *Hypoxis aurea* and *Hypoxis decumbens*.

a) *Hypoxis hemerocallidea* (*Hypoxis rooperi*)

Unspecified parts of *Hypoxis hemerocallidea* have been reported to have anti-tumour activity in humans at a dose of about 20 mg [110]. Even though the identity of this plant was questionable, *Hypoxis hemerocallidea* has been reported to have an IC_{50} of 500 μ g/ml on 5- α -reductase in humans [111]. An unspecified dose of the dried roots of this plant was found to improve benign prostatic hyperplasia (BPH) in humans [91, 112]. Improvement was seen with a three times daily dose of unspecified extract for one hundred days, when given to 98 patients with prostrate adenoma in a double blind study [113]. A review article by Bach (1978) [114], stated that the alcoholic extracts of the dried corms of *Hypoxis hemerocallidea* on 82 patients from both sexes was given for a period of three years and resulted in a symptomatic reduction of inflammation due to rheumatoid arthritis.

Methanolic extracts of the dried tuber from Swaziland showed weak molluscicidal property on *Bulinus africanus* at a concentration of 25 ppm [94]. A six month double blind placebo controlled clinical trial showed significant improvement in the quality of life, peak flow rates and residual urinary volume when infusion of the dried corms were used in the treatment of benign prostatic hypertrophy. It was also found to enhance the production and secretion of plasminogen activators [115]. Hot water extracts of the corms were found to be active against necrodegenerative hepatitis [116]. In mice, the aqueous-ethanolic (1:1) extract of the leaves of this plant was found to be inactive against tumour and non-cytotoxic in cell cultures [117]. A methanolic extract of *Hypoxis hemerocallidea* was found to reduce blood glucose concentrations of normal and diabetic rats. The study compared anti-diabetic effects with those of glibenclamide. Both normoglycaemic and hypoglycaemic male wistar rats showed 46.3% and 68.7% reduction in blood glucose respectively, while administration of a methanolic extract gave corresponding values of 35% and 55%.

The results suggested that *Hypoxis* extracts may have benefit when used by the indigenous community for the treatment of adult-induced diabetes mellitus [118].

Among traditionally used plants of South Africa, aqueous and ethanolic extracts of nearly forty three plants were tested for their therapeutic effect against epilepsy and convulsions using the GABA_A-benzodiazepine binding assay. Three species of *Hypoxis* were tested and the ethanolic extract of the corms of *Hypoxis colchifolia* was found to be effective compared to the extracts of *Hypoxis angustifolia* and *Hypoxis hemerocallidea* [119].

Aqueous and methanolic extracts of *Hypoxis hemerocallidea* were investigated for anti-inflammatory effects on the albumin-induced rat paw oedema model. The methanolic extract was found to produce a greater anti-inflammatory effect than the aqueous extract [120].

Another anti-inflammatory study [121] was done on the inhibitory effect of ethanolic extracts of *Hypoxis hemerocallidea* using COX-1 and COX-2 assays and the ethanolic extract was found to show higher inhibitory effects (>88%) than aqueous extracts (23%) which was in concurrence with previous studies described by Jager *et al* (1996) and Zschoke, van Staden (2000) [122, 123].

In 2005, Steenkamp *et al*, [121] compared the hydroxyl radical scavenging ability of aqueous and ethanolic extracts (4 mg/ml) of AP with vitamin C, using a Fenton type reaction which was measured by the spin trapping method suggested by Janzen, Kotake and Hinton (1992) [124]. Both AP extracts showed significant (>85%) hydroxyl radical scavenging activity.

Gram negative bacteria, *Escherichia coli*, cultured and maintained on blood plates, were used to assess the anti-bacterial activity of an aqueous extract of AP according to the experimental protocol published by Eloff (1998) [125]. The growth of this microorganism was inhibited by an aqueous extract (62.5 µg/ml) that was found to be less potent than ciprofloxacin which was used as the reference [121]. These authors, have claimed that the aforementioned concentration would not yield the necessary anti-microbial activity on consumption by humans, even if 100% absorption is presumed. Their suggestion was based on extrapolated serum concentrations of AP extract which appears to be ill-conceived, since extracts are not absorbed into the blood stream *per se*, and quantitative data should relate to specific phytoconstituents rather than concentrations of the extract. It is highly unlikely that an

analytical method can quantitatively determine the serum concentration of an “extract” as opposed to measuring specific phytochemical constituents.

An interesting study was undertaken to establish whether the ultraviolet reflectance of the flowers of *Hypoxis hemerocallidea* is responsible for the attraction of insect pollinators. Sunscreen solution was applied to the AP flowers and honey bees (*Apis mellifera* sculetta) foraging on the strongly UV-reflecting flowers, rejected those flowers whose UV reflectance had been eliminated by the sun screen coating, but continued to visit control flowers sprayed with solutions that did not contain the UV absorbing compound (**Figure: 2.4.**).

It was suggested that changing the UV reflectance of a flower with sunscreen could be useful for determining the response of a wide range of pollinators to the UV component of spectral reflectance in flowers and could be used to test the functional significance of UV-contrasting nectar guide [126].

Figure: 2.4.

Honey Bees Foraging on the Flowers of *Hypoxis hemerocallidea*



b) Hypoxis obtusa

The methanolic extract of the dried corms, at a dose of 0.5 mg/kg, was found to be ineffective in the reduction of oedema in mouse ear induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). It was weakly active against carrageenan-induced paw oedema at a dose of 100 mg/kg [127].

c) *Hypoxis nyasica*

Acetone extracts of the dried tubers (0.5 g/litre) from Malawi were shown to be active against an unspecified species of molluscs [101].

d) *Hypoxis colchifolia*

Ethanollic and aqueous extracts of the leaves of *Hypoxis colchifolia* were found to inhibit angiotensin converting enzyme (ACE) at a concentration of 333.3 µg/ml whereas aqueous and ethanolic extracts (333.3 µg/ml) of the dried root were inactive and weakly active towards the inhibition of ACE [128], respectively.

e) *Hypoxis aurea*

The ethanolic (95%) extract of dried whole plant was found to be inactive against various types of tumour (melanoma-B16, colon 38, leuk-P388) in mice at concentrations up to 200 mg/kg. The ethanolic extract was found to be cytotoxic at (ED₅₀ = 3.0 µg/ml) against CA-9KB cell lines. The general toxicity of the ethanolic extract was 400 and 900 mg/kg for melanoma-B16 and colon 38, respectively [129].

f) *Hypoxis decumbens*

The dried entire plant at unknown concentration was found to be inactive against various bacterial and fungal strains such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecium*, *Neuspora crassa* and *Mycobacterium smegmatis* [130].

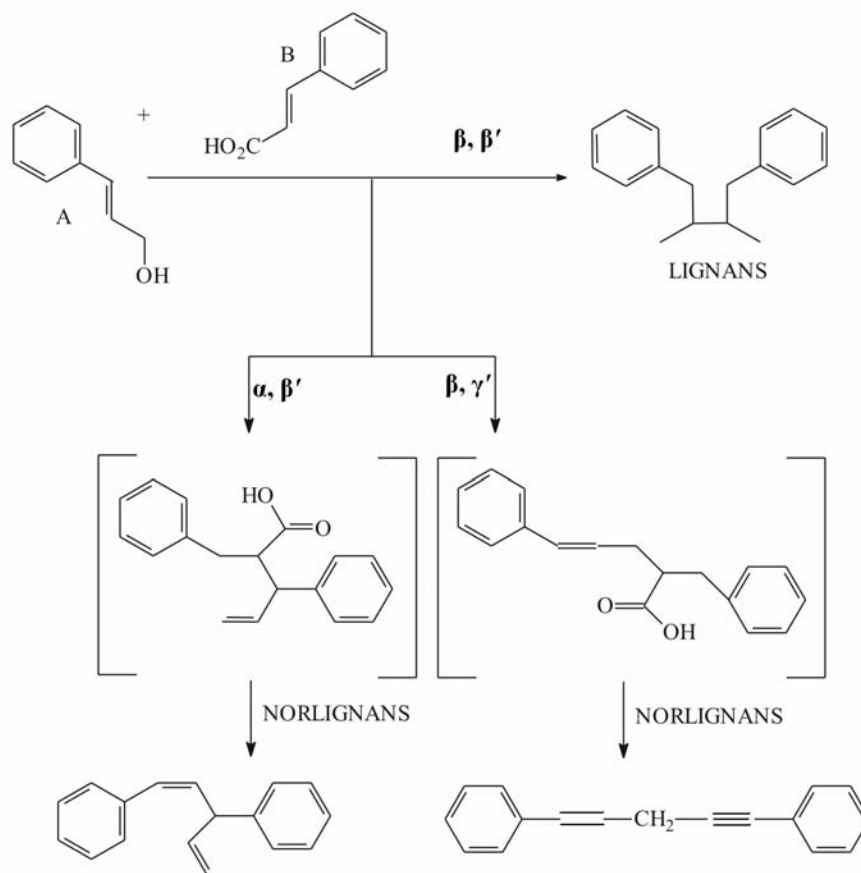
2.3 Phytochemical Constituents in *Hypoxis* Species

‘Norlignans’, also known as non-lignans/norneolignans/conioides are a typical class of compounds present in the *Hypoxis* genus [131]. They have either a Ph-C5-Ph or Ph-C3 (C2)-Ph skeleton, also sometimes referred to C6 (aromatic)-C5-C6 (aromatic) skeleton or C6 (aromatic)-C3-C2-C6 (aromatic) skeleton, respectively and

which are considered to be derived from cinnamic acid and cinnamyl alcohol, but linked other than β - β' position as shown in the **Figure: 2.5**.

Figure: 2.5.

Bio-Synthetic Scheme of Norlignans [76]



A- Cinnamyl alcohol, B- Cinnamic acid

A norlignan di-glycoside namely hypoxoside was the first compound to be isolated from the corms of *Hypoxis obtusa*. The enzymatic hydrolysis of this compound by the action of β -glucosidase yields the aglycone, (E)-1, 5-bis (3', 4'-dihydroxyphenyl)-1-penten-4-yne. This aglycone, termed rooperol, is formed by the acetylenic linkage of a Ph-C2 unit to a Ph-C3 unit at the γ -position of the propenylic

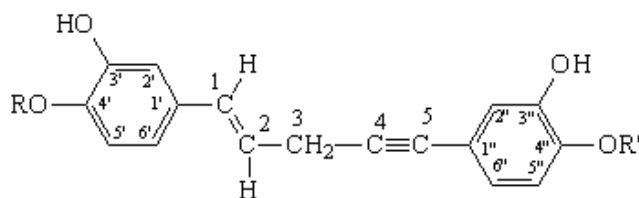
system. From the same source, two more isomeric mono-glycosides, Obtusides A and B were isolated and both these also yield the same aglycone, rooperol following enzymatic cleavage by β -glucosidase [132].

Hypoxis nyasica also has hypoxoside as a constituent along with other similar glycosides, nyasoside, nyasicoside, nyaside and the mononyasines A and B. Nyasoside is the diglucoside of nyasol (i.e., (Z)-1, 3-bis (4'-hydroxyphenyl)-1, 4-pentadiene) which is formed by the olefinic linkage of Ph-C2 unit to a Ph-C3 unit in α -position in the allylic system.

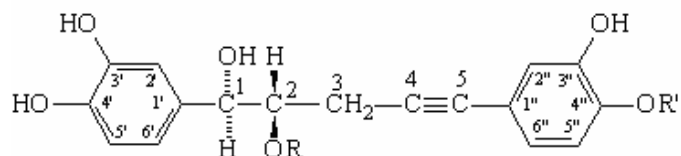
In 1989, Messana *et al* [133] reported mononyasines A and B which are mono-glucosides having the same aglycone of nyasoside, nyasol. Nyaside is a triglucoside, and apart from having the same structure as nyasoside, it also constitutes an additional apiose linked 1-6 to the glucose at 4-phenolic group (**Figure: 2.6.**).

In 1990, Sibanda *et al* [134] isolated nyasol, nyasoside, hypoxoside, nyaside and mononyasines A and B from *Hypoxis angustifoila*. Samples of *Hypoxis obtusa* from Mozambique, however, did not show the presence of nyasoside whereas samples from Kenya and Zimbabwe revealed the presence of both hypoxoside and nyasoside. This could be attributed to the possible presence of apomictic plants in the complex [135]. It was interesting to note that nyasicoside was also isolated from *Curculigo recurvata* from Zaire, which proved that these glucosides are present in other genera besides Hypoxidaceae.

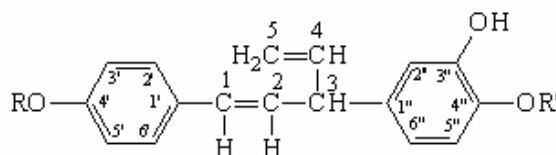
Figure: 2.6.
Structures of Norlignan Glycosides of Hypoxidaceae



Carbon	R	R'	Compound
1	Glucose	Glucose	Hypoxoside
2	H	H	Rooperol
3	Glucose	H	Obtuside A
4	H	Glucose	Obtuside B



Carbon	R	R'	Compound
5	Glucose	H	Nyasicoside
6	p-hydroxycinnamoyl►2-Glucose	Glucose	Interjectin



Carbon	R	R'	Compound
7	Glucose	Glucose	Nyasoside
8	H	Glucose	Mononyasine A
9	Glucose	H	Mononyasine B
10	Glucose	Ap►6-Glucose	Nyaside
11	H	H	Nyasol

The occurrence of pentosans and hexosans in the leaves and corms of *Hypoxis rooperi* from Natal, South Africa have been reported by Bews and Vanderplank in 1930 [136]. Subsequently, in 1970, Morice [137] reported the presence of large amounts of linolenic acids in *Hypoxis pusilla* found in New Zealand. Zeatin and its glycoside from various *Hypoxis* species have also been reported [138]. A new geraniol glycoside namely acuminoside was isolated from *Hypoxis acuminata* Bak. [139]. The major phytochemicals of the family Hypoxidaceae are shown in the **Table**: 2.2.

Table: 2.2.
Major Phytochemicals in Hypoxidaceae

Species	Origin	Constituents
<i>Hypoxis obtusa.</i>	Mozambique	Hypoxoside [140] Obtusides A and B [141] Curculigine [142] Shipamanine [142]
<i>Hypoxis obtusa</i>	Kenya, Mozambique and Zimbabwe	Zeatin [138] Zeatin glycoside [138] Hypoxoside [143] Mononyasines A and B [143] Obtusides A and B [143] Nyasoside [143] Hinokiresinol [143] Hypoxine [143] Dimethyl Hypoxoside [143]
<i>Hypoxis nyasica</i>	Malawi	Hypoxoside [96] Nyasoside [96] Nyasicoside [144] Mononyasines A and B [133] Nyaside [132] Obtuside A and B [145]
<i>Hypoxis angustifolia</i>	Zimbabwe	Hypoxoside [134] Nyasoside [134] Mononyasines A and B [134] Nyaside [134] Nyasol [134] Hinokiresinol [145] Nyasoside [145] Obtuside A and B [145]

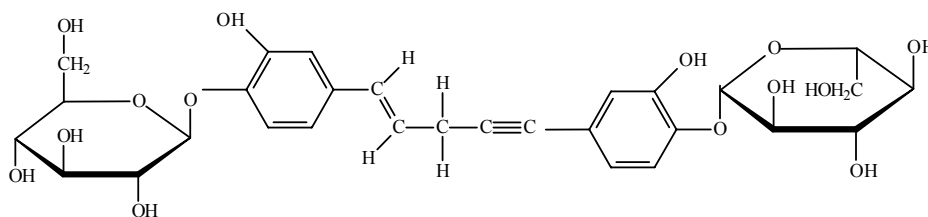
<i>Hypoxis interjecta</i>	South Africa	Hypoxoside [76] Interjectin [76] Nyaside [145] Obtuside A and B [145] Interjectin [145]
<i>Hypoxis decumbens</i>	Brazil	Nyasicoside [145] Nyaside [145]
<i>Hypoxis multiceps</i>	South Africa	Hypoxoside [146] Interjectin [146] Obtusides A and B [146] Nyaside [145] Nyasoside [145]
<i>Hypoxis rooperi</i> (Syn: <i>Hypoxis hemerocallidea</i>)	South Africa	Hypoxoside [147] Daucosterol [148] (Syn. BSSG) β -Sitosterol [114] Zeatin [138] Hexosans [136] Pentosans [136]
<i>Hypoxis acuminata</i>	South Africa	Hypoxoside [149] Acuminoside [139]
<i>Hypoxis nitida</i>	South Africa	Hypoxoside [149]
<i>Hypoxis rigidula</i>	South Africa	Hypoxoside [149]
<i>Hypoxis latifolia</i>	South Africa	Hypoxoside [149]
<i>Hypoxis pusilla</i>	New Zealand	Linoleic acid [150]
<i>Curcilio recurvata</i>	Zaire	Nyasicoside [151]
<i>Spiloxene Schlechteri</i>	South Africa	Hypoxoside [149]
<i>Hypoxis argentea</i>	South Africa	Interjectin [145]

2.3.1 Chemical Properties of Hypoxoside, Rooperol, Sterols, Stanols and Sterolins

Of all the phytochemicals reported in AP (**Figures: 2.7-2.13.**), only a few of them have been claimed to be responsible for the therapeutic effects of AP. The phytochemical, zeatin glucoside though present in AP, is present in negligible quantities and no reports on its pharmacological activity have been published. The therapeutic activity of AP is attributed to other phytochemicals present in it, namely, daucosterol (also called β -sitosterolin, β -sitosterol glycoside, sitosterolin, or BSSG) and hypoxoside. The term sterolin, refers to a sterol glycoside and was the first therapeutically active moiety reported from AP and whose extraction was patented [148, 152-156]. Thereafter, most of the pharmacological studies reported have been based on hypoxoside, and its aglycone moiety, rooperol. There are no reports on the detection or isolation of β -sitosterol, stigmasterol and/or stigmastanol in AP, yet they have been labelled to be the major ingredients in AP formulations. The analysis of AP products for the presence and significance of these sterols and stigmastanol is discussed in Chapter 5.

Figure: 2.7.

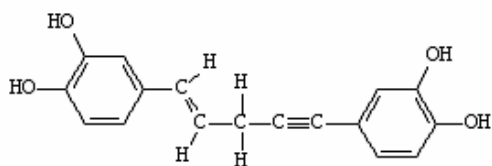
Hypoxoside



<i>Formula</i>	C ₂₉ H ₃₄ O ₁₄
<i>Molecular Weight</i>	606.57*
<i>λ max</i>	258 nm
<i>pKa</i>	8.61±0.35*
<i>Melting point range</i>	149-151° C

Figure: 2.8.

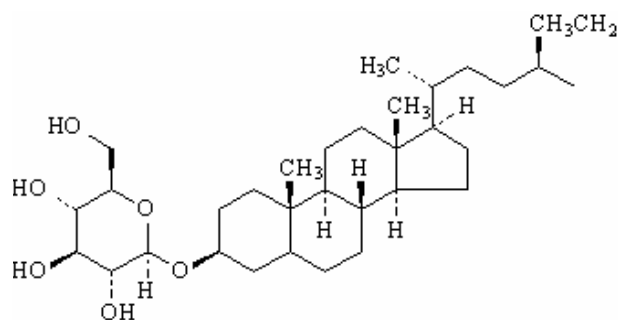
Rooperol



<i>Formula</i>	$C_{17} H_{14} O_4$
<i>Molecular Weight</i>	282.29*
λ_{max}	261 nm
<i>pKa</i>	9.15±0.10*
<i>Melting point range</i>	154-156° C

Figure: 2.9.

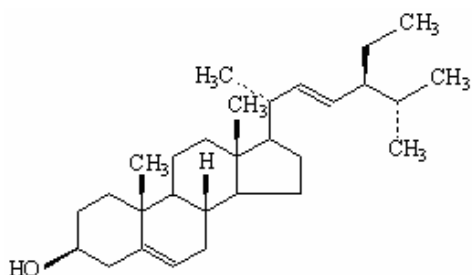
Daucosterol (β-Sitosterol glucoside)



<i>Formula</i>	$C_{35} H_{60} O_6$
<i>Molecular Weight</i>	576.85*
λ_{max}	<210 nm
<i>pKa</i>	12.91±0.70*
<i>Melting point</i>	308° C

Figure: 2.10.

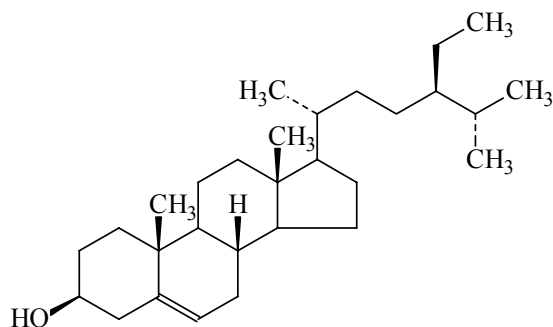
Stigmasterol



<i>Formula</i>	$C_{29} H_{48} O$
<i>Molecular Weight</i>	412.69
λ max	<210 nm
<i>pKa</i>	15.02±0.70*
<i>Melting point</i>	170° C

Figure: 2.11.

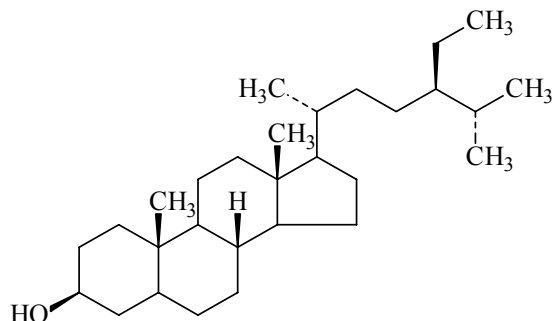
β-Sitosterol



<i>Formula</i>	$C_{29} H_{50} O$
<i>Molecular Weight</i>	414.81
λ max	<210 nm
<i>Melting point</i>	130-145° C

Figure: 2.12.

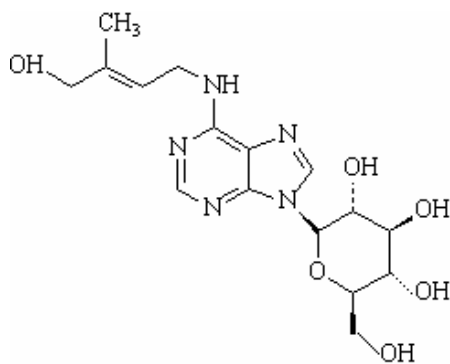
Stigmastanol



<i>Formula</i>	C ₂₉ H ₅₂ O
<i>Molecular Weight</i>	416.93
<i>λ max</i>	<200 nm (no UV absorbing chromophores)
<i>Melting point range</i>	160-164° C

Figure: 2.13.

Zeatin glycoside



<i>Formula</i>	C ₁₆ H ₂₃ N ₅ O ₆
<i>Molecular Weight</i>	381.38
<i>λ max</i>	<210 nm
<i>pKa</i>	12.89±0.70*, 3.61±0.30*

(*Values obtained using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris®
1994-2005 ACD/Labs)

2.4 Pharmacological Properties of Hypoxoside, Rooperol, Sterols, Stanols and Sterolins

2.4.1 Sterols, Stanols and Sterolins

Sterols, sterolins and stanols appear to play an important role in the realm of health supplements with extensive scientific support for their prophylactic and therapeutic use for various medical conditions and ailments such as atherosclerosis [157, 158], benign prostatic cancer [159] and colon cancer [160, 161].

Sterol/sterolin mixtures have been reported to be an effective adjuvant in the treatment of pulmonary tuberculosis [162-164] and were also found to prevent immune suppression in marathon runners [165, 166]. In HIV positive patients, β -sitosterol - β -sitosterol glycoside mixture (BSS-BSSG) showed a significant decrease in plasma viral loads and stabilized CD4 cell counts over a period of 40 months [165]. Feline immune virus (FIV) infected cats maintained stable CD4 cell counts over extended periods of time on treatment with sterol mixtures [167]. Sterols have also been found to be effective in rheumatoid arthritis [168], allergic rhinitis and sinusitis [169].

Phytosterols (plant sterols) are members of the 'terpene' family of natural products which includes more than 100 different phytosterols and more than 4000 other types of triterpenes [170, 171]. Plant membranes contain several types of phytosterols that are similar in structure to cholesterol but include a methyl or ethyl group at C-24. Phytosterols, as with cholesterol in animals, are thought to stabilize plant membranes. An increase in the sterol/phospholipid ratio leads to membrane rigidification [172]. Whilst sterols and their glycosides have been evaluated for a variety of therapeutic activities [173-185], issues of safety/toxicity do not appear to be of concern. This is further substantiated from the fact that these phytosterols and their glycosides (sterolins) are ubiquitously present in most vegetables and fruits, amongst others. On the other hand, however, many reports on the medicinal properties of sterols are largely based on *in vitro* data or unrealistic high *in vivo* doses, making the therapeutic application of these compounds somewhat questionable [186-190].

The average daily human consumption of phytosterols is approximately 250 mg, mostly derived from vegetable oils, cereals, fruits and vegetables [191-194]. For

vegetarians, dietary phytosterols have been estimated to be almost twice this level [195]. Phytostanols are much less abundant in nature than phytosterols and consequently are typically consumed in much lower amounts (~25 mg/day) in human diets [189, 195]. Since the mid-1990s phytosterols have also been used as strategies for lowering cholesterol and for reducing the risk of cardiovascular diseases (CVD) [196, 197]. However, the advent of the ‘statin’ compounds has resulted in a rapidly diminished use of phytosterol products.

In recent years, the increasing interest in functional foods and the use of phytosterols and sterol esters (>2 g/day) for reducing serum cholesterol and increasing immunity has resulted in these compounds regaining considerable interest.

It should be noted that due to poor solubility and bioavailability of free phytosterols, their purported serum cholesterol lowering effects were not always consistent and very high doses (up to 25-50 g/day) appear to be required for efficacy. However, the problems of solubility and bioavailability have led to many confounding results in early clinical studies [189].

A combination of β -sitosterol and its glycoside (BSSG) was claimed to be useful in the treatment of benign prostatic hypertrophy and its action was ascribed to inhibition of 5α -reductase or to diminished binding of dihydrotestosterone within prostate [198]. BSSG and other sterol glycosides (sterolins) were earlier thought to be localized in the plasma membrane involved in the biosynthetic process [199, 200], but later it was reported to serve as a primer of cellulose synthetase in plants [201]. Sterolins are converted to the corresponding sterol in the gastrointestinal tract (GIT) of humans following ingestion and the resulting sterol is thought to be responsible for the purported therapeutic effect [202, 203].

Products containing sterols, stanols and their esters entered the European market through Finland in the mid 1990s and later, the US market in 1999 after considerable regulatory discussions and delay. There are differences between the recommended dosage for phytosterols and phytostanols. The US FDA’s interim rule suggested that more than twice the amount of stanol (3.4 g/day) than sterols (1.3 g/day) were required to ensure a significant reduction in low density lipids (LDL)-cholesterol levels [197]. A recent study stated that a dose of 2 g/day of stanol or sterols could result in an optimum effect and has now been recommended in the latest published National Cholesterol Education Programme guidelines [204]. The serum

cholesterol lowering effect of sterols and stanols are not clear. One of the theories suggested that cholesterol in the intestine, which is marginally soluble, is precipitated into a non-absorbable state in the presence of sterols and stanols. A second theory suggested that cholesterol gets displaced from micelles leading to their very poor absorption [205].

It has been shown that stigmasterol (1 g) reduced cholesterol absorption in human subjects by about 11% and its bioavailability was increased in the presence of lecithin by 34% following a 300 mg dose level [206].

There has been a constant debate over the efficiency of the cheaper free sterols and stanols against their esterified forms. However, head to head comparison of free and esterified forms indicated that their bioavailabilities and efficacies were similar in lowering LDL levels in humans [207-210].

There are many therapeutic claims relating to the use of sterols and/or stanols at relatively higher concentrations, such as 2 g/day of sterols or stanols which has been purported to result in a reduction in the risk of coronary heart diseases by 25% [196].

Several studies have shown that phytosterols are cytotoxic to various forms of cancer such as breast cancer [159, 211], colon cancer [160, 212] and prostate cancer [213]. The data from various studies [157, 197, 214-216] were pooled together and it was stated that [196] plant sterols and stanols lower blood concentrations of β -carotene by about 25%, concentrations of α -carotene by 10% and concentrations of fat soluble vitamin E by 8%. Even though sterols and stanols do not induce malabsorption of fat soluble vitamins and antioxidants in comparison to fatty acid ester forms [217], one of the studies suggested that an increase in dietary carotenoids was effective in maintaining plasma carotenoid levels [218].

In a clinical study involving 2400 subjects dosed with phytosterols and phytostanols (25 g/day or more) showed no adverse effects. A series of studies conducted over four years showed a complete lack of toxicity in animal and human models except for those individuals who had an extremely rare autosomal recessive disorder namely, Phytosterolaemia/Sitosterolaemia [219-221]. It has been suggested that [205, 222] sterols should be substituted with sitostanol in patients with this rare disorder in order to reduce serum cholesterol and free sterol content [223]. Steryl esters have been shown to raise serum phytosterol levels whereas stanols decrease them [208, 224]. Two studies have reported that consumption of free phytosterols and

mixtures of free phytosterols and phytostanols do not result in a change (neither increase nor decrease) in serum phytosterol levels [208, 224].

Early claims relating to the medicinal properties of β -sitosterol and its glycoside in AP have been reported in the literature [148]. Although not substantiated by strong pharmacological data, several patents have been filed on the usage and medicinal claims of sterolin enriched extracts of AP for various therapeutic purposes [148, 152-156].

2.4.2 Hypoxoside and Rooperol

The norlignan glycoside in AP, hypoxoside, possesses low toxicity [147] and undergoes enzymatic hydrolysis in the presence of the enzyme, β -glucosidase, to form its aglycone, rooperol [140]. Many pharmacological studies have been carried out involving hypoxoside and especially its aglycone rooperol. This has lead to several patents of these compounds and their derivatives for various therapeutic use [225-230]. Although hypoxoside has been reported to have limited pharmacological activity, its aglycone, rooperol, has been shown to possess antiphlogistic [231], bacterostatic, bactericidal and various other pharmacological properties [232].

2.4.2.1 Inhibitory Activity on Cell Growth (Cytotoxicity)

The aglycone, rooperol was patented in the US as an anti-cancer agent. Considerable data are available on the pharmacological properties of rooperol and also on the potential of hypoxoside as an oral prodrug for the treatment for cancer therapy [233]. It has also been reported that rooperol can induce apoptosis in HL60 human promyleocytic leukaemia cells [234, 235].

Hypoxoside was found to be cytotoxic toward B16-F10-BL-6 mouse melanoma cells in the presence of the enzyme, β -glucosidase whereas rooperol ($IC_{50} = 20 \mu M$) was found to be cytotoxic *per se* [236]. On the other hand, rooperol metabolites (sulphates and glucuronides), isolated from the urine of humans, have been found to be non-toxic to BL6 melanoma cell cultures even at high concentrations (200 $\mu g/ml$). However, in the presence of a deconjugating enzyme such as β -glucuronidase which facilitates the re-conversion of those metabolites to active

rooperol, the initially observed significant cytotoxicity is re-established, even at low concentrations ($IC_{50} = 75 \mu\text{g/ml}$). Such metabolite conversion could conceivably take place in pathological tissue where the lysosomal deconjugases, β -glucuronidase are present. Furthermore, it is known that many tumours contain necrotic areas where such lysosomal enzymes are also present and release of these enzymes also occurs in inflammatory sites due to macrophage activation [235]. Hence, *in vivo* opportunities exist for the manifestation of cytotoxic effects of rooperol following ingestion of hypoxoside, especially in the vicinity of tumours and associated nearby inflammatory sites.

2.4.2.1.1 Mechanism of Cytotoxic Activity

Light and electron microscopy have revealed that human UCT-Mel 1 melanoma cells when exposed to hypoxoside ($50 \mu\text{g/ml}$) which was deconjugated to form rooperol by endogenous β -glucosidase showed a smooth exterior punctured by holes of $0.5\text{-}2.0 \mu\text{m}$ in diameter or by detaching cytoplasmic protrusions. However, the molecular basis of rooperol cytotoxicity still needs to be clarified. It is possible that the morphological effects described here were triggered by an inhibition of leukotriene synthesis. Biochemical studies have shown that rooperol is a potent inhibitor of leukotriene synthesis in polymorphonuclear leucocytes at a concentration of $1 \mu\text{M}$ or less. The synthesis of cyclo-oxygenase products, TxB_2 and PGD_2 , were inhibited at concentrations of 10 and $100 \mu\text{g/ml}$ and growth inhibition occurred at concentrations ranging from about 0.6 to $0.8 \mu\text{g/ml}$, which is equivalent to about $1\text{-}13 \mu\text{M}$ [237]. It has been reported that inhibition of leukotriene synthesis was also observed in the structurally similar Nordihydroguaiaretic acid (NDGA), which inhibited the growth of HL-60, K-562 and KG-1 human leukaemia cell lines at concentrations ranging from $5\text{-}10 \mu\text{M}$ [238]. A further possibility for the cytotoxicity of rooperol may be due to reactive semiquinone radicals formed during rooperol conversion which could damage cell membranes directly. This possibility cannot be ruled out, since semiquinone radicals have been found to cause lysis of red blood cells under *in vitro* oxidative conditions [237].

A study was conducted to investigate the effect of rooperol on type I collagen synthesis in normal skin and lung fibroblasts and also the cell growth in normal and

transformed fibroblasts. It was shown that low concentrations of rooperol selectively inhibited the growth of transformed cells while stimulating collagen synthesis in normal fibroblasts. Furthermore, normal cells were more resistant to the growth inhibitory effect of rooperol than transformed cells. These findings indicated the possible advantage of rooperol if used in the treatment of cancer by selectively killing cancer cells without seriously affecting the surrounding normal cells. Furthermore, it also suggested that the elevated collagen synthesis and deposition could impede tumour cell invasion and metastasis, thereby indicating rooperol as a useful anti-metastatic agent in the treatment of cancer [239]. All these observations taken together, strongly suggests the potential use of rooperol as an oral prodrug for cancer therapy [233, 235, 236, 240].

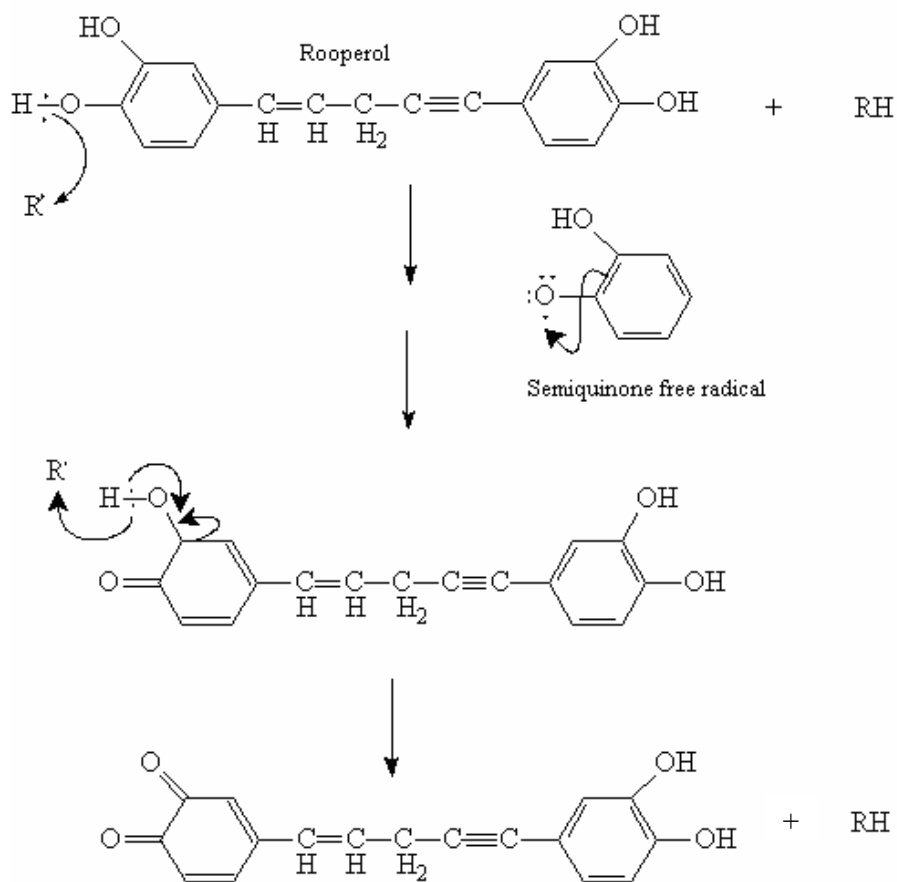
2.4.2.2 Antioxidant Activity

Rooperol has been found to actively inhibit mutagenesis in the Ames test and scavenge free radicals ten times more actively than ascorbate ions [234]. Rooperol and NDGA, the latter being a phytochemical isolated from creosote bush (*Larrea sp.*), have similar structures with two catechols suspended on a carbon bridge. However, NDGA lacks the pent-1-en-4-yne configuration. Thus, rooperol and NDGA differ from each other only in the C-bridge connecting the two catechols. In other words, rooperol has a pent-4en-1-yne-diyl bridge whereas NDGA, a known antioxidant, has a 2, 3-dimethyl-1, 4-butane diyl bridge. NDGA has been shown to inhibit leukotriene synthesis in the polymorphonuclear leukocyte and prostaglandin synthesis in platelet microsomes. It has also been found to act as an antimutagen [241], inhibit the transcription of the HIV gene [242] and induce apoptosis in HL60 cells. Like NDGA, rooperol is a dicatechol, and the latter compound is associated with antioxidant activity and has been found to interact with the oxidative process in human blood. It has been shown that there is a significant difference in the resonance structure of the biologically active semiquinone radicals of rooperol and the known antioxidant NDGA, which makes rooperol and its glycone potential candidates for antioxidant studies [237, 243]. Most of the commonly known antioxidants, vitamin E, flavanoids and catechin have a benzene ring (variously substituted) but at least containing one 1, 4-or 1, 2-dihydroxy group. Interestingly in rooperol the OH groups are 1, 2 relative to

each other. The scheme for the probable free radical scavenging [198] is given in **Figure: 2.14.**

Figure: 2.14.

Rooperol as a Radical Scavenger (Antioxidant)



2.4.2.3 Anti-HIV Activity

Rooperol was found to inhibit the phorbol ester induction of cell line transfected with HIV-LTR-Luc reporter gene [244]. HIV-infected lymph nodes contain active macrophages which release enzymes that could deconjugate rooperol metabolites, thereby inhibiting the expression of the HIV genome. Also, the disulphate metabolite of rooperol has been found to inhibit the *in vitro* proliferation of

HIV-1 [234]. A clinical study was conducted on HIV infected patients where a methanolic extract of AP was administered. During the study, a stable CD4 lymphocyte count and a decrease in serum p24 HIV antigen were observed. Furthermore, there was a reduction in the expression of HLA-DR CD8 lymphocyte activation marker.

2.4.2.4 Analgesic Activity

The antinociceptive activities of hypoxoside were evaluated by tail flick assay, formalin test and phenylquinone writhing test on Swiss-Webster mice (25-30 g). Doses of 5, 10 and 20 mg/kg in saline (5 ml/kg) were used in the above studies. Hypoxoside did not induce gross behavioural alterations; however at the highest dose, it was able to reduce the nociceptive response in the formalin and in the writhing tests whereas, no effect was observed in the tail flick assay [245].

2.4.2.5 Cardiovascular Effects

Hypoxoside (500 mg) and rooperol (240 mg) were dissolved in isotonic saline and separately infused in anaesthetised chacma baboons during 15 min. Hypoxoside had no effect on the cardiovascular system as it was eliminated without significant conversion to the metabolite, rooperol. In contrast, when rooperol was administered, it was rapidly metabolised [235] into several metabolites of which the mixed glucuronide-sulphate was the major one. Five min after starting the rooperol infusion, cardiac output and stroke volume increased significantly. However, they returned to control values within 10 min after stopping the infusion. Cardiac output increased together with both systemic and pulmonary arterial pressure, but these changes were not accompanied by changes in heart rate, vascular resistances or in the filling pressures of the heart. These observations are consistent even though the long-term effects of rooperol on the cardiovascular system are not yet known. Rooperol exerted moderate cardiac stimulation during administration. The above findings are suggestive of increased myocardial contractility, possibly related to rooperol's catechol structure [243].

2.4.2.6 Miscellaneous Activities and Effects

Hypoxoside inhibited the growth of kinetin-induced soya bean callus in tissue culture experiments when included in the nutrient medium. The antagonistic effect of hypoxoside was lost with time and this coincided with the accumulation of the latter in the vacuoles of the soya callus cells. Hypoxoside seemed to have no long-term toxic effects in the callus, yet it inhibited cell division for a narrow period of time. However, the manner in which it inhibits cell division is unclear. The effect of hypoxoside on $[8 - ^{14}\text{C}]$ kinetin metabolism was investigated and hypoxoside appeared to affect the rate of kinetin metabolism [246].

2.5 Pharmacokinetics

2.5.1 Animal Studies

Following oral administration, hypoxoside appeared not to be absorbed *per se* into the blood stream of mice since it was not detected in serum. However, unambiguously it was found that hypoxoside was deconjugated to rooperol in the caecum and colon of the mouse. There is little doubt that this was due to the β -glucosidase enzymes of intestinal bacterial origin because, after oral administration of clindamycin and the subsequent reduction of bacteria, no rooperol but only hypoxoside alone was found in the faeces.

The detection in mouse bile, of new chromatographic peaks with UV absorbance spectra similar to hypoxoside and rooperol indicated that rooperol was absorbed and converted to new conjugates through phase II metabolism for human serum [247].

The presence of metabolites in mouse portal blood suggested that rooperol had also been conjugated in the epithelial cell lining of the caecum and colon. This interpretation is also supported by the absence of rooperol in blood from the general circulation or from the portal vein of the mouse. Human urinary metabolites of rooperol comparable to those found in the bile of the mouse were isolated and incubated with BL6 mouse melanoma cells in the presence and absence of exogenous β -glucuronidase. Inhibitory effects on cell proliferation in the presence of β -glucuronidase was observed [233].

In another study, anaesthetised chacma baboons were used in the evaluation of the pharmacokinetics of hypoxoside and rooperol. Following intravenous infusion of hypoxoside and rooperol over 15 min in separate studies, hypoxoside was eliminated without significant metabolite formation whereas rooperol was metabolised rapidly to nine metabolites. Among those, the major three were the diglucuronide, disulphate and mixed glucuronide sulphates. The concentration-time data from HPLC of arterial blood samples were subjected to non-linear curve fitting to obtain a two-compartment mamillary pharmacokinetics model (**Table: 2.3.**).

Table: 2.3.
Pharmacokinetic Model Parameters

Parameters	Hypoxoside-500 mg (5 Baboons)	Rooperol-240 mg (4 Baboons)
Half-life of the distribution phase- $t_{1/2\alpha}$ (min)	3.68	0.55
Half-life of the elimination phase- $t_{1/2\beta}$ (min)	61.15	3.48
Volume of distribution of the central compartment- V_1 (ml)	1344	709
Volume of distribution of the peripheral compartment- V_2 (ml)	1821	738
Total body clearance- Cl (ml/min)	47	840

The extremely rapid metabolism of rooperol is a notable feature of its pharmacokinetic behavior. Most of the metabolites reached maximum concentration about 4 min after the 15 min infusion of rooperol was completed. The other minor metabolites found were mono-glucuronide, mono sulphate, dehydroxy and bis dehydroxy rooperol respectively [243].

2.6 Clinical Studies

A Phase I clinical study was conducted in which the toxicity and pharmacokinetic parameters were evaluated on 24 lung cancer patients for whom no alternative therapy was available. Out of the 24 patients in the trial, (14 males and 10 females) were selected in the age group of 43 to 77 years (mean age = 56.4 years). Histologically proven diagnoses were adrenocarcinoma (9), large cell carcinoma (9) and squamous cell carcinoma (6). At the time of entering the trial, the average survival time predicted for 19 patients was 4 months and this was in agreement with the prognosis without any therapy. Most of the patients had already developed metastases [235].

In a randomised open study, single doses of 1600, 2400 and 3200 mg of standardised *Hypoxis* extract (200 mg capsules) was administered to three groups of patients (six in each group), whereas the fourth group was treated as control. Similarly, a multiple dose study was also conducted on another group of six patients with 4 capsules (200 mg) three times a day for 11 days. The above standardized extract was reported to contain 50-55% hypoxoside, 8-12% β -sitosterol (BSS), and 0.2-0.3% BSSG. Interestingly, a description of the mode of extraction of the products used was not given and the HPLC method used [247] was cross-referenced to another publication wherein the method of analysis was qualitative and inadequate to support the QC of the aforementioned extract.

Upon oral administration, blood samples were withdrawn at regular intervals and were analyzed by HPLC [248]. The publication describing the HPLC method did not provide validation data and it is unclear how it was used to obtain quantitative data. It was observed that neither hypoxoside nor rooperol appeared in the circulation. This could possibly be due to the phase II biotransformation of rooperol into its various metabolites such as diglucuronide, disulphate and the major glucuronide-sulphate mixtures.

Computer modelling of the observed values showed a single open-compartment model in which the mass of the patient did not influence either the volume of distribution or clearance. The elimination of metabolites was observed to follow first order kinetics with half lives ranging from 50 hr for major metabolite to 20 hr for minor metabolites. Multiple dose studies also showed large inter-patient variation. During the course of the above clinical studies, all patients were subjected

to frequent and extensive laboratory and clinical examinations including computed tomography (CT) scanning.

It was found that maintenance level doses need to be individualised for each patient in order to reach the tumouricidal concentration of rooperol (100 µg/ml), especially after enzymatic deconjugation of its metabolite. It has been suggested that cancer patients who might benefit from hypoxoside are those with relatively slow growing necrotizing tumors that are inoperable and associated with high concentrations of β -glucuronidase and sulphatase enzymes. Similarly, cancer cells such as H552 human adrenocarcinoma have a high sensitivity for rooperol. There is an anecdotal case in which a patient on *Hypoxis* plant extract survived for 10 years after diagnosis with cancer of the pancreas. Since it could be argued that cancer in the pancreas might result in significant release of deconjugating enzymes, this could facilitate the deconjugation of rooperol metabolites to produce sufficient active aglycones to act as effective cytotoxic agents. From the pharmacokinetic data obtained it was observed that most patients need about 2400 mg standardised extract to reach physiological therapeutic concentrations.

In the above study, 19 of the 24 patients survived for less than a year. One patient alone survived for five years before succumbing to tuberculosis. Histological examination of the lung showed the presence of connective tissue and not cancerous cells. During the entire study, no clinical, biochemical or haematological parameters showed toxicity that could be ascribed to the ingestion of hypoxoside. The liver enzymes alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LD) stayed within normal limits, while mean values of enzymes sensitive to metastases were above normal. Serum electrolytes (Na^+ , K^+ , Cl^- , Ca^{2+}), serum creatinine and urea concentrations were mostly within normal limits except for sporadic deviations during the terminal phases.

Patient's hematological profiles confirmed that there was no negative impact of hypoxoside on them. White blood cell (WBC) count stayed constant, while the neutrophil count increased marginally with a slight decrease in the count of lymphocytes. The platelet count stayed remarkably constant. Blood pressure and body temperature showed no abnormalities. One patient experienced possible drug intolerance on 171st day of the trial when the serum concentration of hypoxoside rose to 163 µg/ml. Anxiety, nausea, vomiting diarrhea, dyspnoea and rigors were associated with a doubling in the LD and alkaline phosphatase values. These

symptoms subsided in 4-6 hr after the drug was stopped. No neurotoxicity was observed on two patients who survived the longest [235].

By the end of the study it was concluded that the high hypoxoside doses in long-term therapy (5 years) did not result in any obvious toxicity. Further, a phase II clinical study was also conducted on more than 100 cancer patients for whom no further conventional therapy was available. No toxic effects were observed in these patients. It was noted that the study helped to extend the life span of about sixteen patients with pancreatic cancer to ten months from an expected three months [233, 235, 240].

It should be noted that *Hypoxis hemerocallidea* has been used as an African traditional medicine for a very long time. If it had been found to be toxic following long term use, it would not have continued to be used as a reliable herbal drug by traditional medicinal healers/practioners (*Sangomas*) of Southern Africa [198].

2.7 Patents

There are several patents relating to AP that have been described in the US patent list. Many of these patents, besides being filed in the USA, have also been patented in other countries such as United Kingdom (UK), Italy, Europe, Germany etc. In the following section the relevant patents of AP and its phytochemicals that have been successfully filed, especially in the USA, will be discussed.

In 1969, Liebenberg [249] patented a hydro-alcoholic extract of *Hypoxis* corms for a long list of claims such as anti-inflammatory, antibiotic, antiarthritic, antiateriosclerotic, diuretic, hormonal and muscle stimulant activity.

In 1973, Pegel and Liebenberg [148] applied to patent the extract of members of *Hypoxis* genus for treatment of prostrate hypertrophy. The activity has been correlated to chemically non defined [76] phytosterol glucosides and sitosterins [138, 250]. Subsequently, in 1976, Pegel and Liebenberg [148] patented the extraction of sterolins from AP plant material. Comparisons of and various methods employed in the extraction of sterolins from *Hypoxis* corms were provided. In addition, the beneficial results of enriched products when administered (in capsules and the like) to patients suffering from benign hypertrophy of the prostate gland indicated their therapeutic value and effectiveness. The average quantity of sterolin content was

calculated as β -sitosteryl- β -D-glucoside obtained over a number of experiments. The highest yield of sterolin mentioned was ~9.1 mg/100 g of spray-dried aqueous extract. Interestingly, there was no specific method or reference cited in the entire patent on the determination of the sterolins in the spray dried extract.

In 1980, Pegel, [153] patented sterolins and their use. Sterolins and sterolin esters of natural semi synthetic or synthetic origin were proposed in the treatment of human diseases in daily doses of less than 10 mg and preferably 0.03 to 0.75 mg based on an assumed average human weight of 75 kg. Pharmacological investigations of the anti inflammatory and chronic toxicity of the sterolins were also carried out.

In 1979, Pegel, [154] patented the active plant extracts of hypoxidaceae and their use. Various methods for the extraction of *Hypoxis* corms to formulate them into different dosage forms for their use against various ailments like rheumatoid arthritis, ulcers, benign prostate hypertrophy (BPH), urinary tract diseases (UTI), cardiovascular (CVS) diseases, hyperlipidemia etc., were suggested. All the above studies were based on the sterolin content of these corms. Pharmacological investigation of the anti inflammatory and chronic toxicity of sterolin enriched extracts have also been studied.

In 1981, Pegel and Walker [155] patented the sterol glycoside for activity as a prostaglandin synthetase inhibitor. It described the preparation of medicament with prostaglandin-synthetases inhibiting activity containing sterolglycosides and/or their esters and/or spiroketal steroid glycosides and/or esters as the principle active compounds. Many pharmaceutical dosage forms containing the same ingredients were prepared and their relevant pharmacological and toxicological activities were provided.

In 1981, Pegel and Colin patented [156] sterolin products. This patent relates to dicarboxylic acid derived half esters of steryl glycosides and the salts of these compounds with respect to their synthesis, their incorporation into pharmaceutical and food products as well as the use of these compounds and products.

In 1983, Drewes and Liebenberg [251] patented the crude extract of the rhizomes of *Hypoxis rooperi* for its anticancer property in skin, colon, lung and spine. A dose of 200-1000 mg/patient per day for clinical treatment and 0.4 mg/ml for cytotoxic activity on mouse sarcoma M (52) B cells were given as supportive data.

In 1983, Marini-Bettolo *et al* [252] patented nyasoside from *Hypoxis nyasica* for its promising cytotoxic activity on KB cells ($IC_{50} = 12 \mu\text{g/ml}$) and P-338 doxorubicine-resistant cells ($IC_{50}=8 \mu\text{g/ml}$).

In 1987 Drewes and Liebenberg patented [229], Rooperol and its derivatives for their anti-cancer properties. The inhibitory concentrations (IC_{50}) of rooperol and its derivatives on different cancer cell lines such as mouse sarcoma, mouse melanoma, human oesophageal carcinoma, human cervical cancer, human mesothelioma cells, human derived adenocarcinoma cells etc., were provided.

In 1996, Allison *et al* patented [225] an anti-inflammatory treatment method using the phytochemicals hypoxoside and rooperol. The complete study was based on rooperol, which showed significant anti-inflammatory activity whereas O-glycosylated conjugates of rooperol did not. In this patent, rooperol was prepared from hypoxoside which was isolated from *Hypoxis rooperi*. The methanolic extract of dried shreds of the corms contained approximately 50-55% by weight of hypoxoside which was further purified chromatographically for the study [247].

In 1996, Bouic and Albrecht patented [226] a method and compositions for modulating or controlling immune responses in humans. This study was based on the treatment of humans with a predetermined amount of a phytosterol and phytosterolin such as BSS and BSSG. It was suggested that these two compounds may be added in admixture or sequentially to treat diseases such as viral and bacterial infections, autoimmune diseases, psoriasis, eczema, asthma and cancer. The activities of various combinations of sterol and sterolins were tested on human T-cells, B-cells and NK cells.

In 1997 Liebenberg *et al* patented [230] a method of treating viral infections. The use of the extract of corms from the family Hypoxidaceae (the extract may be of at least one plant species selected from the group consisting of *Hypoxis nitida*, *H. obtusa*, *H. rigidula*, *H. villosa*, *H. interjecta*, *H. multiceps*, *H. nyasica*, *H. rooperi*, *H. accuminata*, *H. latifolia*, *Spiloxene schlechteri* and hybrids thereof and, more particularly, from the group consisting of *H. rooperi*, *H. accuminata* and *H. latifolia*) in the treatment of a viral infection by reducing the rate of decrease of CD4 lymphocyte counts in a subject with a viral infection was claimed. In these extracts, hypoxoside and its congeners were consistently between 45-50% m/m of which hypoxoside was the major (90-95% m/m) component among them [247, 248].

In 1999, Bouic patented [227] treatment of HIV positive patients by using a mixture of β -sitosterol and β -sitosteryl glycoside in a ratio of between 1:1 and 500:1. A FIV (Feline Immune Virus) model was used in this study. It was claimed that beta-sitosterol and beta-sitosteryl glycoside have the effect of stabilizing CD4 cell numbers, decreasing the plasma viral load, degree of apoptosis in, and the serum levels of IL6 in the peripheral blood.

2.8 Formulations and Marketing of AP Products

AP has gained tremendous popularity in Southern Africa. Its usage as a reliable traditional medicine has been promulgated by the various research publications and patents in the recent past. In addition, AP and its formulations have been widely marketed under different trade names for the past few decades. Interestingly, they have often been claimed to be a panacea for many ailments without any substantial scientific data. The common claims advertised for AP products include; cancer, benign prostatic hypertrophy (BPH), enhance endurance in athletes, diabetes, pulmonary tuberculosis, arthritis, immune booster, relieve joint pain/muscular aches/stiffness/sprain/rheumatism/muscle injuries, gout, psoriasis, anti-inflammatory, melanoma, migraine, sinusitis, topical protection, eczema, acne, allergies, ageing, AIDS, alcoholism, asthma, auto immune disorders, scars, boils, burns, cataracts, chicken pox, arteriosclerosis, digestive disorders, poor complexion, dandruff, gum disease, headaches, herpes, infertility, male health problems, measles, pimples, piles, pollution, psoriasis, smoking and drinking, ulcers, loss of vitality, etc.

There has been an increase in the sales of AP products via the internet which is evident from various websites attributed to aggressive sales promotion of the same. Many common references have been cited in such websites to substantiate their therapeutic claims even though comprehensive scientific data are conspicuously absent [89, 163, 234, 253-255].

Commercial AP products date back to 1967, when R. W. Liebenberg, an entrepreneur from South Africa was interested in AP and subsequently launched a commercial product in 1974. This phytomedicine based on AP has been successfully marketed in Germany for the treatment of benign prostate hypertrophy under the trade name 'Harzol' [256, 257]. However, AP and its products were brought into the

limelight when the Minister of Health of South Africa promoted its dietary use as an immune booster for HIV positive patients [258].

In addition, many misleading advertisements are currently being used to market such products (Figures: 2.15a-2.15f.). Similarly, many reports in the media based on anecdotal information have exaggerated the value of such products and proliferated further erroneous information (Figures: 2.16a-2.16h.). The usage and promotion of AP has invited both applause and criticism from many quarters (Figures: 2.17a-2.17c.).

Figures: 2.15a-2.15h.

Advertisements of AP Products

African Potato Cream

Down to Earth produces this natural remedy in cream form, for external use, in the relief of various types of pain. The cream has been enriched by the addition of: **VITAMIN E, EVENING PRIMROSE** and **ALOE VERA**. This combined with the Hypoxis rooperi renews and revitalizes old and damaged skin.

African Potato

The African Potato (*Hypoxis rooperi*) has remained unmodified for over 300 000 years and contains up to 50 000 times as many sterols as modern vegetables.

During excessive physical release of pro-inflammatory factors due to tissue damage, sterols/sterolins have been shown to reverse these processes.

Plant sterols (which possess potent anti-inflammatory properties similar to **CORTISONE**) decreases the blood levels of cortisol as well as the factors which include inflammation, for example, damage, muscular aches and stiffness. The active ingredient of sterols and sterolins is

MOST EFFECTIVE for relief of:

ACHING JOINTS, ARTHRITIS, BLEMISHES, CRACKED SKIN,
COLD SORES, ECZEMA, FIBROSITIS, GOUT, INSECT BITES,
WARTS, MELANOMA, MIGRAINES, MUSCLE INJURIES,
PSORIASIS, RHEUMATISM, SCAR TISSUE, SHAVING RASH,
SINUSITIS, SPRAINS, SUN DAMAGED SKIN AND WRINKLES.

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R55,00 for 125ml and other products available
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(2.15a.)



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The most important supplement for HIV-AIDS sufferers and persons recovering from illness.

ARE YOU TROUBLED BY THE FOLLOWING:

CHRONIC CHEST

CHRONIC ILLNESS & INFECTION

SORE THAT DON'T HEAL

FATIGUE / TIREDNESS

APPETITE DISORDER

PERSPIRATION AT NIGHT

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MIXTURE*

USE OUR MIXTURE OF LIFE AND SEE HOW YOUR HEALTH AND STRENGTH IMPROVES

(CHILDREN ½ DOSAGE)



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XA UHLUTSHWA ZEZIZIFO ZILANDDAYO:

UBUHLUNGU BOMZIMBA

UKUKHOHLELA OKUNGAPHELIYO

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UKUNGATHANDI UKUTYA

UKUBILA EBUSUKU

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AVAILABLE FROM:



(2.15b.)

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- ★ HIV / AIDS
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- ★ Diabetes
- ★ Ulcers
- ★ Sores that do no heal
- ★ Stress fractures
- ★ Cancer
- ★ Arthritis
- ★ Sinusitis
- ★ TB
- ★ Tiredness, Chronic Illness and Infection,
- Perspiration at night, Swollen glands
- ★ Stressful lifestyle

These products can assist you!

Most Affordable!



Imu-tain • Imu-Wize Forté • Imu-Wize • Africa's Solution

The Lesotho Government has approved these for HIV / AIDS treatment!

(TV News)

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Aids treatment helpline 082 579 1569

iMage works (012) 548 4872

(2.15c.)

WHAT IS IMUNO-Active?

IMUNO-Active is an immune system modulator, food supplement and energy provider, all in one!

IMUNO-Active CONTAINS:

- African potato (*Hypoxis hemerocallideae*)
- Aloe Ferox
- Calcium
- Phosphorus
- Protein
- Iron
- Vitamins. A, C, D, E, B₁, B₂, B₃, B₅, B₆, B₁₂
- 20 amino acids including the 8 essential ones
- Sterols and Sterolins
- Energy value (1544.10 kJ/100g)
- Carbohydrates
- Magnesium
- Potassium
- Folic acid
- Zinc and Selenium

HOW SAFE IS IMUNO-Active?

- Imuno-Active is a 100% natural product.
- It contains no preservatives or stimulants such as alcohol or caffeine.
- It can be used with any other medication.
- It is safe for children and pregnant women.

Imuno-Active is not a medicine and its effect may therefore differ from one individual to the next

DOSAGE AND DIRECTIONS FOR USE:

Adults : 3-6(capsules per day) *In divided*
Children : 2-4(capsules per day) *doses.*

IMUNO-Active HELPS FOR:

- All persons wishing to enhance their resistance/Growing children
- Food supplement for the elderly.
- Improves nutritional state and immunity in cancer patients.
- All types of Arthritis and Gout.
- Allergies
- Stress & Sleeplessness
- Muscle pains and Stiffness
- Impotency
- Chronic fatigue
- Memory loss and bad concentration
- Blood pressure
- Bacterial infections: Sinusitis/Tonsillitis
- Viral infections: Coxsackie/Influenza/HIV/AIDS **(incredible results)**
- Poor blood circulation
- Diabetes
- Impaired lung function
- Enlarged prostate/Testicular tumors
- Systemic Lupus Erythematosus

▪ www.imunoactive.co.za ▪

WHAT IS IMUNO-Active ?

Alfred had TB and weighed 34 kg on 10 February 2004. He could not move without assistance. On 8 June 2004 after four months on Imuno-Active his weight increased to 55 kg.



Emmie weighed 39 kg and has a foreign diabetes and a rare bacterial infection in her intestines. Her weight is now 68 kg and she has abundant energy.



IMUNO-Active FIGHTS DISEASES, CANCER & HIV - NATURALLY -

THROUGH YOUR OWN ENHANCED IMMUNE SYSTEM



IMMUNE SYSTEM ENHANCER, FOOD SUPPLEMENT,
ENERGY BOOSTER

VUSELELA
Herbals (Pty) Ltd.
IMUNO-Active
Immune system enhancer
Food supplement
Energy provider

MANUFACTURER:
Vuselela Herbals (PTY) Ltd.
P.O. BOX 18936 PTA NORTH 0116

PROUDLY
SOUTH AFRICAN

(2.15d.)



*Good health
is a gift*

This is how to care
for yours



For it



For your Health

Understanding your immune system

The function of the immune system is to keep you healthy. Like a bodyguard, it protects you from the effects of bacteria, viruses and parasites and eliminates abnormal or diseased cells. It does this very effectively by using specialised cells that work together in a balanced manner.

So, why do we get sick?

The immune system needs to be looked after. Every day, things like stress, environmental pollutants, poor nutrition and simply getting older, can reduce its effectiveness. Catching a cold or flu, painful joints and muscles, cold sores, skin disorders, allergic reactions and chronic fatigue, among others, are indications of an immune system that is not functioning as Nature intended.

What's the answer?

Research has proven that two plant-based compounds, sterols and sterolins, have a positive effect on an unbalanced immune system. They actively stimulate T-cells, making them better prepared to fight and kill invaders like bacteria and viruses, as well as restoring the balance between the different lymphocytes.

Why Moducare?

Moducare capsules contain these sterols and sterolins in a patented ratio of 100:1. Moducare, the original immune booster, has been thoroughly researched and proven to work, to help keep you healthy (see the list of some of the published clinical trials below)

Moducare is virtually for everybody, in any state of health. Whether you want long-term health, or just can't afford to get sick before an exam or holiday, taking Moducare will boost your immune system. Moducare is also one of the few immune boosters that can be taken indefinitely and has no known side-effects.

One pack of 90 capsules should last you a month. Try it. You have nothing to lose.

For more information, speak to your Pharmacist or visit the Moducare website: www.moducare.com



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BOOSTER**



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Moducare™ capsules. Each capsule contains 20 mg plant sterols and 0.2 mg sterolins in a ratio of 100:1. Aspen Pharmacare Ltd, Co. Reg. No. 1808/900352/56. Aspen Pharmacare, Healthcare Park, Woodlands Drive, Woodlands, Tel. (011) 229 3450, Fax: (011) 229 3438.

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(2.15e.)



Importers and Distributors of Products and Equipment

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- DISPOSABLE PAPER PRODUCTS
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G-cide[®]
THE GINOVE CARE SYSTEM

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Permanent Make-Up

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AROMATHERAPY PRODUCTS

Essence of Africa® products capture the healing essences of Africa. For centuries the restorative value of the plants used have been part of the traditional healers wisdom in using plant extracts for healing ceremonies. Our ingredients are soothing, protective and nourishing.

Bath Salts - 200g

A valuable African bath time experience when needing to relax and restore the body. Make bath time a time to honour your Spirit. Pour salts under running water. Close your eyes and connect with the essence within you. Whilst soaking, heal your thoughts, while the plant extracts nurture your body.



Hand & Body Lotion - 100ml

A valuable ritual when needing to soothe and condition the skin while treating skin ailments.



Massage Oil - 50ml

A valuable traditional healing experience when needing to relax and restore the body. Soothes minor burns and irritated skin. Excellent as an all over body massage oil. Whilst massaging, focus on your thoughts and belief systems. With every massage movement, imagine yourself releasing any negativity stored in your body.



Face & Body Scrub - 100ml

A valuable African bath time experience needed to exfoliate lifeless skin cells while nourishing the skin the ancient African way. Gentle enough to use on the face.



Marula Conditioning Oil - 50ml

Blended with Neroli, to soften signs of ageing through it's anti-stress action. Benzoin further acts as an anti-inflammatory. Aids chapped, inflamed and irritated skin. Excellent as an all over body massage oil to condition the skin.



Body Soak - 200ml

Allow the essences from Africa to soothe, hydrate and nourish the skin whilst soaking. Heal your thoughts and allow the plant extracts to nurture your body.



Conditioning Body Fragrance - 50ml

A skin conditioning fragrance that nourishes while uplifting the senses. Contains no alcohol.



Body Soap - 100g

Make bath time a time to honour your Spirit. Close your eyes and connect with the essence within you. Whilst soaking, imagine yourself washing away all negativity. Now, heal your thoughts, and allow the Essence of Africa plant extracts to nurture your body.



AFRICA POTATO contains Rooperols, hypoxide and an abundance of sterols and sterolins. Africa Potato has used traditionally as a strengthening tonic for acne, eczema, psoriasis, rosacea and other skin ailments. MARULA OIL contains natural anti-oxidants and the fatty acid profile has been found to be similar to that of coconut oil. The combination of high nutritional value and excellent stability makes Marula Oil an ideal and innovative for modern cosmetic formulae. Marula Oil softens, soothes and improves skin dehydration and helps fight free radicals. ALOE FEROX is a valuable plant rich in nutritional and healing compounds and minerals, amino acids, lipid enzymes and monosaccharides. Traditional healers apply aloe juice to wounds and is also used internally as a tonic. All over the world Aloe extract is gaining popularity for its benefits in the treatment of burns, bites, eczema, dermatitis, acne and psoriasis.

(2.15f.)

Figures: 2.16a-2.16f.

Media Propaganda on Traditional Medicines and the Use of AP in South Africa (RSA).



You are in: World: **From Our Own Correspondent**
Sunday, 7 November, 1999, 14:53 GMT

Zambia's amazing potato cure



From Our Own Correspondent

Letter From
America
UK
UK Politics
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In the minds of many Zambians the African potato is a wonder-cure

By Ishbel Matheson

I had a rather nasty cold when I went up to the Tuesday vegetable market in Lusaka. This is a wonderful, weekly event where sellers spread out a colourful array of local produce on the ground.

Red tomatoes and chillies gleaming in the sun, deep bags full of beans and nuts, fragile herbs wilting in the heat. Today, though, I was looking for something a little different.

"Have you seen any African potatoes?" I asked the small boy who ran up, and offered to carry my bag. He led me off to a corner of the market, where one trader had laid out a selection of very, curious looking vegetables.

They were large, the size of a small melon, ridged, with tough hairs sprouting on the outer-skin, and shaped like a honey-bee hive.



Location: Sunday 07 Sep 2003 > National arts

A hot potato

spit & polish

The government should put its money where its mouth is and prove that a diet that includes the African potato can help Aids sufferers, writes Barry Ronge

The August 9 edition of The Economist, a magazine that carries some clout in international business and political circles, produced a report on the recent Durban Aids conference. The headline was "Death by dithering", and the subhead was "A health tragedy compounded by government farce".

In a measured tone, it reported the statistics we all know so well. Aids is the top killer of South Africans, accounting for 30% of deaths. The writer estimated that 600 people die from the disease every day and that life expectancy at birth in this country has plummeted from 62 years in 1994 to 51 years today.

The article then reported on how the health minister was widely jeered at the conference, and reviewed the various evasions and inexplicable decisions that have characterised the government's stance on Aids. The story concluded: "Mr (Thabo) Mbeki stayed away from the Durban meeting, and Mrs (Manto) Tshabalala-Msimang flew in only for the opening and closing ceremonies. But there is one reason why

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British Broadcasting Centre (BBC) News
November 07-1999
(2.16a.)

Sunday Times-September 07-2000
(2.16b.)

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/Now

Manto defends herbal research in the fight against Aids

July 24, 2003, 03:31 PM

Manto Tshabalala-Msimang, the Health Minister, has once again come out in defence of her department's initiative to invest in research on traditional herbs to help people living with the HIV/Aids virus.

There has been widespread criticism against recommendations that traditional food combinations have the capacity to arrest the advance of Aids in infected people.

Tshabalala-Msimang believes a mixture of an African potato, garlic, onions and olive oil - can help people fight Aids. "It's not just anti retrovirals...we've got traditional medicines that we know actually averts Aids related diseases and one of it come here from the North-West...we are busy studying it with the Medical Research Council of South Africa and we are seeing excellent results," she said.

However, many such as the Stellenbosch University's Nutrition Information Centre (NICUS) are sceptical about traditional medicines. They claim the African potato can damage bone marrow if given to people with HIV/Aids. Researchers say garlic could also weaken anti-HIV drugs.

The number of South Africans infected with HIV has increased from 24.8% in 2001 to 26.5% in 2002.



/Today

Crime/Justice

* Another christmas tragedy after family shooting

General

* Storm sweeps through Natal town causing havoc
* Fatality free Christmas day in KwaZulu-Natal
* Five pedestrians killed

RELATED STORIES

Traditional leaders reject government proposals (July 23, 2003, 12:38 PM)

First aids vaccine trial in South Africa approved (June 19, 2003, 06:04 PM)

Experts: Aids vaccine likely by 2009 (June 18, 2003, 09:16 PM)

Mandela to continue fighting HIV/Aids, improve schools (July 16, 2003, 10:20 PM)

http://www.sabcnews.com/south_africa/health/0,2172,62793,00.html

*South African Broadcasting Corporation-July24-2003
(2.16c.)*

R6m to study sangomas' Aids cures

YAZEED KAMALDIEN

JOHANNESBURG — The health department has given the Medical Research Council R6 million to check claims by traditional healers that they have found a cure for HIV-Aids, Health Minister Manto Tshabalala-Msimang said yesterday.

The role traditional medicine will play in the government's comprehensive plan for dealing with HIV-Aids is being discussed at a two-day conference in Benoni. The conference ends today.

The minister said she gave the MRC the funds two weeks ago after hearing anecdotal evidence from traditional healers.

"There are traditional healers who know of people who have had HIV-Aids reversed [in their bodies]. People feel better and this is work we must continue to do," said the minister.

"Traditional medicine is in the comprehensive plan [for treating HIV-Aids] and it should be an option available, like antiretrovirals. The MRC is working with traditional health practitioners."

Herbert Vilakazi of the University of Zululand



TRADITION: Manto Tshabalala-Msimang, the health minister, and Andrew Masondo, a traditional healer, spoke on Aids research at a conference yesterday.

PICTURE: LEON SADIQ

yesterday said that he was working with the MRC and traditional healers who say they have cured patients of Aids.

"After observing people who are terminally ill, we have found that a good part (sic) of them have improved. Now we can clinically observe whether or not there is improvement in their

status," said Vilakazi.

Sindephi Spogter, a traditional healer who serves on the Medicines Control Council African traditional medicine committee, said people do not understand sangomas, the indigenous knowledge system or plants.

"They just use needles. We can teach medical doctors how we operate," he said.

"We also need to call traditional medicines by our African names because they have meaning and identity for us."

Wellie Shasha of the UN's World Health Organisation said it encouraged research into traditional medicine.

"Traditional medicine can't be an appendix. It's an entity. The resurgence of traditional medicine has been prompted by the failure of Western medicine," said Shasha.

"Up to 80 percent of Africans depend on traditional medicine for their healthcare needs. In sub-Saharan Africa there is one traditional medicine practitioner for every 500 people, compare with Western medicine practitioners where the ratio is one to 40 000."

Manto has praise for healers

JOHANNESBURG — The status and profile of traditional healers should be raised for the benefit of all South Africans, Health Minister Manto Tshabalala-Msimang said at a Traditional Healers Conference in Benoni yesterday.

"It is unfortunate that we often lose sight of the continuing service that has been offered by this sector which remains largely informal and marginalised in most parts of the world," she said.

Dr Tshabalala-Msimang said she believed traditional medicine could not be adapted to Western medicine. She said she believed traditional and Western medicine should work hand in hand.

The theme of the conference is "Working together with traditional health practitioners in health care delivery". — Sapa

The Herald-2004

(2.16d.)

Health supplements 'falsely labelled'

A CONTROVERSIAL study of health supplements, which finds that only three in nine match their labels, recommends the annual screening of "probiotics" to protect consumers.

Bought over the counter in pharmacies and used to counter the harmful effects of antibiotics and to treat conditions like diarrhoea, probiotics are defined as "live microbial supplements which offer health benefits beyond basic nutrition".

The companies responsible for manufacturing the six probiotics considered substandard have contested

the evaluation, published in the SA Medical Journal this month.

A detailed rebuttal — attacking the impartiality, methodology and results of the study — will be published in the March issue.

The study was initiated by Kim Teversham, the medical adviser to Thebe Pharmaceuticals, which markets BioPro Reuteri products.

The BioPro Reuteri tablets and straws, and Infantforte capsules (marketed by SciPharm) were the three probiotics found to match their labels.

The study's authors, Dr Gene Elliot and Dr Teversham, said their evaluation confirmed that "the contents of several probiotics available in South Africa do not correspond to the label claims".

The study recommended the annual screening of probiotics and the sending of these results to the Medicines Control Council.

Evaluations of probiotics in the US and Europe have also shown a "poor correlation between the label claims and actual contents". —

Claire Keeton

Sunday Times. 23 Feb 2004

Sunday Times-February 23-2004

(2.16e.)

More and more people turning to traditional medicine in city

Situation apparently caused by increase in HIV/Aids infections in Nelson Mandela Bay

By SIPHO MASONDO

THE increase of HIV and Aids in Nelson Mandela Bay has created a boom in the field of traditional medicine.

The growing prevalence of the virus and the difficulty with which patients access anti-retrovirals, coupled with the after effects of the pills, has led to more and more people turning to traditional medicine.

"There are so many people who use the African potato. Even white people use it. We see about 20 people every week," Zwile-based traditional healer Nomathemba Chizama said.

The African potato as well as garlic, onions, beetroot and olive oil, are just some of the remedies that South African Health Minister Manto Tshabalala-Msimang has endorsed as

immune system boosters for HIV-positive people.

Another traditional healer, Nomacamagu Pama, said: "HIV has made my business much better, not mine alone, but the traditional medicine industry at large. Like Manto, I encourage people to use the African potato. But they must get directions on how to use it from us."

She also said: "Not that I am happy because people are dying – it is not about business."

"We are just helping our communities because people are dying."

Orthodox medicine has not been a stumbling block for Pama's business and she encourages her patients to use both traditional and mainstream forms of healthcare.

"They can go to the doctors who will diagnose them. They will then come to us for the

potato, and return to the doctors to evaluate their CD4 count," she said.

Mainstream medicine has frowned on traditional healing in the past, citing a lack of documentation to support its efficacy.

But according to the president of the Traditional Healers Organisation, Nhlavana Maseko, traditional medicine is here to stay, because it also addresses critical ailments such as TB and cancer. Maseko also sits on the World Health Organisation (WHO) as its adviser on traditional medicine.

The South African government has realised the importance of traditional healers.

Last year it adopted a Bill to recognise and regulate them as health-care professionals.

The legislation made provisions for the establishment of a

council, which is currently under way, to oversee the licensing of traditional healers.

The WHO has also endorsed the critical role played by traditional healers in addressing HIV/Aids in South Africa.

"The WHO regional office for Africa has developed generic and disease specific research protocols for the clinical evaluation of traditional medicines. They apply particularly to priority diseases in the region such as HIV/Aids, malaria, hypertension and diabetes," WHO's South African representative, Dr Wellie Shasha, said.

He said this was critically important, specifically in sub-Saharan Africa, as there was one traditional medical practitioner for every 500 people, compared to orthodox medicine practitioners where the ratio was one to 40 000 individuals.

*The Herald-November 08-2005
(2.16f.)*

NEWS FROM THE WORLD OF MEDICINE

AFFORDABLE HIV DRUG

AN AFFORDABLE, natural, non-toxic drug that helps to fight HIV was released for sale to patients in April. Based on extracts from the indigenous African potato, *Hypoxis rooperi*, it has been tested on HIV and AIDS patients since 1992 and has been found to boost the immune system to such an extent that the quality of patients' lives improves markedly. It has proved to stabilize significantly the T-cells of patients and has even caused an increase in these cells and weight gain in some cases.

According to the head of the research team that conducted the trials at Tygerberg Hospital's HIV clinic, immunologist Professor Patrick Bouie of the University of Stellenbosch, the drug has no side effects and is administered in capsule form three times a day. "It will cost the user less than R100 a month and is considerably cheaper than AZT, which, in combination with other drugs, costs about R2 500 a month," he says.

— Adele Baleta in *Saturday Argus*, Cape Town

DREAD THE DRILL?

PAINLESS DRILLING may be coming to a dentist near you. The US Food and Drug Administration (FDA) recently



approved the first laser device for hard-tissue dental procedures. Proponents say that lasers do away with most of the pain and anxiety of dental work because they don't grind in the same ways as drills. And for most people, local anaesthesia isn't necessary. — Rhonda Rundie

in *The Wall Street Journal*

EYE SAVER

ELEVATED pressure inside the eye can cause glaucoma. According to a recent study at the Oregon Health Sciences University in the US, regular exercise can lower eye pressure.

In the study, Dr Michael Passo asked sedentary patients to walk briskly for 30 to 40 minutes four times a week. After three months, those with glaucoma saw a 16-per-cent decrease in eye pressure, those with high pressure saw a 20-per-cent decrease, and those with normal pressure had a nine-per-cent drop.

"We're not recommending walking as a sole treatment for glaucoma," says Passo. "But for most glaucoma patients, walking is a good addition to medication." And for those at risk, it may help prevent or delay onset of the disease. "But before starting a walking programme, patients should consult their eye-care specialist." — Lisa Kaplan in *Walking*

PHOTOGRAPH: © DON CARSTENS

79

butes to the maintenance of healthy skin, teeth, bones and metabolic processes.

The evidence currently available on the therapeutic value of drinking rooibos tea gives some credibility to the anti-ageing claims.

STEROLS AND STEROLINS

Plant fats found in all fruits and vegetables, sterols and sterolins – collectively called phytosterols – have been found to play a unique role in supporting the immune system and cellular function.

The original South African research on these plant fats was conducted on the hypoxis plant, or African pot-

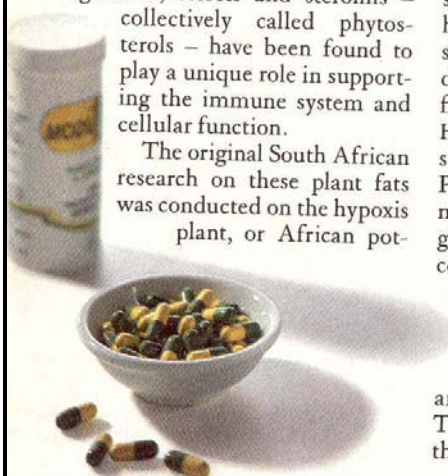
T-lymphocyte cells (which are all-important in the body's defence against invading bacteria, viruses and cancer cells), and cytokines (which are generally associated with cell-mediated immunity). They also enhance the response of the T-cells and stimulate their activity.

Studies have shown that phytosterols may have a positive role in reducing or preventing the progression of asthma, benign prostatic hyperplasia, cancer, chronic fatigue syndrome, coeliac disease, Crohn's disease, type 1 diabetes, eczema, fibromyalgia, hepatitis C, herpes, HIV/AIDS, hives, irritable bowel syndrome, lupus, multiple sclerosis, Parkinson's disease, psoriasis, rheumatoid arthritis, scleroderma, shingles, tuberculosis and ulcerative colitis.

Although phytosterols have been shown to have antitumour properties by enhancing the activity of cancer-killing cells and encouraging the proliferation of T-cells, more research is needed in this field.

Sterols and sterolins also play an important role in offsetting the immunosuppressive effects of conventional chemotherapy and radiation, and in reducing the associated side effects, such as nausea, hair loss and mouth sores.

To order a copy of the Reader's Digest book "The Healing Power of Vitamins, Minerals and Herbs", phone Customer Service on 0800 212020.



Sterols and sterolins – These are available as supplements in the form of capsules

ato. Nowadays, they are extracted from other plants which have been found to have higher concentrations.

Phytosterols may have a role in treating a variety of diseases associated with a defective immune system. They increase the numbers of

48

Readers Digest-November-1999
(2.16h.)

* The above displayed product has been referred as Product N in this thesis
(Figures: 2.15e., 2.18n and Table: 2.4.).

Figures: 2.17a-2.17c.

Media Criticisms on the Use of AP as an Immune Booster for HIV Positive Patients

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Africa: African Potato Hinders Aids Treatment - Study

UN Integrated Regional Information Networks
 February 4, 2005
 Posted to the web February 4, 2005

Johannesburg

Herbal remedies such as the African potato may significantly weaken the power of anti-AIDS drugs, suggests a new study in 'AIDS', a leading medical journal.

A series of tests on the effects of African potato capsules, tablets found that the effectiveness of AIDS drugs was inhibited to such an extent that "extreme caution" is recommended to HIV positive people.

According to a local newspaper, The Sunday Tribune, researchers said that if tests were not carried out on herbal remedies, there could be "poisonous drug interactions, treatment failure and resistance to antiretrovirals".

The potato is among remedies like garlic, onions, beetroot and virgin olive oil, that South African Health Minister Manto Tshabalala-Msimang has recommended as immune system boosters for HIV-positive people.

For an HIV/AIDS excerpt from the 2005 guidebook here. (Adobe Acrobat To buy the book here.)

Relevant Links
 HIV-Aids and STDs

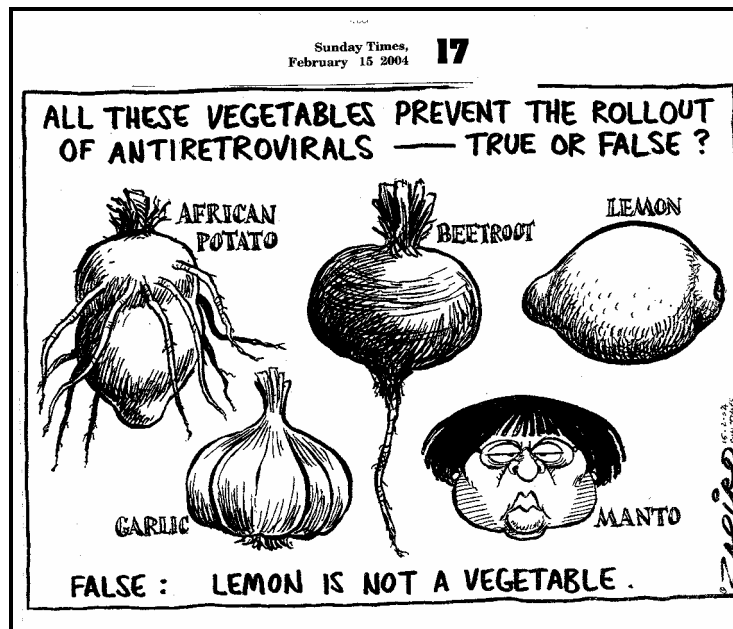
AIDS
 HIV/AIDS 'Indirectly' Responsible for Increased Mortality - Report
 EAC Urged to Scrap Tax On Aids Drugs
 Call for More Aids Drugs As Deaths Rocket
 Government Ready to Issue Aids Drug Tenders
 Academics And Students Thrown Aids Lifeline

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UN Integrated Regional information Network-February 4-2005
(2.17a.)

The above criticism is in response to a preliminary herb-drug interaction study [259] conducted by our research associates and discussed in Chapter 8, Section 8.2 of this thesis.



Sunday Times-February 15-2004.
(2.17b.)



Sunday Times- 2004
(2.17c.)

* The Character Manto in Figures: 2.17b-2.17c., refers to the Minister of Health of the Republic of South Africa (RSA).

There are many formulations on the market being sold for their content of crude AP plant material (**Figures: 2.18a-2.18q.**) These formulations include tablets, capsules, dried powder, (unknown solvent) extracts, solutions, etc.

Figures: 2.18a-2.18q.

Products and Raw Material of African Potato (AP)



Product A (2.18a.)



Product B (2.18b.)



Product C (2.18c.)



Product-D (2.18d.)



Product E (2.18e.)



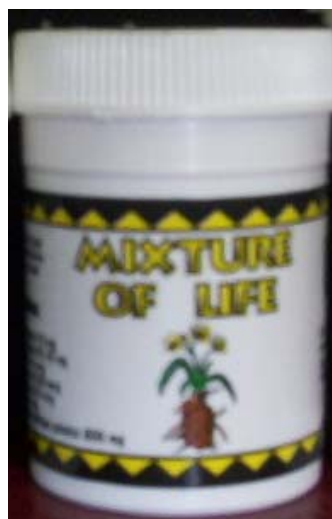
Product F (2.18f.)



Product G (2.18g.)



Product H (2.18h.)



Product I (2.18i.)



Product J (2.18j.)



Product K (2.18k.)



Product L (2.18l.)



Product M (2.18m.)



Product N (2.18n.)



Product O (2.18o.)



Raw Materials of AP (2.18p.)



Traditional Aqueous Extract of AP (2.18q.)

Usually labels on AP products do not provide any details of the form used in the product, viz: extract, plant material etc. (Table: 2.4.).

Table: 2.4.
Products of African Potato (AP)

Manufacturer/Supplier	Name of Product	Dosage Form	Label Claim	Prescription on the Label		
Fine Pharmaceuticals	<i>Sterols and sterolins Hypoxis-African potato</i>	Capsule	Sterols and sterolins 40 mg <i>Hypoxis hemerocallidea</i> 232 mg	1 capsule three times daily		
Swiss garde (Pty) Ltd	<i>Natural remedy</i>	Capsule	<i>Hypoxis</i> extract (African Potato) 15 mg, Sterols and sterolin extracts 30 mg	Take two capsules		
Impilo Drugs (Pty) Ltd	<i>Hi-Vite High impact vitamin</i>	Capsule	African Potato 70 mg	One to three capsules per day		
Nissi Health care	<i>Suprimune forte</i>	Capsule	<i>Hypoxis rooperi</i> 250 mg	Take one capsule daily		
Spiraforce Enterprises	<i>Spiraforce</i>	Capsules	<i>Hypoxis</i> extract 12 mg, Sterols and sterolins 25 mg	One to two capsules per day		
Bermins	<i>Imu-Wize Forte</i>	Tablet	<i>Hypoxis</i> powder (African Potato) 275 mg	One tablet a day		

Table: 2.4. (Continued)

Bermins	<i>Imu-wize</i>	Tablet	<i>Hypoxis</i> powder 200 mg	One tablet a day		
Bermins	<i>Imu/Wize (different package)</i>	Tablet	<i>Hypoxis</i> powder 200 mg	One tablet a day		
-	<i>Mixture of life</i>	Tablet	<i>Hypoxis</i> (African Potato) 200 mg, Plant sterols 60 mg	One tablet daily		
Phyto-force herbal laboratories	<i>Phyto Force</i>	Herbal tincture	<i>Hypoxis rooperi</i> , ethanol (alcohol) and purified water	10 drops		
Bermins	<i>Africa's solution forte</i>	Herbal extract	Each 7.5 ml contains extract of 550 mg <i>Hypoxis</i> , β -sitosterol and sitosterolin 28 mg	Three tea spoons per day		
Bermins	<i>Africa's solution</i>	Herbal extract	Extract of <i>Hypoxis</i> 1000 mg, β -sitosterol 55 mg	Three tea spoons per day		
Pharma Choice HealthCare	<i>Viral Choice</i>	Capsule	Plant sterols and sterolins 25 mg	Take one to two capsules per day		
Aspen Pharma Care	<i>Moducare</i>	Capsule	Plant sterols 20 mg and plant sterolins 0.2 mg	Two capsules three times daily for loading and one capsule three times for maintenance		
Vitamology (Pty) Ltd	<i>Herbology</i>	Tablet	Plant sterols and sterolins 100 mg	Take two tablets daily		

AP Products are generally fortified with many other health supplements such as vitamins, minerals and extracts of other herbal medicines. Among them, free sterols/stanol and sterolins are found to be the most common additives included to fortify products for their intended therapeutic activity. Generally, sterols and stanols, namely, β -sitosterol (BSS), stigmasterol (STG), stigmastanol (STN) and β -sitosterol glycoside (BSSG) are used to fortify such products. Also, there are numerous commercially available formulations in the market containing AP, fortified with additional sterolins. One such formulation of AP is said to contain sterols: sterolins in the ratio of 100:1 (**Table:** 2.4., Product N). This specific ratio has been claimed to enhance the *in vitro* proliferative response of human T cells, more so than the individual compounds at the same concentration [162, 163, 165, 166, 169, 198, 260] and this notion has been used to market and promote sales of this particular product. The absence of strong ethno-medical information relating to the efficacy of the 100:1 ratio of sterols: sterolin coupled with the non-availability of data from other sterol and sterolin ratios raises many questions regarding the validity of the efficacy and therapeutic claims inclusion of this combination in products.

Chapter 3

Extraction, Isolation and Characterization of Hypoxoside

3.1 Introduction

Amongst the various phytochemical components in AP, hypoxoside, a unique norlignan glycoside found only in some members of the Hypoxidaceae family, has been suggested as being the main chemical compound responsible for the therapeutic value of this herbal medicine. As previously discussed in Chapter 2, there is a plethora of research data which have been reported on the pharmacological aspects of hypoxoside and its aglycone, rooperol in comparison to any of the other phytochemical constituents of AP. Hence, hypoxoside is a distinctive choice for use as an active marker for the QC of AP raw material and its formulations/products.

Several methods have been reported for the extraction, isolation and determination of hypoxoside [99, 134, 145] from the genus *Hypoxis*. In 1982, Marini-Bettlo *et al* [99] were the first to report the isolation of hypoxoside from a methanolic extract of *Hypoxis obtusa*. The residue of this extract following liquid-liquid extraction with ethyl acetate and n-butanol was further purified with the help of countercurrent distribution (CCD - 400 transfers) between water: ethylacetate: n-butanol (10: 08: 02, v: v: v). Hypoxoside was isolated following evaporation of the above solvents and its melting point was determined to be 149-151° C. The UV spectrum in methanol revealed absorption peaks at 212, 258 and 291 nm. Various derivatives of hypoxoside were made to obtain NMR data which subsequently facilitated confirmation of its chemical structure.

In 1984, Drewes *et al* [147] isolated hypoxoside from the ethanolic extracts of *Hypoxis rooperi* (*Hypoxis hemerocallidea*). Preparative HPLC with methanol: water (1:1) was used to enrich the extract and the resultant enriched extract was purified with the aid of a 45 mm x 600 mm chromatography column packed with silica gel

(230-400 mesh). Hypoxoside was eluted with 2-butanol: benzene: water: methanol (4:3:2:1) and then purified by crystallization from 2-butanol. The spectral data were found to be in good agreement with that reported by Marini-Bettlo [99].

In 1994, Kruger *et al* [247] isolated hypoxoside from a methanolic extract of dehydrated *Hypoxis* corms using a preparative C₈ HPLC column and an isocratic mobile phase consisting of acetonitrile-water (15:85) at a flow rate of 100 ml/min. A detection wavelength of 260 nm was used to monitor the eluate and 250 ml fractions were collected. The relevant fractions were subsequently further chromatographed on a preparative C₁₈ HPLC column and the pure fraction evaporated to yield hypoxoside. Sibanda in 1990 and Betto in 1992 described methods for the detection and semi-quantitative determination of hypoxoside from other species of *Hypoxis* [134, 145]. These methods were not validated and involved the use of gradient elution.

Although hypoxoside has been identified and isolated from different species of the genus *Hypoxis* (**Table: 2.2.**, Chapter 2), *Hypoxis hemerocallidea* has been the most widely used plant as a traditional medicine. Furthermore, no other species in this genus has been explored for hypoxoside as much as AP. Since one of the main objectives of this research was to develop appropriate QC methods for raw plant material as well as for formulations/products containing AP, hypoxoside was chosen as the active marker for quantitative determination as a QC tool.

In view of the fact that no commercial source could be identified for the supply of hypoxoside in pure form, it was necessary to develop a procedure to extract hypoxoside from plant material and subsequently purify, characterise and qualify such material for use as a reliable reference standard.

3.2 Materials and Methods

3.2.1 Reagents

Acetonitrile and methanol (HPLC grade) were purchased from Romil Ltd, (Cambridge, UK), butanol, ethyl acetate, toluene were purchased from BDL Chemicals Ltd, (Poole, UK) and sulphamerazine (99.1%) was gratefully received as a gift from Orchid chemicals, (Chennai, India). Water was purified in a Milli-Q[®] System (Millipore, Bedford, USA) and Millex HV[®] hydrophilic (PVDF, 0.45 µm)

filters were purchased from the same source. Silica gel 100-200 mesh (75-250 μm) was purchased from Sigma (St. Louis, MO, USA). A sample of hypoxoside (90% purity) was gratefully supplied by Dr.C.F.Albrecht (Research Co-ordinator, CANSA, South Africa) and was used for the qualitative identification of hypoxoside peaks by HPLC.

3.2.2 Instrumentation

Two separate HPLC systems were used for the isolation and quantitative analyses of hypoxoside, respectively. Isolation of hypoxoside was effected by semi-preparative HPLC using a SpectraSYSTEM P2000 pump equipped with an AS 1000 autosampler and UV 1000 variable-wavelength UV detector (all supplied by Thermo Separation Products, Riviera Beach, FL, USA).

Method development and validation for the quantitative analysis of hypoxoside is discussed Chapter 4. The analysis was carried out on an Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 2996 photodiode array (PDA) detector, degasser, column heater and an auto sampler. A Luna[®] C₁₈ (2) (5 μm , 150 x 4.6 mm i.d.) analytical column (Phenomenex, Torrance, CA, USA) was used at $23 \pm 2^\circ\text{C}$ and separation was achieved using a mobile phase composition of acetonitrile: water in isocratic mode (20: 80, v/v) at a flow rate of 1 ml/min using a detection wavelength of 260 nm.

3.3 Experimental

3.3.1 Raw Material Collection

The fresh corms of AP were collected (**Figure: 3.1.**) from Settlers Hill adjoining the botanical gardens of Rhodes University, Grahamstown, South Africa. Samples were authenticated and assigned accession numbers. The vouchers were submitted for recording and storage in the herbarium (Faculty of Pharmacy, Rhodes University).

Figure: 3.1.
Collection of African Potato Corms



3.3.2 Extraction

The corms were carefully washed under running tap water and wiped with tissue paper. The following procedures were carried out under subdued light in order to prevent any possible photo-degradation. The corms were cut into approximately 0.5 cm³ pieces (150 g) and made into fine slurry with 50 ml of methanol using a mechanical stirrer (500-750 rpm). The slurry containing the pulp of the corm and its juice was extracted at room temperature (~23°C) with 500 ml of methanol for 30 min using a magnetic stirrer and strained through a muslin cloth. The filtered methanolic extract was stored separately whilst the remaining pulp was re-extracted with fresh methanol (500 ml).

The above extraction was repeated thrice under photo protective conditions and subsequently, the filtered methanolic extract was passed through Whatman[®] (ashless, grade 44) filter paper, (Fairfield, NJ, USA) to remove fine fibers. The filtered extract was pooled together in a tared round bottomed flask and dried in a rotary evaporator (Buchi, Optalabor, Johannesburg, South Africa.) under vacuum at 45°C until constant mass of about 45 g was achieved.

Approximately 35 g of the above extract was weighed into a 500 ml stoppered conical flask and 250 ml water was added. The suspension was manually shaken for

about 10 min and then again filtered through Whatman[®] (ashless, grade 44) filter paper and the filtrate transferred to a 500 ml separating funnel.

Water saturated ethyl acetate (250 ml) was added to the separating funnel and shaken for about 10 min. The non-polar fraction was separated and a further aliquot 250 ml of water saturated ethyl acetate was added. This procedure was repeated three times and the ethyl acetate portions were discarded.

The residual aqueous portion was partitioned using water-saturated n-butanol (250 ml x three times) and the non-aqueous layer was removed, pooled and subsequently thoroughly dried under vacuum to yield approximately 30 g of enriched extract.

3.3.3 Isolation

A loose slurry of about 200 g of silica gel (60-120 mesh) was made with toluene: butanol: water: methanol (6:4:2:1, v: v: v: v) and loaded into a glass column (300 x 10 mm i.d.) and the first 50 ml that eluted at a flow rate of 0.5 ml/min was discarded. Care was taken that the reservoir attached to the glass column was not allowed to run dry in order to prevent cracking of the column bed. A volume of 10 ml methanol was added to 2 g of the dried butanolic extract, mixed with about 2 g of silica gel and the partially wetted slurry was then loaded onto the column and the first 200 ml of mobile phase was discarded. The column was subsequently further eluted with a different ratio of toluene: butanol: water: methanol (4:4:2:1) at a flow rate of 0.5 ml/min, and again the first 50 ml was discarded and 200 ml fractions were collected and dried in a rotary evaporator at 45°C under vacuum.

A portion of this dried eluate (1 g) was accurately weighed out and dissolved in methanol to obtain 1 mg/ml concentration. These samples were analyzed for hypoxoside and found to have high degree of chromatographic purity (>80%). In order to further purify this enriched eluate, an aqueous solution (5 mg/ml) of this enriched dried eluate was dissolved in 50 ml water and injected (100 µl) onto the semi preparative HPLC as previously described.

Separation was achieved on a Luna[®] C₁₈ (2) 250 x 10 mm i.d. 5 µm semi-preparative column (Phenomenex, Torrance, CA, USA). Samples (100 µl) were injected onto the column which was maintained at a temperature of 23 ± 2° C and the

eluate was monitored at a detection wavelength of 260 nm. An isocratic separation using a mobile phase consisting of acetonitrile: water (18: 82, v/v) was performed at a flow rate of 5 ml/min. An increase in acetonitrile content in the mobile phase resulted in co-elution of other unknown phytoconstituents of AP. A decrease in acetonitrile (10%, v/v) resulted in a longer run time of hypoxoside (21.4 min).

The fractions corresponding to hypoxoside (Retention Time =15.3 min) were collected diligently and combined together. They were then freeze dried (The Virtis Company, NY, USA) to yield a white fluffy solid mass of hypoxoside. Approximately 500 mg of pure white hypoxoside was obtained from each gram of the enriched dried eluate.

3.4 Characterization

Characterization of hypoxoside was undertaken using various techniques such as scanning electron microscopy (SEM) to determine the appearance of the extracted compound, differential scanning calorimetry (DSC) to produce informative thermograms as well as the melting point, fourier transform infrared spectroscopy (FTIR) for functional group analysis and nature of the chemical bonds in the molecule and ultraviolet spectroscopy (UV) to establish the absorption wavelengths and associated characteristic chromophores if present. HPLC coupled with photodiode array (PDA) detection provided information on the chromatographic purity of the molecule as well as information on the presence of impurities whilst liquid chromatography-mass spectroscopy (LC-MS), ^1H , ^{13}C nuclear magnetic resonance spectroscopy (NMR) were used to confirm the molecular structure and purity.

3.4.1 Appearance

When the purified sample of hypoxoside was exposed to light for more than 15 min, it turned to pale yellow in color. Electron micrographs of a sample of hypoxoside dissolved in absolute ethanol which was then evaporated under nitrogen yielded crystalline flakes (**Figure: 3.2.**). Freeze-dried material was found to be quite uniform and fluffy when viewed under the scanning electron microscope (**Figure: 3.3.**)

Figure: 3.2.
Scanning Electron Microscope Image of Hypoxoside Evaporated From an Ethanolic Solution
(12 KV, 10 μ m x 400).

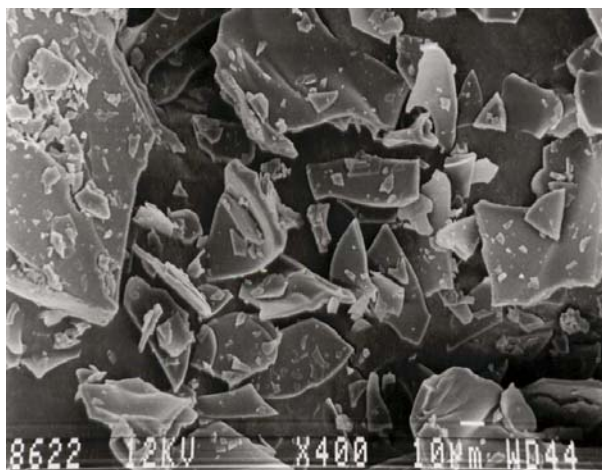
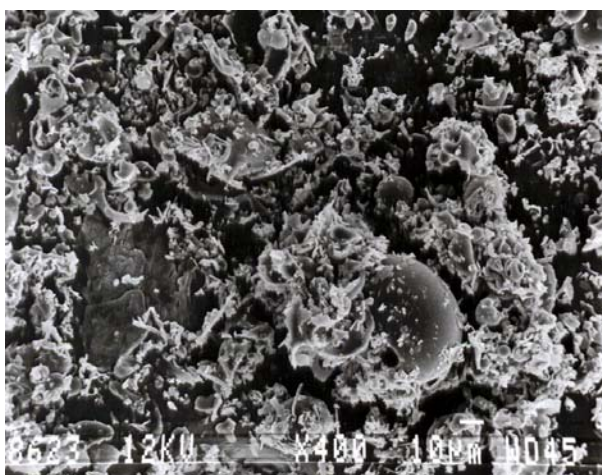


Figure: 3.3.
Scanning Electron Microscope Image of Freeze Dried Hypoxoside
(12 KV, 10 μ m x 400)



3.4.2 Determination of Melting Point

The melting point of the isolated hypoxoside was obtained from Differential Scanning Calorimetry Model DSC7 (Perkin Elmer Inc., MA, USA). DSC is a thermal method whereby the energy necessary to establish a zero temperature difference between the test sample and a reference material is recorded either as a function of temperature or time, while both sample (hypoxoside) and reference material (alumina)

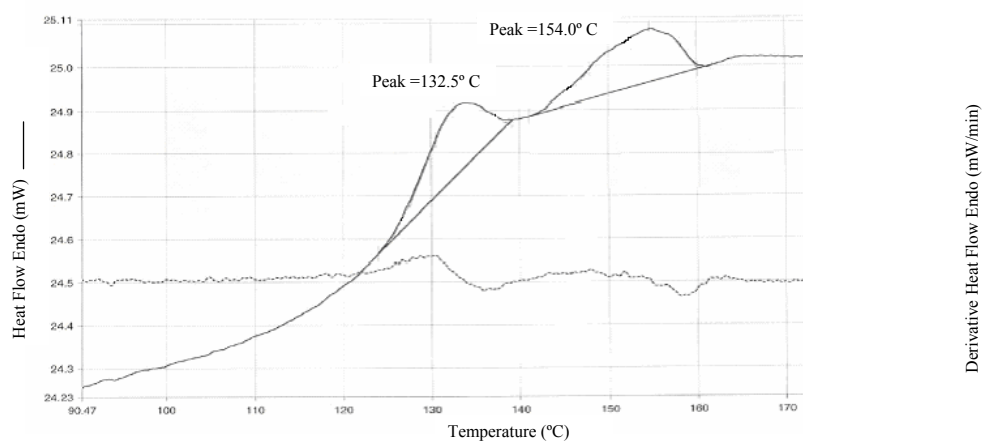
are heated or cooled at a predetermined rate. The DSC measurements (**Figure: 3.4.**) were carried out in a nitrogen filled environment. Hypoxoside (2.1 mg) was weighed and placed in the sample holder before the temperature was increased from 25° C to 200° C at increments of 10° C/min.

The DSC spectrum of the freeze dried hypoxoside showed two broad endothermic peaks, the first at 132.5° C and a second peak at 154.0° C. The first peak could be due to a transition in the crystalline structure of hypoxoside since no melting was observed visibly. The second peak was the decomposition melting point of hypoxoside which was confirmed with a conventional optical melting point apparatus (Mettler Instruments AG, Zurich, Switzerland). The higher melting point observed (154.0° C) compared with the previously reported value (148-152° C) could be attributed to the presence of impurities and/or degradation products which result in a lowering of the melting point of a compound [261]. The melting point of hypoxoside previously reported did not involve freeze drying or isolation and processing under photo protective conditions. Furthermore, the purity of hypoxoside in the reported methods was not provided [99, 147] and it is thus possible that that hypoxoside sample may have contained impurity (ies).

3.4.3 Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectroscopy is one of the most powerful analytical techniques that offer the possibility of chemical identification. One of the most important advantages of infrared spectroscopy over other common spectroscopic methods of analysis is that it readily and rapidly provides useful information about the structure of the molecule being analyzed. This technique is based on the premise that the atomic bonds in a chemical substance vibrate, giving rise to closely-packed absorption bands which result in an IR absorption spectrum extending over a wide wavelength range.

Figure: 3.4.
Differential Scanning Calorimetry (DSC) Thermogram of Hypoxoside



The IR spectrum of hypoxoside (**Figure: 3.5.**) was determined with an FTIR SPECTRUM 2000[®] spectrometer (PerkinElmer Inc., MA, USA) compressed in a potassium bromide disc.

The IR spectrum (**Table: 3.1.**) revealed absorption bands at ~ 780 and ~ 860 cm^{-1} suggesting C-H rocking vibrations. The C-H in plane bending is shown by the absorption band at ~ 1410 cm^{-1} and a C-H stretching vibration at ~ 2835 and 2894 cm^{-1} . The absorption bands at ~ 1270 and 3410 cm^{-1} denote OH bending and stretching respectively. The bands at ~ 860 and 1010 cm^{-1} suggest the C-H out of plane deformation when attached to a carbon by a double bond (C=C-H). The C=C in an aromatic ring is shown by two bands of medium intensity at ~ 1500 and 1560 cm^{-1} . The weak absorption bands at 1636 and 1677 cm^{-1} indicate C=C alkene group. The C $\equiv$ C stretching vibration is shown by absorption bands at ~ 2320 and ~ 2350 cm^{-1} . The resulting IR spectrum correlated with the characteristic Ar-CH=CH-CH₂-C $\equiv$ C-Ar skeleton which is common to the aglycone, rooperol [262]

Figure: 3.5.

Infrared Spectrum of Hypoxoside

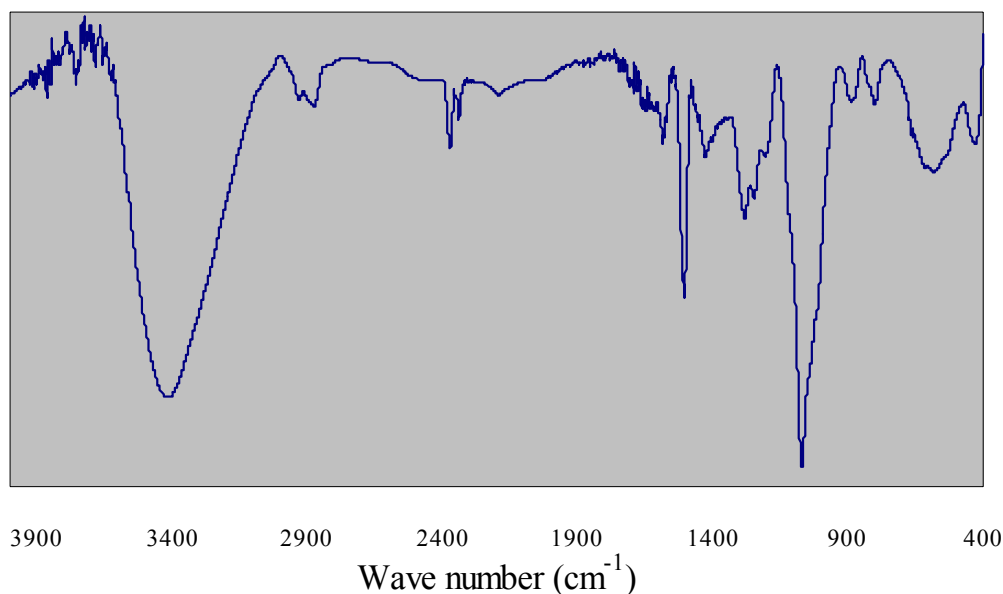


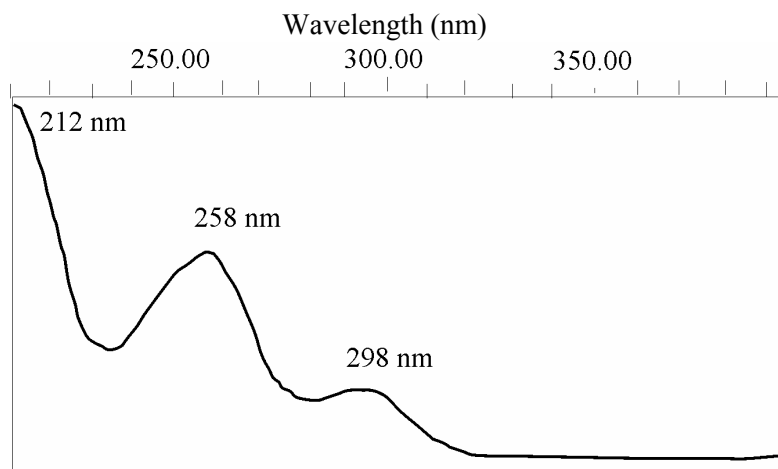
Table: 3.1.
The IR Spectrum Absorption Bands

Observed Wave number (cm ⁻¹)	Group and Comment [263]
781, 863	C-H (600-900) rocking
863, 1008	C=C-H (850-1000) C-H out of plane deformation
1271	OH (1200-1500) bending
1410	C-H (1300-1500) bend in plane
1502, 1567	C=C aromatic (1600-1650) two or three bands of medium intensity
1636	C=C diene (1600-1650) two strong bands
1636, 1677	C=C alkene (1640-1680) often weak
2326, 2356	C≡C stretch (2100-2400)
2835, 2894	C-H stretch (2700-3300)
3417	OH stretch (3000-3700)

3.4.4 UV Absorption Spectroscopy

The isolated compound was readily soluble in methanol and a solution of hypoxoside resulted in an ultraviolet absorption spectrum (from 200-400 nm) which was very similar to the previously reported values [99, 247] (**Figure: 3.6.**).

Figure: 3.6.
UV Absorption Spectrum of Hypoxoside



3.4.5 Chromatographic Purity

Chromatographic analyses under various conditions were used to determine the presence of impurities in a methanolic solution of the hypoxoside sample. Chromatography was carried out using different analytical HPLC columns such as Phenomenex 5 μm Luna[®] C₈ and C₁₈ (150 x 4.6 mm i.d.) and a Synergi Hydro RP[®] Column (4 μm , 4.6 x 50 mm i.d.) (Phenomenex, Torrance, CA, USA) at a temperature of $23 \pm 2^\circ \text{C}$. The analysis was carried out on an Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 2996 photodiode array (PDA) detector, degasser, column heater and an auto sampler previously described. Since no peaks eluted prior to the hypoxoside peak, a linear gradient of water : acetonitrile (0 to 100% for 30 min at 1 ml/min) was used in order to obviate the need of longer run times which would be necessary using an isocratic elution to detect late eluting peaks. This gradient system resulted in the early elution of hypoxoside at approximately 4 min and no further peaks were seen up to 30 min. The PDA detector was used to determine the UV spectrum of the hypoxoside peak and also peak homogeneity. The hypoxoside peak was found to be homogeneous which was evident from the angle of purity being lower than the peak threshold at all instances (Empower[®] software package, Waters Corporation, Milford, MA, USA).

3.4.6 LC-MS Analysis

Liquid chromatography-mass spectrometry (LC-MS) was used to confirm the identity of the hypoxoside peak and also to detect the presence of any non-UV absorbing impurities [264]. LC-MS is the preferred method for qualitative analysis of many compounds. Two ionization techniques are predominately used for the analysis of natural compounds and these involve the ion-spray detection techniques, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The commonly used ion-desorption techniques are fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI) and plasma desorption (PD) [264-266].

LC-ESI-MS was used to study hypoxoside and the technique involves nebulization of the LC effluent to form an aerosol by the application of an electric

field on the metallic capillary of the ESI probe. Depending on the voltage polarity, the aerosol droplets are either positively or negatively charged. This nebulization process may be aided by an auxiliary supply of nitrogen gas which encapsulates the capillary. Desolvation then follows which results in an increase in the charge density on the surface of the droplets and as the droplets reduce further in size, repulsion forces eventually cause a columbic explosion. This results in the suspended ions being transported into vacuum of the mass analyzer.

A Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) coupled to a SpectraSYSTEM P2000 pump equipped with an AS 1000 autosampler and UV 1000 variable-wavelength UV detector (all supplied by Thermo Separation Products, Riviera Beach, FL, USA) was used for the analysis. The instrument was tuned with a 100 µg/ml solution (0.1%, v/v, formic acid) of hypoxoside using positive ion ESI-MS. The MS parameters were optimized by direct infusion of hypoxoside at 4 µl/min into the source and by adjusting the sheath gas flow rate and percentage (%) collision energy.

Hypoxoside sample solutions in methanol were determined by HPLC method according to the previously described analytical method. The mobile phase was slightly modified to include 0.1% formic acid in order to ensure positive ionisation of hypoxoside. Hence the mobile phase consisted of acetonitrile: water (0.1% formic acid) (20: 80, v/v) at a flow rate of 1 ml/min with a detection wavelength of 260 nm and isocratic elution. A single peak corresponding to hypoxoside was observed following injection of the hypoxoside solution under the above conditions and the fragmentation of the corresponding peak yielded a mass/charge (m/e) ratio corresponding to hypoxoside (M^{+1} , m/e 607.17) as shown in **Figure: 3.7**.

Figure: 3.7.
LC-MS Chromatogram of Hypoxoside

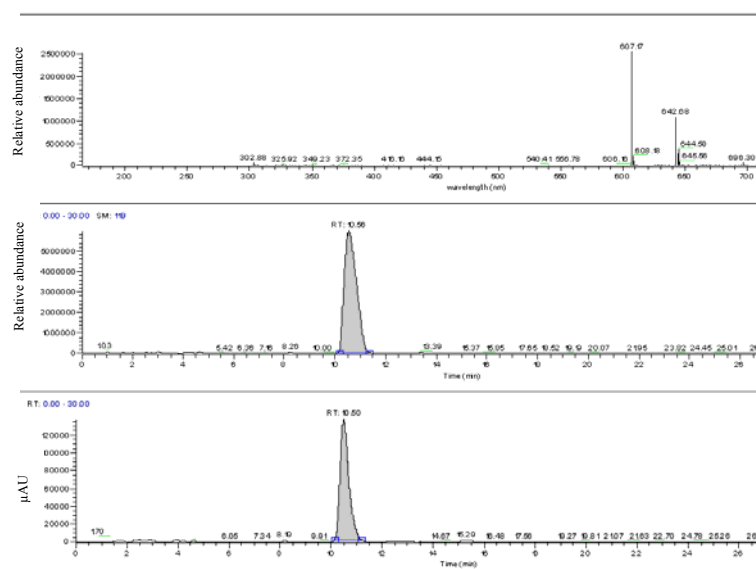


Figure: 3.7. Conditions:- Column: Phenomenex Luna® (5 μm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2°C; Mobile phase: (Acetonitrile 18
Injection volume: 10 μl; Detection λ: 260 nm..

The presence of hypoxoside was further confirmed by LC-MS-MS using negative ion mode, with a collision energy of 25% to fragment the base peak of hypoxoside (M^{-1} , m/e 605.00). The resultant fragments showed (**Figure: 3.8.**) at the loss of first, one glucose ($C_6H_{11}O_5$) moiety (m/e 443.07) and then two glucose ($(C_6H_{11}O_5)_2$) moieties (M^{-1} , m/e 281.16). The loss of two glucose moieties leads to the formation of rooperol ($C_{17}H_{14}O_4$), which is purported to be the pharmacologically active aglycone of hypoxoside ($C_{29}H_{34}O_{14}$). This was confirmed by analysing an aqueous solution of rooperol (100 $\mu\text{g/ml}$) in the positive ion mode adjusted to 35% collision energy to fragment the base peak of rooperol (M^{+1} , m/e 283.16), shown in **Figure: 3.9**. Rooperol, in pure crystalline form was earlier received as a generous gift from Dr. Carl Albrecht (Co-ordinator of Research, CANSA, South Africa).

Figure: 3.8.

LC-MS-MS Chromatogram of Hypoxoside

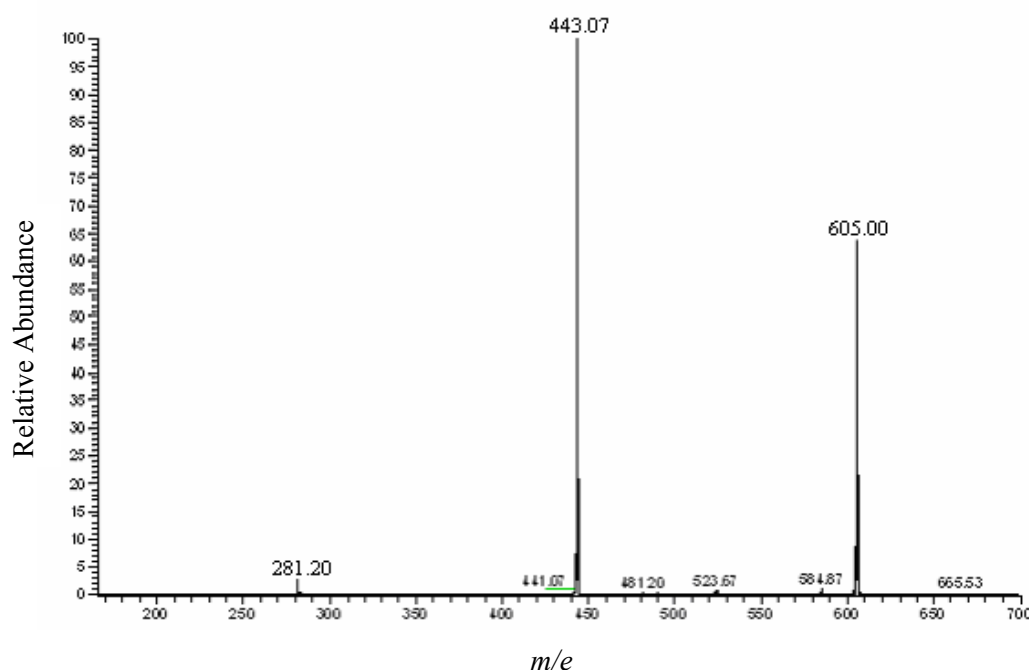
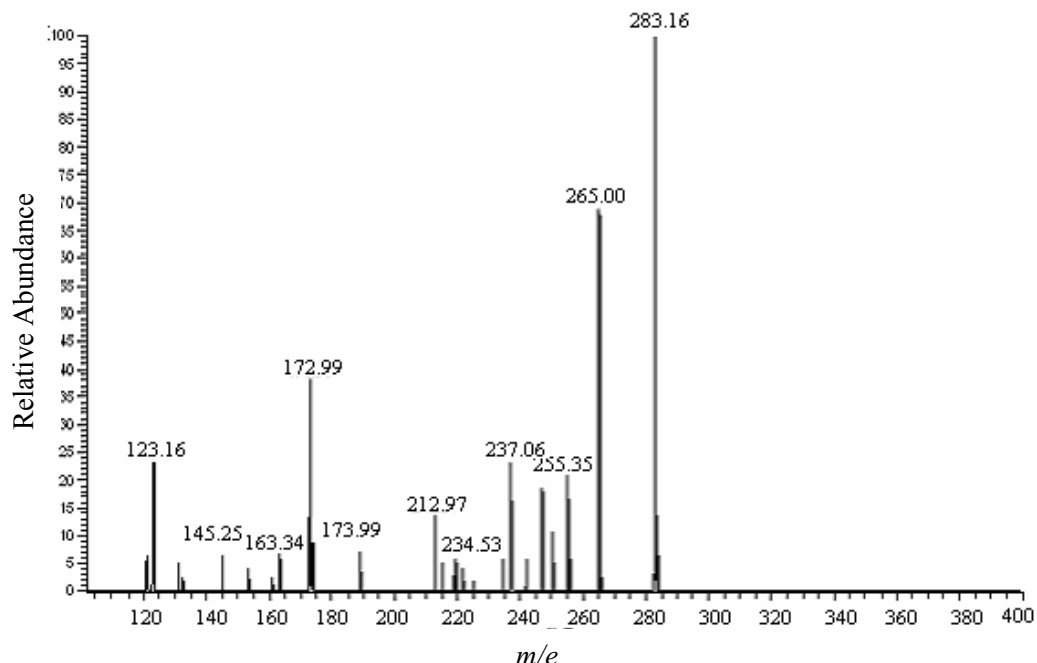


Figure: 3.9.
LC-MS-MS Chromatogram of Rooperol



3.4.7 Nuclear Magnetic Resonance (^1H and ^{13}C NMR)

The ^{13}C NMR analysis was performed on a Bruker Avance DRX 400 MHz NMR spectrometer (Rheinstetten, Germany) by analyzing a 10 mg/ml solution of hypoxoside in deuterated water (D_2O). Interestingly, there has been no published NMR spectral data of hypoxoside *per se*, only ^1H and ^{13}C NMR data for its derivatives. In order to obtain the ^{13}C NMR spectrum of hypoxoside, it was necessary to run repetitive scans over a period of 20 hr corresponding to ~28000 scans. A data acquisition time with a relaxation period of 2 seconds was used between each pulse since the compound gave a weak response. The resulting ^{13}C NMR spectrum was compared with the values reported for the derivatives of hypoxoside (**Table: 3.2.**). The obtained data correlated well and confirmed the presence of the $\text{Ar-CH=CH-CH}_2\text{-C}\equiv\text{C-Ar}$ carbon skeleton of hypoxoside. Further, ^{13}C NMR spectrum was processed with a simulation software package (MestRec, Santiago, Spain) which also confirmed the structure of the compound as hypoxoside. However, it was noted that the chemical

shift values observed for the C (2') and C (2'') positions in the hypoxoside molecule were slightly higher (117.36 and 117.38, respectively) than those observed for the derivatives (**Table:** 3.2.). Although the LC-MS-MS fragmentation data supported the absence of any substituents at these positions, the structure of hypoxoside was confirmed using the Heteronuclear Multiple Bond Correlation (HMBC) spectrum as shown in **Figure:** 3.10. This data indicated the correlation of H (2') and H (2'') with C (1'), C (1'') and C (3'), C (3'').

Table: 3.2.
¹³C NMR Chemical Shifts

No	Dimethyl hypoxoside (D ₂ O)	Isolated Sample (D ₂ O)	Rooperol (Acetone)
1	n.o - C(3)	23.63- C(3)	21.5- C(3)
2	83.7- C(5)	83.42- C(5)	81.5- C(5)
3	87.4- C(4)	87.78- C(4)	83.6- C(4)
4	118.3- C(1'')	114.94- C(1'')	114.2- C(1'')
5	120.4- C(2)	119.93- C(2)	117.7- C(2)
6	126.4- C(6')	124.61- C(6')	120.7- C(6')
7	127.6- C(6'')	125.57- C(6'')	122.9- C(6'')
8	132.5- C(1)	131.26- C(1')	128.8- C(1')
9	133.5- C(1')	133.83- C(1)	129.9- C(1)
10	110.8, 110.8- C(2'), C(2'')	117.36, 117.38- C(2'), C(2'')	111.9, 114.4- C(2'), C(2'')
11	116.2, 116.8- C(5'), C(5'')	117.71, 117.74- C(5'), C(5'')	114.4, 117.4- C(5'), C(5'')
12	146.2, 146.9- C(3'), C(3'')	145.08, 145.87- C(3'), C(3'')	143.7, 143.8- C(3'), C(3'')
13	149.1, 149.4- C(4'), C(4'')	146.39, 146.56- C(4'), C(4'')	144.0, 144.5- C(4'), C(4'')
14	Glucose	Glucose	
15	56.5- OCH ₃	58.43- OH	
16	61.3- C(6)	61.21- C(6)	
17	67.9- C(4)	69.87- C(4)	
18	69.9- C(2)	69.91- C(2)	
19	73.7b- C(5)	73.74- C(5)	
20	76.8b- C(3)	76.87- C(3)	
21	101.4, 101.5- C(1)	102.26, 102.58- C(1)	

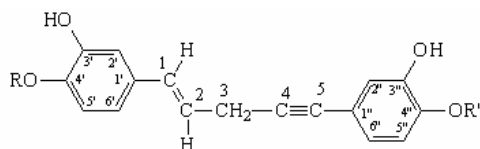
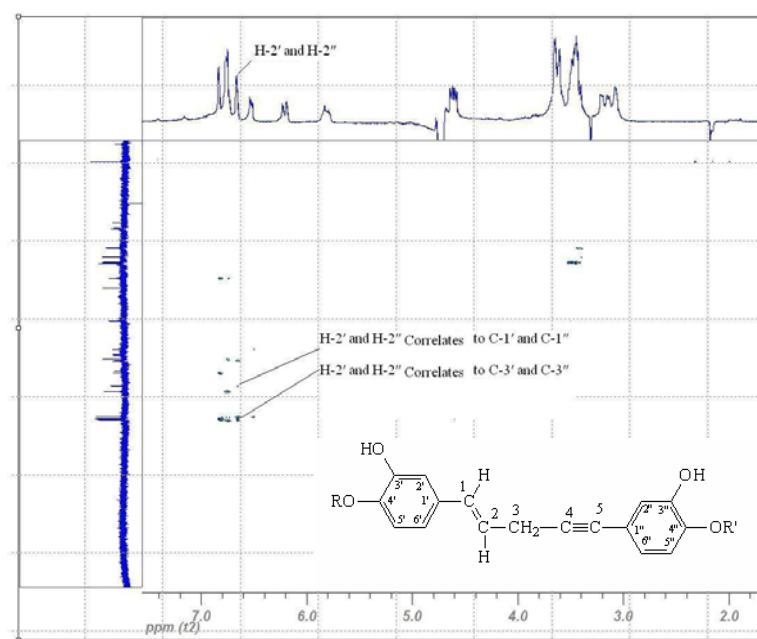


Figure: 3.10.
HMBC Spectrum of Hypoxiside



3.5 Conclusions

Hypoxoside was extracted and isolated from fresh AP corms using a methanolic extract. The % yield of hypoxoside (~6.0%) from these AP corms was found to be higher than the previous methods (~1.2%). The method used involved liquid-liquid extraction procedure followed by column chromatography and semi-preparative HPLC. Previous methods, on the other hand, involved the use of relatively costly reverse phase bonded column packings.

The final purified hypoxoside from enriched extracts were freeze dried and characterized by confirming its purity using a range of chromatographic and spectroscopic techniques which were then correlated with data from various analytical methods reported previously. Scanning electron microscopy showed hypoxoside to be uniformly fluffy. The differential scanning calorimeter yielded a melting point of 154.6° C. The infrared (FTIR) spectrum in the finger print region confirmed the identity from the presence of the functional groups of hypoxoside. The isolated compound was readily soluble in methanol and yielded the same UV absorption spectrum as previously reported [247]. Chromatographic peak purity of hypoxoside using HPLC with PDA detection provided data that confirmed the absence of any UV absorbing impurities. The chromatographic peak of hypoxoside under various conditions was found to be homogenous and absent from impurities. Analysis of hypoxoside by LC-MS using ESI-MS provided a fragmentation pattern showing a characteristic *m/e* peak 607.18 corresponding to the hypoxoside molecule and other prominent *m/e* peaks at 443.56 and 281.02 which indicated the subsequent loss of one and two glucose moieties respectively to form the aglycone, rooperol.

The ¹³C NMR and ¹H NMR analysis yielded data that corresponded to previously reported values for derivatives of hypoxoside [99]. Moreover, the ¹³C NMR spectrum was processed with a simulation software package (MestRec, Santiago, Spain) which confirmed the structure of the compound as hypoxoside. The above information confirmed that the isolated compound was spectrally pure without the presence of any UV absorbing/non-absorbing impurities. The various spectral data supported the use of the isolated hypoxoside as a high purity reference standard (>99% chromatographically pure) for the QC of AP plant material and its formulations.

Chapter 4

High-Performance Liquid Chromatography (HPLC)

4.1 Introduction

Reversed phase HPLC (RP-HPLC) has become a commonly used separation and analytical technique that is economical for routine QC analyses of medicines and associated substances and products. Moreover, HPLC is versatile and not influenced by the volatility or stability of the analyte and therefore can be used to separate and analyze a broad class of analytes such as medicinal compounds, including natural products [261]. From a fingerprinting (qualitative) point of view, this is extremely advantageous since it can usually provide useful content information for profile comparisons [267]. These abovementioned features currently make HPLC the most frequently used method for the analysis of herbal preparations [268, 269]. HPLC coupled with UV-Vis detection is the most common technique used for the analysis of compounds with strong chromophores that absorb light in the wavelength region from about 200 nm-800 nm. Also, there are many HPLC methods which use various other detection modes that are rapidly finding application due to technological advancement. The photodiode array (PDA) detector is an extremely useful multi-wavelength UV detection technique and operates on the principle of reverse optics, such that the UV-Vis absorption spectrum of an eluting compound can be obtained on-line during the analysis. Moreover, it has the additional advantage of determining peak purity as well as peak identification with the aid of PDA spectral libraries. These features are very much in demand these days for analyzing complex matrices such as plant extracts [269]. A recent article has recently promoted the use of HPLC-PDA for the fingerprinting of traditional South African herbal medicines [270].

On the other hand, compounds with differing electroactivity and which may cause characteristic voltametric responses can be selectively measured using

electrochemical detection (ED). Furthermore, compounds that lack the necessary chromophores leading to poor UV-Vis absorption can be detected by a refractive index (RI) or evaporative light scattering detector (ELSD) [269].

At a more advanced and sophisticated level, recent advances in mass spectrometric (MS) instrumentation have paved the way for the incorporation of liquid chromatography coupled with mass spectrometry (LC-MS-MS) for use in routine analysis [271]. In addition, LC-MS facilitates peak identification and confirmation from mass spectral data. From the collision-induced dissociation (CID), intricate structural details such as the glycolysis (loss of sugar moiety) of a molecule can be deduced [264]. The use of single ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes used in the MS detection, facilitate the specific detection and quantification of co-eluting or unresolved peaks [272], especially in complex matrices such as in natural products. This feature, in particular, has distinct advantages over the conventional UV/PDA detection. In particular, a major advantage of LC-MS is its specificity which it provides from the characteristic mass/charge (m/e) data of the eluting peak.

LC-ESI-MS involves nebulization of the LC effluent to form an aerosol by creating a fine spray of highly charged droplets [264-266] in the presence of a strong electric field (typically 3.5 kV). As the droplet decreases in size, the electric charge density on its surface increases. The mutual repulsion between like charges on this surface becomes so great that it exceeds the forces of surface tension, and ions begin to leave the droplet through what is known as a "Taylor cone". Desolvation then follows which results in an increase in the charge density on the surface of the droplets and as the droplets reduce further in size, repulsion forces eventually cause a coulombic explosion and the suspended ions electrostatically are directed into the mass analyzer. Depending on the voltage polarity on the metallic capillary of the ESI probe, the aerosol droplets are then either positively or negatively charged. This nebulization process may be aided by a supply of nitrogen which encapsulates the capillary.

The number of charges retained by an analyte can depend on factors such as composition and pH of the electro-sprayed solvent as well as the chemical nature of the sample. ESI allows for very sensitive analysis of small, large and labile molecules such as peptides, proteins, organometallics, oligosaccharides, and polymers. Another

advantage of ESI-MS is that ions are formed directly from solution, a feature that has established the technique as a convenient mass detector for HPLC [273].

Other, more complex hyphenated techniques have been explored more recently such as HPLC-PDA-MS, HPLC-Nuclear Magnetic Resonance (NMR) spectroscopy, HPLC-PDA-MS-NMR and HPLC-chemiluminescence [269].

4.2 Background and Objectives

Very little information has appeared in the literature to describe methods for the quantitative analysis of hypoxoside. In 1990, Bayley and van Staden [274] reported a colorimetric method for the quantitative determination of hypoxoside. This was done by derivatizing the TLC plate fraction containing hypoxoside with p-nitroaniline reagent followed by measurement of the absorbance at 522 nm using a colorimeter.

In 1990, Vinesi *et al* [100] reported an HPLC method for the separation of the norlignan glycoside fraction from plant material of *Hypoxis obtusa*. Validation data to support the use of this analytical method for the quantitative analysis of hypoxoside was however, not provided.

In 1992, Betto *et al* [145] described the determination of norlignan glycosides by HPLC using a gradient method that was claimed to be useful for the separation of the relevant glycosides and to monitor production of norlignan glycosides in tissue cultures. In 1993 and 1994, Kruger *et al* [247, 248] described the use of guanidine hydrochloride and ammonium sulphate for the sorption enrichment of xenobiotics in biological fluids by HPLC that was subsequently applied to study the chromatographic behavior of hypoxoside and rooperol analogues. The method was also applied to analyze human urine and plasma samples for hypoxoside biotransformation products following the oral administration of hypoxoside. In addition, analysis of methanolic extracts of *Hypoxis rooperi* and *Hypoxis latifolia* were carried out to identify hypoxoside and rooperol analogues using the above analytical method. The applied method however, as in previous published methods for the determination of hypoxoside, also was not validated and no quantitative data were provided.

4.3 Stability Studies

The determination of inherent stability of compounds using stress testing has been in practice for decades and now it is spelt out as a requirement in the international conference on harmonisation (ICH) guidelines, which has been accepted and followed as a standard by industries worldwide. For medicinal compounds, stress testing generally forms part of the chemistry component of drug dossiers but such data, unfortunately are not available in the public domain. Stress studies need to be done on all new drugs whereas some laxity has been considered acceptable for “old” compounds and pharmacopoeial drugs, as suggested by the Committee for Proprietary Medicinal Products (CPMP) guideline QWP556/96 [275]. The ICH guideline, Stability Testing of New Drug Substances and Products (Q1A) [276], requires that stress testing be conducted to elucidate the intrinsic stability of the active substance. These stability tests need to be done to study the effects of temperature, humidity (where ever applicable), oxidation, photolysis and susceptibility to hydrolysis across wide pH ranges. In 2000, Saranjith Singh and Monika Bakshi [277], suggested methods and procedures for the determination of the test parameters, hydrolysis under neutral, acidic, alkaline, oxidation and photolysis.

Generally about 10-20% degradation of the active substance is required for establishing an assay method which is stability-indicating. Greater degradation beyond 20%, however, often results in the formation of secondary degradants that could possibly complicate method development [278]. It has been recommended that higher concentrations of the parent drug in solution should be used to obtain sufficient quantities of degradation products. When stressing samples with either acid or alkali, it is recommended that the degraded solutions be preferably diluted rather than neutralized in order to facilitate analysis.

Photostability studies should be carried out on both solid and solutions of the compound under a lamp that meets the output of D65/ID65 emission standards defined by the International Standard Organisation (ISO) [279].

It is advisable to use four samples for every stress study as follows: The first sample is the treatment blank (no compound) at zero time, the second is the blank under stress conditions in the same manner in which the compound will be exposed

for the specified length of time, third is the sample under the treatment conditions at zero time and the fourth is the sample subjected to the stress treatment condition for the specified length of time.

No information has appeared in the literature that provides data on the stability of hypoxoside under various stress conditions. Furthermore, there has been no reported stability-indicating assay method for hypoxoside.

Hence the main objective was to develop a simple, rapid, accurate, precise and reproducible stability-indicating HPLC method which will be suitable for the assay and QC of plant material, extracts and commercial formulations containing AP.

4.4 Materials and Methods

4.4.1 Instrumentation

A Mettler dual range electronic balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing the standards and samples. A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes. The analysis was carried out on an Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 2996 photodiode array (PDA) detector, a degasser, column heater and an auto sampler. A Luna[®] C₁₈ (2) (5 µm, 150 x 4.6 mm i.d.) column (Phenomenex, Torrance, CA, USA) was used at 23 ± 2° C to achieve chromatographic separation.

The HPLC-MS analyses of hypoxoside and its samples from the stress studies were carried out using A Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) that has previously been described (Chapter 3, Section 3.4.6).

4.4.2 Reagents

Acetonitrile and methanol (HPLC grade) were purchased from Romil Ltd, (Cambridge, UK), butanol, ethyl acetate, toluenes were purchased from BDL Chemicals Ltd, (Poole, UK) and sulphamerazine (99.1%) was gratefully received as a gift from Orchid chemicals, (Chennai, India). Sodium tetraborate, sodium hydroxide pellets (Rochelle Chemicals, Port Elizabeth, South Africa), hydrogen peroxide

solution (30 vol.) (Riedel-de-Haen, Johannesburg, South Africa), hydrochloric acid (Merck KGaA, Darmstadt, Germany), 85% (v/v) ortho-phosphoric acid (Merck, Midrand, South Africa) were of analytical reagent grade and were used without additional purification. Water was purified in a Milli-Q[®] System (Millipore, Bedford, USA) and Millex HV[®] Hydrophilic (PVDF, 0.45 µm) filters were purchased from the same source. Silica gel 100-200 mesh (75-250 µm) was purchased from Sigma (St. Louis, MO, USA). Twelve different formulations were purchased from a local retail pharmacy in Grahamstown, South Africa.

4.5 Experimental

4.5.1 Method Development

Hypoxoside is highly soluble in methanol which prompted the choice of a C₁₈ analytical column for the analytical method development. Furthermore, previous analytical methods involving hypoxoside [100, 145, 246, 247] were based on the use of a C₁₈ column. Initial LC conditions for method development involved the use of a Phenomenex Luna[®] C₁₈ 150 mm x 4.6 mm i.d. column maintained at 23 ± 2 °C. Previous HPLC methods [100, 145, 246, 247] involved the use of buffers for the analysis of norlignan glycosides. In view of the fact that one of the objectives of this work was to develop a stability-indicating assay method which would be compatible with an MS detector, a non-buffer mobile phase was preferred.

Acetonitrile was used in combination with water which provided relatively lower back pressure than a possible alternative solvent, methanol. Although the short run time using isocratic conditions of water-acetonitrile (75:25) at 1 ml/min was achieved (6.4 min) for hypoxoside, exclusive determination of the latter was not possible when extracts of raw material and formulations were analyzed. This was due to interfering peaks which co-eluted with hypoxoside prompting further method development using a PDA detector to confirm peak homogeneity.

An aqueous extract of AP, which exhibited a fairly complex profile, was prepared as traditionally used by the indigenous population of South Africa [121] and the preparation thereof is described in Section 4.5.2.4 of this Chapter. Samples of this preparation were used for the analytical method development.

A decrease in water content of the MP (water-acetonitrile, 65: 35, v/v) resulted in the early elution of hypoxoside closer to the column void volume (2.2 min). Increasing the water content from a ratio of 75:25 to 80:20 considerably increased the retention time of hypoxoside to 11.2 min. With this change in mobile phase composition, interfering peaks were separated and peak homogeneity for hypoxoside was achieved. When extracts of formulations were analyzed, they too did not show any chromatographic interference with the hypoxoside peak. A further increase in the water content of the mobile phase resulted in an unacceptably long retention time (**Table: 4.1.**).

Table: 4.1.
Influence of Mobile Phase on the Retention of Hypoxoside

Water (%)	Acetonitrile (%)	Retention time of Hypoxoside in min
65	35	2.2
70	30	4.1
75	25	6.4
80	20	11.2
85	15	19.7

The effect of flow rate on the elution of hypoxoside was investigated in order to reduce the analysis time without compromising the separation. Although the higher flow rates resulted in shorter elution times they were also associated with slightly higher column back-pressures and did not offer any significant advantages. More importantly, the retention time of 11.2 min using a flow rate of 1 ml/min facilitated the choice of an appropriate internal standard since ample time was available to allow elution of an IS without interference.

Although hypoxoside shows relatively strong UV absorption at 210 nm a detection wavelength of 260 nm was chosen for its specificity in order to avoid chromatographic interference from excipients and other potential impurities. A chromatogram of the well-resolved hypoxoside peak in the traditional AP extract is shown in **Figure: 4.1.**

Figure: 4.1.

Traditional Aqueous Extract of AP

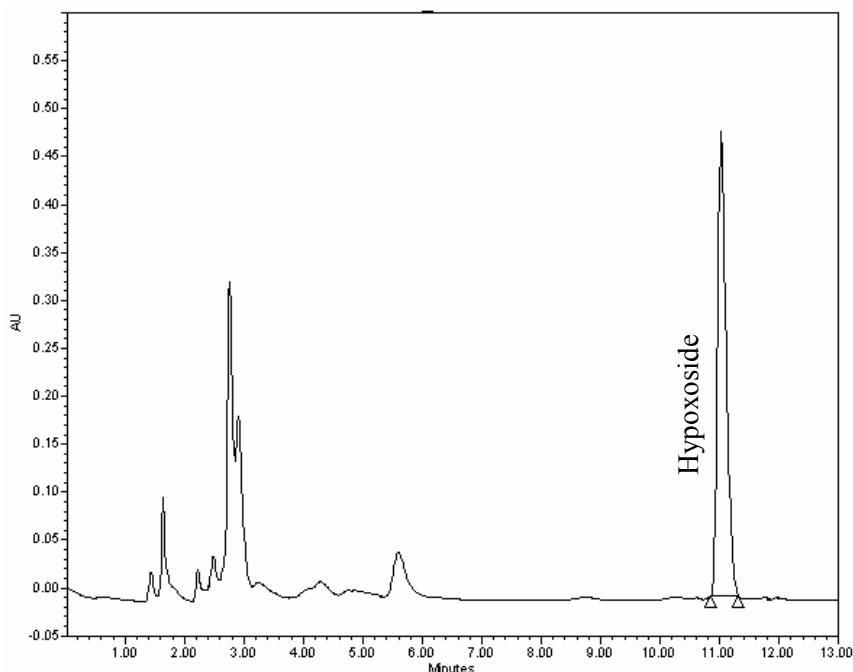


Figure: 4.1. Conditions:- Column: Phenomenex Luna[®] (5 μ m) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 $\pm$ 2 $^{\circ}$ C; Mobile phase: (acetonitrile 20: 80 water) Flow rate: 1 ml/min; Injection volume: 10 μ l; Detection λ : 260 nm.

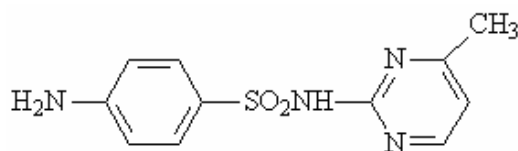
The use of internal standard in an assay procedure helps in compensating possible errors that may arise during the extraction procedure such as solvent spillage/evaporation. Furthermore, it also compensates for any possible errors that may be due to volume differences/losses.

Sulphonamides are a group of compounds which are mostly soluble in methanol, possessing UV absorption at 260 nm. Different sulphonamides were dissolved in methanol to yield solutions with a concentration of approximately 100 μ g/ml and chromatographed under the previously described conditions for hypoxoside.

Three of the sulphonamides (sulphamerazine, sulphafurazole and sulphamethoxazole) eluted at times compatible with the analysis of hypoxoside. An aliquot of about 0.5 ml of the relevant sulphonamide sample solution was then mixed with 0.5 ml of a hypoxoside stock solution (100 μ g/ml) and injected onto the HPLC column. Sulphamerazine (**Figure: 4.2.**) was found to elute at 5.08 min giving a well-resolved peak. Sulphamerazine has a wavelength maximum of 266 nm (**Figure: 4.3.**) and eluted before hypoxoside.

Figure: 4.2.

Chemical Structure of Sulphamerazine

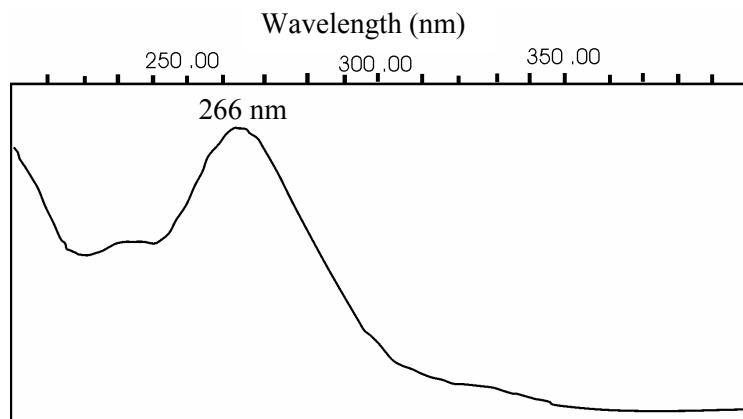


<i>Formula</i>	C ₁₁ H ₁₂ N ₄ O ₂ S
<i>Molecular Weight</i>	264.30*
<i>pKa</i>	6.98±0.30, 1.64±0.10*
<i>Melting point</i>	234-238° C

(*Values obtained using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris®
1994-2005 ACD/Labs)

Figure: 4.3.

UV Absorption Spectrum of Sulphamerazine



Under the above described analytical conditions, the internal standard (IS) eluted at 5.01 min and hypoxoside at 11.47 min (**Figure: 4.4.**). Subsequently, it was also found that sulphamerazine did not interfere with any of the other constituents of the extracts analyzed. Peak purity was confirmed by performing the peak purity test on the relevant peaks, sulphamerazine and hypoxoside (Empower® software package, Waters Corporation, Milford, MA, USA)

Figure: 4.4.

UV Spectra and Chromatogram of Hypoxoside and Sulphamerazine (IS).

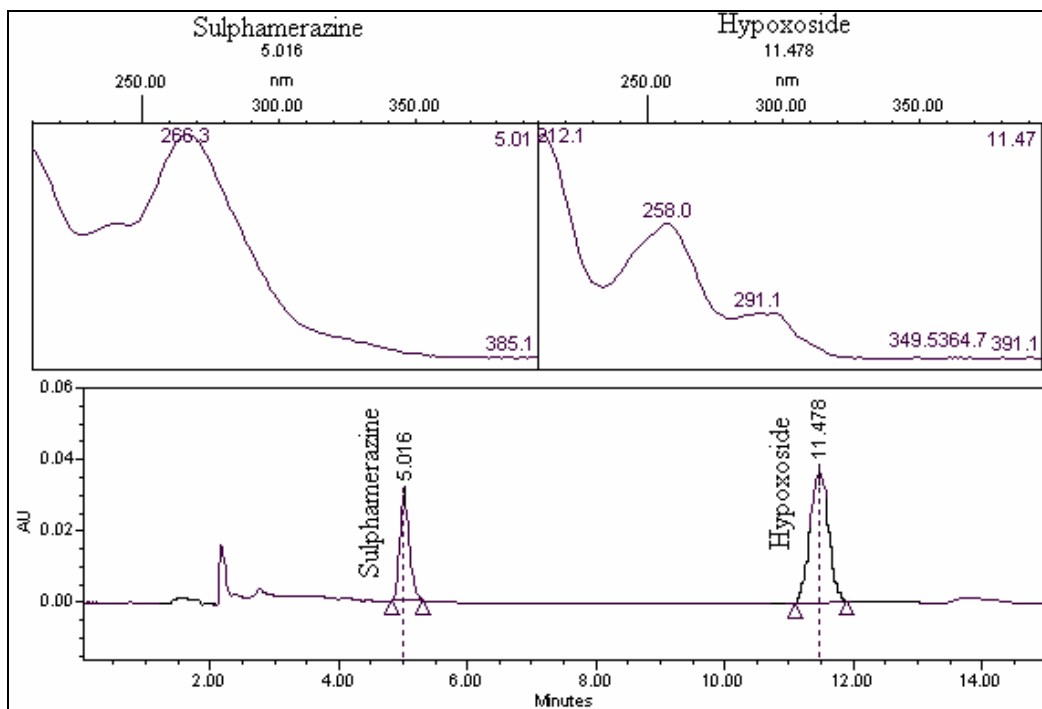


Figure: 4.4. Conditions: - Column: Phenomenex Luna[®] (5 μ m) C₁₈ (150 x 4.5 mm i.d.); Column temperature: 23 $\pm$ 2 $^{\circ}$ C; Mobile phase: (acetonitrile 20: 80 water) Flow rate: 1 ml/min; Injection volume: 10 μ l; Detection λ : 260 nm.

4.5.2 Method Validation

4.5.2.1 Sample Treatment

Fresh corms were carefully washed with water and wiped with tissue paper. They were cut into approximately 0.5 cm³ pieces and segregated into four lots of known weight (ca. 250 g). Replicates of 250 g of fresh samples of AP (cubes) were dried according to the following four procedures until constant mass was achieved.

- Evenly spread and allowed to dry by exposing to sunlight for 8 hr daily for three weeks (SUN)
- Dried under the shade (the same loss as above was reached in a month) (SHD)

- Immediately frozen in liquid nitrogen and lyophilized for 12 hr (FDAP)
- Micro-waved (750 W) for 4 min (high) to assist dehydration (MWD)

In all instances, a constant mass was reached, i.e. 40% of initial mass achieved.

Twelve different commercially available formulations (A-L) were chosen, five (A, B, C, D, E) were hard gelatin capsules, four products (F, G, H, I) were solid dosage forms (tablets) and the remaining three (J, K, L) were in liquid form. Four different batches of Product-D were purchased from local pharmacies to study the inter-batch variability. The average weights of all the solid formulations were calculated.

4.5.2.2 Hypoxoside Reference Standard

Hypoxoside that was isolated as previously described in Chapter 3, was stored in a deep freezer ($-20 \pm 2^\circ \text{C}$) until required for use. Confirmation of the structure and purity of hypoxoside (>99%) was established using spectral data (Chapter 3, Section 3.4).

4.5.2.3 Preparation of Standard Solutions

On each of three days, methanolic stock solutions of hypoxoside (100 $\mu\text{g/ml}$) and the internal standard (IS) sulphamerazine (100 $\mu\text{g/ml}$) were prepared in 10 and 50 ml methanol, respectively. Working solutions of hypoxoside comprising a set of five calibrators in the concentration range of 10-100 $\mu\text{g/ml}$ and containing 10 $\mu\text{g/ml}$ IS were prepared daily by appropriate dilution with methanol. Additional samples were freshly prepared on each day of analysis (30 and 60 $\mu\text{g/ml}$) for use as QC standards.

4.5.2.4 Sample Extraction

Ten hard gelatin capsules of each of Products A, B, C, and D were emptied, separately mixed and aliquots of each powder mix (50 mg) weighed into a tared 10 ml volumetric flask. The flasks were made up to the volume with methanol and sonicated for 20 min. After allowing them to cool for 5 min, 1 ml of each filtered solution was transferred to a 10 ml volumetric flask, 1 ml of the IS solution (100 $\mu\text{g/ml}$) added and

their volumes were made up with methanol. The solutions were filtered using PVDF filters (0.45 μm) prior to injecting 10 μl into the HPLC system.

Ten tablets of each of Products E, F, G, H and I were crushed in a mortar, powdered and well-mixed using a pestle. Approximately 50 mg of each product was accurately weighed in a tared 10 ml volumetric flask, methanol added and treated as described above for capsules.

The liquid formulations, Products J, K and L were well shaken and 1 ml of each preparation was transferred into a 20 ml volumetric flask and the volume was made up with methanol after addition of sulphamerazine as internal standard. Since Product J was found to have a high content of hypoxoside, the above solution of this product was further diluted by 200 times (0.5 to 100 ml) before the addition of internal standard. The sun dried (SUN), shade dried (SHD), freeze dried (FDAP) and microwave dried (MWD) cubes of the AP corms were powdered using a pulverizer (Retsch KG, 5657 Haan, West Germany) fitted with a 40 mesh sieve. In addition, a further two AP raw material samples were commercially procured, one of those was labeled to contain powder of dried AP to be filled in capsules and the other was labeled as containing '*Hypoxis* extract (10:1)'. Separate samples of each powder were well mixed and 100 mg of each was weighed into a tared 20 ml volumetric flask, approximately 15 ml of methanol added, sonicated for 20 min and allowed to cool for 5 min before making up to volume. Thereafter 1 ml of this solution was added to a 10 ml volumetric flask, 1 ml of the IS added and the volume made up to 10 ml with methanol. The solution was vortexed for 1 min before filtering through a PVDF membrane (0.45 μm) prior to analysis by HPLC.

An aqueous decoction of AP was prepared according to the usual method used by Traditional Healers ("*Sangomas*") in the Eastern Cape region of South Africa. Authenticated, fresh AP was peeled and then shredded using a blender (Kenwood Ltd, U. K) and approximately 20 g samples were weighed out into round-bottomed flasks, and 250 ml water added. The mixture was boiled for 20 min, strained through a clean muslin cloth and then filtered through Whatman[®] (ashless, grade 44) filter paper and made up in a measuring cylinder to an approximate volume of 250 ml (weight of AP equivalent to 80 mg fresh AP/ml). One milliliter of this aqueous extract was serially diluted to provide a concentration equivalent to approximately 8 mg of AP/ml, the IS solution added and this solution was then filtered using PVDF filters (0.45 μm) prior to injecting 10 μl for analysis by HPLC.

4.5.2.5 Stability of Hypoxoside Solutions

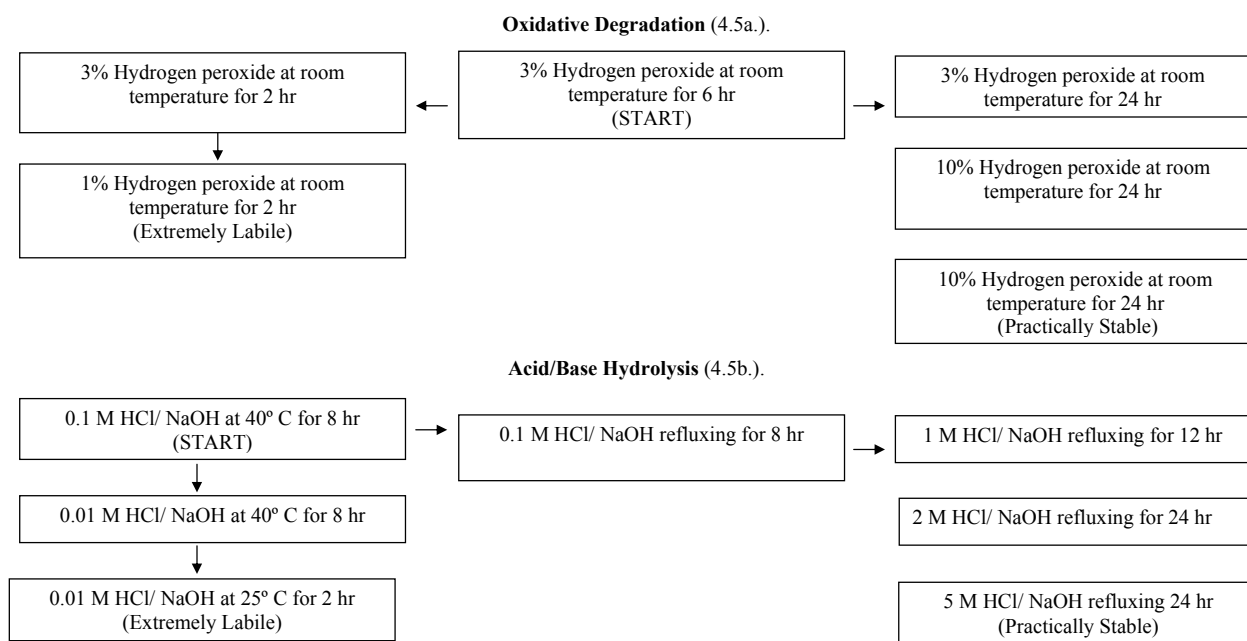
The stability of methanolic solutions of hypoxoside standards and samples were assessed by storing them for 10 days at room temperature (~23°C) and also by storing in the refrigerator at 4° C.

4.5.3 Forced Degradation Studies

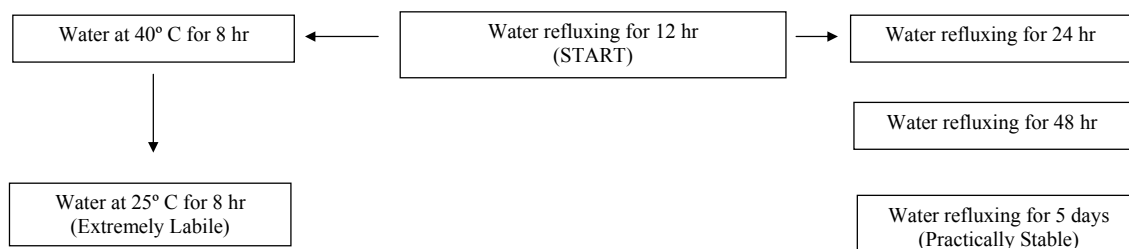
4.5.3.1 Stress Testing of Hypoxoside

Stress studies were undertaken to ensure that the developed method was stability-indicating. Hypoxoside was stressed under various conditions in order to force its degradation using a slight modification of the method reported by Saranjit Singh and Monika Bakshi 2000 [277]. A stock solution of hypoxoside (1 mg/ml) was prepared in methanol and further dilutions were made to prepare test solutions, which were subjected to various stress conditions as shown in the flow charts (**Figures: 4.5a-4.5d.**).

Figures: 4.5a-4.5d.
Flow chart for Stress Studies

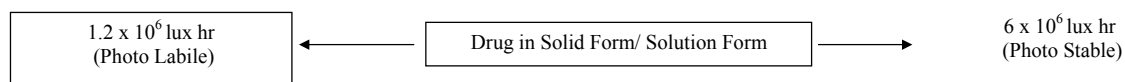


Neutral Hydrolysis (4.5c).



Photolysis (4.5d).

(D65/ID65 Emission standards defined by ISO)



4.5.3.2 Stress Conditions

The method used was based on the procedures of Saranjit Singh and Monika Bakshi 2000 [277]. and in addition, included an intermediate stress condition of 0.1 M HCl and 0.1 M NaOH at 40° C for 8 hr since less harsh stress conditions and longer exposure times avoid total destruction of both parent as well as primary degradation products and may result in the appearance of additional degradation products which are generally not present under the usual (less harsh) practical conditions of storage.

The following describe the various stress conditions used in this study.

- a) Oxidative degradation: Methanolic solutions of hypoxoside (100 µg/ml) were mixed with various concentrations of hydrogen peroxide (1 -10%, v/v), protected from light and stored for 12 hr prior to analysis.
- b) Acid hydrolysis: Methanolic solutions of hypoxoside (100 µg/ml) were mixed with equal parts of 0.2 M hydrochloric acid and placed on a water bath for 12 hr, which was maintained at 40° C prior to analysis.
- c) Alkali hydrolysis: Methanolic solutions of hypoxoside (100 µg/ml) were mixed with equal parts of 0.2 M sodium hydroxide and placed on a water bath for 12 hr, which was maintained at 40° C prior to analysis.
- d) Neutral hydrolysis: A (50%) methanolic solution of hypoxoside (50 µg/ml) was refluxed for 12 hr prior to analysis.
- e) Photolysis: About 5 mg of hypoxoside was spread into a fine layer on a watch glass. Methanolic hypoxoside solutions were prepared (50 µg/ml). All these samples were placed in a light cabinet (Suntest CPS/CPS⁺, Atlas Material Testing Technology, Germany) and exposed to light for 30 hr using an overall illumination of $\geq 1.2 \times 10^6$ lux hr at 35° C. Duplicate samples as controls were also placed in the illuminated cabinet but covered with aluminum foil in order to protect from light. The samples were then analyzed as previously described.

A set of appropriate blank solutions without hypoxoside for each stress condition were also subjected to stress conditions and analyzed at varying times. The system suitability was confirmed during the analysis by injecting the prescribed mixture of

compounds (toluene, xylene, benzene, uracil and naphthalene) using the mobile phase of acetonitrile: water, 35:65, suggested by the column supplier (Phenomenex, Torrance, CA, USA).

4.6 RESULTS AND DISCUSSION

4.6.1 Method Validation

4.6.1.1 Linearity

Calibration curves were constructed by plotting the peak area ratio of hypoxoside/IS versus concentration of hypoxoside on each of three separate days and were linear with determination coefficients higher than 0.99 in all cases.

4.6.1.2 Limits of Detection (LOD) and Quantification (LOQ)

The LOD (signal/noise >3) and LOQ (signal/noise >10) were determined by analyzing serial dilutions of known concentrations of hypoxoside standard solutions. The LOD and LOQ were 0.75 and 3.5 µg/ml respectively.

4.6.1.3 Accuracy and Precision

Accuracy and precision studies were performed and assessed within and between runs using two sets of QC samples that were separately prepared on each of the three days of analysis as previously described. The accuracy of the method was found to be between 97.30 - 103.00 % and the RSD for inter-day precision was better than 3% (**Table: 4.2.**).

Table: 4.2.
Accuracy and Precision of Hypoxoside

Day-1				Day-2				Day-3	
Actual weight (µg/ml)	Calculated weight (µg/ml)	% Accuracy	% RSD	Actual weight (µg/ml)	Calculated weight (µg/ml)	% Accuracy	% RSD	Actual weight (µg/ml)	Calculated weight (µg/ml)
29.68	29.42	99.13	0.24	30.06	30.11	100.19	0.73	30.02	30.18
59.36	57.75	97.30	0.34	60.12	61.92	103.00	0.56	60.04	59.55

4.6.1.4 Recovery

The recovery of hypoxoside was evaluated to assess the extraction efficiency of the method. Product-D and E were chosen for the intra-day and inter-day recovery studies. The first batch of Product-D (D I) was chosen for the recovery studies and contained 16.35 mg hypoxoside per unit whereas product E, which did not have any detectable amount of hypoxoside, presented an ideal opportunity for use as a 'blank matrix'. These formulations were individually and separately spiked with low, medium and high concentrations of hypoxoside, i.e., 15, 30, 45 µg/ml respectively (**Figure: 4.6.**). This was done on three different days and the analyses were performed in triplicate. Recovery values of Product-D I were between 94.39 % and 104.49 % and product E were between 94.18% and 98.53%.

Figure: 4.6.

Chromatogram of Product-D spiked with Low, Medium and High Concentrations of Hypoxoside

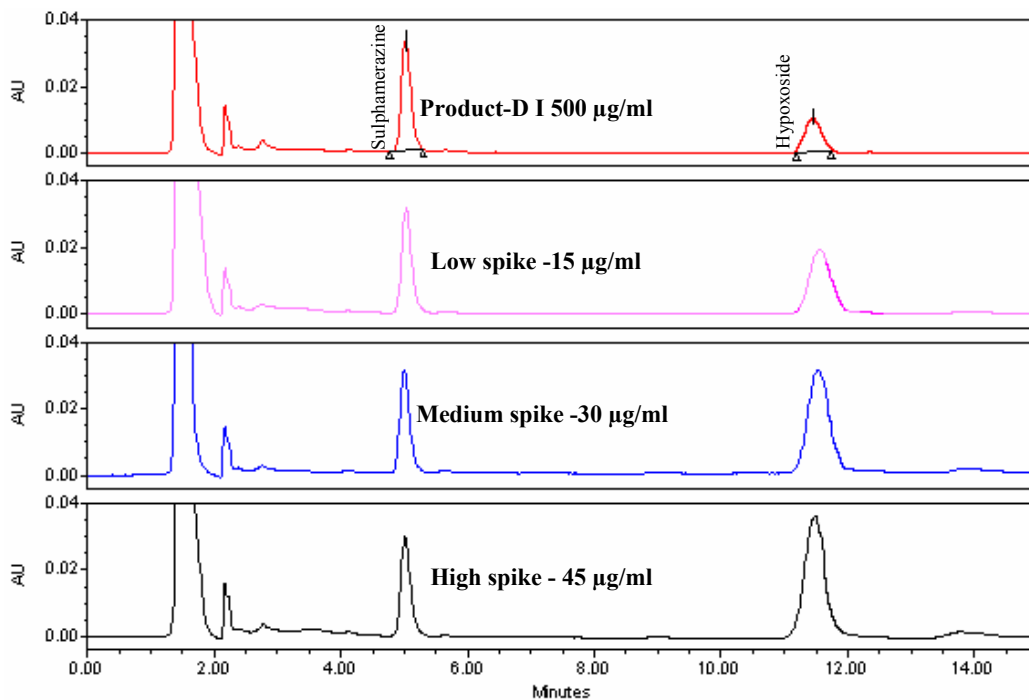


Figure: 4.6. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (acetonitrile 20: 80 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

The intra-day precision for Product-D I, expressed as % RSD was not higher than 6.15 % throughout the 3 days and the inter-day precision was found to be not higher than 5.64%. Similarly, good intra- and inter-day precisions were seen for product E which resulted in % RSD not higher than 3.03 % and 2.34%, respectively (**Table: 4.3.**). Although the spiked samples used for the recovery studies showed slightly higher % RSD values than data obtained from the QC samples, the data are generally in close agreement, indicating that the method is accurate and precise.

4.6.2 Analysis of Samples

4.6.2.1 Extraction Efficiency

Extraction efficiency using methanol was investigated by conducting two parallel studies in which 3 aliquots of separately powdered and well-mixed samples of Product C (representative sample of hard gelatin capsule) and Product F (representative sample of tablet) were used. Samples of about 50 mg were weighed out into tared 20 ml volumetric flasks and sonicated over a period of 1 hr. Aliquots of samples were withdrawn at not less than 10 min intervals before filtering through PVDF filters (0.45 μm) and analyzing them by HPLC. The amount of extracted hypoxoside was calculated by considering the optimum amount extracted at 60 min (maximum time) to be 100%. At 20 min, the samples from products C and F indicated 102.61 and 105.76 % of hypoxoside respectively compared to slightly lower values for the samples analyzed after 10 min and without any significant improvement at the longer time intervals.

From the results obtained, it was found that ultrasonication for 20 min was the minimum time required for optimum extraction of hypoxoside from each of the different formulations. Different solvents were also studied for their extraction efficiency, such as n-butanol and acetone. Although n-butanol was found to be similar to methanol in terms of extraction efficiency, it was not used due to its immiscibility with the mobile phase used.

Table: 4.3.
Recovery of Hypoxoside

Sample	Day- 1				Day- 2				Day-	
	Spiking level (mg/500 mg dosage form)	Observed content (µg/ml)	% Recovery	% RSD	Spiking level (mg/500 mg dosage form)	Observed content (µg/ml)	% Recovery	% RSD	Spiking level (mg/500 mg dosage form)	Obs co (µg)
Product-D I	14.41*	-	-	0.98	14.29*	-	-	0.44	14.66*	
	29.47(+15.06)	30.94	104.49	2.00	29.42(+15.13)	30.60	104.01	6.15	29.77(+15.11)	28
	44.53(+30.12)	44.68	100.33	2.35	44.55(+30.26)	44.13	99.05	2.72	44.88(+30.22)	43
	59.59(+45.18)	59.14	99.24	3.57	59.68(+45.39)	58.15	97.43	3.51	59.99(+45.33)	58
Product-E	-*	-	-	1.98	-*	-	-	-	-*	
	15.06(+15.06)	14.84	98.53	2.00	15.13(+15.13)	14.68	97.02	2.34	15.11(+15.11)	14
	30.12(+30.12)	28.56	94.82	0.94	30.26(+30.26)	28.63	94.61	1.87	30.22(+30.22)	28
	45.07(+45.18)	44.08	97.79	2.25	45.39(+45.39)	42.75	94.18	2.95	45.33(+45.33)	44

* Actual content before spiking the sample 500 mg dosage form
- The amount is below LOD (3.75 mg/ 500 mg)

4.6.2.2 Commercial Formulations

Table: 4.4. shows the assay values and the individual label claims of each of the analyzed commercial products. It should be noted that the labels of each of the products investigated indicate the presence of AP (either as powdered plant material, extract or other unspecified form) and not hypoxoside *per se*. Analysis of products (C, D I, D II, F, G and H) revealed the presence of hypoxoside amongst which, Product H (43.32 mg/dosage unit) was found to have the highest content of that compound. However, hypoxoside was not found in the other products (A, B, D III, D IV, E, I, K and L) in spite of their respective label claims indicating substantial AP content. Furthermore, within different batches of the same Product-D, a difference in hypoxoside content was found, viz: D I (16.35 mg/dosage unit) and D II (21.67 mg/dosage unit). Amongst the products (J, K, and L) in liquid form, only Product J, which according to the label consisted of an aqueous-alcoholic extract of AP, contained hypoxoside (344.07 mg/ml). Products K and L also indicated the presence of an extract of AP but the vehicle was not disclosed (Chapter 2, **Table:** 2.4.). Representative chromatograms of AP products in capsule (Product-D II), tablet (Product H) and liquid (Product J) dosage forms are shown in **Figures:** 4.7a-4.7c.

Table: 4.4.
Assay of AP Commercial Products

Sample No	Sample name	Average weight of the formulation in grams	Labeled content/weight	Hypoxoside (mg/Dosage un
1	Product-A	0.551 g	232 mg <i>Hypoxis hemerocallidea</i>	-
2	Product-B	0.638 g	15 mg <i>Hypoxis</i> extract	-
3	Product-C	0.590 g	African potato 70 mg	12.42
4	Product-D I	0.690 g	<i>Hypoxis rooperi</i> 250 mg	16.35
5	Product-D II	0.660 g	<i>Hypoxis rooperi</i> 250 mg	21.67
6	Product-D III	0.740 g	<i>Hypoxis rooperi</i> 250 mg	-
7	Product-D IV	0.664 g	<i>Hypoxis rooperi</i> 250 mg	-
8	Product-E	0.599 g	<i>Hypoxis</i> extract 12 mg	-
9	Product-F	1.214 g	<i>Hypoxis</i> powder (AP) 275 mg	18.75
10	Product-G	1.117 g	<i>Hypoxis</i> powder 200 mg	14.05
11	Product-H	1.099 g	<i>Hypoxis</i> powder 200 mg	43.32
12	Product-I	1.160 g	<i>Hypoxis</i> (AP) 200 mg	-
13	Product-J	50 ml	<i>Hypoxis rooperi</i> , Ethanol and Water	344.07(mg/ml
14	Product-K	500 ml	Extract of 500 mg <i>Hypoxis</i> /7.5 ml	-
15	Product-L	500 ml	2 Tea spoon/Extract of <i>Hypoxis</i> 1000 mg	-

Figure: 4.7a.
Chromatogram of Product-D II

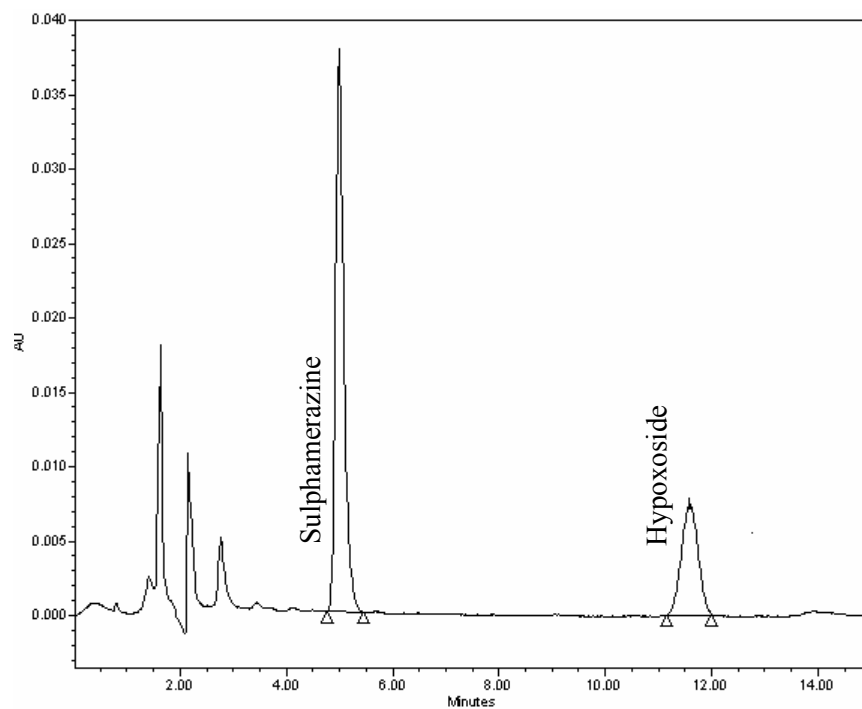


Figure: 4.7b.
Chromatogram of Product-H

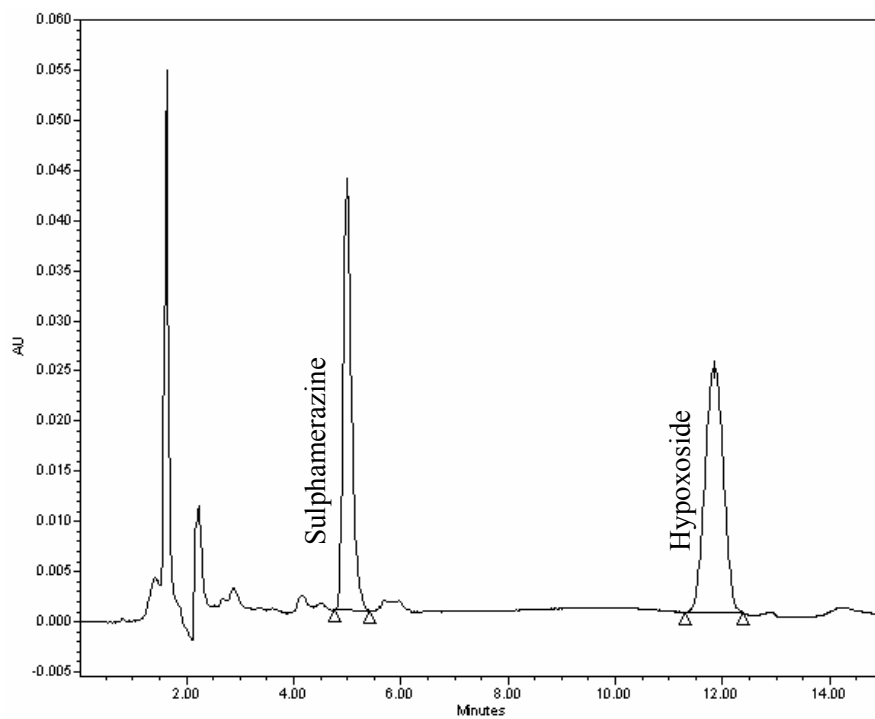
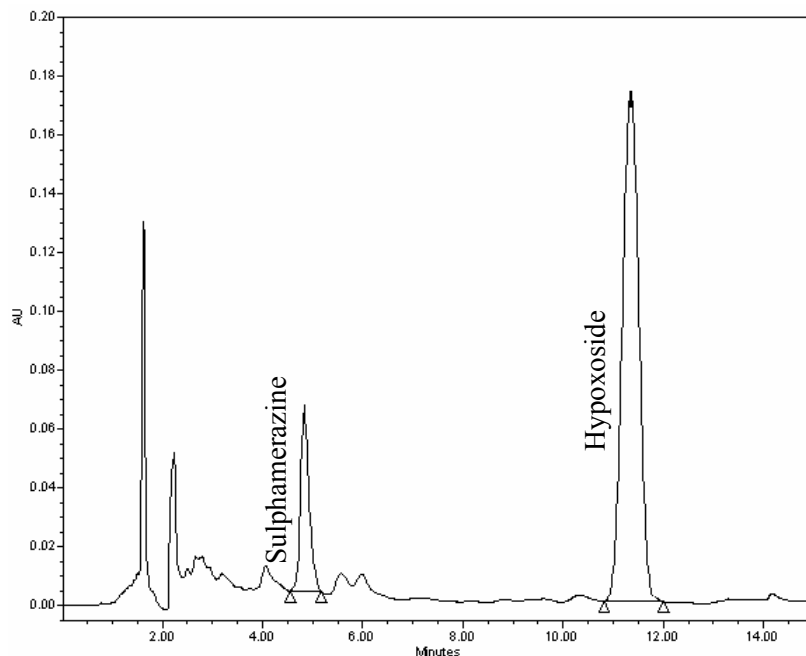


Figure: 4.7c.

Chromatogram of Product-J



Figures: 4.7a-4.7c. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C;
Mobile phase: (acetonitrile 20: 80 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

4.6.2.3 Crude AP Corms

Assay results from the various drying methods are depicted in **Table: 4.5**. Hypoxoside content of the corms subjected to the various drying treatments, expressed in terms of the percentage dried weight (g/100 g, w/w) indicated that lyophilized AP (FDAP) (**Figure: 4.8.**) contained the highest content of hypoxoside (10.18%, w/w) followed by MWD (5.85%, w/w), SHD (2.79%, w/w) and SUN (1.29%, w/w). The commercially procured AP raw materials used in formulations of AP dosage forms were also analyzed. Amongst the four raw materials (1, 2, 3 and 4), only 3 and 4 contained quantifiable amounts of hypoxoside.

Table: 4.5.
Assay of AP Raw Materials

Sample No	Sample name	% Average dry wt (g/100 g) of Hypoxoside	S.D.	% RSD
1	FDAP	10.18	0.52	5.14
2	SHD	2.79	0.12	4.19
3	SUN	1.29	0.03	2.36
4	MWD	5.85	0.29	3.43
5	Commercial AP Decoction	-	-	-
6	Extemporaneous Traditional Decoction	6.13	0.39	6.48
7	Raw material-1	-	-	-
8	Raw material-2	-	-	-
9	Raw material-3	0.61	0.03	5.85
10	Raw material-4	9.79	0.36	3.71

Figure: 4.8.

Chromatogram of Freeze Dried African Potato (FDAP)

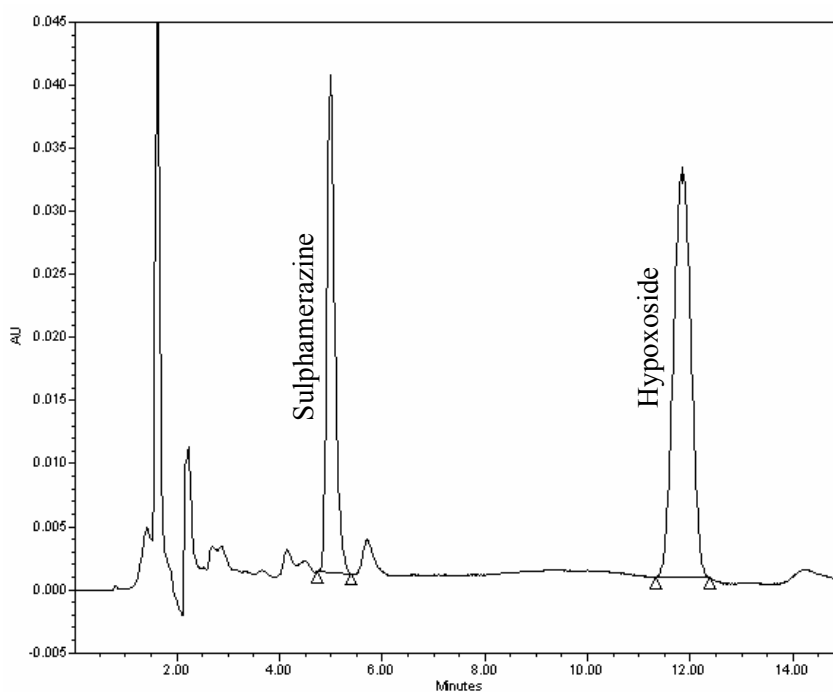


Figure: 4.8. Conditions:- Column: Phenomenex Luna® (5 μ m) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 $\pm$ 2° C; Mobile phase: (acetonitrile 20: 80 water) Flow rate: 1 ml/min; Injection volume: 10 μ l; Detection λ : 260 nm.

4.6.2.4 Traditional Extract of AP

Analysis of the aqueous extract prepared according to the procedure suggested by the traditional doctors (“*Sangomas*”), were also analyzed for hypoxoside content. From a survey carried out on different traditional doctors of this region, who often use and prescribe AP, the dose content of AP in such a traditional decoction was accordingly estimated. Typically, an average amount of ~20 g of freshly shredded AP boiled in 250 ml water is prescribed for daily consumption for patients to boost their immunity against various ailments. In 2005, Steenkamp *et al* [121] mentioned a daily dose intake of approximately 37 mg/ml AP following administration of the decoction three times a day, but did not specify the volume administered. An extract similar to that of Steenkamp *et al* [121] was prepared and analyzed as previously described. The content of hypoxoside was found to be 6.13% (**Table: 4.5.**, sample 6) of the mass of fresh AP corms which equates to an average daily consumption of 1.22 g of hypoxoside/patient. Importantly, hypoxoside was not found in aqueous extracts of AP sold by unqualified vendors, which was meant for ready consumption by patients (**Table: 4.5.**, sample 5).

4.6.2.5 Stability Studies

Methanolic standard solutions containing hypoxoside as well as extracted solutions of commercial samples were found to be stable when stored at room temperature (~23°C) and also when stored at 4° C in a refrigerator for 10 days.

4.6.3 Forced Degradation

When hypoxoside solutions were exposed to light under controlled conditions in a light cabinet, the solutions changed colour from virtually colourless to a pale golden yellow color. Under the same conditions, hypoxoside solid material in powder form turned dark yellow compared to the very pale yellow color of the control sample. Exposure of hypoxoside to alkali, yielded dark yellow solutions compared to the pale yellow colour seen on neutral hydrolysis. All other stressed test solutions remained virtually colourless.

The stressed samples ($n \geq 2$) were analyzed after different exposure time intervals at elevated temperature and compared with the zero time control sample at room temperature ($\sim 23^\circ\text{C}$).

Under conditions of alkali and also neutral hydrolysis, two degradation peaks were observed with similar UV absorption spectra. Since the degradation products were not well separated from each other but did not interfere with the resolution of the hypoxoside peak, the water content of the mobile phase was slightly increased by 2 % (water: acetonitrile, 82:18) in order to resolve the co-eluting degradation peaks. This resulted in increasing the elution time of hypoxoside to ~ 14.5 min instead of the usual retention time of ~ 11.2 min. It was also observed that alkaline hydrolysis samples, when injected, resulted in a slight outward shift (~ 0.5 min) in retention time of hypoxoside compared to the neutral hydrolysis samples. This increase in retention time could be due to the effect of an increase in pH of the analytical sample.

- a) Oxidative degradation: Hypoxoside with H_2O_2 (3%, v/v) resulted in 14.1% degradation after 24 hr whereas it was swiftly and completely degraded with higher concentrations of H_2O_2 (10%, v/v). When the blank H_2O_2 (3 %, v/v) sample at 24 hr was injected, the chromatogram showed 3 peaks. (**Figure: 4.9.**) Following injection of the stressed sample (24 hr), no additional peaks other than three peaks previously observed in the blank H_2O_2 (3 %, v/v) solution were observed (**Figure: 4.10.**). All peaks were well resolved from hypoxoside and their UV absorption spectra along with peak purity determination indicated the absence of any new degradants co-eluting under the same analytical conditions

Figure: 4.9.

UV Spectra and Chromatogram of H₂O₂ (3%, v/v) after 24 hr

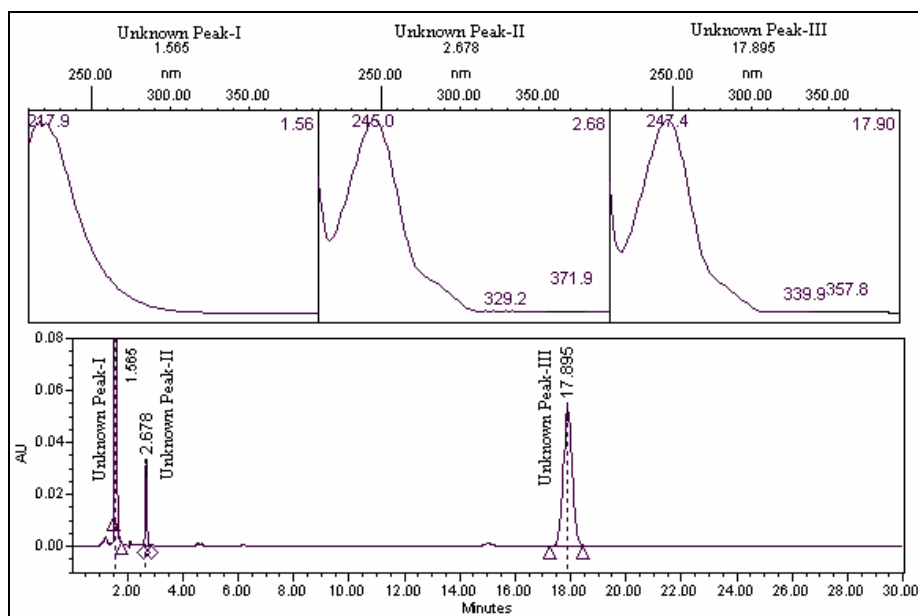
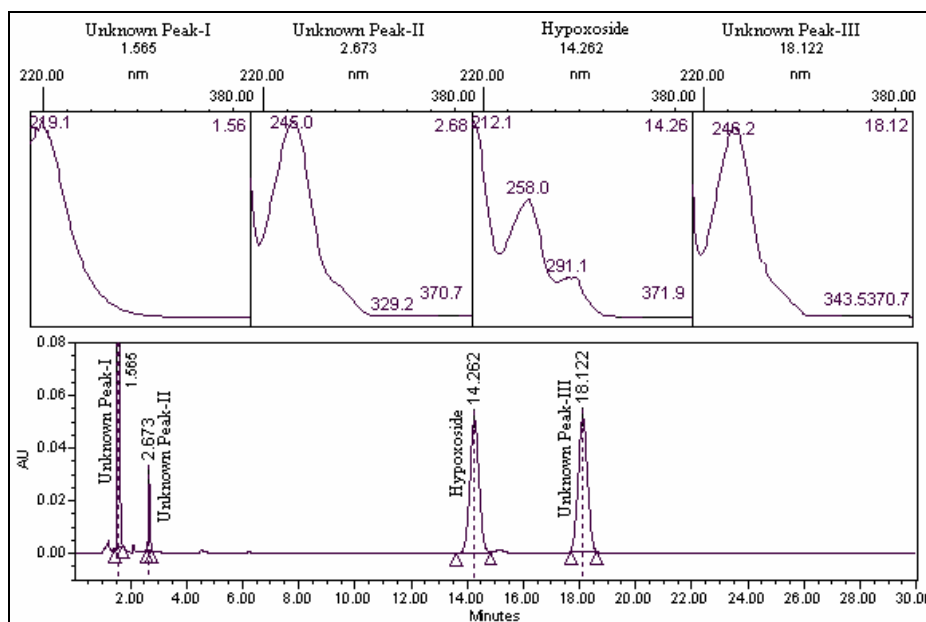


Figure: 4.10.

UV Spectra and Chromatogram of H₂O₂ (3%, v/v) Stress Solution of Hypoxoside after 24 hr



Figures: 4.9. and 4.10. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (acetonitrile 18: 82 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

b) Acid hydrolysis: Hypoxoside was found to be stable under acidic conditions [277] when compared to alkaline stress conditions. With 0.1 M HCl at 40° C for 8 hr, hypoxoside showed 9.52% degradation. A decrease in the hypoxoside peak area was observed when the acidic stress conditions were increased by increasing acid concentration and exposure time under elevated temperature. No additional peaks were seen concurrent with the decrease in hypoxoside peak area. Inspection of the chromatograms in comparison to the control solution did not reveal the presence of additional peaks, either in the void volume or at the end of the run (25 min), (**Figure: 4.11.**). The possible reason for this absence of degradation products may thus be due to those products not having the necessary chromophore for detection under the run conditions, such peaks may be late eluting or their presence is below the LOD.

Figure: 4.11.

UV Spectrum and Chromatogram of Acid Hydrolysis Sample of Hypoxoside Solution in 0.1 M HCl at 40° C after 8 hr

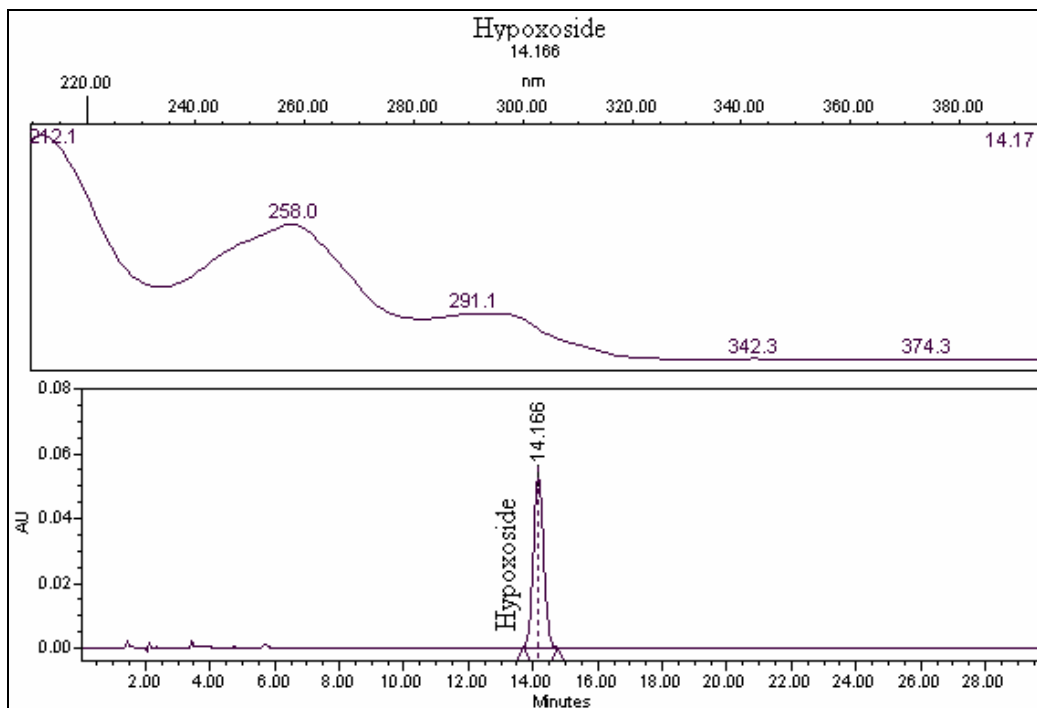


Figure: 4.11. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈(150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (acetonitrile 18: 82 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

c) Alkali hydrolysis: Hypoxoside was found to be labile under alkaline stress conditions (**Figure: 4.12.**). When exposed to NaOH (0.1 M) at 40° C, hypoxoside showed 95.5% degradation at the end of 8 hr. Two unidentified peaks with similar UV absorption spectra were observed at ~23.13 and ~24.01 min respectively. With an increase in temperature and alkaline strength, hypoxoside degraded rapidly as seen by the eventual disappearance of the hypoxoside peak.

Figure: 4.12.

UV Spectra and Chromatogram of Alkaline Hydrolysis Sample of Hypoxoside Solution
0.1 M NaOH at 40° C after 4 hr

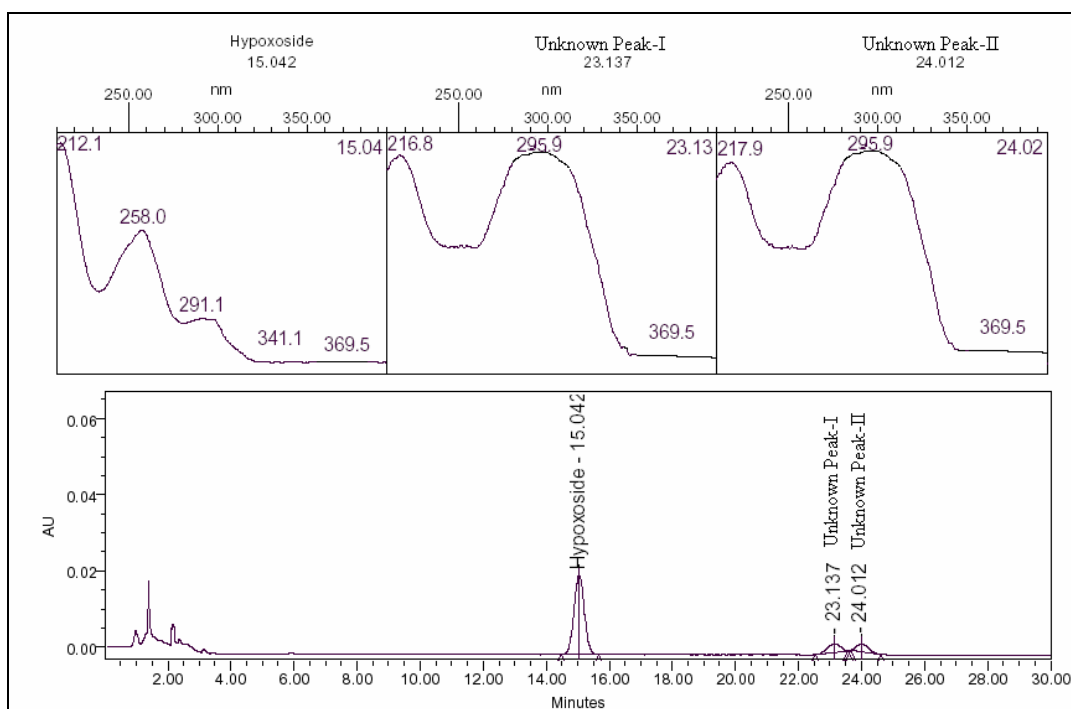


Figure: 4.12. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (acetonitrile 18: 82 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

d) Neutral hydrolysis: The methanolic solution (50%) of hypoxoside (100 µg/ml) was refluxed (>85° C) and these solutions showed more than 70% degradation after 12 hr. After refluxing for 24 hr, no hypoxoside was discernible. Two degradant peaks were observed with similar UV absorption spectra at ~21.73 and ~22.54 min respectively. Interestingly, these two peaks eluted at about the same time as that of

the alkaline degraded products and had similar UV spectra to those which appeared following the alkaline degradation conditions (**Figure: 4.13.**).

Figure: 4.13.

UV Spectra and Chromatogram of Neutral Hydrolysis (reflux)

Sample of Hypoxoside Solution after 8 hr

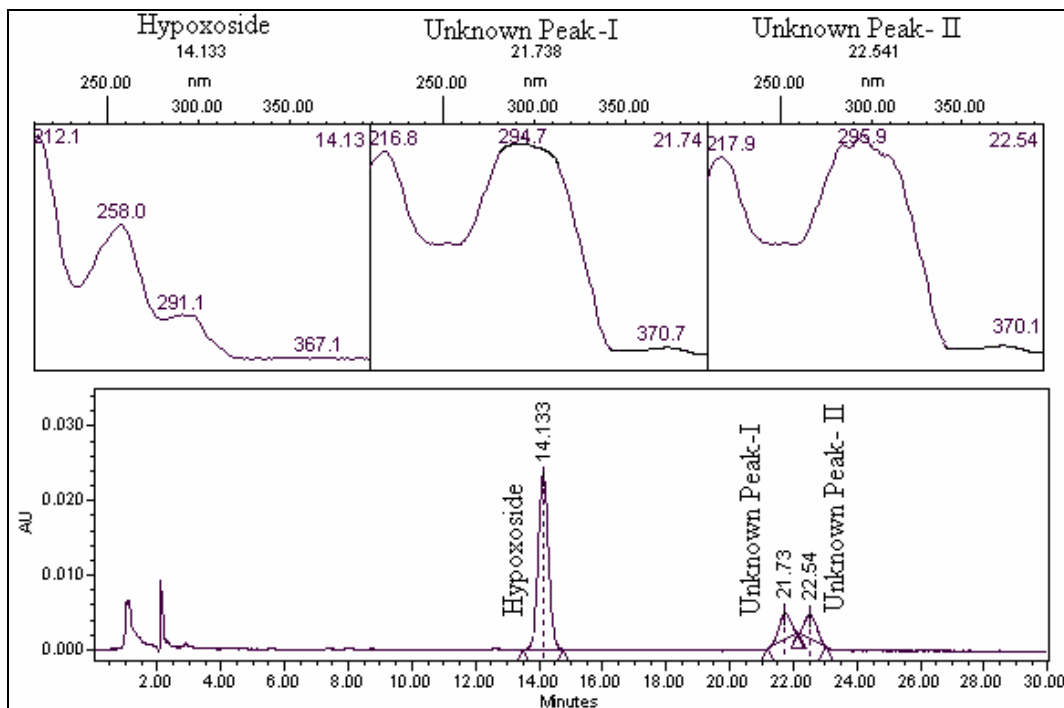


Figure: 4.13. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (acetonitrile 18: 82 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

- b) Photolysis: Photolytic studies were carried out and both dry (powdered) hypoxoside and wet samples (methanolic solutions of hypoxoside) were analyzed. Both the solid and the wet stress samples showed degradation after exposure to an overall illumination of $\geq 1.2 \times 10^6$ lux hr at 35° C. Following HPLC analysis of the solid samples, an unidentified peak which was absent in the control samples, eluted at ~17.76 min (**Figure: 4.14.**) and a similar peak was also seen in the wet sample, both with similar UV spectra. In addition the wet stress sample also showed two additional unidentified (unknown) peaks (**Figure: 4.15.**).

Figure: 4.14.

UV Spectra and Chromatogram of Solid (dry) Photo-hydrolysis Sample of Hypoxoside after 30 hr Exposure ($\geq 1.2 \times 10^6$ lux hr at 35° C)

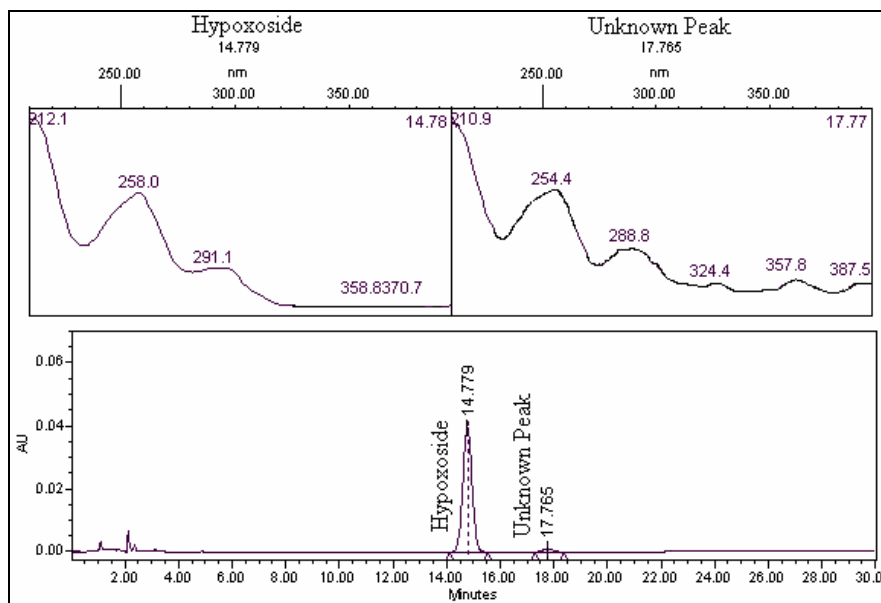
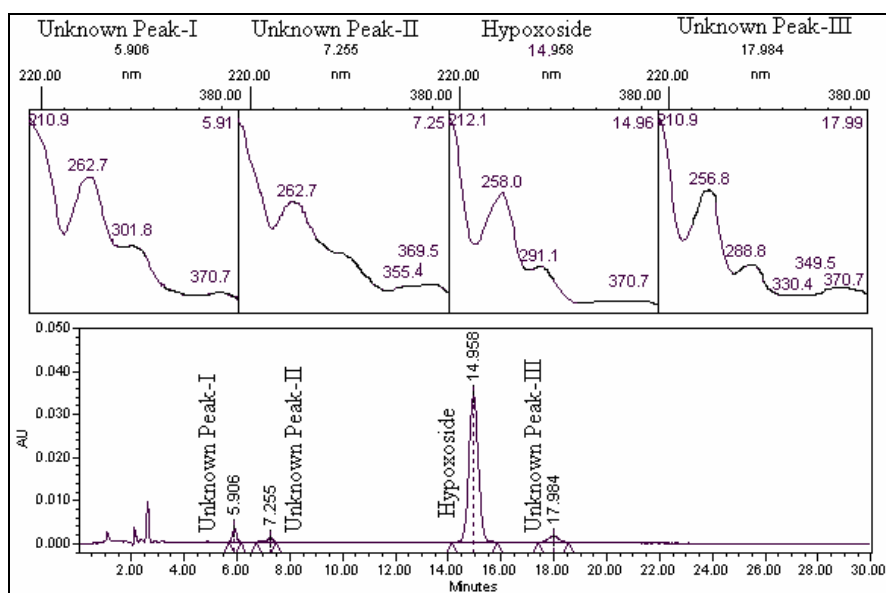


Figure: 4.15.

UV Spectra and Chromatogram of Wet (solution) Photo-hydrolysis Sample of Hypoxoside After 30 hr Exposure ($\geq 1.2 \times 10^6$ lux hr at 35° C)



Figures: 4.14. and 4.15. Conditions:- Column: Phenomenex Luna® (5 μ m) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 $\pm$ 2° C; Mobile phase: (acetonitrile 18: 82 water) Flow rate: 1 ml/min; Injection volume: 10 μ l; Detection λ : 260 nm.

c) Combined degradation mixture: Separate mixtures of blank solutions and stressed sample solutions were prepared and analyzed. These mixtures ultimately consisted of the combined acid, alkali, neutral and photolytic sample solutions (except oxidative stress samples) containing hypoxoside to result in a concentration of approximately 20 µg/ml of the analyte. They were examined for common degradation products and any possible interference from the respective blank solutions which were also subjected to similar stress conditions. No interference was observed from any of the reagents used (Figure: 4.16.).

Figure: 4.16.

**UV Spectra and Chromatogram of Degradation Mixture
(0.1 M HCl, 0.1 M NaOH, Neutral Hydrolysis, Dry and Wet Photo Degradation)**

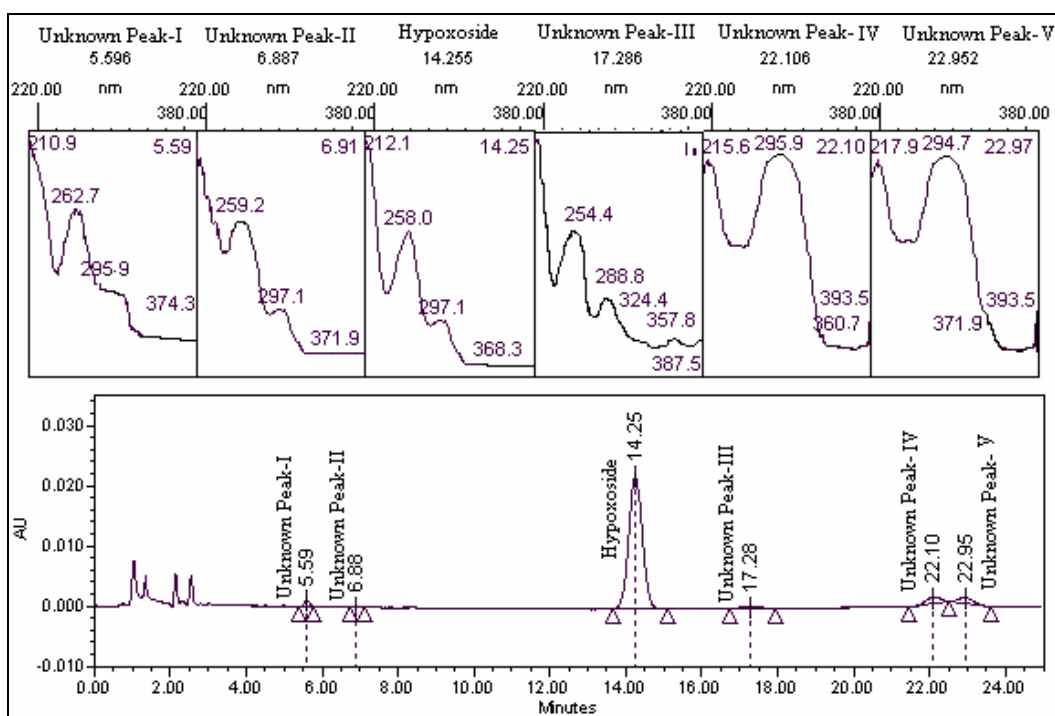


Figure: 4.16. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C;
Mobile phase: (acetonitrile 18: 82 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 260 nm.

The hypoxoside peak was clearly discernible from the five additional (unidentified/degradation products) peaks that eluted during the HPLC analyses. The two unidentified/degradation peaks formed under the alkaline conditions

eluted at the same time as those which formed under neutral conditions which was confirmed following injection of the mixture. PDA peak purity profiling showed that both these degraded products were similar if not identical.

This was further confirmed by analyzing these samples using LC-MS (**Figure: 4.17.**). Although the desired resolution was observed, there was a slight decrease in the retention time of both hypoxoside and the unidentified peaks and this could be attributed to differences in the length of the tubing between the column and the detector and other possible differences in system configuration since a different HPLC system was used for LC-MS compared to the HPLC-UV analytical system.

Tuning was done using positive ion ESI-MS with 25% collision energy. The m/e of both the unknown peaks were 606.49 which is similar to that of the parent molecule, hypoxoside (M^+ , 606.99). However, the similar UV absorption spectra of these unknown peaks were different from that of hypoxoside (showing a bathochromic shift) with a $\lambda_{\max}$ around 295 nm. This gives rise to the possibility of a structural rearrangement suggested by Drewes *et al* [198, 280].

Figure: 4.17. LC-MS Chromatogram of Neutral Hydrolysis Sample of Hypoxoside Solution Subject

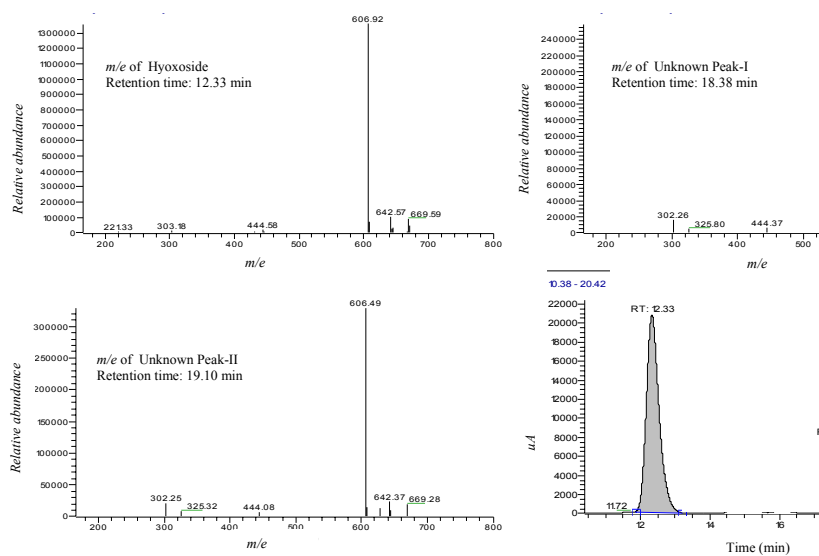
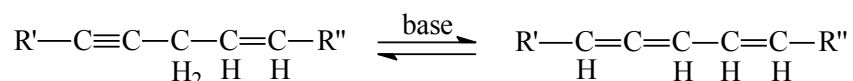


Figure: 4.17. Conditions:- Column: Phenomenex Luna® (5 µm) C₁₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (acetonitrile 18: 82 wa
Detection λ: 260 nm, ESI-full scan MS-25% collision energy.

It has been reported that the -CH₂ in hypoxoside is located between two non-identical moieties (**Figure: 4.18.**) and the two protons are prochiral. This structural feature is present in the pharmacologically active aglycone, rooperol, which allows it to interact in a very specific way with a receptor.

Figure: 4.18.

Structural Re-arrangement of Pentenyne Skeleton



Further evidence of the likelihood that the proposed structural re-arrangement may have occurred under the relevant stress conditions may be gleaned from their chromatographic behaviour. Such a re-arrangement that involves the conversion of the alkene bond to two adjacent alkane linkages and an associated bathochromic shift in UV absorption concurrent with a decrease in polarity is highly likely to result in longer retention of these compounds as observed.

It has been contemplated that the difficulty in synthesizing rooperol may be due to rearrangement of the pentenyne system, especially when the two halves of the molecule with protected phenolic groups are coupled together under basic conditions [198, 280].

4.7 Discussion and Conclusions

A simple, accurate and precise stability-indicating HPLC-PDA method was developed and validated for the quantitative determination of hypoxoside in plant material as well as in formulations and products containing AP. In addition, sample preparation and the assay procedure for formulations and also for AP plant material was rapid and reproducible and can be readily applied for the QC and routine assay of AP and formulations thereof. The results indicated that vast discrepancies in hypoxoside content exist amongst the various materials and commercially available

products that were analyzed. These findings emphasize that suitable QC measures need to be implemented to ensure consistent quality of marketed products.

The LC-MS method developed also has the potential for use in routine analysis of AP and its commercial products. This method was however not used for QC purposes in view of the higher running costs and practical limitations with respect to the relatively higher capital outlay for such hardware and maintenance etc., which could be an impediment in applying it for routine use on an industrial scale.

The study of traditional AP preparations also showed large differences in the content of hypoxoside in commercially available AP preparations compared to an extemporaneously prepared aqueous decoction. A further notable finding is that the content of hypoxoside in the latter preparation, and using the same quantity of AP claimed in the labeled content in the commercially available products, was considerably higher than in any of those formulations analyzed. This is indeed food for thought, considering that if hypoxoside is associated with pharmacological activity, the various commercially available products studied would not be expected to provide the necessary outcomes for the various conditions for which AP is indicated.

A further consideration relates to the findings from the stability studies where two degradation products were formed under alkaline and neutral conditions. Since the traditional AP preparation involves boiling raw AP corms (for 20 min) under conditions that essentially are considered to mimic the neutral stress conditions used in the stability studies, the traditional decoctions of AP should also contain these degradation products at the expense of hypoxoside content. However, analysis of the AP decoctions did not reveal evidence of such degradation products which could be due to the fact that the boiling time of up to 20 min is insufficient to result in any significant degradation of hypoxoside. This was confirmed from the analysis of refluxed samples under the same neutral conditions where samples analyzed at 20 min showed no evidence of degradation. It is therefore important that should AP decoctions continue to be used; heating of the mixture should not be prolonged in order to prevent possible degradation.

Chapter 5

High-Performance Liquid Chromatography with Evaporative Light Scattering Detection.

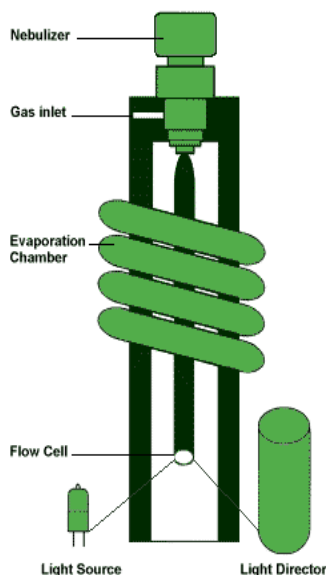
5.1 Introduction

Although HPLC with UV detection is the most commonly used system for quantitative analysis of pharmaceuticals and phytochemicals, compounds that lack the necessary chromophores cannot be detected and assayed by this procedure. Hence, alternative detection systems are required such as the use of refractive index (RI) or evaporative light scattering detector (ELSD)[269]. However, ELSD is considered to be more advantageous considering the relative baseline instability observed with RI detectors that are extremely sensitive to temperature variation and also to gradient elution systems. Moreover, ELSD is compatible with a much wider range of solvents and modifiers and produces stable baselines during gradient elution.

The principle of ELSD (**Figure: 5.1.**) is based on the nebulization of the LC column effluent into droplets by the nebulising gas. On the entrance into a temperature controlled evaporation chamber, the evaporation of mobile phase takes place. The resulting ‘cloud’ of solid micro particles is then directed towards a narrow beam of light in a flow cell. Light scattered by these micro particles is measured by a photo multiplier or photodiode used as a light detector.

The response of ELSD is independent of the spectral properties of the solvent and the analyte and is based approximately upon the absolute quantity of the analyte. Thus, it produces uniform sensitivity for most analytes regardless of their physical or chemical properties. Analytes that lack chromophores can be detected without any need for chemical derivatisation. The uniform response to structurally similar analytes facilitates calibration compared to other light absorbing detectors. The drift tube temperature and the inlet gas flow need to be optimized to obtain an optimum signal to noise ratio.

Figure: 5.1.
Principle of Evaporative Light Scattering Detector (ELSD) [281]



A plot of detector response against analyte concentration is sigmoidal and the peak area (PA) is related to the sample size and shape. The chemical identity of the residual particles passing through the light beam follows the relationship $PA = a m^b$, where (b) is the slope of the response line, (m) is the mass of the compound injected and (a) is the response factor. Plots of peak area ratio versus the analyte concentration using logarithmic coordinates are linear [282].

5.2 Background and Objectives

Initially in the 1970s, it was been claimed that sterols and sterolins present in AP are mainly responsible for the medicinal properties [148, 152-156, 283]. The first commercial product of AP, 'Harzol', launched in Germany in 1974 gained wide acceptance for its combination of β sitosterol and its glycoside. It is well known that sterols and sterolins are ubiquitous in nature, and their presence is not just confined to

AP [76, 250]. Interestingly, at present there are several products of AP on the market that are fortified with additional quantities of sterols and stanols.

The most common sterols in free form, fortified in the formulations are β -sitosterol (BSS) and stigmasterol (STG). The saturated β -sitosterol is called stigmastanol (STN) or sitostanol and is also usually added to such formulations though found abundantly in nature. Compared to phytosterols, the phytostanols are absorbed to a lesser extent from the GIT [205]. Since AP formulations are commonly fortified with BSS, STG and STN, these phyto-constituents were selected as active markers for the QC of the raw material, extracts and commercial products of AP.

In a recent review, Abidi [284] discussed various methods for the analysis of sterols and structurally related compounds. Most of those methods are tedious and mainly involve gas chromatography-mass spectrometry (GC-MS). Other chromatographic methods such as HPLC using UV, MS or ELSD detection involved the use of relatively complex multi-component solvent mixtures as mobile phase [285-287] to determine various sterols and related compounds. However, none of those methods have been used for the simultaneous quantitative analysis of the combination, BSS/STG/STN, purported to be present in preparations containing AP.

The main objective of this study was, therefore, to develop and validate a rapid and efficient analytical method for the simultaneous determination of BSS, STG and STN (Chapter 2, **Figures:** 2.10-2.12.) that were chosen as marker compounds, and which are found to be common constituents in such commercial formulations.

Unlike BSS and STG, STN does not have the necessary chromophore to enable detection by UV. Hence an ELSD detector [281] was coupled to the UV detector to monitor this compound and permit the simultaneous determination of BSS and STG together with STN.

An HPLC-PDA-ELSD method was developed and validated for the quantitative and qualitative analysis of BSS, STG and STN in commercially available oral dosage forms purported to contain AP material or extracts thereof.

In order to confirm the specificity of the method for BSS, STG and STN, LC-MS was used with an atmospheric pressure chemical ionization (APCI) interface. The APCI interface comprises a glass capillary surrounded by a high velocity nebulizing gas as well as a heater block which facilitates the rapid formation of a fine mist of vapor as the LC eluent passes through the capillary. Near the capillary tip, a corona discharge needle causes ionization and reacts chemically with the vaporized analyte molecules before the

transportation of these ions to the mass analyzer. Although both ESI and APCI techniques are classified as soft ionization ESI-MS is well suited for the detection of polar, non-volatile compounds [264] like the norlignan glycoside, hypoxoside which is ionized in solution. APCI, on the other hand, is more suitable for analysing non-polar and non-ionic compounds such as sterols and stanols which are relatively thermostable [266].

5.3 Experimental

5.3.1 Instrumentation

A Mettler dual range electronic balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing the standards and samples. A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used for sonication and extraction purposes. All experiments were performed using a Waters Alliance HPLC system equipped with a separation module (model 2690), a PDA detector (model 2996), an online degasser and an autosampler (Waters Corporation, Milford, MA, USA). A Luna[®] C₈ Column (5 μ m, 50 x 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) was used at 23 $\pm$ 2° C with an injection volume of 10 μ l. After UV detection with a PDA detector, the chromatographic column effluent was passed, on-line, into an ELSD (Alltech 2000, Alltech Associates, Inc., Deerfield, USA). The output of this detector was interfaced, via a SATIN[®] box, to an Empower[®] Chromatographic Manager (Waters Corporation, Milford, MA, USA).

The HPLC-MS analyses of sterols were carried out using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) with APCI as an interface coupled to a SpectraSYSTEM P2000 pump equipped with an AS 1000 autosampler and UV 1000 variable-wavelength UV detector (all supplied by Thermo Separation Products, Riviera Beach, FL, USA).

5.3.2 Reagents and Chemicals

Methanol (HPLC Grade) was purchased from Romil Ltd (Water Beach, Cambridge, UK). Stigmasterol (95%), stigmastanol (95%), β -sitosterol (97%) were purchased from Sigma (St. Louis, MO, USA) and cholesterol from Croda Chemicals Ltd. (North Humberside, UK). Water was obtained from a Milli-Q[®] system (Millipore, Bedford, MA, USA) and all samples were filtered using Millex HV[®] Hydrophilic (PVDF, 0.45 μ m) filters purchased from the same source. Five commercial products (A, B, M, N and O) were purchased from a local pharmacy in Grahamstown, South Africa. The labelled weight/unit of each of the formulations were 400 mg (A), 500 mg (B), 400 mg (M), 200 mg (N) and 1000 mg (O) respectively (Chapter 2, **Table:** 2.4.). Four of the products (Products A, B, M and N) were capsules, containing pulverized AP fortified with additional quantities of sterols and stanols, and one product (Product O) was formulated as a tablet.

5.3.3 Method Development

During the method development, elution of STG and BSS were monitored at a wavelength of 209 nm, which was the lowest wavelength setting provided by the PDA detector. The ELSD was necessary to monitor STN which lacked the necessary chromophore for UV detection. Optimization of the ELSD was effected with the structurally similar STG and BSS since these compounds could be monitored in the UV region and detected with the PDA detector. Interestingly, STG, BSS and STN have similar structures with differences only in the presence of double bonds. STG has double bonds at C-5 and C-22 position, BSS only has one double bond at C-22 whereas stigmastanol does not possess a double bond. Saturation of double bonds make a compound more non-polar and this phenomenon was helpful in the HPLC method development to predict the elution pattern of the analytes with a decrease in polarity (STG>BSS>STN).

The sterols (STG, BSS) and stigmastanol (STN) are soluble in methanol but are relatively non-polar, which prompted the choice of a C₈ analytical column instead

of a C₁₈ analytical column for the analytical method development. Since ELSD was the detector of choice, it was necessary to opt for a non-buffer relatively volatile mobile phase (MP), containing less of the aqueous component so that it would be compatible with the ELSD method.

A mixture of STG, BSS and STN (50 µg/ml each) was used as a standard solution for method development. When a MP with 100% methanol at 1 ml/min was used, all the analytes eluted within 8 min but were not resolved from one another. Hence an increase in polarity of the MP was effected by introducing water into the 100% methanol MP. When methanol (95): water (5), v/v, was used as MP, all three compounds (STG, BSS and STN) were well separated within 12 min at room temperature of 23 ± 2° C and a flow rate of 1 ml/min. The retention times of the analytes under these conditions were ~8.92 min, ~9.85 min and ~11.61 min for STG, BSS and STN, respectively

A methanolic extract of Product N, which exhibited a fairly complex profile, was prepared by ultrasonication for 15 min that was similar to the method described in Section 5.3.7.1 of this Chapter. To this methanolic extract, known concentrations of BSS, STG and STN were spiked for easy identification of the markers. Samples of Product N were used as representative samples to assess the potential interference from excipients and other constituents present in formulations.

An increase in methanol content in the MP reduced the resolution of all the above compounds whereas an increase in the water content moved the peaks further apart from one another with an undesirably longer retention time. Substitution of the mobile phase with acetonitrile-methanol (85: 15, v/v) gave a similar elution pattern, but the methanol: water (95:5, v/v) was used for further analysis considering its lower cost.

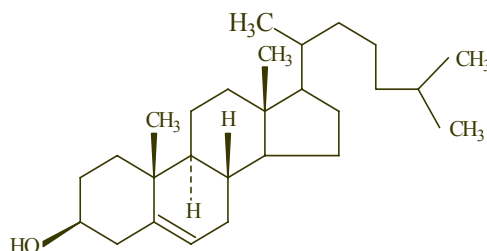
The effect of flow rate on the elution of the relevant compounds was investigated. Although higher flow rates resulted in shorter elution times, they did not offer any significant advantages. They were also associated with higher column back-pressures since methanol is a relatively more viscous solvent when mixed with water. Moreover, the flow rate of 1.0 ml/min facilitated the choice of an appropriate internal standard since ample time was available to allow elution of an internal standard (IS) without interference.

For the assay procedure, an IS was used to minimise any possible errors which might arise during the extraction and analysis of samples. For these purposes,

structurally and physicochemically similar compounds (ergosterol, cholesterol and brassicasterol combine with BSS) were chosen and 0.5 ml aliquot of a solution in methanol (~50 µg/ml) was mixed with 0.5 ml of an analyte mixture (STG, BSS and STN, 50 µg/ml each) and injected onto the HPLC system. Ergosterol was found to elute earlier and co-eluted with the excipients/additives when analysed with Product N (~500 µg/ml), whereas brassicasterol was found to co-elute with STG.

Cholesterol (CHOL) (**Figure: 5.2.**), which has a $\lambda_{\max}$ below 210 nm, eluted at 7.63 min and was well resolved from the other analytes (STG, BSS and STN). Subsequently, it was also found that CHOL did not interfere with any of the constituents of the extracts of the formulations analyzed. Peak purity was confirmed by performing the peak purity test on the relevant peaks (Empower[®] package, Waters Corporation, Milford, MA, USA) shown in (**Figure 5.3.**).

Figure: 5.2.
Structure of Cholesterol



<i>Formula</i>	C ₂₇ H ₄₆ O
<i>Molecular Weight:</i>	386.65*
<i>$\lambda_{\max}$</i>	<210 nm
<i>pKa</i>	15.03±0.70*
<i>Melting point</i>	148° C

(*Values obtained using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris[®]
1994-2005 ACD/Labs)

Figure: 5.3.
UV Spectra and Chromatogram of Standard Solutions

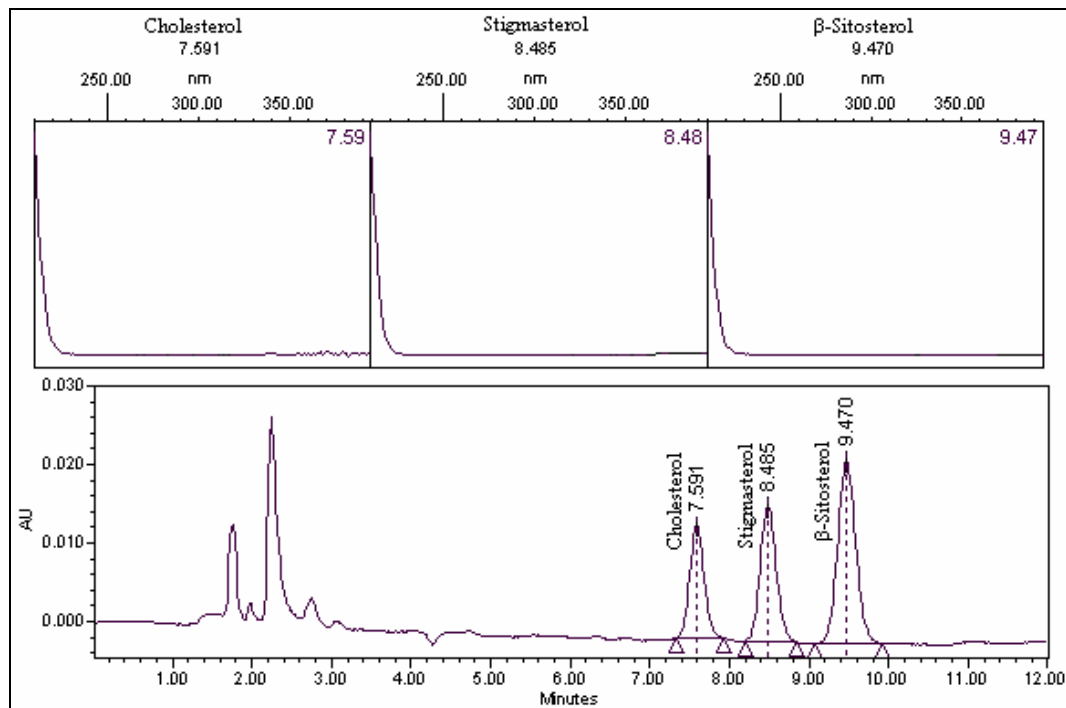


Figure: 5.3. Conditions:- Column: Phenomenex Luna® (5 μ m) C₈ (150 x 4.5 mm i.d); Column temperature: 23 $\pm$ 2° C;
 Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 μ l; λ max: 209 nm

5.3.4 Optimisation of ELSD Conditions

The most important parameters affecting the ELSD response are the nebulizer gas flow rate and the drift tube temperature. The gas flow rate influences the droplet size of the column effluent before evaporation occurs. Higher flow rates result in the formation of smaller aerosol droplets and less scattering of light with subsequent lower sensitivity but increased stability. On the other hand, lower gas flow rates are associated with larger droplet formation, augmented light scattering and therefore a higher response but baseline stability is often compromised. It is therefore pertinent to optimise this parameter to ensure that the optimal signal to noise ratio (S/N) is achieved [281].

The gas flow rate was therefore investigated over the range of 0.5-2.5 L/min in increments of 0.2 L/min. The sensitivity was highest at 0.7 L/min and nebuliser gas flow rates higher than 1.5 L/min decreased the sensitivity. As a result, 0.7 L/min was chosen for an optimal S/N ratio. Mobile phases with high polarity solvents require higher drift tube temperatures than those consisting of predominantly organic non-polar components. Similarly, optimal sensitivity of non-volatile solutes requires higher drift tube temperatures than semi-volatile solutes [281]. It was therefore predicted that a relatively high drift tube temperature would be required for adequate evaporation since the sterols are not highly volatile and moreover the MP consisted of polar solvents. The effect of temperature on sensitivity was determined over the range 50 to 110° C in increments of 10° C and thereafter by increments of 2.5° C for fine tuning.

A drift tube temperature of 100° C was finally selected. Baseline separation of all the marker compounds **Figure: 5.4.** was obtained by nebulisation of the effluent with a stream of pressurised air (0.7 L/min) and evaporated at 100 °C.

Figure: 5.4.

ELSD Chromatogram of Standard Solution

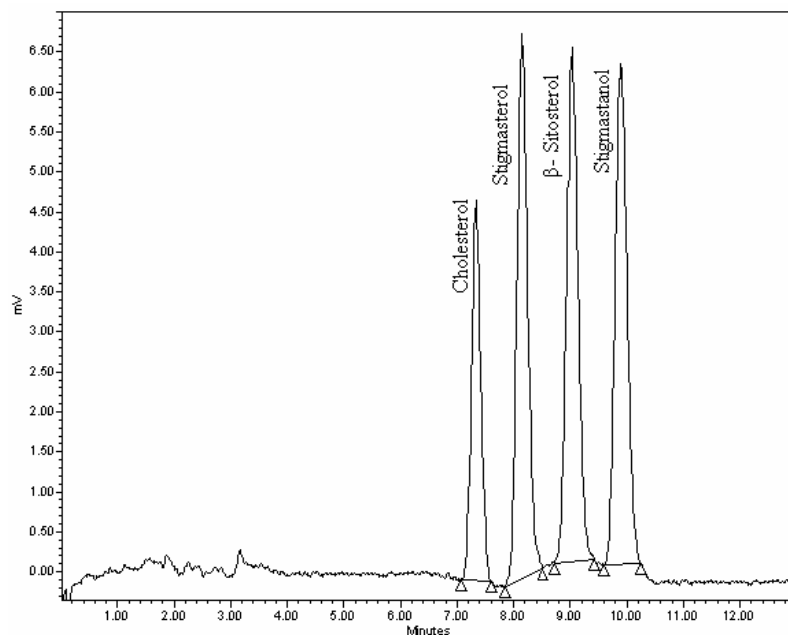


Figure: 5.4. Conditions:- Column: Phenomenex Luna® (5 µm) C₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C; Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Nebulisation air flow: 0.7 L/min; Evaporation chamber temperature 100 °C

5.3.5 Optimization of MS Conditions

The APCI-MS parameters for each compound (BSS, STG, STN and CHOL) were optimized by direct infusion of a separate solution of 50 µg/ml at 5 µl/min into the source. The instrument was adjusted to the following parameters for a good response: nebulizer temperature 400° C; source temperature 120° C; corona discharge, 2.5 µA; cone voltage 20 V; cone gas flow (Nitrogen 99.99%), 160 l/h; desolvation gas flow (Helium 98.6%) 500 l/h. HPLC-MS determinations were performed by operating the mass spectrometer in the positive ion (PI) mode. Full-scan mass spectra were acquired in the 300-450 Daltons range. The LC running conditions and column were the same as those used for HPLC-ELSD.

5.3.6 Preparation of Standard Solutions

Separate stock solutions of each of the reference standards were made by accurately weighing out appropriate amounts into volumetric flasks and filling to volume with methanol. Aliquots of each of the 3 solutions were mixed to prepare a new stock solution that was then serially diluted to yield five working standard solutions in the concentration range of 10-100 µg/ml. These solutions were prepared on three different days and were used for the linearity experiments. On each of these three days, two concentrations (30 and 60 µg/ml) of the mixed standard solutions were separately prepared for use as QC standards in the accuracy experiments. CHOL solution (500 µg/ml in methanol) was added to each final dilution as the internal standard (IS) in order to obtain a final CHOL concentration of 50 µg/ml.

5.3.7 Sample Preparation and Extraction

5.3.7.1 Hard Gelatin Capsules

Fifteen capsules of each product (A, B, M and N) were emptied and separately mixed with a mortar and pestle. Powder (50 mg) was weighed into a tared 10 ml volumetric flask and 8 ml of methanol was added. The mixture was ultrasonicated for 20 min and was allowed to cool for 5 min, thereafter the volume was made up with methanol. One millilitre of this extract was transferred into a 10 ml volumetric flask,

followed by addition of 1.0 ml of a 500 µg/ml CHOL solution (internal standard). The volume was made up to 10 ml with methanol and the solution was vortexed for 1 min before filtering through a PVDF (0.45 µm) membrane and analysed.

5.3.7.2 Solid Oral Dosage Forms (Tablets)

Fifteen tablets (Product O) were weighed and powdered using a mortar and pestle. The powder (50 mg) was extracted as mentioned above for capsules.

5.4 Results and Discussion

5.4.1 Sample Extraction Efficiency

Extraction efficiency using methanol was investigated by conducting two parallel studies in which 3 aliquots of separately powdered and well-mixed samples of Product N (representative sample of hard gelatin capsule) and Product O (representative sample of tablet) were used. The same procedure used for the extraction efficiency investigation of hypoxoside in Chapter 4 was adopted (Section 4.6.2.1).

The samples (~50) mg were accurately weighed out into tared 20 ml volumetric flasks and sonicated for various periods of time as previously described. Aliquots of these solutions were withdrawn at 10 min intervals and filtered through PVDF (0.45 µm) filters prior to analysis by HPLC. Product O did not show the presence of any of BSS, STG or STN even after sonication for 90 min. After 20 min, peak areas of BSS, STG and STN in the samples from Product N were similar to those found at the longer sonication times. The absence of the relevant sterols and STN in Product O could not be due to poor extraction efficiency since complete recovery of these compounds (BSS, STG and STN) were observed following spiking of the product with known quantities of the standard.

Ultrasonication for 20 min was therefore determined to be the minimum time required for optimum extraction of sterols and STN from each of the different formulations.

Different solvents were also studied for their extraction efficiency, such as n-hexane and chloroform. Although these were found to extract the relevant sterols and stanol with an acceptable extraction efficacy, they were not pursued due to their incompatibility with the mobile phase used.

5.4.2 Method Validation

5.4.2.1 Linearity

Data obtained from triplicate injections of the mixture of the working standard solutions over three days were analyzed. Analysis by HPLC-UV resulted in a linear calibration line with determination coefficient ($r^2 > 0.997 \pm 0.002$) over the concentration range 10-100 µg/ml. Analysis by HPLC-ELSD yielded a second order polynomial curve which after log transformation, provided a linear plot (**Table: 5.1.**)

Table: 5.1.

Linearity of STG, BSS and STN

	Constituents	$y = ax + b$	Determination	$Y = AX + B$	Determination
		Linear model	coefficient (r^2)	Log linear plot	coefficient (r^2)
		(PDA detector)	(n=3)	(ELSD detector)	(n=3)
Day-1	Stigmasterol	$y = 0.0245x + 0.0524$	0.9991	$Y = 1.13103X - 1.9355$	0.9987
	β-Sitosterol	$y = 0.213x + 0.0629$	0.9987	$Y = 1.2254X - 1.7701$	0.9947
	Stigmastanol	-	-	$Y = 1.3276X - 1.9183$	0.9989
Day-2	Stigmasterol	$y = 0.025x + 0.0673$	0.9995	$Y = 1.3296X - 1.9112$	0.9915
	β-Sitosterol	$y = 0.0218x + 0.0504$	0.9994	$Y = 1.3364X - 1.9129$	0.9940
	Stigmastanol	-	-	$Y = 1.3069X - 1.8270$	0.9963
Day-3	Stigmasterol	$y = 0.255x + 0.0310$	0.9995	$Y = 1.3398X - 1.9603$	0.9941
	β-Sitosterol	$y = 0.222x + 0.0375$	0.9993	$Y = 1.3318X - 1.9275$	0.9975
	Stigmastanol	-	-	$Y = 1.3529X - 1.9323$	0.9965

PDA, y = peak area ratio, x = concentration;

ELSD, Y = log peak area ratio, X = log concentration.

n = number of injections.

Each concentration (5, 10, 20, 40, 80, 100 µg /ml) was injected three times.

5.4.2.2 Limits of Detection (LOD) and Quantification (LOQ)

The LOD (signal/noise >3) and the LOQ (signal/noise >10) were determined by analysing diminishing concentrations of serial dilutions of a solution containing all the marker compounds. The LOD_{ELSD} and LOQ_{ELSD} were 2 and 5 µg/ml respectively and LOD_{UV} and LOQ_{UV} 5 and 7 µg/ml respectively for all the compounds. The uniformity in ELSD response is due to the uniform response factor towards all structurally similar compounds whereas UV detection relies on the response at a specific wavelength that is a function of the compounds absorbance.

5.4.2.3 Accuracy and Precision

The accuracy and precision of the method were determined by analysing three sets of samples at two different concentrations (30 and 60 µg/ml). The % accuracy was found to be between 97.92 and 104.75 using UV detection and between 97.84 and 102.67 by ELSD. The intra-day precision (% RSD) was between 0.83 and 2.98 by UV detection and between 1.29 and 1.89 using ELSD. The inter-day precision (% RSD; n= 3) was better than 3 % for UV detection and 2.03 % for ELSD (**Table: 5.2.**). It is thus seen that the results are similar using either of the detection systems.

Table: 5.2.

Accuracy and Precision of STG, BSS and STN

Compound and detector used	Day-1				Day-2				I	
	Actual weight (µg/ml)	Calculated weight (µg/ml)	% Accuracy	% RSD (n=3)	Actual weight (µg/ml)	Calculated weight (µg/ml)	% Accuracy	% RSD (n=3)	Actual weight (µg/ml)	Calculated weight (µg/ml)
Stigmasterol (PDA)	30.01	30.28	100.90	1.52	30.12	29.74	98.77	2.98	31.26	30.61
	60.86	61.29	100.72	0.83	61.22	61.11	99.82	2.86	61.32	60.60
Stigmasterol (ELSD)	30.01	30.10	100.29	1.71	30.12	30.85	102.45	1.48	31.26	31.69
	60.86	61.75	101.46	1.58	61.22	62.42	101.96	1.79	61.32	62.96
β-Sitosterol (PDA)	30.23	30.22	99.96	1.32	30.47	30.80	101.11	1.62	30.46	31.15
	60.46	61.22	101.25	2.58	60.94	60.85	99.85	1.51	60.92	63.81
β-Sitosterol (ELSD)	30.23	29.58	97.84	1.47	30.47	29.93	98.23	1.50	30.46	30.92
	60.46	60.60	100.23	1.77	60.94	61.70	101.24	1.79	60.92	60.86
Stigmastanol (ELSD)	30.96	31.14	100.58	1.29	30.56	30.92	101.18	1.68	30.40	30.27
	61.25	62.86	102.62	1.89	62.34	63.86	102.44	1.80	60.52	60.97

5.4.2.4 Sample Analysis

Standard and sample solutions were found to be stable on storage for 10 days at room temperature (~23°C). Product A was found to have the highest content of sterols, whereas sterols and stigmastanol were not detected in Product O in spite of the label claiming a unit content of “plant sterols and sterolins 100 mg”. The other samples (Products B, M and N) contained variable amounts of sterols and/or stigmastanol, non of which complied with label claims. Stigmasterol was only present in a quantifiable amount in Product M (50.7 % of the labelled claim/unit dosage form) whereas Product A was the only product which contained stigmastanol, albeit a relatively small amount at the level of the LOQ. The sterol content of all the products (except Product O) appeared to comprise of mainly BSS and their relative percentages of BSS was expressed as % of total sterol content. The assay values and the individual sterol and stanol content together with their respective % BSS of total sterol content of label claim as analysed by HPLC-ELSD along with the label claims of each of the commercial products are given in **Table: 5.3**. Chromatograms of the various products analyzed are shown in **Figure: 5.5**.

Table: 5.3.

Content of Commercial Products (HPLC-ELSD)

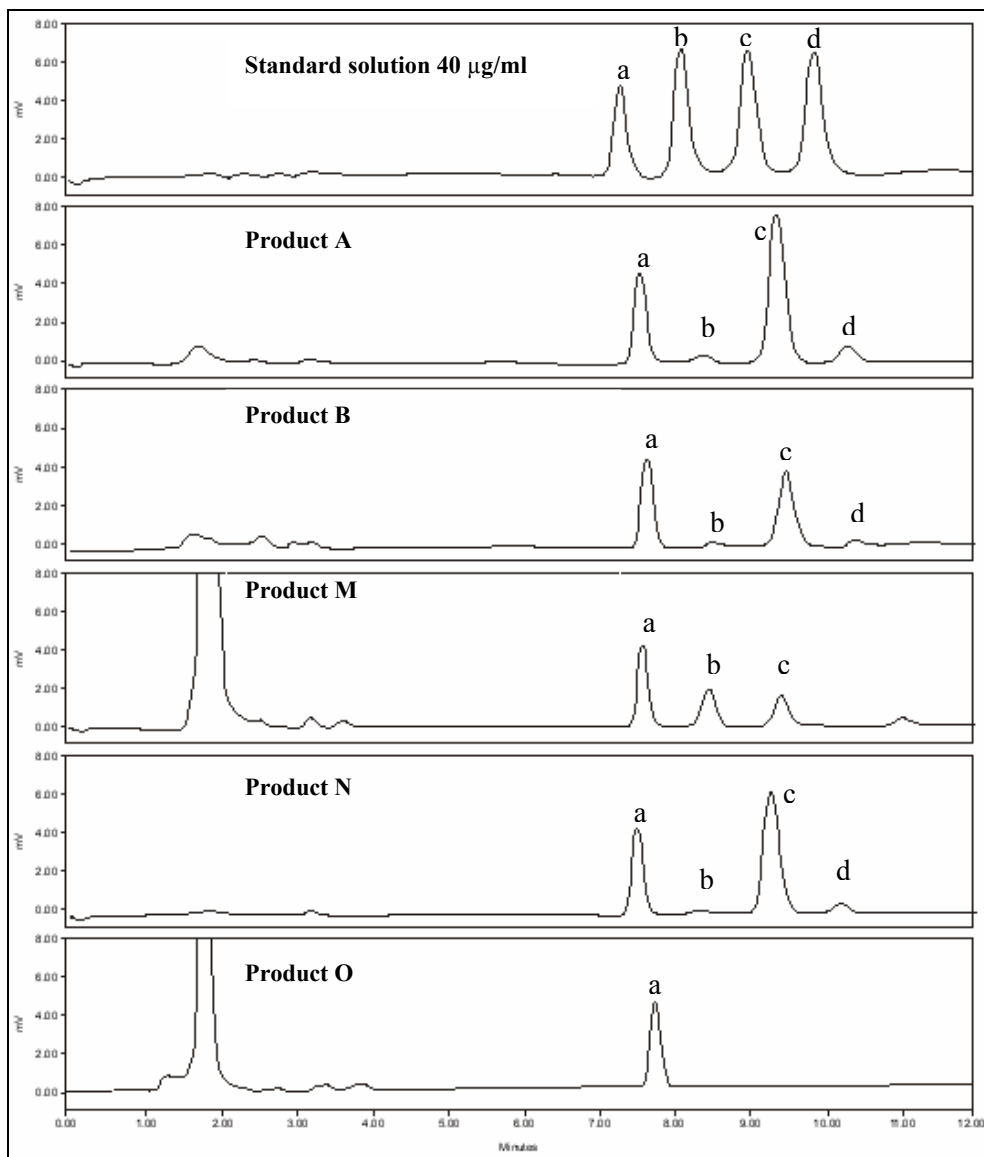
Name of Product	Average weight (mg) per unit	Labeled weight (mg) per unit	Label claim of sterol content (mg) per unit	Amount in mg $\pm$ SD /500 mg dosage form (n=3)			% BSS of total sterol content of label claim
				Stigmasterol	β -Sitosterol	Stigmastanol	
A	520	400	40	+	48.1 $\pm$ 0.4	7.4 $\pm$ 0.7	105.9 $\pm$ 0.7
B	625	500	30	-	22.5 $\pm$ 0.8	+	72.8 $\pm$ 2.5
M	516	400	25	15.2 $\pm$ 0.1	13.1 $\pm$ 0.7	-	94.4 $\pm$ 4.3
N	278	200	20	+	38.1 $\pm$ 0.6	+	86.7 $\pm$ 1.3
O	1073	1000	100	-	-	-	-

+ the amount is between LOD and LOQ (5 mg/500 mg dosage form);

- the amount is below LOD (2 mg/500 mg dosage form)

Figure: 5.5.

HPLC-ELSD Chromatogram of the Commercial Products



a= CHOL, b= STG, c= BSS, d= STN;

Figure: 5.5. Conditions:- Column: Phenomenex Luna[®] (5 µm) C₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C;
Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Nebulisation air flow: 0.7 L/min;
Evaporation chamber temperature 100 °C

5.4.2.5 Recovery

Product N was chosen for intra-day and inter-day recovery studies, since it contained all three marker compounds above the limit of detection (LOD). Each unit

of this product contained, on average, a content of 228 mg. This formulation was spiked with low, medium and high concentrations, i.e., 15, 30, 45 mg/500 mg dosage unit respectively of the three marker compounds (**Figures: 5.6. and 5.7.**) In other words, each unit was spiked to contain the equivalent of ~6.84, ~13.68 and ~20.52 mg each of the three markers. This was done on three different days and the analyses were performed in triplicate. Recovery values (**Table: 5.4.**) were between 95.29 % and 106.88 %. The intra and inter-day precision % RSD; n=3) were better than 5 %. Both these values were slightly worse than the corresponding values in the accuracy experiments which were based on the inclusion of QC samples. This suggests that the presence of excipients and/or other product components, not included in the QC samples but present in the spiked samples, may possibly have had an influence on the extraction efficiency with the resulting small effects on the comparative accuracy and precision data.

Figure: 5.6.

HPLC-UV Chromatograms of Product N Spiked with BSS, STG and STN

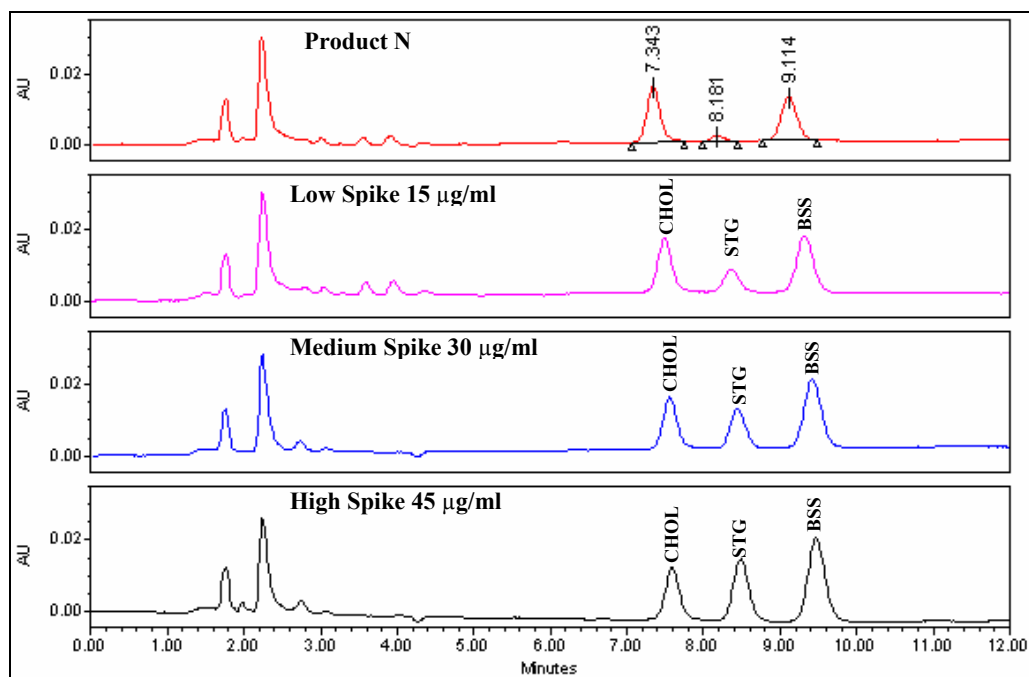


Figure: 5.6. Conditions:- Column: Phenomenex Luna® (5 µm) C₈ (150 x 4.5 mm i.d.); Column temperature: 23 ± 2° C; Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Detection λ: 209 nm.

Figure: 5.7.

HPLC-ELSD Chromatograms of Product N Spiked with BSS, STG and STN

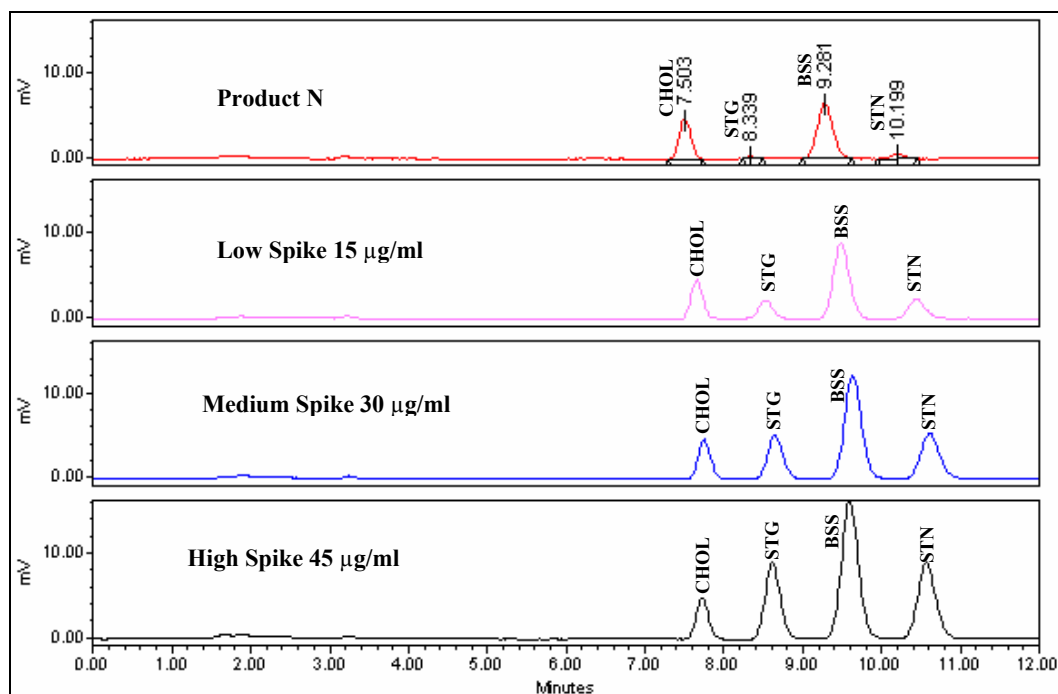


Figure: 5.7. Conditions:- Column: Phenomenex Luna® (5 µm) C₈ (150 x 4.5 mm i.d); Column temperature: 23 ± 2° C;
Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 µl; Nebulisation air flow: 0.7 L/min;
Evaporation chamber temperature 100 °C

Table: 5.4.

Recovery Studies of STG, BSS and STN

Constituent	Spiking level (mg/unit dosage form)	Intra-day % recovery S D (n=3)						Inter-day RSD (%, n=3)
		Day-1		Day-2		Day-3		
		% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	
Stigmasterol (ELSD)	6.85	95.29	3.85	96.86	2.66	96.53	0.44	0.86
	13.71	100.13	3.37	97.93	1.03	100.31	0.68	1.33
	20.57	98.24	4.25	98.12	2.29	98.4	3.03	0.14
β -Sitosterol (ELSD)	6.86	97.34	2.63	95.64	0.39	105.4	2.59	5.21
	13.73	99.52	3.70	95.72	0.87	105.03	3.60	4.67
	20.62	102.23	2.81	97.31	2.31	98.83	1.31	2.56
Stigmastanol (ELSD)	6.89	102.93	4.00	102.26	2.12	96.12	2.11	3.73
	13.78	103.66	2.28	102.81	1.61	100.83	0.44	1.41
	20.67	106.88	1.83	99.35	3.66	97.78	1.85	4.80

5.4.3 LC-MS Analysis

LC-MS studies were performed in full scan mode to ensure the specificity of the method for the separation of the sterols and stanol (STG, BSS and STN). Individual LC-MS scans of the reference standards were conducted for each compound and the retention times for CHOL, STG, BSS and STN were 8.93 min, 10.22 min, 11.14 min and 12.90 min respectively (**Figure: 5.8.**). The LC-MS-MS mode was used to yield diagnostic product ion mass spectra, which were characteristic of the structural moieties of analytes (**Figure: 5.9.**). The scanning ranges for each marker and their characteristic fragments ions are shown in **Table: 5.5.**

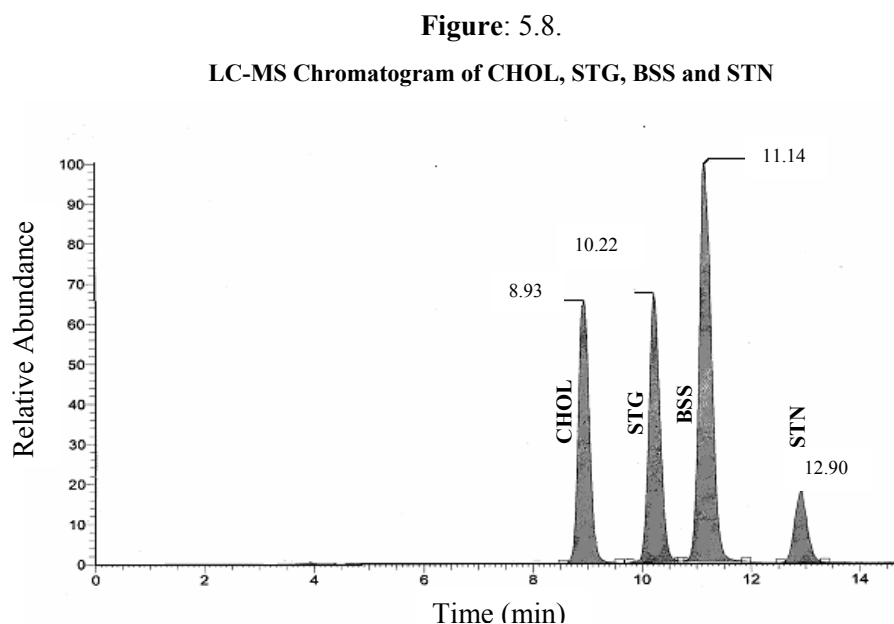


Figure: 5.8. Conditions:- Column: Phenomenex Luna® (5 μ m) C₈ (150 x 4.5 mm i.d.); Column temperature: 23 $\pm$ 2 $^{\circ}$ C; Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 μ l;

Figure: 5.9.

LC-MS-MS Fragmentation Pattern of CHOL, STG, BSS and STN

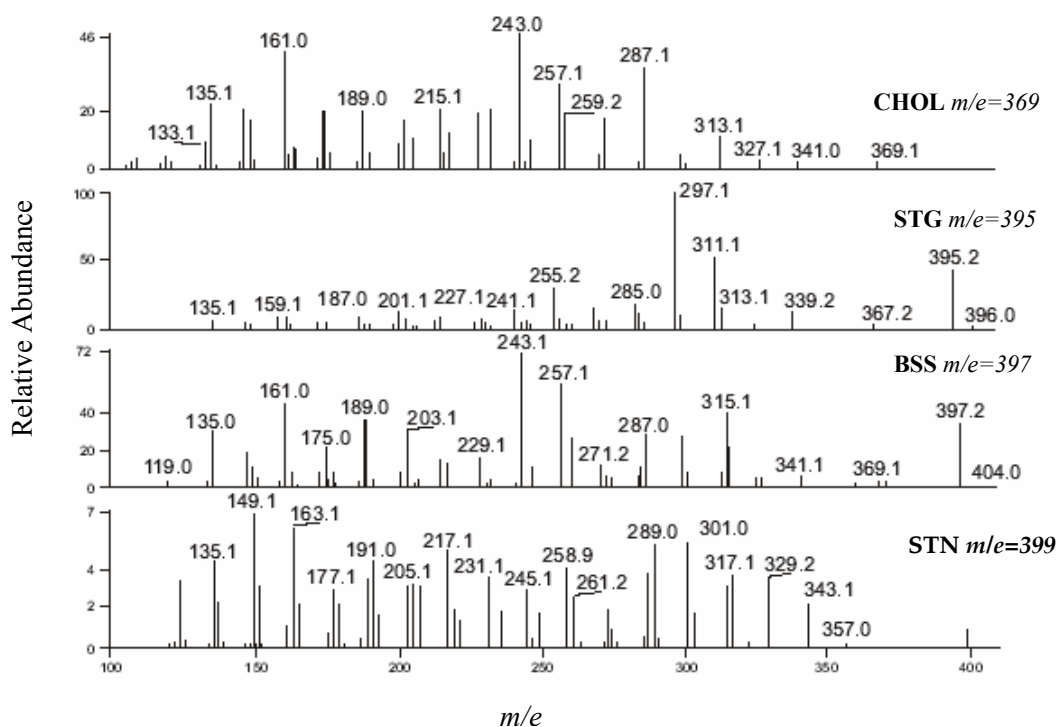


Table: 5.5.

Fragment Ions of CHOL, STG, BSS and STN

Compound	Observed Molecular ion (M^{-17})	Fragment ions (m/e) Scanning range (100-420)
Cholesterol (MW, 386.65)	369	135/161/189/203/243/257/287/369
Stigmasterol (MW, 412.69)	395	135/159/173/187/201/227/241/255/285/313/339/367/395
β -Sitosterol (MW, 414.81)	397	135/161/175/189/203/229/243/257/287/315/341/369/397
Stigmastanol (MW, 416.93)	399	135/163/177/191/205/231/245/259/289/317/343/399

MW= Molecular Weight

The fragmentation of all the compounds gave a common fragment ion m/e at 135 probably indicative of a fragment of the basic isoprene unit common to all these compounds. Moreover, the relevant parent ions were characteristic of their precursor molecules showing the loss of a hydroxyl group (M^{-17}) attached to the C-3 position. This was followed by the loss of an ethyl group ($-\text{CH}_2\text{CH}_3$) and formation of similar ions by both CHOL and BSS that indicated that the fragmentation of this group occurred at the C-24 position. Further fragmentation occurred through the loss of an ethyl group at C-25 and C-26 positions followed by the loss of ($-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_3$) at position C-23. BSS and STG, which possess one and two ($\text{C}=\text{C}$) bonds more than STN, respectively, showed a proportionate decrease (M^{-2} and M^{-4}) in all ions formed until the formation of the common ion at m/e of 135. These observations confirm the specificity of the method for the analysis of each of the compounds, STG, BSS and STN.

5.5 Conclusions

An uncomplicated specific, accurate and precise HPLC-ELSD method for the quantitative determination of STG, BSS and STN was developed and validated. Baseline separation was achieved within a short analysis time using a Phenomenex Luna[®] C₈ (5 μm), 150 x 4.6 mm i.d. column. ELSD was necessary for the detection of stigmastanol, which is transparent in UV region. It is relatively inexpensive and easily operable compared to GC and MS detection. This method was successfully applied for the analysis of several commercially available solid oral dosage forms. The analyses showed that the sterol and stanol contents were quite different between the products analyzed. These results therefore indicate that suitable QC measures need to be implemented to ensure consistent quality and efficacy of marketed products.

The developed LC-MS method has the potential for use in the routine analysis of commercial products of AP containing sterols and stanols. This method was however not used for QC purposes considering the higher capital outlay and running costs, which could be an impediment in applying it for routine use on an industrial scale.

Chapter 6

Capillary Electrophoresis

6.1 Introduction

Capillary electrophoresis (CE) is a relatively new technique that started to gain popularity over the last 2 decades due to its high separation efficiency, short analysis times, wide application and low cost of operation. In CE, under the influence of an applied electric field, separation of chemical components occurs on the basis of differential migration of charged species. The narrow bore capillary (usually 50 -100 μm i.d.) is filled with buffer and very small sample volumes are usually injected onto the anodic end. An electric field is then applied to sweep the charged species of the sample solution towards the cathodic end by a phenomenon called electroosmotic flow (EOF).

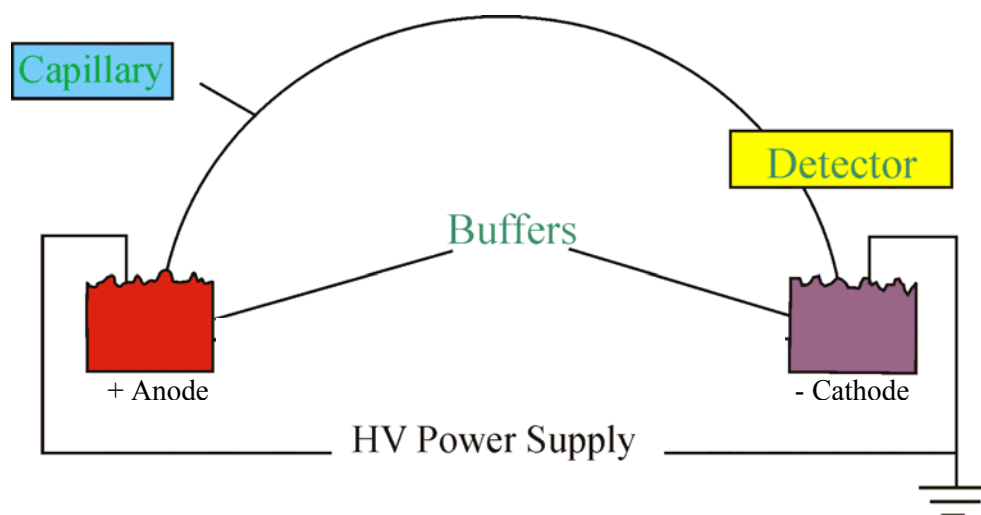
The EOF is caused by the application of an electric field on the counterions (predominantly cations) accumulated on the negatively charged silanol groups of the capillary wall, which form an electrical double layer [288, 289]. In this process, as the solvated cations migrate towards the cathode, they carry the bulk of the buffer solution with them. This facilitates the separation of the chemical components based on their characteristic electrophoretic mobilities which are dependent on the solute's charge and size [288, 289]. The positively charged species that are small in size with a high charge-to-mass ratio are the first to reach the detector at the cathodic end. This is followed by cations with lower ratios (larger size) and then neutral compounds. Next in the sequence are anions with large charge to mass ratio and finally followed by those with small ratios which are the last to be detected since they are the last to be dragged to the cathode by the net bulk flow of the EOF [269].

A schematic representation of a simplified CE system is shown in **Figure: 6.1**. This method is particularly suited for samples such as botanical products that may contain multiple chemical constituents usually requiring lengthy analysis time and complex separation methods [290]. With simple high pressure washing of the

capillary with water in between consecutive injections, the problems associated with the adsorption of highly hydrophobic compounds or unknown natural product constituents can be eliminated, which is an advantage compared with HPLC [291]. This analytical method requires very small volumes of sample (5-10 μ l) injected into the capillary [292]. Such small volumes result in reduced detection sensitivity, thereby necessitating samples which are more concentrated (2-5 times) to generate a similar response usually achieved by HPLC. This may pose problems when analyzing poorly soluble drugs [293, 294].

Recently, various instrumental and operational adaptations have been attempted to overcome limitations arising from the narrow optical path length provided by the capillary tube placed in the detector. Use of bubble cells and Z-cells to increase detector pathlength, sample pre-concentration and sample stacking techniques are a few of the techniques used [295].

Figure: 6.1.
Schematic Diagram of a CE System

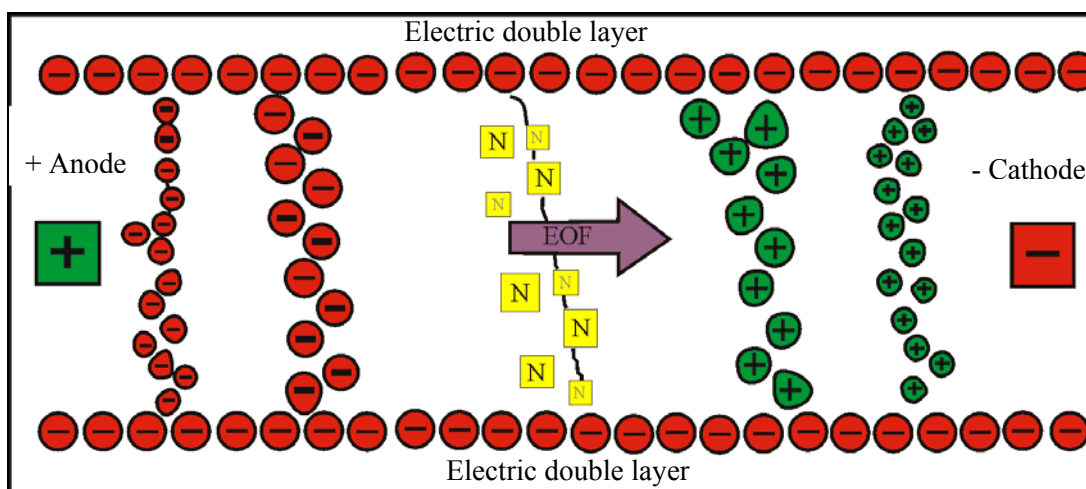


One of the main reasons for the success of CE as an analytical instrument is its various separation modes which work on vastly different operative and separation characteristics. These include capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE),

capillary isotachopheresis (CITP) and capillary isoelectric focusing (CIEF). These various methods facilitate the analysis of distinctly different classes of chemical compounds using the same instrument [288].

CZE is the most frequently used mode of CE due to its simplicity and versatility and has a wide application range which includes the analysis of peptides, amino acids and enantiomers. A simple buffer solution with a suitable pH value is used as an electrolyte and separation takes place in an uncoated polyimide fused-silica column with an internal diameter varying from 50-100 μm . As shown in **Figure 6.2.**, after the sample introduction at the anodic end, charged analyte species separate into spatially discrete zones based on their electrophoretic mobility when an electric potential is applied.

Figure: 6.2.
Capillary Zone Electrophoresis (CZE) Separation Process



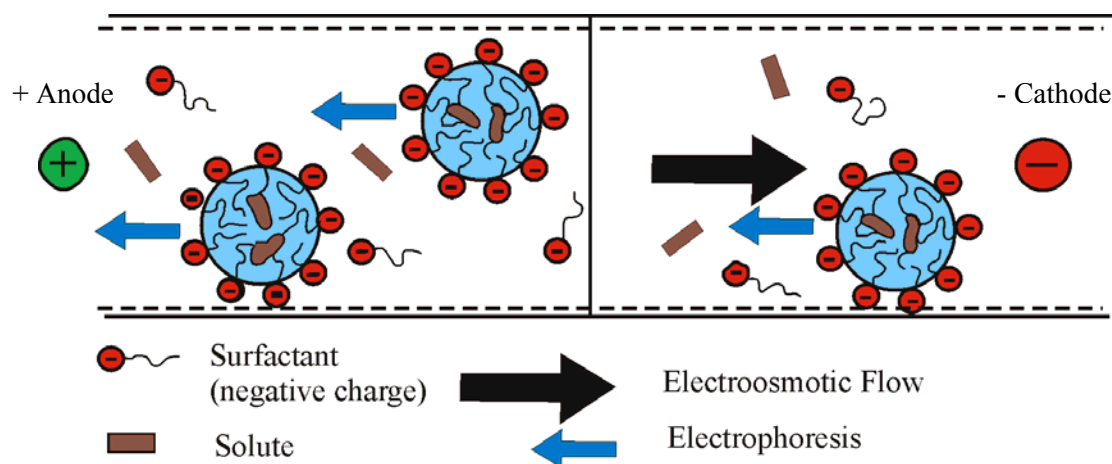
The change in charge-to-mass ratios of ionisable compounds can be easily manipulated by changing the electrolyte pH thereby influencing their electrophoretic mobilities for subsequent separation [288]. Neutral compounds (N) generally co-elute with the EOF since they are not capable of migrating on the basis of charge. In order to separate such neutral and non-ionisable molecules another method, known as micellar electrokinetic chromatography (MEKC) can be employed [288].

The MEKC method, which is a hybrid of both electrophoresis and chromatography, has the capability to separate both neutral and charged solutes within

a single run. In this method an anionic or cationic surfactant is incorporated into the buffer solution at a concentration above the critical micelle concentration (CMC) to form micelles. The neutral solutes become embedded in these micelles and are compelled to migrate at a speed between the EOF and micellar velocity (**Figure: 6.3.**) and this detection time frame is also known as the migration time window [289].

Figure: 6.3.

Micellar Electrokinetic Chromatography (MEKC)



Capillary gel electrophoresis (CGE) involves the separation of macromolecules such as proteins and nucleic acids by electrophoresis of the compounds through a “molecular sieve” consisting of a suitable polymer network. The separation of the analytes is based on the size as they migrate through the gel-filled capillary under the influence of an applied potential, with larger solutes experiencing more hindrance than smaller ones. This technique is suited for analytes whose mass-to-charge ratios change with an increase in size, as seen in DNA and SDS-saturated proteins that have similar mobilities in normal buffer systems [288].

Capillary isotachopheresis (CITP) is often described as a “moving boundary” electrophoretic technique in which two buffers are employed to create leading and terminating electrolytes with higher and lower mobilities than the analyte, respectively. The sample zone is sandwiched between the electrolyte buffer systems and when the electric field is applied, the analytes separate into discrete zones with

each band migrating at the same velocity as determined by the leading electrolyte. However, this analytical method has limited application since simultaneous determination of cations and anions is not possible and the selection of an appropriate discontinuous buffer system at the desired pH is often very tedious [288, 289].

Capillary isoelectric focusing (CIEF) is a “high resolution” technique that has been designed to separate proteins and peptides based on their isoelectric points or isoelectric (pI) values. In this method, ampholytes (zwitterions) are used to construct a pH gradient within the capillary with an acidic solution at the anode and a basic solution at the cathode. As soon as the capillary is filled with a mixture of ampholytes and solutes, an electric field is applied which propels the charged ampholytes and amphoteric proteins to migrate until they reach a region where they are uncharged (at their respective pI values) and remain stationary or “focused”. High resolution is obtained since migration of a solute into a zone of differing pH would cause a disruption in the neutral charge and the solute rapidly migrates back to into its pI zone. The completion of amphoteric focusing is indicated by a break in current. For the mobilization of the zones towards the detector, either external pressure is applied or salt is added to either of the reservoirs. CIEF has been adapted for automated instrumental use [288].

6.2 Background and Objectives

Although several reports for the determination of hypoxoside using HPLC have been published, very little information has appeared in the literature to describe methods, which can be readily used for the quantitative determination of hypoxoside and its subsequent application for QC of AP products [99, 100, 134, 145, 247, 248]. As discussed in Chapter 4 (Section 4.2), most of the previously published HPLC methods were not validated and no quantitative data have been provided.

CZE is finding increasing use for the QC of therapeutic products [296] and many publications have been cited to support this method for quantitative analysis [297-301]. Amongst the various advantages of CZE, the most notable include the avoidance of the use of relatively toxic and expensive HPLC grade solvents, high separation efficiency, improved selectivity, low operational costs and speed of analysis [302-308].

The main objective of this chapter was to develop a rapid, accurate and precise CZE method which is reproducible, stability-indicating [277, 309, 310] and suitable for the assay and QC of commercial formulations containing AP.

6.3 Stability Studies

The procedures used to investigate the forced degradation of hypoxoside for the development of a stability-indicating assay were the same as those discussed in Chapter 4 (Section: 4.3).

6.4 Experimental

6.4.1 Instrumentation

A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes and all weighings of chemicals and reference standards were performed on a Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland). A Crison GLP21 pH Meter (Crison, Barcelona, Spain) was used to measure and adjust relevant pH values.

Analyses were carried out on a Prince CE system (Prince Technologies, Emmen, Netherlands) using a 50 μm i.d. x 363 μm o.d. uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA), total length 71 cm and effective length 58 cm. The length of the capillary was kept as short as possible in order to avoid possible siphoning effects and increased joule heating. The complete analysis was performed at ambient temperature and detection was at 260 nm using a linear UV/Vis Model 200 ultraviolet detector (Linear Instruments Corp., Reno, NV, USA). The output of this detector was interfaced, via a SATIN[®] box, to a Waters Empower[®] Chromatographic Manager (Waters Chromatography Division, Milford, MA, USA).

6.4.2 Reagents and Chemicals

Sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{ H}_2\text{O}$), sodium hydroxide pellets (Rochelle Chemicals, Port Elizabeth, South Africa), hydrogen peroxide solution (30%, v/v) (Riedel-de-Haen, Johannesburg, South Africa), hydrochloric acid (Merck KGa A, Darmstadt, Germany), 85% (v/v) ortho-phosphoric acid (Merck, Midrand, South Africa) were of analytical reagent grade and were used without additional purification. Hypoxoside, used as a reference standard, was isolated, purified and characterized as previously described in Chapter 3. Sulphafurazole (98.4%) was gratefully received as a gift from Orchid chemicals, (Chennai, India). Water was purified in a Milli-Q[®] System (Millipore, Bedford, USA) and Millex HV[®] Hydrophilic (PVDF, 0.45 μm) filters were purchased from the same source. Two different commercial preparations containing AP (Products-D I and G) were purchased from a local pharmacy in Grahamstown, South Africa.

6.4.3 Method Development

6.4.3.1 Capillary Conditioning

Each new capillary was systematically conditioned by washing with 0.1 M sodium hydroxide for 30 min, 1 M NaOH for 1 hr, and deionised water for 15 min. Between each consecutive injection, the capillary was washed with water for 1 min, 0.1 M NaOH for 2 min and running buffer for 4 min to ensure optimal charge density on the capillary wall. For overnight storage, the capillary was sequentially washed with water for 5 min, 0.1 M NaOH for 10 min, 1 M sodium hydroxide for 30 min and finally with water for 10 min. External pressure of 300 KPa was applied from a nitrogen cylinder to facilitate washing the capillary. To ensure reproducibility and prevent effects of pH changes in the background electrolyte due to electrolysis, the electrolyte at the anode and cathode was replaced after every injection

6.4.3.2 Initial Conditions

Preliminary systematic studies were carried out at different pH, ionic strength and separation voltage. Sample introduction into the capillary was effected by hydrodynamic injection at 100 mbar for 0.1 min, which was adequate to load the necessary amount of analyte. Product-D I, was selected for method development since prior analysis by HPLC Chapter 4 (Section 3.1) indicated that this product contained several unknown peaks in addition to hypoxoside, and as such would provide a complex matrix to challenge the selectivity of the method.

6.4.3.3 Optimization of Running Electrolyte

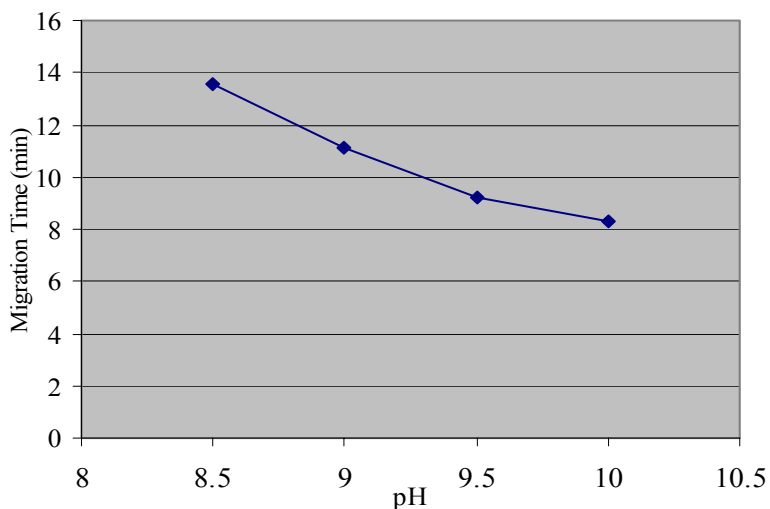
Preliminary systematic studies were carried out at acidic (low) pHs of 25 mM, 50 mM and 100 mM phosphate buffer (pH range 2-5) using the conditions previously described at 10 and 15 kVa. Hypoxoside peaks were not seen under these conditions. Hence a basic pH range of 7–10 was investigated using borate buffer, which similar to phosphates, is transparent in the UV wavelength region and does not contribute to baseline noise, allowing the use of low UV wavelengths such as 190 –200 nm to monitor solutes [305, 311]. An electrolyte containing borate buffer at a pH of 9.2 was used and hypoxoside was detected at 260 nm under the conditions previously described above.

6.4.3.4 Optimization of pH

The electrophoretic migration of hypoxoside was studied at pH values between 8.5 and 10.0 since this pH range is compatible with the optimal buffering capacity of sodium borate (**Figure: 6.4.**). The pH adjustments were made with hydrochloric acid and sodium hydroxide solutions. At pH 10, hypoxoside migrated early along with components of the formulation which interfered with the detection whereas at pH 8.5, hypoxoside migrated at a much slower velocity and was detected at a later time. A pH of 9.2 was subsequently chosen since under these conditions,

hypoxoside could be resolved from the excipients and other interfering components in Product-D I.

Figure: 6.4.
Effect of pH on the Migration Time of Hypoxoside



6.4.3.5 Optimization of Ionic Strength

The effect of the ionic strength of the running buffer on hypoxoside was investigated at concentrations of 15, 25 and 50 mM sodium borate, respectively. At an ionic strength of 50 mM, the electrophoretic mobility of hypoxoside decreased (**Figure: 6.5.**) concomitantly with an increase in current (45 μ A) presumably due to the joule heating effect associated with an increase in temperature. At a lower ionic strength of 15 mM, even though the current and the temperature were comparatively low (16 μ A), a slight distortion in the peak shape of hypoxoside was observed (**Figure: 6.6.**). Hence an optimum 25 mM buffer, which gave a better peak shape and a relatively low current, was chosen for the analysis of hypoxoside.

Figure: 6.5.
Effect of Ionic Strength on the Migration Time of Hypoxoside

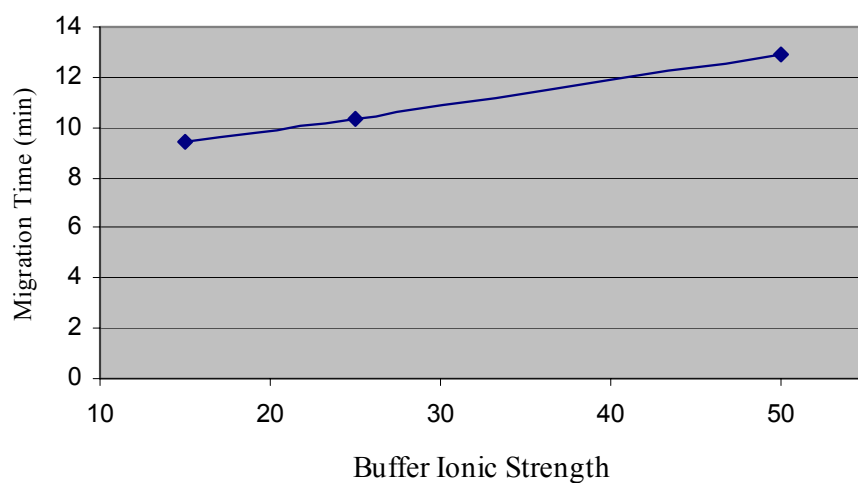


Figure: 6.6.
Electropherogram of Hypoxoside using 15 mM Borate Buffer

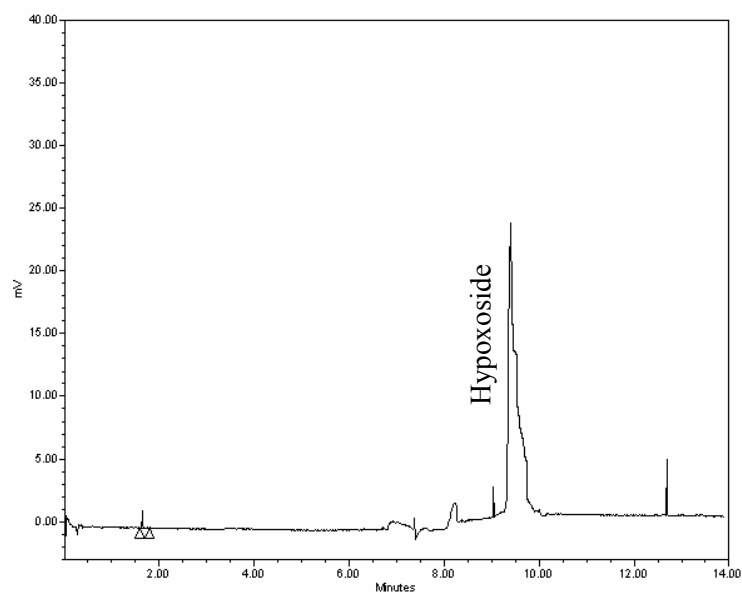


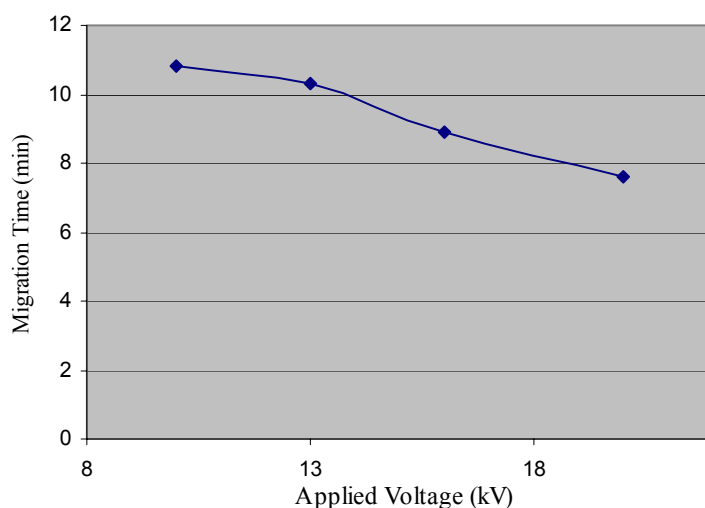
Figure: 6.6. Conditions:- Capillary: 50.0 μm i.d; Total length = 71.0 cm; Effective length = 58.0 cm; Sample buffer: 50:50 methanol: water; Running buffer: 25 mM Sodium tetra borate, (pH 9.2); Hydrodynamic injection: 100 mbar for 0.1 minutes; Voltage: +13 kV

6.4.3.6 Optimization of Applied Voltage

The applied voltage had a proportional effect on the electrophoretic migration of hypoxoside (**Figure: 6.7**). The effect of voltage was studied with between the voltage range of 10 to 20 kV and 13 kV was ultimately chosen since it facilitated the migration of hypoxoside at a satisfactory time with a low current of 26 μ A. Moreover, this voltage was able to resolve the hypoxoside peak from other excipient peaks.

Figure: 6.7.

Effect of Applied Voltage on the Migration Time of Hypoxoside



6.4.3.7 Selection of Internal Standard

The use of an internal standard (IS) in an assay procedure helps to compensate for possible errors, in particular, changes in volume that may arise during the extraction procedure or sample loading.

As mentioned in the Chapter 4 (Section: 4.5.1), some sulphonamides were chosen for investigation since they are soluble in the preferred solvent, methanol, and have good UV absorption at 260 nm. Several of these sulphonamides were dissolved

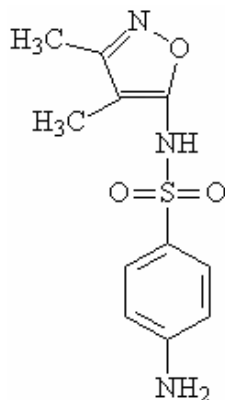
in methanol to yield solutions with a concentration of approximately 100 µg/ml and analysed under the previously described CZE conditions for hypoxide.

All the sulphonamides, sulphadimethoxine, sulphanilamide, sulphafurazole, sulphamethoxazole and sulphadiazine migrated at times compatible with the analysis of hypoxoside.

An aliquot of about 0.5 ml of each of these sulphonamide sample solutions were then mixed separately with 0.5 ml of a hypoxoside stock solution (100 µg/ml) and hydrodynamically injected onto the capillary. Whereas sulphadimethoxine, sulphanilamide and sulphafurazole were resolved from hypoxoside and other components of Product-D I, sulphamerazole and sulphadiazine interfered with some of the components. Sulphafurazole (**Figure: 6.8.**) was ultimately chosen as internal standard since it migrated after hypoxoside, was well-resolved and yielded good absorbivity at comparatively low concentrations.

Figure: 6.8.

Chemical Structure of Sulphafurazole



<i>Formula</i>	C ₁₁ H ₁₃ N ₃ O ₃ S
<i>Molecular Weight</i>	267.30*
<i>Pka</i>	4.83±0.50, 1.52±0.10*
<i>Melting point</i>	183 °C

(*Values obtained using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris®
1994-2005 ACD/Labs)

6.4.3.8 Summary

Data obtained from the above studies were used to optimize the conditions of the CZE assay. A running buffer of 25 mM borate at a pH of 9.2 was chosen and samples were hydrodynamically loaded onto the capillary at 100 mbar for 0.1 min. A typical current level of about 26 μ A was observed throughout the study when a constant voltage of 13 kV was applied to obtain the desirable separation of hypoxoside and sulphafurazole (SF) at $\sim$ 10.3 min and $\sim$ 11.9 min, respectively (Figure: 6.9.).

Figure: 6.9.

Electropherogram of Hypoxoside and Sulphafurazole (SF)

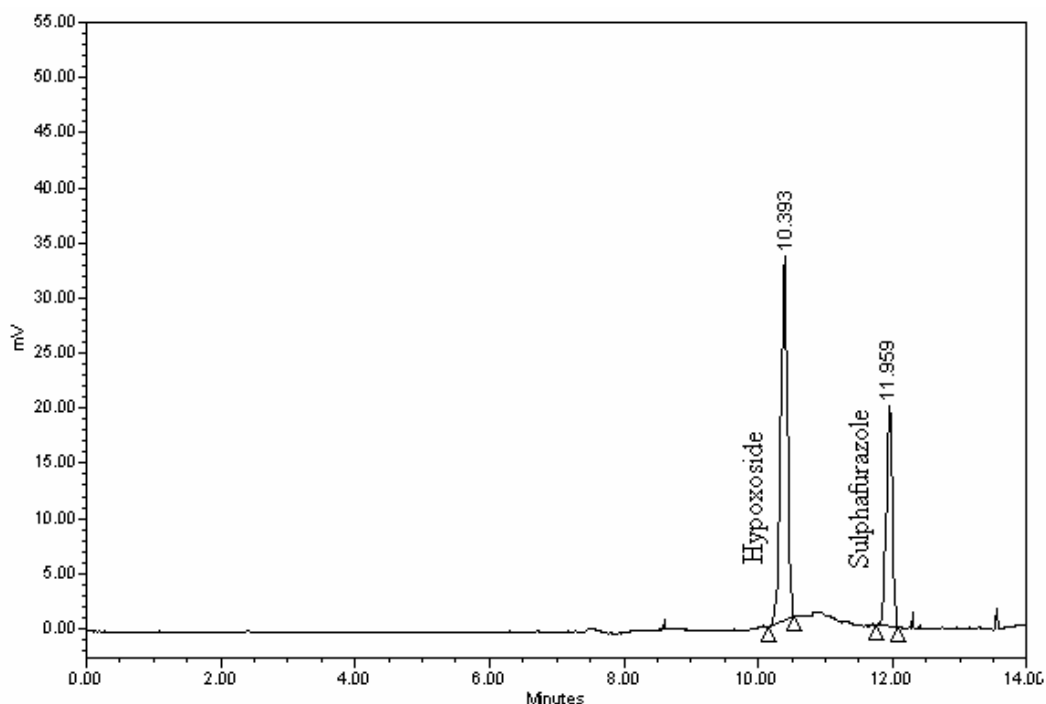


Figure: 6.10. Conditions:- Capillary: 50.0 μ m i.d; Total length = 71.0 cm; Effective length = 58.0 cm; Sample buffer: 50:50 methanol: water; Running buffer: 25 mM Sodium tetra borate, (pH 9.2); Hydrodynamic injection: 100 mbar for 0.1 minutes; Voltage: +13 kV

6.4.4 Method Validation

6.4.4.1 Preparation of Reference Standards

Methanolic solutions of hypoxoside (1 mg/ml) and the internal standard, sulphafurazole (SF) (1 mg/ml) were prepared and their stability was assessed by storing diluted solutions (100 µg/ml) of each in a refrigerator at 4° C and for 10 days at room temperature (~23°C). All solutions were found to be stable under the relevant conditions of storage.

6.4.5 Working Standards

On each of five days, standard solutions containing hypoxoside and internal standard, SF were prepared from the stock solutions. Working standard solutions comprising a set of five calibrators in the concentration range of 5-60 µg/ml of hypoxoside containing 20 µg/ml Sulphafurazole as IS were prepared daily by appropriate dilution with methanol. Additional samples (30 and 50 µg/ml) for use as QC standards were freshly prepared on each day of analysis.

6.4.6 Preparation of Sample Solutions

Two different commercially available AP products formulated as hard gelatin capsules (Product-D I) and tablets (Product-G) were chosen. The content of ten of each the formulations were separately mixed well with a pestle in a mortar and aliquots of the powder mix (~50 mg) accurately weighed into a 10 ml volumetric flask. The flasks were made up to volume with methanol and sonicated (Cole-Parmer Instrument Company, Chicago, Illinois, USA.) for 20 min. After allowing them to cool for 5 min, 1 ml of each filtered solution was transferred to a 10 ml volumetric flask, 2.0 ml of the IS solution (100 µg/ml) was added and their volumes were made up with methanol. The solutions were filtered using PVDF filters (0.45 µm) prior to analysis.

6.4.7 Stress Testing of Hypoxoside - Forced Degradation Studies

Hypoxoside was stressed under various conditions that have been discussed in Chapter 4 (Section: 4.3), in order to force degradation. This was done to ensure that the analytical method is stability-indicating. A stock solution of hypoxoside (1 mg/ml) was prepared in methanol and further dilutions were made to prepare test solutions, which were subjected to various stress conditions.

6.5 Results and Discussion

6.5.1 Method Validation

6.5.1.1 Linearity

Calibration curves constructed for hypoxoside were linear over the entire concentration range of 5 to 60 µg/ml. Two sets of capillaries were used to show that the method was robust and repeatable under the specific conditions of analysis. Calibration curves were constructed by plotting the peak area ratio of hypoxoside/IS versus concentration of hypoxoside on each of five separate days and were linear with determination coefficients greater than 0.99 on all five days of analysis (**Table: 6.1.**).

Table: 6.1.
Linearity of Hypoxoside

Day	$y=ax + b$ Linear model	Determination coefficient (r^2) (n=3)	Concentration range (µg/ml)
Day-1 ^a	$y = 0.0363x - 0.0241$	$r^2 = 0.9996$	5, 10, 20, 40, 60 µg/ml
Day-2 ^a	$y = 0.0377x - 0.0493$	$r^2 = 0.9991$	
Day-3 ^a	$y = 0.0371x - 0.0240$	$r^2 = 0.9991$	
Day-4 ^b	$y = 0.0361x - 0.0305$	$r^2 = 0.9983$	
Day-5 ^b	$y = 0.0346x - 0.0011$	$r^2 = 0.9993$	

a = Capillary 1

b = Capillary 2

n = number of samples

6.5.1.2 Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOD (signal/noise >3) and LOQ (signal/noise >10) were determined by analyzing serial dilutions of known concentrations of hypoxoside standard solutions. The UV spectrum of both hypoxoside and the IS showed good absorbance at 260 nm, which was used as the detection wavelength throughout the analyses. The LOD and LOQ were 0.5 and 2 µg/ml respectively.

6.5.1.3 Accuracy and Precision

Accuracy and precision studies were performed and assessed within and between runs using two sets of QC samples, which were separately prepared on each of the three days of analyses as previously described. The results were obtained by interpolation of replicate (n=3) peak area ratios from a calibration curve prepared as described previously. The accuracy of the method was found to be between 98.35 - 103.43 % and the % RSD for inter and intraday precision was better than 5% (**Table: 6.2.**).

6.5.1.4 Recovery

The recovery of hypoxoside was evaluated to assess the extraction efficiency of the method. Product-D I, which contained, on average, 18.83 mg hypoxoside/dosage unit (capsule) was chosen for the intra-day and inter-day recovery studies. The formulation was spiked with low, medium and high concentrations equivalent to 13.67, 27.62 and 41.57 mg of hypoxoside/dosage unit, respectively (**Figure: 6.10.**). This was done on three different days and the analyses were performed in triplicate. Recovery values of hypoxoside were between 98.79 % and 104.29 % and the inter- and intra-day precision, yielded in RSDs better than 3 % and 5.2%, respectively (**Table: 6.3.**) .

Table: 6.2.
Accuracy and Precision of Hypoxoside

Day-1				Day-2				Day-3	
Actual concentration (µg/ml)	Calculated concentration (µg/ml)	% Accuracy	% RSD (n=3)	Actual concentration (µg/ml)	Calculated concentration (µg/ml)	% Accuracy	% RSD (n=3)	Actual concentration (µg/ml)	Calculated concentration (µg/ml)
30.24	30.37	100.43	3.517	30.18	29.76	98.60	0.76	30.33	30.2
50.40	49.57	98.35	1.171	50.45	50.46	100.01	0.38	50.55	49.7

Table: 6.3.
Recovery of Hypoxoside

Sample	Day-1				Day-2				Hypoxoside content (µg/500 µg/ml)
	Hypoxoside content (µg/500 µg/ml)	Observed content (µg/500 µg/ml)	% Recovery	% RSD (n=3)	Hypoxoside content (µg/500 µg/ml)	Observed content (µg/500 µg/ml)	% Recovery	% RSD (n=3)	
Product-D I	13.44			4.01	14.11			2.22	13.96
Low	(+10.11)23.55	23.99	104.29	4.06	(+10.11)24.21	24.32	101.14	5.19	(+10.11)24.07
Med	(+20.22)33.66	34.12	102.21	0.71	(+20.22)34.32	34.08	98.79	4.94	(+20.22)34.18
High	(+30.33)43.77	44.43	102.14	4.43	(+30.33)44.43	44.08	98.82	2.33	(+30.33)44.29

n= number of samples, Stock solution = 1.011 mg/ml.

Figure: 6.10.
Recovery Studies on Product-D I

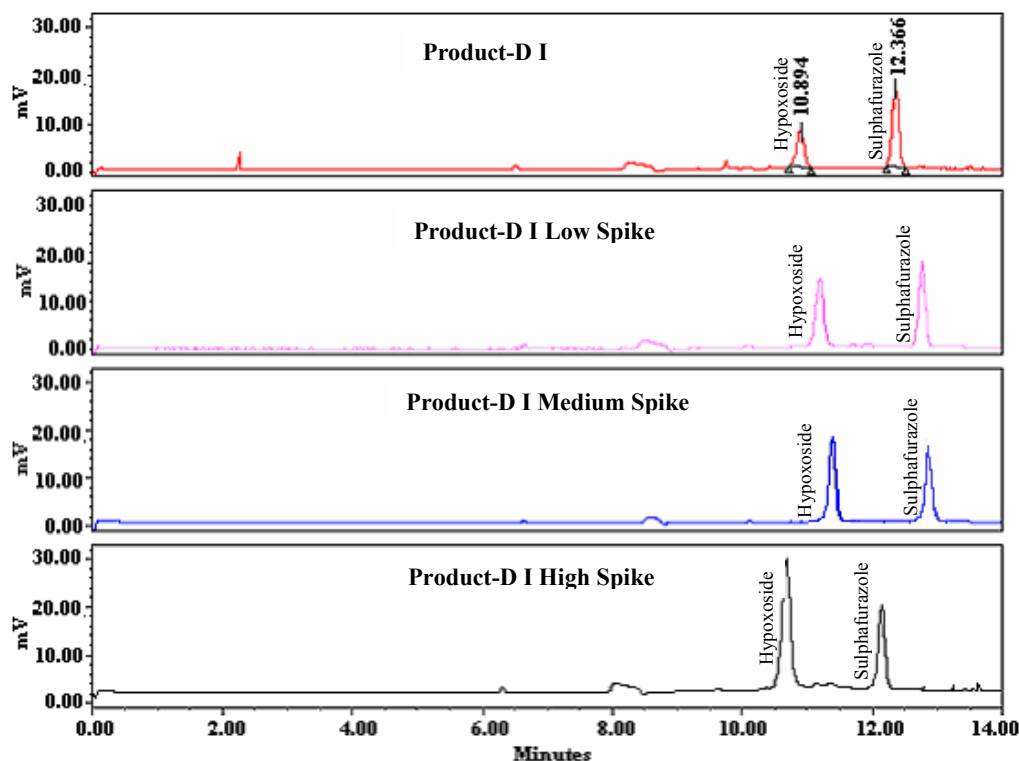


Figure: 6.10. Conditions:- Capillary: 50.0 μm i.d; Total length = 71.0 cm; Effective length = 58.0 cm; Sample buffer: 50:50 methanol: water; Running buffer: 25 mM Sodium tetra borate, (pH 9.2); Hydrodynamic injection: 100 mbar for 0.1 minutes; Voltage: +13 kV

6.5.2 Analysis of Samples

6.5.2.1 Extraction Efficiency

Extraction efficiency of the formulations used for the CZE assay method was investigated earlier when analyses of these samples were performed using HPLC as discussed in Chapter 4 (Section: 4.6.2.1). There was no need to modify the above method since the method was found to be reproducible without any incompatibilities.

6.5.2.2 Analysis of Commercial Products

The assay values and the individual label claims of each of the analyzed commercial products are given in **Table: 6.4**. The hypoxoside content of Product-D I (18.83 mg/dosage unit) and Product-G (11.24 mg/dosage unit) were determined. The

intraday and interday % RSD with each of the capillaries used were better than 4.01% and 2.66% respectively. It should be noted that the labels claim the presence of AP in one or other form and not hypoxoside *per se*.

Comparison of the assay results by HPLC for these products are in good agreement where the percentage hypoxoside content of each of the Products-D I and G were found to be 2.89 and 1.26 compared to 2.76 and 1.06 respectively, found by the CZE assay, expressed as the average dry weight of hypoxoside (g/100 g).

Table: 6.4.
Assay of Formulations

Sample name	Average weight of the formulation in grams	Labeled content/ dosage unit	Day	% dry wt of Hypoxoside (g/100 g)	Intraday % RSD (n=3)	Hypoxoside (mg/dosage unit)	Interday % RSD (n=4)
Product-D I	0.690	<i>Hypoxis rooperi</i> 250 mg	Day-1^a	2.68	4.01	18.83	2.66
			Day-2^a	2.82	2.21		
			Day-3^a	2.79	2.91		
			Day-4^b	2.65	2.01		
Product-G	1.117	<i>Hypoxis</i> powder 200 mg	Day-1^a	1.06	1.54	11.84	-

a = Capillary 1

b = Capillary 2

n = number of samples

6.5.2.3 Selectivity

A high degree of selectivity was observed from the results of the stress studies on hypoxoside. The physical changes in the appearance of the samples subjected to stress are the same as mentioned in Chapter 4 (Section 4.6.3).

The electropherogram of the hypoxoside sample from the oxidation study showed interference by hydrogen peroxide. This can be seen from the electropherogram of a blank solution containing hydrogen peroxide (10%, v/v), (**Figure: 6.11.**). Neither the alkali nor acid reagents resulted in interference (**Figure: 6.12.**). Each of the acid, alkali, neutral and photolytic samples containing hypoxoside were mixed to provide a concentration of 20 µg/ml of the analyte and this mixture

was subsequently analyzed and examined for possible interference by degradation products. No interference was observed either from the appearance of degradation products nor from any of the reagents used and the hypoxoside peak is clearly discernible at 10.64 min (**Figure: 6.13.**).

Figure: 6.11.

Electropherogram of Blank Hydrogen Peroxide Solution (10%, v/v)

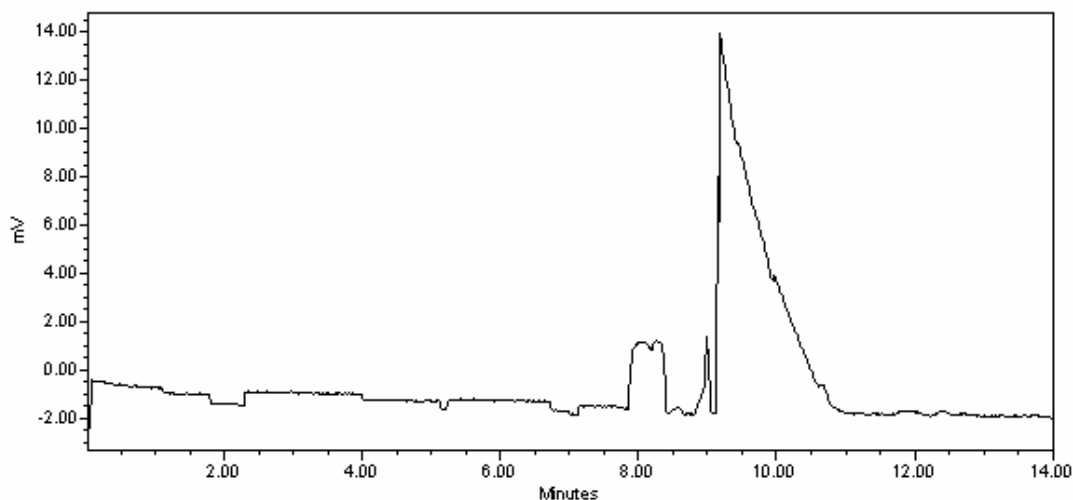


Figure: 6.12.

Electropherogram of a Mixture of Stress Treated Blank Solutions Used for Acid, Alkali and Neutral Hydrolysis

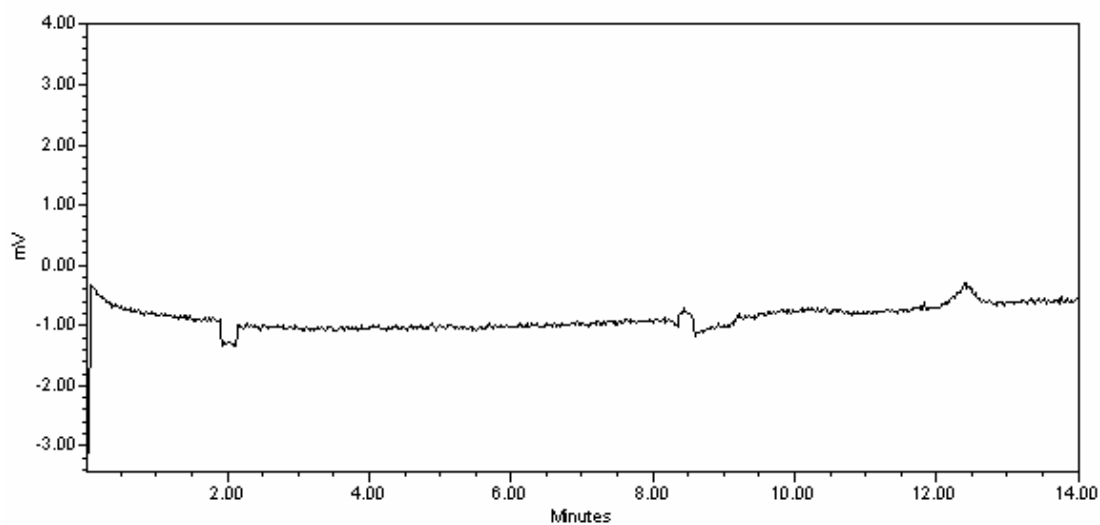


Figure: 6.13.

Electropherogram of a Mixture of Stress Treated Sample Solutions after Acid, Alkali and Neutral Hydrolysis

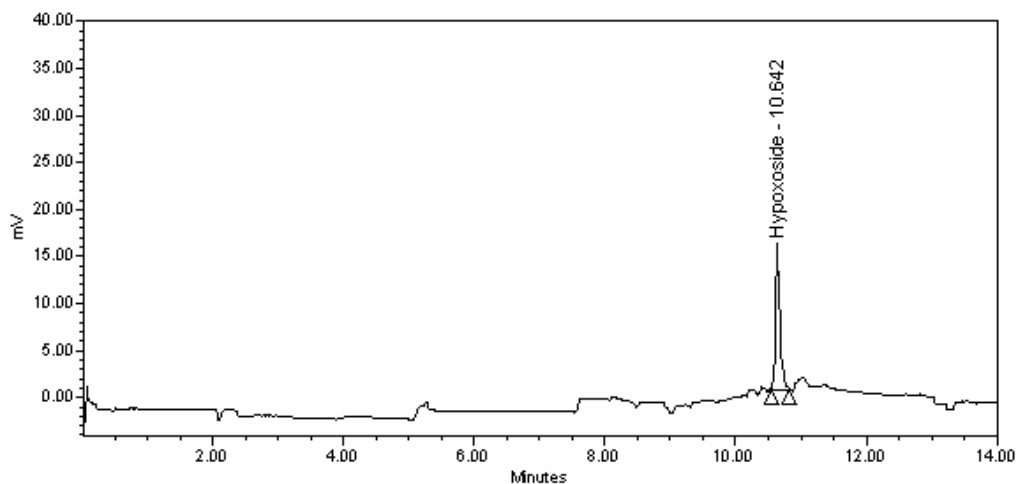


Figure: 6.13 .Conditions:- Capillary: 50.0 μm i.d; Total length = 71.0 cm; Effective length = 58.0 cm; Sample buffer 50:50 methanol: water; Running buffer: 25 mM Sodium tetra borate, (pH 9.2); Hydrodynamic injection: 100 mbar for 0.1 minutes; Voltage: +13 kV

6.6 Conclusions

This chapter describes a rapid, accurate, precise and stability-indicating CZE method for the quantitative analysis of hypoxoside in some commercially available formulations containing AP. Analysis of the commercial formulations showed discrepancies in hypoxoside content amongst the products analyzed. Product-D I claimed that each capsule contained 250 mg *Hypoxis rooperi*/0.69 g unit weight whereas Product G was labeled to contain 200 mg *Hypoxis* powder/1.117 g unit weight. Assay results revealed that the unit capsule hypoxoside content of Product-D I was 18.83 mg whereas the unit capsule hypoxoside content of Product G was 11.24 mg.

These results clearly suggest that suitable QC measures need to be implemented to ensure consistent quality of marketed products containing AP.

Chapter 7

Antioxidant Activity of Hypoxoside, Rooperol and Extracts of African Potato.

7.1 Introduction

In recent years there has been an increased interest in antioxidants which have the ability to scavenge free radicals, such as the superoxide radical, the hydroxyl radical and others that have been implicated in a number of degenerative diseases including cancer [312], cardiovascular disease [313], cataracts [314], macular degeneration [315], impaired wound healing [316], gastrointestinal inflammatory diseases [317] and other inflammatory processes. Phytomedicines usually contain natural antioxidants such as vitamin C, vitamin E and carotenoids, for example, which can scavenge free radicals. These dietary antioxidants have generated particular interest for use as a defence against degenerative diseases [318-322].

Antioxidants are of interest because they may help to protect the human body against damage by reactive oxygen species (ROS). ROS are potentially harmful and are generally produced as a consequence of normal aerobic metabolism.

Compounds can exert antioxidant activity by inhibiting the generation of ROS or by directly scavenging free radicals. When ROS are generated *in vivo*, a wide variety of physiological antioxidants such as superoxide dismutase (SOD), glutathione peroxidase, catalase, caeruloplasmin etc., come into play.

There are several methods that can be used to assess the various antioxidant mechanisms. In order to establish specific antioxidant mechanisms however, several different approaches are usually necessary. However, such a comprehensive approach may be tedious, expensive and time consuming. There are however some relatively simple methods that can be performed to examine the direct antioxidant ability *in vitro* and to test for possible pro-oxidant effects on different molecular targets. *In vitro* antioxidant activity should be screened first before proceeding to *in vivo* studies. The

reason for such an approach is that if a compound is found to be poorly effective *in vitro*, it is highly unlikely to be any better *in vivo* [323].

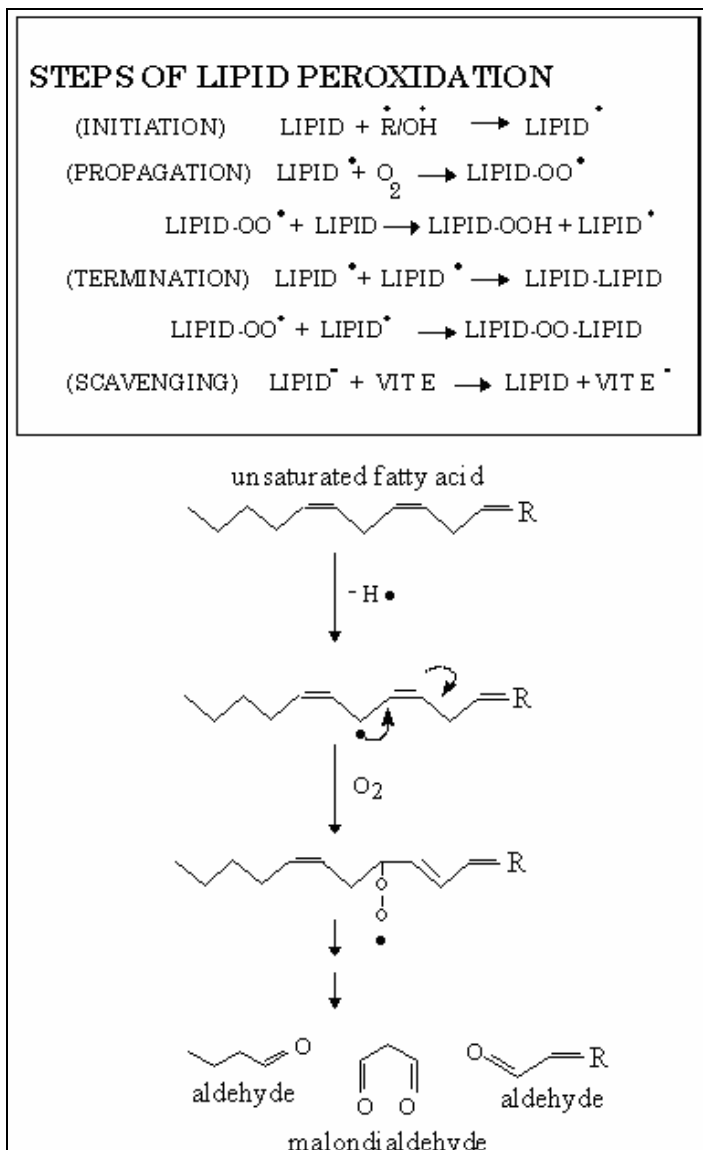
There are several methods to measure total antioxidant capacity of potential candidates, amongst them a few are considered to be very valuable in providing information on the antioxidant properties of samples. Tests which measure the combined antioxidant effect of compounds absorbed into biological fluids may be useful in providing information on their ability to protect the body from oxidative damage. Most of these tests are based on the measurement of the ability of a plasma sample to withstand the oxidative effects of reactive species purposefully generated in the reaction test mixture [324]. The utilization or depletion of the antioxidant is generally denoted by a signal such as rate of oxygen utilization or chemiluminescence. The assessment of free radical scavenging activity using the 1, 1-diphenyl-2-picryl-hydroazyl (DPPH) assay is an example of such a test.

A biological antioxidant is usually a substance, when present at low concentrations that significantly delays or prevents the oxidation of an oxidisable substrate. Some antioxidants prevent the generation of ROS by methods such as metal chelation or by enzyme-catalysed removal of the potential oxidant that is associated with a redox reaction. In such reactions, the oxidizing species reacts with the antioxidant instead of the substrate thereby reducing the antioxidant. This indirectly means that the antioxidant power of a substance or its “reducing ability”, could be measured by using reductants in a redox-linked colorimetric method employing an easily reduced oxidant in stoichiometric excess. An antioxidant assay known as “ferric reducing ability of plasma” (FRAP), which assesses the ‘antioxidant power’ of a substance by the reduction of ferric to ferrous ion is an example of such a colorimetric method.

Cell membrane lipids, such as cholesterol and in particular, polyunsaturated fatty acids (PUFAs) are often the most avid targets of oxygen radical-induced injury. The process of lipid damage by oxygen radicals is known as lipid peroxidation (LP). In view of the process of first pass hepatic metabolism, liver tissue is considered to be highly susceptible to LP. LP is initiated when a free radical attacks PUFAs, where a hydrogen atom is removed resulting in a lipid-derived free radical (**Figure: 7.1.**).

Figure: 7.1.

Schematic Diagram of Lipid Peroxidation [325].



The propagation step begins with the rapid addition of molecular oxygen to the lipid-derived free radical resulting in a lipid peroxyl radical. This lipid peroxyl radical can attack other lipids that results in the generation of lipid-derived free radicals and a lipid hydroperoxide. This whole process is a chain reaction and once

initiated, termination is extremely difficult [326] unless two free radicals react together to form a non-radical product [327]. Using antioxidants can also limit the ROS-induced LP [327].

The measurement of known elevated end products of lipid peroxidation in animal material is probably the evidence most frequently quoted in support of free radical induced tissue damage [328]. In this respect, the thiobarbituric acid test is the most widely used assay for measuring lipid peroxidation [328].

Occasionally under normal biological conditions, oxygen cannot accommodate a spin-matched pair, so it manages to procure electrons from other molecules, one at a time by auto-oxidation. The breaking up of electron pairs results in free radical formation and such a one-electron product of oxygen is known as a superoxide radical, ($O_2^{\cdot -}$) [329]. The $O_2^{\cdot -}$, generated via multiple enzymatic and non-enzymatic pathways, often starts the oxidative stress cascade. In an environment where the pH is approximately 7.4, the $O_2^{\cdot -}$ is partially protonated to form the hydroperoxyl radical ($HO_2^{\cdot}$), which is a more reactive species [330]. In a study by Antunes *et al* in 1996 [331] the hydroperoxyl radical was estimated to inflict more than 5 times the damage of a hydroxyl radical. The protective measures that the cells employ in response to such damage are usually sufficient, except during excessive production of ROS, where biological defenses are overwhelmed, leading to oxidative stress [332].

The cell has its own coping mechanism to prevent this free radical induced damage. Once formed, the $O_2^{\cdot -}$ quickly undergoes dismutation to generate H_2O_2 and this reaction is markedly accelerated by a family of enzymes known as the superoxide dismutases (SOD) [333, 334]. Therefore any agents, which enhance the activity of this important antioxidant enzyme (SOD) could aid in the prevention of this free radical induced damage. Another defense mechanism is by using antioxidants that scavenge the $O_2^{\cdot -}$ free radical thereby limiting the damage that may be caused by this free radical.

7.2 Background and Objective

Hypoxis hemerocallidea, commonly known as African potato (AP) is also known as *Hypoxis rooperi*. It has a long history of traditional use for a diversity of ailments [99] and more recently has been the subject of several scientific studies [76].

Details on previous studies relating to the potential antioxidant activity of rooperol, the aglycone of the unique compound hypoxoside present in AP has been discussed in Chapter 2 (Section 2.4.2.2).

The wide use of AP for the treatment of various ailments and the preliminary scientific data from previous antioxidant studies [121, 237, 243] AP extract and its phytoconstituents, support the need to pursue further antioxidant studies on AP and its phytoconstituents.

In the present study the antioxidant properties of hypoxoside, rooperol and an AP extract were examined, initially as free radical scavenging activity using the 1, 1 diphenyl-2-picryl hydrazyl (DPPH) assay. This method is useful to determine the hydrogen donating capacity of a molecule, [335-338] but not the possible reactions with free radical intermediates and the production of oxidative chain reactions. In addition, the total antioxidant capacity of all the samples was studied using the ferric reducing ability of plasma (FRAP) assay which involves a redox-linked colorimetric method to assess non-enzymatic antioxidant properties [324].

The thiobarbituric acid (TBA) test was used to colorimetrically measure the extent of lipid peroxidation, which leads to cell death because membranes are damaged by reactive oxygen species (ROS) [328, 339].

Superoxide (O_2^-), which can be generated through multiple enzymatic and non-enzymatic pathways, is often the start of the oxidative stress cascade [333, 340, 341]. The superoxide anion free radical scavenging activity of AP extracts, hypoxoside and rooperol were determined using the nitroblue tetrazolium assay (NBT) [333, 342-344].

An important part of the evaluation of plant derived medicines is to phytochemically characterize the material under investigation. Since no HPLC analytical method has previously been published to simultaneously determine hypoxoside and rooperol in a plant extract using an isocratic mobile phase, an additional objective of this study was also to develop a rapid and reliable HPLC

method to qualitatively determine these compounds in the lyophilized forms of AP in order to confirm their presence in the materials tested.

7.3 Experimental

7.3.1 Instrumentation

A Mettler dual range electronic balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing the standards and samples. A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes. The UV-Vis absorption was measured with a Hitachi, U-2000 spectrophotometer (San Jose, CA, USA).

The chromatographic analysis was carried out on an Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a 2996 photodiode array (PDA) detector, degasser, column heater and auto sampler. A Synergi Hydro RP[®] Column (4 μ m, 4.6 x 50 mm i.d.) (Phenomenex, Torrance, CA, USA) was used at $23 \pm 2^\circ$ C.

7.3.2 Reagents and Chemicals

DPPH (1, 1-diphenyl-2-picryl hydrazine), TPTZ (2, 4, 6-tri pyridyl-S-triazine), QAC (quinolinic acid), BHT (butylated hydroxytoluene), TBA (2-thiobarbituric acid), NBD (nitroblue diformazan), NBT (nitroblue tetrazolium) and 1, 1, 3, 3-tetramethoxypropane (99%), quercetin and ascorbic acid were obtained from Sigma Chemical Co (St. Louis, MO, USA). Glacial acetic acid, sodium acetate and ferric chloride were of analytical grade (BDH chemicals, Poole, UK) and HPLC grade solvents were purchased from Romil Ltd, (The Source, Water Beach, Cambridge, UK). TCA (trichloroacetic acid), ethanol and butanol were purchased from Saarchem, Johannesburg, South Africa. Water was purified in a Milli-Q[®] system (Millipore, Bedford, MA, USA) and all samples were filtered using Millex HV[®] Hydrophilic (PVDF, 0.45 μ m) membranes purchased from the same source.

7.3.3 Plant Material and Phytochemicals

AP corms were collected on Settlers Hill adjoining the botanical garden of Rhodes University, Grahamstown, South Africa. The voucher specimen (AP08/2003) was submitted to the herbarium, Faculty of Pharmacy, Rhodes University for the records. Hypoxoside was isolated and characterized and its purity confirmed by spectroscopy. These data have been described Chapter 3. Crystalline rooperol (99.1%) was gratefully received as a gift from Dr. Carl Albrecht, (Research Co-ordinator, CANSA, South Africa).

7.4 Experimental

7.4.1 HPLC Method Development

Hypoxoside is soluble in water whereas rooperol is soluble in warm water at 60° C. Previously, an HPLC analytical method was developed and validated for the quantitative determination of hypoxoside using a Luna[®] C₁₈ analytical column (Chapter 4). The retention time for hypoxoside was 11.2 min with good specificity but could not be used for the simultaneous determination of rooperol, since the latter was highly retained and only eluted at ~40 min due to its non-polar nature. In order to reduce the elution time of rooperol, the acetonitrile content of the MP was increased by 20% from water: acetonitrile, 80:20, v/v) to water: acetonitrile, (60: 40, v/v). However, under these conditions, hypoxoside eluted near the void volume whereas rooperol eluted at 22 min. Methanol was substituted in place of acetonitrile but this did not provide the requisite result.

A more polar Luna[®] C₈ (5 µm) (Phenomenex, Torrance, CA, USA) column was thus used and although the retention time for rooperol was improved (~9 min) using a MP consisting of water: methanol (35:65, v/v), this system was still inadequate since the more polar compound, hypoxoside again eluted near the void volume (~2 min). A similar trend in the elution pattern with this column was observed even on substitution with acetonitrile in the place of methanol.

These observations prompted the investigation of alternative columns, such as a Synergi Polar-RP[®] (4 µm, 80 Å, 4.6 x 150 mm i.d.) (Phenomenex, Torrance, CA, USA), and a Hypersil C₁₈ Column (5 µm, 4.6 x 150 mm i.d.) (Phenomenex, Torrance, CA, USA) but similar unsuitable elution patterns for hypoxoside and rooperol were obtained using an isocratic mobile phase.

Subsequently, a Synergi Hydro-RP[®] column (4 µm, 80 Å, 4.6 x 150 mm i.d.) (Phenomenex, Torrance, CA, USA) which is a C₁₈ column with polar end capping and recommended for non polar as well as highly polar compounds was chosen. Method development was initiated with methanol: water (50:50) at a flow rate of 1.0 ml/min and this resulted in the separation of both hypoxoside and rooperol at 3.9 and 13.4 min respectively. The methanol content was subsequently decreased to increase the retention time of hypoxoside using methanol: water (45:55) and this resulted in retention times of 6.8 and 17.4 min hypoxoside and rooperol respectively. The mobile phase was further adjusted to a final composition of methanol: water (48:52) at a flow rate of 1.0 ml/min in isocratic mode yielding retention times for hypoxoside and rooperol at 4.37 and 15.32 min, respectively (**Figure: 7.2.**). Throughout the method development, the temperature was maintained at 23°C ± 2°C and 10 µl samples of each reference compound, hypoxoside and rooperol, were injected. The eluate was monitored by UV detection at a detection wavelength of 260 nm at which both hypoxoside and rooperol provided a good UV response.

The effect of flow rate on the elution of hypoxoside and rooperol were investigated. Although higher flow rates resulted in shorter elution times, they did not offer any significant advantages. Moreover, the higher flow rates were also associated with higher column back-pressures; hence a flow rate of 1.0 ml/min was chosen and provided ample time to allow elution of any polar phytoconstituents from AP aqueous extract without interference with the analyte peaks. The peak purity of both these peaks were confirmed with the PDA detector (Empower[®] package, Waters Corporation, Milford, MA, USA).

Decreasing concentrations of serial dilutions of solutions of hypoxoside and rooperol solutions were prepared in order to establish the LOD (signal/noise >3) and LOQ (signal/noise >10) for each standard.

Figure: 7.2.

HPLC Chromatogram of Hypoxoside and Rooperol

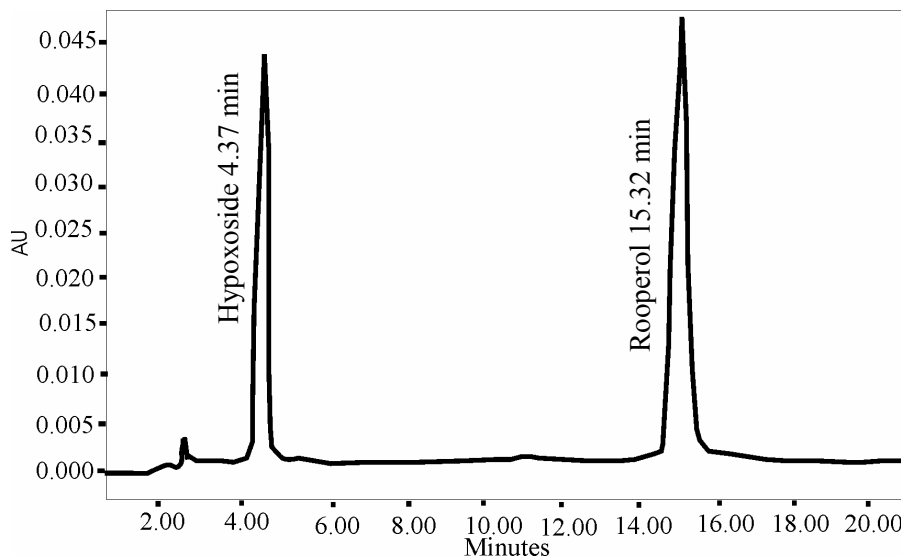


Figure: 7.2. Conditions:- Column: Synergi Hydro-RP[®] (4 μ m) C₁₈ 80 A, (150 x 4.6 mm i.d.); Column temperature: 23 $\pm$ 2 $^{\circ}$ C; Mobile phase: (methanol 48: 52 water) Flow rate: 1 ml/min; Injection volume: 10 μ l; Detection λ : 260 nm.

7.4.2 Sample Extraction

Freshly collected corms of AP were thoroughly washed under running water. They were then sliced into uniform cubes of approximately 0.5 cm³ and flash-frozen with liquid nitrogen before being lyophilized for 12 hr *in vacuo*. The lyophilized material was milled and sieved (40 mesh size) and aqueous preparations were prepared by weighing out ~1 g of the powdered material and extracting with hot water. An aqueous extract was preferred since AP is widely consumed as a traditional aqueous decoction. The mixture was vortexed for 1 min and then sonicated for a pre-determined time of 20 min and subsequently centrifuged (1000 x gr) for 2 min. The supernatant was filtered through Millex HV[®] Hydrophilic (PVDF, 0.45 μ m) filters before further diluting to the desired concentrations. This extraction procedure was conducted under less harsh conditions than those used to prepare a traditional decoction of AP when the aqueous preparation is usually boiled for 20-30 min.

7.4.3 Preparation of Solutions for Antioxidant Investigations

A solution of 5 mg/ml of hypoxoside was prepared with Milli-Q[®] water at room temperature (~23°C), whereas, the solution of rooperol (2.5 mg/ml) was made at 60° C to facilitate its solubilization. Both the solutions were sonicated for 2 min before serially diluting to the desired concentrations for the antioxidant studies.

7.4.4 Antioxidant Investigations

7.4.4.1 Free Radical Scavenging Ability

The DPPH radical scavenging activity of the samples was determined using the method proposed by McCune and Jhons, 2002 [345]. The extinction of the purple color of the DPPH due to the presence of an odd electron gives a strong absorption band at 517 nm. When this free electron gets paired off in the presence of a free radical scavenger, the absorption decreases and the resulting decolourization process is stoichiometric.

To 1.5 ml of DPPH (0.0394 mg/ml in methanol) 0.25 ml of test solution was added. The decrease in absorbance was measured for 30 min at 10 min intervals. The AP aqueous extract (0.1, 0.5, 1.0 mg/ml), hypoxoside (32 µg/ml) and rooperol (4, 8, 16, 32 µg/ml) were compared to quercetin solutions (4, 8, 16, 32 µg/ml), a well-known antioxidant [345]. Experiments were conducted in quadruplicate and are represented as the percentage (%) inhibition of the DPPH radical.

7.4.4.2 Ferric Reducing Activity of Plasma (FRAP)

FRAP was used to determine the total antioxidant potential of the samples [324]. In this assay the electron donating capacity of the antioxidant was measured by the change in absorbance at 593 nm when a blue colored Fe²⁺-tripyridyltriazine (Fe²⁺ TPTZ) compound is formed from a colourless oxidized Fe³⁺ form. Calibration curves were prepared from aqueous solutions of FeSO₄ at different concentrations ranging from 100-1000 µmol/litre. Working reagent, 0.9 ml (25 ml of acetate buffer, 2.5 ml of

TPTZ solution, and 2.5 ml of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution) were mixed with 30 μl of each plant extract and 90 μl of distilled water and the change in absorbance was used to calculate the antioxidant efficiency of the test samples.

The results from AP aqueous extracts (0.1, 0.5, 10 mg/ml), hypoxoside (32 $\mu\text{g/ml}$) and rooperol (4, 8, 16, 32 $\mu\text{g/ml}$) were compared to the antioxidant activity of vitamin C (8, 16, 32 $\mu\text{g/ml}$) at 10 and 20 min. Care was taken that all the solutions were diluted to fit within the linearity range and were used on the same day of preparation. These experiments were also conducted in quadruplicate.

7.4.4.3 Animal Care and Homogenate Preparation

Adult male Wistar rats, weighing between 250-300 g were purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The animals were housed in a controlled environment with a 12-hr light: dark cycle, and were given access to food and water *ad libitum*. The Rhodes University animal ethics committee approved protocols for the experiments. For the purpose of lipid peroxidation and superoxide anion assays, rats were sacrificed by cervical dislocation and the livers were removed and perfused with saline. Livers were homogenized (10%, w/v) in 0.1 M phosphate buffered saline (PBS), at pH = 7.4 and used immediately for the assay.

7.4.4.4 Lipid Peroxidation Assay

The elevated amount of known end products of lipid peroxidation in animal material is probably the evidence most frequently quoted in support of free radical induced tissue damage [328]. The thiobarbituric acid test is the most widely used assay for measuring lipid peroxidation [328] and involves the reaction between malondialdehyde (MDA), an end product of lipid peroxidation, and thiobarbituric acid to yield a pink chromogen, which is measured colorimetrically at 532 nm using a spectrophotometer [328].

Lipid peroxidation was determined according to a modified method of Placer *et al* (1966) [346]. Homogenate (1.0 ml), containing 1 mM QAC alone or in combination with either hypoxoside (12.5, 25, 50 µg/ml), rooperol (7.5, 15, 30 µg/ml) or aqueous extracts of AP (1.25, 2.5, 5.0 mg/ml) were incubated at 37° C in a shaking water bath for 1 hr. At the end of the incubation period 0.5 ml BHT (0.5 mg/ml in ethanol) and 1.0 ml 25% TCA was added. The tubes were sealed and heated for 10 min in a boiling water bath to release protein bound MDA, the product of lipid peroxidation. To avoid adsorption of MDA to insoluble proteins, the samples were cooled to 4° C and centrifuged at 2000 x gr for 20 min. Following centrifugation, 2.0 ml of the protein free supernatant was removed from each tube and 0.5 ml aliquots of 0.33% TBA was added to this fraction. All tubes were heated for 1 hr at 95° C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2.0 ml butanol. The absorbance was read at 532 nm and MDA results were determined from a standard curve generated from 1, 1, 3, 1-tetramethoxypropane. Final results were presented as nmoles MDA/mg tissue.

7.4.4.5 Superoxide Anion Assay (Nitroblue Tetrazolium Assay)

A modification of the assay procedure of Das *et al.* (1990), which involves the reduction of the nitroblue tetrazolium (NBT) dye by the superoxide anion to the insoluble nitroblue diformazan (NBD) was employed and the NBD was extracted with glacial acetic acid and measured colorimetrically [342-344]. It is known that cyanide is an inhibitor of complex IV of the mitochondrial electron transport chain and distal inhibition of the electron transport chain by cyanide augments reactive oxygen species production [347]. The primary mechanism of action of cyanide involves the inhibition of cytochrome oxidase a_1a_3 , the terminal oxidative enzyme of the electron transport chain [348, 349]. These electrons have the capacity to leak out of the mitochondria and come in close proximity to O₂ resulting in the generation of the superoxide anion.

Homogenate (1.0 ml) containing KCN (1 mM) alone or in combination with either hypoxoside solutions (50, 25, 12.5 µg/ml), rooperol solutions (30, 15, 7.5 µg/ml) or aqueous extracts of AP (5, 2.5, 1.25 mg/ml) were incubated with 0.4 ml of 0.1% NBT in a shaking water bath for 1 hr at 37° C. Termination of the assay and extraction of the reduced NBT (NBD) was carried out by centrifuging the samples for

10 min at 2000 x gr followed by re-suspension of the pellets with 2.0 ml of glacial acetic acid. The absorbance was measured at 560 nm and converted to micromoles diformazan using a standard curve generated from NBD. Final results are expressed as μ moles diformazan/mg tissue.

7.4.5 Statistical Analysis

All experimental data were analyzed using the one way analysis of variance (ANOVA) and significant differences among the means from quadruplicate analysis at ($p < 0.05$) were determined by the Student-Newman-Keuls Multiple Range Test.

7.5 Results and Discussion

7.5.1 HPLC Analysis

The HPLC method gave well-resolved peaks of both hypoxoside and rooperol in a single determination. Retention times were 4.34 min and 15.32 min respectively (**Figure: 7.2.**). The LOD and LOQ of both hypoxoside and rooperol were 1 and 4 μ g/ml respectively. The lyophilized AP (FDAP) was extracted separately with methanol as well as warm (60° C) water and was subjected to analysis using the HPLC method. Both extracts of FDAP, at 500 μ g/ml concentration, did not show any detectable rooperol peak. This was expected since rooperol does not exist in free form unless hypoxoside is glycolysed by the action of β -glycosidase as occurs in the gastrointestinal tract [243]. Also, rooperol, when present can be recovered with warm water (60° C). The amount of hypoxoside in FDAP was determined as 10.2% (dry weight, w/w) as previously determined (Chapter 4, **Table: 4.5.**)

7.5.2 Antioxidant Investigations

7.5.2.1 Free Radical Scavenging

In the present study the free radical scavenging effects of the aqueous extract of AP (FDAP), hypoxoside and its aglycone, rooperol were compared with that of quercetin. (**Table: 7.1.**) The free radical scavenging activity of the aqueous extract

using the DPPH assay showed high levels of activity that increased with increasing concentration. Rooperol showed free radical scavenging activity comparable to quercetin at concentrations above 4 µg/ml suggesting it is a relatively potent free radical scavenger at higher concentrations. The prodrug, hypoxoside, even at the higher concentration of 32 µg/ml showed no significant free radical scavenging activity. Some antioxidant activity was observed from the extracts albeit quite low at high concentrations.

Table: 7.1.

Comparison of Antioxidant Activities Using Quercetin as Control (DPPH assay)

Sample name	Concentration in µg/ml	% inhibition of DPPH @ 10 minutes mean ± SD	% inhibition of DPPH @ 20 minutes mean ± SD	% inhibition of DPPH @ 30 minutes mean ± SD
Aqueous extract of FDAP	100	10.46 ± 0.41	12.75 ± 0.40	14.65 ± 0.42
	500	31.78 ± 3.11	37.87 ± 3.31	42.07 ± 3.42
	1000	59.84 ± 2.62	66.43 ± 1.09	71.86 ± 1.28
Hypoxoside	32	0.78 ± 0.15	-1.04 ± 0.24	-1.45 ± 0.23
Rooperol	4	2.87 ± 0.70	4.47 ± 0.31	6.03 ± 0.09
	8	24.96 ± 4.96	29.22 ± 5.21	32.79 ± 5.44
	16	43.15 ± 1.99	49.97 ± 2.17	54.87 ± 2.27
	32	75.23 ± 2.36	80.32 ± 0.22	82.94 ± 0.34
Quercetin	4	14.59 ± 0.33	16.65 ± 0.33	18.55 ± 0.31
	8	23.77 ± 1.50	25.73 ± 5.21	27.38 ± 1.36
	16	43.15 ± 1.99	49.73 ± 2.17	46.92 ± 0.48
	32	75.23 ± 2.36	87.62 ± 0.78	90.04 ± 0.63

(Rooperol vs Quercetin @30 min, (***) 4µg/ml, (***) 8 µg/ml, (***) 16µg/ml, (***) 32µg/ml),
(*p<0.05, **p<0.01, ***p<0.001, ns- p>0.05)

7.5.2.2 Ferric Reducing Activity of Plasma (FRAP)

Ferric reducing activity plots were highly linear ($r^2 > 0.999$). As shown in **Table: 7. 2.**, the Fe^{2+} formed by the aglycone, rooperol at equivalent ascorbic acid concentrations, was significantly ($p < 0.001$) higher than that produced by the standard. At 20 min, the aqueous extract of AP at 1000 µg/ml gave a FRAP value of

approximately 804 $\mu\text{mol/litre}$ compared to 471 $\mu\text{mol/litre}$ which resulted at the highest ascorbic acid concentration of 32 $\mu\text{g/ml}$. Since hypoxoside, considered to be the main component in AP, did not show any significant antioxidant activity in this assay, the relatively low antioxidant activity observed at the highest concentration of aqueous extract of FDAP (1000 $\mu\text{g/ml}$) must be due to some other chemical components present in the extract.

Table: 7.2.

Comparison of Antioxidant Activities Using Ascorbic Acid as Control (FRAP assay)

Sample name	Concentration in $\mu\text{gm/ml}$	FRAP $\mu\text{mol/litre}$ @ 10 minutes mean $\pm$ SD	FRAP $\mu\text{mol/litre}$ @ 20 minutes mean $\pm$ SD
Aqueous extract of FDAP	100	71.33 $\pm$ 2.36	89.81 $\pm$ 2.57
	500	338.19 $\pm$ 7.17	429.71 $\pm$ 9.24
	1000	645.00 $\pm$ 7.49	803.81 $\pm$ 11.21
Hypoxoside	32	2.97 $\pm$ 1.29	0.77 $\pm$ 0.16
Rooperol	4	104.42 $\pm$ 2.05	126.33 $\pm$ 2.05
	8	213.76 $\pm$ 6.81	259.16 $\pm$ 7.21
	16	402.95 $\pm$ 11.46	490.66 $\pm$ 14.69
	32	703.96 $\pm$ 9.33	860.82 $\pm$ 7.14
Ascorbic acid	4	62.37 $\pm$ 0.825	99.92 $\pm$ 2.67
	8	195.01 $\pm$ 1.43	198.56 $\pm$ 2.42
	16	216.9 $\pm$ 3.49	288.56 $\pm$ 4.85
	32	442.89 $\pm$ 2.33	471.01 $\pm$ 7.26

(Rooperol vs Ascorbic acid, (***) 4 $\mu\text{g/ml}$, (***) 8 $\mu\text{gm/ml}$, (***) 16 $\mu\text{gm/ml}$, (***) 32 $\mu\text{gm/ml}$)
 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns- $p > 0.05$)

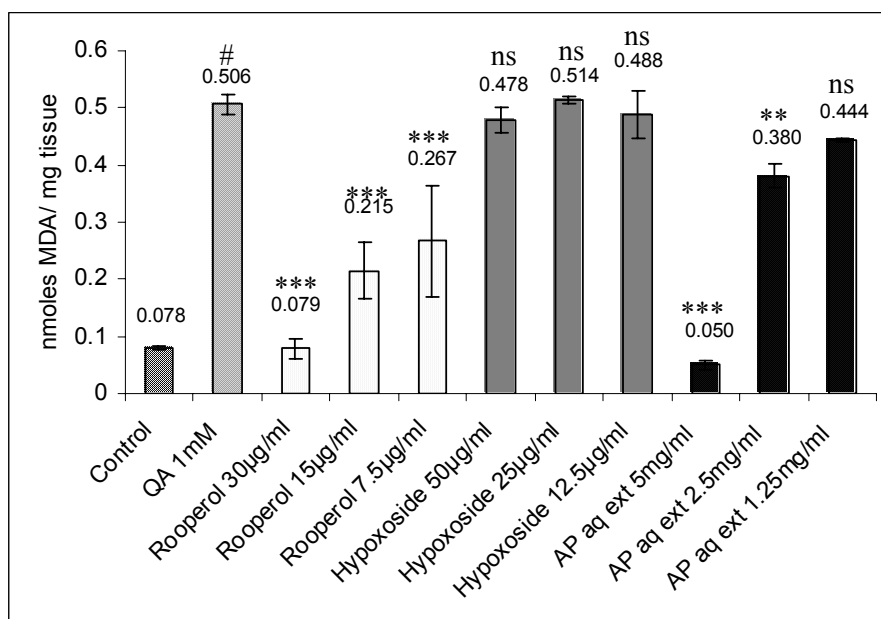
7.5.2.3 Lipid Peroxidation Assay

Quinolic acid (QAC) chelates ferrous ions and it is the QAC-iron complex that generates free radicals and acts as a pro-oxidant by initiating lipid peroxidation [350]. The present study demonstrated that the aqueous extracts of AP and also rooperol significantly ($p < 0.001$) reduced QAC-induced lipid peroxidation in liver homogenates with rooperol being the more potent antioxidant. It was noted that at a concentration

of 30 $\mu\text{g/ml}$, rooperol shows no significant difference from the control, which means it completely abolished the QAC-induced lipid peroxidation (**Figure: 7.3.**). This could be through scavenging the free radicals that are formed, or by interacting with the ferrous ions, thereby preventing the formation of the QAC-ferrous ion complex [323]. However, hypoxoside even at a higher concentration (32 $\mu\text{g/ml}$) did not reduce the QAC-induced lipid peroxidation.

Figure: 7.3.

Effect of Increasing Concentration of Rooperol, Hypoxoside and AP on QAC (1 mM)-Induced Lipid Peroxidation in Rat Liver Homogenates



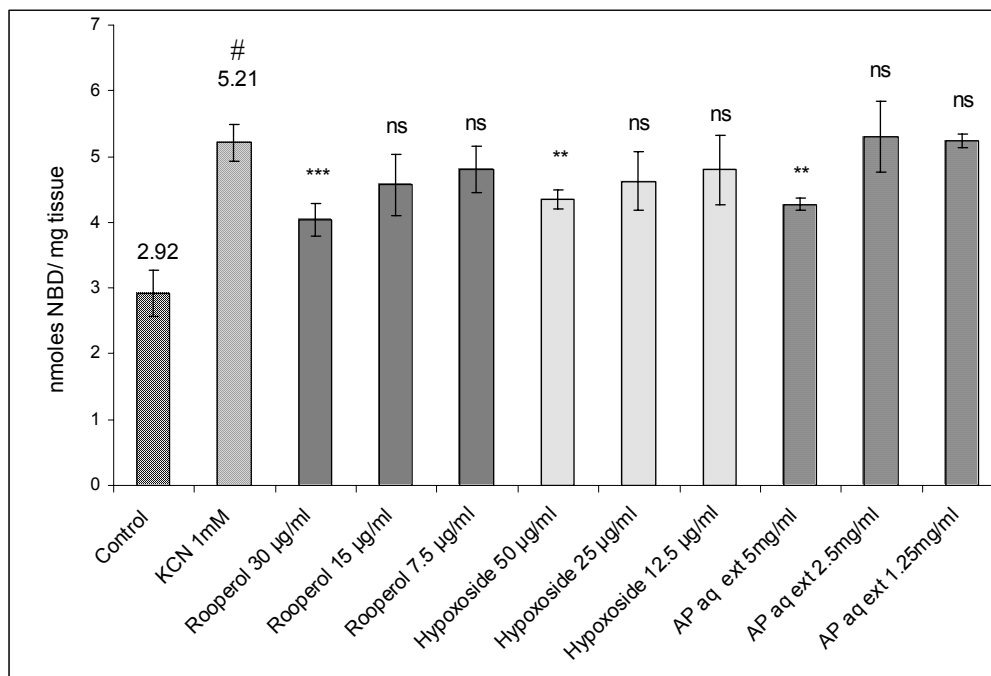
Each bar represents the mean $\pm$ SD (n=4), # ($p < 0.001$) compared to control. ns (not significant), ** ($p < 0.01$), *** ($p < 0.001$) in comparison to 1 mM QAC induced lipid peroxidation.

7.5.2.4 Superoxide Anion Assay (Nitroblue Tetrazolium Assay)

The results show that only the higher concentrations of AP extract ($p < 0.01$), hypoxoside ($p < 0.01$) and rooperol ($p < 0.001$) scavenge the superoxide anions thereby curtailing the reduction of nitroblue tetrazolium to nitroblue diformazan (**Figure: 7.4.**).

Figure: 7.4.

Effect of Increasing Concentrations of Rooperol, Hypoxoside and Aqueous Extract AP on 1 mM KCN Induced Super Oxide Radical in Rat Liver Homogenate.



Each bar represents the mean $\pm$ SD ($n=4$). # ($p < 0.001$) in comparison to controls, ** ($p < 0.01$), *** ($p < 0.001$) in comparison to 1 mM KCN induced group.

7.6 Conclusions

The objective of these studies was to develop an isocratic HPLC method for the determination of hypoxoside and rooperol and to assess their antioxidant activity as well as that of an aqueous extract of AP. Hypoxoside, a known constituent of AP did not show any significant antioxidant activity whereas its aglycone, rooperol, which is not present in AP *per se*, was shown to have significant antioxidant activity. The latter is probably due to the dicatechol-like structure similar to the known antioxidant compound, nordihydro-guaiaretic acid. Interestingly, the aqueous extract of AP showed increased free radical scavenging activity with higher concentrations. This could be due to various other phytochemicals present in AP. Aqueous extracts of AP have good potential for increased antioxidant activity *in vivo* since in addition to the

extract's confirmed *in vitro* antioxidant activity, the rich inactive content of hypoxoside (10.2 %, w/w) is readily converted to rooperol in the gastrointestinal tract of humans. Whereas rooperol is converted to its glucuronides and sulphates as it crosses the gastrointestinal membrane and enters into the systemic circulation, these metabolites are subsequently converted back to rooperol by tumor cells which secrete deconjugative enzymes [243]. These features indicate that AP could have value as an antioxidant pro-drug.

Chapter 8

Drug Interaction Studies

In vitro Evaluation of Human Cytochrome P450

Enzymes and P-glycoprotein Interactions

8.1 Introduction

The physico-chemical properties of drugs play an important role both in absorption and elimination from the human body. The elimination of drugs normally involves their metabolic conversion to hydrophilic compounds. This process is often the major determining factor defining the pharmacokinetics of drugs, which in turn can influence the efficacy and side-effect profile of medicinal compounds. There are many enzymes involved in the various metabolic pathways to effect elimination. These enzymes are subject to genetic polymorphisms demonstrating marked species differences that can be affected by diet or by the co-administration of other drugs, amongst other factors. Although drug metabolising enzymes are present throughout the body, the liver and intestine are significant organs of metabolism for most foreign compounds. Extra-hepatic sites are normally less important for drug metabolism and their role is determined by the route of administration of the drug [351].

Drug metabolising enzymes are usually classified as either Phase I or Phase II which relate to the reactions that they catalyse. During Phase I reactions, enzymes take part in the introduction or production of functional groups such as -OH, -SH, -NH₂, -COOH for example, into the molecule. Oxidation and reduction are the two most common Phase I reactions, whereas Phase II biotransformations involve conjugation of a suitable chemical group of the molecule with entities such as glucuronic acid, sulphate, glutathione and amino acids (glycine, taurine, glutamine) which are meant to increase the water solubility of the molecule.

Phase II reactions rarely precede Phase I reactions since the hydrophilicity of Phase II biotransformed products make themselves poor substrates for the Phase I enzymes to act upon [351-353].

From a survey of the elimination pathways of approximately 450 drugs marketed in the USA and Europe, 75% of these drugs are cleared by metabolism of which approximately 75% is mediated by Cytochrome P450 enzymes. Cytochrome P450 enzymes are ubiquitous in humans which has necessitated a systematic nomenclature system. Cytochrome (CYP) P450 enzymes consist of a more than 40% amino acid sequence homology and are placed in the same family designated by an Arabic numeral such the “3” in CYP3A4, for example. When two or more subfamilies are known to exist within the family, then enzymes with greater than 60% homology are placed in the same subfamily, designated with a letter, such as “A” in the example above. Finally this is followed by an Arabic number “4” shown in CYP 3A4 representing the individual enzyme and is assigned in order of discovery. At least 36 of the P450 enzymes described in man are likely to be of clinical relevance and only those belonging to the families 1, 2 and 3 appear to be responsible for the metabolism of drugs. Amongst these 18 enzymes in the above families, perhaps 5 or 6 enzymes such as CYP3A4, CYP3A5 and CYP19 are quantitatively relevant and significant in the drug metabolism [351-354].

There are many drugs which form substrates as well as have an effect on these enzymes by inducing or inhibiting their activity. Such an interaction is generally pharmacokinetic and not pharmacodynamic, and results in an expected response but at an unexpected dose. It has often been reported that the dramatic pharmacokinetic changes caused by inhibition/induction of CYP enzymes end up as adverse drug reactions (ADRs) and in extreme cases fatalities [355]. There is currently a growing awareness that herbal remedies and other phytochemicals could severely affect the disposition of concomitantly administered conventional drugs. Well-known examples of such inhibition and induction of CYP enzymes are by grape fruit juice and St. John’s wort, respectively.

Enzyme induction and inhibition activities of a large number of compounds and extracts can be conveniently studied by the use of *in vitro* assays involving CYP enzymes. Most of these assays are based on the determination of enzyme activities which either requires HPLC separation of the metabolites for quantitation or a relatively simpler and rapid microtiter plate based on fluorometric assay.

Notwithstanding, in order to overcome assumptions concerning the effects on pharmacokinetic parameters such as dose, bioavailability of the active components, distribution and clearance, it is pertinent to carry out *in vivo* studies in animals and in humans.

Besides these possible effects on drug metabolizing enzymes, drug transporters also play a major role in drug interactions. There are several transporters in diverse organism such as yeast, bacteria and also in humans. ATP binding cassette (ABC) transporters are one such class of energy dependent transporters. The genes of the multi-drug resistance (MDR) transporters which belong to this class, encode P-glycoprotein (P-gp), have wide tissue distribution in humans [356-359] and are over-expressed in tumours. The wide expression of P-gp occurs not only in malignant cells but also in normal human tissues causing implications for drug therapy. In tissues, such as intestines, P-gp limits the bio-availability of orally delivered drugs such as paclitaxal, for example, effluxing the drug back into the gut lumen.

Drugs can be substrates of P-gp, thereby inducing or inhibiting the function of these drug transporter proteins. This effects the change in concentration of either the substrate or other concomitantly administered drugs, thus resulting in drug-drug interactions [360]. The effect of drugs on P-gp is normally studied on transfected or over-expressed cell lines derived from tumour cells or animal tissue. The determination of the intracellular accumulation/efflux of a fluorescence P-gp substrate such as rhodamine 123 in the presence of the test sample is a preferred method.

8.2 Background and Objectives

The South African National Department of Health (DoH) has recently accredited 27 facilities whose mandate is to provide AIDS care. One of the strategies involves the provision of intervention that is purported to delay the progression of the disease, such as nutritional and micronutrient supplementation and also the use of African Traditional Medicines (ATMs) and complementary/alternate (CAMs) medicines. The DoH, along with its member states and non-government organisations (NGOs) have endorsed the use of ATMs as HIV/AIDS remedies [258]. AP is such an ATM that has been specifically promoted by the South African Minister of Health for this purpose [258]. AP, *Hypoxis hemerocallidea*, also known as *Hypoxis rooperi* of the family Hypoxidaceae, is of great

medical interest [76] and has been used for a variety of ailments as discussed in Chapter 2. More recently, AP has been widely consumed in South Africa especially by patients who are undergoing anti-retroviral therapy in AIDS related therapeutic programmes such as HAART (Highly Active Anti-Retroviral Therapy). The concurrent use of herbal products and health supplements containing AP in combination with other therapeutic products is also widespread [361].

The effect of such concomitant administration of ATMs and other herbal medicines on the safety and efficacy of conventional medicines has not been fully determined. This is evident from medical and scientific reports which suggest that concomitant oral administration of ATMs and natural products in general, with prescription medicines or over the counter products may have significant effects on human metabolism and increase the risk for serious clinical adverse reactions including a reduction in efficacy [24, 25, 27, 362-372].

The metabolism of a large proportion of medicines is mediated by CYP P450 enzymes, in particular, CYP3A4 which has broad substrate specificity. Significant inhibition of this enzyme can have a detrimental effect on safety. Since these drug interaction studies have been focused on AP which is indigenous to the Southern part of Africa, it was of primary interest to investigate potential interactions with CYP3A5 enzymes whose incidence is characteristically higher among the population of this region [373].

CYP19 is also known as the enzyme, aromatase and is responsible for the conversion of the C₁₉ steroids into C₁₈ estrogens. The inhibition of this enzyme could lead to estrogen insufficiency resulting in increased adiposity, truncal obesity and uneven fat deposition such as the 'bull hump' seen in HIV patients under HAART [374].

The wide expression of the drug transporter protein, P-gp, generally occurs in tissues, such as the intestines. The effect of medicinal/phytochemical compounds on P-gp limits the bioavailability of orally delivered medicines thereby effluxing the compound back into the gut lumen or by increasing their intracellular concentration. Thus P-gp can have considerable implications for the safety and efficacy of medicines [360].

In a preliminary study [259] the potential for clinically significant drug interactions by AP was identified. This was evident from the *in vitro* inhibition of CYP3A4 and P-gp expression at 100 mg/ml concentration of aqueous and alcoholic extracts of AP. The

data suggested that the co-administration of AP with antiretroviral agents may result in early inhibition of drug metabolism and transport. Since only CYP3A4 was investigated and the range of AP samples used was relatively small, a more comprehensive study was undertaken.

The present study included several different forms of AP such as sun-dried (SUN), shade dried (SHD), freeze dried (FDAP), traditional aqueous extract (TRAD), commercially available raw material (RM 1, 2 and 3) and 6 commercially available AP formulations (Products-A, C, D I, F, M and N), β -sitosterol (BSS), stigmasterol (STG) and stigmastanol (STN), hypoxoside and rooperol. In addition, the range of CYP enzymes studied was extended to include CYP3A4, 3A5 and CYP19 as well as P-gp.

8.3 Materials and Methods

8.3.1 Instrumentation

A Mettler dual range electronic balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing the standards and samples. A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes.

The analyses of the phytochemicals were carried out using the methods and equipment previously discussed in Chapters 4, 5 and 7. In addition, the fluorescence measurements on the microtiter plates were made with the aid of a 4000 series Cytofluor microplate reader (PerSeptive Biosystems, MA, USA).

8.3.2 Reagents and Chemicals

Commercial products containing AP (capsules and tablets) were procured from a local pharmacy in Grahamstown, South Africa. The CYP P 450 enzymes CYP3A4, 3A5, CYP19 and the marker substrate, dibenzyl fluorescein (DBF) were obtained from Gentest Corporation, Woburn, MA, USA. Dulbecco's modified eagle medium, trypsin-EDTA, penicillin-streptomycin-glutamine and fetal bovine serum

were all obtained from Gibco (Invitrogen, Burlington, Ontario). CaCo-2 (6CC-colon carcinoma cells) were procured from ATCC, (American Type Culture Collection, Manassas, USA) Nicotinamide adenine dinucleotide phosphate (NADPH-N-1630), β -sitosterol (BSS), stigmasterol (STG), stigmastanol (STN), 40% β -sitosterol with dihydrobrassicasterol and campesterol (BSS 40%) were purchased from Sigma Chemical Co, St Louis, MO, USA. Water was purified in a Milli-Q[®] System (Millipore, Bedford, USA) and Millex HV[®] Hydrophilic (PVDF, 0.45 μ m) filters were purchased from the same source. Acetonitrile and methanol (HPLC grade) were purchased from Romil Ltd, (Cambridge, UK) and all other chemicals and solvents were of analytical grade. Hypoxoside was extracted and isolated from AP (AP) plant material and characterised using chromatographic and spectroscopic analyses as described in Chapter 3. Rooperol was gratefully received as a gift sample from Dr. Carl Albrecht (Research Co-ordinator, CANSA, South Africa).

8.4 Experimental

8.4.1 Sample Preparation

8.4.1.1 AP Raw Material

Fresh AP corms were collected, dried and processed to yield shade dried (SHD), sun dried (SUN) and freeze dried (FDAP) crude plant material as elaborated in Chapter 4 (Section 4.5.2.1). About 100 mg of each of the well-triturated samples was accurately weighed out in 5 ml Falcon polypropylene tubes (BD Biosciences, San Jose, CA, USA). Four millilitres of HPLC grade water was added to each tube and sonicated for 5 min at 50° C. The commercially available crude raw materials of AP (RM-1, 2 and 3) were procured from various suppliers and were extracted as previously described.

An aqueous decoction of AP (TDAP) was prepared according to the usual method used by Traditional Healers ("*Sangomas*") as discussed in Chapter 4 (Section 4.5.2.4). This aqueous extract was diluted to yield a concentration equivalent to approximately 25 mg of AP/ml.

8.4.1.2 Commercial Products

Amongst the six formulations chosen (**Table: 8.1.**), four were hard gelatin capsules (Products A, C, D I, M and N) and one was a tablet (Product F). For the formulation in tablet dosage form, average weight of twenty five tablets was calculated before powdering them in a mortar using a pestle. The hard gelatin capsules were emptied and triturated separately. One hundred milligrams of each of the powdered products were well-triturated and quantitatively transferred to a 10 ml Falcon centrifuge tube and the volume was made up with HPLC grade water. The samples which were studied for their purported sterols and stanol content (Products A, M and N) were separately prepared by replacing water with methanol (HPLC grade) as solvent. The tubes were sonicated for 20 min before centrifuging and aliquots of the supernatant (10 mg/ml) were filtered (PVDF, 0.45 μ m) before use.

8.4.1.3 Phytochemicals

Hypoxoside, rooperol and STG, STN, BSS and BSS (40%) stock solutions of varying concentrations were prepared using either water or methanol. The hypoxoside (5 mg/ml) and rooperol (2.5 mg/ml) stock solutions were prepared in HPLC grade water, the latter solution being facilitated by heating to 60° C. Separate stock solutions (2.5 mg/ml), each containing STG, STN, BSS and BSS (40%) were prepared in HPLC grade methanol. All stock solutions were freshly prepared prior to use. The stock solutions were either used as such or diluted to the relevant concentration and filtered through PVDF (0.45 μ m) filters.

Table: 8.1.**Contents of Commercial Products**

Product	Contents
Product-A	Sterols and sterolins 40 mg, <i>Hypoxis hemerocallidea</i> -232 mg
Product-C	African Potato 70 mg, Siberian Ginseng 40 mg, Selenium 100 µg, Vitamin A 1320 I.U, Vitamin B1-2 mg, Vitamin B2- 2 mg, Vitamin B6-1 mg, vitamin B12- 1 µg, Vitamin-60 mg, Vitamin-D-100 µg, Vitamin E-10 mg, Nicotinamide-15 mg, Calcium D pantethenate-400 µg, Folic acid- 400 µg, Iron-1 mg, Calcium-12 mg, Copper- 1 mg, Potassium-4 mg, Manganese-1 mg, Zinc-1 mg, Lecithin-50 mg, L-Methionine (Amino acid)-25 mg, L- Glutamine (Amino acid)-25 mg, Chromium picolinate-100 µg.
Product-D I	<i>Hypoxis</i> Rooperi-250 mg, Grapefruit extract-45 mg, Proanthocyanidin-50 mg, Spirulina-2 mg, Beta carotene-2 mg, Thiamine-7.5 mg, Pyridoxine HCl-15 mg, Riboflavin-7.5 mg, Nicotinamide-10 mg, Vitamin B12-4 µg, Folic acid-600 µg, Lipoic acid-20 mg, Zinc AAC-15 mg, Selenium AAC2%-100 µg, Ascorbic acid-60 mg, Vitamin E-18 mg, White Willow bark-50 mg, olive leaf extract- 20 mg, Silicon Dioxide- 12 mg.
Product-F	<i>Hypoxis</i> powder (African Potato)-275 mg, Biocytin-30 mg, Olive leaf extract-50 mg, Ginkgo biloba-40 mg, Grape fruit (Pomelo) seed extract- 5 mg, Vitamin E (di- α -Tocopherol)- 7.5 I.U, Vitamin B2 (Thiamine HCl)-3.5 mg, Vitamin B2 (Riboflavin)-3.5 mg, Niacin (Nicotinamide)-3.5 mg, Vitamin B5 (Pantothenic acid)-3 mg, Vitamin B6 (Pyridoxine HCl)-7.5 mg, Biotin-0.05 mg, Vitamin C (Ascorbic acid)-10 mg, Folic acid-0.6 mg, Vitamin B12 (Cobalamine)-0.005 mg, Zinc AAC-15 mg, Selenium AAC2%-0.05 mg, Soy protein-370 mg.
Product-M	Plant sterols and sterolins-25 mg, Garlic-50 mg, Vitamin C- 150 mg, Vitamin A-333RE, Vitamin E- 10 mg, Vitamin D-4 µg, L-Arginine-10 mg, L-Methionine-33.3 µg, Folic acid-250 µg, Vitamin B12-4 µg, Vitamin B6-3 mg, Biotin-100 µg, Iron-2 mg, Zinc-3 mg, Selenium-5 µg, Copper-0.33 mg, Absorption enzymes-5 mg.
Product-N	Plant Sterols-20 mg, Sterolins-0.2 mg

8.4.2 Cytochrome P450 Assay

An *in vitro* fluorometric microtiter plate assay was modified from the one reported by Crespi *et al* (1997) [375] and Gentest (1998) [376] that was used to assess inhibition of CYP3A4, CYP3A5 and CYP19 by the various extracts, commercial formulations and relevant phytochemicals.

For the assay, 10 µl of sample solution was added to each well of the clear-bottomed opaque walled microtiter plates (96 well, Corning Co-star, model CS00-3632, Corning, NY) followed by 100 µl Milli-Q[®] water, 50 µl buffer (0.5 M phosphate, pH 7.4), 10 µl DBF, 20 µl NADPH (Nicotinamide adenine dinucleotide phosphate, reduced form) and the relevant enzyme (10 µl). Control wells contained the sample, buffer, DBF, NADPH, Milli-Q[®] water and the corresponding denatured

CYP enzyme. For the blank, the corresponding sample was added with Milli-Q[®] water, buffer (0.5 M Phosphate, pH 7.4), DBF, NADPH and the relevant solvent used in the preparation of the sample under investigation.

The samples were maintained at 37° C and fluorescence was read after 30 min for the CYP3A4/3A5 studies and for 50 min for CYP19 interactions. Nine wells were used for each of the samples, their respective controls and blanks.

All the enzymes solutions were freshly thawed before adding to the wells and care was taken that no enzyme was subjected to more than two freeze thaw cycles. The process was carried out under golden fluorescent light (Industrial Lighting, Ottawa, ON, Canada).

8.4.3 P-Glycoprotein Assay

The effect of the samples on P-gp was studied using the method adopted and modified by Parasurampuria *et al* (2001) [360]. The principle used to investigate the functional activity of P-gp in CaCo-2 cells was based on measuring Rhodamine 123 (Rh123) retention/efflux in the presence of P-gp modulators. Rh123 is a cationic, fluorescent dye taken up by the cells and actively pumped out of the cells by P-gp [377]. The ability of the CaCo-2 cells to efflux Rh123 in the presence of P-gp inhibitors is decreased [378, 379] resulting in an increased intracellular accumulation of Rh123 which reflects the inhibitory effect of the samples on P-gp mediated transport and is determined using a fluorescence microplate reader.

The Caco-2 cells were thawed by rapidly increasing the temperature to 37° C. The cell viability was assessed and optimised by ethidium bromide/acridine orange uptake [360]. The Caco-2 cells were then cultured in multi-well (24) cell culture plates (Falcon BD, BD BioSciences, SanJose, CA, USA) for at least 2 days at 37° C in a sterile environment till the cells became confluent. Once the viable cells were calculated, 12 µl Rh123 (1.25×10^{-5} g/ml) was added to a positive control consisting of 50 µl ritonavir solution in methanol (1 mg/ml) and to the test wells containing 50 µl of the relevant test sample solution. In addition, blank solutions containing either methanol or water (50 µl) and Caco-2 cells were used and Rh123 added.

Duplicate cell plates were prepared and 1 plate measured after incubation for 1 hr whilst the second plate was retained in the incubator (37° C) under a 50% CO₂

atmosphere and measured after 24 hr. After each incubation period, cell plates were washed with ice-cold phosphate buffer saline (PBS, pH 7.4) before the addition of 250 µl trypsin-EDTA solution. The cells were again incubated at 37° C for 10 min to separate them from the base of the cell plates. Seven hundred and fifty microlitres of PBS were added to each well and mixed thoroughly. Three hundred microlitres of this cell suspension was transferred in triplicate to a 96 well plate platform (Fischer Scientific-Costar Corning, Pittsburg, USA) before measuring the fluorescence using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

For both the CYP P450 and P-gp assay procedures, all samples were prepared in triplicate with the resultant percent inhibition calculated based on the differences in fluorescence between the test and the blank wells and the mean difference between each control and blank well. Thus nine experimental values were obtained for each sample.

8.4.4 Phytochemical Analysis

The extracts were analyzed for the various phytochemicals of interest using the analytical methods that were optimised and fully validated for all the adequate parameters such as linearity, accuracy, precision, intra and inter-day recovery, specificity, LOD and LOQ.

The extracts and the formulations were assayed for the various phytochemicals purported to be present. Three separate analytical methods and two different extraction procedures were used as previously described (Chapters 4, 5 and 7). The raw material (RM) and the relevant products were analyzed for hypoxoside using the assay method discussed in Chapter 4, the sterols, BSS and STG and stigmastanol (STN) were analyzed with the method discussed in Chapter 5 and rooperol was analyzed using the semi-quantitative method described in Chapter 7 (**Table: 8.2.**).

Table: 8.2.
Phytochemical Content

S No	Sample Name	Concentration in % dry weight, (w/w)				
		Hypoxoside	Rooperol	β -Sitosterol	Stigmasterol	Stigmastanol
1	Product-C	2.60%	-	-	-	-
2	Product-D I	2.40%	-	-	-	-
3	Product-F	1.59%	-	-	-	-
4	Product-A	-	-	9.61%	+ (<1%)	1.51%
5	Product-M	-	-	2.63%	3.04%	-
6	Product-N	-	-	7.61%	+ (<1%)	1.10%
7	FDAP	10.18%	-	-	-	-
8	TDAP	6.96%	-	-	-	-
9	SUN	1.29%	-	-	-	-
10	SHD	2.79%	-	-	-	-
11	RM-1	-	-	-	-	-
12	RM-2	0.61%	-	-	-	-
13	RM-3	9.79%	-	-	-	-

RM= Raw Material

8.4.5 Statistical Analysis

All experimental data were analyzed using the one-way analysis of variance (ANOVA) and significant differences between the means from quadruplicate analysis at ($p < 0.05$) were determined by the Student-Newman-Keuls Multiple Range Test.

8.5 Results

All extracts, four out of six formulations, hypoxoside, rooperol and stigmasterol exhibited inhibitory activity on the CYP P450 enzymes studied. Effects on the transporter protein, P-gp were shown by hypoxoside, 3 of the formulations containing AP and AP plant material. The amounts of the various phytochemical components in the extracts, plant materials and formulations are shown in **Table: 8.2**.

8.5.1 Cytochrome P450 Assay

Phytochemical components which affected CYP P450 enzymes showed increased inhibition with increasing concentration. Aliquots of the sample solutions of AP raw material as well as commercial products containing AP inhibited the activity of all the CYP enzymes (**Table: 8.3.**). Hypoxoside, at a concentration of 3.6 mg/ml showed approximately 33 % inhibition of CYP 3A4 and 11 % inhibition of CYP 3A5 enzyme, with a maximum inhibitory effect of 41 % on the CYP 19 enzymes. In contrast, the aglycone rooperol, showed very high inhibitory activity (>98 %) on CYP enzymes 3A4 and 3A5, even at a low concentration of 50 µg/ml. At 2.5 µg/ml, rooperol showed a 32 % inhibitory effect on CYP19. It should be emphasized that the inhibitory effects elicited by the various AP plant materials, extracts and formulations could not be due to rooperol, since the latter was not present in any of those samples. Among the AP plant materials, FDAP was found to have the highest content of hypoxoside. At a concentration of 25 mg/ml, FDAP significantly inhibited (>70 %) the three CYP enzymes tested with highest effect on CYP 19 (>93 %). The SUN and SHD samples had similar inhibitory activities on all the three enzymes. The traditional aqueous decoction provided lower (20-40 %) inhibition compared to FDAP, SUN and SHD.

The Products (C, D I and F) that contained hypoxoside showed relatively high inhibitory activity. Amongst them, Product-D I exhibited the least inhibitory activity (<50 %) towards all the CYP enzymes. The sterols and STN were individually studied for their inhibitory activity on CYP enzymes at a concentration of 2.5 mg/ml using methanol as solvent. STG was the only compound to show inhibitory activity. The formulations purported to contain sterols and stanols were also studied and only one, Product M, showed inhibitory activity against all CYP enzymes. It was interesting to note that this particular formulation contained quantifiable amounts of STG whereas Products A and N, which did not exhibit inhibitory activity did not contain this particular sterol in quantifiable amounts.

Table: 8.3.

The % Inhibition of Human Cytochrome Enzymes (n ≥3; mean ± SD)

Samples	Concentration	CYP 3A4 % Inhibition ± SD	CYP 3A5 % Inhibition ± SD
FDAP	25 mg/ml	71.1 ± 1.3	77.5 ± 0.6
TDAP	25 mg/ml	20.7 ± 2.3	40.4 ± 1.7
SHD	25 mg/ml	61.2 ± 0.1	70.2 ± 0.8
SUN	25 mg/ml	61.9 ± 1.3	74.4 ± 1.0
RM-1	25 mg/ml	27.6 ± 3.0	29.1 ± 0.5
RM-2	25 mg/ml	69.8 ± 0.5	72.7 ± 0.1
RM-3	25 mg/ml	43.9 ± 1.5	63.5 ± 0.7
Hypoxoside	3.6 mg/ml	32.9 ± 3.3	10.2 ± 0.7
Rooperol	50 µg/ml	>98 ± 0.0	>98 ± 0.0
β-Sitosterol	2.5 mg/ml	0 ± 0.0	0.0 ± 0.0
Stigmasterol	2.5 mg/ml	67.7 ± 1.6	30.6 ± 1.1
Stigmastanol	2.5 mg/ml	0.0 ± 0.0	0.0 ± 0.0
40% β-Sitosterol	2.5 mg/ml	0.0 ± 0.0	0.0 ± 0.0
Product-C	10 mg/ml	62.2 ± 1.6	73.1 ± 3.8
Product-D I	10 mg/ml	48.5 ± 0.9	17.4 ± 0.7
Product-F	10 mg/ml	78.2 ± 1.7	18.9 ± 0.7
Product-A	5 mg/ml	0 ± 0.0	0.0 ± 0.0
Product-M	5 mg/ml	40.9 ± 1.3	84.9 ± 4.8
Product-N	5 mg/ml	0.0 ± 0.0	0.0 ± 0.0

8.5.2 P-Glycoprotein Assay

The effects of the above samples on P-gp were examined using ritonavir (1 mg/ml) as the positive control. Since only five sets of samples (in triplicate) could be studied on a plate using a single control, three plates were used (**Table: 8.4.**). The induction of P-gp was denoted by a negative value (loss of Rh123 from the cells) and inhibition was shown by a positive value (retention of Rh123 in the cells). Ritonavir showed initial (1 hr) induction of P-gp protein followed by inhibition (24 hr).

The results of the P-gp studies were in contrast to those observed on the CYP enzymes. Whilst hypoxoside showed low inhibitory activity on all the CYP enzymes, this compound, at a concentration of 5 mg/ml showed remarkably high induction of P-gp at one hr (-16.5%) and at 24 hr (-39%).

Table: 8.4.
The % Induction and % Inhibition of P-glycoprotein (n ≥6)

Sample name	Extraction solvent	Concentration	1 hr % ± S.D	24 Hr % ± S.D
Plate No:01				
Ritonavir	55% Methanol	1 mg/ml	0.9 ± 2.8	31.3 ± 10.1
FDAP	Water	25 mg/ml	-11.9 ± 2.2	-31.4 ± 1.2
SHD	Water	25 mg/ml	-2.3 ± 1.9	-21.9 ± 2.1
SUN	Water	25 mg/ml	-11.5 ± 2.6	-8.2 ± 1.4
RM-2	Water	25 mg/ml	-2.0 ± 2.5	-6.4 ± 1.9
RM-3	Water	25 mg/ml	13.4 ± 3.9	-10.2 ± 3.4
Plate No:02				
Ritonavir	55% Methanol	1 mg/ml	-3.0 ± 2.1	39.2 ± 3.1
TDAP	Water	250 mg/ml	-21.1 ± 1.5	-38.0 ± 2.8
Product-C	Water	10 mg/ml	-20.3 ± 1.4	-38.8 ± 2.1
Product-D I	Water	10 mg/ml	-23.4 ± 3.1	-40.8 ± 3.6
Product-F	Water	10 mg/ml	-18.8 ± 2.5	-40.9 ± 7.1
Hypoxoside	Water	5 mg/ml	-16.5 ± 1.2	-39.0 ± 5.5
Plate No:03				
Ritonavir	55% Methanol	1 mg/ml	-5.0 ± 1.9	30.8 ± 6.6
β-Sitosterol	Methanol	2.5 mg/ml	-1.9 ± 1.6	-4.7 ± 3.0
Stigmatsterol	Methanol	2.5 mg/ml	-5.5 ± 2.5	-1.7 ± 5.4
Stigmastanol	Methanol	2.5 mg/ml	-4.8 ± 4.9	-7.6 ± 3.5
Rooperol	Water	10 µg/ml	-2.1 ± 2.0	-1.3 ± 2.4

The SUN and the SHD samples did not show any significant induction of P-gp which concurred with their relatively low hypoxoside content whereas the traditional extract of AP showed high (-38%) induction of the protein transporter. It was interesting to note that the commercially available AP raw material (RM-3), in spite of its significant content of hypoxoside (~9%, w/w), showed inhibition (13.4%) after one hr and induction at twenty four hr (-10.2%). Moreover, the three AP Products, (C, D I and F) each containing similar amounts of hypoxoside (1.5 –2.5%) to that of SUN and SHD showed significant induction at one (>20%) and 24 hr (-38 to -41%) of the study. Rooperol, in contrast to its high inhibitory activity on CYP enzymes showed negligible induction of P-gp (<2%).

At a concentration of 2.5 mg/ml, BSS induced P-gp at a level of -3.5%, STG at -5.5% and STN at a level of -7.6%. Recall that only STG showed activity against CYP enzymes.

8.6 Discussion

The effects of AP extracts (25 mg/ml) on CYP3A4 and P-glycoprotein were in agreement with data obtained from a preliminary study [259] using a lower concentration that used previously (100 mg/ml).

Hypoxoside did not show any significant inhibitory effect on any of the CYP enzymes at a relatively high concentration of 3.6 mg/ml. Interestingly, the various AP samples, FDAP, SUN, SHAD, RM 1, 2 and 3 as well as a freshly prepared traditional extract (25 mg/ml each) all showed pronounced inhibitory effects on CYP enzymes, even at a lower content of hypoxoside than that tested in pure form. This phenomenon was also observed with the Products of AP (C, D I and F), which contained varying content of hypoxoside (1.6-2.6%, w/w). A possible explanation for this inhibitory activity may be due to other phytoconstituents/ingredients present in these samples and thus not related to hypoxoside content. The CYP inhibitory activity of both SHAD and SUN samples were very similar and irrespective of the hypoxoside content in the SHAD being approximately double that in the SUN sample. This reinforces the probability that there are other photo-stable phytoconstituents present in AP, which contribute to the inhibitory activity.

Whilst hypoxoside did not show any significant inhibitory activity of CYP enzymes, its aglycone, rooperol, which is not present in AP *per se*, was shown to have significant inhibitory activity even at a very low concentration (50 µg/ml). Rooperol is converted to its glucuronides and sulphates as it crosses the gastrointestinal membrane and enters into the systemic circulation. These metabolites are subsequently converted back to rooperol by deconjugative enzymes [243]. Hence the consumption of traditional extracts of AP could result in a reinforced degree of CYP enzyme inhibitory activity since the rich inactive content of hypoxoside would be converted to rooperol in the gastrointestinal tract of humans and combined with the other phytoconstituents should complement the inhibitory process.

Among the sterols and STN, STG (2.5 mg/ml) was the only compound found to act on the CYP enzymes. An inhibitory effect was also observed in one of the Products, (Product M), which was found to have the highest content of STG. However, this is a relatively low sterol content (~0.3 mg) compared to the concentration of 2.5 mg/ml STG (95%) used *per se*. Hence, any observed inhibitory effect of this formulation is unlikely to be due to STG content only. Furthermore, since garlic, one of the many constituents of this formulation, has previously been shown to inhibit CYP enzymes [25], the garlic effect cannot be precluded.

Hypoxoside induced P-gp efflux of Rh123 cationic dye (-39%) at a concentration of 5 mg/ml. The percentage induction of P-gp by the various AP samples (FDAP, SUN, SHD, RM 1 and 2) as well as the traditional extract was significant and in proportion to their hypoxoside content. FDAP and the traditional extract of AP showed the highest induction of P-gp that correlated with their high content of hypoxoside compared to the other samples. SHD produced double the % induction compared to SUN also correlating with hypoxoside content. Whilst the results of most of the raw material samples were in congruence with their respective hypoxoside content, only RM-3 exhibited intriguing results. Although this sample had the second highest hypoxoside content, it initially produced a ~13.4% inhibition of P-gp and subsequently caused a ~10.2% induction. The three commercial products used (C, D I, F) which contained similar hypoxoside content, showed significant induction of P-gp (>38%), which was higher than the values exhibited by pure hypoxoside alone. Once again, there appears to be synergistic activity due to the presence of other phytochemical constituents/ingredients in these formulations.

Unlike its effect on CYP enzymes, the aglycone, rooperol did not show any notable effect on P-gp even at relatively high concentration. Similarly, no effect on P-gp was observed with the sterols and the stanol, STN.

8.7 Conclusions

Hypoxoside, the important phytochemical component in AP does not inhibit CYP 3A4, 3A5 or CYP 19. On the other hand, the aglycone, rooperol is quite active and implies that traditional extracts of AP, when consumed in the usual daily dose (200 ml containing >37 mg AP/ml [121], should provide a significant *in vivo* concentration of active inhibitory substance. STG had a relatively minor inhibitory effect whereas no inhibitory effect was shown by either BSS or STN. Three formulations which contained hypoxoside showed inhibitory activity whilst amongst a further three formulations which contained BSS but no hypoxoside, only one which in addition contained a quantifiable amount of STG showed an inhibitory effect on the CYP enzymes. This effect was probably mainly due to the garlic content in that particular formulation, which was absent in all the other formulations.

Hypoxoside has been shown to induce the transporter protein, P-gp but neither BSS, STG nor STN showed any effect. Rooperol did not show any activity on P-gp and all AP samples, formulations and traditional extracts that contained hypoxoside were also active.

These *in vitro* results suggest that concurrent use of AP together with other medication requires a degree of caution especially when treating patients with medicines which can act as substrates for CYP enzymes and P-gp. Even though such possible interactions will need to be confirmed by appropriate *in vivo* studies, the *in vitro* results suggest that the concomitant use of AP along with anti-retrovirals in the treatment of HIV positive patients could be detrimental leading to several clinical ramifications.

Chapter 9

In Vitro Dissolution Studies

9.1 Introduction

Recently there has been an increase in the popularity and use of ATMs and CAMs, however, control of their quality, safety and efficacy have not received the necessary attention by many regulatory authorities around the world. Unlike orthodox medicines, many ATMs and CAMs appear to be unregulated and come to market without complying with well-established criteria for quality let alone safety and efficacy.

In vitro dissolution testing is an important component of pharmaceutical development and quality control since it provides guidance in the design and assessment of new formulations. The robustness and uniformity of manufacturing batches are also determined by *in vitro* dissolution tests. *In vitro* dissolution testing is an important tool to monitor the release of relevant component (s) from a dosage form and possibly, also to predict the *in vivo* availability from orally administered solid dosage forms. Whereas assay methods for use in QC of AP products have been developed, validated and described in this thesis, a further important QC criterion, namely, dissolution testing needs to be addressed. This is so since in addition to confirming content and compliance with label claims for solid oral dosage forms, it is also necessary to determine the release properties required to enable adequate absorption from the gastro-intestinal tract (GIT) following administration.

The maintenance of the release properties required to effect absorption from the GIT is considered as an important feature as a QC parameter. The safety and efficacy of drugs can be guaranteed only when this specific parameter is reliable and reproducible from batch to batch. In comparison to chemically defined compounds used in orthodox medicines, most of the herbal medicinal products (HMPs) are poorly characterised in terms of their pharmaceutical properties. Often, plant extracts have been assumed entirely as an active moiety since it is usually difficult to identify the individual components responsible for the pharmacological activity as well for their

synergistic action. However, for those with identified active components, it should be possible to compare the products with respect to their biopharmaceutical properties.

The guidelines for dissolution testing of oral dosage forms have been developed by the FDA and the FIP [380] but the application of such guidelines to HMPs has been complicated by the lack of data to confirm the utility/activity of components in many of herbal extracts and products. In a few countries such as Germany, the quality requirements for dosage forms containing single active entities are also applicable to HMPs whereas in USA, HMPs are generally considered as nutritional supplements. A chapter has been included in the United States Pharmacopoeia (USP) which provides guidelines for dissolution testing [381] of such nutritional supplements. According to its requirement, more than 75% of the label strength of the substance must be released under the test conditions chosen. However, this requirement is mainly intended for products containing vitamins and minerals where the manufacturer is only required to characterise the dissolution of one representative substance.

CAMs are complex, consisting of multiple components that may be active individually or in combination. The “actives” in CAMs are generally defined to be the whole herbal preparation, e.g. the extract in its entirety. The USP [381] suggests that for botanicals, compliance with dissolution testing is performed by testing six or more dosage units individually in each vessel and measuring one or more index marker compounds or the extract specified in the individual monograph. This non-specific dissolution testing is meant to be diagnostic of known technological problems that may arise due to coatings, lubricants, and other substances inherent in the manufacturing process.

The dissolution measurement also helps in the assessment of the extent of decomposition that may have been produced by excessive drying or other manipulations used in the manufacture or preparation of the botanical extracts. It is assumed through this procedure that if the index/marker compounds or the extract is demonstrated to have dissolved within the time frame and under the conditions specified, the dosage form does not suffer from any of the above formulation or manufacturing related problems.

The USP [381] also suggests that disintegration testing could be used to check compliance of nutritional supplements and botanical products to individual class monographs. Disintegration testing applies only to uncoated/plain coated tablets and

to soft/hard gelatin capsules and not to tablets or capsules designed to liberate vitamin or mineral content over an extended period or where the label states that the dosage form is to be chewed.

In Europe, the European Agency for the Evaluation of Medicinal Products (EMA) proposes three categories of HMPs based on the characterization of their active components [382].

Category A: Standardised extracts

The HMPs for which an active moiety/moieties has/have been definitively identified. Standardisation of such products may be achieved by adjusting the level of actives by the addition of extracts or inert excipients that have higher or lower levels of the desired active compounds.

Examples: silymarin, aescin and sennoside [383].

Category B: Quantified extracts

The HMPs for which the active ingredient (s) has/have not been clearly identified which are known to contribute to the pharmacological or synergistic activity. Standardisation of these products may be achieved by blending batches of either raw botanical material or herbal preparations of higher or lower quality, however, the addition of inert excipients is not performed.

Examples: ginsenosides, procyanidines, flavonoides and hyperforin [383].

Category C: Unknown active ingredients

The HMPs for which no individual active ingredients have been identified, but have a traditional place in the therapy of certain diseases. Here, chemical compounds which may not contribute to any pharmacological activity are selected as markers for Good Manufacturing Practice (GMP).

Examples: extracts of valerianae and echinaceae [383].

The EMA exempts dissolution tests for HMPs falling under the categories B and C if the product is being formulated as an immediate release. The dissolution test could be substituted with a disintegration test if the active ingredient is known (category A) to be soluble in aqueous solutions at pH values typical to the GIT [384].

However, in Germany, it is required that all the HMPs under category A be subjected to dissolution tests, but the guidelines for categories B and C remains the same as that of EMEA.

For the purpose of evaluation of pharmaceutical and biopharmaceutical quality, dissolution testing can be readily applied to those products for which the components that contribute to the activity have been identified. Hence, dissolution testing is seen as appropriate for HMPs under category A or B. A typical example of such a product under category B is St. John's Wort, where one of the important components with purported activity, hyperforin, was tested for dissolution using biorelevant dissolution media [382] such as fasted state simulation fluid (FaSSIF) and fed state simulation fluid (FeSSIF).

The FDA dissolution guidance for industry [380] recommends the use of buffers in the range of pH 1.2 to 7.5 for initial dissolution method development. Generally, the development of *in vitro* dissolution test methods have been based on a trial and error approach. The biopharmaceutics classification system (BCS) has made this process easier by providing information on the classification of drugs and suitable dissolution tests for them.

Class I drugs with high solubility and permeability are those which can readily dissolve in aqueous media in a pH range of 1 to 8. In Class II, drugs that have low solubility but a high permeability, the choice of medium plays an important role in their dissolution since their solubility in aqueous media is insufficient to dissolve the whole drug under the usual aqueous conditions of the GIT [385]. It is considered that the dissolution of these drugs is the rate limiting step in their absorption, hence a wide variety of factors such as surfactants, pH, buffer capacity, ionic strength and volume of the media have to be adjusted to simulate the conditions of the upper GIT. Considering the abovementioned physiological conditions, several dissolution media such as milk, simulated gastric fluid (SGF), simulated intestinal fluid (SIF), fasted state simulation fluid (FaSSIF, **Table: 9.1.**) and fed state simulation fluid (FeSSIF, **Table: 9.2.**), have been introduced to better predict the *in vivo* performance of Class II drugs.

FaSSIF and FeSSIF are considered suitable for the dissolution of lipophilic compounds such as sterols and stanol in view of the content of lecithin and bile salts included in the dissolution medium [206].

Table: 9.1.
Fasted State Simulating Fluid (FaSSIF)

Content	Quantity/Concentration
Potassium di hydrogen phosphate	3.9 g
Sodium hydroxide (to adjust)	pH –5.3, 6.5, 7.4, 8.0
Sodium taurocholate	3 mM
Lecithin	0.75 mM
Potassium Chloride	7.7 g
Water	1000 ml

FaSSIF has usually been used at a pH of 6.5 because this particular pH was intended to simulate intestinal pH and provide useful information to predict *in vivo* dissolution. Although bicarbonate ions are generally present in human GIT fluids where it provides a buffering role, phosphate buffer is used in FaSSIF in order to avoid pH instability which would occur with the inclusion of a bicarbonate buffer. Bile salts and lecithin are typical for the fasted state medium and is adjusted with potassium chloride to reach an optimum iso-osmolarity.

Table: 9.2.
Fed State Simulating Fluid (FeSSIF)

Content	Quantity/Concentration
Acetic acid	8.65 g
Sodium hydroxide (to adjust)	pH –5, 8.0
Sodium taurocholate	15 mM
Lecithin	3.75 mM
Potassium Chloride	15.2 g
Water	1000 ml

FeSSIF is slightly different from FaSSIF in that that medium contains an acetate buffer instead of phosphate buffer in order to simulate the conditions of the upper small intestine at fed state. In the FeSSIF medium, the buffer capacity is 75 mEq per liter per pH unit, which is higher than that of FaSSIF. Bile salt and lecithin

are also higher in quantity with an osmolarity of 600 mOsm compared to FaSSIF. These features differentiate FaSSIF from FeSSIF by reflecting the contributions of food and other secretions. The preferred pH 5.0 of FeSSIF is considered to simulate the fed state of the intestine in human body [386].

9.2 Background and Objectives

Whenever the components that contribute to the activity of HMPs have been identified, it should be required that dissolution testing be performed to evaluate the pharmaceutical and biopharmaceutical quality of those products. Sterols (BSS, STG), sterolins (BSSG) and STN are known/accepted for their pharmacological activity and this infers that AP products containing labelled content of sterols and stanols should be classified as category A. On the other hand, hypoxoside is considered to be a unique active marker of AP and its formulations, but the pharmacological activity of this glucoside is largely unknown when compared to its aglycone rooperol, the latter compound not being present in AP. Considering the above, AP and its formulations which have not been fortified with sterols, sterolins or stanol (no label claim for such content) could be included under category B of the EMEA guideline.

There is currently no monograph or QC method for commercial formulations containing AP. Similarly, to-date there have been no reported methods to evaluate the *in vitro* dissolution profile of AP products based on their sterols (BSS and STG) or STN content. There are also no methods or data on the *in vitro* dissolution profile of AP products on the basis of hypoxoside content.

From the results obtained when some commercially available AP products were assayed (Chapter 5) it was found that BSS was the major component present in some AP products when compared to their STG and STN content. This sterol was consequently chosen as the marker for the dissolution testing of AP products with labelled content of BSS. In addition, hypoxoside, was chosen as a marker for products which were assayed (Chapter 4) and found to contain quantifiable amounts of this norlignan component.

The prescribed dissolution conditions according to the USP were used to monitor the release of the relevant components of AP. However, in view of the fact

that BSS is relatively insoluble under aqueous conditions, the usual conditions for dissolution testing appeared to be inappropriate to monitor the release of this component from AP products. Hence, biorelevant dissolution media such as FaSSIF and FeSSIF containing lecithin as emulgent and bile salts as solubilizer were chosen as an option to monitor the release of BSS from the AP products studied.

9.3 Materials and Methods

9.3.1 Instrumentation

A Hanson SR8 PLUS AutoplusTM, MultifillTM and maximiser syringe fraction collector (Chatsworth, CA, USA) were used for the dissolution studies. A Mettler dual range electronic balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing the standards and samples. A Crison GLP21 pH Meter (Crison, Barcelona, Spain) was used to measure and adjust relevant pH values.

Analyses of the phytochemicals were carried out using the equipment and validated analytical methods previously discussed in Chapters 4 and 5.

9.3.2 Reagents and Chemicals

In addition to the reagents and chemicals mentioned in Chapter 4 and 5 the following chemicals and reagents were used. Sodium taurocholate (>98%) and egg-phosphatidylcholine (99.1%) were purchased from Sigma (Missouri, USA). Potassium dihydrogen phosphate, sodium hydroxide pellets, sodium dihydrogen phosphate and potassium chloride, all analytical grade from Rochelle Chemicals (Port Elizabeth, South Africa). Acetic acid of analytical grade was purchased from BDL Chemicals Ltd, (Poole, UK).

Seven formulations containing AP were purchased from a local retail pharmacy in Grahamstown. South Africa. The labeled weight/unit of each of the formulations were 400 mg (A), 500 mg (B), 500 mg (C), 600 mg (D II), 1000 mg (F) 400 mg (M) and 200 mg (N) respectively (Chapter 2, **Table:** 2.4.). Four of the products (Products A, B, C, D II, M and N) were capsules and only Product F was in tablet form. Amongst these products, A, B, M and N were purported to be fortified

with additional quantities of sterols and sterolins (although no hypoxoside was detected in any of these products, Chapter 4), where the release of BSS was monitored. Products C, D II and F (no label claims made for BSS content) were used to monitor the dissolution of hypoxoside.

9.4 Experimental

9.4.1 Dissolution Conditions

Dissolutions tests were performed using the USP-2 (Paddle) apparatus of the USP [381] for Product F (tablet dosage form) whereas Type 1 (Basket) apparatus of the USP was used for all the other capsule dosage forms (Products A, B, C, D II, M and N). Each of the vessels were filled with 900 ml of the appropriate dissolution medium and the temperature of the vessel contents were maintained at $37 \pm 0.5^\circ \text{C}$. The rotation speed of the paddle as well as the basket were set at 100 rotations per min (rpm). Volume adjustments of the media were made by replacement of the withdrawn sample volume with fresh buffer at the same pH stored in a reservoir at the same temperature.

9.4.2 Analyses of the Dissolution Samples

Samples of about 2 ml were withdrawn from the dissolution vessels through 0.2 μm in-line filters at various time intervals 0.2 ml of relevant internal standard was added to 1.8 ml of each sample. Sulphamerazine (10 $\mu\text{g}/\text{ml}$) was used as internal standard for the hypoxoside measurements and cholesterol (50 $\mu\text{g}/\text{ml}$) was used for the samples analyzed for BSS. Adjustments in the calculations were done to compensate for dilutions of dissolution media as a result of volume replacement following sample withdrawal and the cumulative amount (mg) of drug released at different time intervals was calculated. These values were then converted to % cumulative drug release with respect to the assay value of the respective formulations and the relevant dissolution profiles were established.

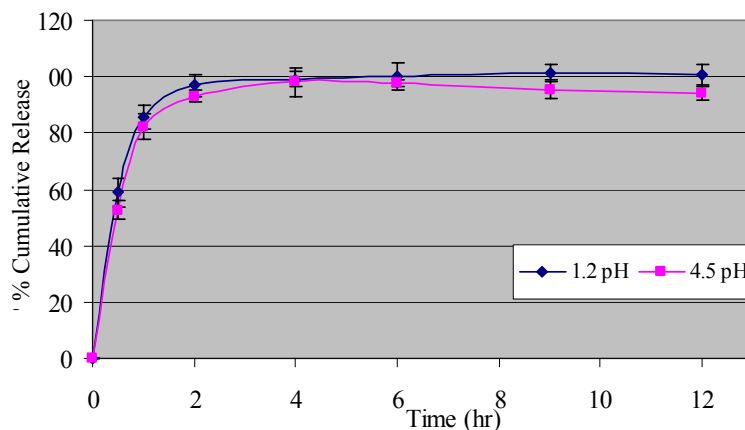
9.4.3 In Vitro Dissolution Method Development

9.4.3.1 Products Containing Hypoxoside

The US-FDA dissolution guidance for industry [380] recommends the use of buffered dissolution media in the range of pH 1.2 to 7.5. However, since hypoxoside is relatively unstable at higher pH values, dissolution media at two pH values (1.2 and 4.5) were used for these studies. The dissolution medium at pH 1.2 was made using hydrochloric acid (100 mM), whereas the dissolution medium at pH 4.5 was prepared with 100 mM phosphoric acid and adjusted with sodium hydroxide pellets. Product-D II was chosen to establish the optimum pH for dissolution. This product was chosen since it was previously assayed to have a high content of hypoxoside (21.67 mg/dosage unit). Four units of the product were added to each of two dissolution vessels containing 900 ml of the respective dissolution medium. Samples were withdrawn at intervals of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 12.0 hr and were analyzed using the validated HPLC method (Chapter 4). The results of the study revealed similar profiles for Product-D II in both pH 1.2 and 4.5 where ~99% hypoxoside was released after 4 hr at pH 1.2 and a slightly lower value at pH 4.5 (**Figure: 9.1.**). From the results of these studies, dissolution media at either pH 1.2 or 4.5 appeared to be suitable for use in dissolution testing of hypoxoside.

Figure: 9.1.

Dissolution of Product-D II at pH 1.2 and 4.5



9.4.3.2 Products Containing β -Sitosterol

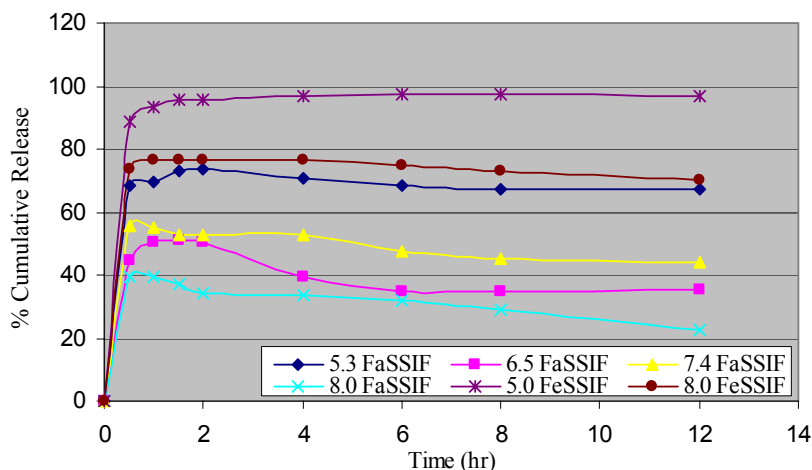
BSS is relatively insoluble in aqueous media and samples of AP product containing BSS were exposed to 4 different dissolution media (pH 1.2, 4.5, 6.8 and 7.8) and analyzed after 12 hr. BSS was not be detected in any of the 12 hr samples, thereby confirming the compound's aqueous insolubility.

As a result, alternative dissolution media were considered for the dissolution testing of BSS. FaSSIF (**Table: 9.1.**) is a dissolution medium which has been proposed by Dressman *et al* (2000) as a medium which represents the fasted state in the proximal small intestine of humans [386]. Although FaSSIF is generally used at a pH of 6.5, four different pHs, 5.3, 6.5, 7.4 and 8.0 were used for these dissolution studies since the current objective was not to attempt to correlate *in vitro-in vivo* results but rather to establish an appropriate discriminating dissolution medium for use with a relatively insoluble compound such as BSS, for the QC of products containing this sterol.

Samples were withdrawn at regular intervals and following analysis by HPLC, BSS dissolution was found to be slightly higher (~70%) when compared to pH 6.5 (~60%) and decreased further with increasing pH (**Figure: 9.2.**). The FeSSIF was subsequently investigated to determine whether it could provide dissolution conditions to effect a higher degree of dissolution.

Whereas the use of FeSSIF is usually conducted at a preferred pH of 5.0, a pH of 8.0 was also investigated. Analysis of the samples of Product A indicated that ~88 and ~93% of BSS was released in the first 30 min and one hr respectively at pH 5.0 compared to the release at the higher pH of 8.0, where ~73 and ~78% of BSS was released at 30 min and one hr respectively. It is thus apparent that the inclusion of bile salts and lecithin facilitate the dissolution of BSS in addition to the effects of pH. Justification for the inclusion of bile salts and lecithin can be gleaned from previously reported data that the bioavailability of stigmastanol, which has similar physicochemical properties to BSS, increases in the presence of lecithin by 34% following a 300 mg dose level [206].

Figure: 9.2.
Dissolution of Product-A



It is therefore seen that in view of the fact that the usual aqueous dissolution media are not applicable to determine the dissolution of BSS, the alternate dissolution medium of FeSSIF at pH 5.0 was an acceptable choice. Hence, this medium was selected to study the *in vitro* release profile of different products containing BSS.

9.4.4 Dissolution of Products Containing Hypoxoside

The dissolution of the different products containing AP were conducted at pH 1.2 under the previously mentioned conditions using three dissolution vessels for each of Products C and F and two vessels for Product-D II which was again included. Fresh dissolution medium at 37° C from the reservoir was used to replace the volume removed by sampling and corrections for dilutions were made prior to the calculation of the cumulative percentage (%) release of hypoxoside.

9.4.5 Dissolution of Products Containing β -Sitosterol

The dissolution of the different products containing BSS were conducted at pH 5.0 under the previously mentioned conditions using FeSSIF as dissolution medium. Two to four units of each product were placed in two dissolution vessel/product and

samples were withdrawn at regular intervals. Fresh dissolution medium at 37° C from the reservoir was used to replace the volume removed by sampling and corrections for dilutions were made prior to the calculation of the cumulative % release of BSS.

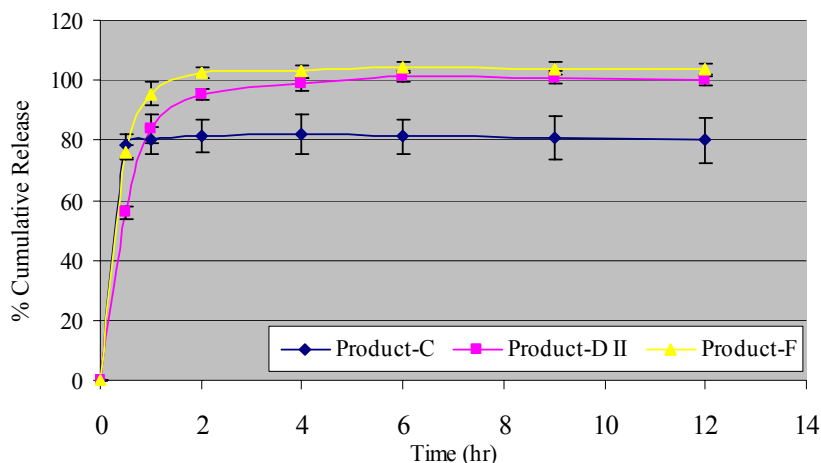
9.5 Results and Discussion

9.5.1 Dissolution Profiles of Products containing Hypoxoside

The samples of different products (C, D II and F) were analyzed at pH 1.2 and their % cumulative release was calculated. (Figure: 9.3.). All the products under this condition released more than 75% hypoxoside within one hr of the study. Product-D II (capsule dosage form) showed similar release of hypoxoside as was previously shown during the development studies (~83.6 % in one hr).

Figure: 9.3.

Dissolution Profiles of Different Products containing Hypoxoside in Phosphate Buffer (pH 1.2)



Product F (tablet dosage form), on the other hand showed a much greater percentage release after one hr (~95.1). Product C released an average of ~79.9% of hypoxoside at one hr and the degree of dissolution did not increase further as seen by the plateau on that product's profile. This could be due to possible variation in content

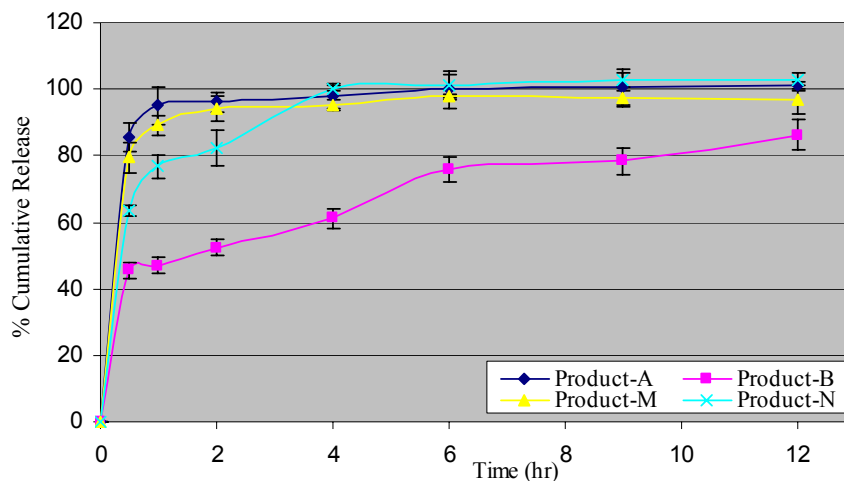
uniformity, interaction (binding/adsorption) of excipients and/or other components present in the raw material used in preparing the capsule dosage form.

9.5.2 Dissolution Profiles of Products containing β -Sitosterol

The dissolution profiles in FeSSIF medium (pH 5.0) of products A, B, M and N containing BSS are shown in **Figure: 9.4**. All the products except Product B released at least 75% of BSS after one hr. Only ~47% BSS was released from Product B after one hr but increased to ~86% at 12 hr. Release of BSS from Product N was greater than any of the other products (~102 % at 12 hr) even though there appeared to be a slight lag in dissolution between 0.5 -2 hr. Dissolution of BSS from Products A and M reached a plateau after two hr (>80 % after 1 hr) where dissolution was seen to be >90%. This feature augurs well for the quality of these products, especially with respect to absorption of BSS following administration.

Figure: 9.4.

Dissolution Profiles of Different Products containing β -Sitosterol in FeSSIF (pH 5.0)



9.6 Conclusions

Over the past few decades, *in vitro* dissolution testing has been considered to be an important tool to assess and control variables associated with formulation which

may alter the release characteristics of the active moiety from the dosage form. Notwithstanding, *in vitro* dissolution testing is one of the most important and useful test methods for assuring product quality, which often aids in guiding the selection of prototype formulations and helps to determine optimum amounts of known ingredients needed to achieve requisite drug release profiles. This feature helps in assuring product quality and batch-to-batch consistency. It is also a valuable tool to assess drug product stability and shelf-life since the dissolution characteristics of products may change over time.

Considering the above advantages, dissolution testing could be used as an important component in the QC of CAMs since it provides information on the rate and amount of active compound (s) which is/are released over a specific period of time.

In the present study, the release profiles of the different products under study indicated that they were intended for immediate release since most of them (except Product B) released 75% of hypoxoside or BSS within one hr under their respective dissolution conditions. This was expected since the formulations were in capsule form (except Product F) and not labeled for extended release.

In vitro dissolution for medicinal products is considered pertinent, both from the QC point of view and also from bioavailability considerations. Furthermore, considering the above features, *in vitro* dissolution could be a useful predictor of bioavailability and the subsequent clinical performance of products provided that an acceptable *in vitro-in vivo* correlation (IVIVC) has been established.

Chapter 10

Sterols and Sterolins in African Potato; Fact or Fiction?

10.1 Introduction

Beta-sitosterol glycoside (BSSG) which is a member of the group of sterol glycosides commonly also known as sterolins, together with other such sterol glycosides have been purported to be present in African Potato (AP). Amongst the various claims made, sterolins have been specifically indicated for the treatment of benign prostate hypertrophy whose action has been ascribed to the inhibition of 5α -reductase or to diminished binding of dihydrotestosterone within the prostate [387]. Various commercial preparations have been marketed as containing AP extracts or plant material, specifically for their sterol/sterolin content. As previously mentioned in Chapter 2, this specific ratio has been claimed to enhance the *in vitro* proliferative response of human T cells, more so than individual sterols at the same concentration and has been used to market and promote sales of this particular product [162-166, 198, 226, 227, 260]. However apart from such reports that have emanated mainly from the single source outlined above the published literature is conspicuously absent in this regard.

Early claims relating to the medicinal properties of β -sitosterol and its glycoside in AP have been reported in the literature [148]. Although not substantiated by strong pharmacological data, several patents have been filed on the usage and medicinal claims of sterolin enriched extracts of AP for various therapeutic purposes [152-156] and have been discussed in Chapter 2 (Section 2.7). In particular, a patent filed by [155] claimed that the potency of an extract from *Hypoxis* species, AP, in the

treatment of prostate hypertrophy was correlated to chemically non-defined phytosterol glucosides, (sterolin such as BSSG).

The highest yield mentioned in the U S patent entitled “Extraction of sterolins from plant material ”, was about 9.01 mg sterolin in 100 g of enriched aqueous extract of AP [148, 152, 153]. The extractive value of the above aqueous extract, defined as the content of extractable matter in mg per gram of plant material, was not mentioned in the patent. Repeated extracts of AP conducted in our laboratories consistently yielded on average, approximately 30% w/w dry aqueous extract from fresh corms of AP. From the above data, the 9.01 mg of sterolin (BSSG) in 100 g of aqueous extract of AP can thus be seen to emanate from approximately 300 g of fresh AP plant material.

Sterolins are converted to their corresponding sterols in the gastrointestinal tract (GIT) of humans following ingestion and the resulting sterol is thought to be responsible for the purported therapeutic effect [193]. Considering the molecular weight ratio of BSSG (molecular weight, 576.85) to BSS (molecular weight, 414.65), 9.01 mg is stoichiometrically converted to about 6.5 mg β -sitosterol which is far lower than reported therapeutic concentrations [191, 193-195, 388]. To our knowledge, to-date, except for the claims made in the patent there have been no publications describing the isolation and analysis of sterols or sterolins in AP nor any scientific data to substantiate the amount of these phytoconstituents required for therapeutic activity and /or their presence in therapeutic concentrations in any species under the genus *Hypoxis*. The foregoing has been substantiated by Nicoletti *et al* (1992) [76] and Hostetmann *et al* (2000) [250].

Referral to the original patent which describes the extract of sterolins from AP corms, however does not provide scientific information relating to the methods used for qualitative and quantitative analysis of those compounds nor were any associated scientific data included to confirm their presence or otherwise. Hence the objective of this study was to re-visit the patent procedures and attempt to reproduce the information provided in that patent in order to confirm the presence and quantity of sterolins, particularly BSSG in AP corms. For this purpose AP corms were harvested and extracted as described in the patent which is similar to the procedure used to prepare a traditional extract of AP.

10.2 Experimental

10.2.1 Instrumentation and Analytical Method

The instrumentation and the analytical method for both the HPLC-UV method as for analysis by HPLC-ELSD were the same as that used for the analysis of sterols and stigmasterol reported in Chapter 5 and recently accepted for publication [389].

10.2.2 Reagents and Chemicals

All reagents and chemicals used were the same as previously reported (Chapter 5, Section 5.3.2). BSSG (>90%) was gratefully received as a gift sample from Dr. Carl Albrecht (Research Co-ordinator, CANSA, South Africa). The exact purity of this sample could not be ascertained since an appropriately qualified BSSG reference standard was not available.

10.2.3 Preparation of Standard Solutions

A stock solution was prepared by accurately weighing out an amount of BSSG into volumetric flasks and filling to volume with methanol to result in a concentration of ~10 µg/ml. Similarly a standard mixture of BSS and STG at a concentration of 10 µg/ml was also used to monitor their presence following injection of the prepared extract.

10.2.4 Sample Preparation

10.2.4.1 Aqueous Extract

In order to establish the content of sterols (β -sitosterol and stigmasterol) and BSSG in AP, fresh corms were used to prepare an extract in accordance with the procedure outlined in the patent [148]. This type of preparation is similar to the AP decoctions prepared according to the usual method used by Traditional Healers (“*Sangomas*”) in the Eastern Cape region of South Africa.

Fresh AP corms were peeled, shredded and approximately 20 g samples were weighed out and mixed with 200 ml water. The mixture was boiled for 60 min, strained through a clean muslin cloth and then filtered and made up to the original approximate volume of 200 ml (weight of AP equivalent to 100 mg fresh AP/ml). This solution was then filtered using PVDF filters (0.45 μm) prior to injecting 10 μl for analysis by HPLC.

10.3 Results

The aqueous extract derived from 100 mg/ml of fresh AP corms did not show any detectable quantities of BSSG (**Figure: 10.1.**) and this was confirmed by spiking the extracts with known methanolic solutions of BSS (10 $\mu\text{g/ml}$) and BSSG (2 $\mu\text{g/ml}$). The method used was sufficiently sensitive to detect the presence of BSSG, STG and BSS had they indeed been present.

Figure: 10.1.

HPLC-ELSD Chromatogram of Extract of AP

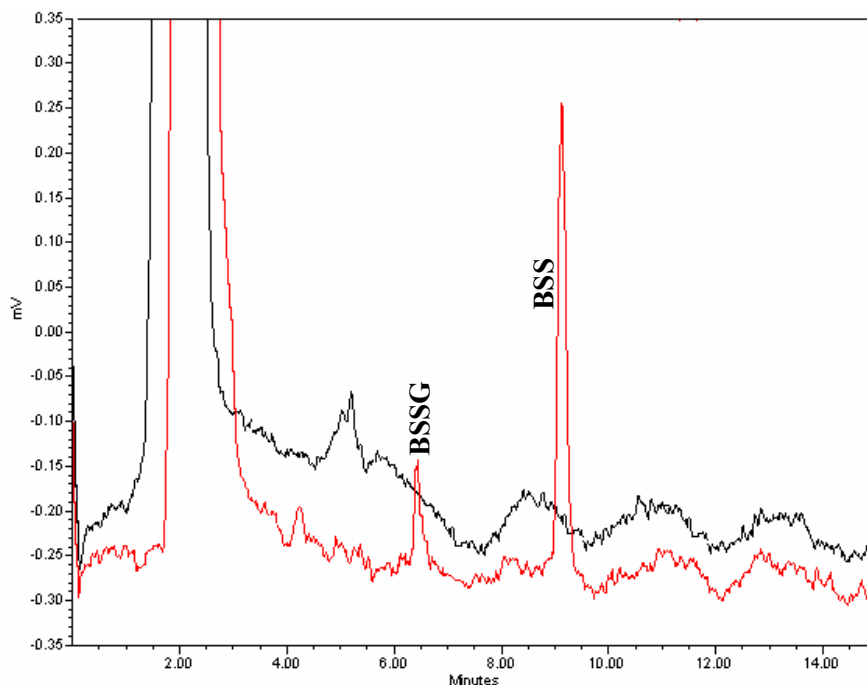


Figure: 10.2. Conditions:- HPLC-ELSD Chromatographic conditions: Column: Phenomenex Luna[®] (5 μm) C₈ (150 x 4.5 mm i.d); Column temperature: 23 $\pm$ 2 $^{\circ}\text{C}$; Mobile phase: (methanol 95: 05 water) Flow rate: 1 ml/min; Injection volume: 10 μl ; Nebulisation air flow: 0.7 L/min; Evaporation chamber temperature 100 $^{\circ}\text{C}$

10.4 Conclusions

An HPLC-ELSD method was used to determine the content of BSSG, STG and BSS in AP corms following their extraction according to the patent procedure [148]. The analytical method had the requisite sensitivity to confirm the presence of BSSG at the concentration claimed in the patent. The amount of BSSG, according to the patent, should have been not less than $\sim 3 \mu\text{g/gm}$ of fresh AP corm, when made into a decoction. The results of this analysis were not in agreement with the patent claims relating to BSSG content since it was not detected in the extract. Furthermore, considering that the daily consumption amongst consumers of AP is about 200–500 ml of such aqueous decoctions of AP (usually containing about 37 mg fresh AP/ml) [121], the daily intake of BSS and BSSG, even if present in the decoction in the amount suggested by the patent, ($<0.01\%$) will be negligible and unlikely to contribute to any therapeutic effect.

General Conclusions

Hypoxoside was isolated from African Potato (AP) corms, purified and characterized using spectroscopic methods and subsequently used as a reference standard in the various assay methods for quality control (QC) purposes. Since AP contains several components which have been purported to be associated with biological activity, assay methods for each of these were developed and validated. These methods include, HPLC with UV detection for hypoxoside, HPLC with evaporative light scattering detection for sterols and stigmastanol and capillary zone electrophoresis for qualitative and quantitative analysis of hypoxoside.

In addition, the antioxidant properties of AP were investigated and these studies revealed that whilst an aqueous extract of AP showed some antioxidant activity, the aglycone, rooperol is associated with significant activity.

In view of the general concomitant use of AP together with antiretroviral therapy, recommended by the South African Minister of Health and endorsed by the National Department of Health, investigations of possible drug-interactions were undertaken. *In vitro* results suggest that concurrent use of AP together with other medications requires a degree of caution especially when treating patients with medicines which can act as substrates for Cytochrome P450 enzymes and the drug transporter protein (P-glycoprotein).

From the analyses of various AP products, discrepancies in the content label claims of products were observed, thus clearly suggesting that suitable quality control measures need to be implemented to ensure consistent quality of marketed products.

This thesis emphasises the development of essential QC methods and criteria that are necessary to ensure the quality, safety and efficacy of products containing AP. In addition, it also reveals that concomitant administration of AP together with other medicines, in particular anti-retroviral drugs, should be contraindicated since *in vitro* studies suggest the possibility of significant interactions that may occur and consequently interfere with the safety and efficacy of the intended therapy.

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