ISOLATION, STRUCTURAL ELUCIDATION, QUANTIFICATION AND FORMULATION OF THE SAPONINS AND FLAVONOIDS OF THE SEEDS OF *GLINUS LOTOIDES*

ISOLIERUNG, STRUKTURAUFKLÄRUNG, QUANTIFIZIERUNG UND FORMULIERUNG DER SAPONINE UND FLAVONOIDE DER SAMEN VON *GLINUS LOTOIDES*

DISSERTATION

der Fakultät für Chemie und Pharmazie
der Eberhard-Karls-Universität Tübingen
zur Erlangung des Grades eines Doktors
der Naturwissenschaften

2005

vorgelegt von
ABEBE ENDALE MENGESHA

Tag der Prüfung   29. August 2005

Dekan: Prof. Dr. S. Laufer

1. Berichterstatter: Prof. Dr. P. C. Schmidt

2. Berichterstatter: Prof. Dr. I. Merfort

Kommissionsmitglieder   Prof. Dr. R. Daniels

ACKNOWLEDGMENTS

Last, but not least, I thank my family: my parents, Endale Mengesha and Etagegnhu Natee, for giving me life in the first place, for educating me and for unconditional support and encouragement to pursue my interest. I thank my brother Samuel Endale for his advice and e-mails that were very useful and all my sisters too.

RESÜMEE

Glinus lotoides wird in Äthiopien traditionell als Anthelminthikum eingesetzt. Das Ziel der vorliegenden Arbeit ist es, Auszüge von *G. lotoides* herzustellen, diese zu charakterisieren und zu standardisieren. In einem weiteren Abschnitt sollen aus den Auszügen Arzneiformulierungen hergestellt werden. Um die Ziele der Arbeit zu erreichen, muss ein standardisiertes Extraktionsverfahren entwickelt werden. Des Weiteren ist ein umfangreiches Screening der Inhaltsstoffe durchzuführen, was die Isolierung und Identifizierung der pharmakologisch aktiven Substanzen einschließt.

Die Isolierung und die Strukturaufklärung der Saponine und der Flavonoide als wirksame Inhaltsstoffgruppen von *G. lotoides* liefern vier neue Hopan-Saponine, die Glinuside F, G, H und K, das bekannte Succulentosid B sowie zwei bekannte Flavonoidglykoside; 5,7,4 '-trihydroxyflavon-6,8-di-C-glucosid (Vicenin-2) und 5,7,4 '-trihydroxy-flavon-8-C-sophorosid (Vitexin-2"-*O*-glucosid). Die Strukturen der Saponine werden mittels NMR, HRESI Massenspektroskopie und Zucker-Analytik als 3β-*O*-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22-hydroxy-hopan (Glinusid F), 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α,16β-dihydroxy-22-*O*-α-L-rhamnopyranosyl-hopan (Glinusid G), 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-hydroxy-22-*O*-α-L-rhamnopyranosyl-hopan (Glinusid H) and 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22-hopan (Glinusid I) aufgeklärt.

Die Gesamtsaponin- und Favonidgehalte der Samen von *G. lotoides* werden mittels RP-HPLC in den Extrakten und in den Tabletten bestimmt. Die quantitative Bestimmung

der Saponine wird nach saurer Hydrolyse (3M HCl, 100 °C für 1 Stunde) durchgeführt, wobei Mollugogenol B entstehen. Für die quantitative Bestimmung der Flavonoide wird Vicenin-2 als externer Standard eingesetzt. Mittels DC und HPLC unter verschiedenen Bedingungen wird eine mindestens 97 %ige Reinheit des isolierten Vicenin-2 und Mollugogenol B gefunden. Die Berechnung der Konzentrationen erfolgt über die Peakflächen von Vicenin-2 und von Mollugogenol B nach der Methode des externen Standards. Der Gesamtflavonoidgehalt und der Gesamtsaponingehalt von *G. lotoides* werden über die Gesamtpeakfläche des jeweiligen Chromatogramms ermittelt.

Im Rahmen der Validierung der Gehaltsbestimmungsmethode erfolgt die Bestimmung von Selektivität, Widerfindung, System- und Methodepräzision, Bestimmungsgrenze und Nachweisgrenze. Als Sekundärstandard wird der aufgereinigte Extakt F charakterisiert. Der Flavonoid- und Saponingehalt der Tabletten beträgt zwischen 95 und 103 % für die Flavonoide und zwischen 94 und 98 % für die Saponine.

Eine zur Formulierung von Tabletten geeignete Extraktzubereitung wird durch Mischung des Extraktes mit Aeroperl® 300 Pharma als Träger-Material hergestellt. Die benötigte Menge von Aeroperl® 300 Pharma wird durch Untersuchungen zur Wasseraufnahme und REM-Untersuchungen optimiert. Hierbei ergibt eine Extraktzubereitung mit 30% (m/m) Aeroperl® 300 Pharma die besten Eigenschaften im Hinblick auf die weiter Verarbeitung. Zusätzlich, durch Walzen-Kompaktierung der Extrakt-Aeroperl-Mischung wird die Fließfähigkeit der Tablettiermischung und den Zerfall der Tabletten verbessert.

Eine geeignete Tablettenformulierung, die 947 mg Extraktzubereitung, 363 mg Avicel® PH101, und 90 mg Ac-Di-sol enthält, wird durch mehrere Optimierungsschritte

entwickelt. Diese Tabletten zeigen einen Zerfall der Tabletten innerhalb von 2,4 Minuten bei einer Härte von 73 N.

Zusätzlich erhalten die Oblong-Tabletten einen Magensaftresistenten Überzog, der den Arzneibuchanforderungen entspricht. Dieser Überzug wird mittels der hier für geeigneten Filmbildner Eudragit® L 100/55 oder Kollicoat® MAE 100P hergestellt. Der Filmüberzug enthält Propylenglykol und Acetyltributylcitrat als Weichmacher. Durch die Prüfung der Zerfallzeit wird sichergestellt, dass die überzogenen Tabletten in künstlichen Magensaft Ph. Eur. (0,1 M Salzsäure, pH 1,0) innerhalb 2 Stunden nicht zufallen jedoch anschließend in künstlichen Darmsaft (Phosphat-Pufferlösung, pH 6,8) innerhalb von 15 Minuten zufallen.

Zusammenfassend kann festgestellt werden, dass es gelungen ist, einen Auszug von *G. lotoides* herzustellen, dieser zu einer zur Formulierung von Tabletten geeigneten Extraktzubereitung zu verarbeiten, eine Tablettenformulierung zu entwickeln und die Tabletten mit einem magensaftresistenten Überzug zu versehen.

CONTENTS

ACRONYMS

CID   Collision Induced Dissociation

DIN   Deutsche Industrie Norm

DEPT   Distortionless Enhancement by Polarization Transfer

ESIMS   Electron Spray Ionization Mass Spectrometry

et al.   et alii

HMBC   Heteronuclear Multiple Quantum Correlation

                       (^{1}H-detected heteronuclear multiple-bond spectroscopy)

HMQC   Heteronuclear Multiple Quantum Coherence

                       (^{1}H-detected heteronuclear one-bond spectroscopy)

HPLC   High Performance Liquid Chromatography

HRESIMS   High Resolution Electrospray Ionization Mass Spectrometry

i.e.   id est

N   Newton

NMR   Nuclear Magnetic Resonance

MS   Mass Spectroscopy

Pa   Pascal

Ph. Eur.   Pharmacopea Europea

rpm   revolution per minute

RSD   relative standard deviation

SD   standard deviation

TLC   Thin Layer Chromatography

UV   Ultra Violet Spectroscopy

Chapter 1

OBJECTIVE OF THE STUDY

Herbal medicines are widely used in conventional as well as alternative medical practices in many countries, both developed and developing. Although herbal medicines have been used by the Ethiopian people for centuries, they have not yet been developed to a level as to serve as a substitute or complementary to synthetic drugs (Bishaw, 1991; Gedif and Hahn, 2003).

However, incorporation of the traditional medicine into the health care system prior to systematic investigation, standardization and proper formulation may cause problems, such as inaccurate dosage, lack of proof of safety and efficacy and/or interaction risk with modern drugs. Standardization and formulation of traditional herbs into modern phytopharmaceuticals shall provide the solution to most of these problems of traditional medicine (Kroll, 2001; Gogtay et al., 2002).

With this respect, the aim of this study is to standardize and formulate the extracts of the seeds of *Glinus lotoides* into enteric coated tablets. In order to achieve this goal, the following four major objectives were set and the experiments were carried out.

1. Extraction, isolation and structural elucidation of saponins and flavonoids from the seeds of *Glinus lotoides*

Qualitative and quantitative characterization of the extracts or the traditional preparations is indispensable to confirm the safety and efficacy of medicinal plants and formulations thereof. Hence, extraction, isolation and structural elucidation of the major

compounds of the seeds of *G. lotoides* are the first objective of the study. In this, the major saponins and flavonoids will be isolated using chromatographic methods such as open column chromatography, TLC and HPLC and their structures will be elucidated using spectral like UV, MS and NMR analyses.

2.   Quantitative determination of saponins and flavonoids of *Glinus lotoides*

Quality control of raw materials, crude extracts, intermediate and finished products and manufacturing parameters require suitable quantitative determination methods. Accordingly, the second objective of the study is the development of HPLC methods for the quantitative determination of the total saponins and flavonoids of the seeds of *G. lotoides* using the isolated compounds as standards.

3.   Preparation and physicochemical characterization of the dry extract preparation for tablet formulations

Preparation of the dry extract and characterization of its physicochemical properties are the third objective of the study. In this, the crude extract will be converted into a free flowing and less hygroscopic powder using Aeroperl$^{®}$ 300 pharma as an inert adsorbent. Physical and chemical characteristics of the dry extract preparations will be determined before embarking upon formulation. Various physicochemical properties such as, particle size and size distribution, densities, hygroscopicity, water content, flow properties, compatibility and stability of the saponins and flavonoids in the dry extract preparations at different storage conditions will be investigated.

4. Dry granulation by roller compaction, tablet preparation and enteric coating

The fourth objective of the study is the granulation of the dry extract preparation and formulation into enteric coated tablets. Successful formulation of a stable and effective dosage form depends on appropriate selection of excipients and manufacturing processes. On this account, the effects of various excipients and manufacturing parameters, such as compression force will be investigated. Formulations of the core as well as enteric coated tablets will be optimized and characterized.

Chapter 2

GLINUS LOTOIDES- MOLLUGINACEAE

2.1   Introduction and botanical description

2.1.1   Introduction

Glinus lotoides L. (Molluginaceae), locally known as "Mettere" is an annual or short-living perennial prostrate herb (Edwards et al., 2000). It is found mostly in tropical and subtropical region such as Ethiopia, Sudan, Uganda, Egypt, India, Pakistan and South America. As it is found in different countries, its traditional uses also differ. In India and Pakistan, for instance, the plant is used as antifungal and antitumor agent and for fishing (Chopra et al., 1956; Kavimani et al., 1999). In Ethiopia, the seeds of *G. lotoides* are traditionally used as anthelminthic for the prevalent tapeworm infection.

There are well over 25 plants in Ethiopia that are traditionally used against tapeworm infection (Kloos et al., 1978). However, literature reveals that most of them are toxic. For instance, several clinical symptoms and deaths have been attributed to the use of *Embelia schimperi* locally known as "*Enkoko*", which contains embolic acid (Watt and

Breyer-Bandwijk, 1962). On the other hand, blurred vision and blindness, effects on the cardiovascular, respiratory and central nervous systems have been reported when people took overdoses of *Hagenia abysinica* locally known as "*Kosso*" (Rokos, 1969; Klitina, 1965). *H. abysinica* was also found to be neurotoxic in mice (Tsega et al., 1978). Since *G. lotoides* is claimed to have little or no side effects, it enjoys a considerable reputation as a taenicide in the Ethiopian traditional medicine.

2.1.2   Classification

G. lotoides is classified under the family molluginaceae and order centrospermea. The order centrospermea contains 13 families, one of which is molluginaceae. The family molluginaceae contains 16 genera and about 100 species. The family molluginaceae was often included within the family Aizoaceae, but now it is generally accepted that the 16 genera of molluginaceae are not related to the other genera of Aizoaceae (Edwards et al., 2000).

G. lotoides was known by different botanical synonyms such as *Glinus dicatamoides*, *Glinus setiforous*, *Mollugo glinus*, *Mollugo hirta* and *Mollugo lotoides*.

2.1.3   Botanical description

G. lotoides is widely distributed throughout the highlands and the banks of rivers and lakes of Ethiopia. The taxonomic features of the plant show that it is glabrous or densely short hairy. The leaves are opposite or clustered at nods and apparently verticillate, narrowly elliptic to subround. The plant contains stipules and the flowers are bisexual and clustered in axillary (Edwards et al., 2000).

2.2   Pharmacological activities of the seeds of *Glinus lotoides*

The seeds of *G. lotoides* are traditionally used in Ethiopia for the treatment of prevalent tapeworm infection (Pankhurst, 1965; Kloos et al., 1978). The taenicidal activities have already been shown in vitro against *Tenia saginata* (Djote, 1978) and *in vitro* as well as in vivo against *Hymenolepis nana* worms (Endale et al., 1997; 1998). The ethanolic extract of the seeds of the plant has been shown to be highly active in vitro against *H. nana* worms with an approximate 50% effective dose (ED_{50}) of 0.003 mg/ml. It has been shown that a concentration of 1 mg/ml of the extract exhibited comparable in vitro taenicidal activity with 0.05 and 0.01 mg/ml niclosamide and praziquantel, respectively (Endale et al., 1997).

The in vivo anthelminthic activity of the extract has been determined in albino mice infested with *H. nana* worms and has been found to be active when given both as a single dose as well as multiple doses. In single dose treatment, higher concentrations of the extract (as much as 3.3 g per kg, i.e., 100 mg per 30 g mouse) were required to expel all the worms, whereas the total multiple dose that cured the mice completely amounted to only 0.6 g per kg (i.e., 3 mg a day for 6 days per 30 g mouse). In the control experiments, a single dose of praziquantel (0.6 mg per mouse) resulted in 100% cure within 48 hrs while a single dose of niclosamide (0.5 mg) had only 73.3% efficacy (Endale et al., 1998).

The antitumor activity of the methanolic extract of *G. lotoides* has been evaluated against Dalton's ascitic lymphoma (DAL) in Swiss albino mice. A significant enhancement of mean survival time of tumor bearing mice and peritoneal cell count in normal mice was observed with respect to the control group receiving 5-Flourouracil (20 mg/kg). Tumor cell growth was inhibited in albino mice treated with extract of *G. lotoides* (50 mg/kg) after intra peritoneal inoculation with DAL cells. After 14 days of

inoculation, the extract of the plant is able to reverse the changes in the haemotological parameters, such as total white blood cells count, protein and packed cellular volume consequent to tumor inoculation (Kavimani et al., 1999).

Preliminary pharmacological studies undertaken on the plant indicate that the extract does not affect the blood pressure, heart rate or the ECG of anaesthetized rabbits, bile production in guinea pigs, or contraction of frog isolated heart after oral administration (Arragie and Chernishov, 1977; Chernishov et al., 1978).

2.3   Chemical constituents of the seeds of *Glinus lotoides*

The pharmacological activities of the seeds of *G. lotoides* have been attributed to its saponins and flavonoids. (Pankhurst 1965; Kloos et al., 1978; Djote, 1978; Endale et al., 1997; 1998; Kavimani et al., 1999). Abegaz and Tecle (1980) have isolated one triterpenoidal saponin with an oleanane skeleton. The chemical structure has been determined to be 3-*O*-[ß-D-glucopyranosyl-(1→4)-α-L-arabinopyranosyl]-oleanolic acid-(28→1)-ß-D-glucopyranosyl ester (Fig. 2.1). Acid hydrolysis of the saponin with 8% methanolic HCl yielded oleanolic acid, D-glucose and L-arabinose and the ratio of glucose to arabinose has been found to be 2:1.
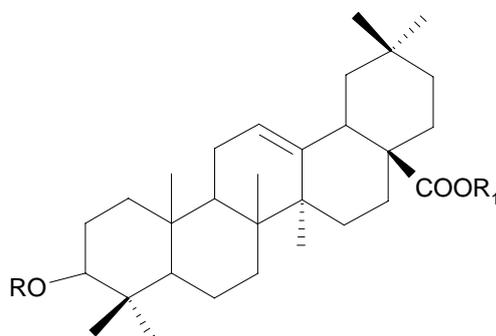
R = glucose 1 → 4 arabinose; R_1 = glucose

Figure 2.1: Chemical structure of oleanolic saponin from the seeds of *G. lotoides.*

Five hopane triterpenoidal saponins (glinusides A – E) are known from the aerial parts
of *G. lotoides* var. dictamnoides growing in Egypt (Hamed et al., 1996; Hamed and El-
Emary, 1999). The chemical structures of those saponins have been elucidated as (**A**) 3-
O-ß-L-arabinopyranosyl-22-*O*-ß-D-glucopyranosyl   (4←1)-α-L-rhamnopyranosyl-15ß-
hydroxyhopan-6-one;   (**B**)   3-*O*-ß-L-arabinopyranosyl-15-*O*-ß-D-glucopyranosyl-22ß-
hydroxyhopan-6-one; (**C**) 3-*O*-ß-D-glucopyranosyl-(4←1)-ß-L-arabinopyranosyl-22ß-
hydroxyhopan-6-one; (**D**) 3-*O*-ß-L-arabinopyranosyl-22-α-L-rhamnopyranosyl-(1→4)-
ß-D-glucopyranosyl-5ß-H-16-ß-hydroxyhopane; and (**E**) 3-*O*-ß-L-arabinopyranosyl-22-
O-ß-d-glucopyranosyl-(1→4)-ß-D-glucopyranosyl-5ß-H-16-ß-hydroxyhopane(Fig. 2.2).
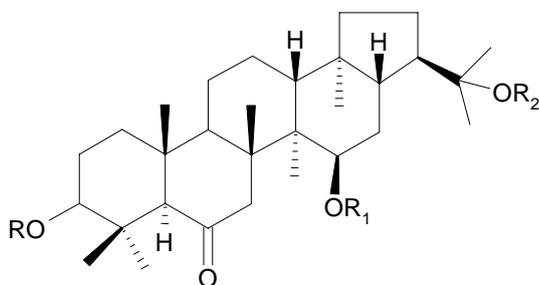
A: R = arabinose; R_1 = H R_2 = rhamnose 1→ 4 glucose
B: R = arabinose; R_1 = glucose R_2 = H
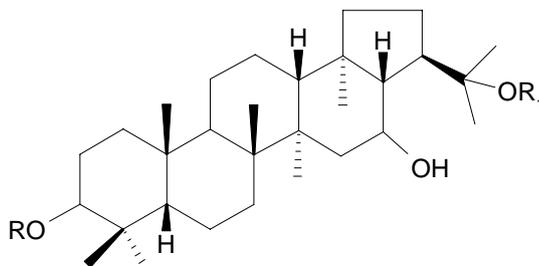
C: R = arabinose 1→ 4 glucose
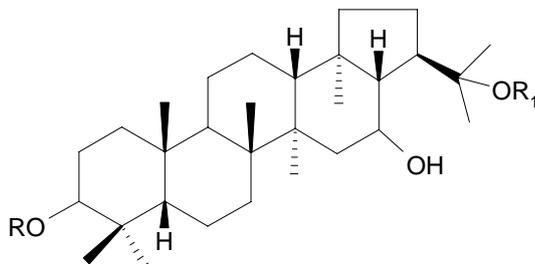
D: R = arabinose R_1 = rhamnose 1 → 4 glucose
E: R = arabinose R_1 = glucose 1 → 4 glucose

Figure 2.2: Chemical structure of glinuside A – E isolated from the seeds of *G. lotoides*.

Recently, seven hopane-type saponins (lotoideside A - F and succulentoside B) have been isolated from *Glinus lotoides* (Biswas et al., 2005) and the structures were elucidated using 1D and 2D NMR, ESIMS and chemical evidences as lotoideside (**A**) 3-*O*-β-D-xylopyranosyl-(1 → 2)-α-L-rhamnopyranosyl-6α-*O*-β-D-xylopyranosyl-22-β-*O*-β-D-glucopyranosyl-16β-hydroxyhopane; (**B**) 3-*O*-β-D-xylopyranosyl-(1 → 2)-α-L-rhamnopyranosyl-22-β-*O*-β-D-glucopyranosyl-6α,16β-dihydroxyhopane; (**C**) 3-*O*-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22β-hydroxyhopane; (**D**) 3-*O*-β-D-xylopyranosyl-16β-*O*-α-L-arabinopyranosyl-6α,22-β-dihydroxyhopane; (**E**) 3-*O*-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16 β,22-β-dihydroxyhopane; and (**F**) 3-*O*-β-D-xylopyranosyl-22-β-*O*-β-D-glucopyranosyl-16β-hydroxyhopan-6-one (Fig.2.3). The known compound succulentoside B has also been identified.
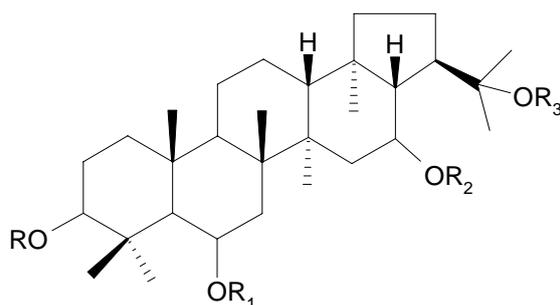
A:	R = xylose 1 → 2 rhamnose	R_1 = xylose	R_2 = H	R_3 = glucose
B:	R = xylose 1 → 2 rhamnose	R_1 = H	R_2 = H	R_3 = glucose
C:	R = xylose	R_1 = xylose	R_2 = xylose	R_3 = H
D:	R = xylose	R_1 = H	R_2 = arabinose	R_3 = H
E:	R = xylose	R_1 = xylose	R_2 = H	R_3 = H
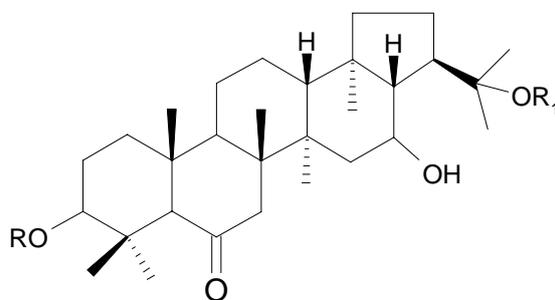
F:	R = xylose	R_2 = glucose

Figure 2.3: Chemical structures of lotoideside A – F isolated from *G. lotoides*.

Phytochemical investigation of *G. lotoides* growing in Egypt afforded three flavonoids which are identified as apigenin-7-*O*-glucoside, isovitexin, and luteolin-7-*O*-glucoside and three isoflavonoids namely 5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)isoflavone, 5,7,4'-trihydroxy -6,3'-di-(3,3-dimethylallyl)isoflavone, and 5,7,2',4'-tetrahydroxy-6,3'-di-(3,3-dimethylallyl) isoflavone. Moreover, ß-amyrin, campesterol, α-spinasterol, ß-sitosterol and lupeol are isolated form the plant (Mohamed, 1998).
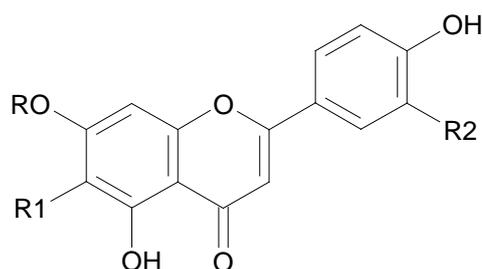
Apigenin-7-O-glucoside:	R = glucose	R_1 = H	R_2 = H
Isovitexin:	R = H	R_1 = glucose	R_2 = H
Lutenol-7-O-glucoside:	R = glucose	R_1 = H	R_2 = OH
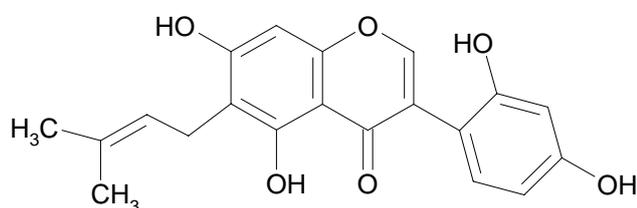
5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)isoflavone
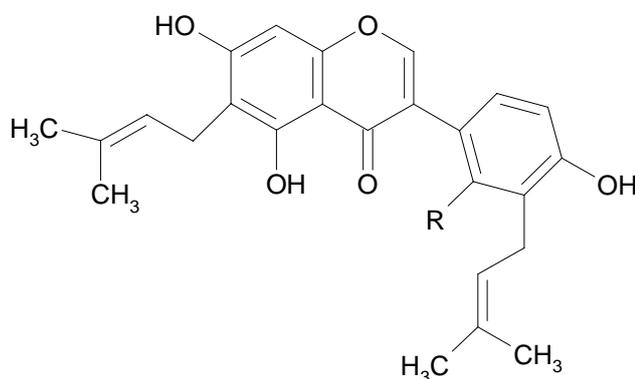
| 5,7,4'-trihydroxy -6,3'-di-(3,3-dimethylallyl)isoflavone | R = H |
| 5,7,2',4'-tetrahydroxy-6,3'-di-(3,3-dimethylallyl) isoflavone | R = OH |

Figure 2.3: Chemical structures of the flavonoids and isoflavonoids of *G. lotoides*.

Two phenylpropanoid glucosides, dictamnoside A (isolated for the first time), and the known compound, citrusin C have been isolated from a chloroform extract of *G. lotoides* L. var. dictamnoides (Hamed et al., 1997). The structures of the phenylpropanoid glucosides are shown in figure 2.4.
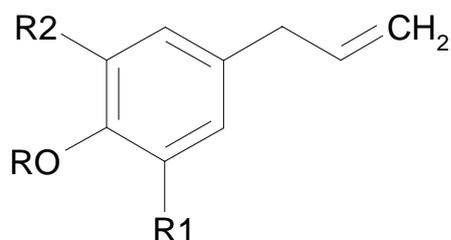
Dictamnoside A: R = glucose $R_1 = OCH_3$ $R_2 = OCH_3$
Citrusin C: R = glucose $R_1 = OCH_3$ $R_2 = H$

Figure 2.4: Chemical structure of phenylpropanoid glucosides of *G. lotoides*

The seeds of *G. lotoides* contain 14% oil. The etherial composition of the plant contains fatty acids, glycosides of sitosterols, stigmasterol and waxes (Biftu et al., 1979). On the other hand, from the saponifiable matter of the extract of *G. lotoides*, various fatty acids such as palmitic acid, stearic acid, behenic acid, linoleic acid, and oleic acid have been isolated (Mohamed, 1998). Singh, et al. (1982) have isolated fifteen alkanes and six isoalkanes from *G. lotoides* and *Mollugo pentaphylla*.

The known *C*-glycoflavonoids, vicenin-2 and vitexin (Nair and Gunasegaran, 1983) and apigenin-7-rhamnoglucoside, pelargonidin-3-sophoroside-7-glucoside (Singh et al., 1982) have been isolated from the fresh aerial parts of *Mollugo hirta*, which is a synonym of *G. lotoides*. Moreover, seven triterpenoidal sapogenins (mollugogenol A-G) have been isolated from *Mollugo hirta* (Chakrabarti et. al., 1969; Chakrabarti, 1969; Chakrabarti and Sanyal, 1969; 1970; Chakrabarti and Choudhury, 1973; Barua et al., 1976a; 1976b; 1989; Choudhury and Chakrabarti, 1975; 1976; 1979; Choudhury et al., 1995).

In this study, four new hopane saponins and two *C*-glycoside flavones were isolated and the structures were elucidated.

2.4   Traditional mode of application

The traditional mode of administration of *G. lotoides* is to orally consume about 15-20 g of capsulated powdered seeds with some food to make it palatable or its "*Tella*" (a home-made beer) extract. This amount is believed to remove the tapeworms completely from the intestine (Pankhurst, 1965; Kloos et al., 1978).

Based on the traditional mode of administration, a solvent system composed of 5% ethanol in water, that approximate the alcohol content of the home-made beer was utilized to estimate the traditional dose of the plant.

Chapter 3

EXTRACTION, ISOLATION AND STRUCTURAL ELUCIDATION OF THE SAPONINS AND FLAVONOIDS OF THE SEEDS OF *GLINUS LOTOIDES*

3.1   Introduction

The major constituents of the seeds of *G. lotoides* are hopane-type triterpenoidal saponins and *C*-glycoside flavones, which are the main bioactive components of the herb, contributing to the cestocidal and pharmacological activities (Pankhurst 1965, Kloos *et al*. 1978, Djote 1978, Endale et al. 1997, 1998, Arragie and Chernishov 1977, Chernishov et al. 1978). In this chapter, the extraction, isolation and structural elucidation of five hopane-type saponins (glinusides F, G, H, I, and succulentoside B) and two flavonoids (vicenin-2 and vitexin-2″-*O*-glucoside) from the seeds of the plant are described.

3.2   Extraction of the seeds of *Glinus lotoides*

The extraction and fractionation of the seeds of *G. lotoides* provided seven extracts (**A**–**G**), Scheme 7.1. The saponins and flavonoids in extracts **A** – **G** were evaluated on thin layer chromatography (TLC) using different solvent systems. Figure 3.1 shows a typical RP-18 TLC of extracts **A** – **G**. Extracts **A**, **D** and **F** show a high concentration of saponins compared to extracts **B**, **C** and **G**. Moreover, as shown in figure 3.1, the seeds of *G. lotoides* contain a large number of saponins, which are well separated on the RP-18 TLC plate. The presence of saponins in extract **E** indicated that the amount of methanol used to dissolve extract **D** was not sufficient (Scheme 7.1).
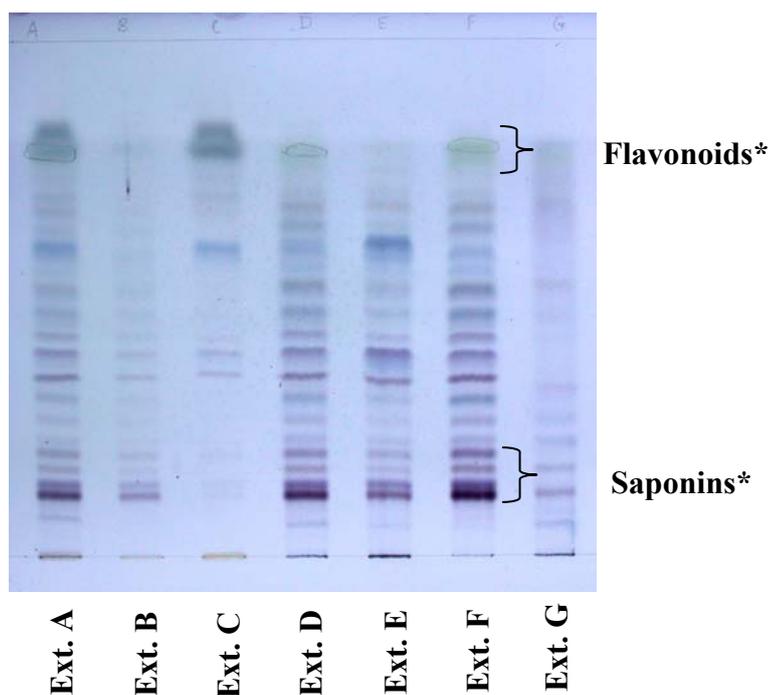
Figure 3.1: Typical RP-18 Thin Layer Chromatography of the extracts A–G of the seeds
of *G. lotoides* [Solvent system: methanol: water (4:1); detection: 5% vanillin
reagent]. *Saponins and flavonoids isolated in this work.

In addition to saponins, the extracts of the seeds of the plant contain flavonoidal
glycosides, which produce a fluorescent complex with the natural product-PEG reagent
(NPR).

3.3   Isolation of saponins and flavonoids

3.3.1   Column chromatography fractionation

Open-column chromatography is often used as a first fractionation step for a crude
extract, which provides a partial separation of the different groups of the constituents. In
order to simplify the isolation of saponins and flavonoids, extract **F** was fractionated
using RP-18 and Sephadex LH-20 column.

Figure 3.2 depicts TLC of 14 fractions (Fr. I - XIV) of extract **F** using RP-18 column chromatography. As shown in the figure, fraction I and II contain flavonoids which were identified using NPR-reagent.
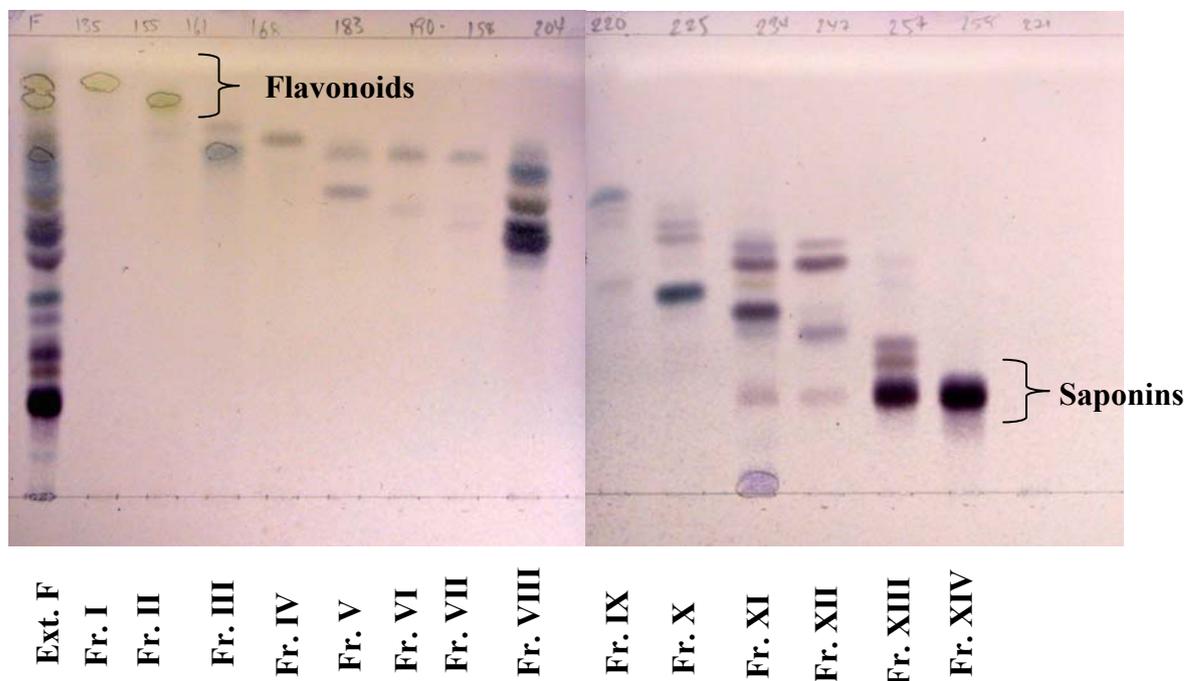
Figure 3.2: TLC of the fractions of extract **F** collected from RP-18 column chromatography [TLC: RP-18; solvent system: methanol: water (4:1); detection: 5% vanillin reagent].

One of the major problems in column chromatography is the length of time required to perform the separations with large numbers of fractions to be analyzed. The RP-18 column provided 280 fractions that were combined after TLC evaluation. Moreover, the yield of the saponins in the fractions was minimal. Hence, sephadex LH-20 column chromatography was used for the fractionation extract **F**. Unlike the RP-18 column, the sephadex LH-20 gel chromatography provides a small number of fractions and the separation was completed in a relatively short time.

Figure 3.3 depicts the major fractions (6 out of the 20 fractions) containing the saponins and flavonoids. After TLC control, these fractions were combined into two major

fractions: Fr. 1 (yield, 352 mg) which contains mainly flavonoids and Fr. 2 (yield, 600 mg) containing saponins.
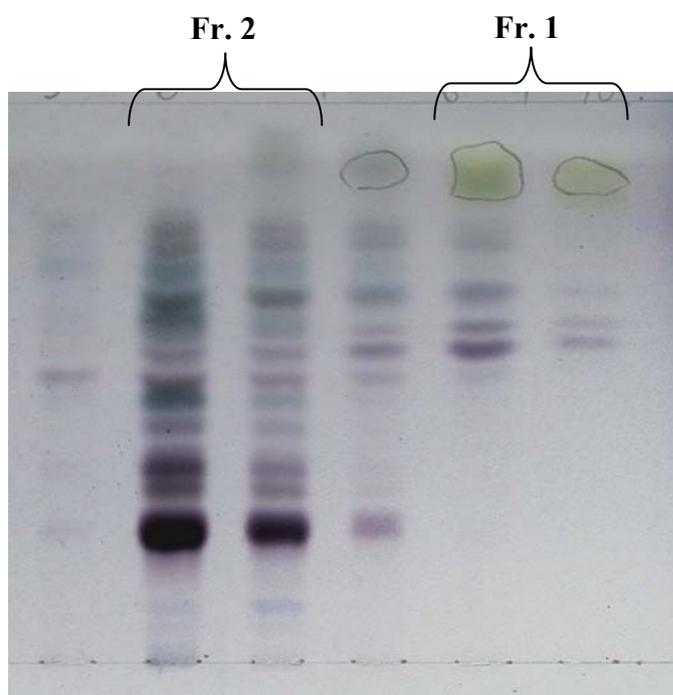
Figure 3.3: TLC of the major fractions of extract **F** collected from sephadex LH-20 column chromatography [TLC: RP-18; solvent system: methanol: water (4:1); detection: 5% vanillin reagent].

3.3.2   High Performance Liquid Chromatography (HPLC)

Fractions (Fr. 1 and Fr. 2) collected from the sephadex LH-20 column chromatography were further purified using reversed phase HPLC to isolate the saponins and flavonoids of *G. lotoides*.

Figure 3.4 shows the analytical (Fig. 3.4 a) and preparative (Fig. 3.4 b) HPLC chromatogram of Fr. 1. The compounds at retention time of 8.2 min (F1/1) and 17.9 min (F1/2) were isolated and the structures were elucidated using spectral analysis including UV, MS and NMR as vicenin-2 and vitexin-2″-*O*-glucoside, respectively. The structural elucidation of F1/1 and F1/2 has been described under **3.4.2**
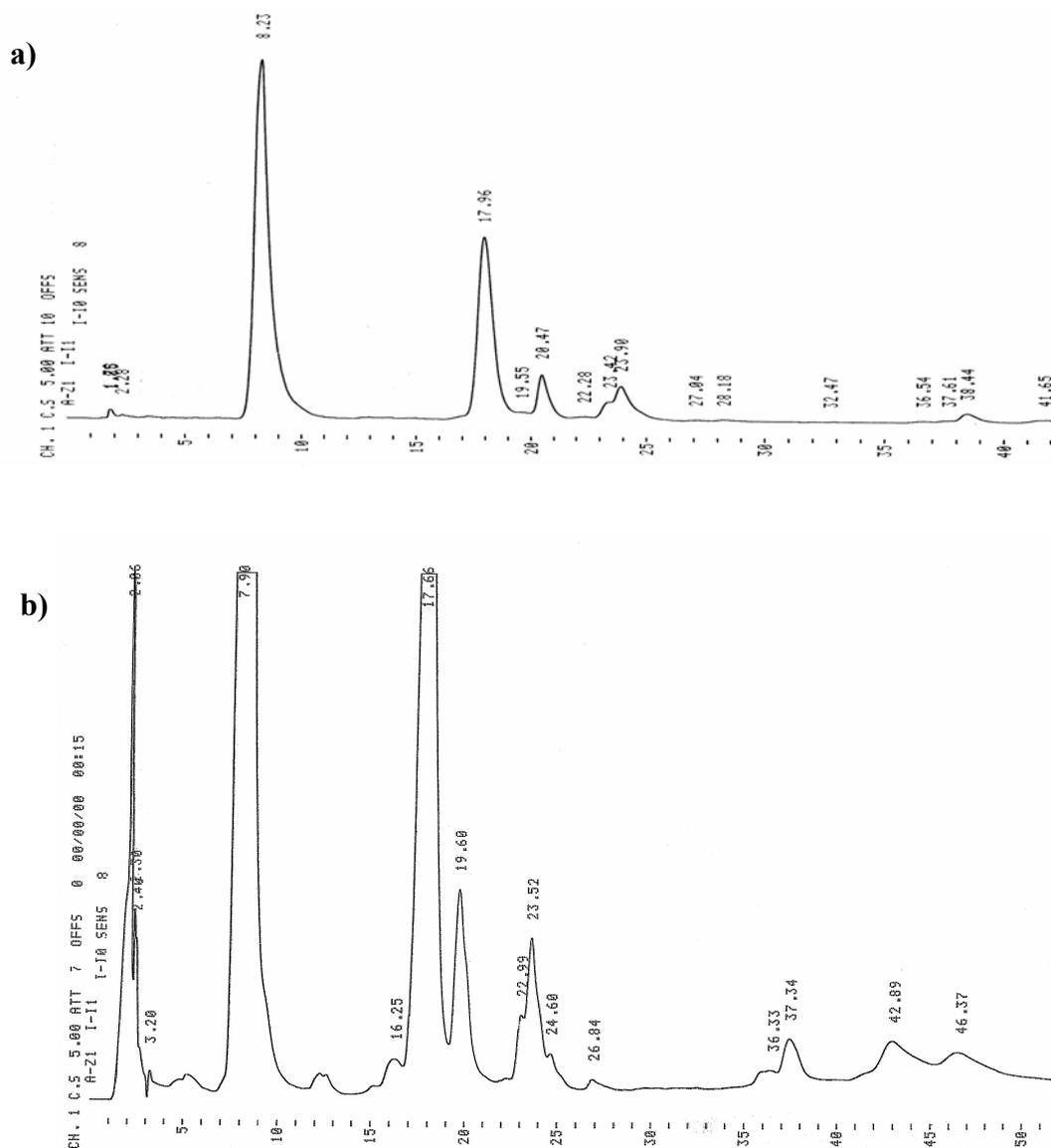
Figure 3.4: HPLC isolation of the flavonoids from Fraction 1. [Column: CC250/4 Nucleosil 100-7 C_{18}; mobile phase: 15% CH_3CN; flow rate: 1 mL/min; detection: UV-360 nm; Injection volume: a) analytical, 20µL and b) preparative, 250µL].

Similarly, fraction 2 (480 mg) containing mixtures of saponins was separated by RP-18 HPLC. Figure 3.5 a) shows the analytical HPLC separation of the saponins S1 and S2 which after preparative scale up (Fig. 3.5 b) was used to isolate the major saponin

fractions. The two major peaks at retention time of 18.8 (S1) and 23 (S2) min were isolated.
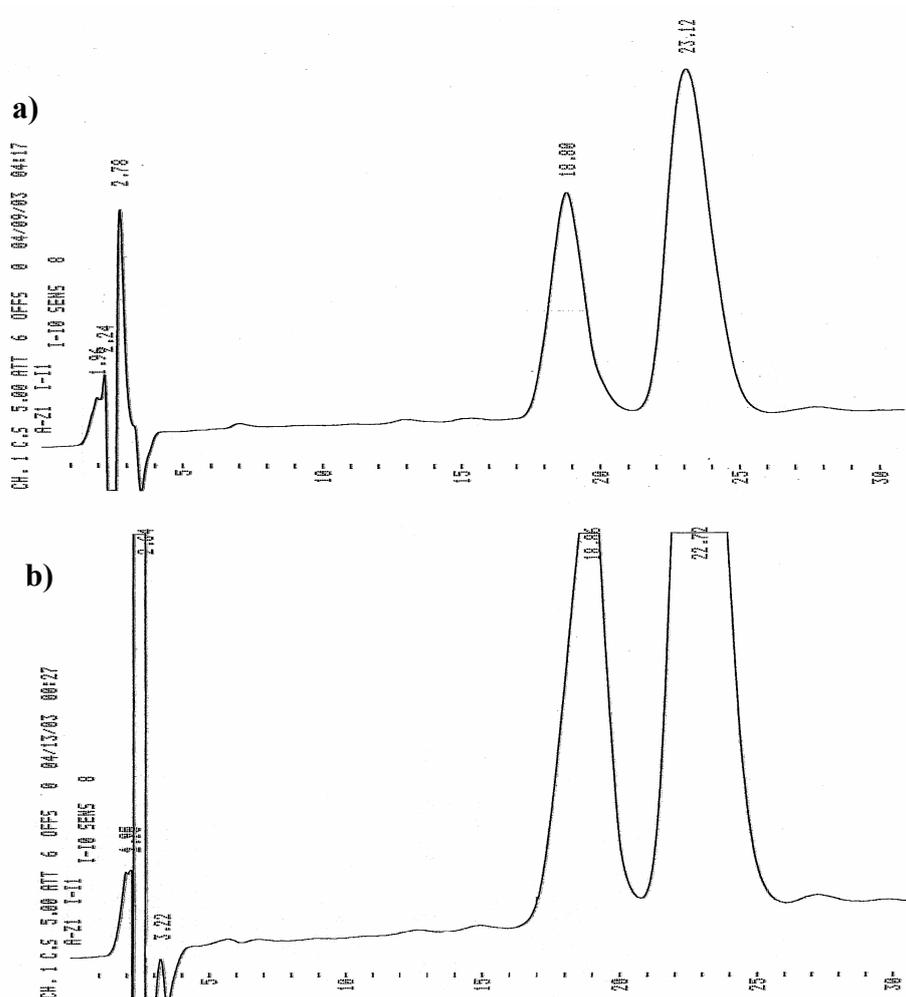
Figure 3.5: HPLC isolation of the saponin fractions (S1 and S2) from fraction 2. [Column: CC250/4 Nucleosil 100-7 C_{18}; mobile phase: 75% MeOH; flow rate: 1 mL/min; detection: RID; Injection volume: a) analytical, 20µL and b) preparative, 250µL].

However, TLC examination of the S1 and S2 (Fig. 3.6) showed that the isolated compounds were composed of a group of saponins, which were not separated by HPLC. As shown in figure 3.6 increasing the sample volume form 1 to 5 µL revealed the presence of additional minor components in S2 fraction. Isocratic elution was not satisfactory for such saponins due to a very similar polarity and gradient elution was not

possible due to the nature of the refractive index detector. Hence, preparative TLC was utilized to isolate the saponins as described below.
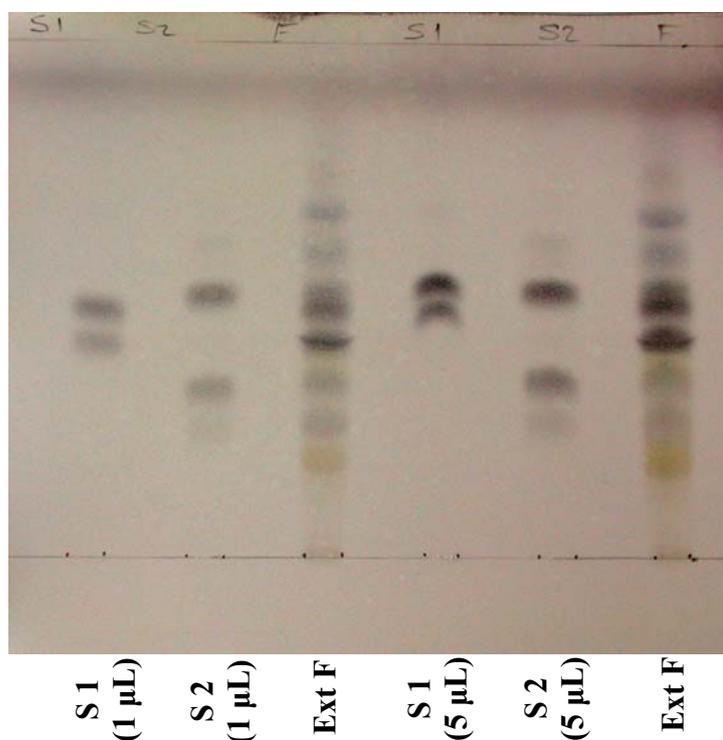
Figure 3.6: TLC of S1, S2 and Extract F. [TLC: Silica gel 60; solvent system: $CHCl_3$-MeOH-H_2O (70:50:4); detection: 5% vanillin reagent].

3.3.3   Preparative thin layer chromatography

Preparative TLC of S1 afforded two fractions, S1/1 ($R_f = 0.48$) and S1/2 ($R_f = 0.41$) and S2 gave two fractions S2/1 ($R_f = 0.52$) and S2/2 ($R_f = 0.33$) (Fig. 3.6) The structure of the isolated saponins has been elucidated using spectral analysis as described below.

3.4   Structural elucidation of the saponins and flavonoids of *Glinus lotoides*

3.4.1   Structural elucidation of the saponins

The structure of the isolated saponin S1/1 was elucidated as 3ß-*O*-ß-D-xylopyranosyl-6α-*O*-ß-D-xylopyranosyl-16ß-*O*-ß-D-xylopyranosyl-22-hydroxyhopane, which is referred to as glinuside F. S1/2, was identified as 6α-*O*-α-L-arabinopyranosyl-3ß-*O*-ß-D-

xylopyranosyl-16ß-*O*-ß-D-xylopyranosyl-22-hydroxyhopane,   succulentoside   B,   a
saponin that was isolated from *Polycarpon succulentum* (Meselhy and Aboutabl, 1997).
The structure of S2/1 was elucidated as 3ß-*O*-α-L-rhamnopyranosyl-(1→2)-ß-D-
xylopyranosyl-6α,16ß-dihydroxy-22-*O*-α-L-rhamnopyranosyl-hopane   (glinuside   G).
S2/2 provided two saponins and their structures were elucidated in a mixture as 3β-*O*-α-
L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-hydroxy-
22-*O*-α-L-rhamnopyranosyl-hopane (glinuside H) and as 3β-*O*-α-L-rhamno-pyranosyl-
(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22-
hopane (glinuside I). Figure 3.7 depicts the chemical structures of the glinusides F, G,
H, I and succulentoside B. The structural elucidation of the glinusides F, G, H and I
using spectral analysis has been described below.

3.4.1.1   General introduction

Saponins are high-molecular weight glycosides, consisting of a sugar moiety linked to a
triterpene or steroid aglycon. Saponins exhibit a wide spectrum of biological activities
and they are constituents of many herbal drugs and folk medicines (Wagner et al., 1985;
Bader and Hiller 1987; Bader et al., 1995; Bialy et al., 1999; Liou and Wu, 2002). Of
these, the most important include: *Saponariae radix*, *Hippocastani semen*, *Primulae
radix*, *Hederae helices herba*, *Senegae radix*, *Liquiritiae radix* and *Ginseng radix*. The
cestocidal and pharmacological activities of the seeds of *G. lotoides* are probably due to
its saponins. Anthelmintic activity has been demonstrated for other triterpene saponins
(Carpani et al., 1989; Enwerem et al., 2001).

The triterpene saponins could be mono-, bi- or tridesmosidic. As monosaccharide units,
D-glucose, L-rhamnose, L-arabinose and D-xylose occur widely and D-apiose, D-ribose,
D-quinovose and D-allose occur rarely (Hostettmann and Marston, 1995).
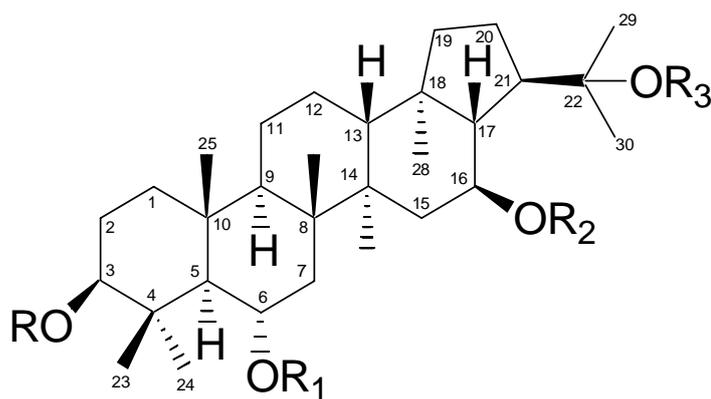
Saponins/Aglycon	R	R1	R2	R3	Formula	Mol. Weight
Mollugogenol A	H	H	H	H	$C_{30}H_{49}O_4$	473
Glinuside F	Xyl	Xyl	Xyl	H	$C_{45}H_{76}O_{16}$	872
Glinuside G	Rha-Xyl	H	H	Rha	$C_{47}H_{80}O_{16}$	900
Glinuside H	Rha-Xyl	Xyl	H	Rha	$C_{52}H_{88}O_{20}$	1032
Glinuside I	Rha-Xyl	Xyl	Xyl	H	$C_{51}H_{86}O_{20}$	1018
Succulentoside B	Xyl	Ara	Xyl	H	$C_{45}H_{76}O_{16}$	872

Abbreviations: Xyl, xylose; Rha, rhamnose and Ara, arabinose.

Figure 3.7: Chemical structures of the saponins of *Glinus lotoides* and the aglycon, mollugogenol A.

Mass Spectrometry (MS)

The choice of ionization method in MS depends on the polarity, liability and molecular weight of the saponin to be analyzed. The so-called 'soft' ionization techniques are employed to obtain molecular weight and sugar sequence information. (Wolfender et al, 1992; Schöpke et al, 1996). ESIMS and HRESIMS methods have been used to characterize the molecular weight and formula as well as information on the sugar nature.

Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR spectra of saponins are complex and tedious to analyze. The vast majority of proton resonances of the sugar moiety appear in a very small spectral width of 3.0 – 4.2 ppm, with subsequent problems of overlapping. 2-D NMR analysis provides a method of overcoming the problem of spectral crowding in these regions, thus simplifying the assignment of saccharide protons. Under favorable conditions, all the protons present in a given sugar residue can be identified.

The methyl peaks of triterpenes are readily discernible and informative. Some useful data can also be obtained from ^{1}H NMR spectra for the anomeric configurations and linkages of the sugar chain. The coupling constant of the C-1 proton of α-linked sugar units is approximately 3 Hz, while β-linked units have a coupling constant of 6-7 Hz (Agrawal, 1992).

The number of anomeric carbons in saponin is readily determined by ^{13}C NMR, defining the number of individual sugar residues present. By comparing the chemical shifts with appropriate model sugars, the ring size (furanose or pyranose form) and type of each monosaccharide can be established (Seo et al., 1978; Bock and Pedersen, 1974; 1983). The anomeric carbon atoms in pyranoses resonate at 90-110 ppm, while carbon atoms bearing secondary hydroxy groups in pyranoses give signals at 65-85 ppm; carbon atoms carrying primary hydroxy groups are found at 60-64 ppm (Bock and Pedersen, 1983).

^{13}C NMR, DEPT, HMQC, and long-range COSY were applied to the structural elucidation of the saponins in order to resolve the problems caused by overcrowding of proton spectrum.

Sugar analysis

NMR and MS information may sometimes not be confirmatory. In this case, additional sugar analysis is necessary. One of the most important procedures for the determination of interglycosidic linkages is to carry out GC-MS analysis of the alditol acetates of the sugars from the premethylated saponins. The premethylated saponin was hydrolyzed, reduced and subsequently acetylated, giving the corresponding monosaccharide derivatives, which were analyzed and compared with the data from authentic samples (Plock et al., 2001).

Microhydrolysis of saponins using a hydrochloric acid vapor on TLC was also used to identify the monosaccharides (Meselhy and Aboutabl, 1997; Meselhy, 1998). Authentic sugars such as arabinose, xylose and rhamnose were utilized as reference monosaccharides.

3.4.1.2   Glinuside F

Glinuside F was isolated as a white amorphous powder. The TLC analysis of glinuside F using a solvent system composed of $CHCl_3$-MeOH-H_2O (70:50:4) provided a spot at R_f –value of 0.48.

The electrospray ionization mass spectrometry (ESIMS) of glinuside F (Fig. 3.8 a and b) in the positive and negative mode showed quasimolecular ion peaks at m/z 872.7 $[M+H]^+$ and 871.6 $[M-H]^-$, respectively, consistent with the molecular formula, $C_{45}H_{76}O_{16}$. This was confirmed by its HRESIMS, which showed a $[M+Na]^+$ at m/z 895.507 (calculated for $C_{45}H_{76}O_{16}$+Na, 895.5031).
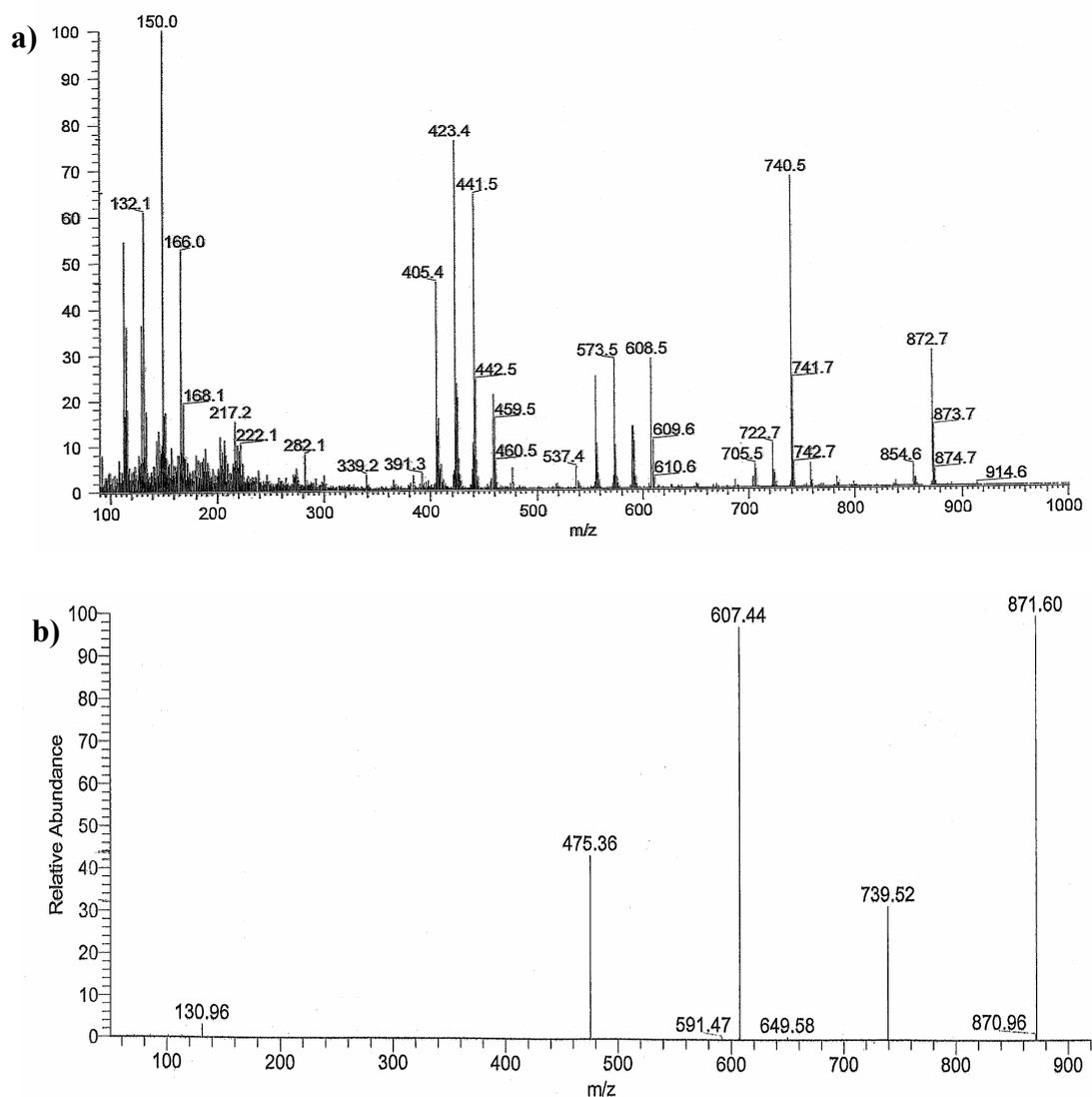
Figure 3.8: ESIMS collision induced dissociation spectra of glinuside F in a) positive
          mode and b) negative mode.

The ^{1}H NMR spectrum (CD$_3$OD) showed signals characteristic for eight tertiary methyl
groups as singlets at δ 0.88, 0.96, 1.10, 1.11, 1.13, 1.15, 1.30 and 1.42, and three
anomeric protons at δ4.30 (d, J = 7.8 Hz), 4.45 (d, J = 7.8 Hz), and 4.50 (d, J = 7.8 Hz)
(Table 3.1) demonstrating the presence of ß-anomers of three sugar units.

The ^{13}C NMR spectrum of glinuside F (CD$_3$OD) analyzed by the aid of distortionless
enhancement by polarization transfer (DEPT) and ^{1}H-detected heteronuclear one-bond
spectroscopy (HMQC) revealed the presence of 45 carbon atoms in the molecule (Table

3.2). DEPT was used for the determination of carbon multiplicities in the ^{13}C NMR. Thirty carbon signals were indicated for the aglycon part with signals characteristic for eight tertiary methyls, eight methylenes, eight methines and six saturated quaternary carbons, including a carbon signal at δ 74.6 for a quaternary carbinol carbon.

From the above data and by comparing the ^{13}C NMR data of glinuside F with literature reports for triterpenoids, the aglycon was identified as mollugogenol A, a triterpene from the hopane type isolated form *Mollugo pentaphylla* L (Hamburger et al., 1989; Mahato and Kundu 1994).

Fifteen carbon signals were left for the sugar moieties including three anomeric carbons at δ 107.6, 106.0, and 104.1, (Table 3.2) from which the presence of three pentose-monosaccharide moieties was indicated. Accordingly, the ESIMS of glinuside F in the positive mode showed fragment ion peaks at *m/z* 740 [(M+H)-132]$^+$, 608 [(M+H)-2(132)]$^+$, and 476 [(M+H)-3(132)]$^+$ (Fig. 3.8 a), due to subsequent loss of three pentose units. Consequently, the spectra in the negative mode produced fragment ions at *m/z* 739 [(M-H)-132]$^-$, 607 [(M-H)-2(132)]$^-$, 475 [(M-H)-3(132)]$^-$ (Fig. 3.8 b).

Acid hydrolysis of glinuside F on TLC plate in a hydrochloric acid vapor afforded only xylose, which was supported by GC-MS analysis of the partially methylated alditol acetates as described by Plock *et al* (2001). The identities of the monosaccharides were confirmed by a detailed inspection of 1D and 2D NMR experiments (^{1}H–^{1}H COSY and HMQC) where information of the respective sugar residues and the anomeric configurations could be obtained.

As shown in Table 3.2, the downfield shift of three oxygen-bearing methines C-3 (δ 90.9), C-6 (δ 81.1) and C-16 (δ 80.8), indicated the sites of glycosylation (Hamburger et al, 1989; Mahato and Kundu 1994).

The above proposed interglycosidic linkages were further substantiated by the long-range correlation observed in the [1]H-detected heteronuclear multiple-bond spectroscopy (HMBC) spectra of glinuside F. Cross peaks between H-1'/C-1' (δ 4.30/ δ 107.6) of one xylose unit and C-3/H-3 (δ 90.9/ δ 3.10) of the aglycon showed one xylose to be bound to C-3 of the aglycon. Similarly, H-1''/C-1'' (δ 4.45/ δ 106.0) of the second xylose unit correlated with C-6/H-6 (δ 81.1/ δ 4.05), and H-1'''/C-1''' (δ 4.50/ δ 104.1) of the third xylose correlated with C-16/H-16 (δ 80.8/ δ 4.30) of the aglycon.

The ^{1}H and ^{13}C NMR data (Table 3.1 and 3.2) indicated the ß-configuration at the anomeric positions for the xylose units ($^3J_{H1,H2}$ = 7.8 Hz) (Hamburger et al, 1989). The multiplicities and coupling constants of the axial protons at C-3 ($J_{2a,3\alpha}$ = 12.0; $J_{2e,3\alpha}$ = 4.5 Hz), C-6 ($J_{5a,6\beta}$ = 12.0; $J_{5e,6\beta}$ = 4.5 Hz) and C-16 ($J_{15a,16\alpha}$ = 10.0; $J_{15e,16\alpha}$ = 4.0; $J_{17a,16\alpha}$ = 10.0 Hz), confirmed the equatorial orientation of all three substitutents in the aglycon part (Hamburger et al, 1989; Breitmaier 1993).

Further analysis of the HMBC spectrum provided independent confirmatory evidence for the nature of the aglycon and allowed unambiguous assignment of both the ^{13}C and ^{1}H NMR chemical shifts of most of the relevant signals (Plock *et al*, 2001). On the basis of the above evidence, the structure of glinuside F was characterized as 3ß-*O*-ß-D-xylopyranosyl-6α-*O*-ß-D-xylopyranosyl-16ß-*O*-ß-D-xylopyranosyl-22-hydroxyhopane.

3.4.1.3   Glinuside G

Glinuside G was isolated as a white amorphous powder. The TLC analysis of glinuside G using a solvent system composed of $CHCl_3$-MeOH-H_2O (70:50:4) provided a spot at R_f–value of 0.52 (Fig. 3.6).

The ESIMS of glinuside G (Fig. 3.9) in the negative mode showed quasimolecular ion peaks at m/z 899 [M-H]$^-$. The molecular formula of glinuside G was calculated as $C_{47}H_{80}O_{16}$ from its HRESIMS, which showed a [M+Na]$^+$ at m/z 923.538 (calculated for $C_{47}H_{80}O_{16}$+Na, 923.5344).
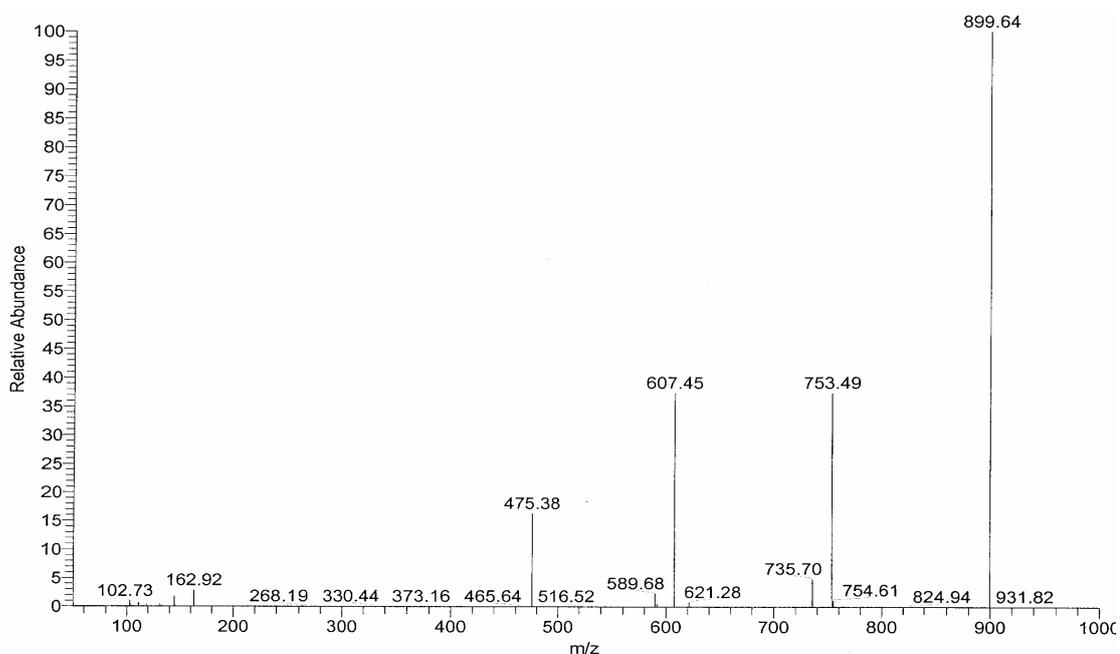
Figure 3.9: ESIMS collision induced dissociation spectra of glinuside G in the negative mode.

The ^{1}H, ^{1}H COSY, ^{13}C, DEPT and HMBC spectra suggested the aglycon to be mollugogenol A, the same moiety as in glinuside F. The only differences were changes in the chemical shifts associated with differences in the glycosylation pattern at C-6, C-16, and C-22.

Sugar analysis of glinuside G revealed two rhamnose moieties and one xylose moiety as sugar components. The occurrence of these sugars was further supported by the fragmentation pattern in the ESIMS of glinuside G, taken in the negative mode, which showed fragment ions at m/z 753 [(M–H)-146]$^-$, 607 [(M–H)-(146+146)]$^-$, and 475 [(M–H)-(146+146+132)]$^-$, due to the subsequent loss of rhamnose, and two molecules of rhamnose and xylose, respectively (Fig. 3.9). Additionally, the presence of these sugars was confirmed from their ^{1}H and ^{13}C NMR data as shown in Table 3.1 and 3.2 (Breitmaier, 1993).

Glycosylation at C-3 with xylose was deduced from the correlation in the HMBC spectrum between the signals at δ_C 106.5/ δ_H 4.42 of xylose and the signal at δ_C 90.2/ δ_H 3.09 (C-3) of the aglycon moiety. The downfield shift of C-2 of xylose at δ_C 78.6 and δ_H 3.47 suggested further glycosylation at this position. Long-range correlations between C-2 (δ 78.6) of xylose and H-1^{IV} (δ 5.36) of rhamnose confirmed the linkage at this position. The second rhamnose moiety was attached to C-22 which is deduced from the long-range correlation between the signals at δ_C 97.0/ δ_H 5.21 from C-1 of rhamnose and δ_C 84.5 of the aglycon at C-22.

Analysis of the HMBC spectrum afforded confirmatory evidence of the structure and allowed assignment of the ^{13}C and ^{1}H NMR data (Plock et al., 2001). Accordingly, glinuside G was identified as 3ß-*O*-α-L-rhamnopyranosyl-(1→2)-ß-D-xylopyranosyl-6α,16ß-dihydroxy-22-*O*-α-L-rhamnopyranosyl-hopane.

3.4.1.4   Glinuside H

S2/2 provided a mixture of two saponins (glinuside H and I) and their structures were elucidated in this mixture due to the small amount obtained. The presence of a mixture was confirmed from inspection of the 1D ^{1}H NMR spectrum where the intensities of the

signals for the anomeric protons indicated a 2:1 mixture of glinuside H and I, respectively. This difference in the proportion of the two saponins in the mixture made possible the identification of the saponins in a mixture. The TLC analysis of the mixture using a solvent system composed of $CHCl_3$-MeOH-H_2O (70:50:4) provided Rf values of 0.33 and 0.25 for glinuside H and I, respectively.

The HRESIMS of the major component (glinuside H) showed a $[M + Na]^+$ at m/z 1055.581, with the molecular formula of $C_{52}H_{88}O_{20}$ (calculated for $C_{52}H_{88}O_{20}$+Na, 1055.5766) indicating the presence of two rhamnose and two pentose sugar moieties. Sugar analysis of the mixture revealed the presence of rhamnose and xylose.

Inspection of the 1D and 2D COSY spectra of the anomeric protons indicated that glinuside H possessed two rhamnose units from signals at δ_H 5.53 and 5.21 and two xylose units at δ_H 4.41 and 4.36 (Table 3.1). Comparison of the data with glinuside G suggested that glinuside H had a rhamnose (1→2) xylose unit at C-3 of the aglycon while analogous comparison with glinuside F suggested a β-xylopyranosyl unit at C-6.

Long-range C-H correlations in the 2D HMBC spectrum provided further evidence for the substitution pattern of the aglycon. Thus, in glinuside H, H-1 of the xylose moiety at δ_H 4.41 correlated with C-3 (δ_C 91.1) of the aglycon as identified from its correlations with H-23 (δ_H 1.42) and H-24 (δ_H 1.10).

Similarly, H-1 of the xylose at δ_H 4.36 correlated with C-6 at δ_C 81.4 as in glinuside F, and the rhamnose moiety at δ_H 5.53 correlated with a carbon signal at δ_C 77.9 attributed to C-2 of the same xylose moiety as shown for glinuside G. H-1 of the rhamnose moiety at δ_H 5.21 correlated with C-22 (δ_C 84.4) of the aglycon as determined from its

correlations with H-30 (δ_H 1.43), H-29 (δ_H 1.36), H-17 (δ_H 1.52), and H-21 (δ_H 2.59). Form the above data, glinuside H was identified as 3β-O-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-O-β-D-xylopyranosyl-16β-hydroxy-22-O-α-L-rhamnopyranosyl-hopane.

3.4.1.5   Glinuside I

The HRESIMS of the minor component of the mixture (glinuside I) showed a [M+Na]$^{+}$ at *m/z* 1041.565 indicating a molecular formula of $C_{51}H_{86}O_{20}$ (calculated for $C_{51}H_{86}O_{20}$+Na, 1041.5610) with three pentose and one rhamnose sugar units. Sugar analysis revealed rhamnose and xylose in the mixture.

The integrals of the 1D and 2D COSY signals of glinuside I had only one rhamnose signal at δ_H 5.53 and two separate signals for xylose units at δ_H 4.39 and 4.37 (Table 3.1). According to the integration, a third xylose signal overlapped with that of the major compound (glinuside H) at δ_H 4.41.

Comparison of the data with glinuside G suggested that glinuside I had a rhamnose (1→2) xylose unit at C-3 of the aglycon and similar comparison with glinuside F suggested a β-xylopyranosyl unit at C-6. Long-range C-H correlations in the 2D HMBC spectrum provided further evidence for the substitution pattern of the aglycon. Thus, in glinuside I, H-1 of the xylose moiety at δ_H 4.41 correlated with C-3 (δ_C 91.1) of the aglycon as identified from its correlations with H-23 (δ_H 1.42) and H-24 (δ_H 1.10). Similarly, H-1 of the xylose at δ_H 4.36 correlated with C-6 at δ_C 81.4 as in glinuside F and the rhamnose moiety at δ_H 5.53 correlated with a carbon signal at δ_C 77.9 attributed to C-2 of the same xylose moiety as shown for glinuside G.

The remaining signal for glinuside I of H-1 of the xylose moiety at δ_H 4.39 correlated with a carbon signal at δ_C 80.7, compatible to C-16 of the aglycon compared with glinuside F. Hence, glinuside I was identified as 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22-hopane.

Table 3.1: ^{1}H NMR spectral data of glinuside F, G, H and I in CD$_3$OD

Aglycon	F	G	H[*]	I[*]
1	1.00 m; 1.70 m	1.01 m; 1.70 m		
2	1.84 m; 1.70 m	1.86 m; 1.70 m	1.87 m; 1.71 m	
3	3.10 dd (12, 4.5)	3.09 dd (12, 4.5)	3.06 dd (11.6, 4.5)	
5	0.95 d (10.5)	0.94 d (10.5)	1.12 d	
6	4.05 ddd (10, 10, 4)	4.03 ddd (11, 10.5, 4)	4.01 m	
7	1.62 m; 1.95 m	1.70 m; 1.58 m	1.62 m; 1.95 m	
9	1.30 m	1.30 m		
11	1.65 m; 1.65 m	1.65 m; 1.65 m		
12	1.60 m; 1.60 m	1.60 m; 1.60 m		
13	1.45 dd (12, 4)	1.43 dd (12, 4)		
15	1.75 m; 1.90 m	1.72 m; 1.33 m	1.29 m; 1.71 m	1.45 m; 1.91 m
16	4.30 ddd (10, 10, 4)	4.14 ddd (10, 10, 4)	4.14 ddd (11.6, 9.3, 4.1)	4.30 ddd (11.6, 9.3, 4.1)
17	1.45 dd (10, 10)	1.55 dd (10, 10)	1.52 m	
19	1.10 m; 1.60 m	1.10 m; 1.60 m		
20	1.90 m; 1.60 m	1.90 m; 1.60 m	1.56 m; 1.92 m	
21	2.60 ddd (10, 10, 9)	2.60 ddd (10, 10, 9)	2.59 m	
23	1.41 s	1.38 s	1.42 s	
24	1.10 s	1.07 s	1.10 s	
25	0.98 s	0.96 s	0.98 s	0.98 s
26	1.13 s	1.14 s	1.14 s	
27	1.11 s	1.13 s	1.11 s	
28	0.87 s	0.88 s	0.88 s	0.87 s
29	1.15 s	1.37 s	1.36 s	1.15 s
30	1.29 s	1.42 s	1.43 s	1.29 s

[*] Only the signals that could be ambiguously assigned are given.

Table 3.1: ^{1}H NMR spectral data of glinuside F, G, H and I in CD$_3$OD (cont'd)

Sugars	F	G	H	I
Xyl C-3				
1′	4.30 d (7.8)	4.42 d (6.6)	4.41 d (7.5)	4.41 d (7.5)
2′	3.25 m	3.46 m	3.51 m	3.51 m
3′	3.39–3.34m	3.47 m	3.51–3.47 m	3.51–3.47 m
4′	3.52–3.48 m	3.52 m	3.48[a] m	3.48[a] m
5′ A	3.91–3.85 m	3.90 dd (5.0, 11.4)	3.82[b] m	3.82[b] m
5′ B	3.26–3.20 m	3.22 dd (9.3, 11.4)	3.22[c] m	3.22[c] m
Xyl C-6				
1″	4.45 d (7.8)		4.36 d (7.5)	4.37 d (7.5)
2″	3.25 m		3.28 m	3.28 m
3″	3.39–3.34 m		3.34 m	3.34 m
4″	3.52–3.48 m		3.49[a] m	3.49[a] m
5″ A	3.91–3.85 m		3.88[b] m	3.88[b] m
5″ B	3.26–3.20 m		3.22[c] m	3.22[c] m
Xyl C-16				
1‴	4.50 d (7.8)			4.39 d (7.5)
2‴	3.25 m			3.20 m
3‴	3.39–3.34 m			3.35 m
4‴	3.52–3.48 m			3.49[a] m
5‴ A	3.91–3.85 m			3.88[b] m
5‴ B	3.26–3.20 m			3.22[c] m
Rha (1-2)				
1 IV		5.36 d (2)	5.53 d (2)	5.53 d (2)
2 IV		3.98 m	3.93 m	3.93 m
3 IV		3.79 m	3.78 m	3.78 m
4 IV		3.43 dd (9, 9)	3.44 m	3.44 m
5 IV		3.77 dq (9, 6.5)	4.09 m	4.09 m
6 IV		1.29 d (6.5)	1.26 d (6.5)	1.26 d (6.5)
Rha C-22				
1 V		5.21 d (2)	5.21 d (2)	
2 V		3.78 m	3.77 m	
3 V		3.67 dd (9, 3.5)	3.67 dd (9; 3.5)	
4 V		3.42 dd (9, 9)	3.41 dd (9; 9)	
5 V		4.00 dq (9, 6.5)	3.77 m	
6 V		1.26 d (6.5)	1.28 d (6.5)	

[a, b, c] Assignments of values with the same superscript may be interchanged;

Table 3.2: ^{13}C NMR spectral data of glinuside F, G, H and I in CD$_3$OD

Aglycon	F	G	H[b]	I[c]
1	40.1 t	40.1 t	40.0 t	
2	26.8 t	26.9	(26.9) t	
3	90.9 d	90.2	91.1 d	91.1 d
4	40.8 s	40.9 s	40.8 s	
5	61.3 d	61.9 d	61.8 d	
6	81.1 d	69.0 d	81.4 d	81.4 d
7	45.1 t	46.1 t	(44.9) t	
8	44.0 s	44.8 s	(43.9) s	
9	49.9[a] d	50.6 d	50.7 d	
10	39.9 s	39.9 s	40.0 s	
11	22.0 t	22.0 t	(22.1) t	
12	24.6 t	24.7 t	(24.7) t	
13	50.6[a] d	50.0 d	50.2 d	
14	44.0 s	43.9 s	(43.9) s	
15	43.4 t	44.2 t	(44.0) t	
16	80.8 d	67.8 d	(67.9) d	80.7 d
17	59.9 d	62.7 d	62.7 d	59.9 d
18	47.5 s	46.6 s	46.7 s	47.4 s
19	42.6 t	42.7 t	42.6 t	
20	29.1 t	28.7 t	(28.7) t	
21	53.4 d	51.5 d	51.5 d	53.5 d
22	74.6 s	84.5 s	84.5 s	74.6 s
23	31.2 q	31.2 q	31.3 q	
24	16.8 q	17.0 q	16.8 q	
25	17.6 q	17.6 q	(17.7) q	
26	17.6 q	17.8 q	(17.7) q	
27	18.5 q	18.7 q	(18.7) q	
28	18.7 q	18.7 q	(18.8) q	
29	30.7 q	25.5 q	25.4 q	30.6 q
30	27.1 q	26.1 q	26.0 q	27.1 q

[a] Assignments with the same superscript may be interchanged.
[b] The shifts in parentheses were assigned from the 1D ^{13}C spectrum by comparison with F and G.
[c] Only the signals that could be unambiguously assigned are given. It is assumed the other signals overlap with those of the major component, glinuside H, in the mixture.

Table 3.2: ^{13}C NMR spectral data of glinuside F, G, H and I in CD$_3$OD (cont'd)

Sugars	F	G	H	I
Xyl C-3				
1′	107.6 d	106.5 d	106.8 d	106.8 d
2′	75.5[a] d	78.6 d	77.9 d	77.9 d
3′	78.3[b] d	78.9 d	79.4 d	79.4 d
4′	71.3[c] d	71.6 d	71.8 d	71.8 d
5′	66.7[d] d	66.3 d	66.6 d	66.6 d
Xyl C-6				
1″	106.0 d		106.6 d	106.6 d
2″	75.0[a] d		75.0[a] d	75.0[a] d
3″	77.9[b] d		78.5 d	78.5 d
4″	71.0[c] d		71.1 d	71.1 d
5″	66.8[d] d		66.6 d	66.6d
Xyl C-16				
1‴	104.1 d			104.1 d
2‴	75.2[a] d			75.1[a] d
3‴	78.0[b] d			78.3 d
4‴	71.1[c] d			71.0 d
5‴	67.2[d] d			66.6 d
Rha (1-2)				
1[IV]		102.0 d	101.4 d	101.4 d
2[IV]		72.1 d	72.4 d	72.4 d
3[IV]		73.1 d	71.8 d	71.8 d
4[IV]		74.0 d	74.3 d	74.3 d
5[IV]		70.4 d	69.7 d	69.7 d
6[IV]		18.0 q	18.0 q	18.0 q
Rha C-22				
1[V]		97.0 d	96.9 d	
2[V]		73.9 d	73.1 d	
3[V]		72.2 d	72.3 d	
4[V]		74.0 d	73.9 d	
5[V]		70.1 d	70.5 d	
6[V]		18.0 q	18.0 q	

[a-d] Assignments with the same superscript may be interchanged.

3.4.2   Structural elucidation of the flavonoids

3.4.2.1   General introduction

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones (Harborne, 1988; Markham and Geiger, 1994). Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest because of their potential beneficial effects on human health as antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant (Tsai et al., 1996; Kuti and Konuru, 2004; Lombardi-Boccia, 2004; Wei et al., 2004; Hsiao et al., 2005).

The structures of the two isolated flavonoids, F1/1 and F1/2, were elucidated using spectral analysis including UV, MS and NMR as 5,7,4′-trihydroxyflavone-6,8-di-*C*-glucoside (vicenin-2) and 5,7,4′-trihydroxy-flavone-8-*C*-sophoroside (vitexin-2″-*O*-glucoside), respectively. The chemical structure of vicenin-2 and vitexin-2″-*O*-glucoside are shown in Figure 3.10.

Vicenin-2   Vitexin-2″-*O*-glucosid
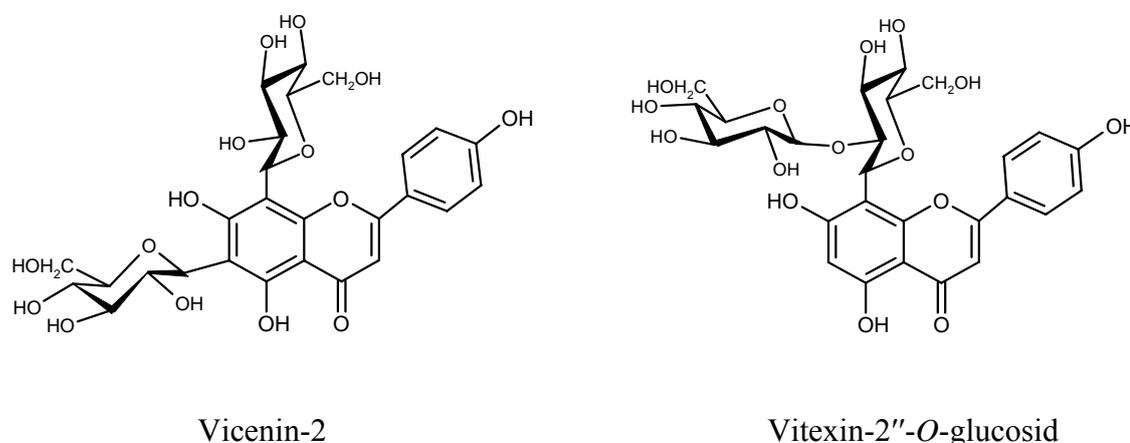
Figure 3.10: Chemical structures of the flavonoids of *Glinus lotoides*.

The structural elucidations of F1/1 and F1/2 are described below.

3.4.2.2   Ultraviolet Spectroscopy (UV)

Figure 3.11 depicts the methanol spectra of F1/1 and F1/2 in the region 240-400 nm,

showing two major absorption peaks at 272 and 330 nm for F1/1 and 270 and 331 for

F1/2. These peaks are characteristics for flavones and flavonols (Fig. 3.12) which show

two major absorption peaks in the region 240-400 nm. These two peaks are commonly

referred to as band I (300-380 nm) and band II (240-280 nm) (Mabry et al, 1970).
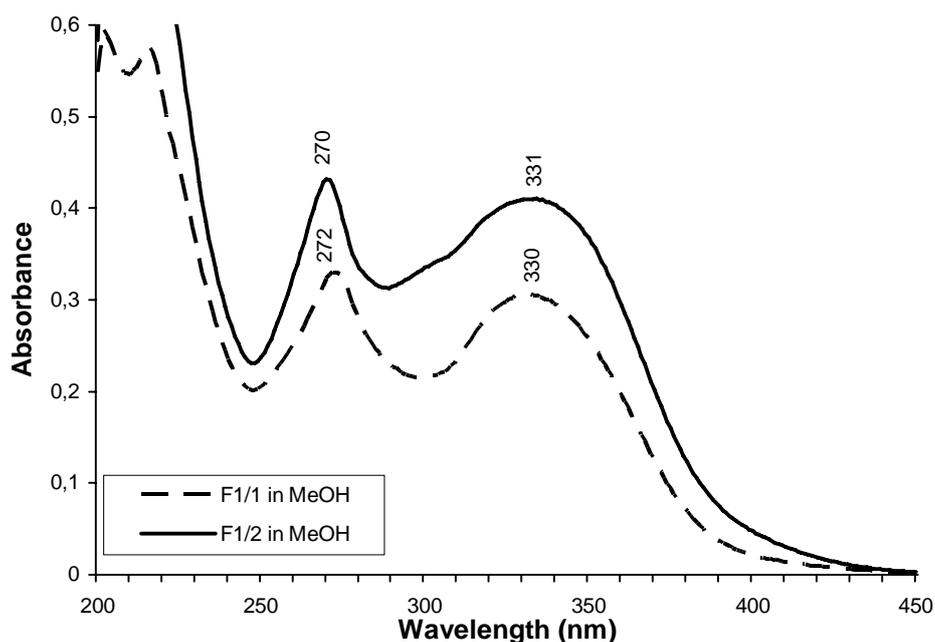
Figure 3.11: The UV spectra of F1/1 and F1/2 in methanol.

Band I is considered to be associated with the absorption due to the B-ring (cinnamoyl

system) and band II with the absorption involving the A-ring, benzoyl system (Fig.

3.12). Particularly, the position of band I in the methanol spectrum provides information

about the type of flavonoid as well as its oxidation pattern. The position of band I

distinguish between flavones and flavonols (3-hydroxyflavones). Band I of flavones

occurs in the range 304-350 nm whereas band I of flavonols appears at a longer

wavelength (352-385 nm).
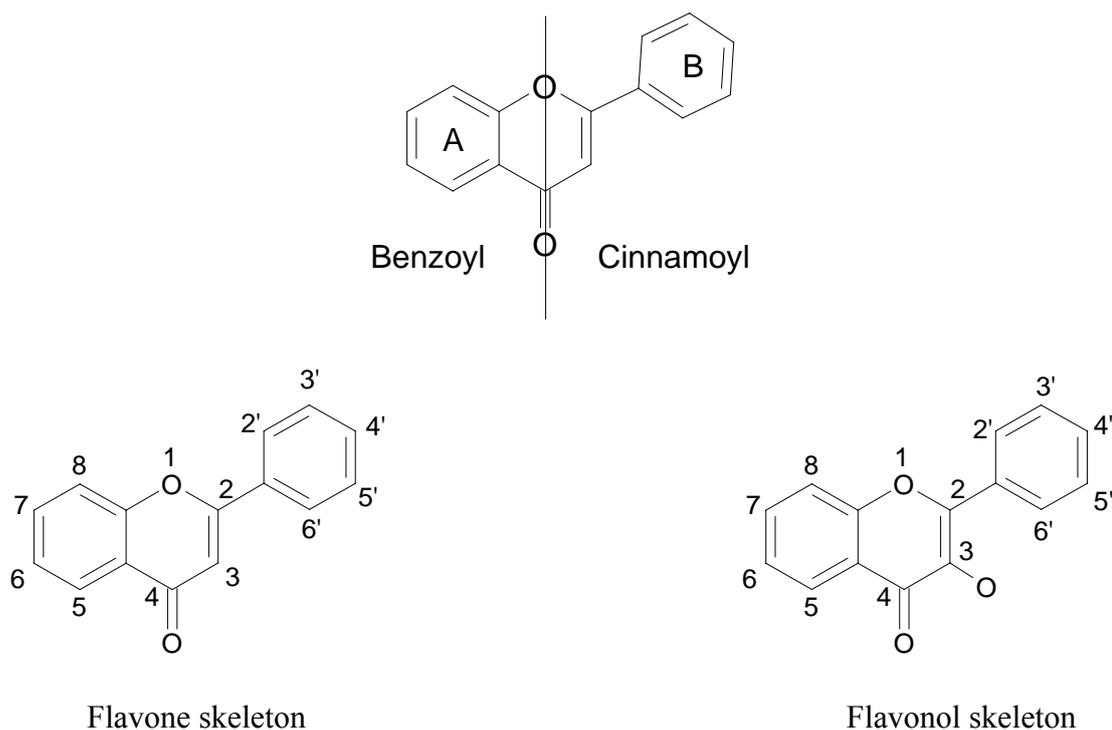
Figure 3.12: Flavone and flavonol skeletons with benzoyl and cinnamoyl moieties.

As shown in Figure 3.11, the maximum of band I absorption in methanol UV spectra of F1/1 and F1/2 appear at 330 and 331 nm, respectively, indicating that F1/1 and F1/2 may belong to flavone groups. However, in flavonols with a substituted 3-hydroxy group (methylated or glycosylated), band I appears in the range 328-357 nm which overlaps the region of band I in flavones, and the general shape of the spectral curves approach those of flavones.

Oxidation pattern of the B-ring in flavones could be identified from the nature of the peaks. In this, $3′, 4′-$ or $3′, 4′, 5′-$ oxygenated flavones usually exhibit two absorption peaks (or one maximum with a shoulder) between 250-275 nm, while the $4′-$oxygenated equivalents have only one peak in this range. The methanol UV spectra of F1/1 and F1/2 (Fig. 3.11) showed a single peak at 272 and 270 nm, respectively, indicating that the B-rings contain only $4′-$OH group.

The UV spectra of flavones and flavonols in the presence of sodium methoxide (NaOMe) provide information on the hydroxylation pattern of the flavonoid. Sodium methoxide is a strong base and ionizes to some extent all hydroxy groups of the flavonoid nucleus. The addition of NaOMe to flavones in methanol usually produces bathochromic shifts in all absorption bands. However, a large bathochromic shift of band I of about 40-65 nm, with an increase in intensity, is diagnostic for the presence of a free 4´-hydroxy group.

As shown in Figure 3.13 the addition of NaOMe to methanol solution of F1/1 produced bathochromic shift of band I by 69 nm (from 330 to 399) with an increase in the intensity of absorption, confirming the presence of free 4´-hydroxy group in F1/1. Similar result was obtained for flavonoid F1/2, which showed a bathochromic shift of band I by 65 nm. The NaOMe spectra of F1/1 and F1/2 were stable for 5 min confirming the absence of free 3-OH group.
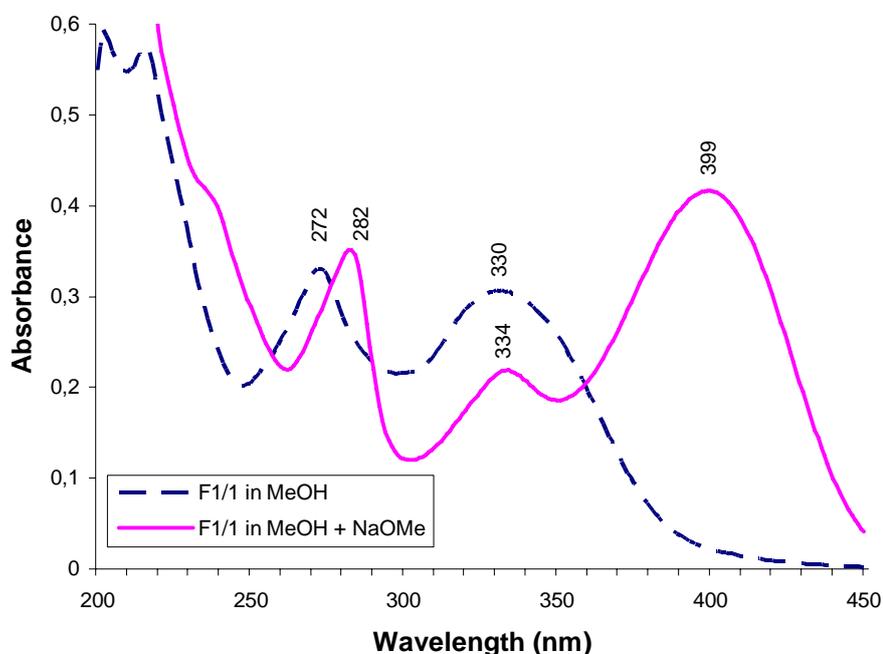
Figure 3.13: Bathochromic shift of the methanol spectrum of F1/1 due to sodium methoxide.

Sodium acetate is a weaker base than NaOMe, and as such ionizes only the more acidic hydroxy groups in flavones and flavonols, i.e., the 3-, 7- and 4′-hydroxy groups. The ionization of the 7-hydroxy group mainly affects band II, whereas the ionization of the 3- and/or 4′-hydroxy groups mainly affects band I. Particularly, NaOAc is a useful diagnostic reagent for the specific detection of 7-hydroxy groups. The UV spectra of flavones containing free 7-hydroxy groups exhibit a diagnostic 5-20 nm bathochromic shift of band II in the presence of NaOAc. However, when 6 and 8 oxygen substituted, the bathochromic shift with NaOAc is often smaller presumably because of the reduced acidity of the 7-hydroxy group. Certain 3′, 4′-dioxygenated derivatives without the 7-OH group showed bathochromic shifts of 20-25 nm.

The NaOAc UV spectra of F1/1 and F1/2 showed bathochromic shift of band II by 10 nm (from 272 to 282) and (from 270 to 280), respectively indicating the presence of free 7-hydroxy group. Figure 3.14 depicts the bathochromic effect of NaOAc on the UV spectrum of F1/1 in methanol.
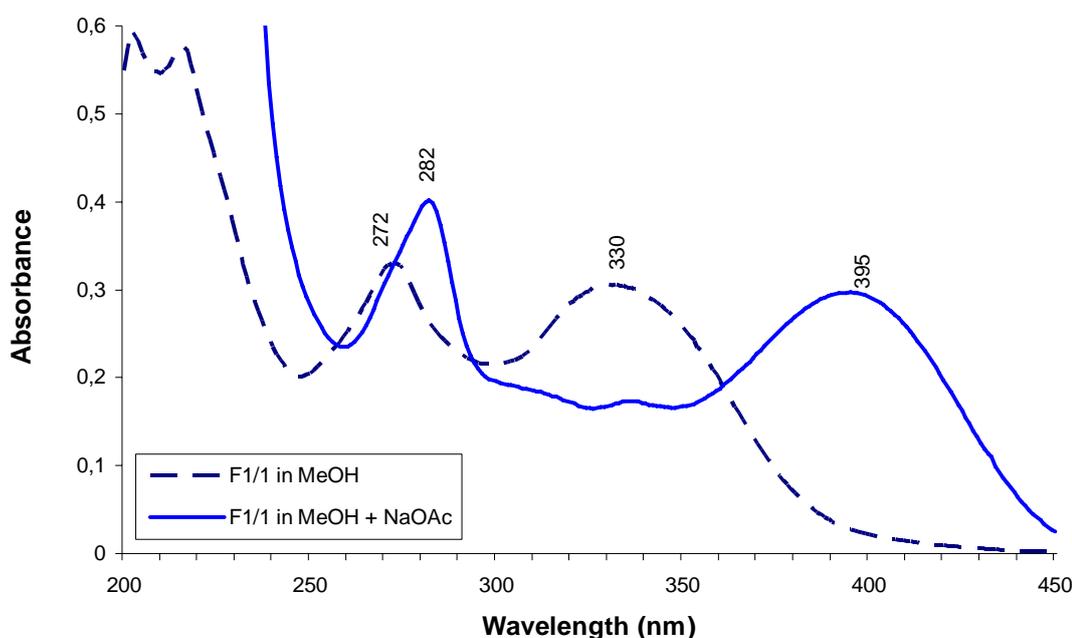
Figure 3.14: The effect of NaOAc on the MeOH UV spectrum of F1/1.

The presence or absence of an ortho-dihydroxy group at all locations of the flavonoid nucleus could be detected from the effect of $NaOAc/H_3BO_3$ in the UV spectrum. In the presence of NaOAc, boric acid forms chelate complexes with ortho-dihydroxy groups. Flavones containing a B-ring ortho-dihydroxy group show a consistent 12-30 nm bathochromic shift of band I in the presence of $NaOAc/H_3BO_3$, whereas a bathochromic shift of about 5-10 nm in band I in the presence of $NaOAC/H_3BO_3$ is diagnostic for the presence of an ortho-dihydroxy group (at C-6, 7 or C-7, 8) in the A-ring. In F1/1 and F1/2, the NaOAc spectra reduced the intensity without bathochromic shifts on addition of boric acid. The disappearance of band I peak in $NaOAc/H_3BO_3$ spectra of F1/1 and F1/2 indicated the absence of ortho-dihydroxy groups in A- and B-rings.

Flavones and flavonols which contain hydroxy groups at C-3 or C-5 form acid stable complexes with aluminum chloride, whereas the aluminum chloride complexes with ortho-dihydroxy groups are not stable. The presence of an ortho-dihydroxy group in the B-ring of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid in the presence of $AlCl_3$ with that obtained in $AlCl_3/HCl$. 30-40 nm hypsochromic shift observed in band I of the $AlCl_3$ spectrum on the addition of acid results form the decomposition of the complex of $AlCl_3$ with the ortho-dihydroxy group. The presence of three adjacent hydroxy groups in the B-ring gives only a 20 nm hypsochromic shift on the addition of acid to the $AlCl_3$ solution.

Figure 3.15 illustrates the $AlCl_3$ and $AlCl_3/HCl$ spectra of F1/1. As shown in the figure, the addition of acid to the $AlCl_3$ containing flavonoid solutions produced no significant hypsochromic shift of band IIb indicating that there is no ortho-dihydroxy group in F1/1. Similar results were obtained for F1/2.
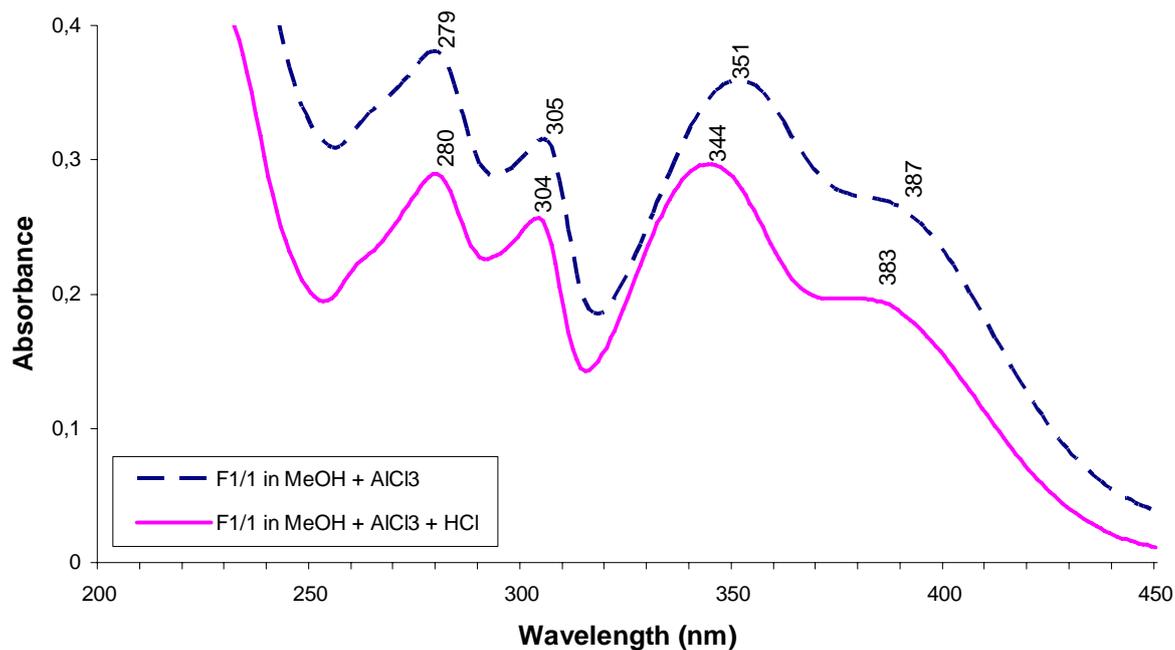
Figure 3.15: The UV Spectra of F1/1 in MeOH in the presence of AlCl$_3$ and AlCl$_3$/HCl.

The addition of acid to a methanolic solution of flavones or flavonols which already contain AlCl$_3$ decomposes the complexes between AlCl$_3$ and ortho-dihydroxy groups. Hence, any shift still remaining in band I or band II relative to the methanolic spectrum will be due to the presence of free 3- and/or 5-hydroxy groups in the flavonoid. Regeneration of the methanol spectrum on the addition of acid indicates that both the 3- and 5-hydroxy groups are either absent or substituted.

The AlCl$_3$/HCl spectrum of a 5-hydroxyflavone typically consists of four major absorption peaks, band Ia, Ib, IIa and IIb, which are all bathochromically shifted relative to their band of origin (presumably Ia and Ib originate from I; and IIa and IIb from II). As shown in Figure 3.15, the AlCl$_3$/HCl spectra of F1/1 depict four major peaks which are bathochromically shifted relative to their respective band in the MeOH spectrum (Fig. 3.11).

The two major flavonoids of *G. lotoides* (F1/1 and F1/2) belong to the family of flavone glycosides with free 4´-, 5- and 7-hydroxy groups. F1/1 and F1/2 do not have ortho-dihydroxy and 3´- or 5´-OH groups. Table 3.3 summarizes the UV spectral data of F1/1 and F1/2.

Table 3.3: UV spectral data of F1/1 and F1/2 in methanol and with diagnostic reagents

Diagnostic reagents	F1/1 (λ_{max} nm)	F1/2 (λ_{max} nm)
MeOH	272, 330	270, 331
MeOH + NaOMe	282, 334, 399	280, 331, 396
MeOH + NaOAc	282, 395	280, 310s, 389
MeOH + NaOAc + H_3BO_3	284, 320, 406	272, 322
MeOH + AlCl$_3$	279, 305, 351, 387	276, 304, 350, 382
MeOH + AlCl$_3$ + HCl	280, 304, 344, 383	277, 302, 342, 378

3.4.2.3   Mass Spectroscopy (MS)

Mass spectroscopy has been successfully employed for the structure determination of flavonoids (Frański, et al., 1999). ESIMS of F1/1 and F1/2 were recorded on a Thermo Finnigan TSQ Quantum triple-quadrupole mass spectrometer.

ESIMS full-scan spectra of F1/1 showed quasimolecular ions at *m/z* 594.9 $[M+H]^+$ (Fig. 3.16 a) and 593.2 $[M-H]^-$ (Fig. 3.16 c) with positive and negative modes, respectively. The collision induced dissociation spectra of the flavonoid provided further characteristic fragmentation patterns at *m/z* 475$[(M+H)-120]^+$, 433$[(M+H)-162)]^+$, 355$[(M+H)-2(120)]^+$, 313$[(M+H)-(120+162)]^+$, 271$[(M+H)-2(162)]^+$ in the positive mode (Fig. 3.16 b) and *m/z* 473$[M–H–120]^-$, 383$[M–H–120–90]^-$, 353$[M–H–2(120)]^-$ in

the negative mode (Fig. 3.16 d) indicating the presence of two hexose sugars, which are

C-glycosides at C-6 and C-8 (Qimin et al, 1991).
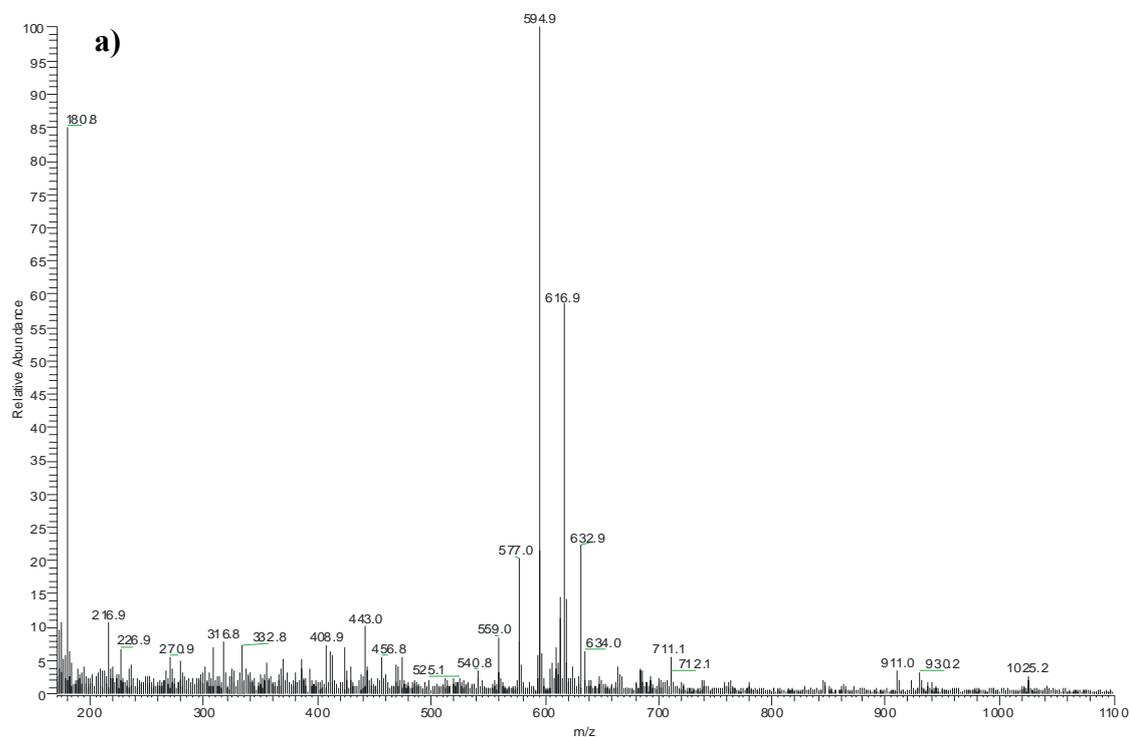
Figure 3.16 a): The ESI-mass spectra of F1/1 [full scan positive mode].
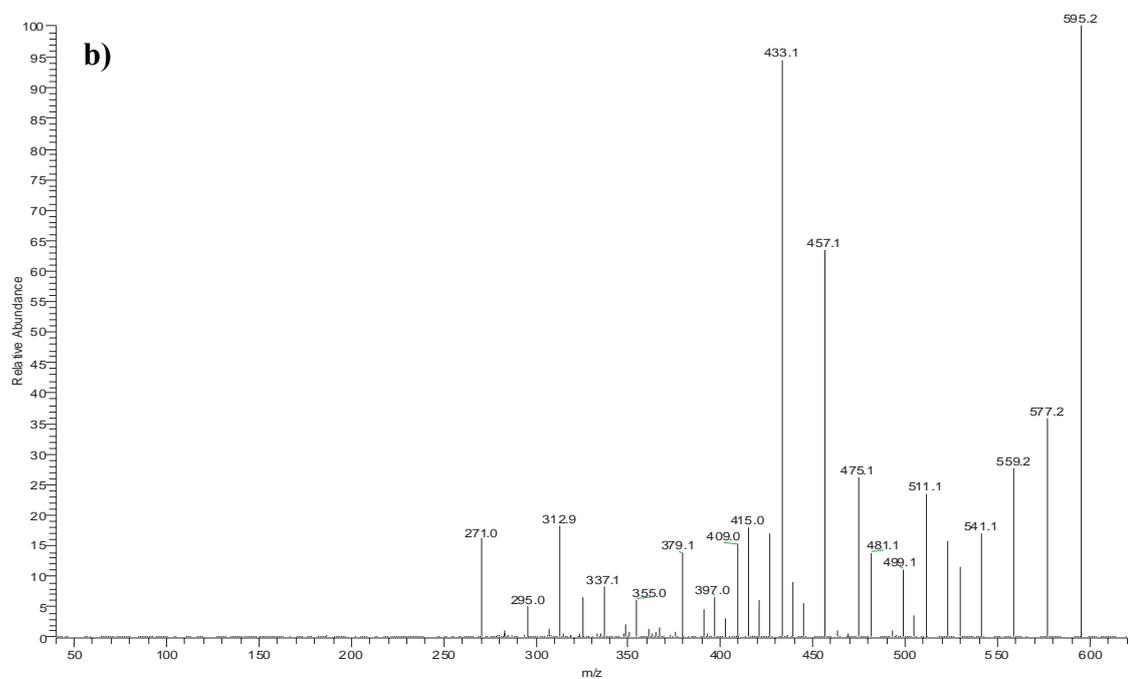
Figure 3.16 b): The ESIMS collision induced dissociation (CID) spectrum of F 1/1 at

m/z 595.
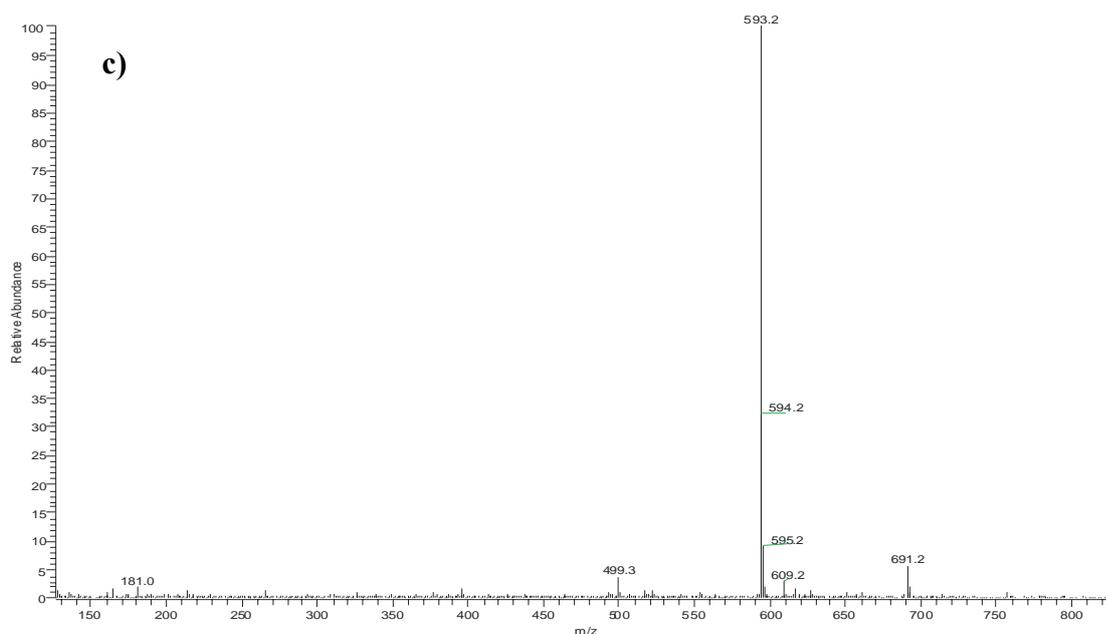
Figure 3.16 c): The mass spectra of F1/1 [full scan negative mode].
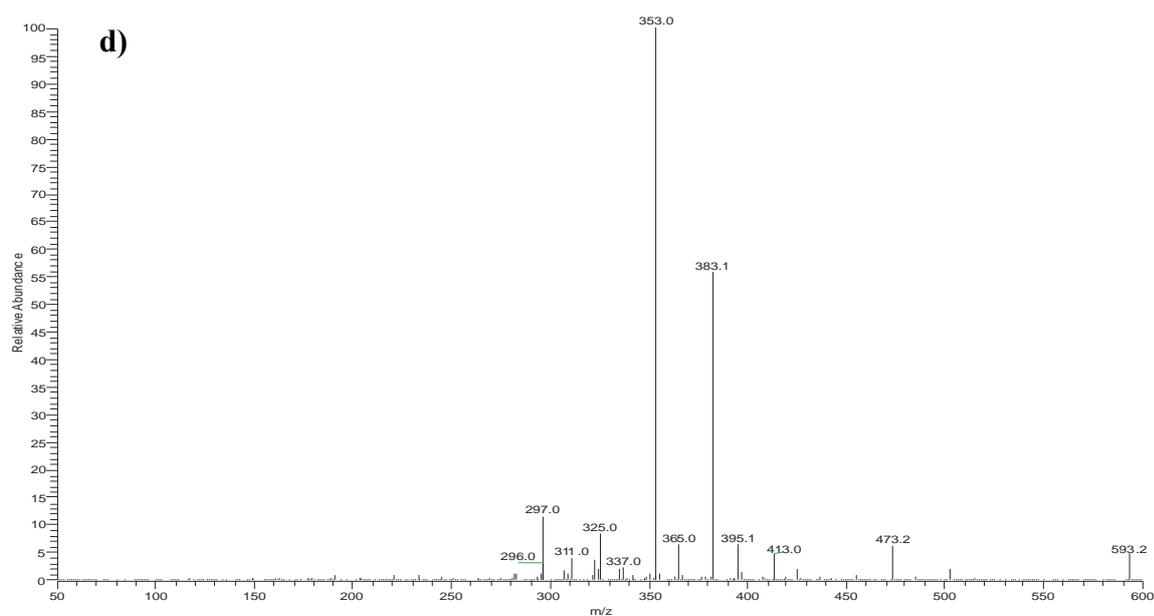
Figure 3.16 d): The ESIMS collision induced dissociation (CID) spectrum of F 1/1 at

m/z 593.

ESIMS of F1/2 provided quasimolecular ions at m/z 595 [M+H]$^+$ and 593 [M-H]$^-$ (Fig.

3.17 a) with positive and negative modes, respectively. The ESIMS CID spectra of F1/2

in negative mode provided additional fragment ions at m/z 413[M-H-180]$^-$ and 293 [M-

H-180-120]$^-$, indicating the presence of two hexose sugar moieties (Fig. 3.17 b).

Similarly, the ESIMS CID in the positive mode provided fragment ions at m/z 595 [M+H]$^+$; 433 [M+H-162]$^+$; 415 [M+H-180)]$^+$; 313[M+H-(120+162)]$^+$; 271[M+H-2(162)]$^+$ (Fig. 3.17 c). The removal of a fragment of 180 depicted that one of the hexose is attached to another sugar molecule. The difference of 120 and 162 showed that the second hexose is *C*-glycoside.
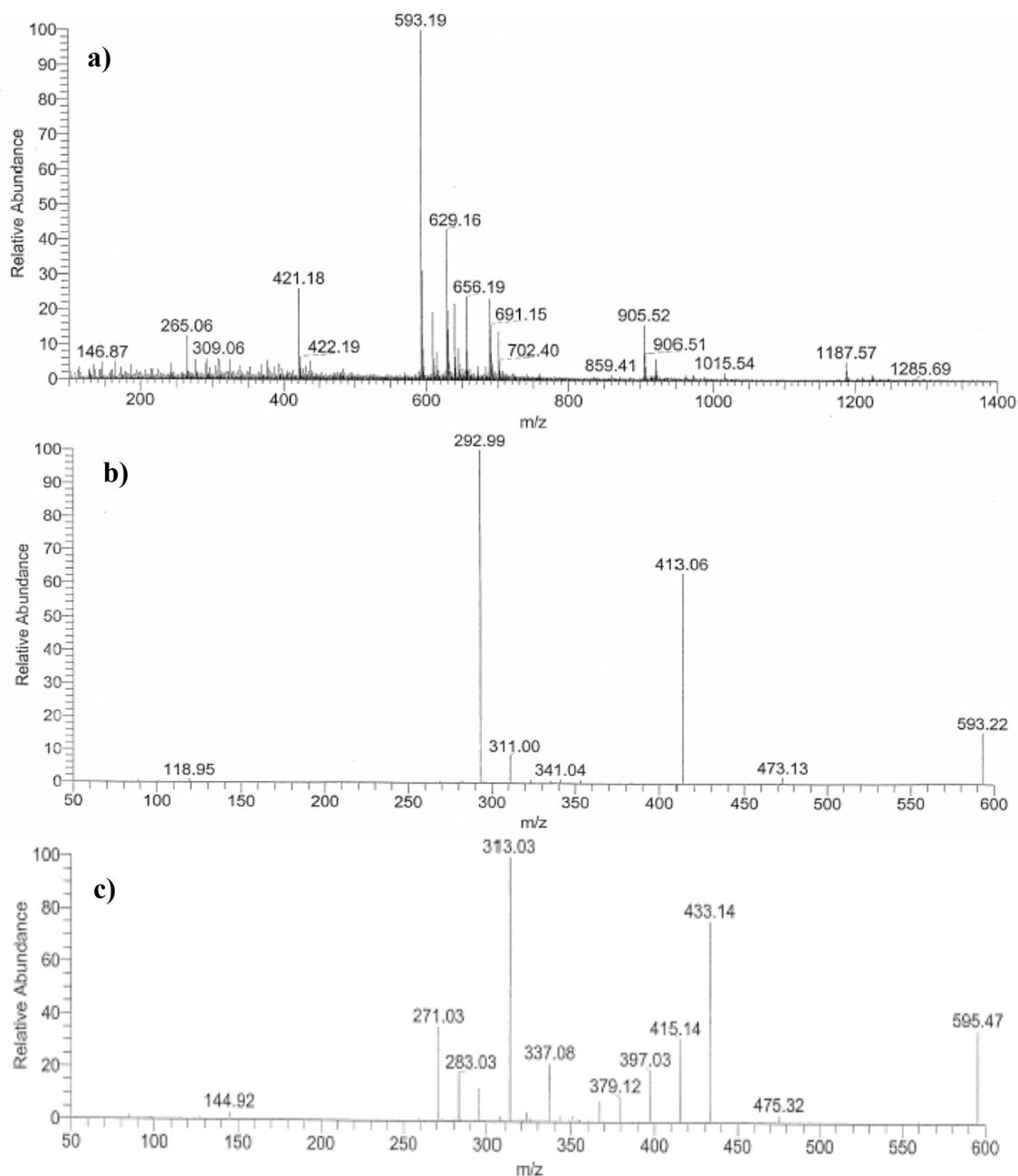
Figure 3.17: The mass spectra of F1/2 a) full scan negative mode; b) CID-fragmentation at *m/z* 593 and c) CID-fragmentation at *m/z* 595.

3.4.2.4　　　Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H NMR

The ^{1}H NMR spectrum of flavonoids appears predominantly in the range 0-10 ppm downfield. The chemical shift establishes the nature of the hydrogen, and coupling constants determine the presence of the ortho, meta and vicinal protons. The ortho and meta couplings have ranges of 6.5-9.0 Hz and 1.5-2.5 Hz, respectively and are of value in establishing the aromatic substitution pattern.

As shown in Figure 3.18, the ^{1}H NMR of F1/1 in (DMSO-d$_6$) showed signals at δ_H 6.78 (s) characteristic for H-3 as well as 6.88 (d) and 7.97 (d) indicating ring B contains para-hydroxy group (4'-OH). The signals at δ_H 6.88 and 7.97 were assigned to H-3'/H-5' and H-2'/H-6', respectively. The broad singlet at δ_H 13.7 was due to the -OH groups which forms hydrogen bonding. The anomeric proton signals of the sugars of F1/1 appeared at δ_H 4.66 (d) and 4.83 (d).
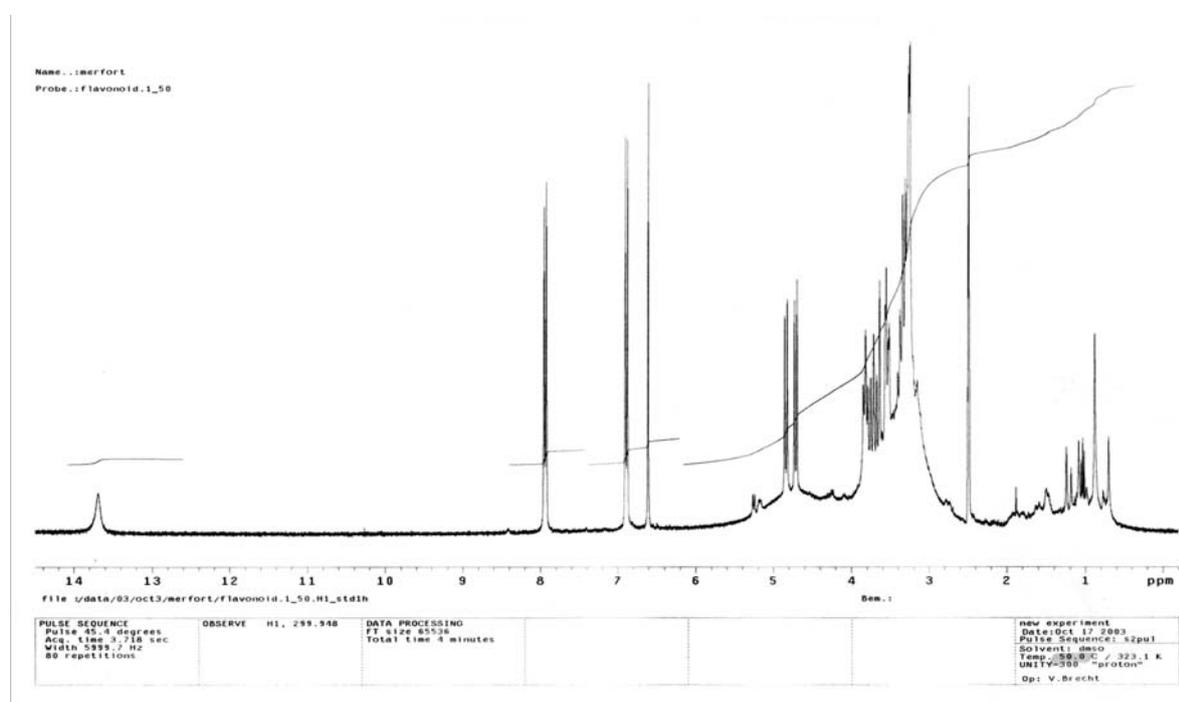
Figure 3.18: ^{1}H NMR of F1/1

^{1}H-^{1}H COSY of F1/1 showed correlations between the signals at δ_H 6.88 and 7.97, indicating ring B contains para-hydroxy group (Fig. 3.19). There was no correlation between these signals and the signal at δ_H 6.78, due to H-3. The ^{1}H NMR results obtained were identical to the published data for the *C*-flavone glycoside, vicenin-2 (Österdahl 1979).
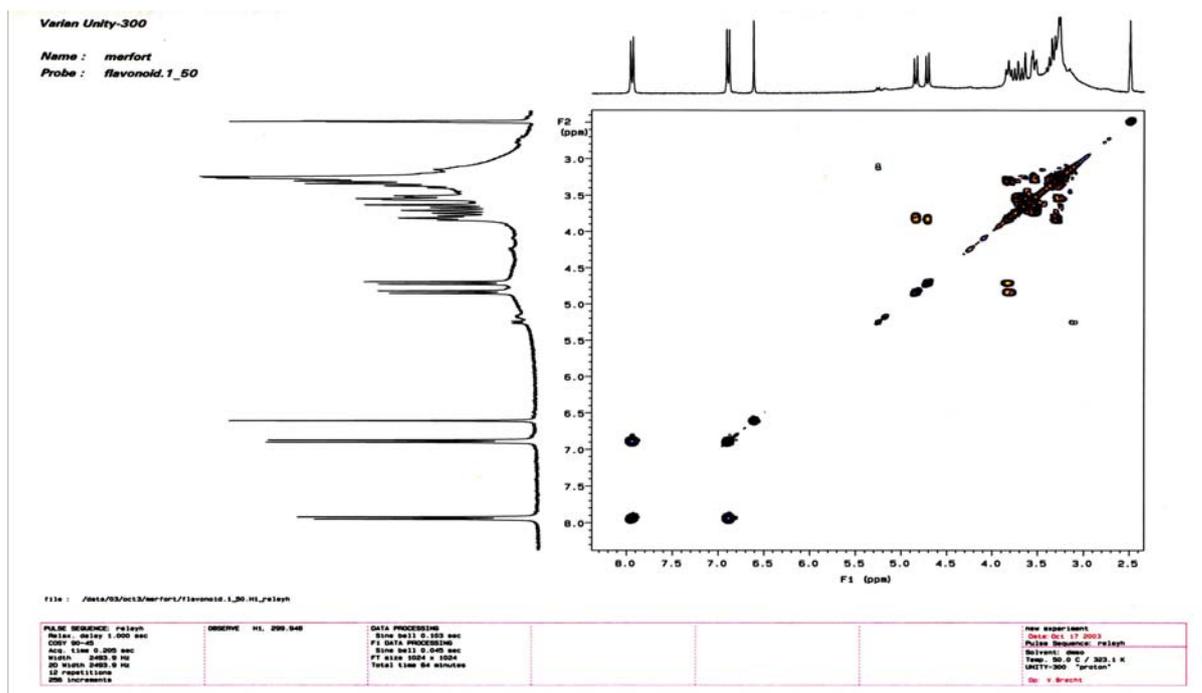
Figure 3.19: ^{1}H-^{1}H COSY of F1/1

The ^{1}H NMR (DMSO-d$_6$) of F1/2 showed signals at δ_H 6.69 (singlet) due to H-3. Comparison of the signals with F1/1 indicated the presence of 4'-OH group in F1/2 due to the signals at δ_H 6.90 for H-3'/H-5' and δ_H 7.91 for H-2'/H-6'. As shown in Figure 3.20, the ^{1}H NMR of F1/2 showed an additional signal at δ_H 6.17 (singlet) for either H-6 or H-8 of ring A, indicating that the *C*-glycoside linkage in F1/2 in only in one position. Moreover, only one anomeric proton signal was observed. The NMR result was in good agreement with the MS which indicated that the two hexose sugars are attached to one another.
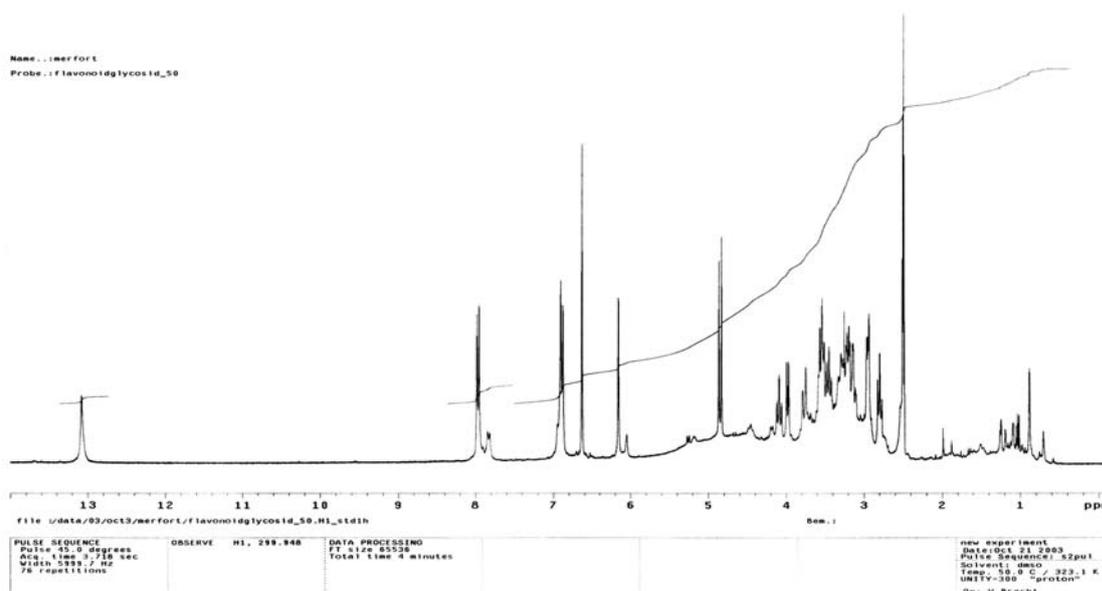
Figure 3.20: ^{1}H NMR of F1/2

^{13}C NMR

In general, ^{13}C NMR spectra are recorded in the proton-noise (broad band) decoupled mode in order to avoid the extensive signal overlap which can occur due to the large one bond carbon hydrogen coupling constants (ca. 120-250 Hz). The multiplets are thus reduced to single lines and it is possible to determine the number of carbons.

Usually, the carbon resonances representing the A- and C-rings are not superimposed and give rise to independent signals. However, the six B-ring carbons can give rise to fewer than six signals when the B-ring is unsubstituted or possesses symmetrical substitution.

Table 3.4 depicted the ^{13}C NMR data of F1/1 and F1/2. Based on the above results and comparing with published data for vicenin-2 (Österdahl, 1979; Sakakibara et al., 1977), F1/1 was identified as 5,7,4'-trihydroxyflavone-6,8-di-*C*-glucoside (vicenin-2). Similarly, the NMR data obtained for F1/2 were identical with published data for vitexin-2''-*O*-glucoside (Markham et al., 1984). Hence, F1/2 was identified as 5,7,4'-trihydroxy-flavone-8-*C*-sophoroside.

Table 3.4: ^{13}C NMR of F1/1 and F1/2.

C	F1/1	F1/2
2	162.72	163.43
3	101.64	102.42
4	181.11	181.62
5	160.78[a]	160.49[a]
6	108.32	98.52
7	160.77[a]	161.11[a]
8	102.03	103.75
9	155.01	156.21
10	104.45	103.17
1'	121.70	121.64
2'	128.35	128.71
3'	115.65	115.81
4'	159.25[a]	160.49
5'	115.65	115.81
6'	128.35	128.71
Sugars	*Glucose at C-6*	*Glucose at C-8*
1''	74.00	71.50
2''	71.10	81.15
3''	78.77	78.26
4''	70.39	70.15
5''	81.47	81.65
6''	61.08	60.38[b]
	Glucose at C-8	*Glucose at C-2''*
1'''	74.27	104.89
2'''	71.43	74.31
3'''	78.77	75.95
4'''	69.86	69.46
5'''	80.87	76.23
6'''	60.60	60.98[b]

[a, b] Assignments with the same superscript may be interchanged.

3.4.2.5   Acid isomerization of the flavonoids

The acid isomerization gives one or more by products owing to the superposition of Wessely-Moser transposition and sugar ring isomerization (Besson and Chopin, 1983). This chemical method, associated with suitable chromatographic systems, remains an

important technique for distinguishing mono-*C*-glycosylflavones, di-*C*-glycosylflavones
and *O*-glycosyl-*C*-glycosylflavones (Maurice, 1994).

F1/1 was stable in acid indicating that it is a di-*C*-glycosylflavones. Acid hydrolysis of
F1/2 yielded vitexin accompanied by small amounts of its Wessely-Moser isomer
(isovitexin) that are identified by HPLC with spiking the sample with authentic
standards of vitexin and isovitexin (Extrasynthese) (Fig. 3.21). The result confirmed
that F1/2 contains an *O*-glycoside linkage (Tomczyk et al. 2002).
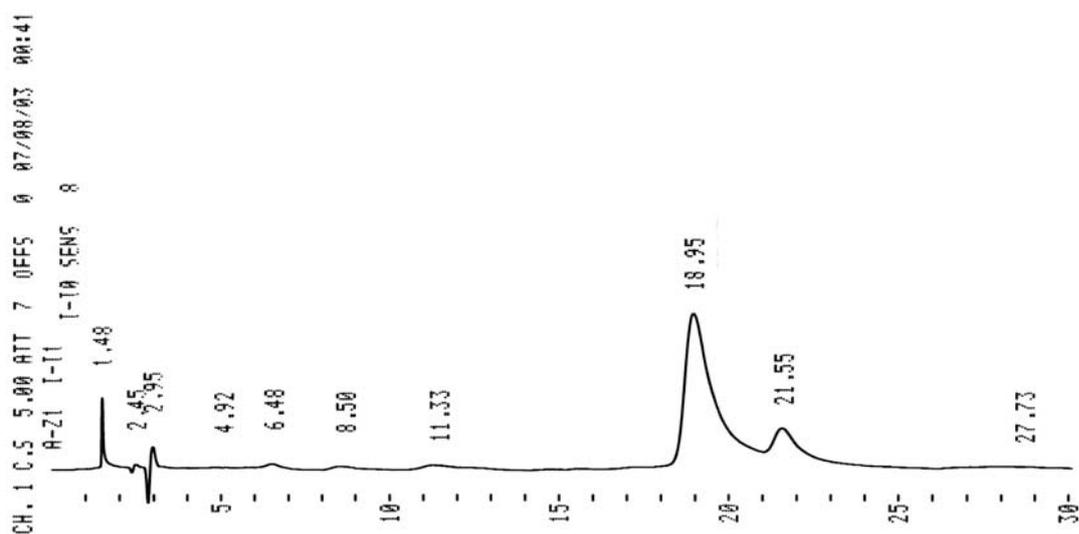
Figure 3.21: HPLC of the acid hydrolysis product of F1/2. [Column: CC250/4 Nucleosil
          100-7 C18; mobile phase: 15% CH_3CN; flow rate: 1 mL/min; detection:
          UV 360 nm] Peaks at retention time of 18.95 and 21.55 min were
          identified as vitexin and isovitexin, respectively.

Chapter 4

QUANTITATIVE DETERMINATION OF THE SAPONINS AND FLAVONOIDS OF *GLINUS LOTOIDES*

4.1 General introduction

The determination of the phytochemical constituents of plant extracts is essential in order to ensure the reliability and repeatability of pharmacological and clinical research, to understand their bioactivities and possible side effects of active compounds and to enhance product quality control. High-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) fingerprints have been applied for this kind of documentation (Gurfinkel and Rao, 2002; Zhang et al., 2005; Ji et al., 2005).

HPLC and TLC methods are generally used for the analysis and identification of pharmaceuticals based on their chromatographic retention data. However, identification problems arise when standards are not available, which is the case for most phytopharmaceutical preparations. In an effort to standardize the crude extract of the seeds of *G. lotoides*, colorimetric and UV-spectrophotometric methods have been developed for quantitative determination of total saponins in the crude extracts using a purified extract and β-escin, a mixture of triterpenoidic saponins obtained from *Aesculus hippocastanum*, as standards (Endale et al., 2000).

In this work, separate HPLC methods were developed for the quantitative routine analysis of the saponins and flavonoids of *G. lotoides*.

4.2   Quantitative determination of the saponins

Different methods have been employed for the qualitative and quantitative determination of saponins. The classical methods like haemolysis, surface activity, fish toxicity have been replaced by chromatographic and photometric methods such as TLC-densitometry and HPLC. High performance liquid chromatography on reversed-phase columns remains the best technique for saponin determination and is the most-widely used method for this group of compounds (Park et al., 2000; Li and Fitzloff, 2001). However, the lack of chromophore allowing detection in UV, limits the choice of gradient and detection method (Oleszek, 2002). Hence, the saponins of *G. lotoides* were quantified after acid hydrolysis using mollugogenol B as an external standard. Ireland and Dziedzic (1985) have described a method for estimation of the total saponin of soybean by HPLC after hydrolysis of the triterpene glycosides.

Due to the absence of commercial standards, mollugogenol B was isolated and used as reference substance for the aglycons of the saponins of *G. lotoides*. The identity of the isolated standard was confirmed with UV and NMR spectral analyses.

4.2.1   Preparation of a standard

Triterpene saponins isolated from the seeds of *G. lotoides* are derivatives of the same aglycon, mollugogenol A, differing only in their sugar moieties and position of substitution (Fig. 3.7, page 21). Therefore, they were quantified after acid hydrolysis using mollugogenol B as an external standard. Unlike the native saponins of *G. lotoides*, mollugogenol B is UV-active due to the formation of a diene chromophore in ring D and E (Fig. 4.1). The isolation and structural confirmation of mollugogenol B are described below.
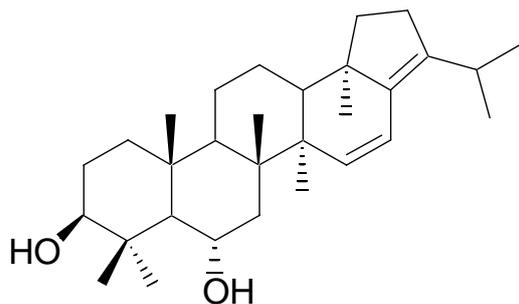
Figure 4.1: Chemical structure of mollugogenol B.

4.2.1.1   Isolation of mollugogenol B

The acid hydrolysis of the saponins of the seeds of *G. lotoides* provided aglycons which are UV-active. Scheme 4.1 shows the acid hydrolysis and extraction of the aglycons from the purified extract of the seeds of *G. lotoides* (Extract F). Similarly, saponin fractions (S1 and S2) were hydrolyzed to identify the hydrolysis products from the major saponins of *G. lotoides*. The optimal acid hydrolysis conditions were determined as described under **4.2.2.**

Scheme 4.1: Acid hydrolysis and extraction of the aglycons of the saponins of *G. lotoides*
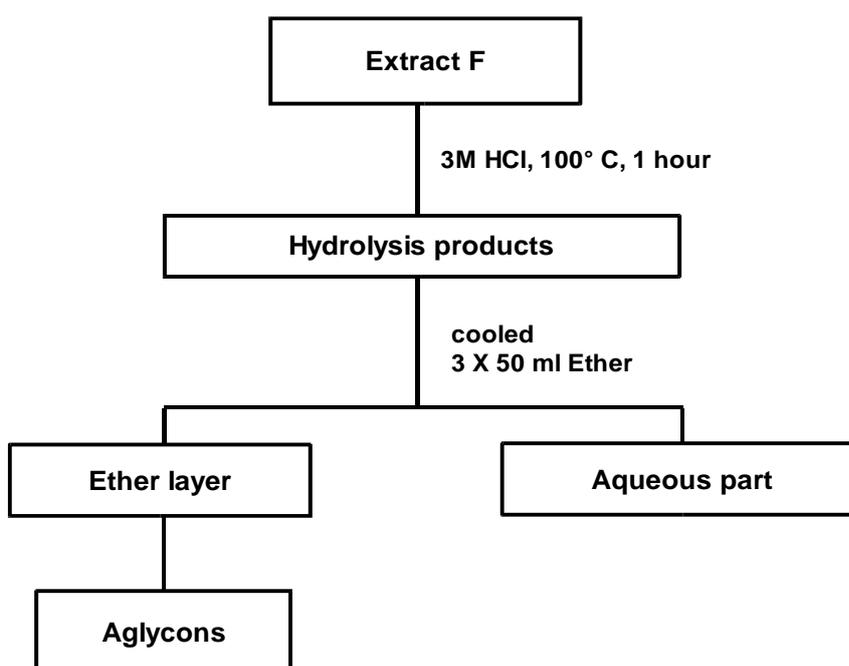
As shown in the scheme 4.1, the purified extract was hydrolyzed with 3M HCl at 100°C for 1 h and the aglycons of the saponins were extracted three times with diethyl ether.

The major hydrolysis product was isolated by RP-HPLC. Figures 4.2 a) and b) depict the HPLC chromatograms of the aglycons from extract F (Fig. 4.2 a) and from the saponin fraction, S1 (Fig. 4.2 b). A similar HPLC chromatogram was obtained after acid hydrolysis of S2.

The acid hydrolysis of extract F gave two major hydrolysis products, aglycons A1 and A2 at retention times of about 13 and 15 minutes, respectively. However, the saponin fractions (S1 and S2) provided only one major aglycon at retention time of 13 min indicating that the major saponins of *G. lotoides* provided only one aglycon, A1. S1 and S2 are HPLC fractions of saponins which provided the five hopane-saponins, glinuside F – I and succulentoside B (Fig. 3.7; Chapter 3).

The acid hydrolysis product at retention time of 13 min was isolated after preparative HPLC scale-up of the injection volume to 250 μL. The structure of aglycon A1 was identified as mollugogenol B by NMR spectral analysis. The isolation and structural identification of aglycon A1 are described below.

TLC analysis of the hydrolysis products of extract F, S1 and S2 as well as the isolated aglycons **A1** and **A2** also confirmed that aglycon A1 is the major hydrolysis product of the saponins of *G. lotoides* (Fig. 4.3).
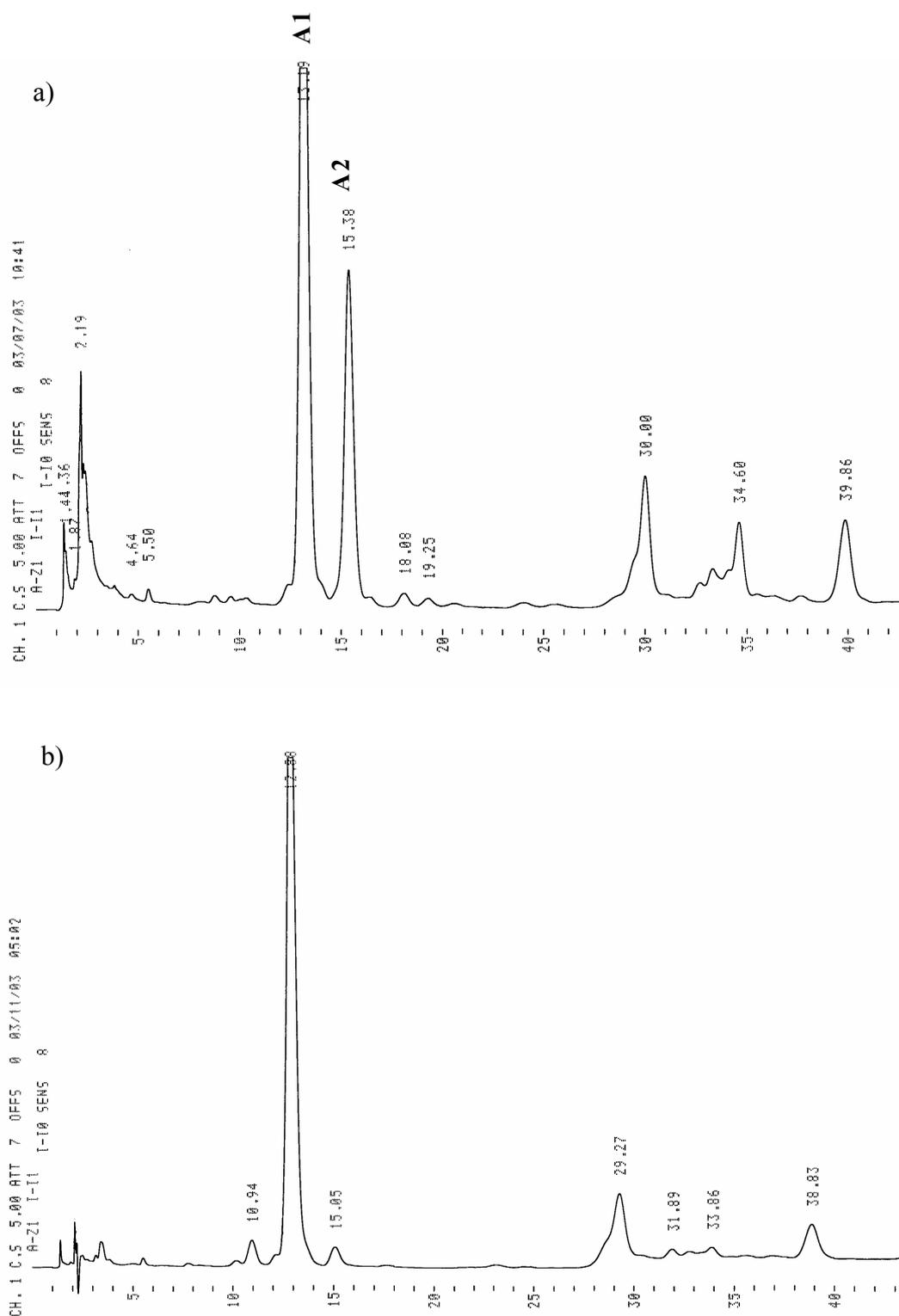
Figure 4.2: HPLC chromatograms of the acid hydrolysis products from a) extract F and b) saponin fraction, S1 [Column: CC250/4 Nucleosil 100-7 C_{18}; mobile phase: acetonitrile; detection: UV-254 nm; flow rate: 1.0 ml/min; injection volume: 20 µL].

Mollugogenol B

S1 S2 A1 A2 Ext. F
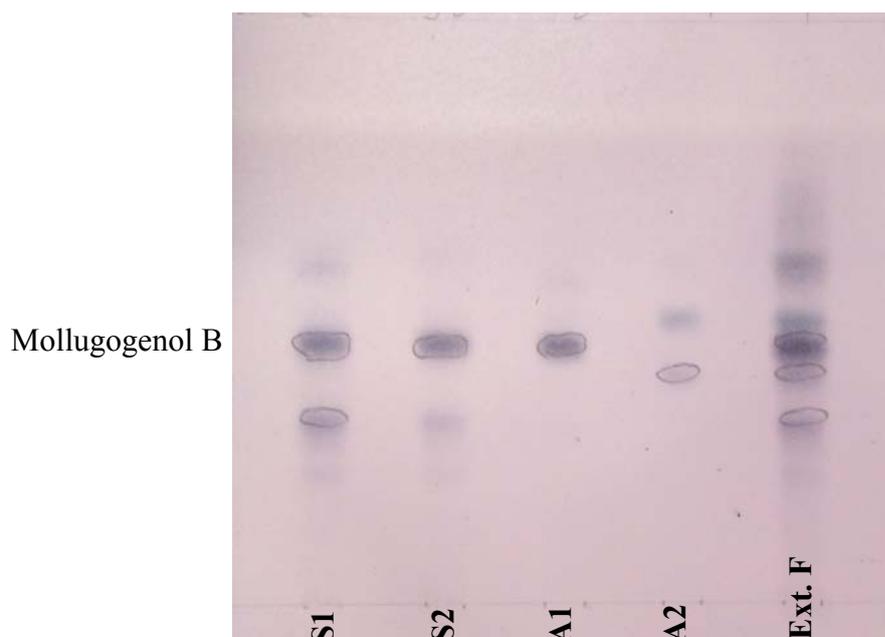
Figure 4.3: TLC analysis of the acid hydrolysis products of Extract F, S1 and S2 and the
isolated aglycons A1 and A2. [RP-18 F_{254s} TLC, Merck; solvent system:
methanol: water (9:1); detection: 5% vanillin in EtOH with H_2SO_4;
mollugogenol B: $R_f = 0.43$]

4.2.1.2   Structural confirmation of mollugogenol B

The structure of the isolated aglycon A1 was confirmed with NMR spectral analysis and
by comparison with published data as mollugogenol B. Table 4.1 provides the ^{1}H and
^{13}C NMR data of mollugogenol B which is in good agreement with previous report
(Hamburger et al., 1989).

4.2.1.3   Determination of the purity of mollugogenol B

The purity of the isolated mollugogenol B was determined by TLC, HPLC and UV-
spectrophotometry.

Table 4.1: ^{1}H and ^{13}C NMR of mollugogenol B

	^{1}H	^{13}C
1		41.3[a] t
2		27.8 t
3	3.15 dd (10.5, 6)	79.6 d
4		40.5 s
5		61.8 d
6	4.04 ddd (10, 10, 4)	69.1 d
7		46.0 t
8		47.2 s
9		51.3 d
10		39.9 s
11		22.8 t
12		23.4 t
13		49.0 d
14		43.5 s
15	5.68 d (10)	136.0 d
16	6.28 d (10)	120.7 d
17		140.3 s
18		49.0 s
19		41.3[a] t
20	2.42 ddd (16, 10.5, 6); 2.25 dd (16, 9)	29.0 t
21		141.7 s
22	2.82 qq	28.0 d
23	1.33 s	31.5 q
24	0.98 s	16.1 q
25	0.92 s	17.2 q
26	1.03	18.7 q
27	1.24 s	18.0 q
28	0.93 s	19.2 q
29	1.02 d (7)	22.0 q
30	1.04 d (7)	21.6 q

[a] Assignments with the same superscript may be interchanged.

Thin Layer Chromatography (TLC)

Figure 4.4 depicts a typical TLC plate of mollugogenol B with only one major spot at R_f value of 0.56. The purity of the standard was evaluated qualitatively with the absence of another major spots.
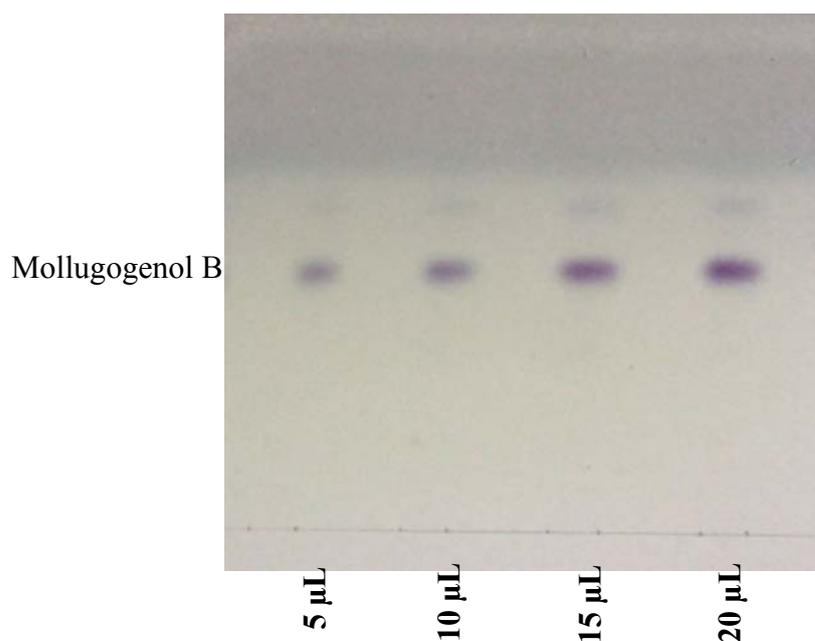
Figure 4.4: TLC of mollugogenol B [RP-18 F_{254s} TLC, Merck; solvent system: methanol; detection: 5% Vanillin in EtOH with H_2SO_4; sample solution: 237 mg/100 ml].

High Performance Liquid Chromatography (HPLC)

A high concentration of mollugogenol B (237 mg/100mL) was injected into HPLC and developed using various solvent systems. A typical HPLC is depicted in Figure 4.5. Peak area of mollugogenol B at retention time of 8.12 min (Fig. 4.5) was compared to the total peak area of the chromatogram and the purity of mollugogenol B was calculated as 98%. As shown in the chromatogram, except for a minor peak at retention time of 14.56 min there is only a peak for mollugogenol B.
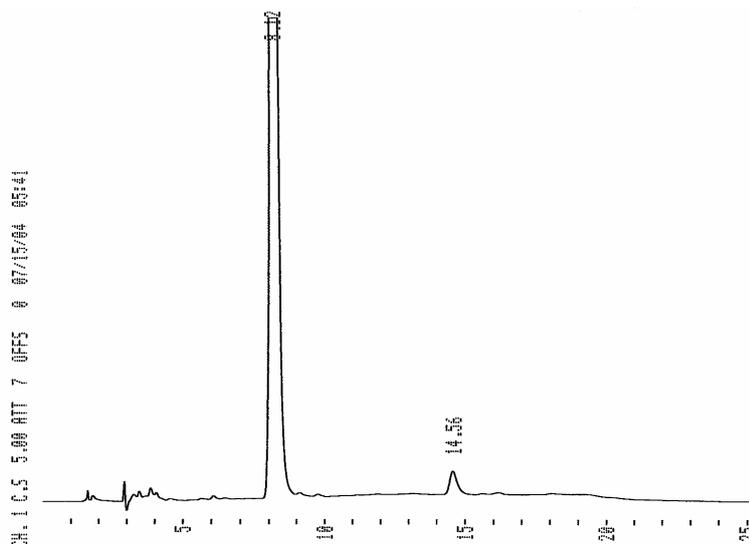
Figure 4.5: HPLC chromatogram of mollugogenol B [Column: CC250/4.6 Nucleosil 100-5 C_{18}; mobile phase: methanol-water gradient system (see under **4.2.3**); detection: UV-254 nm; flow rate: 1.0 ml/min; injection volume: 20 µL of 237 mg/100ml].

Ultraviolet Spectroscopy (UV)

Figure 4.6 depicts the UV spectrum of mollugogenol B. The molar absorptivity (log ε) of the methanol solution of mollugogenol B at concentration of 0.948 mg/100ml was found to be 3.98, 4.16 and 4.12 at λ_{max} of 260sh, 251 and 243 nm, respectively, which is in good agreement with literature values (Hamburger et al. 1989), indicating the purity of mollugogenol B.
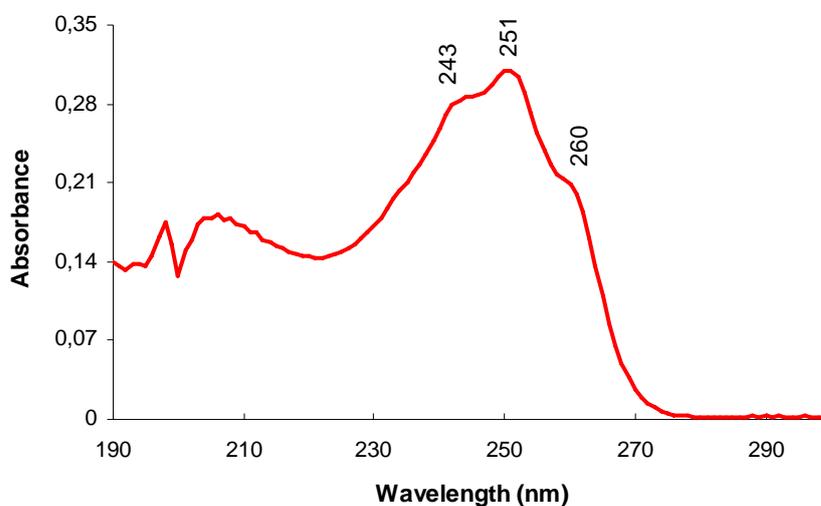
Figure 4.6: UV spectrum of mollugogenol B [0.948 mg/100ml MeOH].

4.2.2   Optimization of the acid hydrolysis of the saponins

During acid hydrolysis, a number of saponins generate not only the genuine aglycon but also artifacts, which can influence the final results. Acid-catalyzed double-bound migration, epimerization and dehydration are often observed during hydrolysis (Potterat et al. 1992). Besides, structurally different saponins show different rates of hydrolysis (Ireland and Dziedzic, 1986). Hence, optimization of the acid hydrolysis conditions is indispensable before development of quantification method. As shown in Figure 4.7, the acid hydrolysis of the saponins using 3M HCl at 100°C for 1 hour provided the maximum yield of mollugogenol B.
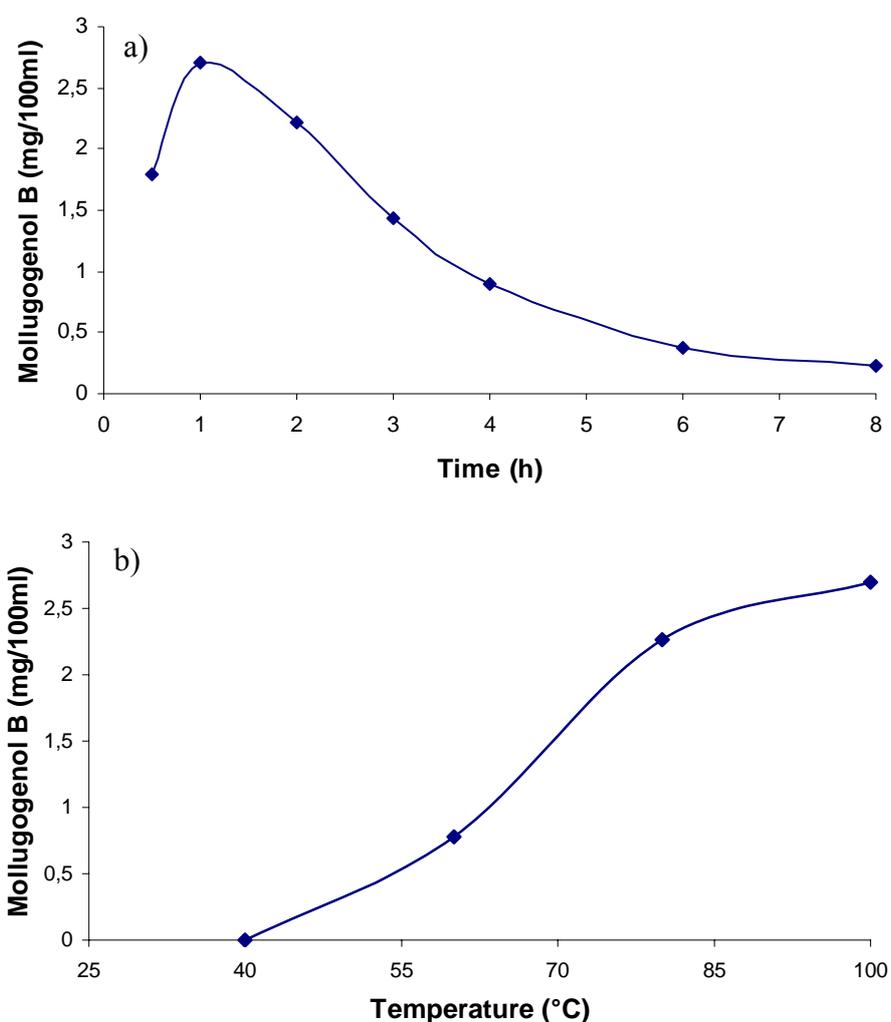
Figure 4.7: Effect of time a) and temperature b) of acid hydrolysis of the saponins of *G. lotoides* on the mollugogenol B yield. Hydrolysis conditions: a) 3M HCl at 100 °C; b) 3M HCl for 1 h.

The optimum time of hydrolysis was determined by analyzing the hydrolysis products at different time intervals (Fig. 4.7 a). As shown in the figure, the formation of mollugogenol B reached the maximum after 1 hour and subsequent heating under the hydrolysis conditions resulted in a loss of mollugogenol B.

The maximum amount of mollugogenol B was obtained using a hydrolysis temperature of 100°C (Fig. 4.7 b). Lower acid concentrations (0.5; 1 and 2M HCl) required longer time to achieve the maximum concentration of mollugogenol B, whereas higher acid concentrations (4 and 5 M HCl) produced artifacts, which reduced the yield of mollugogenol B.

4.2.3   HPLC analysis of the aglycons of the saponins

Figure 4.8 shows typical HPLC chromatograms of mollugogenol B as external standard (Fig. 4.8 a); acid hydrolysis products of extract A (crude extract of the seeds of *G. lotoides*) (Fig. 4.8 b) and tablet formulation of extract A after acid hydrolysis (Fig. 4.8 c). As shown in the figures, separation of the aglycons was completed within 25 min and the major peak at 8 min belongs to mollugogenol B. The other peaks were not identified.

The crude extract of the seeds of *G. lotoides* (extract A) was used for the formulation of tablets. Hence, the analytical method developed should determine the total saponins the extract as well as the tablet formulations thereof. The preparation of the dry extract and the tablet formulations thereof are described under Chapter 5 and 6, respectively.
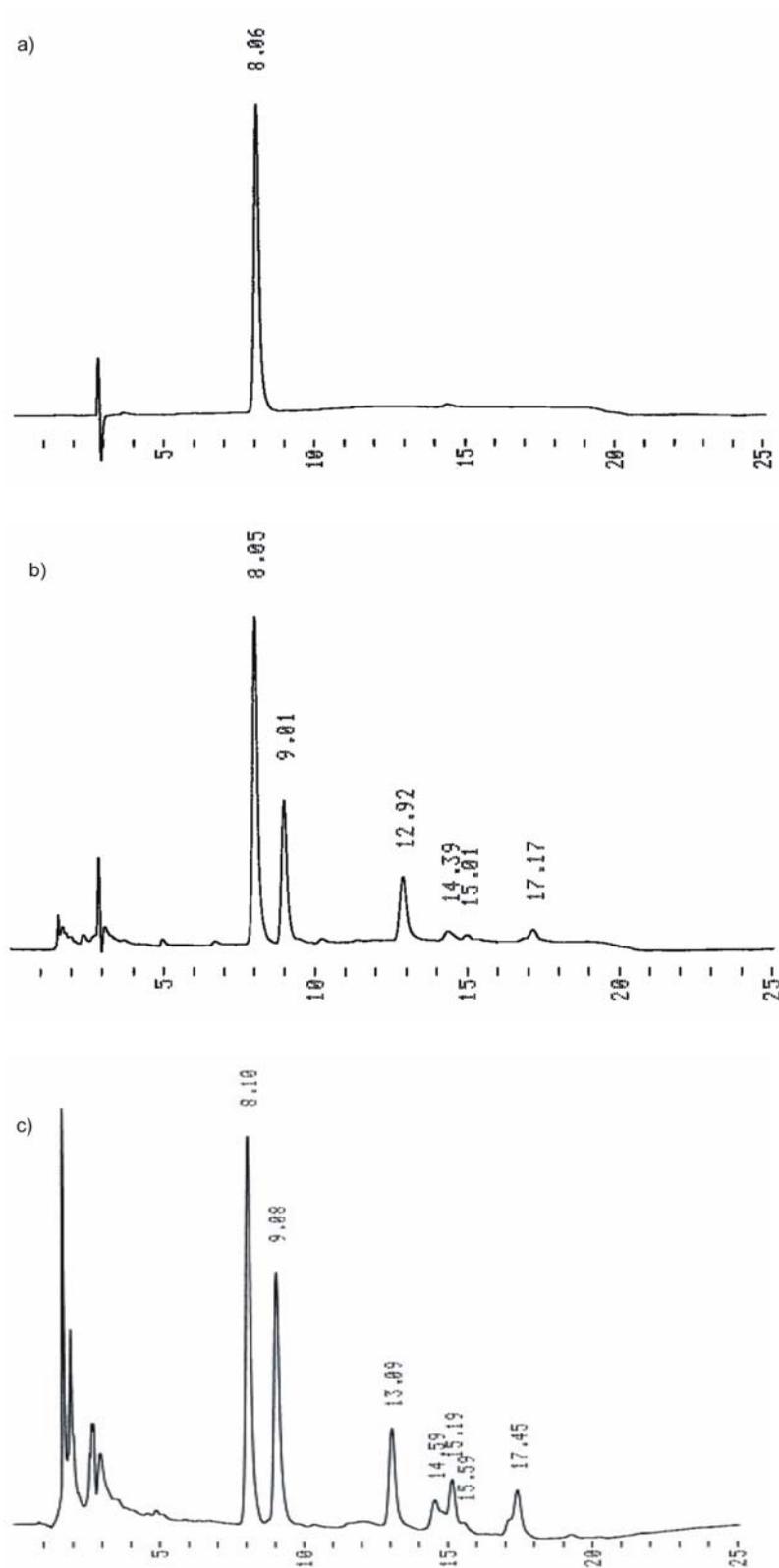
Figure 4.8: HPLC Chromatogram of a) mollugogenol B as external standard, b) acid
hydrolysis products of the crude extract of the seeds of *G. lotoides* and c)
tablet formulation of the extract after acid hydrolysis. [Column: CC 250/4.6
Nucleosil 100-5 C_{18}; mobile phase: see under 7.**5.1.5** (Table 7.8); flow rate
1.0 mL/min; detection: 254 nm; sample volume: 20 µL].

4.2.4   Preparation of the calibration curve

The calibration curve of mollugogenol B provided a linear relationship between the peak area (Y) and the concentrations of mollugogenol B injected (X) in mg/100ml with a regression equation of $Y = 376738 X - 703.7$ ($r^2 = 0.9998$) in the concentration range of 0.237 to 2.37 mg/100mL (Fig. 4.9). As shown in Figure 4.10 the residuals points of the calibration points were well distributed within acceptable limits.

The total amounts of saponins of the crude extract and tablet formulation were determined as glinuside G equivalent. The molecular weight of glinuside G (900) approximates the average molecular weight of the saponins of *G. lotoides* (Fig. 3.7). Before determination of the total saponins, an equimolecular mollugogenol A (molecular weight = 476) was calculated from mollugogenol B (molecular weight = 440).

4.2.5   Method validation

Validity of an analytical method can be verified by establishing several analytical and statistical parameters (Table 4.2). The linearity of the detector response for mollugogenol B was confirmed between 0.237 – 2.37 mg/100ml. The calibration curves (Fig. 4.9), residuals (Fig. 4.10) and standardized residuals were inspected to assess linearity. The calibration curves were analyzed using a linear regression model and correlation coefficients (Table 4.2).

The intra- and inter-day repeatability of the method was evaluated using multiple preparations of the sample with relative standard deviation of 0.5 and 1.17%, respectively. The limits of detection (LOD) and limits of quantification (LOQ) were calculated according Gottwald (2000) and were found to be 0.027 and 0.082

mg/100mL, respectively, which were confirmed with serial dilutions.
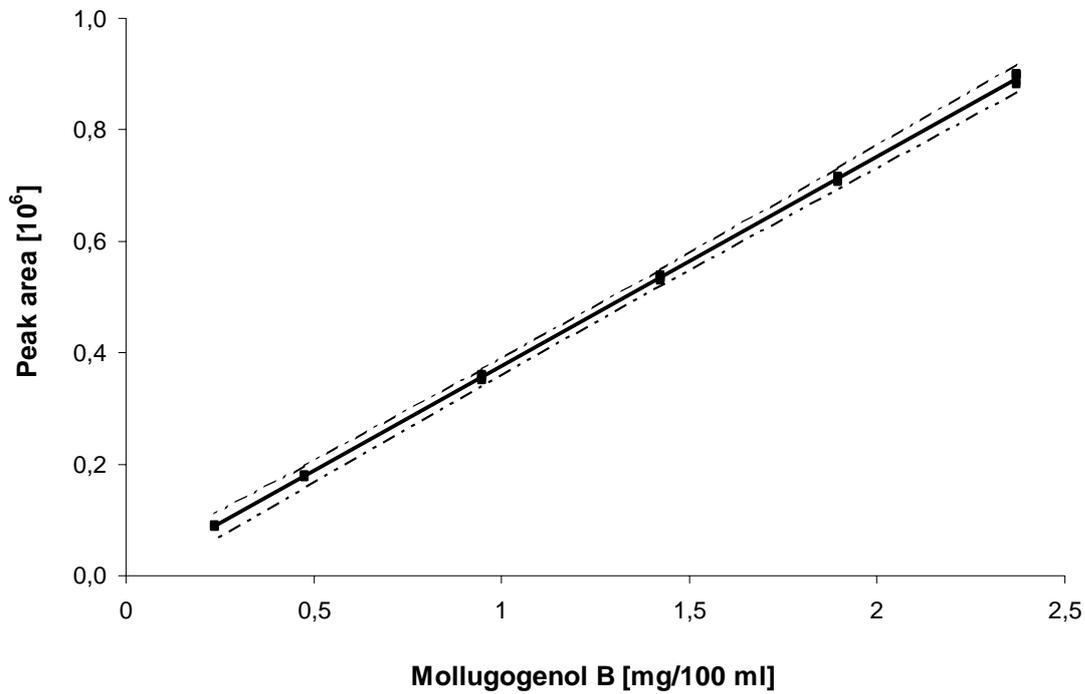
Figure 4.9: HPLC calibration curve of mollugogenol B [■ Data points; ——— Linear

------   regression; 95% Confidence interval (10 times)].
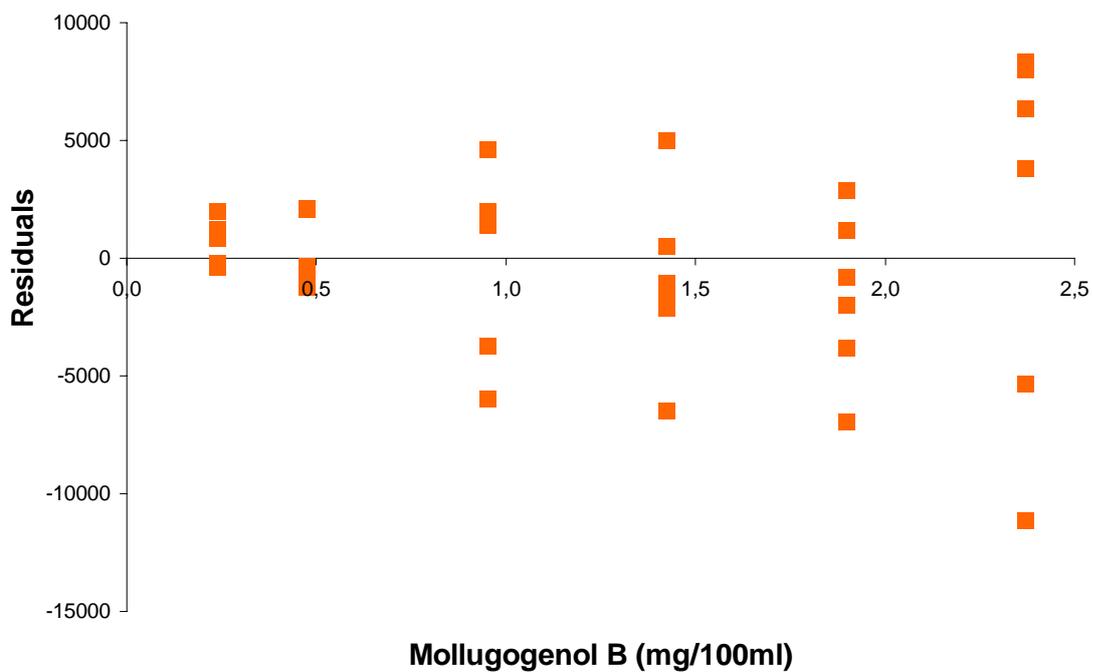
Figure 4.10: Residual plots of the HPLC calibration curve of mollugogenol B

Table 4.2: Statistical validation of the calibration data for quantitative determination of

mollugogenol B

Parameter	Mollugogenol B
Concentration range [mg/100ml]	0.237 – 2.37
Number of concentration	6
Average values	$x = 1.225$ $y = 460\ 807.222$
Correlation coefficient	0.9998
Relative standard deviation (%)	0.90
Calibration equation	$f(x) = 376\ 738x - 703.66$
LOD [mg/100ml]	0.027
LOQ [mg/100ml]	0.082
System precision	1.17% (n = 6)
Method precision	0.50% (n = 9)

4.3   Quantitative determination of the flavonoids

Several liquid chromatographic (LC) methods with UV-Vis absorption (Gil et al., 1995; Hertog et al., 1992; Blouin and Zarins, 1988) or diode-array ultraviolet (DAD-UV) (Mouly, 1998; Mateos, et al., 2001; Bramati, et al., 2002), fluorescence (Hollman et al., 1998) and mass spectrometric (Raffaelli et al., 1997; Justesen, 1998) have been developed for the analysis of flavonoids. Merken und Beecher (2000) have reviewed the various HPLC and sample preparation methods that have been employed to detect and quantify flavonoids.

Quantitative determination of individual flavonoid glycosides is difficult because most reference compounds are not commercially available. Hence, the total flavonoids of the

seeds of *G. lotoides* in the crude extract as well as in tablet formulations were determined by HPLC using vicenin-2 as an external standard.

4.3.1   Preparation of a standard

The isolation and structural confirmation of vicenin-2 from the purified extract of the seeds of *G. lotoides* have been described under section **3.3.2** and **3.4.2,** respectively.

4.3.2   Determination of the purity of vicenin-2

The purity of the isolated vicenin-2 was determined using TLC and HPLC methods as described below. The TLC and HPLC were loaded with high concentrations of vicenin-2 to evaluate the degree of purity.

4.3.2.1   Thin layer chromatography

Figure 4.11 shows a TLC plate of vicenin-2. Overloading the TLC plates up to 39 μg showed no major impurity. However, due to the overloading, the spots at high concentration of vicenin-2 (19.5 and 39 μg,) were diffused.
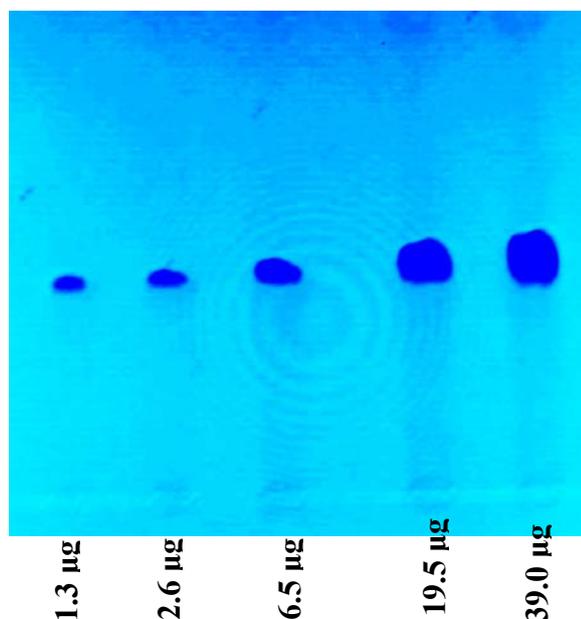
Figure 4.11: TLC of vicenin-2 [RP-18 TLC plates; solvent system $CH_3CN:H_2O$ (15:85); sample concentration: 130 mg/100 ml; detection: NPR (1% Diphenyl-boryloxyethylamin/ MeOH and 5% PEG 400 in EtOH), UV-366nm].

4.3.2.2   High performance liquid chromatography

As shown in Figure 4.12, the HPLC of vicenin-2 provided only one major peak at retention time of about 20 min. The purity of vicenin-2 was calculated from the peak area as 97%.
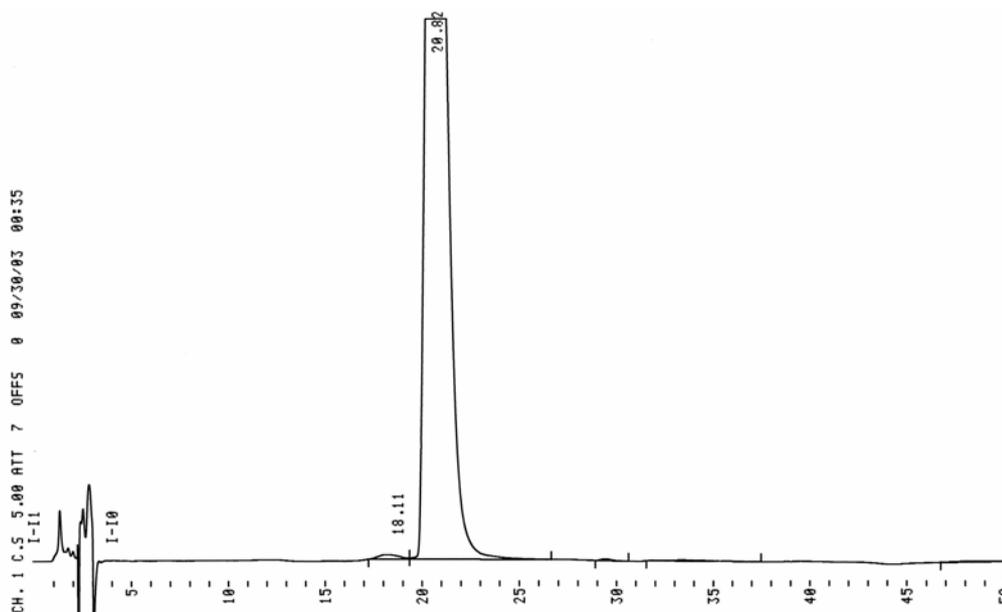
Figure 4.12: HPLC chromatogram [CC 250/4.6 Nucleosil 100-5 C_{18}; mobile phase: $CH_3CN:H_2O$ (1:9); flow rate 1.0 mL/min; detection: UV-360 nm; sample volume: 20 µL of 130 mg/100ml]

4.3.3   HPLC analysis of the flavonoids

Figures 4.13 (a, b, and c) depict chromatograms of vicenin-2 (Fig. 4.13 a), crude extract of *G. lotoides* (Fig. 4.13 b) and tablet formulation of the extract (Fig. 4.13 c). The flavonoids at retention time of 8 and 10.7 minutes were identified as vicenin-2 and vitexin-2″-*O*-glucoside, respectively. The concentration the total flavonoids of the seeds of *G. lotoides* were calculated from total peak area of the chromatogram (Fig. 4.13 b and c) and expressed as vicenin-2 equivalent.
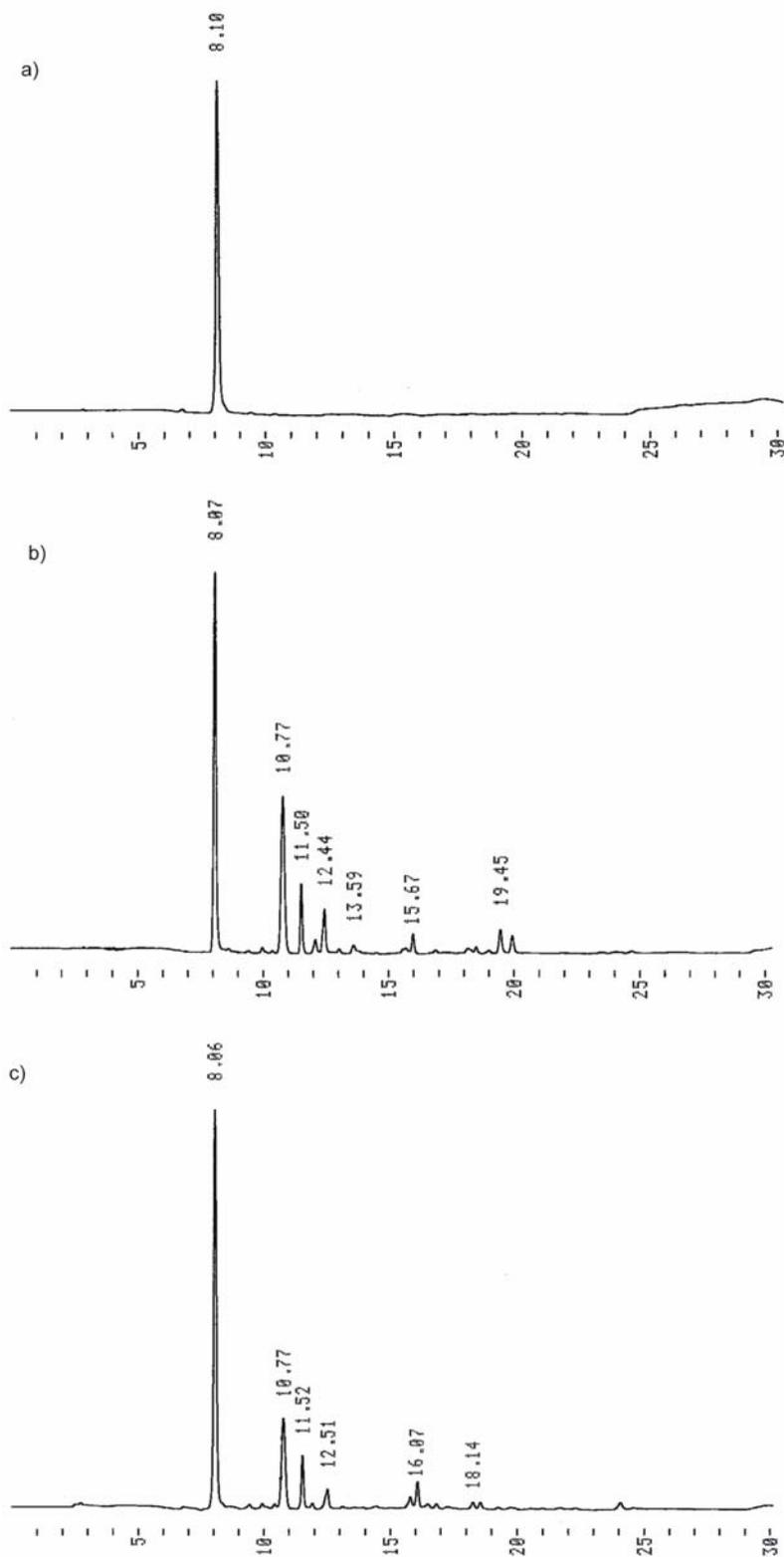
Figure 4.13: Chromatograms of a) vicenin-2 standard, b) crude extract of the seeds of *G.*

lotoides and c) tablet formulation of the extract separated by RP-18 HPLC.

[Column: CC 250/4.6 Nucleosil 100-5 C_{18}; mobile phase: see under flow

rate 1.0 mL/min; detection: 360 nm; injected sample volume: 20 µL].

Six tablets were analyzed and the content of the flavonoids was determined. As shown in Figure 4.13 c, the HPLC chromatogram of the tablets showed similar flavonoids pattern compared to the crude extract (Fig. 4.13 b) indicating that the HPLC method was not affected by the tablet excipients and manufacturing processes (Chapter 6).

4.3.4   Preparation and validation of the calibration curve

Within the concentration range of 0.583 to 5.83 mg/100mL, the relationship between peak area of vicenin-2 (Y) and concentration in mg/100mL (X) was linear with a regression equation Y = 260009X – 5250.8. The linearity of the calibration curve was verified by the correlation coefficient ($r^2 = 0.9997$) as well as inspection of the residuals (Fig. 4.14 and 4.15). Table 4.3 provides the calibration data of vicenin-2.
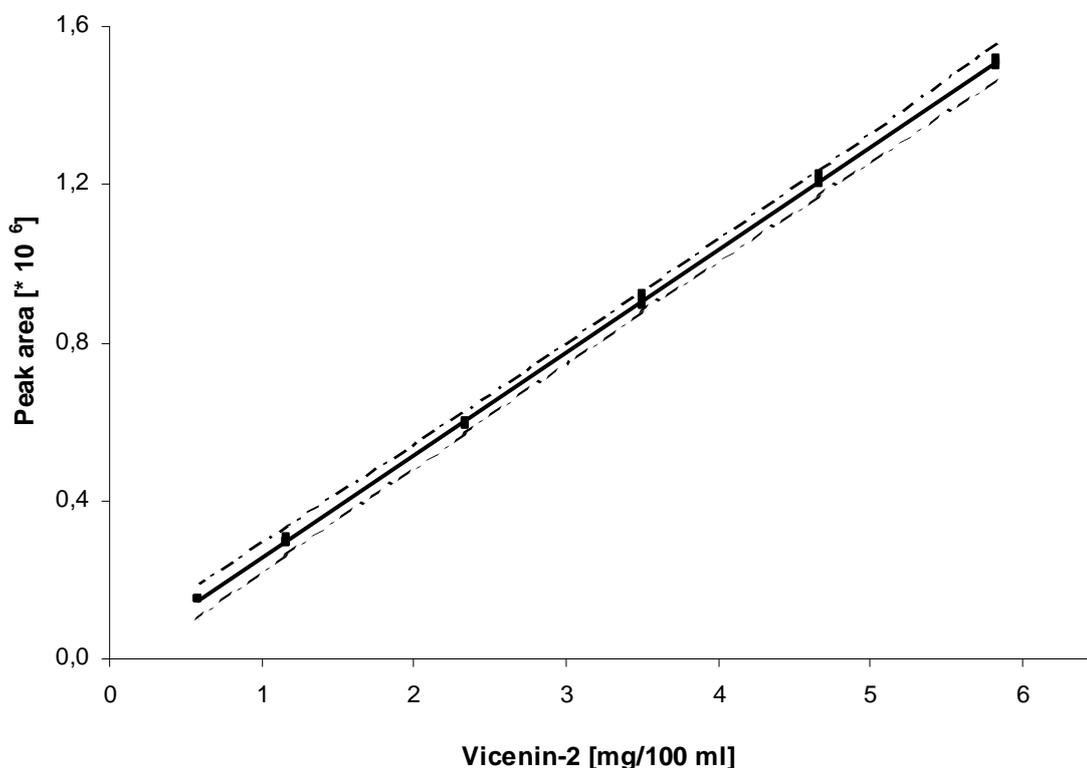
Figure 4.14: HPLC calibration curve of vicenin-2   [■ Data points; ——— Linear
----- regression; 95% Confidence interval (10 times)].
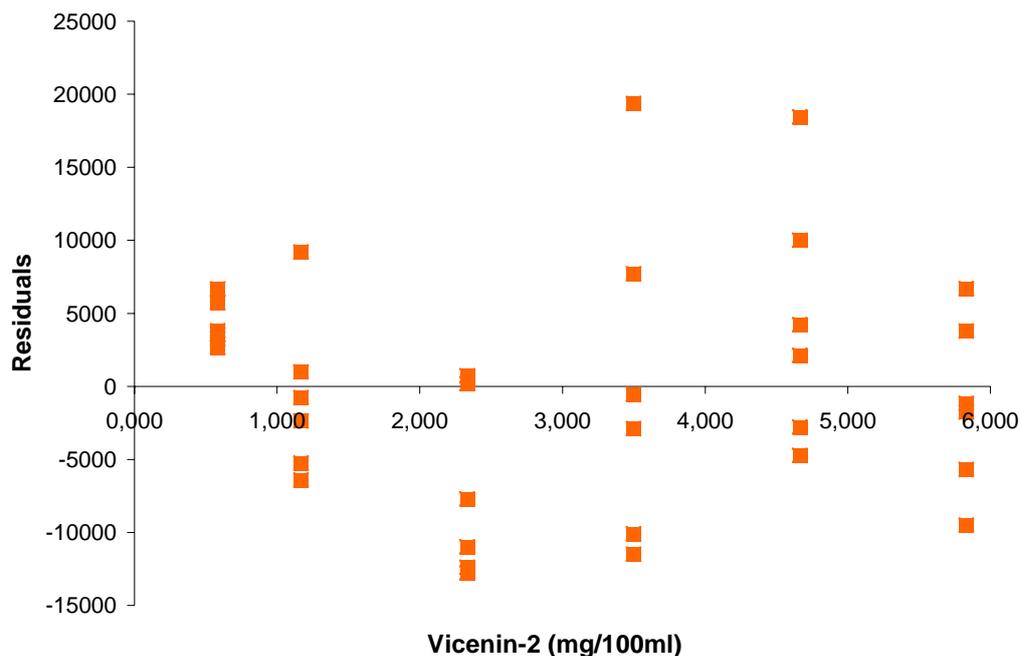
Figure 4.15: Residual plots of the HPLC calibration curve of vicenin-2

Table 4.3: Calibration data and statistical validation of the HPLC quantitative determination of vicenin-2

Parameter	Vicenin-2	
Concentration range [mg/100ml]	0.583 – 5.830	
Number of concentration	6	
Average values	$x = 3.012$	$y = 777\ 940.361$
Correlation coefficient	0.9997	
Relative standard deviation	1.01%	
Calibration equation	f(x) = 260009x – 5250.8	

The LOD and LOQ were calculated according to Gottwald (2000) and were found to be 0.075 and 0.225 mg/100mL, respectively. Similar LOD and LOQ results were obtained using serial dilution of the standard solution. Reproducibility of the methods was verified by determining six extracts with relative standard deviations of 0.95% and the

intra-day variations (n = 9) were found to be 1.86%. The values are in good agreement with USP requirements for a validated method.

4.4   Preparation of secondary standard

Characterization and utilization of a secondary standard is necessary as extraction and isolation of the pure standards are time consuming and expensive for routine quality control of the plant extracts. Hence, the purified extract of the seeds of *G. lotoides* (extract F) was prepared and characterized. The extraction and purification of extract F have been described in Scheme 7.1 under Experimental section. The contents of total flavonoids and saponins in extract F were determined using the HPLC methods detailed above.

Accordingly, the secondary standard was found to contain 10% vicenin-2 and 21.3% total flavonoids calculated as vicenin-2 equivalent. The amount of saponins in the secondary standard was 6.3% mollugogenol B and 12.4% total aglycons calculated as mollugogenol B, 14.2% glinuside G and 25.4% total saponins calculated as glinuside G equivalent.

4.4.1   Preparation of calibration curve using the secondary standard

Figures 4.16 and 4.17 depict the calibration curve of extract F for the saponins and flavonoids, respectively. The HPLC quantitative methods described above were adopted. The peak areas of mollugogenol B and vicenin-2 were taken to calculate the calibration equation. These equations were then used to determine the total saponins and flavonoids from the total peak area of the respective chromatogram. Table 4.4 shows the calibration data of extract F as a secondary standard for the saponins and flavonoids of the seeds of *G. lotoides*. The total saponins and flavonoids in extract F were calculated as glinuside G and vicenin-2, respectively.
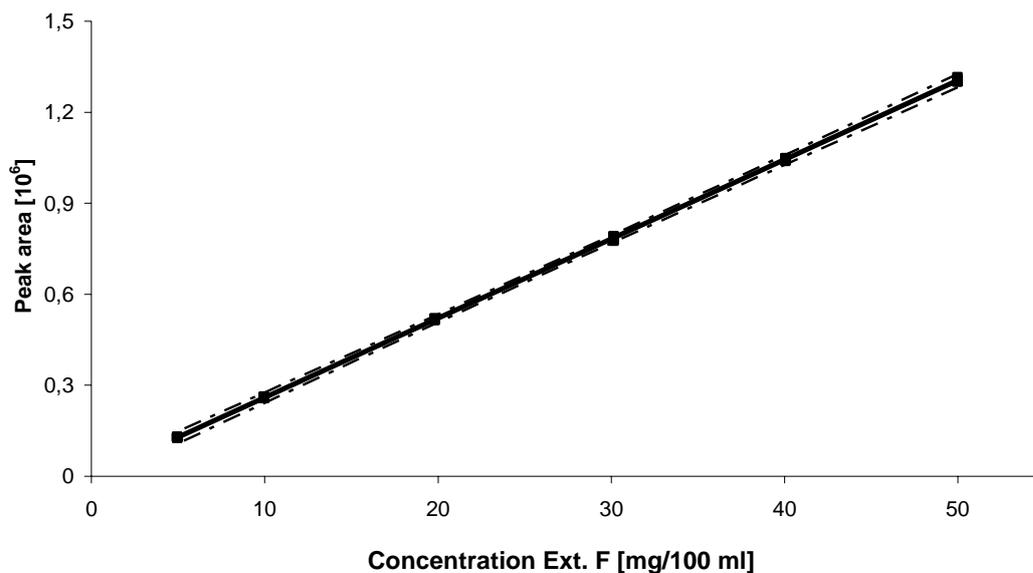
Figure 4.16: HPLC calibration curve of extract F, as secondary standard for the determination of saponins. [■ Data points; —— Linear regression; - - - - 95% Confidence interval (10 times)]. Data points represent the peak area of mollugogenol B in extract F.
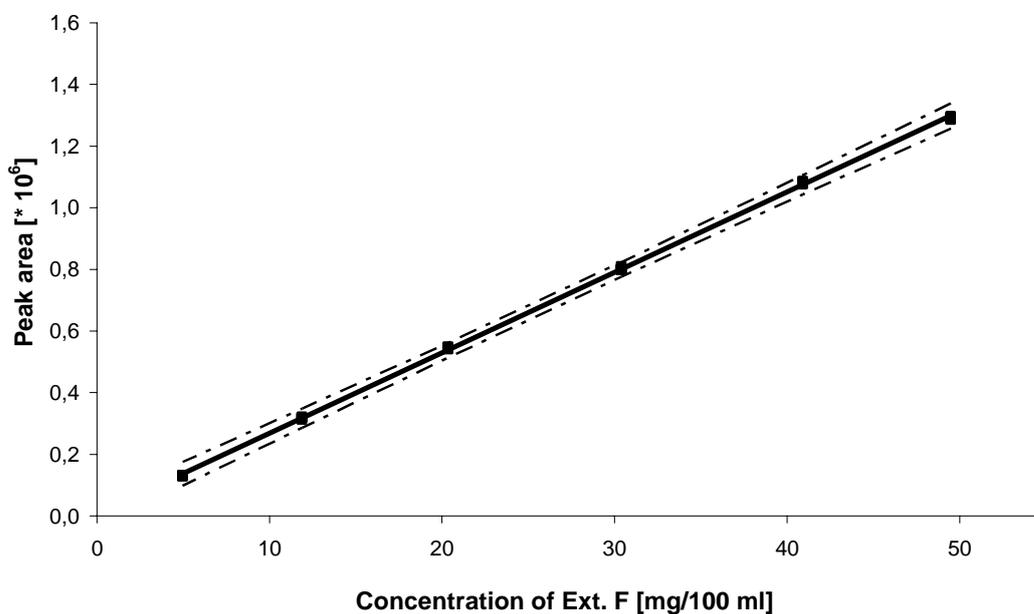
Figure 4.17: HPLC calibration curve of extract F, as secondary standard for the determination of flavonoids. [■ Data points; —— Linear regression; - - - - 95% Confidence interval (10 times)]. Data points represent the peak area of vicenin-2 in extract F.

Table 4.4: Calibration data of quantitative determination of extract F using
mollugogenol B and vicenin-2

Parameter	Mollugogenol B	Vicenin-2
Concentration range [mg/100ml]	4.95 – 49.98	4.96 – 49.49
Number of concentration	6	6
Average values	$x = 25.812$ $y = 673230.75$	$x = 26.327$ $y = 694350.722$
Correlation coefficient	0.9999	0.9997
Relative standard deviation	0.53%	0.98%
Calibration equation	$f(x) = 26117x - 880.48$	$f(x) = 26097x + 7316.3$
LOD [mg/100ml]	0.339	0.635
LOQ [mg/100ml]	1.017	1.906

Accuracy of the analytical method was determined by spiking 32.8 mg of extract F with standard solutions to produce 50, 75, 100, 125 and 150%. The recovery of vicenin-2 was found to be between 98.8 and 101.6% (Table 4.5).

Table 4.5: Statistical data for validation of the HPLC quantitative method using extract
F as secondary standard.

Parameter	Theoretical value [%]	Concentration [mg/100ml]	RSD [%]	Recovery [%]
System precision		4.96 – 49.49	0.95 (n = 6)	
Method precision		33.51 (n = 9)	1.86	
Recovery	50	16.4	1.85 (n = 3)	99.0
	75	24.6	0.68 (n = 3)	99.2
	100	32.8	0.50 (n = 3)	98.8
	125	41.0	1.26 (n = 3)	101.6
	150	49.2	1.94 (n = 3)	99.8

Table 4.6: The contents of vicenin-2 and mollugogenol B standards, total flavonoids and saponins of *G. lotoides* in the crude extract, secondary standard and tablet formulation.

Compound(s)	Crude extract	Secondary standard	Tablet formulation[c]
Vicenin-2	4.4%	10.0%	4.3%
Total flavonoids[a]	9.3%	21.3%	9.1%
Mollugogenol B	3.1%	14.2%	3.0%
Total saponins[b]	10.8%	25.4%	10.4%

[a]calculated as vicenin-2 equivalent

[b]calculated as glinuside G equivalent

[c]1.4 g oblong tables containing 650 mg extract of the seeds of *G. lotoides*

4.5   Determination of the traditional dose

The traditional dose of *G. lotoides* was determined by HPLC analysis of the contents of the saponins and flavonoids in the 5% ethanol extract. Extraction of 20 g powdered seeds of *G. lotoides* (traditionally recommended quantity) with 500 ml of 5% EtOH yielded 1.06 g dry extract.

As shown in Table 4.7, the traditional dose contains 3.35% vicenin-2 and 4.99% total flavonoids of *G. lotoides* calculated as vicenin-2 equivalent. Similarly, the contents of mollugogenol B, mollugogenol A, glinuside G and total saponins of *G. lotoides* as glinuside G were found to be 2.16, 3.83. 4.40 and 7.83%, respectively.

The traditional dry extract and the crude extract (extract A) were then compared with respect to the contents of saponins and flavonoids and it was found that 650 mg of extract A was equivalent to 1060 mg of the 5% EtOH dry extract, i.e., the traditional

dose. Accordingly, a dry extract preparation containing 650 mg of extract A was used for the tablet formulation.

Table 4.7: The contents of vicenin-2 and total flavonoids in the traditional dose

5% EtOH extract (mg/100ml)	Vicenin-2 %, (RSD)	Total flavonoids[a]
10.54	3.35 (0.73)	4.95
39.79	3.34 (1.42)	5.20
60.20	3.37 (1.54)	4.85
89.25	3.36 (0.85)	4.98
Average	**3.35**	**4.99**

[a]calculated as vicenin-2 equivalent from the total peak area of the HPLC chromatogram

Chapter 5

PREPARATION AND PHYSICOCHEMICAL CHARACTERIZATION OF THE DRY EXTRACT PREPARATION FOR TABLET FORMULATIONS

5.1   General introduction

The preparation of a dry extract by adsorption of liquid plant extract on an inert excipient, such as fumed silica has been described by Palma et al. (1999). In this, the performance of some common excipients to be used as support for the extractive products, such as lactose, starch, dicalcium phosphate and fumed silica have been evaluated. Diluting dry extract with starch yielded materials with poor flow properties indicating that the selection of suitable inert material for adsorption is fundamental (Palma et al., 1999).

The crude extracts (extract **A**) of the seeds of *G. lotoides* have been reported to be highly hygroscopic liquefying at higher relative humidity (65% and above). Dry extract preparations containing various concentrations of Aeroperl® 300 Pharma were prepared to improve the hygroscopicity and poor flow property of the crude extract of the seeds of *G. lotoides*.

Aeroperl® 300 Pharma is a granulated high purity colloidal silicon dioxide for use in pharmaceutical products. Aeroperl® 300 Pharma is highly porous, spherical, dust free and easy in handling. It has high purity and safety which is tested according to Ph. Eur. and USP/NF. Aeroperl® 300 Pharma has been effectively utilized as carrier for liquid and pasty active ingredients and covert them into free-flowing powders. It has huge surface area and cavities for adsorption. Aeroperl® 300 Pharma shows an excellent flow both loaded and unloaded.

Aeroperl® 300 Pharma is white, odorless powder and 40 g/l suspension has pH value between 3.5 and 5.5. The bulk density of Aeroperl® 300 Pharma is 280 g/l and true density of 2.2 g/cm³ at 20 °C. It is practically stable with decomposition temperature above 2000 °C.

This chapter described the preparation and physicochemical characterization of the dry extract preparation using Aeroperl® 300 Pharma as inert sorption material which, later on, was formulated into enteric coated tablets.

5.2   Preparation of the dry extract preparation

The seeds were defatted three times with *n*-hexane. The presence of fats in the starting plant materials not only interferes with the extraction and drying process but also with formulations. The amount of fat removed at three successive defatting steps was found to be 62.5%, 23.6% and 9.7%, respectively, with a total yield of 95.8% of the fats (Endale et al., 2004). The total fat content was determined by exhaustive defatting of the seeds (10 successive defatting with *n*-hexane) and was reported to be 14% (Biftu et al., 1979, Endale et al., 2004).

The solvent system, 60% methanol in water, has provided the maximum amount of saponin-containing extract (Endale et al., 2004). Previous extraction works conducted on the seeds of G. lotoides have utilized 80% methanol in water (Djote, 1978; Endale et al., 1997; 1998) which yields less than 60% of the total saponins.

Admixing of the liquid extract with Aeroperl® 300 Pharma as an inert carrier material and drying the mixture subsequently provided a non-adherent and free-flowing powder. The amount of Aeroperl® 300 Pharma required was optimized to 30% after investigation of the scanning electron micrographs and water-sorption properties of the dry extract preparations containing 10, 20, 30, 40, 50 and 60% w/w Aeroperl® 300 Pharma. An excess amount of

Aeroperl® 300 Pharma increases the bulk volume and the tablet weight, whereas an insufficient amount leads to extract preparations with unacceptable physical properties.

5.3   Physicochemical characterization of the dry extract preparations

Assessment of the physicochemical properties of drug powder whether it is a pure chemical or an extract is a primary task during preformulation studies (Wells, 1988). Physicochemical properties can be related to the pharmaceutical and pharmacological properties of a drug. Particle size may influence number of important factors that can relate to the manufacturing of pharmaceuticals (Pitkin and Carstensen, 1973). Hygroscopicity contributes to poor fluidity and adversely influences both physical and chemical stability of many powders particularly plant dry extracts. Hence, investigation of some physicochemical properties of the dry extract preparation is imperative before commencing the formulation.

In this respect, physical properties of the dry extract preparations of *G. lotoides* which are useful for the formulation of tablets, such as particle size and size distribution, morphology, water sorption pattern, bulk, tapped and true densities, flow properties (angle of repose and flow rate) and compaction profiles were investigated. Based on the density results, physical properties like porosity, Carr's consolidation index and Hausner ratio were calculated.

5.3.1   Particle size distribution

Figure 5.1 depicts the particle size distribution of the dry extract preparations (DEP) containing 10 – 60% Aeroperl® 300 Pharma. As shown in Table 5.1, the average particle sizes (d_{50}) of the particle sizes of the dry extract preparations decreased with increasing the amount of Aeroperl® 300 Pharma.
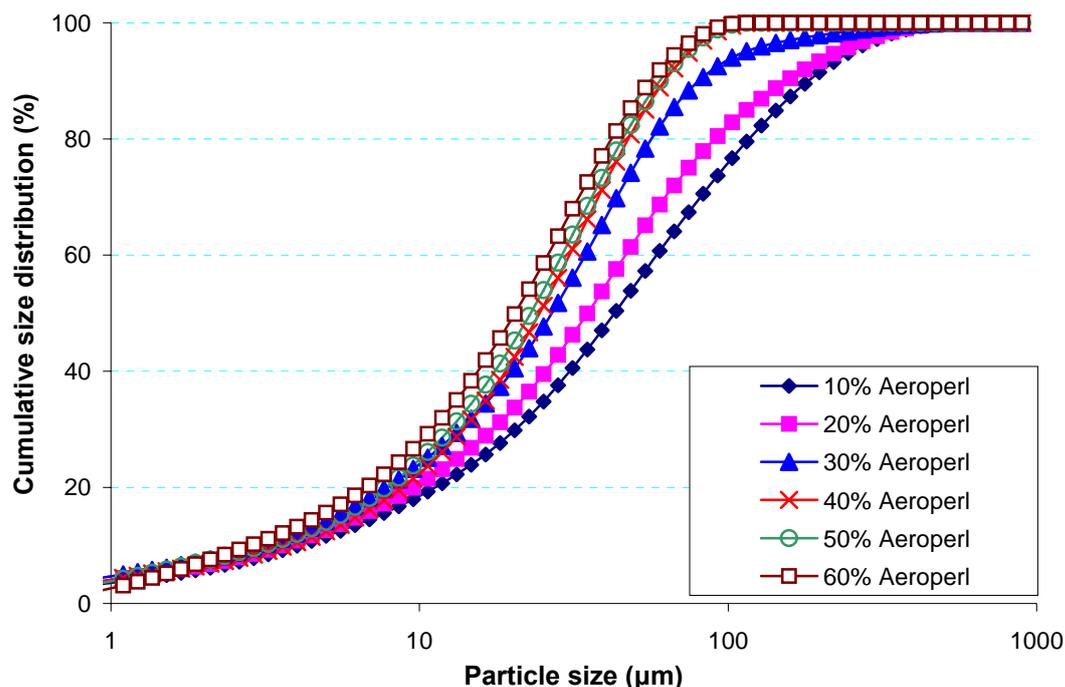
Figure 5.1: Cumulative particle size distribution of the dry extract preparations of *G. lotoides* containing 10 – 60% Aeroperl® 300 Pharma.

Table 5.1: Average cumulative particle size (d_{10}, d_{50} and d_{90}) of the dry extract preparations of *G. lotoides* containing 10 – 60% Aeroperl® 300 Pharma.

DEPs	d_{10} μm (rel. S.D., %)	d_{50} μm (rel. S.D., %)	d_{90} μm (rel. S.D., %)
10% Aeroperl	4.4 (2.22)	46.4 (2.20)	194.2 (4.43)
20% Aeroperl	3.9 (0.57)	38,7 (1.30)	170.2 (2.67)
30% Aeroperl	3.5 (0.82)	30.1 (1.06)	90.3 (2.08)
40% Aeroperl	4.1 (1.23)	27.4 (0.54)	69.8 (0.58)
50% Aeroperl	3.5 (1.97)	25.8 (0.44)	67.3 (0.57)
60% Aeroperl	3.2 (1.24)	23.1 (1.20)	63.7 (1.61)

The average particle size the crude extract (extract A) has been reported to be 68.39 μm (Endale et al., 2004). Hence, the dry extract preparations provided smaller particles.

5.3.2 Scanning electron microscopy

Surface structures of the crude extract (extract A), Aeroperl® 300 Pharma, and the DEPs containing 10 – 60% Aeroperl® 300 Pharma were visualized using a scanning electron

microscope (Fig. 5.2 a – m). The accelerating voltage and the magnification are shown on the micrographs. Figures 5.2 (a) and (b) show the scanning electron micrographs of the vacuum oven dried crude extracts of the seeds of *G. lotoides* at magnifications of 500 and 2000 times, respectively. The particles are irregular in shape and relatively compact masses with sharp edges.

On the other hand, the surface of Aeroperl® 300 Pharma particles appeared relatively uniform, smooth and regular. Figures 5.2 (c) and (d) showed the micrographs at magnifications of 100 and 3000 times, respectively. The cavities shown in Fig 5.2 (d) depicted the porosity Aeroperl® 300 Pharma, indicating the enormous free surface area for the adsorption of liquid extracts.

Scanning electron micrographs of the dry extract preparations revealed that the morphology of extract A as well as Aeroperl® 300 Pharma were changed, due to the adsorption of the extract onto Aeroperl® 300 Pharma (Fig. 5.2 e-m). Mixing the crude extract with Aeroperl® 300 Pharma had improved the irregularities of the particles of the crude extracts. Moreover, the formation of compact masses with sharp edges was decreased when the extract is dried together with Aeroperl® 300 Pharma. These improvements of the surface properties of the extract were found to be a function of the amount of Aeroperl® 300 Pharma added.

Dry extract preparation (DEP) containing 10% Aeroperl® 300 Pharma (Fig. 5.2 e and f) showed surface structure similar to the crude extract, indicating that the amount of Aeroperl® 300 Pharma added was not enough to alter the surface structure of the extract completely. Figure 5.2 f (high resolution, 1000 times) shows the formation of aggregates of the extract and the Aeroperl® 300 Pharma particles.

a)

b)
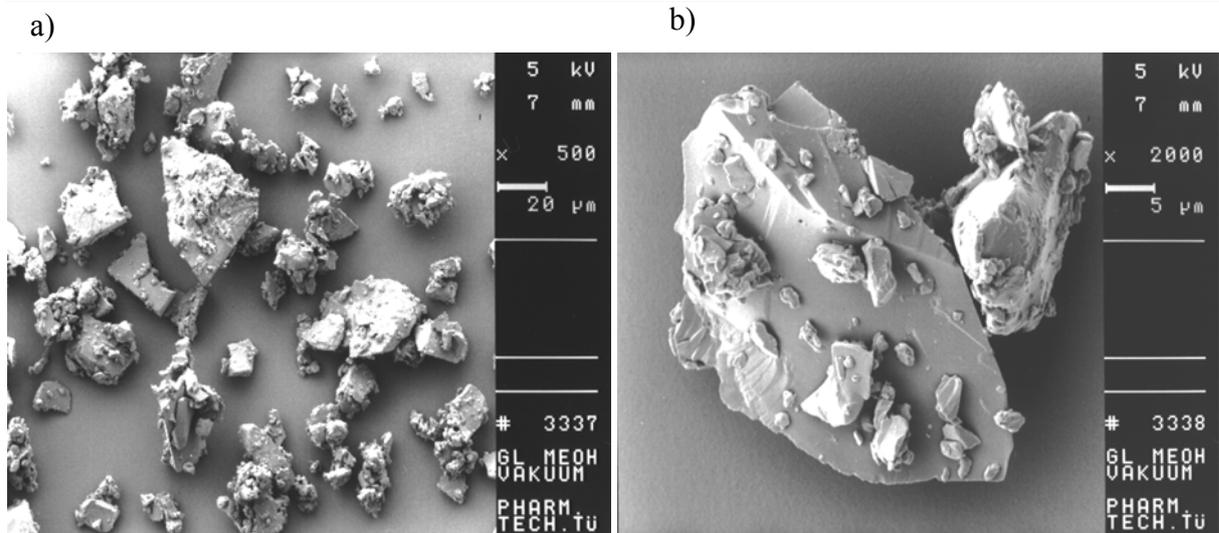
Figure 5.2: Scanning electron micrographs of the crude extract of the seeds of *G. lotoides* at magnifications of a) 500 times and b) 2000 times.

c)

d)
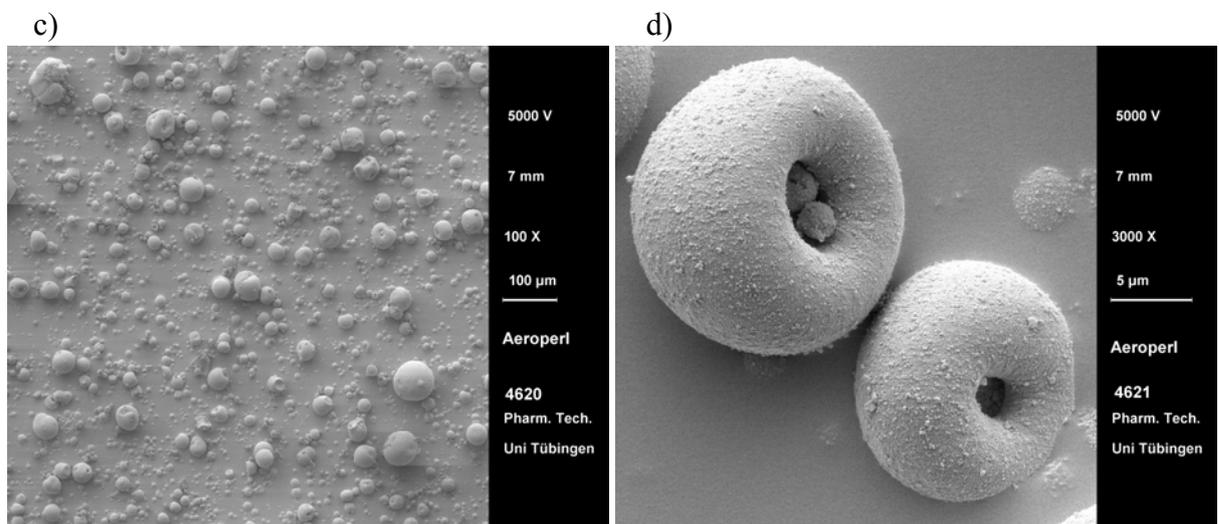
Figure 5.2: Scanning electron micrographs of Aeroperl® 300 Pharma at c) 100X and d) 3000X.
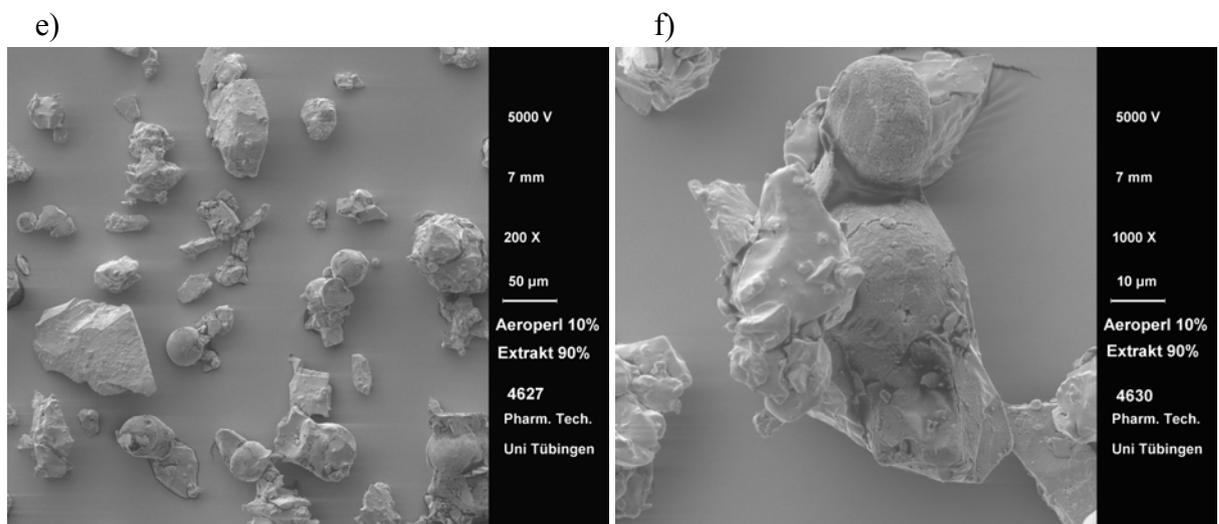
e)

f)

Figure 5.2: Micrographs of the dry extract preparation (DEP) containing 10% Aeroperl® 300 Pharma at e) 200X and f) 1000X.

g)

h)
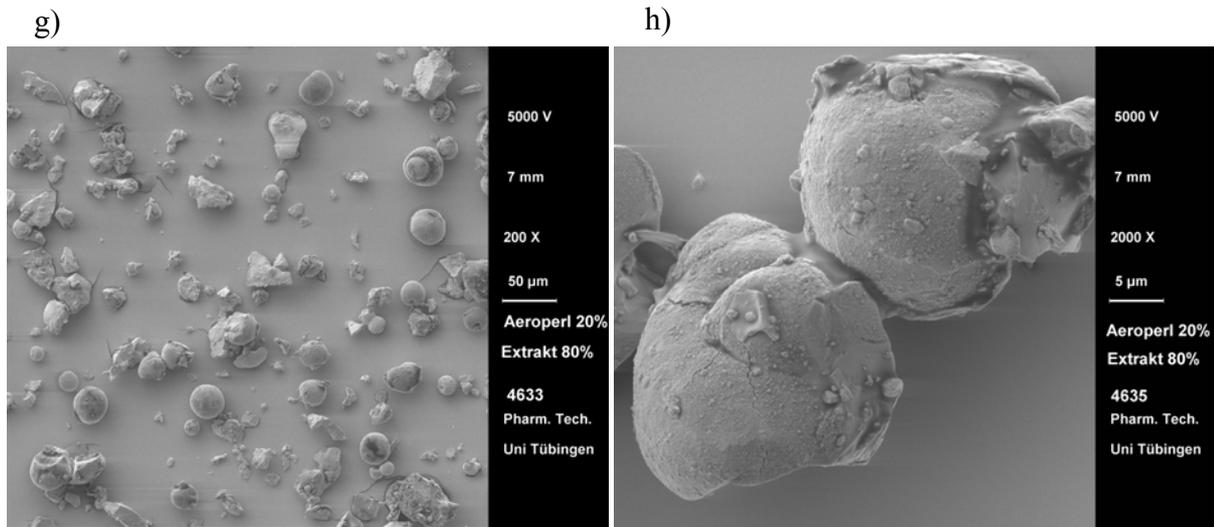
Figure 5.2: Micrographs of the dry extract preparation (DEP) containing 20% Aeroperl® 300
Pharma at g) 200X and h) 2000X.

i)

j)
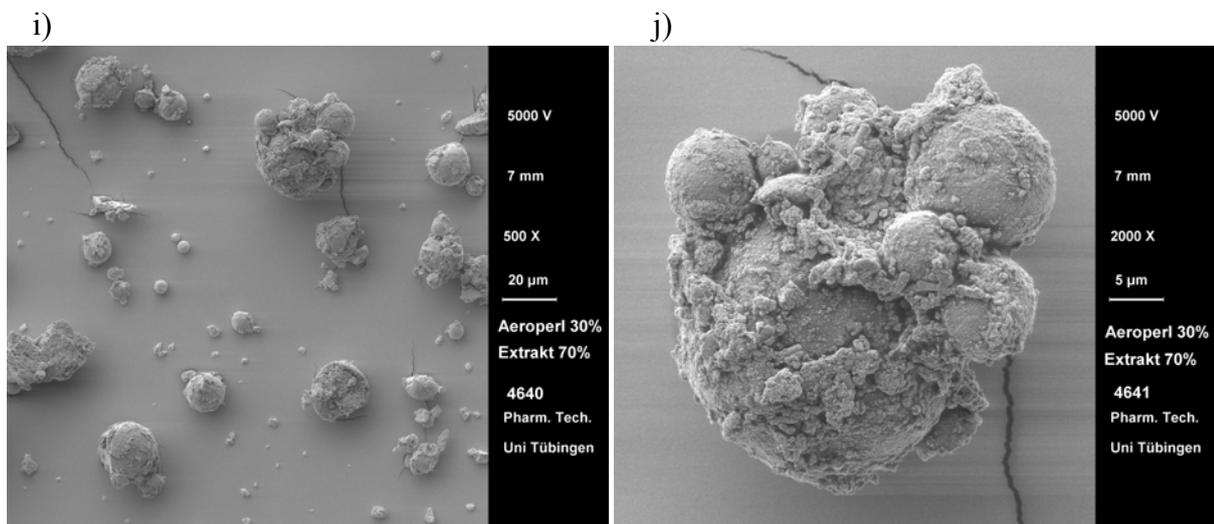
Figure 5.2: Micrographs of the dry extract preparation (DEP) containing 30% Aeroperl® 300
Pharma at i) 500X and j) 2000X.

k)

l)
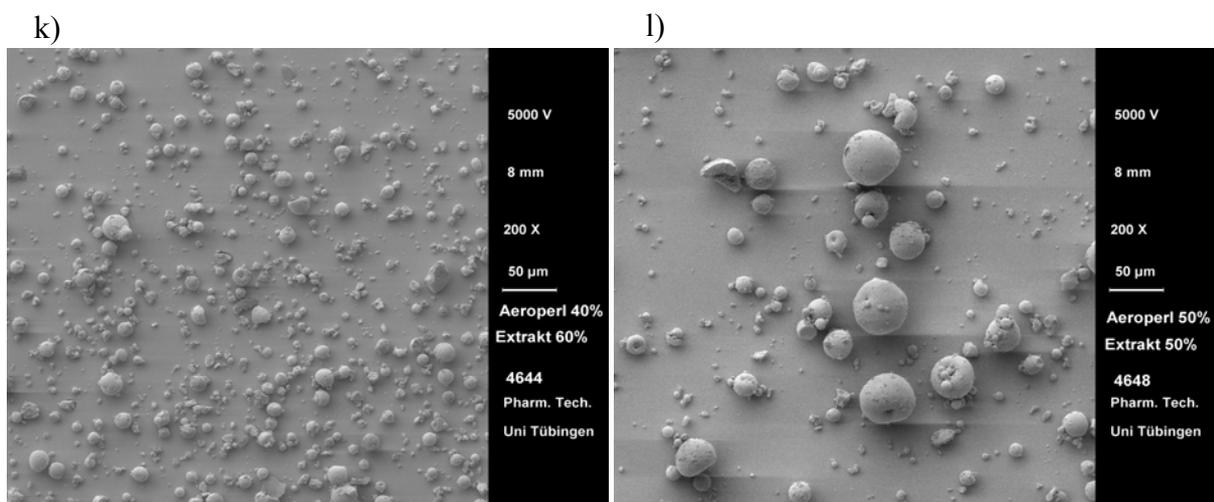
Figure 5.2: Micrographs of the dry extract preparation (DEP) k) containing 40% Aeroperl®
300 Pharma at 200X and l) containing 50% Aeroperl® 300 Pharma at 200X.

m)
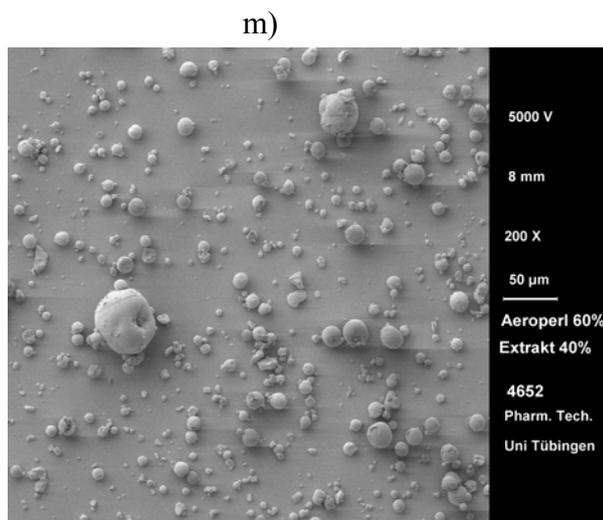
Figure 5.2: Micrographs of the dry extract preparation (DEP) m) containing 60% Aeroperl®
        300 Pharma at 200X

Figures 5.2 (g) and (h) depict the SEM of the DEP containing 20% Aeroperl® 300 Pharma at
magnifications of 200 and 2000, respectively. As shown in the figures, some proportion of the
crude extract particles were not adsorbed on Aeroperl® 300 Pharma. However, the particle
size of the extract in the DEP were smaller than the corresponding crude extract particles (Fig
5.2 a) as well as that of DEP containing 10% Aeroperl® 300 Pharma (Fig 5.2 e). This
indicates, even though, the particle size and shape of the crude extract had improved with the
addition of 20% Aeroperl® 300 Pharma, it was not sufficient to adsorb all the extract particles.

Dry extract preparation containing 30% Aeroperl® 300 Pharma showed an aggregation of
particles (Fig. 5.2 i and j) where the crude extract was adsorbed on the surface of Aeroperl®
300 Pharma as small particles. The typical compact and sharp edged particles of extract A
(Fig. 5.2 a and b) were not formed.

Figures 5.2 (k), (l) and (m) show the SEM of DEPs containing 40, 50 and 60% Aeroperl® 300
Pharma, respectively. As shown in the figures, the proportion of the Aeroperl® 300 Pharma
increased with maximum for the DEP containing 60% Aeroperl® 300 Pharma. Hence, in these

preparations the amount of the Aeroperl® 300 Pharma was more than required. Based on the comparison of the SEM, the optimum amount of Aeroperl® 300 Pharma for the dry extract preparation was set to 30%. The result obtained was further supported by the water uptake studies of the various dry extract preparations as described below.

5.3.3   Water uptake study

Many drug substances, particularly plant extracts, have a tendency to adsorb moisture. The adsorption and equilibrium moisture content can depend upon different factors such as the atmospheric humidity, temperature, surface area, exposure time and the mechanism for moisture uptake.

With most hygroscopic materials, changes in moisture level can greatly influence many important parameters, including chemical stability, flow properties and compatibility (Van Campen, et al., 1983). The effects of moisture on various physical properties such as angle of repose, bulk and true densities, porosity and compression properties of various plant extracts have been reported with different authors (Rai and Kumar, 1995; Mishra, et al., 1996; Murthy and Bhattacharya, 1998).

The water sorption pattern of the dry extract preparations (DEP) containing 10 – 60% Aeroperl® 300 Pharma was investigated in humidity chambers (desiccator method) as well as using a Krüss, Processor Tensiometer K12 (Krüss GmbH). In these methods, various relative humidity levels were realized using saturated salt solutions as described under experimental section (Table 7.9). Water uptake of Aeroperl® 300 Pharma alone was determined at various relative humidity and the water uptake due to the extract was calculated.

Figures 5.3 a – g show the kinetic moisture sorption profiles of the dry extract preparations (DEPs) containing 10 – 60% Aeroperl® 300 Pharma.
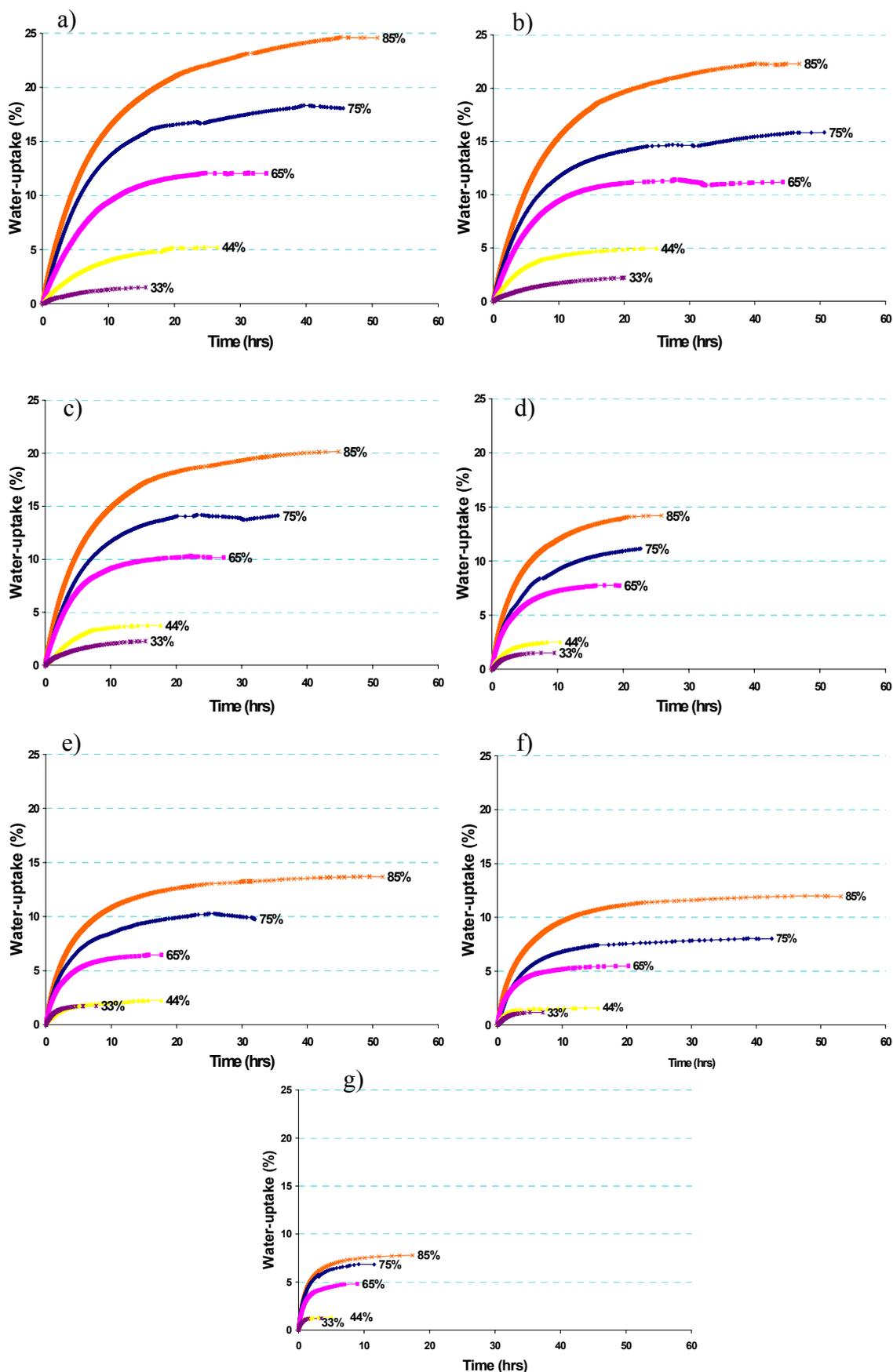
Figure 5.3: Water uptake profiles of the dry extract preparations containing Aeroperl® 300 Pharma a) 10%; b) 20%; c)30%; d) 40%; e) 50%; f) 60% and g) 100% Aeroperl® 300 Pharma at 33, 44, 56, 75 and 85% relative humidity levels at 20°C.

As shown in the figures, the percent water uptake by the DEPs increased with increasing the relative humidity. Moreover, the DEPs required longer time to reach a maximum and equilibrium water uptake at higher relative humidity levels.

Similar water uptake profiles were obtained using the desiccator method. A typical profile for DEP containing 30% Aeroperl® 300 Pharma is depicted in Figure 5.4.
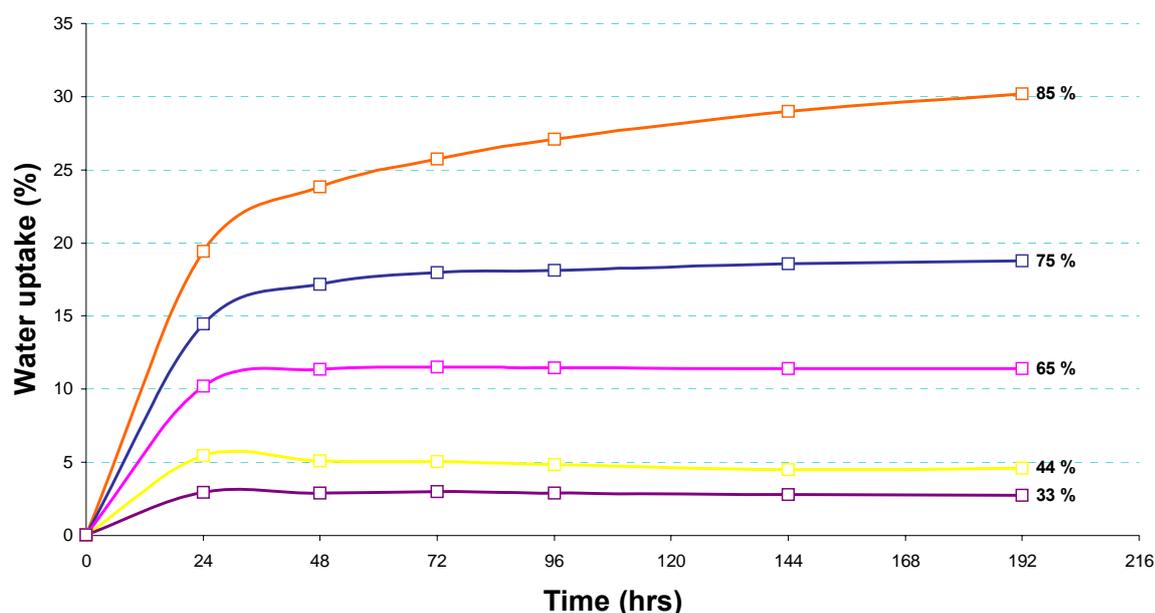
Figure 5.4: Water uptake profiles of the dry extract preparation containing 30% Aeroperl® 300 Pharma at 33, 44, 56, 75 and 85% relative humidity levels at 20°C [desiccator method].

The water uptake profiles of the DEP containing 30% Aeroperl® 300 Pharma were compared using the two methods (Figure 5.5.) A higher water uptake were observed using the desiccator method indicating that the DEPs require longer time to reach equilibrium than the ones determined by the Krüss Tensiometer.

Figure 5.6 depicts the water sorption isotherms of the DEPs at 20° C. The values were taken from the equilibrium moisture uptake from Fig. 5.3. The addition of Aeroperl® 300 Pharma to

the crude extract decreases the hygroscopicity. Moreover, the sorption isotherms of the crude extract show a marked water uptake increase above 65% relative humidity, indicating that, the hygroscopicity of the DEPs are manageable at lower relative humidity.
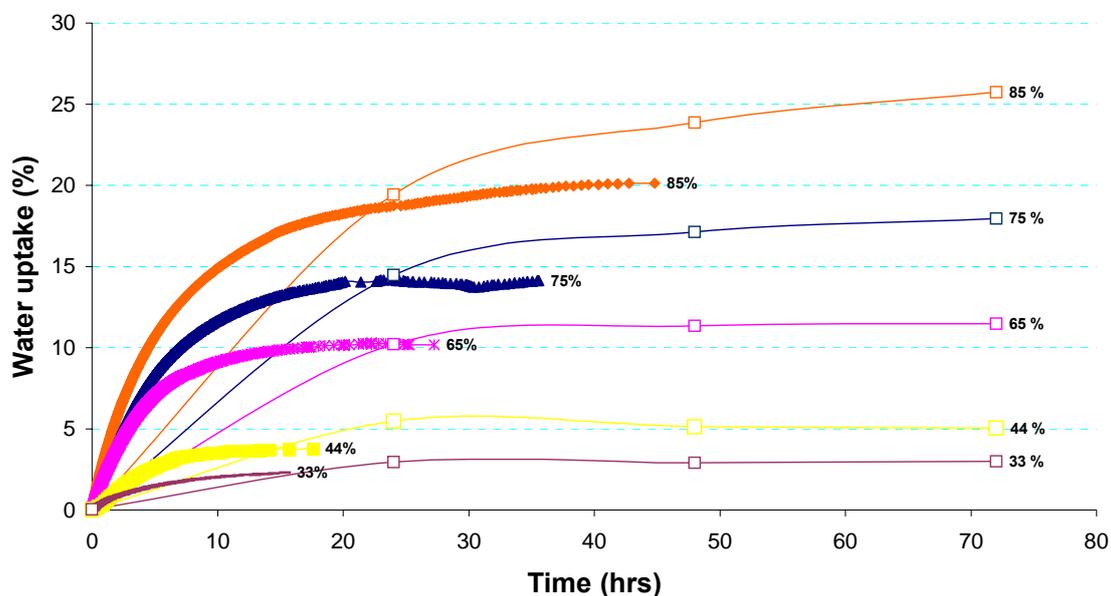
Figure 5.5: Water uptake profiles of the dry extract preparation containing 30% Aeroperl® 300 Pharma (——□—— desiccator method; other symbols: Krüss Tensiometer)
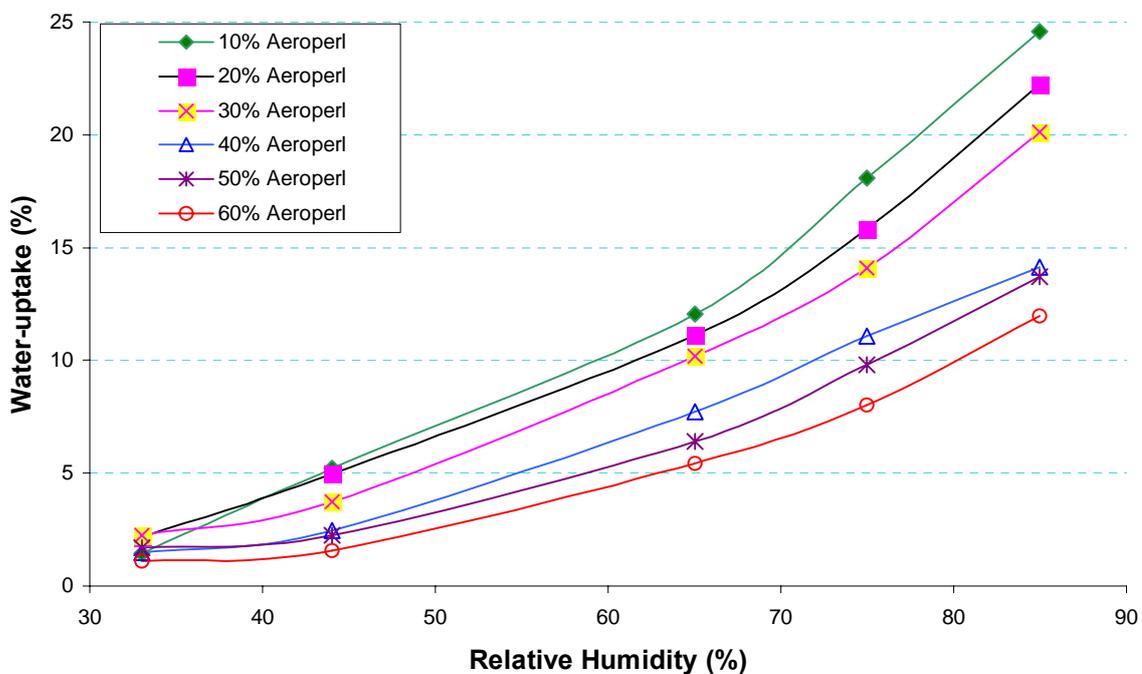
Figure 5.6: Water sorption isotherm of the DEPs containing 10 – 60% Aeroperl® 300 Pharma [using a Krüss Tensiometer K12].

Powder beds of 1 g of the dry extract preparations containing 10 – 60% Aeroperl® 300 Pharma were prepared in petri dishes and stored at 20° C over the saturated salt solutions. The moisture content of the various extract preparations was measured gravimetrically at 24 hrs intervals for 15 days. Dry extract preparations containing 10% and 20% Aeroperl® 300 Pharma were deliquesced at relative humidity levels of 65% and above (Fig. 5.7). However, as shown in the picture, even though DEP containing 30% Aeroperl® 300 Pharma adsorbed 20% water at 85% relative humidity (Fig. 5.3c, 5.4, 5.5 and 5.6), the preparation remained powder.
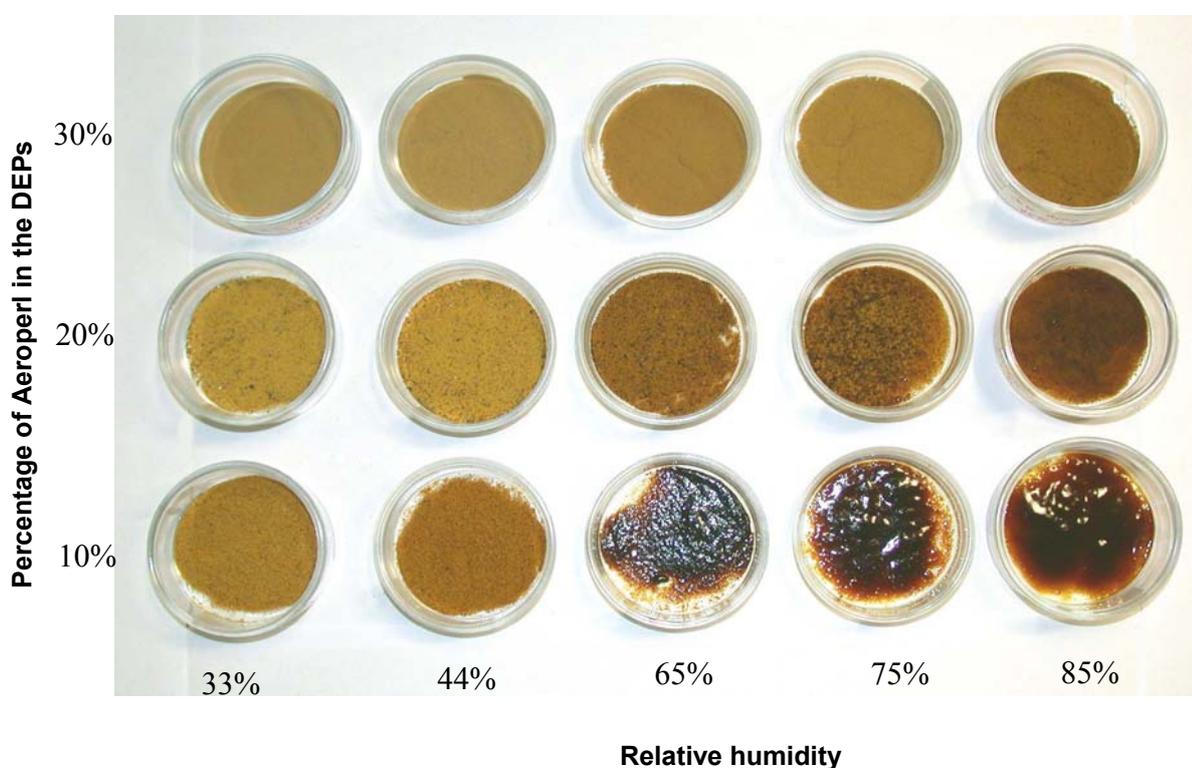
Figure 5.7: Physical appearance of the DEPs containing 10%, 20% and 30% Aeroperl® 300 Pharma after 15 days in 33, 44, 65, 75 and 85% relative humidity chambers.

5.3.4   Water content determination

Table 5.2 shows the water content of the DEPs containing 10 – 60% Aeroperl® 300 Pharma which were determined using Karl Fischer titration. The results indicate that, the water content of the dry extract preparations varies with the content of Aeroperl® 300 Pharma.

Increase in Aeroperl® 300 Pharma decreases the water content of the dry extract preparations.

Table 5.2: Water content of the dry extract preparations

DEPs containing	Water content (%)	SD
10% Aeroperl® 300 Pharma	5.1	0.54
20% Aeroperl® 300 Pharma	4.8	0.43
30% Aeroperl® 300 Pharma	3.5	0.23
40% Aeroperl® 300 Pharma	3.3	0.15
50% Aeroperl® 300 Pharma	3.3	0.28
60% Aeroperl® 300 Pharma	3.1	0.12

Based on the results of the SEM and water uptake studies, the dry extract preparation containing 30% Aeroperl® 300 Pharma was selected for tablet formulation. Hence, density, flow property and compactability of this DEP were further investigated. The term dry extract preparation (DEP) refers to the dry extract preparation containing 30% Aeroperl® 300 Pharma.

5.3.5   Density

The bulk, tapped and true densities of the dry extract preparation containing 30% Aeroperl® 300 Pharma were found to be 0.697, 0.870 and 1.547 gm/ml, respectively. The crude extract of the seeds of *G. lotoides* (extract A) showed a bulk, tapped and true densities of 0.66, 0.94 and 1.51 g/ml, respectively. Hence, due to the addition of Aeroperl® 300 Pharma, the bulk density increased while the tapped density decreased. The true density had also showed a tendency of increasing.

Porosity, consolidation index (Carr compressibility index) CC % = [(tapped density – bulk density)/bulk density]*100 and Hausner ratio, HR = [tapped density/bulk density] which are measures of compressibility of powders were calculated from the density results. Table 5.3 summarizes the results of porosity, consolidation index and Hausner ratio of the dry extract

preparation and the crude extract of the seeds of *G. lotoides* (extract A).

Compressibility indices less than 15% are indicative of free-flowing powders; indices greater than 40% usually correspond to very poor flow (Carr, 1965). As shown in the table, the consolidation index had decreased form 29.8 to 19.9 indicating the improvement of the flow properties due to the formulation of the crude extract in to dry extract preparation using Aeroperl® 300 Pharma.

Table 5.3: Porosity, consolidation index and Hausner ratio of the dry extract preparation
(DEP) as well as the crude extracts of the seeds of *G. lotoides* (extract A).

	Porosity (%)	Consolidation (Carr's) index (%)	Hausner ratio
DEP	55.0	19.9	1.25
Extract A	56.0	29.8	1.42

The HR is a simple test usually used to evaluate fluidity, where values less than 1.25 indicate good flow properties and values greater than 1.5 indicate poor flow properties (Fonner et al., 1966). As shown in the table, the dry extract preparation decreased the HR from 1.42 to 1.25, indicating the improvement of flowability after mixing with Aeroperl® 300 Pharma.

5.3.6 Powder flow properties

Angle of repose and flow rate (timed delivery through an orifice) are the most simple and commonly used methods to evaluate powder flow properties. The flow properties of powders vary considerably, ranging from those that flow readily and uniformly to those that resist free flow. Particulate solids resist flow because of the frictional and cohesive forces in powders which are usually reflected in the angle of repose.

The values of angle of repose obtained may be used as a guide to the type of flow expected. Angle of repose less than 25° indicates the powder has excellent flow properties, whereas 25°-30° good, 30°-40° passable (fair) and greater than 40° very poor (Wells and Aulton, 1988).

Dry plant extracts usually show poor flowability. The poor flow property of the crude extract of the seeds of *G. lotoides* has been reported earlier. The crude extract of *G. lotoides* did not flow through a funnel with a 10-mm aperture under gravitational force and showed an angle of repose of 43.7° (Endale et al., 2004). A number of reasons have been postulated for the poor flow properties of the crude extract including the rough and irregular particle shape, hygroscopicity and the very fine particles which lead powder packing and densification (Endale et al., 2004).

The dry extract preparation showed an angle of repose 36.2° which is classified as passable (fair) flow according to Wells and Aulton (1988) and the flow rate was found to be 7.9 gm/sec. Scanning electron micrographs of the DEP (Fig. 5.2 i and j) showed that the rough and irregular particle shape of the crude extract have been improved when the liquid extract was adsorbed on the surface of Aeroperl® 300 Pharma. DEP was found as aggregated spherical particles with less contact angle and hence less frictional force.

5.3.7   Compactability study of the dry extract preparation

The relationships between the compression pressure and the radial tensile strength as well as the porosity of the tablets prepared from the dry extract preparation alone are depicted in Figure 5.8. The radial tensile strength was calculated from the thickness, diameter and crushing strength of the tablets, whereas the porosity of the tablets was calculated from the true and apparent densities of the extract. The apparent density was calculated form the

thickness out of die, weight and diameter of the tablets.

As shown in the figure, the tensile strength of the tablets increased with increase in compression pressure up to 260 Mpa. The radial tensile strength of the tablets increased minimal with increase in compression pressure beyond 260 Mpa. The tablets showed no sign of capping and lamination at higher compression pressures indicating the high plastic deformation of the dry extract preparation.
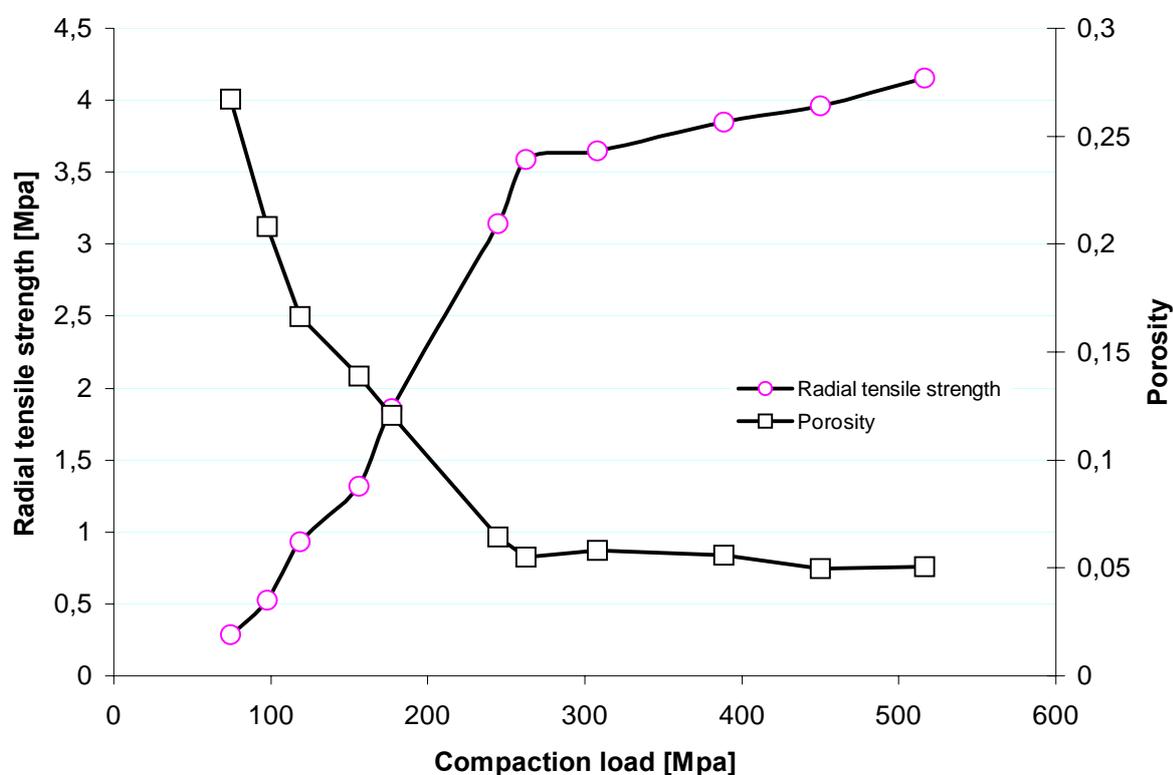
Figure 5.8: Radial tensile strength and porosity of the dry extract preparation at various compression pressures.

On the other hand, the porosity decreased with compression pressure which remained constant at 0.05% after the compression pressure of 260 MPa.

The Heckel plots of the dry extract preparation at compression pressures of 118, 262 and 515

Mpa are depicted in Figure 5.9. The mean yield pressures (reciprocal of the slope), which show the minimum pressure required to cause deformation, were found to be 661, 808 and 874 at compaction pressure of 118, 262 and 515 MPa, respectively. Large values of the Heckel constant indicate the susceptibility of the dry extract preparation to plastic deformation at low pressures.
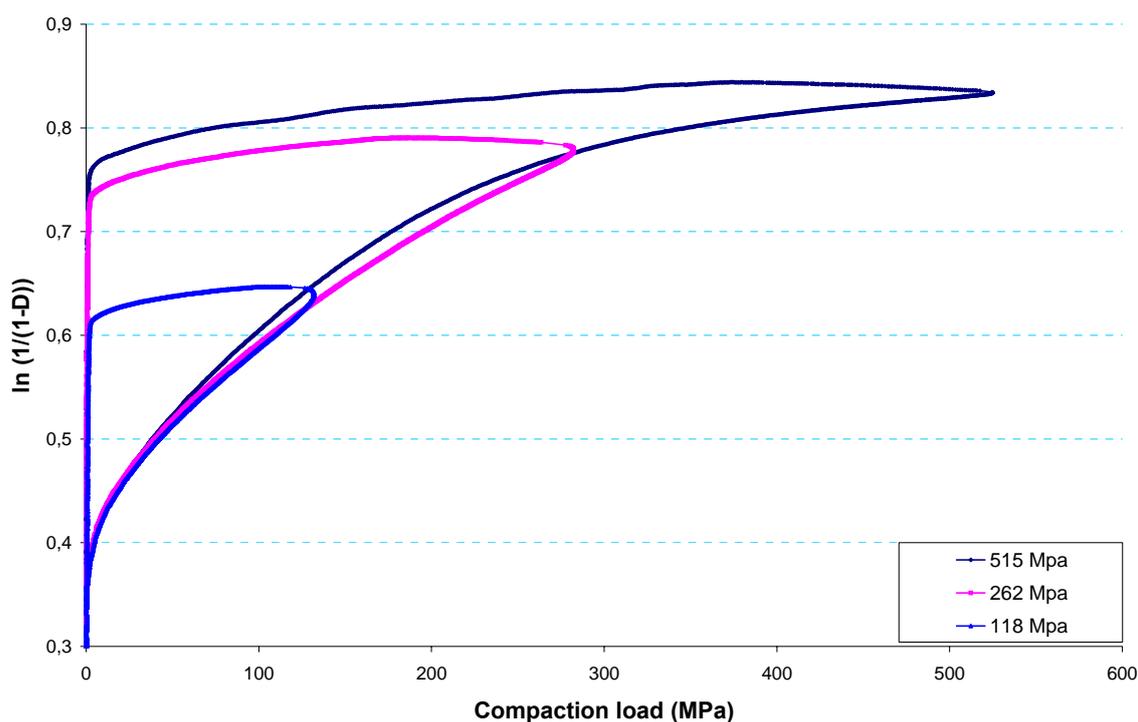
Figure 5.9: Heckel plots of the dry extract preparation at compression pressure of 118, 262 and 515 MPa.

Paronen and Ilkka (1996) described the process of volume reduction of a powder mass under pressure and enumerated different stages: die filling, rearrangement of particles, deformation by elastic changes, permanent deformation by plastic flow or particle failure by brittle fracturing. Numerous mathematical expressions dealing with the characterization of tablet dimensions and changes involved in the densification process including Heckel, Kawatika and Cooper Eaton methods have been suggested.

5.4   Stability of the saponins and flavonoids in the dry extract preparation

The stability of the saponins and flavonoids in the dry extract preparation was determined at 25 and 40° C for a period of 6 months. Figure 5.10 depicts the data. The amounts of vicenin-2 as well as the total flavonoids were determined by HPLC using the method described under **4.3**. The measurements were done in triplicate and the percent recovery was calculated form the amount of flavonoids in the dry extract preparation at the beginning of the stability study.

As shown in the figure, the amount of flavonoids under both storage conditions was more than 95% during the six months indicating a good stability at the specified storage conditions. The phytochemical profiles of the HPLC figureprints of the dry extract preparation stored at 25 and 40° C were not altered significantly during the 6 months.
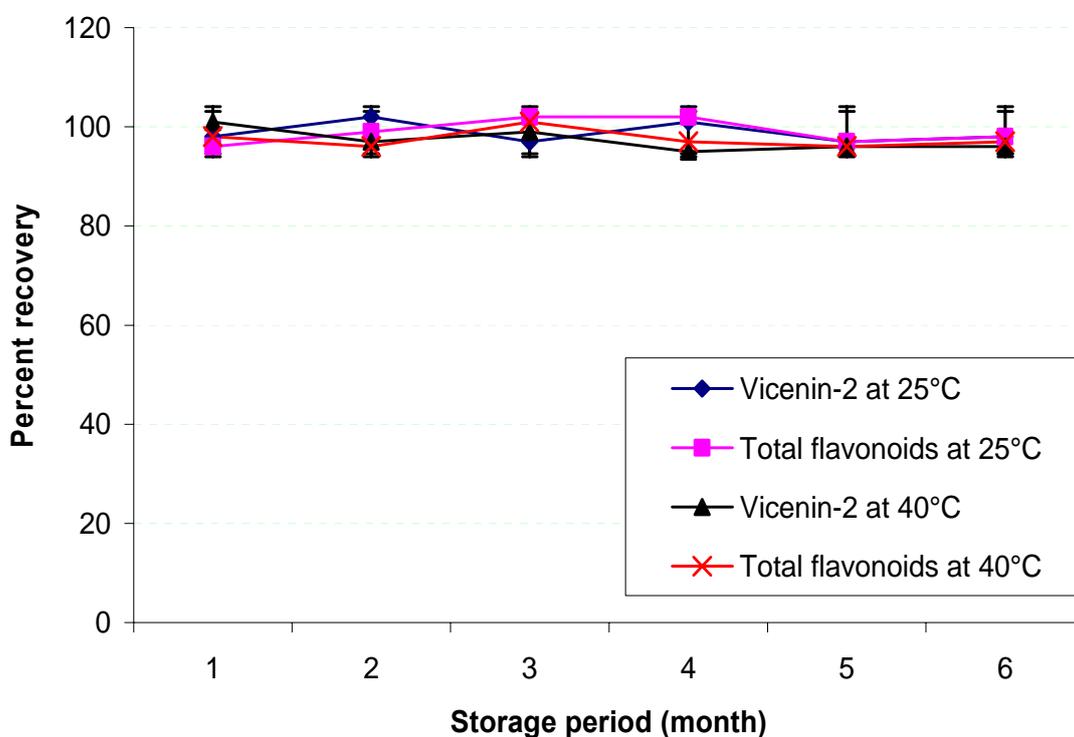
Figure 5.10: Stability of the flavonoids of *G. lotoides* in dry extract preparation stored at 25 and 40°C for six months.

The stability of the saponins in the dry extract preparations during the storage conditions was confirmed by TLC comparison. A freshly prepared dry extract preparation was used as a standard (Figure 5.11). As shown in the figures, the major figureprints of the TLC plates were not altered significantly.
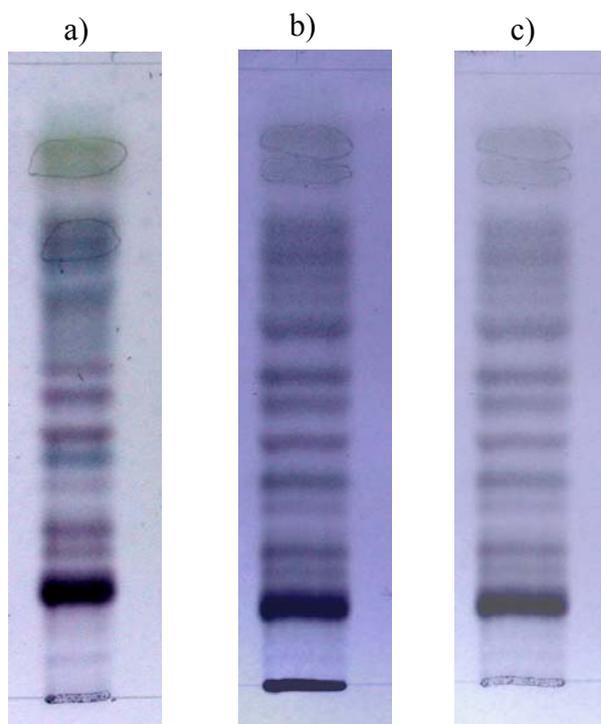
Figure 5.11: TLC of the dry extract preparation a) freshly prepared b) stored at 25°C for 6 months and c) stored at 40 °C for 6 months.

Moreover, the amount of saponins in the dry extract preparation stored at 25 and 40°C was analyzed by HPLC after acid hydrolysis. Mollugogenol B was determined and the total saponins of *G. lotoides* were calculated as glinuside G as described under **4.2.** Figure 5.12 shows the data of the saponins. As shown in the figure, the amount of saponins remaining after 6 months were found to be 97 and 94% at 25 and 40°C, respectively. Hence, the saponins were found to be stable and compatible with Aeroperl® 300 Pharma in the dry extract preparation at the specified storage conditions.
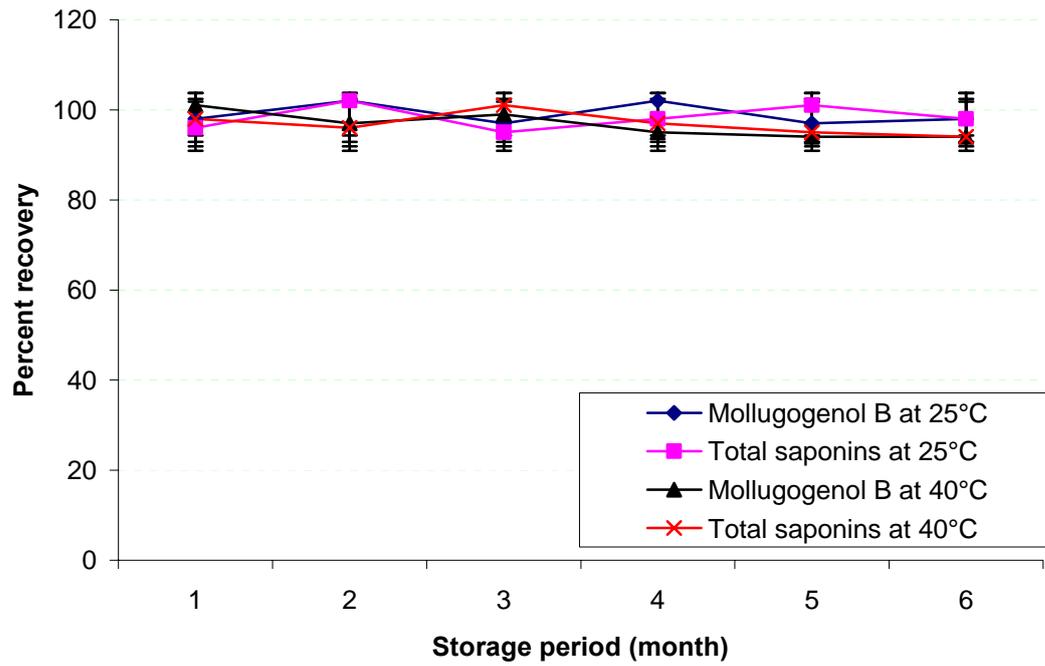
Figure 5.12: Stability of the saponins of *G. lotoides* in dry extract preparation stored at 25 and

40°C for six months.

Chapter 6

DRY GRANULATION BY ROLLER COMPACTION, TABLET PREPARATION AND ENTERIC COATING

6.1 Dry granulation by roller compaction

6.1.1 General introduction

Dry plant extracts usually lack good flow properties to be processed by direct compression. In addition, because the active components of the extracts are diluted by coextracted substances, high dosages are required. This is in conflict with the limited proportion in which the extracts can be incorporated into the final mixture for tablet compression (Vennat et al., 1993). Numerous reports have addressed techniques used to solve these problems, such as wet granulation with non-aqueous solvents (Diaz et al., 1996), direct compression of spray-dried extracts (Plazier-Vercamen and Bruwier, 1986) and selection of suitable excipients for the formulation of dry plant extracts in direct compression tablets (Renoux et al., 1996).

Processing of powders into granules or aggregates is commonly performed in order to modify the flowability and compactability drugs and excipients as well as the disintegration time of the tablets thereof (Carstensen, 1980). One of the processes of densification of powders is granulation by roller compaction, which improves flowability, compressibility and disintegration time (Parrott, 1981).

Moreover, compaction-granulation of powders could have one of the following objectives: obtain products of the same size, volume and weight; obtain a specified and constant product size-range; reduce the volume of a powder; stabilize mixtures of powders during handling and transport, provide uniformity of density, shape and size of

the particles, minimize segregation, control the dispersion rate of granules; solve the problems of powder caking during storage and eliminate the problems of dust generation during handling. Granulation by roller compaction of powders usually requires no binder and the bonding of particles is ensured by the mechanical pressure exerted on the product being compacted (Gerad and Zak, 1995).

The crude extract (Extract A) of the seeds of *G. lotoides* has shown a poor flowability and a prolonged disintegration time after tabletting (Endale et al., 2004). Moreover, large amounts of the dry extract preparation were required to achieve the traditional dose. These conditions necessitate the need for granulation of the dry extract preparation by compaction. Rocksloh et al. (1999) have reported that granulation by roller compaction of plant extracts before compression had improved both disintegration time and crushing strength of the tablets.

6.1.2   Granulation and characterization of the dry extract preparation (DEP)

The dry extract preparation was blended with 2% magnesium stearate and compacted using a Gerteis Micro-Pactor® (Gerteis AG) at a compaction force of 12 kN. The ribbons formed were grinded into granules of the required size using a mill called '*Zahnscheibenmühle'* (BECO).

6.1.2.1   Particle size distribution of the DEP granules

Figure 6.1 shows the particle size distribution by sieve analysis of the granulation of the dry extract preparation. As shown in the figure, the majority of the particles (more than 90%) have particle sizes between 250 and 710 µm. The amount of fine powders (less than 250 µm) and particles greater than 1.00 mm was found to be 6.3 and 1.4%, respectively.
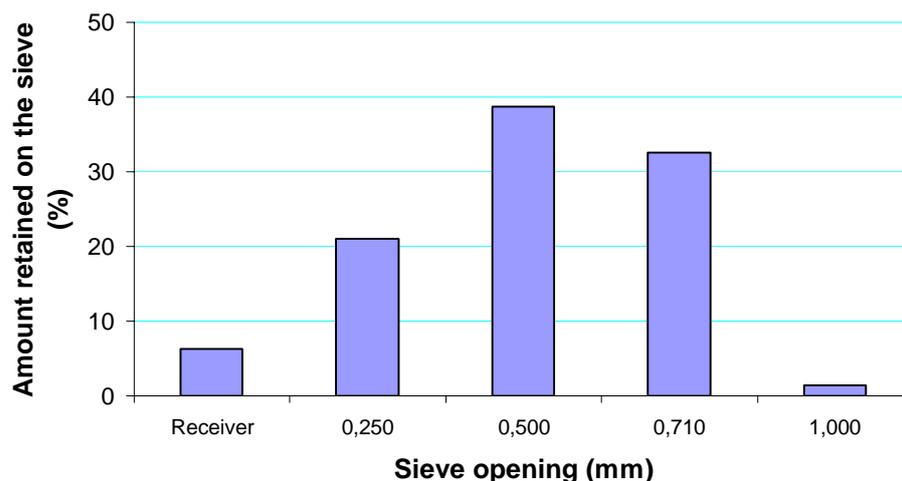
Figure 6.1: Sieve analysis of the granules of the dry extract preparation prepared by

      roller compaction.

6.1.2.2   Compressibility and flow properties of the DEP granules

The flowability, as measured by the angle of repose, flow rate and compressibility index, was greatly improved by the granulation process due to the increase of particle size. Table 6.1 depicts the angle of repose, flow rate and density results of the powders and granules of the dry extract preparation.

The granules of the dry extract preparation exhibited relatively high bulk and tapped densities, and the compressibility index was 7.3%, which is considered as an excellent flowable material (Table 6.1). The powdered dry extract preparation showed a compressibility index of about 20% which is categorized as fair (passable) flow properties (Fonner et al., 1966). The roller compaction of the dry extract preparation decreased the angle of repose from 36 to 31 degrees and the Hausner ratio from 1.25 to 1.07. It is well known that powder flowability is size-dependent, and as particle size increases, the total surface area decreases, resulting in reduction of frictional forces between particles which improves the flow properties (Amidon 1999; Parrott, 1981; Arnaud et al., 1998).

Table 6.1: Compressibility and flow properties of the dry extract preparation and the
granulations by roller compaction.

Properties	Dry extract preparation	
	Powders	Granules
Densities (gm/ml)		
Bulk	0.697	0.742
Tapped	0.870	0.796
Angle of repose (°)	36.24	31.2
Flow rate (gm/sec)	7.94	10.33
Hausner ratio	1.25	1.07
Carr's compressibility index (%)	19.89	7.3

6.2   Formulation and characterization of DEP tablets

6.2.1   Formulation of tablets

The tablets were prepared from the granulated dry extract preparation (DEP) and
contain 947 mg of DEP per tablet. The 2% magnesium stearate, which was included
into the dry extract preparation before compaction was sufficient to prevent sticking of
the tablet mixture to the punch faces. Tablet hardness of the different formulations was
maintained in the range of 70 to 80N. The tablet formulations and properties of the
tablets are given in Table 6.2.

As shown in table 6.2, tablet formulations I, II, and III compare the effect the amount of
Avicel® PH 101 on the disintegration time of the tablets. The disintegration time of the
tablets decreased from 8.6 to 2.4 min with increase in the amount of Avicel® PH101
from 163 to 363 mg with comparable tablet hardness.

Comparison of tablet formulation I, IV and V revealed that Kollidon® CL as
disintegrant provides tablets with faster disintegration time (30 sec). Whereas, a

formulation with Explotab showed a disintegration time of 9.2 min with comparable tablet hardness. Ac-Di-Sol has shown a disintegration time of 2.4 min.

Table 6.2: Composition and characterization of the various tablet formulations

Constituents	Tablet formulations (mg)						
	I	II	III	IV	V	VI	VII
DEP (granules)	947	947	947	947	947	947	947
Avicel® PH 101	363	263	163	363	363	-	-
Avicel® PH 200	-	-	-	-	-	363	-
Cellactose® 80	-	-	-	-	-	-	363
Ac-Di-Sol®	90	90	90	-	-	90	90
Kollidon® CL	-	-	-	90	-	-	-
Explotab®	-	-	-	-	90	-	-
Properties							
Tablet weight (mg)	1400	1300	1200	1400	1400	1400	1400
Tablet hardness (N)	73	81	80	77	71.3	72	78
Disintegration time (min)	2.4	4	8.6	0.5	9.2	4	12.2

However, tablets prepared with Kollidon® CL as disintegrant were unstable at relative humidity levels of 65% and above. The tablets prepared with Kollidon® CL adsorb moisture and disintegrate prematurely.

On the other hand, Formulations I, VI and VII depicted the effect of filler/binder such as Avicel® PH 101, Avicel® PH 200 and Cellactose® 80, on the disintegration time of the tablets prepared with Ac-Di-Sol® as disintegrant. Tablets with disintegration times of 2.4, 4.0 and 12.2 min were obtained with Avicel® PH 101, Avicel® PH 200 and Cellactose® 80, respectively.

Based on the preliminary investigation (Table 6.2), tablet formulations containing dry extract preparation (947 mg), Avicel® PH101 (363 mg) and Ac-Di-sol (90 mg) was selected. The effect of compression force on crushing strength, disintegration time and friability of the tablets was evaluated by compressing the formulation at approximately 13.5, 16.1, 18.4, 20.6, 23.7 and 27.9 kN.

6.2.2   Characterization of the DEP tablets

Figure 6.2 depicts the effect of compression force on the crushing strength, disintegration time and friability of the DEP tablets. Due to the complex geometry of the oblong tablets, compression pressure, porosity and tensile strength were not calculated.
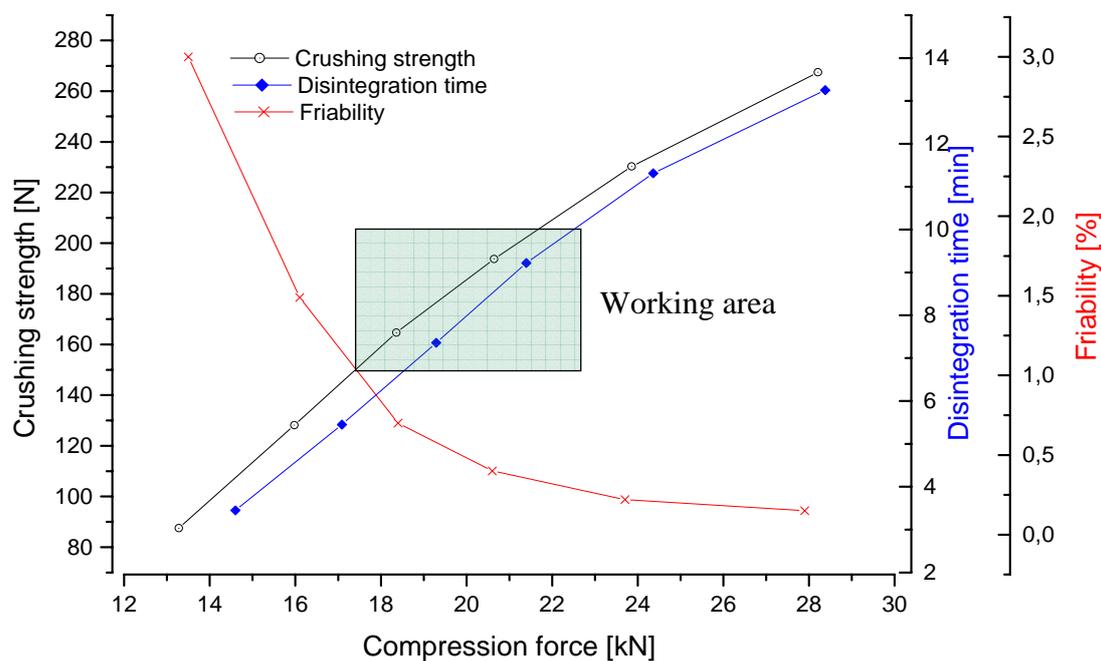
Figure 6.2: Effect of the compression force on the tablet hardness, disintegration time and friability of DEP tablets.

As shown in figure 6.2, the crushing strength of the tablets increased with increasing compression force without any sign of capping or lamination at higher compression forces.

On the other hand, friability decreased rapidly as the compression force increased. The target friability of less than 1% was obtained at a compression force of 18.4kN. This result confirmed that compression force of 18 kN could provide tablets, which were not easily broken during enteric coating. No substantial difference in friability was found above compression force of 23.7 kN. A disintegration time of less than 10 min was set as a target which together with the friability defines the working area (shaded part in Figure 6.2)

G. lotoides tablets prepared from the crude extract have shown slow disintegration times irrespective of the type of disintegrants used (Endale et al., 2004). The tablets exhibited surface erosion (i.e., dissolution) rather than spontaneous disintegration. This phenomenon could be explained or attributed to the presence of hygroscopic extract in the formulation (Lowenthal, 1973; Erdös, 1986; Lieberman and Lachman, 1989).

The delaying effect of hygroscopic ingredients on the disintegration times of tablets containing super disintegrants has been reported (Gordon, 1987). This is so, because the hygroscopic ingredients promote dissolution rather than disintegration of the tablet. The rate of water penetration into tablets was also shown to decrease as the solubility of the drug increased due to competitive inhibition of the disintegrant for locally available water (Graf, 1982).

6.2.3   HPLC analysis of the DEP tablets

The contents of the saponins and flavonoids in the tablet formulation were determined by HPLC as described under **4.2** and **4.3**, respectively. Six tablets were analyzed and the contents of the saponins and flavonoids were calculated as glinuside G and vicenin-2 equivalents, respectively. Accordingly, the content of total saponins of the six single tablets was between 94 to 98% of the claimed amount. Whereas, the content of flavonoids of the six single tablets was found to between 95 and 103%.

6.3   Enteric coating

6.3.1   General introduction

Enteric or sustained release coating of tablets, capsules, granules, pellets, crystals and other drug-loaded cores serves to ensure their physical and chemical stability, to enhance patient compliance and to further improve their therapeutic efficacy. The efficiency of a pharmaceutical dosage form depends not only on the active ingredients it contains but also on the formulation and processing technique (Bauer et al., 1998). Enteric coating of *G. lotoides* tablets provides an immediate release of the active components in the small intestine, where it acts as anthelmintic agents and it also protects the tablets for atmospheric humidity.

Anionic acrylic polymers such as EUDRAGIT® L carry carboxyl groups. They are insoluble in acidic medium, i.e. resistant to gastric fluid, and dissolve only in the neutral to weakly alkaline medium of the small intestine. Medications that are instable in gastric fluid, or cause incompatibilities in the stomach, i.e. irritate the gastric mucosa or provokes nausea and sickness, can be coated with enteric films to pass the acid environment of the stomach unchanged and release the active ingredient only after entering the small intestine (Bauer et al., 1998).

Enteric coating materials should swell as little as possible in the acid environment of the stomach at pH 1 to 4 and remain largely impermeable for several hours, as large cores may take longer to pass the stomach. The film coat should form a coherent layer that is largely impermeable to penetrating gastric fluid and emerging active ingredients.

The pharmacopoeial specifications allow some softening of enteric-coated cores. Testing for gastroresistance must confirm that the films remain intact and that the diffusion of active ingredient in gastric fluid remains within tolerable limits.

Enteric polymer films are expected to dissolve between pH 5.5 and 6.5, on the one hand to avoid premature disintegration in the stomach and, on the other hand, to ensure rapid release of the active ingredients in the intestinal tract.

6.3.2   Formulation of tablet cores

Tablet cores having the necessary hardness to be coated were prepared at a compression force of 20 kN as described earlier. These tablets showed a crushing strength of 195 N, a disintegration time of 9 min and a friability of 0.4%.

Placebo oblong tablets were prepared from Ludipress® (360 mg), Avicel® PH 101 (352 mg), Aeroperl® 300 Pharma (40 mg), Ac-Di-Sol (40 mg) and magnesium stearate (8 mg). The composition of the placebo tablets were selected which could produce tablets with similar properties like the core DEP tablets. The placebo core tablets showed a crushing strength of 150 N, friability of 0.25% and disintegrate in less than a minute. The placebo tablet cores were used to increase the batch volume of the core tablets in the coating pan. The ratio of placebo to *G. lotoides* tablet cores was maintained at 4:1.

6.3.3   Preparation and characterization of enteric coated *Glinus lotoides* tablets

6.3.3.1   Enteric coating of *Glinus lotoides* tablets

EUDRAGIT® L 100-55 which dissolves from pH 5.5 upwards was used as enteric coating polymer for *G. lotoides* tablets. The polymer was dissolved in isopropyl alcohol and acetone and after the addition of plasticizer, glidant (talc), lubricants and pigment, the mixture was used as final enteric coat on *G. lotoides* core tablets. The formulation of the enteric coating suspension is given in Table 7.13 under experimental section.

Flößer et al. (2000) have evaluated the effect of variations in plasticizer type, quantity and talc concentration on the preparation, processing and properties of isolated films and film-coated caffeine tablets. Plasticizers such as propylene glycol (PG), polyethylene glycol (PEG) 400, PEG 6000, PEG 1500, and triethyl citrate (TEC) were investigated at various concentrations. With all the plasticizers investigated, the minimum film-forming temperature (MFT) decreases as the amount of plasticizer increased. Of all the variants investigated, the formulation with 20% PG performed the best.

EUDRAGIT® L 100-55 requires the addition of plasticizer and talc. Usually, the amounts of polymer required for enteric coating ranges from 4 to 6 mg/cm² of the tablet surface. Depending on the quality of the tablets (hardness, friability), higher amounts of polymers may be necessary. However, higher amounts of polymer may increase the disintegration time in the intestinal fluid. Incorporated pigments (TiO_2, color lakes) may increase the permeability of the coating and require higher amounts of applied polymer.

6.3.3.2 Characterization of the enteric coated *Glinus lotoides* tablets

Disintegration time

The enteric coated tablets resisted disintegration or softening in simulated gastric fluid (0.1 M HCl) for a minimum of 2 hours. The gastro-resistance test was performed in a tablet disintegration tester according to the EP. The tablets were then inspected for signs of disintegration and cracks that might lead to premature release of the active ingredients.

Subsequently, the test fluid is replaced with phosphate buffer solution pH 6.8 as simulated intestinal fluid. The enteric coated tablets disintegrated within 15 min whereas the enteric coated placebo tablets disintegrated within 4 min, which is in compliance with the EP specifications. There was no significant difference observed between Eudragit® L 100/55 and Kollicoat® MAE 100P. Both propylene glycol and acetyl tributyl citrate were efficient as plasticizers at concentration of 11% in dry film.

Physical assessment of the coated tablets

Table 6.3 depicts the average specifications of the core and enteric coated *G. lotoides* tablets.

Table 6.3 Average specifications of the core and enteric coated tablets.

	Parameter				
	Weight, mg (n = 10, RSD, %)	Crushing strength, N (n = 6, RSD, %)	Dimension, mm (n = 6)		
			Length (RSD, %)	Width (RSD, %)	Thickness (RSD, %)
Core tablets	1380 (0.53)	195 (3.8)	20.29 (0.12)	8.24 (0.30)	8.43 (0.26)
Enteric coated tablets	1531 (0.67)	380 (2.3)	20.49 (0.45)	8.56 (0.42)	8.81 (0.40)

6.3.3.3   Stability of the enteric coating

Figure 6.3 shows the increase in tablet weight due to water absorption. As shown in the figure, the core tablets absorb up to 10% w/w water at 26°C and 60% relative humidity. The adsorption equilibrium was reached within three days.

On the other hand, the water uptake of the enteric coated *G. lotoides* tablets at 26°C and 60% relative humidity was found to be 6% in 15 days. As shown in the figure, the equilibrium was not achieved during this period. Similar water adsorption pattern was observed at 21° C and 45% relative humidity but the water uptake was reduced to 4% and less than 2%, for the core and enteric coated tablets, respectively.
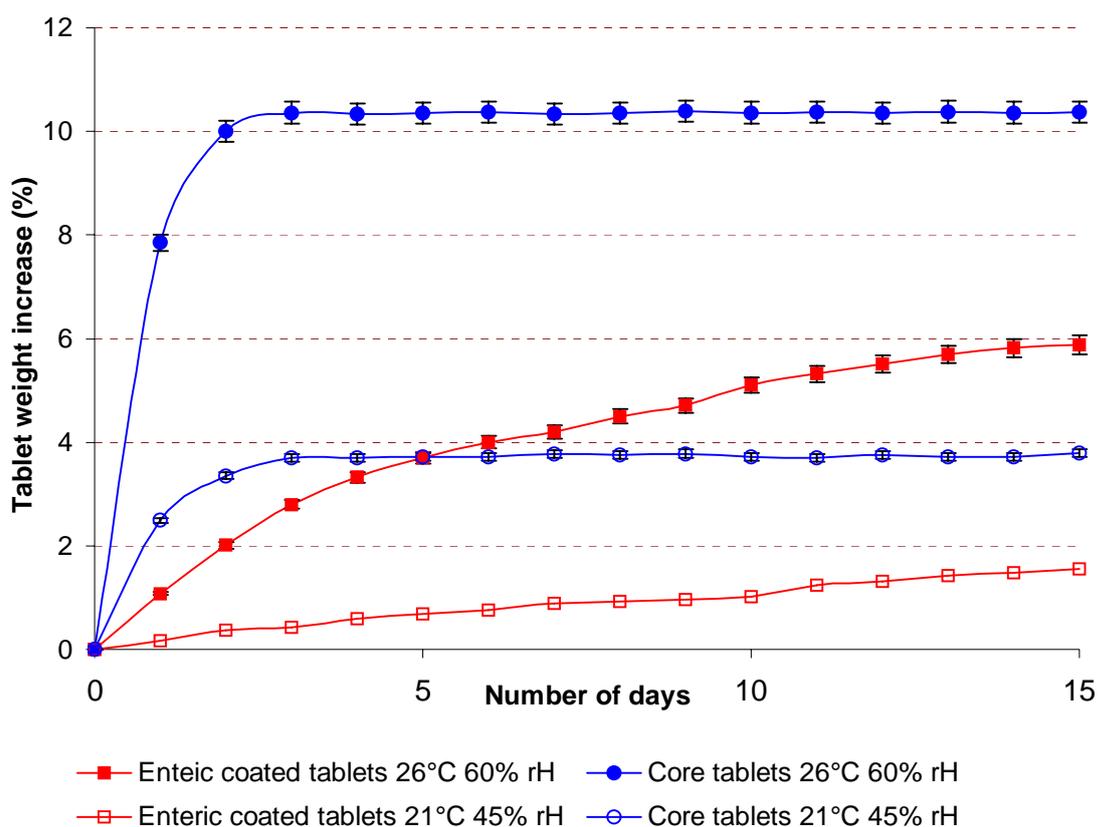
Figure 6.3: Water uptake of the core and coated tablets at 21°C in 45% relative humidity and at 26°C in 60% relative humidity.

Similar pattern was observed with the increase in tablet thickness due to water uptake (Figure 6.4.) In addition to the increase in tablet thickness, the color of the core tablets changed which is more pronounced at 26 °C and 60% relative humidity level. At a relative humidity of 60% at 26 °C the thickness of the enteric coated tablets increased continuously with time.
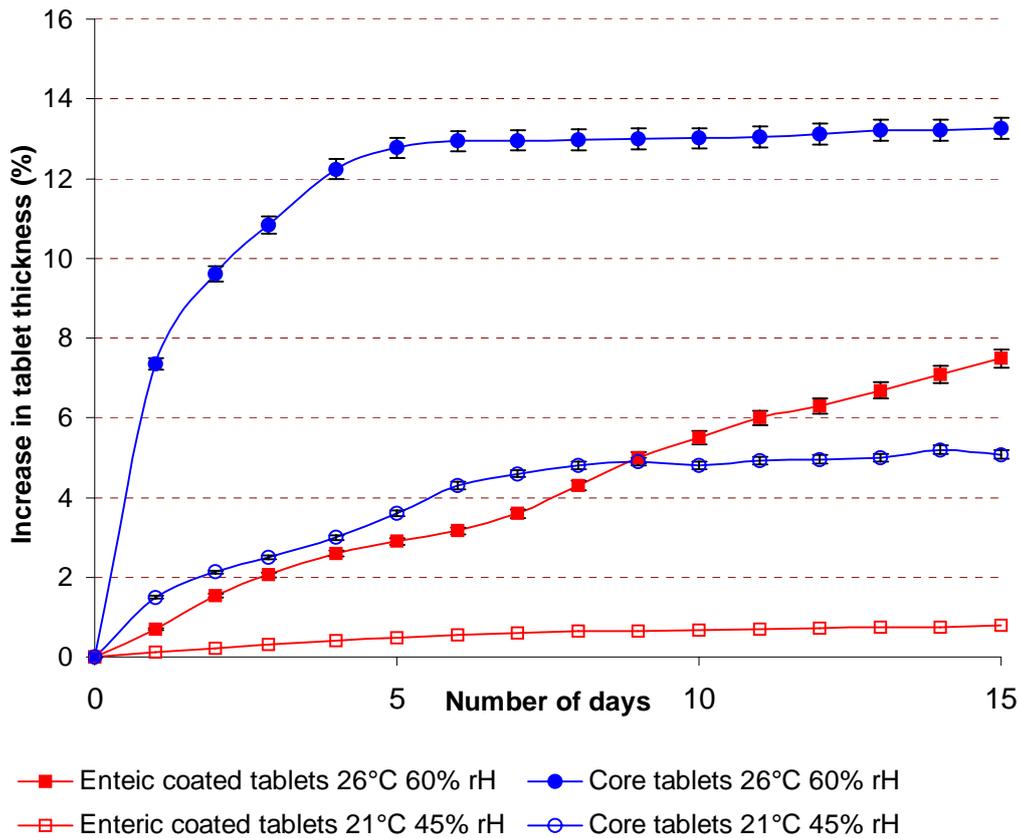
Figure 6.4: Increase in tablet thickness of the core and coated tablets at 21°C in 45% relative humidity and at 26°C in 60% relative humidity due to water uptake.

Chapter 7

EXPERIMENTAL SECTION

7.1   Materials

Plant Material

Fruits of *Glinus lotoides* L. were purchased from a local market in Addis Ababa, Ethiopia, in September 2001. The identity was confirmed by the National Herbarium, Department of Biology, Faculty of Science, Addis Ababa University, Ethiopia. A voucher specimen (No. 003444) has been deposited at the National Herbarium, Department of Biology, Faculty of Science, Addis Ababa University, Ethiopia.

Table 7.1: Solvents and chemicals

Substance	Charge number	Manufacturer/Supplier
Acetic acid glacial	K23614063651	Merck KGaA
Acetone	1056180	Fluka Chemie AG
Acetonitrile	9012	J. T. Baker
Aluminum chloride	06220	Fluka Chemie AG
p-Anisaldehyde	53615208235	Merck KGaA
Boric acid	K25574065843	Merck KGaA
n-Butanol	21500	Fluka Chemie AG
Citric acid monohydrate	K91649444 025	Merck KGaA
Chloroform	442392	Fluka Chemie AG
1,2-Dichloroethane	K27929255046	Merck KGaA

Substance	Charge number	Manufacturer/Supplier
Ethyl acetate	K27290323	Merck KGaA
Ethyl methyl ketone	K13788608	Merck KGaA
Formamide	20210	Riedel-de Haën
Formic acid	K27372532	Merck KGaA
Hydranal composite 2	33410	Riedel-de Haën
Hydrochloric acid	K27984217	Merck KGaA
Magnesium chloride	TA743633	Merck KGaA
Methanol	0377501	Fischer Scientific GmbH
PEG 400	51632267	BASF AG
Phosphoric acid (85%)	K17169073	Merck KGaA
Potassium carbonate	A493528	Fluka Chemie AG
Potassium chloride	K24252236	Merck KGaA
iso-Propanol	K2935696	Merck KGaA
Sodium chloride	K 31900304	Merck KGaA
Sodium nitrite	S30973034	Merck KGaA
Sodium acetate	82810	Riedel-de Haën
Sodium bromide	K30615060	Merck KGaA
di-Sodium hydrogen phosphate	K25613676841	Merck KGaA
Sodium tartarate dihydrate	6664	Merck KGaA
Sulphuric acid 95-97%	K27582431010	Merck KGaA
Vanillin	K25739410840	Merck KGaA

Table 7.2: Standard substances

Substance	Charge number	Manufacturer/Supplier
Arabinose	10860	Fluka Chemie AG
Xylose	L328889	Merck KGaA
Rhamnose	1.0736	Merck KGaA
Vitexin	95042502	Extrasynthese
Isovitexin	94111405	Extrasynthese

Table 7.3: Excipients for extract preparation, tablet formulation and enteric coating

Substance	Charge number	Manufacturer/Supplier
Aeroperl® 300 Pharma	9125063	Degussa AG
Avicel® PH 101	6350	FMC Biopolymer
Ac-Di-Sol®	T112C	FMC Corporation
Magnesium stearate	0266	Baerlocher GmbH
Talc	92092223	Norwegian Talc GmbH
Oleic acid	K91145571916	Merck KGaA
Explotab®	E7094	Mendell GmbH
Avicel® PH 200	X119	FMC Biopolymer
Kollidon® CL	19-2143	BASF AG
Ludipress	72-1281	BASF AG
Kollicoat® MAE 100 P	83802009T0	BASF AG
1,2-Propylene Glycol	50027101	BASF AG

Substance	Charge number	Manufacturer/Supplier
CITROFOL® B II	3002862/05.05	Jungbunzlauer GmbH
Eudragit® L 100-55	B030804067	Röhm GmbH
Titan dioxide	60180	Kronos Titan GmbH
Sicovit Braun 70 E 172	68M6160	BASF AG
Glycerol monostearate	0047064	Henkel KGaA

Table 7.4: Chromatography and Miscellaneous materials

Substance	Charge number	Manufacturer/Supplier
TLC, Silica gel 60 F_{254}, Art. No. 1.0554	940389246	Merck KGaA
TLC, RP-18 F_{254s}, Art. No. 1.0559	0B076562	Merck KGaA
TLC plates, SIL G-100	004104	Macherey-Nagel GmbH
Sephadex LH-20, Art. No. 17-0090-01	292931	Amersham Biosciences AB
Silica gel 60, CC	TA288385 715	Merck KGaA
Wacker® antifoam emulsion	12131 EK	Drawin GmbH
Helium gas, Type 5.0	0238	Messer Griesheim GmbH
Cellulose acetate filter, 0.2 μm	16534	Sartorius AG
Depth filter, T 1000	D105	Pall Corporation

7.2   Equipment

Table 7.5: List of equipment

Equipment	Manufacturer/Supplier
Analytical balance, model AE 200	Mettler Toledo GmbH
Balance, model PG 4002-S	Mettler Toledo GmbH
Beckman air comparison pycnometer, model 930	Beckman Instruments GmbH
Centrifuge, Megafuge 1.0R	Heraeus Instruments
Dial high gauge, model No. 192-106	Mitutoyo Messgeraete GmbH
Ph. Eur. Settling apparatus, JEL	Engelsmann AG
Filter Press, Type A 20 Z	Seitz-Werke GmbH
Freeze dryer, LYOVAC GT 2	FINNA-AQUA GmbH
Gerteis Micro-Pactor$^{®}$ 1079/2	Gerteis AG
Hydraulic Press 5 L	Fischer & Co. KG
Karl Fischer apparatus	Deutsche Metrohm GmbH & Co.
Korsch EK II tablet press	Korsch GmbH
Korsch EK0 tablet press	Korsch GmbH
Krüss Processor Tensiometer K12	Krüss GmbH
Mastersizer 2000	Malvern Instruments GmbH
Oven, model T5050	Heraeus Holding GmbH
Ball mill	Erweka GmbH
Vacuum rota vapor	Büchi Labortechnik GmbH
TBH-30 hardness tester	Erweka GmbH

Equipment	Manufacturer/Supplier
Turbula mixer T2C	Bachofen AG
Ultra-Turrax T 45/N	IKA-Werk GmbH
Ultra-Turrax T25	IKA-Werk GmbH
UV-Vis Spectrophotometer 550S	Perkin-Elmer GmbH
Vacuum drying oven, VDL 53	Binder GmbH
Water distillation apparatus	Wagner & Munz GmbH
Fraction collector, UltroRac® II	LKB-Produkter AB

7.3   Data processor

Computer: AMD Athlon (TM) XP1600+, 523 MB RAM, 60 GB hard disk

Table 7.6: List of general software

Software	Manufacturer/Supplier
Microsoft word	Microsoft
Microsoft excel	Microsoft
Microsoft power point	Microsoft
Krüss Processor Tensiometer K12 software	Krüss GmbH
Messfix 2.3	Developed in the Institute
Malvern system software	Malvern Instruments GmbH
Catman 3.1	Hottinger-Baldwin Messgeraete GmbH

7.4   Extraction, isolation and structural elucidation of the saponins and flavonoids of the seeds of *Glinus lotoides*

7.4.1   Extraction of the seeds of *Glinus lotoides*

The seeds of *G. lotoides* were separated from the fruits, powdered and extracted as shown in scheme 7.1. 500 g seeds of *G. lotoides* were defatted three times with 5 L *n*-hexane in 10-L beaker for 15 min using an Ultra-Turrax (Type T 45/N, IKA-Werk GmbH) at 10000 rpm. The *n*-hexane extracts were filtered and the solvent was removed under reduced pressure in a rotavapor (Büchi Labortechnik GmbH). The amount of fat removed was found to be 66.5 g, which is equivalent to 13.3% of the dried powdered seeds. The defatted powdered seeds of *G. lotoides* (433.5 g) were stored at -20°C and used as starting plant material for the isolation and quantification of the saponins and flavonoids.

The defatted powdered seeds (5 g) were extracted three times with 50 mL 60% methanol in water using an ultra-turrax (IKA-Werk GmbH) at 9500 rpm for 10 min. The extracts were filtered, concentrated under reduced pressure and dried in a freeze-dryer (FINNA-AQUA GmbH) using a 24-hrs drying program: -30° C for 8hrs; 0° C for 4hrs; 10° C for 6hrs and 20° C for 6hrs. This dried extract was referred to as extract **A** and was found to be 0.9 g, which is equivalent to 18% of the defatted dry powdered seeds.

Exhaustive extraction of the seeds of *G. lotoides* (10 successive extractions with 80% methanol) has yielded 18% crude extract (Extract **A**) calculated on the dry defatted seeds. The extraction method used in this study has provided an extract containing comparable amount to the exhaustive extraction reported earlier (Endale *et al*. 2004).

0.9 g of extract **A** was dissolved in 30 ml water and extracted three times with an equal volume of *n*-butanol. The *n*-butanol fractions were combined and dried which gave 160 mg (3.2% of the defatted dry seeds). This extract was referred to as extract **D** and contains the saponins and flavonoids of *G. lotoides*. The remaining aqueous part was freeze-dried (extract **B**) as described above. Between the *n*-butanol and the aqueous layer, an emulsified intermediate layer was separated and dried (extract **C**). The amount of extract **C** decreased significantly with repeated extraction with fresh *n*-butanol and water.

Extract **D** (160 mg) was suspended in a relatively small volume of methanol (2 ml) and allowed to settle. The undissolved part was separated and dried (extract **E**) and the dissolved part was added to a large volume of diethyl ether (40 ml). The precipitate formed was separated by centrifugation (Megafuge 1.0R, Heraeus Instruments) at 2000 rpm for 20 min at 5 °C and dried (120 mg, 2.4% of the defatted dry seeds). This extract was referred to as extract **F** and was used as a starting material for the isolation of the saponins and flavonoids and later on as a secondary standard for the routine quality control of the crude extract and the tablet formulations thereof. After centrifugation, the supernatant was dried which gave an extract **G**.

Scheme 7.1: Extraction of the seeds of *G. lotoides*

Scheme 7.1: Extraction of the seeds of *G. lotoides* (Cont'd)

```
                    ┌─────────────────────────┐
                    │ 5 g Defatted dry powder │
                    │   seeds of G. lotoides  │
                    └─────────────────────────┘
                              │  Extracted (3 x 50 ml 60% MeOH, Ultra-Turrax
                              │      9500 U/min; 10 min, at room temp.)
                              │  Filtered
                    ┌─────────────────────────┐
                    │  60% Methanol extract   │
                    └─────────────────────────┘
                              │  Concentrated in rota vapor at 40 °C
                              │  Freeze dried
                    ┌─────────────────────────┐
                    │ Dry extract, 0.9 g (Extract A) │
                    └─────────────────────────┘
                              │  Dissolved in 30 ml Water
                              │  Partitioned between n-butanol (1:1)
         ┌────────────────────┼────────────────────────┐
  ┌──────────────┐    ┌──────────────┐         ┌──────────────────┐
  │ Aqueous layer│    │ n-Butanol    │         │   Emulsified     │
  │              │    │   layer      │         │ intermediate layer│
  └──────────────┘    └──────────────┘         └──────────────────┘
       │ Freeze dried       │ Concentrated            │ Dried
       │                    │ Dried
  ┌──────────────┐    ┌──────────────┐         ┌──────────────┐
  │  Extract B   │    │ Extract D    │         │  Extract C   │
  │              │    │  (160 mg)    │         │              │
  └──────────────┘    └──────────────┘         └──────────────┘
                              │  Suspended in 2 ml MeOH
            ┌─────────────────┴─────────────────┐
   ┌──────────────────┐              ┌──────────────────┐
   │ Undissolved Part │              │  Dissolved Part  │
   └──────────────────┘              └──────────────────┘
            │                               │ Added into 40 ml ether
   ┌──────────────┐               ┌──────────────────┐
   │  Extract E   │               │   Precipitate    │
   └──────────────┘               │    formation     │
                                  └──────────────────┘
                                          │ Centrifugation
                                          │ 2000 rpm, 20 min, 5 °C
            ┌─────────────────────────────┤
   ┌──────────────┐               ┌──────────────────┐
   │ Supernatant  │               │   Precipitate    │
   └──────────────┘               └──────────────────┘
            │                               │
   ┌──────────────┐               ┌──────────────────┐
   │  Extract G   │               │   Extract F      │
   └──────────────┘               └──────────────────┘
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7.4.2   Fractionation and isolation of the saponins and flavonoids

7.4.2.1   Column chromatography fractionation

Sephadex LH-20 column chromatography

100 g of Sephadex LH-20 (Amersham Biosciences AB) were packed manually into a column of 28 cm x 1.5 cm i.d. The specified amount of sephadex LH-20 was allowed to swell in MeOH overnight and cautiously filled into the column using a funnel. The gel was then allowed to settle and pack for 24 hrs. The entrapment of air was avoided and care was taken not to dry the column.

Extract **F** (1.2 g) was dissolved in a 2 ml methanol and carefully applied on the top of the column. The extract was eluted with methanol at a rate of 4 ml/min and 20 fractions were collected using UltroRac$^®$ II (LKB-Produkter AB). The fractions were combined into two fractions, Fr. 1 (352 mg) and Fr. 2 (600 mg) after controlling on analytical TLC plates (pre-coated RP-18 F_{254s} aluminum sheets, Merck KGaA) using a mixture of MeOH:H_2O (4:1) as eluent and 5% vanillin-H_2SO_4 reagent for detection. The solvent system composed of $CHCl_3$-MeOH-H_2O (70:50:4) was used for the flavonoids with fluorescence detection after spraying with Natural Products Reagent (5 mL of solution I (1% diphenylboryloxyethylamine in MeOH) followed by 5 mL of solution II (5% PEG 400 in EtOH).

Silica gel 60 and RP-18 column chromatography

Similarly, silica gel 60 (Merck KGaA) and RP-18 (Merck KGaA) column were utilized for fractionation of the purified extract (extract **F**). The experimental conditions of the RP-18 column chromatography are given in Table 7.7. Up to 280 fractions were collected which were combined into 14 fractions after TLC examination.

Table 7.7: RP-18 column chromatography conditions of extract F

Parameter	
Column size	18 cm x 1.5 cm i.d.
Mobile phase	H$_2$O:MeOH in the following order
	[(100:0); (95:5); (85:15); (70:30); (50:50); (30:70)(0:100)]
Fractions volume	6 mL (200 drops)
Flow rate	6 mL/ 30min
Number of fraction collected	280
Fractions analysis	RP-18 TLC plate, MeOH:H$_2$O (4:1), 5%vanillin/H$_2$SO$_4$

7.4.2.2   High Performance Liquid Chromatography (HPLC)

Fractions collected from sephadex LH-20 column chromatography were further purified on HPLC to isolate saponins and flavonoids. Fraction 1 (256 mg) containing mainly flavonoids was separated by reversed-phase HPLC using 15% CH$_3$CN in water as a mobile phase to provide two flavonoids, F. 1/1 (18.3 mg) and F. 1/2 (10.8 mg) which later on the structures were elucidated using spectral analysis as vicenin-2 and vitexin-2"-*O*-glucoside, respectively.

Fraction 2 (480 mg) containing mixtures of saponins, was separated by RP-18 HPLC using 75% MeOH in water as a mobile phase. Refractive index detector was employed for the saponins due to the lack of a suitable chromophore for UV detection. The RP-HPLC provided two fractions, S1 (24 mg) and S2 (29 mg) which are composed of at least two major components as identified using TLC. Preparative TLC of S1 and S2 provided five saponins.

HPLC conditions for the isolation of flavonoids and saponins

Equipment:

Pump:	LC-10AD, Shimadzu Liquid Chromatography, Shimadzu Co.	
Injector:	Rheodyne, 7021, Rohnert Park, CA.	
Detector:	For flavonoids:	LC Spectrophotometer, MILLPORE® Waters, Model 481, Milford.
	For saponins	Shimadzu RID-10A refractive index
Integrator:	Chromato-Integrator, D-2000, Merck.	

Column: CC 250/4 Nucleosil 100-7 C_{18} Cat. No. 721609.40, Ser. No. 1096117, Charge/Batch 21401011, Macherey-Nagel

Guard column: LichroCART® 4-4, Lichrospher® 100 RP-18, 5 μm, Merck

Mobile phase: For flavonoids: 15% CH_3CN in water, Isocratic

                  For saponins: 75% MeOH in water, Isocratic

Injection volume: 150 μL

Flow rate: 1 mL/min

7.4.2.3   Preparative Thin Layer Chromatography (TLC)

Preparative silica gel TLC (Sil G-100, 1.0 mm, Macherey-Nagel GmbH) with solvent system of $CHCl_3$-MeOH-H_2O (70:50:4) was used to isolate saponins from S1 and S2 HPLC fractions. One small edge of the TLC plates was sprayed with vanillin reagent and the regions containing the saponins were scratched. The saponins were then extracted with MeOH. S1 afforded two saponins, S1/1 (2.2 mg) and S1/2 (1.9 mg). Similarly, S2 gave two fractions S2/1 (2.4 mg) and S2/2 (3.0 mg). The structures of the isolated compounds were elucidated as described below.

7.4.3   Structural elucidation of the saponins and flavonoids

7.4.3.1   Structure analysis of flavonoids by Ultraviolet Spectroscopy

UV-Vis spectra of the flavonoids were recorded in methanol and after the addition of various diagnostic reagents into the methanol solutions using Perkin-Elmer Lambda 16 UV/Vis spectrometer (Perkin-Elmer GmbH).

Preparation of diagnostic reagents

I.   Sodium methoxide (NaOMe): 2.5 g freshly cut metallic sodium were added cautiously in small portions to 100 mL dry spectroscopic methanol.

II.   Aluminum chloride (AlCl₃): 5 g fresh anhydrous AlCl₃ were added cautiously to 100 mL spectroscopic methanol.

III.   Hydrochloric acid (HCl): 50 ml concentrated HCl was mixed with 100 mL distilled water.

IV.   Sodium acetate (NaOAc): Anhydrous powdered reagent grade NaOAc was used.

V.   Boric acid (H₃BO₃): Anhydrous powdered reagent grade H₃BO₃ was used.

Measurements of the spectra

Stock solutions of the isolated flavonoids (F 1/1 and F 1/2) were prepared by dissolving 0.5 mg in 50 ml MeOH. The concentration was then adjusted so that the optical density of the major absorption peak between 250 and 400 nm gave an optical density reading in the region 0.6 to 0.8. The methanol spectrum was measured using 3 ml of the stock solution of the flavonoid. The NaOMe spectrum was measured immediately after the addition of three drops of the NaOMe stock solution to the methanol solution. After 5 min, the spectrum was re- recorded to check the stability of the flavonoids. The AlCl₃ spectrum was measured immediately after the addition of six drops of the AlCl₃ stock solution to 3 ml of fresh methanol solution of the flavonoid. The AlCl₃/HCl spectrum was then recorded after the addition of three drops of HCl solution to the AlCl₃ solution.

The NaOAc spectrum of the flavonoid was determined as follows. Excess coarsely powdered, anhydrous reagent grade NaOAc was added with shaking to a cuvette containing 3 ml of fresh MeOH solution of the flavonoids. About 2 mm layer of NaOAc remained on the bottom of the cuvette. A second spectrum was run after 10 min to check for decomposition. The NaOAc/H_3BO_3 spectrum was determined immediately after the addition of sufficient powdered anhydrous reagent grade H_3BO_3 to the cuvette containing the NaOAc to give a saturated solution.

7.4.3.2   Mass Spectrometry[1]

The mass spectra of the substances were obtained using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan) equipped with an electrospray interface coupled to an integrated syringe pump system working with a flow rate of 10-20 µl/min. The electrospray interface was locked in a 90° position toward the orifice and operated at 3.8 kV in positive ionization mode and at 2.6 kV in negative ionization mode. Capillary temperature and sheath gas were optimized at 280°C and 30 (arbitrary) units, respectively. Auxiliary gas was not used. The collision induced dissociation spectra (CID-spectra) were recorded with collision energy 20 and 35 eV for positive and negative modes, respectively. Source CID voltage was set to a constant value of 10V for both Q1 and Q3 full scan and CID spectra, and the argon collision gas for MS/MS experiments was maintained at a pressure of 1.3×10^{-6} bar.

7.4.3.3   Nuclear Magnetic Resonance (NMR)[2]

1D (^{1}H, ^{13}C and DEPT-135) and 2D (^{1}H COSY, HMQC and HMBC) NMR spectra were obtained at 300 °K on Bruker AVANCE DMX-600 or ARX-400 NMR

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1 Measurement was conducted at the Institute of Pharmacology and Toxicology, Division of Clinical Pharmacology, University Hospital of Tübingen, by Dr. Bernd Kammerer

2 NMR Spectra were measured at the Institut für Pharmazeutische Wissenschaften, Universität Freiburg, by Prof. Irmgard Merfort und GBF-Gesellschaft für Biotechnologische Forschung, Braunschweig, by Dr. Victor Wray

spectrometers (Bruker) locked to the major deuterium signal of the solvent, CD$_3$OD. Chemical shifts are reported in δ ppm and coupling constants in Hz.

7.4.3.4   Sugar analysis of the saponins of *G. lotoides*

Acid hydrolysis on TLC plate

Glinuside solutions (5 µL of 0.08 mg/mL corresponding to 0.4 µg glinuside) were applied on pre-coated silica gel 60 F$_{254}$ aluminum sheet TLC plates and left in a hydrochloric acid atmosphere at 100 °C for 1 h HCl vapor was then eliminated under hot ventilation and the authentic sugars, namely arabinose (Fluka Chemie AG), xylose (Merck KGaA), and rhamnose (Merck KGaA) were applied. The TLC plate was developed with H$_2$O-MeOH-CH$_3$COOH-C$_2$H$_4$Cl$_2$ (10:15:25:50) and visualized after spraying with *p*-anisaldehyde-H$_2$SO$_4$ reagent followed by heating at 110 °C for 10 min. Arabinose, xylose and rhamnose were observed at R_f values of 0.55, 0.65 and 0.68, respectively.

Methylation analysis of the sugar constituents[3]

Sugar-linkages were analyzed with GC-MS system of the partially methylated alditol acetates as described by Plock *et al* (2001).

7.5   Quantitative determination of the saponins and flavonoids for routine quality control purposes

7.5.1   Quantitative determination of the saponins

7.5.1.1   Preparation of a standard

Isolation of mollugogenol B

500 mg of extract **F** were hydrolyzed with 3M HCl at 100°C for 1 h and the aglycons of the saponins were extracted with diethyl ether. The major hydrolysis product (A1, 30

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3 Analysis was conducted at the GBF-Gesellschaft für Biotechnologische Forschung, Braunschweig, by Dr. Victor Wray

mg) was isolated using RP-HPLC (CC250/4 Nucleosil 100-7 C_{18} Macherey-Nagel, GmbH & Co. KG) with a guard column (ChroCART 4-4 Lichrospher 100 RP-18, 5 μm, Merck KGaA) using acetonitrile as a mobile phase and UV detection at 254 nm.

Structural confirmation of mollugogenol B

The structure of the isolated aglycon was elucidated by NMR spectral analysis as mollugogenol B. The NMR analyses were performed as described under section **7.4.3.3**.

Determination of the purity of mollugogenol B

The purity of mollugogenol B was determined using HPLC, TLC and UV-spectrophotometry. The pre-coated RP-18 TLC plates were loaded with 5, 10, 15 and 20 μL of mollugogenol B solutions which were equivalent to 11.85, 23.70, 35.55 and 47.40 μg of mollugogenol B, respectively. The TLC plates were then developed with various solvent systems. The appearance of minor spots in high concentrations was noted. Similarly, 23.7 mg/100 mL mollugogenol B was injected into HPLC and the percentage of the mollugogenol B peak was calculated as 98%. Stock solutions of mollugogenol B in MeOH (2.37 mg/100 mL) were prepared and the absorbance was measured at λ_{max} 244, 251 and 261 nm. The molar absorptivity (log ε) was calculated and compared with literature values.

7.5.1.2 Optimization of the acid hydrolysis of the saponins

Optimal hydrolysis conditions were investigated using various acid concentrations (0.5 – 5.0 M HCl), hydrolysis temperature (25 – 100°C) and hydrolysis duration (0.5 – 8 h) and a method was selected that provided the maximum amount of mollugogenol B with minimum artifacts.

7.5.1.3   Sample preparation for HPLC analysis

Analysis of the crude extract

G. lotoides saponins were analyzed after extract A (the crude extract) was hydrolyzed with 3M HCl at 100°C for 1 h. The hydrolysis products were cooled and the aglycons were extracted three times with diethyl ether. The ether layers were combined, dried and dissolved in methanol. The solution was filtered through a 0.2 μm cellulose acetate filter (Sartorius AG) and injected into HPLC. Calculation of the total saponins was based on glinuside G (mol. Wt., 900), which possesses an average molecular weight of *G. lotoides* saponins.

Analysis of tablet formulation

Oblong tablets (1.4 g) containing 650 mg extract A were crushed using mortar and pestle and 215.4 mg of the tablet powder, equivalent to 100 mg of extract A, were hydrolyzed with 100 mL of 3M HCl at 100 °C for 1 h. The aglycons were extracted three times with 100 mL diethyl ether. The ether layers were combined, dried and dissolved in 100 mL methanol. The solution was filtered and injected into HPLC. Six tablets were analyzed and the content of the saponins was determined.

7.5.1.4   Preparation of the calibration curve

An amount of 2.37 mg mollugogenol B was accurately weighed and dissolved in 100 mL of methanol (stock solution [2.37 mg/100mL]). Five additional calibration levels (1.896; 1.422; 0.948; 0.474 and 0.237 mg/100mL) were prepared by dilution of the stock solution with methanol. The standard solutions, stored at 4 °C, were stable for at least 3 months, as confirmed by re-assaying the solutions.

7.5.1.5   HPLC conditions for the analysis of aglycons of the saponins

Equipment:

Pump:	LC-10AD, Shimadzu Liquid Chromatography, Shimadzu Co.
Injector:	Rheodyne, 7021, Rohnert Park.
Detector:	LC Spectrophotometer, MILLPORE® Waters, Model 481, Milford.
Integrator:	Chromato-Integrator, D-2000, Merck
Column:	CC 250/4.6 Nucleosil 100-5 C18 Cat. No. 721662.46, Ser. No. 4056737, Charge/Batch 21304011 Macherey-Nagel
Guard column:	CC 8/4 Nucleosil 100-5 C18, Cat. No.: 721602.40, Ser No., 2035906, Batch 2019 Macherey-Nagel
Mobile phase:	25 minutes gradient flow of methanol -water solvent system as indicated in Table 7.8

Table 7.8: Gradient flow for the aglycons

Time (min)	Solvent system	Condition
0 – 15	95% MeOH to 100% MeOH	Linear
15 – 20	100% MeOH to 95% MeOH	Linear
20 – 25	95% MeOH	Isocratic

The solvents were degassed with helium (Type 5.0, Messer Griesheim GmbH). The injection volume and the flow rate were adjusted to 20 µL and 1 mL/min, respectively. The detection was UV at 254 nm.

7.5.1.6   Method validation

Linearity

The calibration curves were analyzed using a linear regression model and correlation coefficients. The calibration curves, residuals and standardized residuals were inspected to assess linearity.

Repeatability

The intra- and inter-day repeatability of the method was evaluated using multiple preparations of the sample.

Limits of detection (LOD) and limits of quantification (LOQ)

The LOD and LOQ were determined from serial dilution and were calculated according Gottwald (2000).

7.5.2   Quantitative determination of the flavonoids

7.5.2.1   Preparation of standard

Isolation of vicenin-2

Vicenin-2 was isolated from the purified extract of the seeds of *G. lotoides* as mentioned under section **7.4.2**.

Structural confirmation of vicenin-2

The structural elucidation of vicenin-2 was performed as mentioned under section **7.4.3.**

Determination of the purity of vicenin-2

The purity of the isolated vicenin-2 was determined using HPLC and TLC methods. In this, the TLC plates were loaded with 1, 2, 5, 15 and 30 μL of vicenin-2 standard solutions which corresponds to 1.3, 2.6, 6.5, 19.5 and 39.0 μg, respectively. The TLC plates were then developed with various solvent systems. Similarly, 130 mg/100ml of vicenin-2 was injected into HPLC and the percentage of vicenin-2 was calculated as 97%.

7.5.2.2   Sample preparation for HPLC analysis

Analysis of the crude extract

The extract of the seeds of *G. lotoides* (extract A) was dissolved in water and filtered through a 0.2 μm cellulose acetate filter (Sartorius AG) before injection into HPLC. Total flavonoids were calculated as vicenin-2 equivalent.

Analysis of the tablet formulation

Oblong tablets (1.4 g) containing 650 mg extract A were crushed using mortar and pestle and 120 mg of the tablet powder were suspended in a 100 mL volumetric flask with water. The mixture was sonicated for 30 min and a portion of the supernatant was filtered through a 0.2 μm cellulose acetate filter before injection into HPLC. Six tablets were analyzed and the content of the flavonoids was determined.

7.5.2.3   Preparation of calibration curve

An amount of 5.83 mg vicenin-2 was accurately weighed and dissolved in 100 mL of water (stock solution [5.83 mg/100mL]). Five additional calibration levels (4.664; 3.489; 2.332; 1.166 and 0.583 mg/100mL) were prepared by dilution of the stock solution with water. The standard solutions, stored at 4 °C, were stable for at least 3 months, as confirmed be re-assaying the solutions.

7.5.2.4   HPLC conditions for the analysis of flavonoids

Equipment:

Pump: LC-10AD, Shimadzu Liquid Chromatography, Shimadzu Co.

Injector: Rheodyne, 7021, Rohnert Park.

Detector: LC Spectrophotometer, MILLPORE® Waters, Model 481, Milford.

Integrator: Chromato-Integrator, D-2000, Merck

Column: CC 250/4.6 Nucleosil 100-5 C18 Cat. No. 721662.46, Ser. No.

                           2017025, Charge/Batch 21301173 Macherey-Nagel

Guard column: CC 8/4 Nucleosil 100-5 C18, Cat. No.: 721602.40, Ser No.,

                           2035906, Batch 2019 Macherey-Nagel

Mobile phase: 30 min gradient flow (Table 7.9) composed of two solvent

                           mixtures

Mixture A:	Acetonitrile:	130
	0.1 M Phosphoric acid:	24
	Water:	825
Mixture B:	Acetonitrile:	120
	0.1 M Phosphoric acid:	6
	Water:	187

Table 7.9: Gradient flow for flavonoids

Time (min)	Solvent system	Condition
0 – 20.0	Mixture A to Mixture B	Linear
20.0 – 25.0	Mixture B to Mixture A	Linear
25.0 – 30.0	Mixture A	Isocratic

The solvents were degassed with helium (Type 5.0, Messer Griesheim GmbH). The sample volume and the flow rate were adjusted to 20 µL and 1 mL/min, respectively. LC-UV at 360 nm was used for the detection of the flavonoids.

7.5.2.5   Method validation

Accuracy

Accuracy of the analytical method was determined by spiking 32.8 mg of extract F with vicenin-2 standard solutions to produce 50, 75, 125 and 150%. The samples were analyzed with HPLC as described above and the recovery of vicenin-2 was determined.

Linearity

The calibration curves were analyzed using a linear regression model and correlation coefficients. The calibration curves, residuals and standardized residuals were inspected to assess linearity.

Repeatability

The intra- and inter-day repeatability of the method was evaluated using multiple preparations of the sample.

L*imits of detection (LOD) and limits of quantification (LOQ)***

The LOD and LOQ were determined from serial dilution and were calculated according Gottwald (2000).

7.6 Preparation and physicochemical characterization of the dry extract preparation for tablet formulations

7.6.1 Preparation of the dry extract preparation

500 g of defatted powder seeds of *G. lotoides* were extracted with 5 L 60% MeOH in a 10-L beaker for 15 min using an Ultra-Turrax (Type T 45/N, IKA-Werk GmbH) at 10000 rpm. The mixture was passed through a cotton bed and the residue was compressed using a hydraulic press 5-L (HAFICO, Fischer & Co KG). The extract was filtered using a filter press (A-20Z, Seitz-Werke GmbH) through filter sheets (T 1000, Pall Co.) and concentrated using a rotavapor at 50 °C to a volume of 2-L. The amount of the dry extract was determined and the specified amount of Aeroperl$^®$ 300 Pharma was added. The mixture was further concentrated to a viscous mass and dried in a vacuum oven (VDL 53, Binder GmbH) at 40 °C and 125 mbar for 24 hours. Various concentrations of Aeroperl$^®$ 300 Pharma (10, 20, 30, 40, 50 and 60% (w/w)) were

utilized as an inert adsorbent to prepare the dry extract. The dry extract preparations were pulverized in a ball-mill and passed through a sieve size of 250 μm. The physicochemical properties of these dry extract preparations were characterized as described below.

7.6.2   Physicochemical characterization of the dry extract preparations

7.6.2.1   Particle size distribution

The particle size distribution of the dry extract preparations (10-60% (W/W) Aeroperl$^{®}$ 300 Pharma) were measured by laser diffraction spectrometry (Mastersizer 2000, Malvern Instruments GmbH) using the dry-dispersing system Scirocco 2000 (Malvern Instruments GmbH). Extract preparations were measured using a dispersing air pressure of 4 bars. Data were collected directly by means of the system software (Malvern Instruments GmbH). The particle sizes were analyzed according to *Mie*-theory at particle refractive index and absorption of 1.300 and 0.1, respectively. The mean values of five measurements were calculated.

7.6.2.2   Scanning electron microscopy

Surface structures of the dry extract preparations were determined using a scanning electron microscope Zeiss DSM 940 A (Carl Zeiss). The pictures were taken with a camera (model Contax M 167 MT, Yashica-Kyocera) and were digitalized using the Orion system (Orion 5, E.L.I. sprl). The dry extract preparations were fixed on an aluminum pin using double-adhesive tape (Tempfix) and then coated with a thin gold layer using a Sputter Coater (model E 1500, Bio-Rad). The samples were sputtered four times for 60 sec and exposed to 20 mA current and 2.1 kV acceleration voltages in a vacuum of 0.02-0.03 mbar. The micrographs were taken at various magnifications using 5 and 10 kV.

7.6.2.3   Water uptake study

Moisture sorption study in humidity chambers

Pyrex desiccators containing appropriate saturated salt solutions (Table 7.10) in distilled water were prepared to create chambers with different levels of relative humidity. All desiccators were kept at 20 ± 0.5°C in an oven (model T5050, Heraeus Holding GmbH) to maintain the desired relative humidity level.

Table 7.10: Saturated salt solutions used for different relative humidity levels

Saturated salt solution	Relative Humidity at 20°C (%)
Magnesium chloride	33
Potassium carbonate	44
Sodium nitrite	65
Sodium chloride	75
Potassium chloride	85

Powder beds of 1 g of the dry extract preparations (10-60% W/W Aeroperl® 300 Pharma in extract) were prepared in petri dish and stored at 20° C over the saturated salt solutions. The moisture content of the various extract preparations was measured gravimetrically at 24 hrs intervals for 15 days.

Kinetic moisture sorption study using a Krüss Tensiometer K12

The kinetics of the water sorption of the dry extract preparations was determined using a Krüss, Processor Tensiometer K12 (Krüss GmbH). Various relative humidity levels were realized in the chamber using saturated salt solutions as shown in Table 7.10. A powder bed of 1 g of the extract preparations was placed on a balance hanged in the humidity chamber. The weight gain per unit time was recorded and analyzed with the processor. Prior to the measurements, the extract preparations were stored in a desiccator over activated silica gel for one week. Water uptake of Aeroperl® 300

Pharma was determined at various relative humidity and the water uptake due to the extract was calculated.

7.6.2.4   Water content determination

The moisture content of the various extract preparations was measured using a Karl Fischer water-titration set-up (702 SM Titrino, Deutsche Metrohm, GmbH & Co.). In this, 35 mg of the extract preparations were dissolved in methanol:formamide (2:1) and titrated with Hydranal® composite 2, (one component reagent for volumetric Karl Fischer titration). The amount of Hydranal® composite 2 consumed was noted and the water contents of the extract preparations were calculated using a program called "702 SM Tritrion". Standard sodium tartarate dihydrate, which contains 15.66% water, was used as standard.

Based on the results of the water uptake and SEM studies, the dry extract preparation containing 30% Aeroperl® 300 Pharma was selected for tablet formulation. Hence, density, flow property and compactability were investigated only for this extract preparation which is referred to as dry extract preparation.

7.6.2.5   Density

Bulk (poured) density

Bulk density was measured according to the European Pharmacopoeia 5th Edition. 150 g of dry extract preparation were filled without compacting into a 250 mL graduated cylinder using a powder funnel and was weighed to $\pm$ 0.1 g (m) (model PG 4002-S, Mettler Toledo GmbH). The unsettled volume (V_0) was read to $\pm$ 1 mL and the bulk density was calculated as the quotient m/V_0. Measurements were performed in triplicate and the mean value was calculated.

Tapped density

A settling apparatus (model STAV 2003, J. Engelsmann AG) was used to measure the tapped density. The settled volume was read after 500 (V_{500}) and 1250 taps (V_{1250}). Another 1250 taps were carried out when the difference between V_{500} and V_{1250} was greater than 2 mL. The tapped density (g/mL) was expressed as the quotient of m/V_{1250} or m/V_{2500}. Measurements were performed in triplicate and the mean value was calculated.

Carr compressibility index, CC % = [(tapped density – bulk density)/bulk density]*100 and Hausner ratio, HR = [tapped density/bulk density] were calculated from the bulk and tapped density results.

True density

The true density was determined using a Beckman air comparison pycnometer (model 930, Beckman Instruments) at room temperature. Atmospheric air was used as a comparison gas. An amount of powder representing 80% (V/V) of the sample cup was weighed exactly (model AE 200, Mettler Toledo GmbH) and the volume was measured. The starting number and the zero measurement were checked before measurements. The true density [g/mL] was calculated as the quotient of weighed substance to true volume. The mean value of three measurements was taken.

7.6.2.6   Powder flow properties

Angle of repose

Angle of repose was determined by pouring 150 ml of the dry extract preparation though a funnel of 10 mm pore size, adjusted at 75 mm height from the base, into 10 cm diameter plate placed below the tip of the funnel. The height (h) of the powder cone was measured with a dial high gauge (model No. 192-106, Mitutoyo Messgeraete GmbH).

The angle of repose (α) was calculated using the following equation:

$$\tan \alpha = h/r$$

The mean value of three measurements was taken.

Flow rate

The flow rate of the dry extract preparation was determined by pouring 100 gm of the extract preparation though a funnel of pore size 10 mm with closing end. The amount of the extract preparation passing per unit of time under gravitational force was recorded.

7.6.2.7   Compactibility study of the dry extract preparation

The dry extract preparation was compressed on an instrumented single punch tablet press Korsch EK II (Korsch Pressen,) using round flat tooling of 10 mm in diameter. Compaction pressure was measured by a full Wheatstone bridge circuit of strain gauges (type 6/120 LY 11, Hottinger Baldwin Messtechnik (HBM)) at the upper punch holder and by a piezo-electric load washer (type 9041, Kistler) mounted directly below the lower punch (Herzog 1991). For the measurement of residual and ejection forces, a camshaft switch was installed on the drive shaft of the tablet press. The camshaft switched on, as soon as the lower punch started to eject the tablet and allowed a precise measurement of the force in Newton range.

Data were acquired using the MGC Plus system including a ML 10 B voltage amplifier (HBM) and the Catman software. The filling height of the die was determined and the Heckel plots were calculated using the corrected upper punch displacement data. The slope of the linear compression part of each Heckel plot and the yield pressure (its reciprocal value) were determined using a stepwise linear regression from 300 data points, which gave the best fit for the linear part of the curve. The residual force was

measured by the lower punch sensor and the ejection force was determined as the highest value measured by the lower punch sensor (Dressler 2002).

The radial tensile strength (S) of the tablets was calculated from the thickness (H), diameter (D), and crushing strength (F_c) using the equation

$$S = 2F_c/\pi DH$$

The porosity of the tablets was calculated from the true density (ρ_t) and the apparent density (ρ_a) of the extract. The apparent density was calculated form the thickness out of die, weight and diameter of the tablets

$$\varepsilon = 1 - (\rho_a/\rho_t)$$

7.6.3 Stability of the saponins and flavonoids in the dry extract preparation

The stability of the saponins and flavonoids in the dry extract preparation was determined at 25 and 40° C for a period of 6 months. 10 gm of the dry extract preparation were filled and sealed in vials of 25 mL. The vials were stored in an oven at 25 and 40° C. One vial was taken every month from both ovens and the amount of saponins and flavonoids were analyzed according to the methods described under **7.5**. The phytochemical profiles of the dry extract preparation were compared with the corresponding TLC and HPLC-profiles of the freshly prepared extract.

7.7 Dry granulation by roller compaction, tablet preparation and enteric coating

7.7.1 Dry granulation by roller compaction

7.7.1.1 Mode of operation of Gerteis Micro-Pactor®

The dry extract preparation was granulated by roller compaction using Gerteis Micro-Pactor® (Gerteis AG) before tablet formulation. Micro-Pactor® is a small scale

compactor which allows predicting, if a given substance, or a blend can be granulated with the roller compaction technology. Minimum quantity (2 – 5 g) is required for the compaction study. The mode of operation of the machine is shown schematically in Figure 7.1.
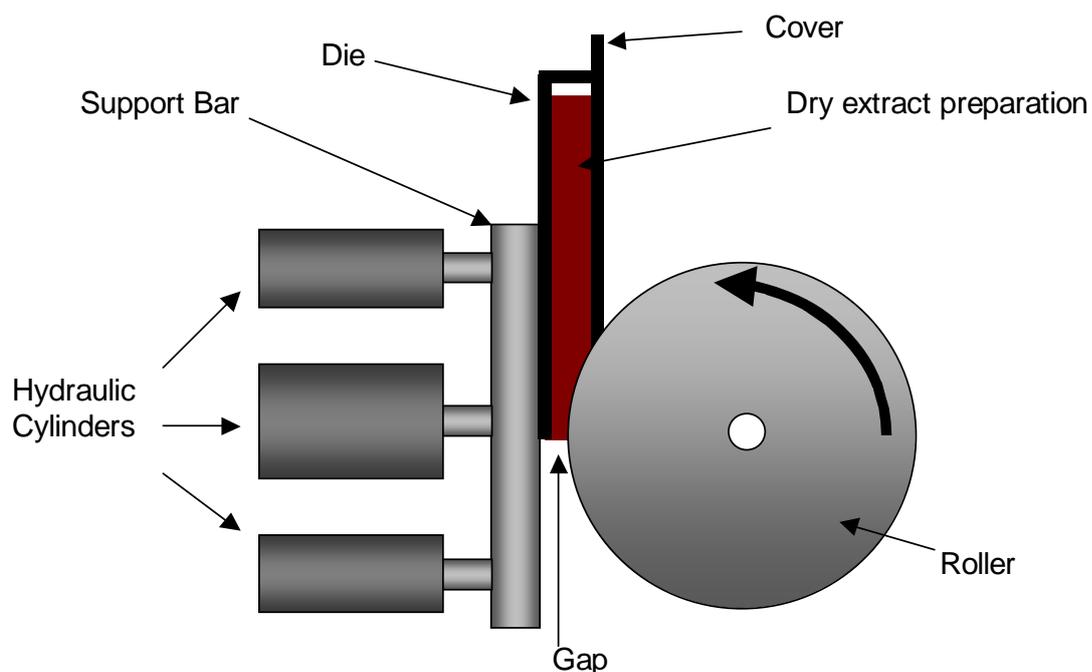
Figure 7.1: Mode of operation of Gerteis Micro-Pactor®

The filling depth, the roller speed, force and the gap of the machine is given in Table 7.11.

Table 7.11: Parameters of the Micro-Pactor®

Parameter	Minimum*	Maximum*	Normal*
Roller speed	0.001 m/s	0.1 m/s	0.05 m/s
Press force	2 kN	20 kN	10 kN
Hydraulic pressure	15 bar	160 bar	70 bar
Gap	0.5 mm	5 mm	2.5 mm
Filling depth	4 mm	8 mm	6 mm

*These values are given by the company.

7.7.1.2   Compaction with Gerteis Micro-Pactor®

Preparation of mixture for compaction

The dry extract preparation was mixed with magnesium stearate (2%) in Turbula mixer T2C (Bachofen AG) at 42 rpm for 5 min. Before mixing, the magnesium stearate was passed through a sieve size 315 μm on the dry extract preparation.

Compaction

The 8-mm die was filled with 7-8 g the mixture for compaction (dry extract preparation containing 2% magnesium stearate) at the horizontal position. The die with the cover was shifted into its guidance and the die was inserted into the machine (Figure 7.1). The compaction force and the roller speed were adjusted to 12 kN and 0.05 m/s, respectively. After having passed the roller, the die falls down and the roller stops automatically. The ribbon was taken out of the die and stored in desiccator over activated silica gel.

Preparation of granules

The ribbons were crushed into granules using a hand driven mill called '*Zahnscheibenmühle*' (BECO). The mill was previously adjusted to produce granules less than 1.000 mm. The granule size and distribution were analyzed by sieve analysis method.

7.7.2   Tablet preparation

Granules prepared from the dry extract preparation by roller compaction were formulated into tablets using an instrumented eccentric tablet machine Korsch EK0 (Korsch GmbH) fitted with oblong punches (20 x 8.25 mm). The lower punch holder was instrumented with strain gauges (3/120 Ly11, Hottinger Baldwin Meßtechnik). Signal transfer was achieved by a carrier frequency bridge (PR 9307, Philips GmbH).

Compression data were collected and analyzed using a program called 'messfix' (Herzog 1991). A calibration factor of 3.444 kN/V was used with a sensitivity of 2 mV/V.

7.7.2.1   Determination of the traditional dose

20 g of the powdered seeds were extracted with 500 ml of 5% EtOH by maceration for 24 hrs. The extract was filtered, concentrated in rota-vapor and dried to constant weight. The yield and the contents of saponins and flavonoids in the dry extract were determined by HPLC.

7.7.2.2   Tablet formulation

Direct compression excipients such as Avicel® PH101, Avicel® PH 200, Cellactose® 80 and disintegrants (Ac-Di-Sol®, Kollidon® CL and Explotab®) were investigated and tablet formulation was developed as shown in Table 7.12.

Table 7.12: Tablet formulation of the dry extract preparation

Substance	Mass in mg/tablet	Percentage
Dry extract preparation (granules)	947	67.64
Avicel® PH 101	363	25.93
Ac-Di-Sol®	90	6.43
Total weight	**1400 mg**	

7.7.2.3   Compression of tablets

The tablet mixture was weighed and mixed in a turbula mixer T2C (Bachofen AG) at 42 rpm for 5 min. Formulations were compressed into oblong tablets at various compression forces (13.5, 16.1, 18.4, 20.6, 23.7 and 27.9 kN) using an instrumented

eccentric tablet machine EK0 (Korsch GmbH). Batch sizes of 500 g were used. The compression force-tablet hardness and compression force-disintegration time profiles were evaluated. For formulation of enteric-coated tablets a compression force of 20 kN was used which provide tablets with the required strength (150-200 N) for coating. Due to the relatively complex geometric shapes of the oblong tablets, the compression pressure was not calculated.

7.7.2.4   Characterization of the tablet formulations

The following characteristics of the tablets were determined 24 hours after tablet compression.

Tablet mass

The tablet mass, as a parameter for weight uniformity, was determined from 10 randomly selected tablets. The tablets were weighed on an analytical balance (AE 200, Mettler Toledo GmbH) and the mean, standard deviation and relative standard deviation were calculated.

Crushing strength

The crushing strength of the tablets was determined according to the European Pharmacopoeia 5[th] Edition using a crushing strength tester TBH-30 (Erweka GmbH). The crushing strength was measured after 24 hrs to allow for completion of elastic deformation and particle rearrangement (consolidation). Tablets fragments were removed from the jaws before the next measurement. Mean, standard deviation and relative standard deviation were calculated.

Friability

The tablet friability was measured according to the European Pharmacopoeia 5[th] Edition. In this, 10 tablets were tested using a friability tester (Type PTF 1, Pharmatest

Apparatebau) for 4 minutes at 25 rpm. Loose dust particles were removed from the tablets with air pressure before and after the test. The tablet friability was calculated from the loss of mass and expressed as the percentage of the initial mass.

Disintegration time

The disintegration time of the tablets was determined with disintegration tester (Type PTZ 1, Pharmatest) according to the European Pharmacopoeia 5[th] Edition. In 1-liter beaker, the temperature of the distilled water was maintained at 36-38° C and the disintegration of 6 tablets was measured. The disintegration time was defined as the time required for the tablets to disintegrate and pass through the sieve openings.

7.7.3   Enteric coating of *G. lotoides* tablets

7.7.3.1   Enteric coating formulation

Tablets were enteric coated in a pan coater (15-L) with the coating suspension as shown in Table 7.13.

Table 7.13: Formulation of enteric coating suspension

Substance	Amount in g	Percentage	Percent in dry film*
Eudragit® L 100-55	125	8.33 %	55.5 %
1,2-Propylene glycol	25	1.67 %	11.1 %
i-PrOH	637.5	42.5 %	-
Acetone	637.5	42.5 %	-
Talc	42	2.8 %	18.7 %
Titan dioxide	10	0.67 %	4.4 %
Iron oxide (brown)	15	1.0 %	6.7 %
Magnesium stearate	8	0.53 %	3.6 %
Total	**1500g**	**100%**	

*Calculated from the total dry weight of the coating suspension.

Eudragit® L 100-55 (125 g) was dissolved in the mixture of the solvent system (*i*-propanol and acetone) and 1,2-propylene glycol (25 g) was dissolved in the polymer solution.

The insoluble ingredients (talc, titan dioxide, iron oxide and magnesium stearate) were mixed with *i*-propanol and grinded in a ball mill (Erweka GmbH) for 24 hrs. The required amount of the pigment suspension was then added to the polymer solution and stirred for 2 hrs before coating.

A batch of 1.5 kg tablets composed of *G. lotoides* and placebo core tablets (Table 7.14) was used. The cores were prewarmed to about 25 °C and the coating solution was sprayed on the core with a pressure of 1.2 bar using a two-substance nozzle (1.2 mm diameter, Schlick GmbH). The drying temperature was set at 40 °C and the 1.5 kg coating solution was sprayed within 90 min (Schiller 2002).

After coating the tablets were dried at 30 °C for 24 hrs in hot air oven (Memmert GmbH). The effects of plasticizers (propylene glycol and acetyl tributyl citrate) and lubricants (talc, glycerol monostearate with and without magnesium stearate) were evaluated. Two commercial products namely, Eudragit® L 100/55 (Röhm) and Kollicoat® MAE 100P (BASF) were compared.

Compression of placebo core tablets

Due to shortage of extract, Ludipress placebo core tablets were prepared using the formulation shown in Table 7.14. The placebo formulation was compressed into oblong tablets (19 x 7 mm) with one sided breaking notch which was used to identify the tablets after coating. Compression force of 8 kN was used after investigation of the

tablet hardness and friability. The tablets mass was adjusted to 800 mg which provide

tablets with less problem of sticking and twins formation during coating.

Table 7.14: Formulation of the placebo tablet cores

Substance	Mass in mg/tablet	Percentage
Ludipress	360	45 %
Avicel PH 101	352	44 %
Aeroperl® 300 Pharma	40	5 %
Ac-Di-Sol	40	5 %
Magnesium stearate	8	1 %
Total weight	**800 mg**	

7.7.3.2   Disintegration time of the enteric coated tablets

The disintegration time of the enteric coated tablets was determined with disintegration

tester (Type PTZ 1, Pharmatest) according to the European Pharmacopoeia 5th Edition.

Six coated tablets were first tested in 0.1 M HCl medium for 2 hours without disks.

Then the rigid rack of the apparatus was removed and the tablets are inspected for signs

of disintegration.

Subsequently, the test fluid was replaced with phosphate buffer solution pH 6.8 and a

loading disk was placed on top of each tablet in the tube. The apparatus was then started

and the disintegration time of the tablets was noted. The temperature of the fluids was

maintained at 36 to 38 °C.

7.7.3.3   Stability of the enteric coating

The enteric coated tablets were stored at 21° C in 45% rel. humidity and 26° C in 60% rel. humidity which approximate the various climate zones. Six enteric coated tablets were placed in each desiccator containing potassium carbonate and ammonium nitrate saturated salt solutions to create relative humidity of 45 and 60 %, respectively. The weight and thickness of the coated-tablets were measured accurately every 24 hrs for 15 days. The changes in weight were calculated and expressed as percentages.

Chapter 8

CONCLUSION

Suitable extraction methods for the seeds of *Glinus lotoides* have been developed and the major saponins and flavonoids have been isolated. In this, four new hopane-type saponins, glinusides F, G, H and K, and the known succulentoside B as well as the two known flavones, 5,7,4'-trihydroxyflavone-6,8-di-*C*-glucoside (vicenin-2) and 5,7,4'-trihydroxy-flavone-8-*C*-sophoroside (vitexin-2″-*O*-glucoside), have been isolated from the seeds of the plant. Based on the spectral analyses including 2D NMR and HRESI mass spectroscopy, the new structures have been characterized as 3β-*O*-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22-hydroxy-hopane (glinuside F), 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α,16β-dihydroxy-22-*O*-α-L-rhamnopyranosyl-hopane (glinuside G), 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-hydroxy-22-*O*-α-L-rhamnopyranosyl-hopane (glinuside H) and 3β-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-6α-*O*-β-D-xylopyranosyl-16β-*O*-β-D-xylopyranosyl-22-hopane (glinuside I).

The total flavonoids and saponins of the seeds of *G. lotoides* in the crude extracts and tablet formulations thereof have been quantified by reversed-phase high performance liquid chromatographic (RP-HPLC) methods with UV detection. The saponins were analyzed after acid hydrolysis in 3M HCl at 100°C for 1 hour. Vicenin-2 and mollugogenol B have been isolated and used as reference substances for the quantification of total flavonoids and saponins, respectively. The identities of vicenin-2 and mollugogenol B have been confirmed using UV, MS and NMR spectral analyses

and comparison with respective published data. The purity of the isolated mollugogenol B has been determined by TLC, HPLC and UV spectrophotometry as 98%, calculated from HPLC peak area. The molar absorptivity of the methanol solution of mollugogenol B at a concentration of 0.948 mg/100ml was found to be 3.98, 4.16 and 4.12 at λ_{max} of 260sh, 251 and 243 nm, respectively, which is in good agreement with literature values, indicating the purity of mollugogenol B. Similarly, the purity of vicenin-2 has been determined as 97% using HPLC, which is confirmed by TLC and UV methods.

Satisfactory separation of the components of the flavonoids and saponins compounds has been obtained in less than 30 and 25 min, for the flavonoids and saponins, respectively. The HPLC methods were validated for linearity, repeatability, limits of detection (LOD) and quantification (LOQ). Repeatability (inter- and intra-day, n = 6 and 9, respectively) showed less than 2% relative standard deviation (RSD). The LOD and LOQ were found to be 0.075 and 0.225 mg/mL, respectively, for vicenin-2 and 0.027 and 0.082 mg/100mL, respectively, for mollugogenol B.

The total amounts of saponins of the crude extract and tablet formulation have been determined as glinuside G equivalent (mol. Wt. 900) which approximates the average molecular weight of the saponins of *G. lotoides*.

The content of flavonoids and saponins of six single tablets has been found to be between 95 and 103% for flavonoids and 94 to 98% for saponins. The validated HPLC methods could be employed to standardize a laboratory produced purified extract, which could be used as a secondary standard for the routine quality control. Accordingly, the secondary standard (extract F) has been found to contain 10% vicenin-2 and 21.3% total flavonoids calculated as vicenin-2 equivalent. The amount of saponins in the secondary standard was 6.3% mollugogenol B and 12.4% total aglycons

calculated as mollugogenol B, 14.2% glinuside G and 25.4% total saponins calculated as glinuside G equivalent.

Admixing of the crude liquid extract of the seeds of *G. lotoides* with Aeroperl® 300 Pharma, as an inert carrier material, and subsequent drying the mixture has provided a non-adherent and free-flowing powder. The required amount of Aeroperl® 300 Pharma has been optimized as 30%, based on the physicochemical properties such as particle size distribution, surface morphology, moisture content and sorption kinetics of the dry extract preparations containing 10, 20, 30, 40, 50 and 60% w/w Aeroperl® 300 Pharma.

A tablet formulation containing granules of the dry extract preparation (947 mg), Avicel® PH101 (363 mg) and Ac-Di-sol (90 mg) provided the best tablet properties such as disintegration time of 2.4 min at a tablet hardness of 73 N. Granulation by roller compaction of the dry extract preparation has improved the disintegration time of the tablets. Oblong tablets which could administer the traditional dose as a single tablet have been developed and enteric coated.

Enteric coated tablets of *G. lotoides* with pharmacopoeial requirements could be prepared using methacrylic acid/ ethylacrylate co-polymer (Eudragit® L 100/55 or Kollicoat® MAE 100P). Propylene glycol and acetyl tributyl citrate could be used as plasticizers at concentrations of about 11% in the dry film.

Chapter 9

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Chapter 10

APPENDIX

10.1 Calibration and validation data of the quantitative determination of the saponins of *Glinus lotoides* by HPLC

Table 10.1: Calibration data of Mollugogenol B.

	Conc. Mollugogenol B [mg/100ml]	Peak area	Conc. Mollugogenol B [mg/100ml]	Peak area
	0.237	88421	0.474	180087
	0.237	89899	0.474	177247
	0.237	88274	0.474	180064
	0.237	90616	0.474	177044
	0.237	89459	0.474	177615
	0.237	88399	0.474	176714
Average		89178		178129
SD		965		1536
RSD		1.08		0.86
	0.948	358008	1.422	540262
	0.948	350665	1.422	528796
	0.948	352901	1.422	535757
	0.948	361214	1.422	533127
	0.948	358598	1.422	534223
	0.948	358630	1.422	533563
Average		356669		534288
SD		4006		3738
RSD		1.12		0.70
	1.896	710120	2.37	900534
	1.896	716780	2.37	896353
	1.896	707003	2.37	887221
	1.896	711949	2.37	898923
	1.896	713132	2.37	881455
	1.896	715095	2.37	900912
Average		712347		894233
SD		3507		8040
RSD		0.49		0.90

Table 10.2: Calibration data of Extract F as secondary standard for determination of the

saponins of *G. lotoides* after acid hydrolysis as Mollugogenol B.

	Conc. Extract F [mg/100ml]	Peak area Mollugogenol B	Conc. Extract F [mg/100ml]	Peak area Mollugogenol B
	4.95	127929	9.95	262614
	4.95	129389	9.95	259217
	4.95	127147	9.95	258811
	4.95	129595	9.95	258247
	4.95	129836	9.95	256981
	4.95	126174	9.95	258240
Average		128345		259018
SD		1496		1916
RSD		1.17		0.74
	19.81	515730	30.13	784497
	19.81	519452	30.13	790157
	19.81	519455	30.13	790585
	19.81	516402	30.13	787807
	19.81	518091	30.13	775957
	19.81	514365	30.13	782428
Average		517249		785239
SD		2086		5549
RSD		0.40		0.71
	40.05	1044109	49.98	1303034
	40.05	1048680	49.98	1301862
	40.05	1048774	49.98	1315613
	40.05	1041979	49.98	1306789
	40.05	1043566	49.98	1302771
	40.05	1039244	49.98	1300780
Average		1044392		1305142
SD		3759		5517
RSD		0.36		0.42

Table 10.3: Determination of the method precision of the acid hydrolysis of Extract F.

	Conc. Extract F [mg/100ml]	Peak area Mollugogenol B	Conc. Extract F [mg/100ml]	Peak area Mollugogenol B
	24.62	637096	27.42	724586
	24.62	637793	27.42	713016
	24.62	639076	27.42	714585
Average		637988		717396
SD		1004		6276
RSD		0.16		0.87
	24.37	620266	23.41	580293
	24.37	622698	23.41	581559
	24.37	623025	23.41	584370
Average		621996		582074
SD		1507		2086
RSD		0.24		0.36
	25.93	685862	28.31	736512
	25.93	683032	28.31	752337
	25.93	679649	28.31	739143
Average		682848		742664
SD		3110		8479
RSD		0.46		1.14

Table 10.4: Data for the determination of system precision of the HPLC method.

	Conc. Extract F [mg/100ml]	Peak area Mollugogenol B	Conc. Extract F [mg/100ml]	Peak area Mollugogenol B
	25.93	685704	25.93	678448
	25.93	682848	25.93	682231
	25.93	683717	25.93	683533
	25.93	682936	25.93	679044
	25.93	675383		
Average	681678.7			
SD	3405			
RSD	0.500			

Table 10.5: Data for the determination of saponins in 5% EtOH extract (extract to

determine the traditional dose).

	Conc. 5% EtOH extract [mg/100ml]	Peak area Mollugogenol B
	104.26	843856
	104.26	842817
	104.26	859941
Average		848871.3
SD		9600.7
RSD		1.13

10.2 Calibration and validation data of the quantitative determination of the flavonoids of *Glinus lotoides* by HPLC

Table 10.6: Calibration data of vicenin-2.

Conc. vicenin-2 [mg/100ml]	Peak area	Conc. vicenin-2 [mg/100ml]	Peak area
0.583	152072	1.166	298923
0.583	150148	1.166	291526
0.583	149874	1.166	292675
0.583	149604	1.166	307117
0.583	148986	1.166	295563
0.583	153011	1.166	297144
Average	150616		297158
SD	1568		5597
RSD	1.04		1.88
2.332	601820	3.498	894170
2.332	590096	3.498	911971
2.332	588314	3.498	901377
2.332	601289	3.498	903740
2.332	593381	3.498	923637
2.332	588699	3.498	892774
Average	593933		904612
SD	6169		11631
RSD	1.04		1.29
4.664	1204642	5.83	1501111
4.664	1209573	5.83	1509448
4.664	1211650	5.83	1514393
4.664	1202719	5.83	1508848
4.664	1217450	5.83	1517291
4.664	1225890	5.83	1504927
Average	1211987		1509336
SD	8589		5936
RSD	0.71		0.39

Table 10.7: Calibration data of Extract F as secondary standard for determination of the

flavonoids of *G. lotoides* as vicenin-2.

	Conc. Extract F [mg/100ml]	Peak area Vicenin-2	Conc. Extract F [mg/100ml]	Peak area Vicenin-2
	4.96	131487	11.88	321198
	4.96	131078	11.88	318711
	4.96	131071	11.88	318127
	4.96	128474	11.88	318101
	4.96	129827	11.88	318333
	4.96	127980	11.88	312101
Average		129986		317762
SD		1238		3011
RSD		0.95		0.95
	20.34	549680	30.39	801647
	20.34	546577	30.39	798251
	20.34	545047	30.39	801146
	20.34	546762	30.39	798879
	20.34	540739	30.39	797558
	20.34	544989	30.39	810210
Average		545632		801282
SD		2941		4662
RSD		0.54		0.58
	40.90	1078782	49.49	1285794
	40.90	1084506	49.49	1294153
	40.90	1078577	49.49	1289314
	40.90	1085842	49.49	1296740
	40.90	1075539	49.49	1289559
	40.90	1079402	49.49	1290445
Average		1080441		1291001
SD		3925		3879
RSD		0.36		0.30

Table 10.8: Data for the determination of the recovery of vicenin-2 in Extract F

Theoretical Conc. [mg/100ml]	16.4	24.6	32.8	41.0	49.2
Theoretical percentage [%]	50	75	100	125	150
Peak area of vicenin-2	422640	639321	853484	1101796	1304771
	438440	647942	852612	1086134	1272433
	432127	645038	853213	1096232	1292048
Average	431069.0	644100.3	853103.0	1094720.7	1289750.7
RSD [%]	1.85	0.68	0.05	0.73	1.26
Calculated Conc. [mg/100ml]	16.2	24.4	32.4	41.67	49.1
Recovery	99.0	99.2	98.8	101.6	99.9

Table 10.9: Data for the determination of the method precision.

	Conc. Extract F [mg/100ml]	Peak area Vicenin-2	Conc. Extract F [mg/100ml]	Peak area Vicenin-2
	30.42	825473	30.39	801647
	30.42	822083	30.39	798879
	30.42	829311	30.39	810210
Average		825622		803579
SD		3616		5907
RSD		0.44		0.74
	33.60	872794	32.43	872374
	33.60	889188	32.43	888158
	33.60	884407	32.43	891475
Average		882130		884002
SD		8430		10206
RSD		0.96		1.15
	31.26	832567	32.85	857985
	31.26	825468	32.85	875648
	31.26	849568	32.85	885264
Average		835868		872966
SD		12384		13835
RSD		1.48		1.58

Table 10.10: Data for the determination of system precision of the HPLC method.

Conc. Extract F [mg/100ml]	Peak area Vicenin-2	Conc. Extract F [mg/100ml]	Peak area Vicenin-2
33.51	875463	33.51	900913
33.51	905638	33.51	878016
33.51	884002	33.51	879046
33.51	877170	33.51	915387
33.51	901804		
Average	890325.5		
SD	16529		
RSD	1.86		

Table 10.11: Data for the determination of the amount of vicenin-2 and total flavonoids

in the 5% EtOH extract (extract to determine the traditional dose).

Conc. 5% EtOH extract [mg/100ml]	Peak area Vicenin-2	Conc. 5% EtOH extract [mg/100ml]	Peak area Vicenin-2
10.54	89015	39.79	344799
10.54	89937	39.79	352468
		39.79	354085
Average	89476		350450.7
SD	652		4960.8
RSD	0.73		1.42
60.20	547895	89.25	804698
60.20	534986	89.25	797965
60.20	532469	89.25	791072
Average	538450		797911.7
SD	8275.9		6813.2
RSD	1.54		0.85

10.3 List of suppliers

Amersham Biosciences AB, SE 751 84, Uppsala

Willy A. Bachofen AG, Utengasse 15-17, CH-4002 Basel

BASF AG, Carl-Bosch-Str. 38, D-68056 Ludwigshafen

Bärlocher GmbH, Riesstr. 16, D-80992 München

Beckman Instruments Inc., Frankfurter Ring 115, D-80807 München

Binder GmbH, Bergstr. 14, D-78532 Tuttlingen

Büchi Labortechnik GmbH, Am Porscheplatz 5, D-45127 Essen

Degussa AG, Bennigsenplatz 1, D-40474 Düsseldorf

Deutsche Metrohm GmbH & Co., In den Birken 3, D-70794 Filderstadt

Drawin Vertriebs-GmbH, Rudolf-Diesel-Str. 15, D-85521 Ottobrunn

Engelsmann AG, Frankenthaler Str. 137-141, D-67059 Ludwigshafen

Erweka GmbH, Ottostr. 20-22, D-63150 Heusenstamm

Extrasynthese, B.P. 62, F-69730 Genay, Frankreich

FINNA-AQUA Santasalo-Sohlberg GmbH, Kalscheurener Str. 92, D-50354 Hürth

Fischer Scientific GmbH, Liebigstr. 16, D-61130 Niederau

Fluka Chemie AG, Industriestr. 25, CH-9470 Buchs, Schweiz

FMC Biopolymer, Wallingston, Little Island, IRL-Cork

Gerteis Maschinen + Processengineering AG, Stampfstr. 74, CH-8645 Jona, Schweiz

Henkel KGaA, Postfach 10 1100, D-40002 Düsseldorf

Heraeus Holding GmbH, Haraeusstr. 12-14, D-63450 Hanau

Heraeus Instruments GmbH, Postfach 1561, D-63450 Hanau

Hottinger-Baldwin Messgeraete GmbH, Im Tiefen See 45, D-64293 Darnstadt

IKA-Werk GmbH & Co., Janke & Kunkel Str. 10, D-79219 Staufen

J. T. Baker, H.-S.-Richardson Str. 1, D-64521 Groß-Gerau

Jungbunzlauer GmbH, Dr.-Albert-Reimann-Str. 18, D-68526 Ladenburg

Kistler Instruments GmbH, Eulachstr. 22, CH-8408 Winterthur

Korsch Pressen GmbH, Breitenbachstr. 1, D-13509 Berlin

Kronos Titan GmbH & Co. OHG, Postfach 10 07 20, D-51307 Leverkusen

Krüss GmbH, Borsteller Chaussee 85-99a, D-2000, Hamburg

LKB-Produkter AB, S-161 26, Bromma, Sweden

Macherey-Nagel GmbH, Valencienner Str. 11, D-52355 Düren

Malvern Instruments GmbH, Rigipsstr. 19, D-71083 Herrenberg

Meggle GmbH, Megglestr. 6-12, D-83512 Wasserburg

Memmert GmbH & Co. KG, Äußere Rittersbacher Str. 38, D-91126 Schwabach

Mendell GmbH, Röpckes Mühle 2, D-25436 Uetersen

Merck KGaA, Frankfurter Str. 250, D-64293 Darmstadt

Messer Griesheim GmbH, Füttingsweg 34, D-47805 Krefeld

Mettler Toledo GmbH, Ockerweg 3, D-35396 Gießen

Microsoft Deutschland GmbH, Konrad-Zuse-Str. 1, D-85716 Unterschleißheim

Mitutoyo Messgeraete GmbH, Borisigstr. 8-10, D-41469 Neuss

Norwegian Talc Deutschland GmbH, Rückmühlenweg 1, D-63628 Bad Sonden-Salmünster

Pall Seitschenk, Filtersystems GmbH, Planiger-Str. 137, D-55543 Bad Kreuznach

Perkin-Elmer GmbH, Postfach 10 11, D-88662 Überlingen

Pharmatest Apparatebau, Postrfach 11 -50, D-63512 Hainburg

Rheodyne LLC, 600 Park Court Rohnert Park, CA 94928 USA

Riedel-de Haën, Wunstorfer Str. 40, D-30926 Seelze

Röhm GmbH, Kirschenallee, D-64293 Darmstadt

Sartorius AG, Weender Landstr. 94-108, D-37075 Göttingen

Düsen-Schlick GmbH, Hutstr. 4, D-96253 Untersiemau/Coburg

Seitz-Werke GmbH, Bad Kreuznach, Rheinland

Shimadzu Europa GmbH, Albert-Hahn-Str. 6-10, D-47269 Düisburg

Wagner & Munz GmbH, In der Rosenau 4, D-81829 München

Waters GmbH, Hauptstr. 87, D-65760 Eschborn

Yashica-Kyocera GmbH, Eifflerstr. 76, D-20537 Hamburg

Carl-Zeiss GmbH, Carl-Zeiss-Str., D-72447, Aalen-Oberkochen

Meine akademischen Lehrer waren die Herren:

Albert,

Gebre-Mariam,

Schmidt,

Wahl,

Zeller

LEBENSLAUF

21. Juli 1971	Geboren in Addis Abeba, Äthiopien
1977 -1984	Grundschule, Addis Abeba, Äthiopien
1984 - 1988	*High School* (Gymnasium), Addis Abeba, Äthiopien
Juli 1988	*ESLCE, Ethiopian School Leaving Certificate Examination*
Sept. 1988 - Nov. 1993	*Bachelor of Pharmacy (B. Pharm.), School of Pharmacy*, Addis Abeba Universität, Addis Abeba, Äthiopien.
06. Mai 1999	Heirat mit Chora Bogale
Kinder	Ananiya Abebe Endale (geb. 04. Nov. 1999)
	Azariya Abebe Endale (geb. 15. März 2002)
Sept. 1998 - Juli 2000	*Master of Science (M.Sc) in Pharmaceutics*, School of Pharmacy, Addis Abeba Universität, Addis Abeba, Äthiopien.
Okt. 1999 – März 2000	Forschungsaufenthalt im Rahmen der Master-Arbeit am Lehrstuhl für Pharmazeutische Technologie der Universität Tübingen unter der Leitung von Herrn Prof. Dr. P. C. Schmidt
Okt. 2001 – März 2002	6-monatiger Deutsch-Sprachkurs am Goethe-Institut in Freiburg Abschluss: DSH
April 2002	Beginn der Dissertation „Isolierung, Strukturaufklärung, Quantifizierung und Formulierung der Saponine und Flavonoide der Samen aus *Glinus lotoides*" am Lehrstuhl für Pharmazeutische Technologie der Universität Tübingen unter der Leitung von Herrn Prof. Dr. P. C. Schmidt