ADDIS ABABA UNIVERSITY

SCIENCE FACULTY

DEPARTMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION ON THE STEMS OF *RHAMNUS PRINOIDES*

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JUNE, 2010

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Table of contents

List of schemes

Pages

List of Figures

Pages

List of Tables

Pages

List of appendices

Acknowledgements

I wish to express my profound gratitude to my advisor Dr. Gizachew Alemayehu for his conception of the problem and his courteous approach, advice and encouragement through out the course of this work.

I would like to convey my sincere thanks to W/t Senait Dagne and Ato Yosef Atilaw for their encouragement and technical support.

1. Introduction

1.1 General

Natural products have been in use since ancient times as medicines insecticides, natural dyes and spices, and the use of herbal remedies and dietary supplements (Cowan MM. 1999). It was estimated that about 80% of all the world medicines are originally derived from plant sources, especially those found in tropical regions. However, many of the plants with in these often remote regions of the world have yet to be identified as species and only about 15% of the known angiosperm species in this region were examined for their medicinal potential. Therefore, there are most definitely a large number of plants derived medicines and other useful compounds that have to be discovered and characterized around the world (Cseke, H., et al., 2006).

Therefore, plant secondary metabolites are currently the subject of much research interest but their extraction as part of phytochemical or biological investigations presents specific challenges that must be addressed through out the solvent extraction process. Successful extraction begins with careful selection and preparation of plant samples, and thorough review of the appropriate literature for indications of which protocols are suitable for particular classes of compounds or plant species. During extraction of plant materials it is important to minimize interference from compounds that may be co-extracted with the target compounds, and to avoid contamination of the extract, as well as to prevent decomposition of important metabolites or artifact formation as a result of extraction conditions or solvent impurities (William, P., et al.).

1.2 The Genus Rhamnus

Taxa belonging to the genus Rhamnus are found in all tropical, subtropical and temperate regions. There are above 150 taxa in this genus and only two, namely, *R. prinoides* and *R. staddo* occur in Ethiopia. *R. staddo*, like *R.*

prinoides is used to make the traditional drinks of 'Tej' and 'Tella' but it is not recommended for 'Tella' although it is some times used. One general feature of the genus is; it contains anthraquinones and some flavonoid glycosides, in addition to a variety of anthrones and their dimeric derivatives (Kebede, T. 1994).

1.3 Rhamnus prinoides

R. Prinoides L'herit, Amharic name 'Gesho', family Rhamnaceae order Rhamnales, is a dicotyledonous angiosperm plant cultivated in Ethiopia. It is a shrub or tree which grows up to 6 meters and is also known to occur in Cameron, Sudan, through out [east Africa to South Africa and Angola and in Arabia (Thulin, R. 1988)]. In Ethiopia the plant is used to add flavor to the fermented drinks 'Tella' and 'Tej'.

One of the early scientific reports on it is that of Salgue (1962) and Coady (1965). Salgue described the presence of inorganic cations, organic acids, and the flavonanoid derivative rhamnetin rhamonside. He also made some toxicological studies of the plant tissues (Salgue, R., 1962).

The fungicidal effect of the extracts of *R. prinoides* fruits were investigated (Biftu, et al., 1979) and the minimum concentration responsible for this effect was reported. In a study made invivo, anti-malarial activity of aqueous extracts from leaves and root barks of *R. prinoides* and their chloroquine (CQ) potential effects against a blood-induced CQ-resistant rodent parasite in mice showed high chemosuppression in the range 51% -75% (Muregi F. et al.,2007).

1.4 Bitter Sources in 'Tella' and 'Tej'

It is assumed that *R. prinoides* maintains acidic pH during 'Tella' fermentation so as to modify the nature of mesh and inhibits the growth of undesirable micro-organisms (Kleyh, et al., 1971).

R. prinoides imparts a bitter taste to 'Tella' and 'Tej' and the bitterness of 'Tella' is directly related to the amount of *R. Prinoides* added during brewing (Sahle

and Abegaz, 1991). In another study made in order to identify the specific compound which possesses bitter properties and rendering bitter taste to 'Tella' and 'Tej', it was found that β-sorigenin-8-O-β–D-glucoside which is named as geshoidin is the one with these properties (Abegaz, B., and Kebede, T. 1995). It was also reported that the above compound is found in greater proportion with in the leaves of *R. prinoides* relative to the identified secondary metabolites.

1.5 More on Goshoidin

β-Sorigenin-8-0-β-D-glucoside which is named as geshoidin were isolated and characterized from the leaves of *R. prinoides* [Abegaz, B et al.,1995) . Organoleptic evaluation of geshoidin revealed its bitter flavor irrespective of its glucosidal nature. Quantitatively geshoidin is the major secondary metabolite of the leaves of *R. prinoides.* Both its bitter flavor and its occurrence as a major metabolite of the leaves indicates that geshoidin is an indispensable ingredient which imparts the characteristic bitterness of 'Tella' and 'Tej'.

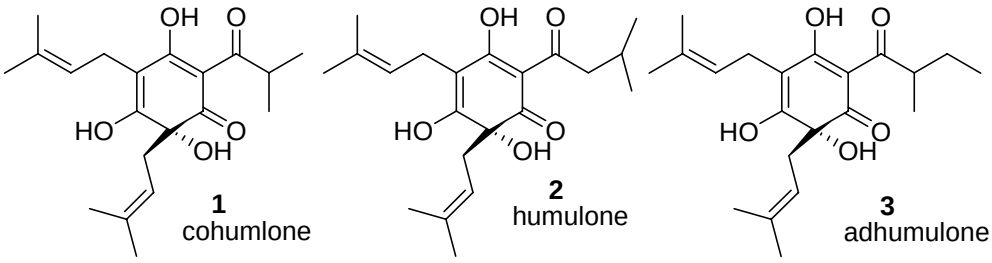
In research done on geshoidin for the purpose of assessing any risks or benefits it has on humanbeings, it was investigated as potential inhibitor and in activator of human recombinant glutathione transferase (GSTs) which are multifunctional enzymes that catalyze conjugation of a wide variety of electrophilic endogenous and exogenous compounds with the tripetide glutathione (GSH). The study has shown that geshoidin inhibit human recombinant GSTs. This observation may be of importance indicating that geshoidin has some anticancer potential due to its various biological activities which maximized its benefits (Hayeshi, R. et al, 2004).

1.6 Comparing the role of hops in beer and 'Gesho' in 'Tella' and 'Tej'

Hops or hop products account for the bitter taste and the flavor of beer. In addition hops have a favorable influence on the stability of beer foam [Cooman et al., 1998) and contribute to the microbiological stability of beer [Miszobuchi and sato, 1985). The common ingredient of the hop products which account for

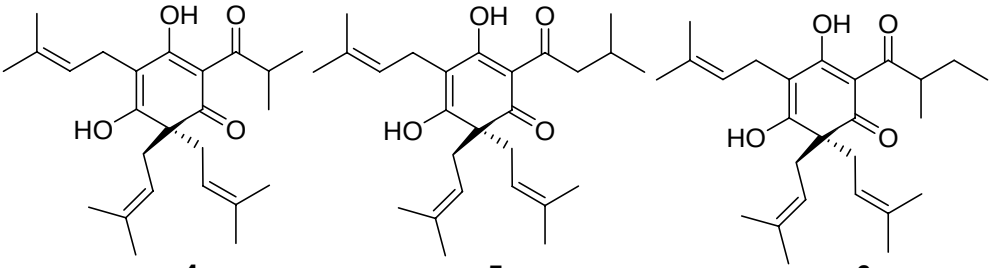
the bitter taste of beer are α and β-hop acids. The same ingredients are also responsible for microbiological stability of beer. The flavonoids of the hop, quercetin and kaempferol, render beer its characteristics color and flavor. And essential oils of the hop have a favorable influence on the stability of beer foam. The main ingredients of the Hop products (*Humulus lupulus L.*) have been summarized below.

A) Hop α-acids



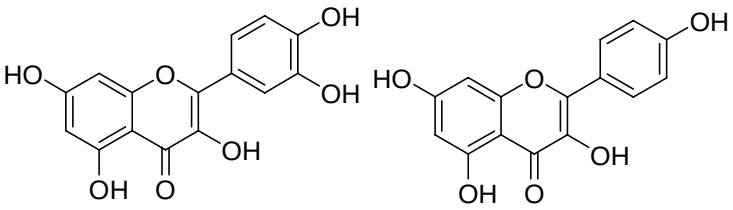
1 cohumlone **2** humulone **3** adhumulone

B) Hop β-acids



4 colupulone **5** lupulone **6** adhupulone

C) Hop flavonoids



7 quercetin **8** kaempferol

In the same way *R. prinodes* ('Gesho') has been used as a bittering agent, in the regulation of microbial flora and in coloring and flavoring of Tella and

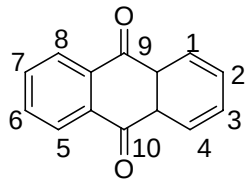
Tej (Sahle& Abegaz 1991) . Recently a naphthalenic glucoside named geshoidin has been identified as one of the ingredients which is responsible for the bitter attribute of the plant in Tella and Tej (Abegaz & Kebede,1995). Other secondary metabolites in particular anthraquinone and flavonoids may have crucial role for microbactieral action and for the characteristics color of Tella and Tej.

From the above it seems that *R. prinodes* can serve the same purpose as hops serve in beer except lacking components which have a favorable influence on foam stability. Of course this idea has received positive assessment as commercial hopping agent for beer [Tessema, 1994).

1.7 Anthraquinones of Rhamnaceae

Anthraquinone derivatives physcion, emodin, emodinathrone, biantrone and prinoidin were among the isolated secondary metabolite, of *R. prinoides*.

The largest group of natural quinone is made up of the anthraquionne. The fundamental anthraquinone structure with the ring-numbering system is shown in figure 1.



9

Fig1. The carbon skeleton of anthraquinones

Rhamnaceae is one of the plant families that are known to contain anthraquinones. Four of the 58 genera of Rhamnaceae are certainly known to contain anthraquinones. Genus *Rhamnus* is the best studied genus of the Rhamnaceae. The well known purgative extracts of *R. frangula, R. purshina* and *R. catharticus* contain anthraquinone derivatives as their active constituents. A unique feature of the genus *Rhamnus* is probably its tendency to elaborate anthraquinone glycosides. Nevertheless, a variety of anthrones,

anthraquinones and bianthrones have been isolated and characterized in more than 15 *Rhamnus* species (Mammo, W. 1989).

1.7.1 **Identification of anthraquinones**

Anthraquinones involve conjugated double bonds in their structures which enable them to absorb UV and visible light. This property serves as a fundamental principle to develop identification methods. Phenol functional group is another common characteristic of anthraquinone. In basic and acidic media they exhibit different colors since on deprotonation the alkoxy part participates in conjugation.

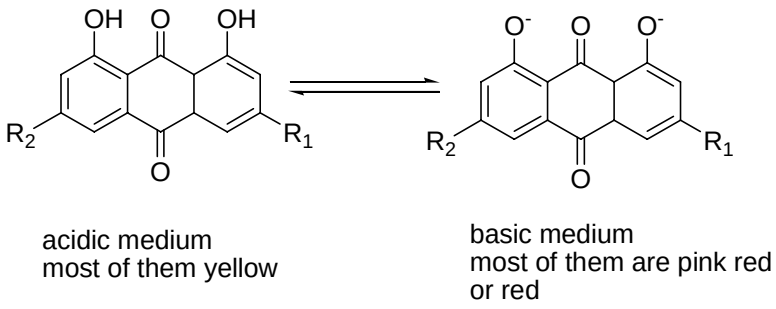


Fig.2 Anthraquinones in acidic and basic medium

1.7.2 Spectroscopy Method

Spectral measurements are essential for identification of quinone structures. The UV and visible spectra indicate the class of quinone present, since the number and position of bands increase with complexity of structure.

The spectra of benzoquinones characteristically have one strong band between 260 and 290 nm and another band of less intensity between 375 and 410 nm.

All naphthaquinones have three or four spectral maxima; one or two below 300nm and other two at 330 to 340 nm and above 400 nm.

Anthraquinones can be distinguished from other classes of quinones by the fact that they have four or five absorption bands in the UV and visible regions. At lest three of these lie between 215 and 300 nm and other one, lie above 430

nm. The pattern in the UV- region is not strongly affected by substitution. On the other hand, absorption in the visible region is influenced by the number of alpha hydroxyl groups. The influence of beta hydroxyl is much weaker except when adjacent to an alpha hydroxyl. Additional alpha hydroxyl results in bathochromic shift of the longer wave absorption (Kebede, T. 1994).

IR spectra

The carbonyl stretching vibration frequencies are useful aids in structural determination of anthraquinones. Examining the various functional groups stretching and bending vibrations of anthraquinones in IR spectrum region is another source of vital information to identify antraquinones. Finally the NMR spectra of each antraquinones serve as its finger print to fully characterize its structure (Kebede, T. 1994).

1.7.3 Spray reagents

As general test, anthraquinones show color change when the medium is changed from acid to base. Therefore, by spraying TLC plates with 10% methanolic KOH solution, the original yellow and yellow brown colors of anthraquinones on chromatography plates change to red, green or purple. The reaction can be done on crude extracts, purified materials or chromatograms (Kebede, T. 1994).

1.7.4 The biosynthesis of anthraquinones

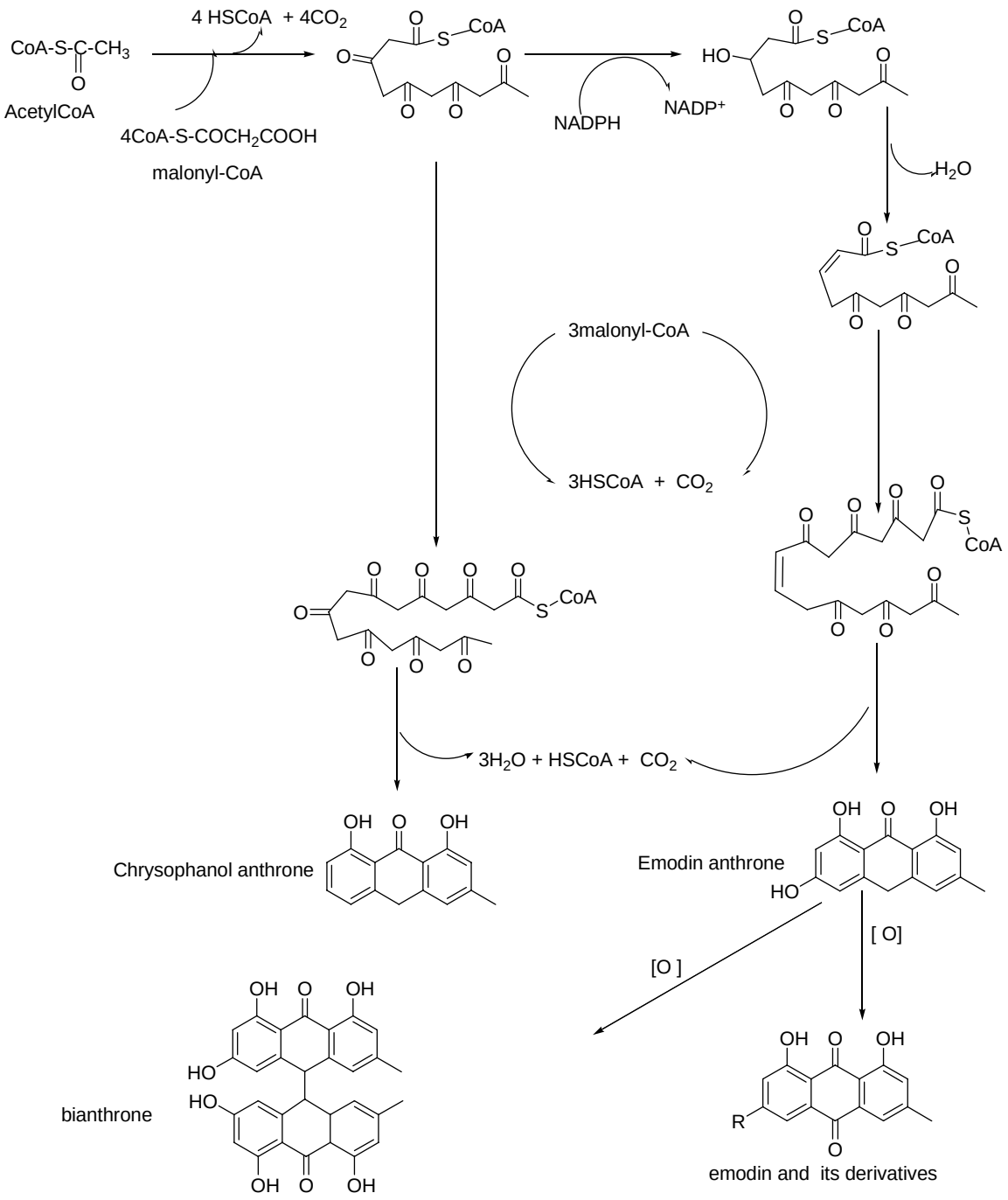
The biosynthesis of anthraquinones in Rhamnaceae appears to proceed via the acetate malanote pathway. The majority of the anthraquinones isolated from the Rhamnaceae are of the emodin type and they appear to arise by suitable folding and condensation of polyketide chain derived from eight acetate units. Polyketides are naturally occurring compounds that contain multiple ketone groups. Plants and marine invertebrates produce three diverse groups of

polyketides (polycyclic aromatics, macrolides, and polyethers (Torsell, K., 1977)).

Even though polyketides are chemically diverse, the mechanism by which they are synthesized is one of the most widespread routes used in nature. Using enzymes called polyketide synthases (pKSs), polyketides are produced in the cytosol via the acetate pathway through the condensation of a starter (usually acetyl CoA) and extensor molecules (usually malonyl CoA), resulting in chain with carbonyl groups present. These enzymes catalyze the intital stages in polyketide formation. Other enzymes can modify the polketide to produce an array of chemical diversity (Hophwood 1997, Shen, 2000).

Structural variations of this basic structure may arise from O-methylation, side chain oxidation, dimerization and the introduction or elimination of nuclear hydroxyl groups (Torsell, G.1997).

The isolation of rhamnalpinoside and its aglycone from *R. fallax* demonstrated a second mode of Cyclization of an octaketide in Rhamnaceae (Mammo, W. 1989). Scheme 1 on the next page shows proposed biosynthetic pathway of anthraquinones.



Scheme1. Proposed pathway for biosynthesis of anthraquinones (Torsell, 1997)

1.7.5 The use of anthraquinones

Anthraquinones have been used as mordant dyes (Alizarin from *Rubia tinctorum*) and purgatives (emodin from *Rheum, Rhumex* or *Rhamnus spp*) (Torsell, K., **1997**). However, they lost their importance like so many other

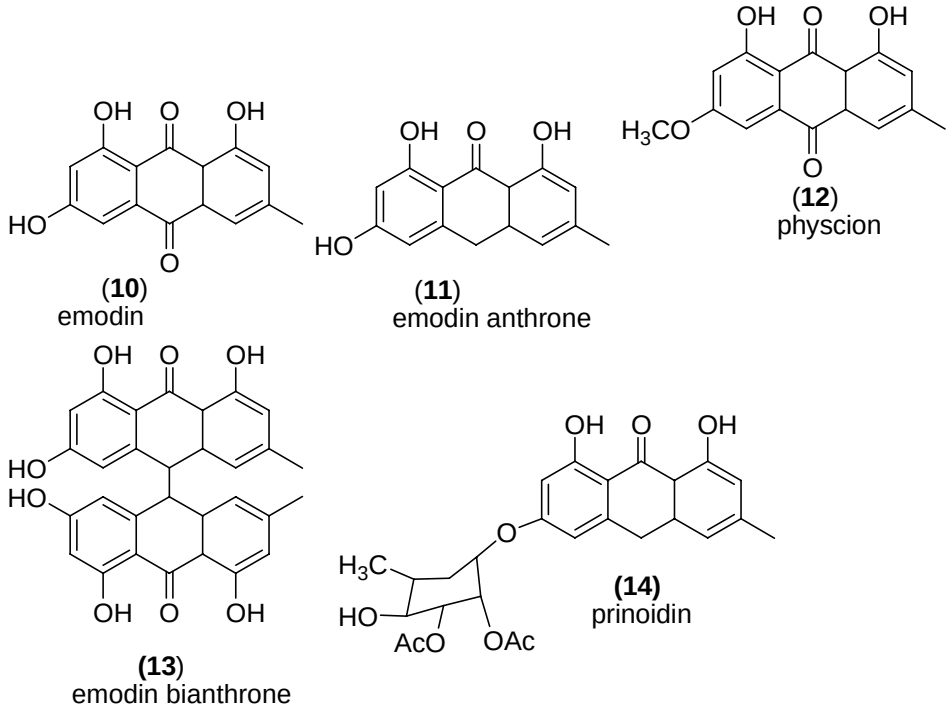
natural dyes, with the development of the synthetic industry. Anthraquinones, long known for their effects on the bowel, appear to have other activities as well. Anthraquinones from the roots of *Daylies, Hemorocallis fulva* var.' kwanzo' were isolated and tested as cancer cell growth inhibitors.

They inhibited the proliferation of human breast, central nervous system, colon, and lung cancer cells with GI_{50} values between 1.8 and 21.1 $\mu gmL^{-1.}$

Co incubating a combination of the anthraquinones with vitamin C and E demonstrated synergic anticancer activities (Cichewcx, R., et al, 2004).

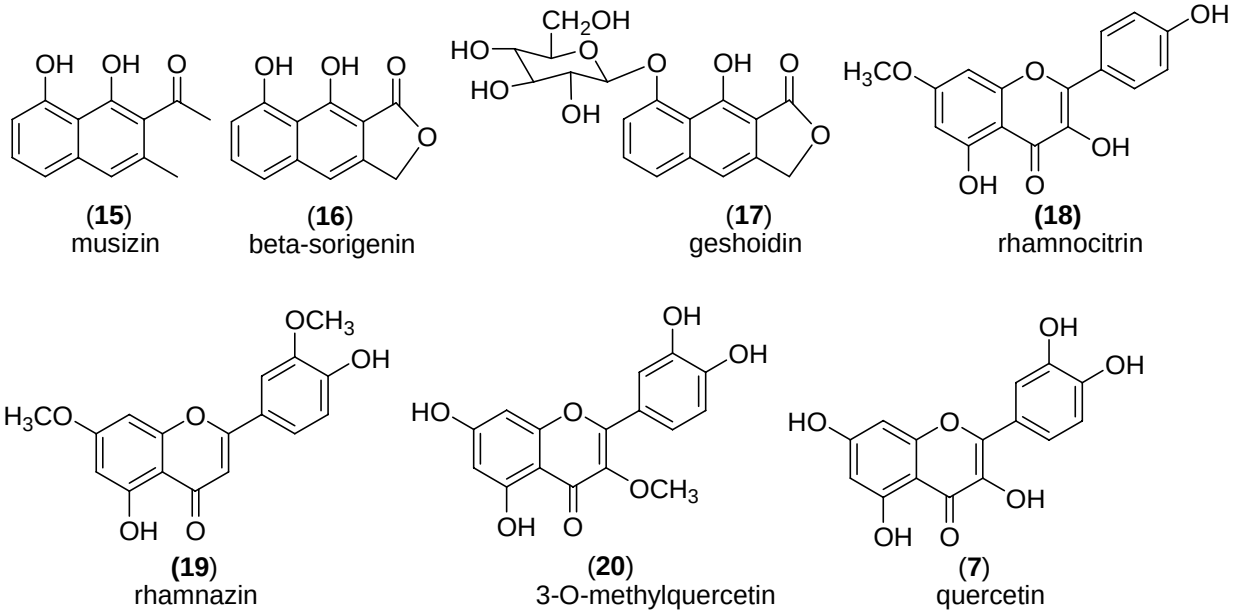
1.8 Summary of compounds reported from the fruits and leaves of R. prinoides

Five antraquinones and their derivatives were reported from the chloroform extracts of the fruits of *R. prinoides* in 1988 whose structures are listed below (Dagne, E. & Abegaz, B.).

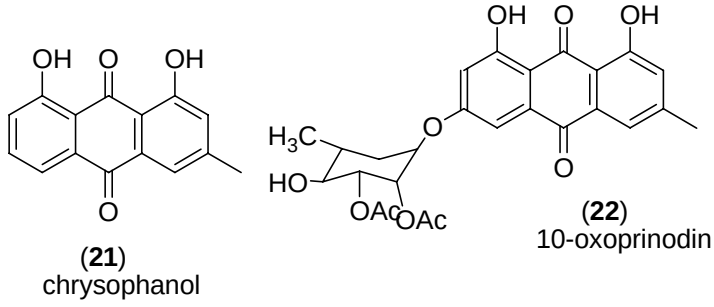


And in an experiment made in 1995, seven years later, on the leaves of the same plant other 9 compounds and two of the above (physcion and emodin) were reported (Abegaz, B., and Kebede, T. 1995). Among these four of them were flavonoids.

The structures of the compounds that were isolated from the leaves are listed below.



Other two compounds, chrysophanol (**13**) and 10-oxoprinoidin (**14**) have been reported from the leaves of *R. prinoides* in same year. (Abegaz,B.& Peter, M. 1995). One of this, 10-oxoprinoidin is different from the previously reported prinoidin, in the latter case; the 10th position has been oxidized to ketone.



Scheme 2. The structure of compounds reported from the leaves and fruits of *R. prinoides*

1.9 Aim of the project

β-Sorigenin-8-O-β-D-glucoside is believed to be the bitter principle in the leaves of R. prinoides (Abegaz, B., and Kebede, T., 1995). Gesho (*R. prinoides*) stems are used in the making of another traditional beverage known as Tej.

To date there is no any phytochemical work done on the stems of *R. prinoides*. The main objective of this work was to do phytochemical work on the stems of *R. prinoides*. Such work will help to compare the constituents of the leaves and the stems and also to see if the bitter principle isolated from the leaves, β-sorigenin-8-O-β-D-glucose, is also present in the stems.

Specific objectives

- To isolate and characterize bitter principle ot Tella, geshoidin, from leaves and stems of R. prinoides
- To study the physical and chemical properties of geshoidin.
- To isolate and characterize some major secondary metabolites from the stems of *R. prinoides*.

2. Results and Discussion

During the course of this chemical study four compounds were isolated and fully characterized from the stems of *R. prinoides.* One of these compounds (geshoidin) has also been isolated and characterized from the leaves of the same plant. The compounds were: β-sorigenin-8-O-D-glucoside (geshoidin), physicion, chrysophanol, and emodin. Prior to this study geshoidn, had been isolated from the leaves of *R. prinides* (Abegaz, B. & Kebede, T., 1995). The other three (chrysophanol, physcion, and emodin) had been isolated from both the fruits and leaves of the same plant (Abegaz & Dange 1988, Abegaz & Kebede 1995). All the compounds were characterized by spectroscopic methods including ^{1}H and ^{13}C NMR and other physical data. ^{1}H NMR and ^{13}C NMR spectroscopic were used for structural elucidation of all the above four Compounds.

2.1 Characterization of Compounds

In order to characterize the isolated compounds from the leaves and stems of *R. prinodes* the melting point, the R_f value, and NMR spectroscopic data of the compounds were utilized. The collected data were analyzed and compared with literature values for the reported compounds.

2.2 Characterization of Compound 17

Compound **17** was adull yellow crystalline material isolated from the leaves and the stems of R.prinodes. It was detected as pink red spot under UV lamp and became blue-green when sprayed with 4% vanillin-H_2SO_4, its R_f value was 0.19 in $CHCl_3$-MeOH (3:1) and its melting point was determined to be 160-162 ^{o}c.

^{1}H NMR spectroscopic data of **17** consisted of multiplets at δ=3.3-3.8 (6H, Glc), doublet at δ=5.3 (1H) for the anomeric proton of glucose, singlet at δ=5.4 (2H)

for lactone's protons, and in aromatic region other four peaks; at δ=7.4 (2H, triplet) atδ=7.5 (1H, triplet) and at δ 7.6 (1H, doublet) and finally one weak broad band at δ=10.5 for the chelated phenolic proton. ^{13}C NMR spectroscopic data showed 6 carbons at δ=61.2-78.3, among which two of them were methylene carbons (point downward on Dept-135) which corresponds to the glucose and lactone's carbon. At δ=103.3 there was one carbon whose chemical shift data agreed with anomeric carbon chemical shift. In the aromatic chemical shift region only 9 carbons were seen, among which three of them bear proton and the rest are quaternary carbons. At δ=168.8 there was lactone's carbonyl carbon. When all the above data were analyzed and compared with literature values **17** was found to be geshoidin (Table1). The ring-numbering system of geshoidin used in this manuscript is one shown below.

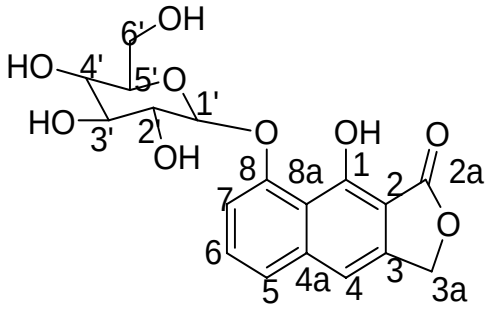


Figure 3. The ring-numbering system in geshoidin

Table1. The ^{1}H NMR spectroscopic data of geshoidin

Observed			Reported (Kebede, T., **1994)**	
$\delta^{1}H$	multiplicity	description	$\delta^{1}H$	multiplicity
3.3-3.8	multiple	Glucose protons	3.3-3.7	
5.15	doublet	anomeric H	5.2	doublet
5.4	singlet	Lactones H	5.4	singlet
7.4	multiplet	H-4&H-7	7.4-7.5 (H-4&H-7)	multiplet
7.5	triplet	H-6	7.5 (H-6)	triplet
7.6	doublet	H-5	7.6(H-5)	doublet
10.5	br. singlet	1-OH	10.5	br. singlet

2.2.1 DEPT spectrum

A distortionless enhancement by population transfer (the DEPT) ^{13}C spectrum is designed to display separate spectra for CH, CH_2, and CH_3 carbon signals. In DEPT spectra signal intensity (i.e., sensitivity) arise by polarization transfer. In those spectra, data are collected in such way that the resulting signal is either positive (CH& CH_3) or negative (CH_2) depending on the number of proton attached (Macomber, S., 1998). In accordance with this principle, Dept-135 spectrum of geshoidin showed two negative (CH_2) carbons and 7 positive (CH) carbons. 8 Carbons of ^{13}C spectrum were not seen on Dept-135 spectrum revealing that these were quaternary carbons as shown in Table 2.

Table2. The^{13}C NMR spectroscopic data of geshoidin

No.	Observed		Reported (Kebede, T., **1994**)		Types of carbons
	δ ^{13}C	Dept-135	δ^{13}C	Dept-135	
1’	103.3	+	102.9	“	“
2’	73.8	+	72.3	“	“
3’	78.2	+	77.8	“	“
4’	70.3	+	69.9	+	methine
5’	76.7	+	73.4	“	“
6’	61.2	-	60.8	-	methylene
1	156.4		155.8		“
2	114.7		114.3		quaternary
3	143.5		142.9		“
4&7	106.4		105.9		quaternary
4a	140.0		139.5		quaternary
5	123.7	+	123.0	+	methine
6	130.3	+	129.5	+	methine
8	155.8		155.3		“
8a	111.6	+	111.0	+	methine
3a	68.6	-	67.9	-	methylene
2a	168.8		168.8		“

2.2.2 The 2D spectra of geshoidin

2D NMR (COSY, HMQC, and HMBC) of geshoidin (**17**) was not reported before. But these spectra are vital to assign unambiguously overlapping ^{1}H NMR spectrum of glucose unit and also the rest part of the compound clearly. There fore, the COSY, HMQC and HMBC spectrum of geshoidin (**17**) have been included in this manuscript.

COSY

The correlation spectroscopy (COSY) correlates the different protons in a given spectrum that are coupled to each other. It is a two dimensional intensity plot; where the normal spectrum is on the diagonal and additional signals, the "cross peaks" appear when ever two protons with different chemical shifts have a coupling in common. In general, each cross peak in COSY represents a correlation due to either two or three bond H-H coupling. The information in COSY is used to show the connectivity of the carbon skeleton of molecules except at quaternary carbon where the correlation disappeared (Cichewcx, R., et al., 2004).

Accordingly, the COSY of geshoidin showed coupling between adjacent protons on the aromatic and the glucose skeleton. These correlations are summarized in Table 3.

HMQC

Heteronuclear multiquantum coherence (HMQC) spectrum is used to see correlations (couplings) between protons and carbon (^{1}H-^{13}C). With respect to

the information content, HMQC spectra offer the advantage that due the large chemical shift range of carbon, the proton spectroscopic information is spread out and overlap is much less likely (Cichewcx, R., et al., 2004).

Accordingly the HMQC spectrum of geshoidin (**17**) clearly resolves the overlapping resonance of the glucose unit (3.2-3.8ppm) of ^{1}H NMR spectrum. This region, in the HMQC spectrum of geshoidin, their correlation peaks are spread out from 61-78ppm (17ppm) in the carbon range. This also offers interesting structural information because a part of the structure is now characterized by two data points (^{1}H-shift and ^{13}C-shift), which enables one to resolve ambiguous assignments of protons in glucose part of geshoidin (**17**).

The HMBC

The main advantage of hetronuclear multibond correlation (HMBC) is to see long range couplings. HMBC is used to connect fragments already identified by COSY and HMQC spectra. Correlations observable in COSY typically end at quaternary carbons; so HMBC serves as an important tool to connect these "independent" spins systems with each other (Cichewcx, R., et al., 2004).

In the case of geshoidin correlations are observed from the anomeric proton of the glucose to the phenolic carbon of the β-sorigenin part and from the lactones proton to the carbonyl carbon of the lactone unit. These and all other important correlations have been summarized in the Table 3.

Table 3. The 2D spectroscopic data of geshoidin.

No.	$\delta^{13}C$	Dept-135	HMQC	COSY	HMBC
1’	103.3	+	5.2(CH)	With 3.4	with C-14&15
2’	73.8	+	3.4(CH)		
3’	78.2	+	3.5(CH)		
4’	70.3	+	3.0(CH)		
5’	76.6	+	3.4(CH)		
6’	61.1	-	3.8,3.5(CH_2)		
1	156.4				
2	114.68				
3	143.6				
4&7	106.4				
4a	140.0				
5	123.7	+	7.6(CH)		with C-8a&2
6	130.3	+	7.5(CH)	With 7.4	WithC-4a,8,1&3
8	155.8				
8a	111.6	+	7.4(CH)	With 5.4	with C-la,2,5,4a,8&1
3a	68.6	-	5.4(CH_2)		with C-4,8a,4a,3&2a
2a	168.8				

The main uses of data in the above table were to explore the possibility of symmetry in the isolated compound. Accordingly, on COSY spectrum the anomeric proton has been coupled with adjacent proton at δ=3.45 attached to C-2' (δ=73.8)

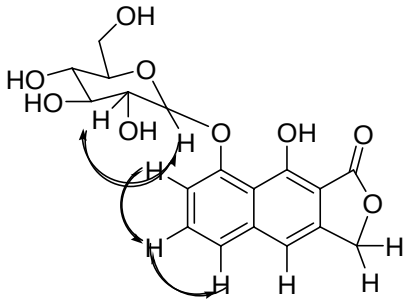


Figure4. COSY of geshoidin

And from HMBC spectrum the anomeric proton has been coupled to C-1(δ=156.8) which provide good information about the glycoside linkage. On the same spectrum the lactone's proton has coupled with C-8a (δ=111), C-4a (δ=140), C-4(δ=106), C-3(δ=143), and the lactone's carbonyl carbon (δ=168.8).

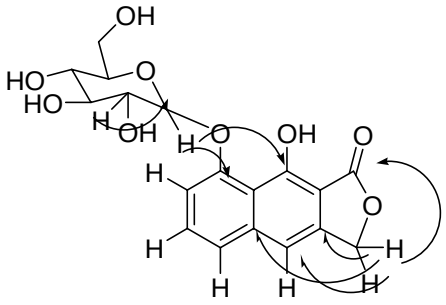
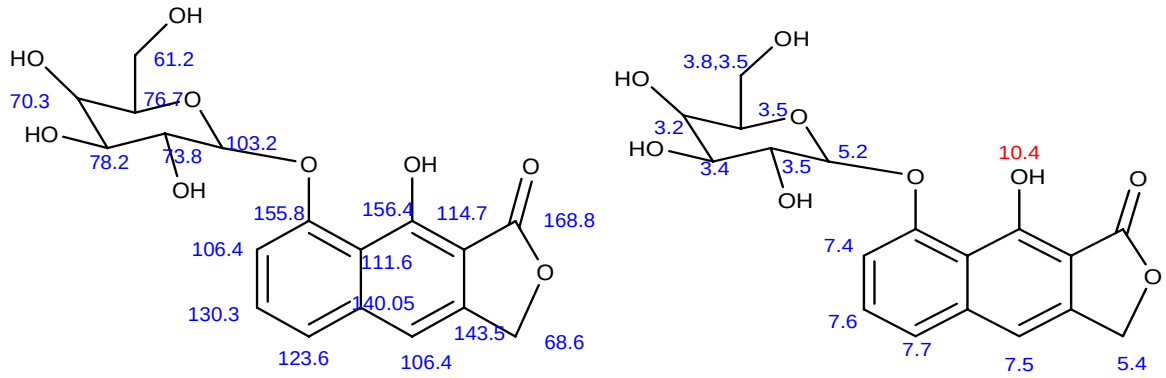


Figure5. HMBC of geshoidin

With all the above information and comparing it with spectroscopic data of geshoidin clearly indicates about the connection of the glucose unit.



17

Figure 6. Spectroscopic data assignment of geshoidin

As further evidence, the above compound was hydrolyzed by refluxing in acidic medium for 10 hrs. And the CH_2Cl_2 extracted β-sorigenin was characterized by ^1H NMR.

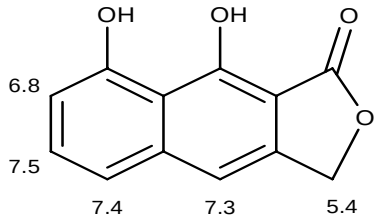
2.3 Characterization of hydrolysis product of 17

β-Sorigenin obtained by hydrolysis of geshoidin was a faint yellow crystal which melts at 239-242 ^oc. It became green on TLC with $CHCl_3$-MeOH (3:1) when sprayed with vanillin-H_2SO_4. ^{1}HNMR (DMSO-d_6) of this compound displayed singlet at δ=5.4(2H) for the lactone's proton, doublet at δ=6.8 (1H) for H-7, singlet at δ=7.3(1H) for H-4, another doublet at δ=7.4(1H) for H-5, and triplet at δ=7.5(1H) for H-5. In addition to the above, there was broad multiplet band at δ 7.7. The two hydroxyl groups, even though, expected at δ=10.4 for chelated one and at δ=5-7 for phenolic hydroxyl, these were not observed on the spectrum. The broad peak at δ=7.8 presumably attributed to these protons. The change in chemical shift may be due to hydrogen bonding with the NMR solvent.

The spectroscopic data of the observed and reported values have been listed in Table 4.

Table4. ^{1}H NMR spectroscopic data of β- sorigenin

Observed		Reported	
$\delta^{1}H$	multiplicity	$\delta^{1}H$	multiplicity
5.4 (lactone's H)	singlet	5.4	singlet
6.8(H-7)	doublet	6.8	doublet
7.3 (H-4)	singlet	7.3	singlet
7.4 (H-5)	doublet	7.4	doublet
7.5 (H-6)	triplet	7.5	triplet
-	-	10.4	br. Singlet



β-sorigenin (**16**)

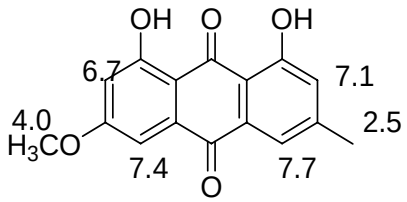
2. 4 Characterization of Compound 12

Compound **12** was isolated from the stems of *R. prinodes*. It was orange yellow crystal and became pink red (when observed with TLC developed with system petrol-EtOAc (4:1)) when sprayed with 5% methanolic KOH solution. The melting range of this compound is 205-207 ^{o}C. $^{1}HNMR$ spectrum of **12** showed singlet methyl proton (3H) at δ=2.5, methoxy protons (3H) at δ=4.0 and other

4H in the aromatic chemical shift regions; at δ=6.6 narrow doublet, at δ=7.0 broad singlet, at δ=7.3 narrow doublet, and at δ=7.6 broad singlet respectively. The broad peaks at δ=7.0 and 7.6 indicate the allylic coupling with methyl protons at C-3. At lower end of the spectrum two chelated protons were seen at δ=12.2 and 12.4 as two separated singlet peaks. When all the above data were compared with literature value, **12** was identified as physcion and the spectroscopic data has been described in Table 5.

Table5. ^{1}H NMR spectroscopic data of physcion

Observed		Reported (Kebede, T., **1994**)	
$\delta^{1}H$	multiplicity	$\delta^{1}H$	multiplicity
2.5(CH_3)	singlet	2.4	singlet
4.0(OCH_3)	singlet	3.9	singlet
6.7(H-7)	doublet	6.6	doublet
7.1(H-2)	br. singlet	7.0	singlet
7.4(H-5)	doublet	7.3	doublet
7.7(H-4)	br. singlet	7.6	br. singlet
12.2&12.4(1.8-OH)	two br. singlet	12.1&12.3	two br. singlet



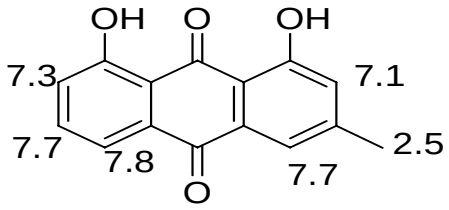
(**12**)
physcion

2.5 Characterization of Compound 21

Compound **21** was another compound isolated from the stems of *R. prinodes*. It is a yellow compound which melts at 193-195 ^{0}C and became red on TLC when sprayed with 5% methanolic KOH solution. ^{1}HNMR data of **21** consisted of five protons in the aromatic chemical shift region and other three types of protons out of the aromatic chemical shift region. These were at δ=2.5(3H) singlet methyl protons, at δ=7.1(1H) broad singlet, at δ=7.3 (1H) triplet, at δ=7.7 multiplet, which showed overlap of H-4 with H-6, and at δ=7.8 broad doublet. And other two chelated protons appeared at δ= 12.1&12.2. With all the above data and comparison with literature value (Abegaz, B., and Kebede, T. 1995) mentioned in Table 6 below confirmed that the compound is chrysophanol.

Table 6. ^{1}H NMR spectroscopic data of chrysophanol

Observed		Reported (Kebede, T., **1994**)	
δ^{1}H	multiplicity	δ^{1}H	multiplicity
2.5(CH3)	singlet	2.5	singlet
7.1(H-2)	br. singlet	7.1	br. singlet
7.3(H-7)	triplet	7.3	triplet
7.7(H-5)	multiple	7.6	multiplate
7.7(H-4)	br. triplet	7.7	br. singlet
7.8(H-5)	doublet	7.8	triplet
12.1&12.2(1,8-di-OH)	two br. singlet	12.0&12.2	two br. singlet



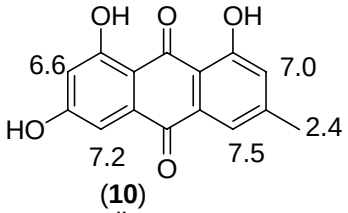
(**21**)
chrysophanol

2.6 Characterization of Compound 10

Compound **10** was orange red compound isolated from the stems of the above plant. The melting range of this compound is 252-254 ^{0}C. On TLC it became red when sprayed with 5% methanolic KOH solution. ^{1}H NMR of **10** showed singlet methyl protons (3H) at δ=2.4, and other four aromatic protons; at δ=6.6 doublet, at δ=7.0 singlet broad peak, at δ=7.3 doublet, and at δ=7.5 broad singlet. And other two chelated hyrdoxyl protons even though expected around (δ=12._) BUT these were not observed in this spectra this may be due to some hydrogen bonding which cause change in chemical shift. When all these data were compared with literature values as drown in table 7 be and **10** was proved to be emodin

Table 7. ^{1}H NMR spectroscopic data of emodin

Observed		Reported (Kebede, T., **1994**)	
δ^{1}H	multiplicity	δ^{1}H	multiplicity
2.4(CH_3)	singlet	2.4	singlet
6.6(H-7)	doublet	6.6	doublet
7.0(H-2)	br. singlet	7.1	br. singlet
7.2(H-5)	doublet	7.2	doublet
7.5(H-4)	br. singlet	7.5	br. singlet
-	-	12.1&12.0	two singlet



(**10**)
emodin

3. Experimental Sections

3.1 General

Instruments

For ^{1}H NMR: Bruker Avance at 400MHz was used.

Melting point was determined using Thiele tube.

Chromatography:

For column chromatography silica gel 60, particle size 0.063-0.200 mm (70-230 mesh ASTM) was used.

For analytical TLC, silica gel 60PF_{254} (Fluka) coated on aluminum sheet, 0.20mm thickness was utilized.

Silica gel 60 PF254+366(merk)1 mm, 0.75 mm, 0.50 mm (70-230 mesh ASTM) was used for preparative TLC. And Sephadex LH-20 (pre-packed column) has been used repeatedly.

Impregnation of silica gel

Silica gel for flash column chromatography was impregnated with 5%oxalic acid and activated by heating at 100 ^oC overnight.

Spray Reagent

5% KOH methanolic solution (Borntrager reagent) and 4% vanillin in sulphuric acid were used as spray reagent.

3.2 Plant Material

The leaves and the stems of *R. prinoides* (Gesho) were bought from Shola market in Addis Ababa. Both the leaves and the stems were dried in air.

3.3 Extraction, fractionation and isolation from leaves

Dried powder leaves (1216) were soaked in 3 L methanol for 72 hrs. The solvent was removed using rotavapor and 248 g of gummy dark residue was obtained, which is 20.5% of the dry weight. TLC with petrol EtOAc (7:3) showed multiple spots when 4% vanillin in H_2SO_4 was used for spray. 30g of this extract was subjected to 350g oxalic acid impregnated silica gel flash

chromatography using solvent systems given in table 8 and 102 fractions each with 200mL were collected.

Table 8. Fractions collected from flash chromatography

Solvent system	Ratio	Fractions collected
$CHCl_3$	100%	1-62
$CHCl_3$ – EtOAc	9:1	63-73
$CHCl_3$- EtOAc	4:1	74-86
EtOAc- MeOH	2:1	87-100
EtOAc- MeOH	1:1	101 and 102

Among $CHCl_3$ fractions those from 1-24, showed three spots on TLC with petrol – $CHCl_3$ (4:1) when 4% vanillin in H_2S0_4 was used for spray. The solvent was removed using rotavapor. Similarly fractions from 25-67, showed more than three spots on TLC with solvent system $CHC1_3$–MeOH (3:1) and with the above spray reagent. Up on concentrating these fractions, they formed precipitate whose melting point was 99-100^{0}c.

This precipitate was soluble in cold methanol and exhibited the physical properties of oxalic acid which was discarded at the end. The next fractions from 68-91 showed multiple points on TLC in the above solvent system and contain oxalic acid.

Fractions from 88-102 also precipitated upon concentrating. And TLC analysis with $CHCl_3$-MeOH (3:1) of the concentrated solution showed again multiple spots when visualized with UV-lamp. The precipitate was collected, recrystallized from methanol and analyzed with TLC in above solvent system (and purified until it gives a single spot on TLC). The purified precipitate melted at 160- 162^{o}c. 30 mg of this product was sent for NMR and comparison of its ^{1}HNMR and ^{13}C NMR with that of geshoidin (spectrum from literature) showed the isolated compound was geshoidin whose total amount is 800mg.

3.4 Extraction, fractionation and Isolation from the stem

A dried powdered stem (2.3 Kg) of R. prinodes was soaked in 6 L methanol for 72 hrs. The solvent was removed by rotavapor and 137 g gummy residue was collected. This was 6.0 % of the dry weight. This crude extract showed 3 spots on TLC plate that turned red when sprayed with 5% methanolic KOH solution [with solvent system $CHCl_3$-EtOAC (9:1)]. The crude extract (40g) was applied on 350 g oxalic acid impregnated silica gel flash chromatography and 46 fractions, each with volume of 200ml were collected with the solvent system shown in Table 9.

Table9. Fractions collected from flash chromatography of the stems.

Solvent System	Ratio	Fractions collected
$CHC1_3$	100%	1-28
$CHC1_3$ – EtOAc	4:1	29-32
	7:3	33-36
	1:1	37
EtOAc- MeOH	2:1	38-44
EtOAc- MeOH	1:1	45
MeOH	100%	46

3.5 TLC analysis of collected fractions

Fractions 1-10 showed three spots on TLC with petrol-$CHCl_3$ (4:1) (when 4% vanillin in H_2S0_4 was used for the spray). The solvent was removed and total dry weight was found to be 450 mg. The next fractions from 11 to 15 showed again three spots which differ from the former in R_f values. When these fractions were concentrated, they formed precipitate. The precipitate contained oxalic acid and some other compounds. The compounds were extracted from the oxalic acid with $CHCl_3$ – petrol (7:3) and the total amount of this mixture

was 200 mg. Fractions from 16-28 showed similar characteristics on TLC with $CHCl_3$- MeOH (9:1) and with the above spray reagent.

They were put together for further analysis after removing the solvent and the oxalic acid. The next fractions from 33-39 also showed multiple spots on TLC with the above solvent system and spray reagent. Fraction 40 formed precipitate up on standing. The precipitate was crystallized from methanol to yield 15mg of geshoidin. It's TLC, melting point and ^{1}HNMR is identical with that of geshoidin.

3.6 Isolation of anthraquinones from the stems

One of the properties of anthraquinones which is important to identify them from other compounds is their exhibition of different color in acidic and basic medium. Generally anthraquinones exhibit pink red or red color when treated with 5%methanolic KOH solution. In accordance with this principle one of the spots in fractions from (1-10) became pink red and from fractions (11-15) became red with 5% methanolic KOH solution. And these anthraquinones have been isolated with procedure below.

3.6.1 Isolation of physcion (12) and chrysophanol (21) from fractions (1-10)

450 mg from fractions (1-10) was subjected to Sephadex LH-20 column and eluted with $CHCl_3$-MeOH (2:1). Five fractions were collected. Among the fractions, the last fraction had the properties of anthraquinone on TLC with petrol-EtOAc (4:1) when sprayed with 5% methanolic KOH solution. However, additional spots were visualized under UV-lamp. In order to isolate anthraquinone from this mixture, 80 mg of mixture was applied on 15 g silica gel packed column and elution was done with Petrol–EtOAc (4:1). Seven fractions were collected. Among the fractions those from 3-6 showed a single spot on TLC with petrol –EtOAC (4:1) and became pink red when sprayed with 5% methanolic KOH solution. When the solvent was freed, it has 15 mg weight.

However, this product displayed two closely spaced spots when analyzed on TLC with petrol-EtOAc (9.5:0.5). The two compounds were separated by applying the above product on 15 g silica gel packed column and elution was done with solvent system show in Table

Table10. Fractions collected on further isolation of fractions (1-10)

Solvent system	Ratio	Fractions collected
Petrol	100%	1.(100mL)
Petrol-EtOAc	9.8:0.2	2 (50mL)
Petrol-EtOAc	9.5:0.5	3-5(50mL)
Petrol-EtOAc	8.5:1.5	6-7(50mL)

And the characteristics of these fractions when analysed with petrol – EtOAc (9.6:0.4) have been summarized in Table 11on the next page.

Table11. TLC analysis of fraction 1-10

Fr1actions	Number of spots	The color of the spots when sprayed
1	1	No change
2	2	One pink red & other no change
3-6	1	Pink red
7	2	pink red
8	1	pink red
9-10	No	No

The amount of fractions 3-6 was 7 mg and the amount of fraction 8 was 4 mg when they became free of the solvent. These products were sent for analysis and the resulting NMR spectra revealed the above two compounds were chrysophanol and physcion respectively.

3.7 Isolation of emodin and other compounds from fractions (11-16)

From fractions (11-16) (200mg) was applied on Sephadex LH- 20 with solvent $CHC1_3$-MeOH (2:1) and 5 fractions were collected. Among these fractions, the 5^{th} fraction showed three spots with petrol-EtOAc (7:3) and one of the spots had characteristics of anthraquinone (became red when sprayed with 5% methanolic KOH solution). The other spots became yellow with the spray reagent. 175 mg of this fraction was applied on 30 g silica gel packed column and eluted with the solvent system shown in Table 12.

Table12. Fractions collected in isolation from fractions (11-16)

Fractions	Ratio	Fractions collected
Petrol	100	1 (50mL)
Petrol – EtOAc	9.5:0.5	2(50mL)
Petrol – EtOAc	9:1	3&4 (50mL)
	8:5:1:5	5&6 (50mL)
	8:2	7,8&9 (50mL)

Among the above fractions, 5 and 6 displayed a single spot on TLC with Petrol –EtOAc (4:1) and became red when sprayed with 5% methanolic KOH solution. This product had 15 mg weight when it became free of the solvent. This product was further purified by washing with hot hexane. At the end 8 mg of this product was ascertained by NMR spectroscopic and the compound was proved to be emodin.

Fraction 7, 8 and 9 appeared as a single spots on TLC with petrol-EtOAc (4:1) but separated into three spots when developed with $CHCl_3$-MeOH (9.6:0.4). The attempt to separate these components using column chromatography with above solvent system was not successful. However, the components were separated on preparative TLC as follows. 20mg of the sample was dissolved in $CHCl_3$-MeOH (9.6:0.4) and applied on 20x20cm PTLC in a line at 1.5 cm from the bottom. The TLC was developed in 100mL $CHCl_3$-MeOH (9.6:0.4) using

appropriate TLC jar. This separated the sample in to three different bands. Each of these bands was collected separately by scratching from the glass plate and washing from silica gel by the above solvent mixture. The solvent was removed by rota vapor. The amount of the first band was 15mg, the 2nd was 9mg and the 3rd band was only trace amount. The extract of 1st band was analyzed by NMR spectroscopic analysis and but it was not fully interpreted.

3.8. Spectroscopic data of compounds

β-Sorigenin-8-O-β-glucose: dull yellow leaflet, blue-green with vanillin, R_f 0.19 in(I), melting point 160-162 ^oC, ^1H NMR (DMSO-d_6): δ 3.2-3.8 (6H,Glc), 5.15 (1H, d, H-1 Glu), 5.4 (2H lactone, CH_2),7.4-7.45 (s and d,2H,H-4 ad H-7), 7.6 (1H, t, H-6), 7.65 (1H, d, H-5), 10.5 (s 1-OH) and ^{13}C NMR (DMSO-d_6): listed in Table 1

Physcion: orange yellow leaflets, pink-red with KOH, R_f 0.56 in (II), melting point 206-208^{o}c ^1H NMR ($CDCl_3$): δ 2.45 (3H, s, Me), 3.92 (3H, s, MeO), 6.6 (1H, d, H-7), 7.0 (1H, br.s, H-2), 7.3 (1H, d, H-5), 7.6 (1H,br. s,H-4), 12.1 and 12.3 (s, 1,8-di-OH) respectively.

Chrysophanol: yellow leaflets, pink red with KOH, R_f 0.65 in (II), melting point 193-195^{o}c, 1H NMR ($CDCl_3$) δ 2.5 (3H, s, Me), 7.1 (1H, br. S, H-2), 7.3 (1H, t, H-7), 7.58 (1H, m, H-5, 7.65 (1H, br. S, H-4), 7.75 (1H, d, t ,H-5) 12.02 and 12.15 (s, 1,8-di-OH)respectively.

Emodin: orange red leaflets, red with KOH, R_f 0.20 in (II), melting point 250-254$_o$c, 1H NMR (Me_2CO-d_6) δ 2.4 (3H, s, Me), 6.6 (1H, d, H-7), 7.1 (1H, br. S, H-2), 7.2 (1H, d, H-5, 7.5 (1H, br. S. H-4), 12.1 and 12.0 (s 1,8-di-OH respectively.

β-Sorigenin: faint yellow needles, green with vanillin-H_2SO_4 R_f 0.41 in (II), melting point 240-243^{o}c, 1H NMR (DMSO-d_6) δ 5.3 (2H, s, CH_2), 6.8 (1H, dd, H-7), 7.3 (1H, s, H-4) 7.4 (2H, t, H-5 & H-6) 10.4 (s 1-OH) respectively.

Solvent system

I: $CHCl_3$-MeOH (3:1)

II: Petrol-EtOAc(4:1)

5. Conclusion

In the course of this chemical study four compounds were isolated from the stems of *R. prinoides.* All of these compounds were fully characterized. These compounds were isolated before this study from the leaves and fruits of the same plant by other researcher team (Abegaz, B., and Kebede, T., 1995**)**

Other three compounds were also isolated from the stems of the plant but these were not fully characterized and they were not included in this manuscript.

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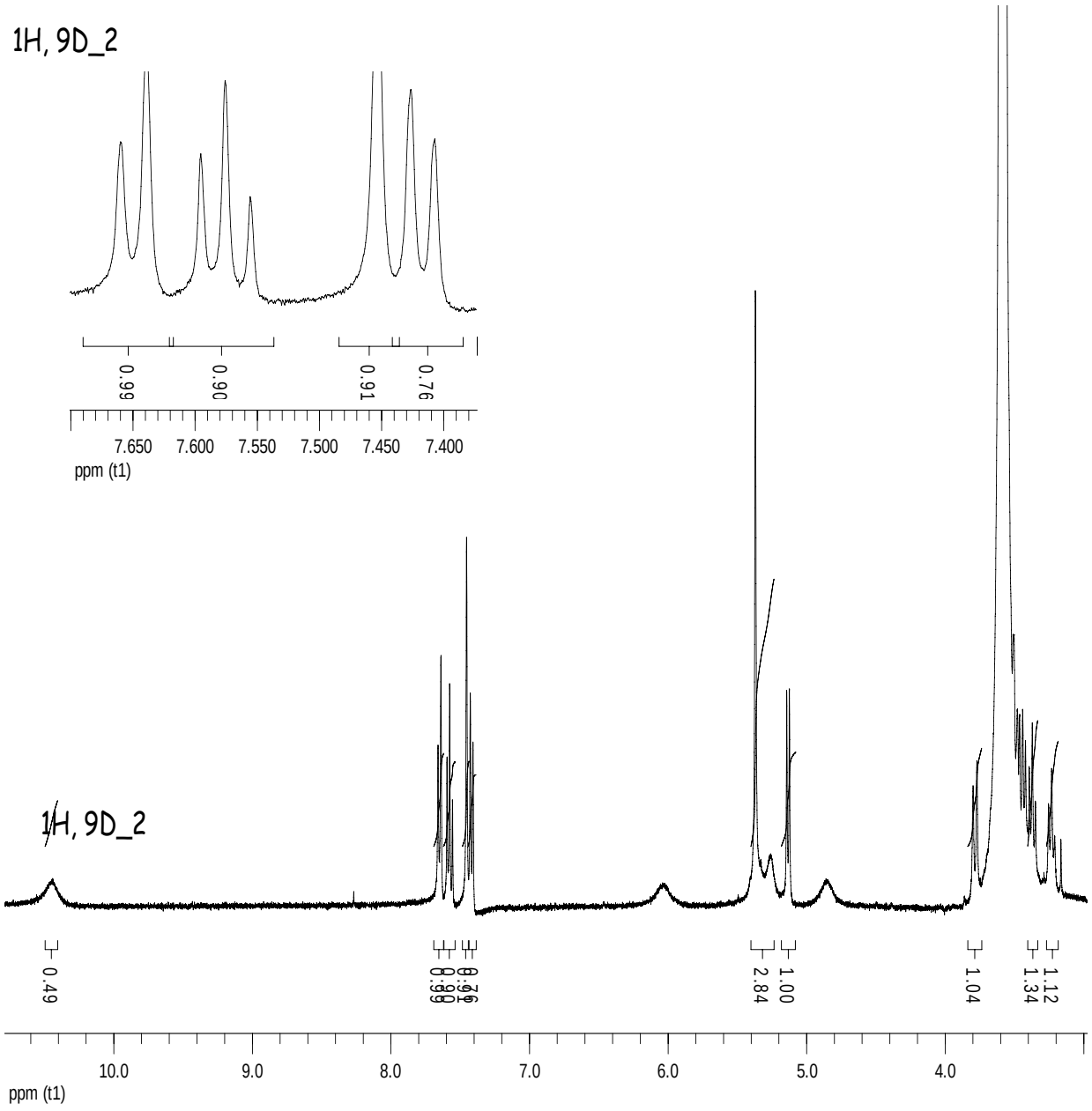
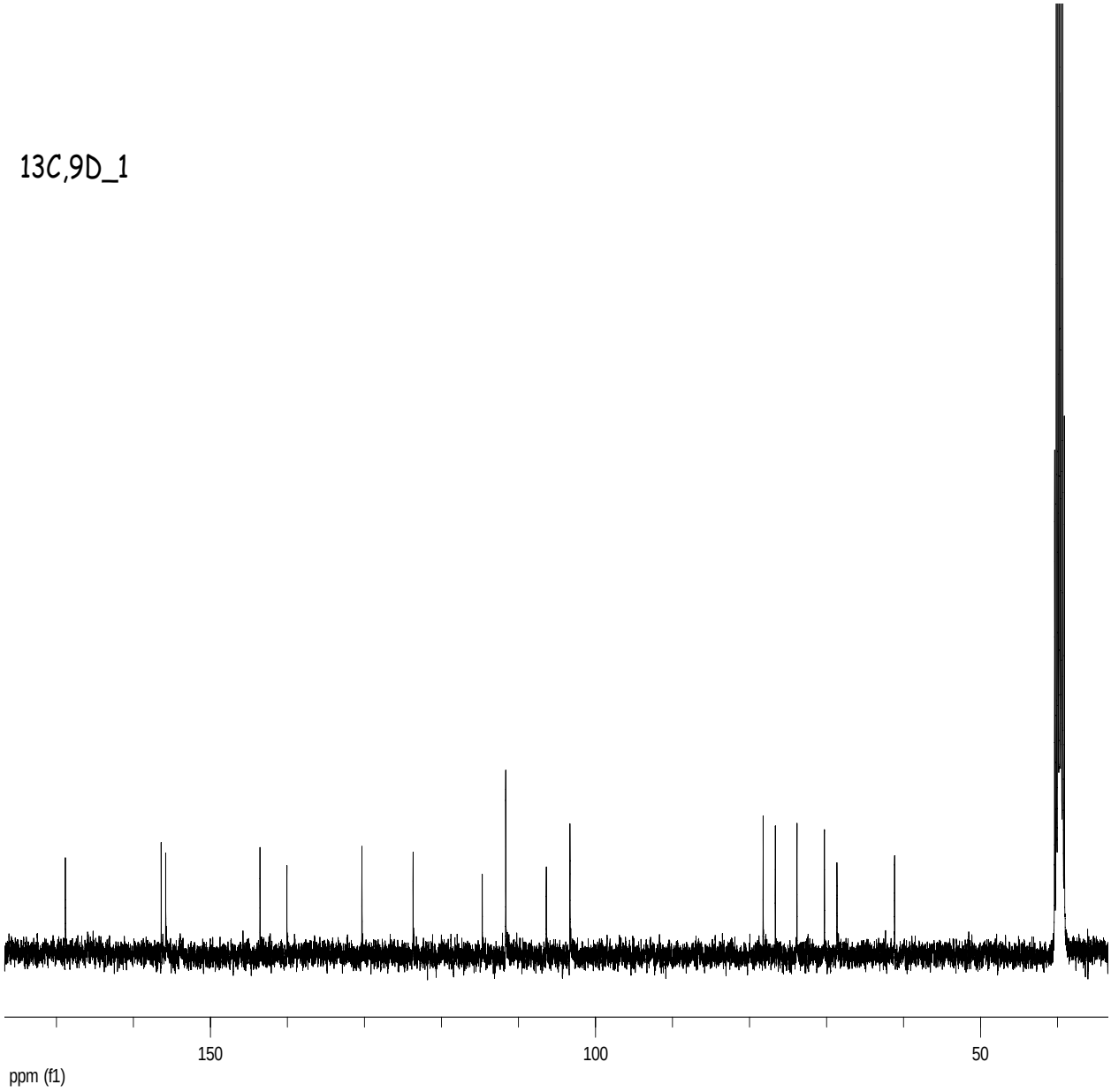
Appendices

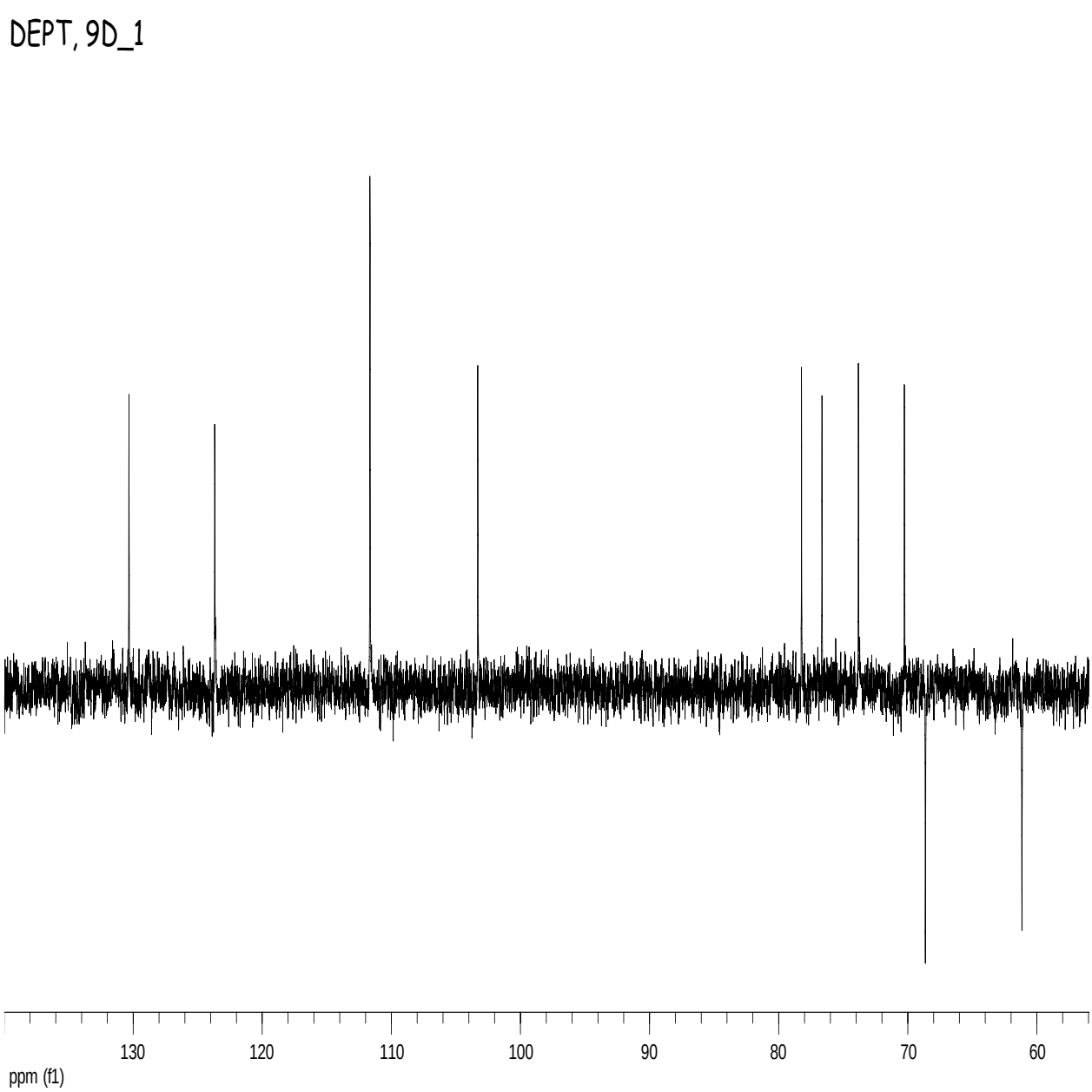
[1]HNMR (DMSO) spectrum of **17**



^{13}CNMR (DMSO) spectrum of **17**



Dept-135 (DMSO) spectrum of **17**

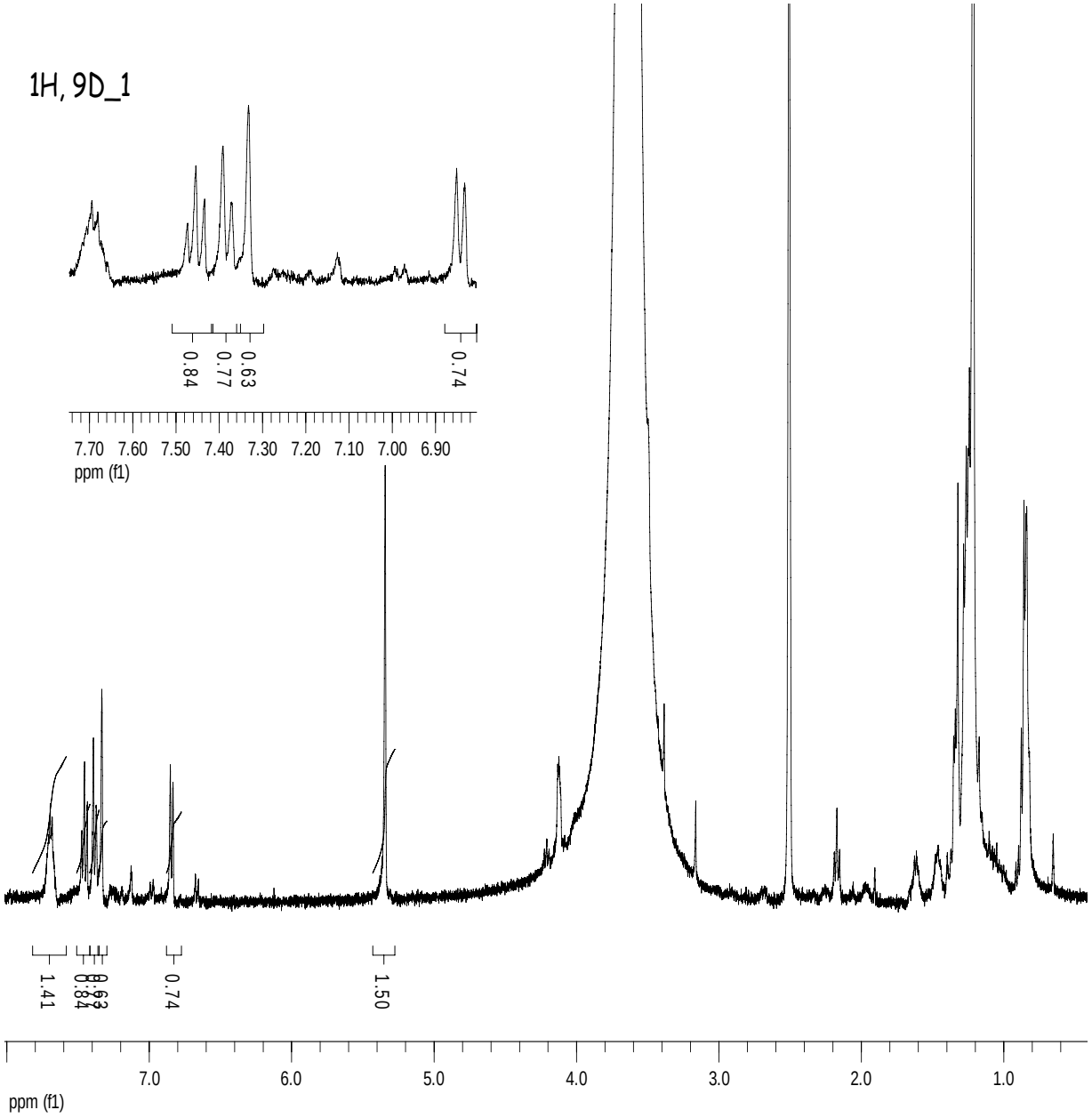


COSY (DMSO) spectrum of 17

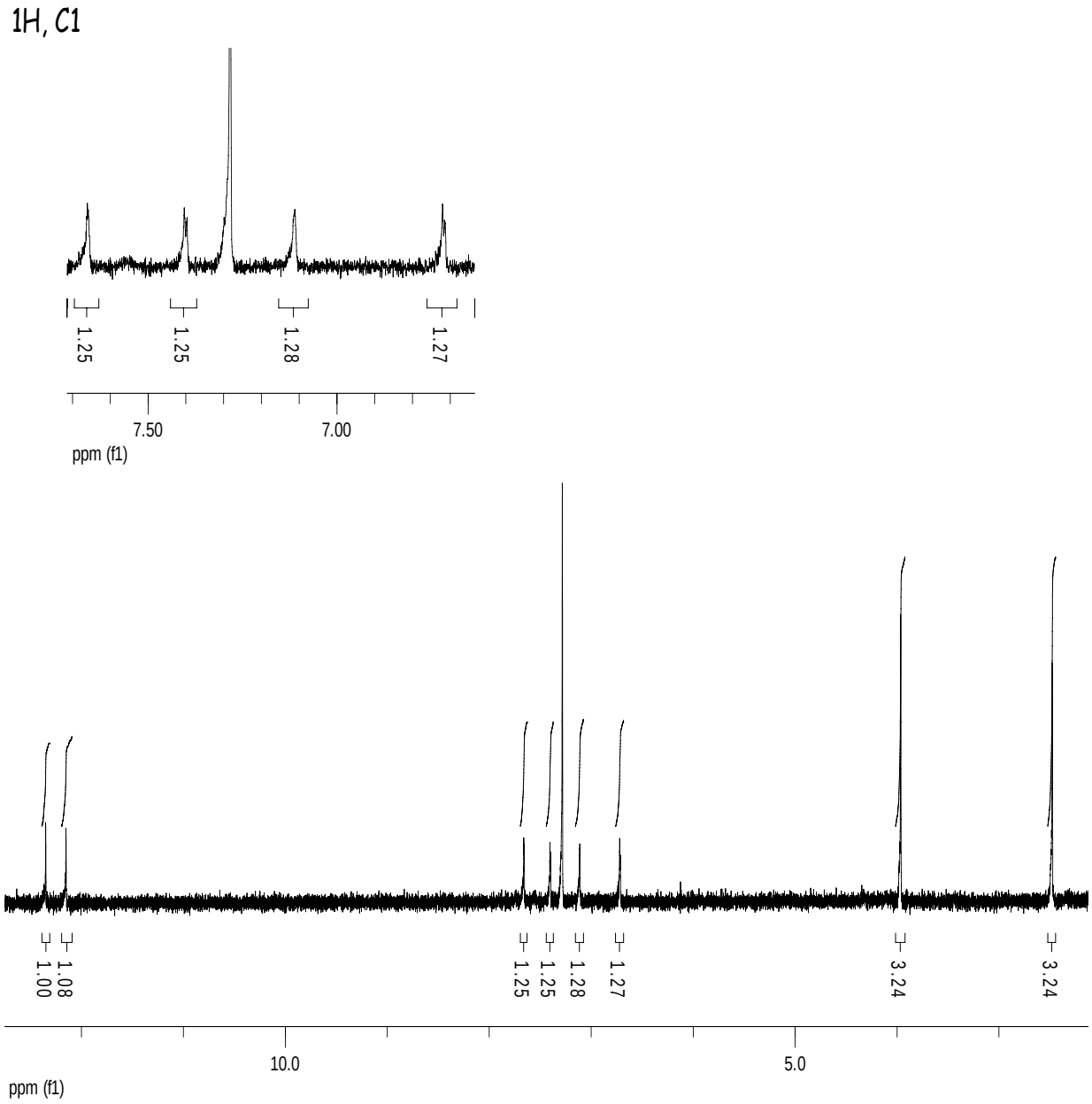
HMQC (DMSO) spectrum of 17

HMBC (DMSO) spectrum of 17

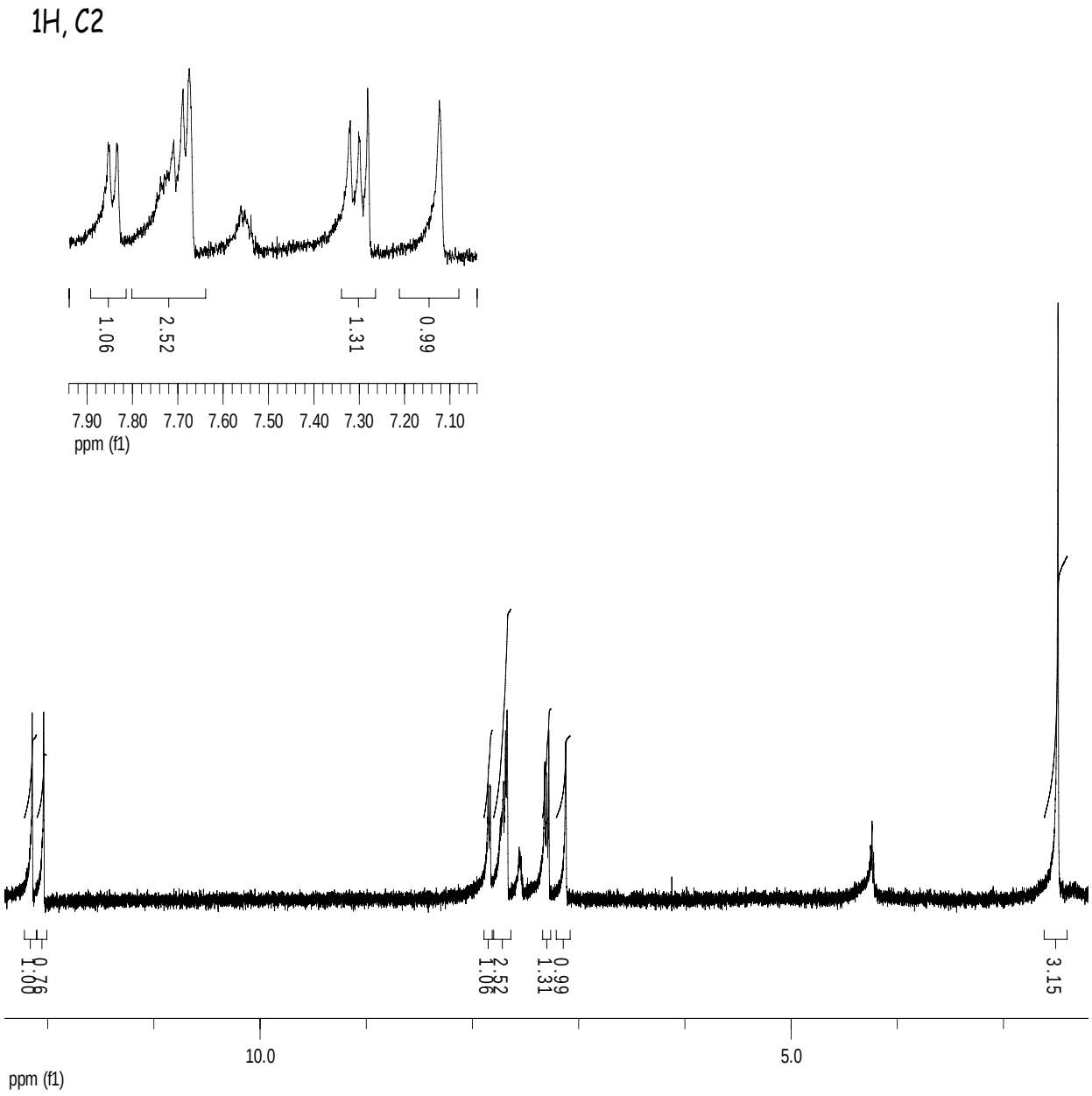
^{1}HNMR (DMSO) spectrum of β-Sorigenin



^{1}HNMR ($CDCl_3$) spectrum of 12



^{1}HNMR ($CDCl_3$& acetone-d_6) spectrum of 21



^{1}HNMR ($CDCl_3$) spectroscopic data of 10