Extraction, separation and identification of compounds from leaves of *Lycium schweinfurthii var.schweinfurthii* (Solanaceae)

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ABSTRACT

Aim of the present study was to identify some phenolic compounds isolated from the dried powder leaves of *Lycium schweinfurthii var.schweinfurthii* (Solanaceae family) collected from alkom alakhdar islet, alborolos lake in Egypt. The investigation of phenolic compounds in *L. schweinfurthii var. schweinfurthii* leaves by different chromatographic methods of separation showed the presence of Naringrin, 3-hydroxy-3,4-methoxy flavone, 7-hydroxy flavone, vitexin and Hisperitine-7-rutinoside. The structures of these flavonoids were characterized on the basis of their UV, NMR and MS spectroscopic data.

Key words: *Lycium schweinfurthii*, Solanaceae, Flavonoids and Phytochemical.

Introduction

The Solanaceae, or nightshades are plant family contains about 94 genera (*Hepper, 1998*) and comprises more than 3000 species (*Quattrocchi, 2000*), herbs, shrubs or small trees. The chief centers of distribution are Central and South America, but it is generally distributed in tropical and temperate regions all over the world (*Hepper, 1998; Quattrocchi, 2000*). The nightshade family is certainly one of the most economically important plant families as food (e.g. potato and tomato genus), medicine (e.g. Atropa Belladonna), ornaments (e.g. Physalis Alkekengi), and several noxious weeds (*Siddiqi, 1978; Hickey and King, 1981; Hussein, 1985*). The family contains a wide range of alkaloids which are of great taxonomic interest. Types of alkaloid recorded are tropane, alkaloidal amine indole, isoquinoline, purine, pyrazole, pyridine, pyrroli-dine, quinazolizidine, steroid alkaloids and glycoalkaloids. Other constituents include steroidal saponins, coumarins, flavones, carotenoids and anthraquinones (*Evans, 2002*). The genus of Lycium is one of the most important genera of family Solanaceae which has been the source of many active constituents. So we interest to choose *Lycium schweinfurthii var. schweinfurthii* which belongs to family Solanaceae.

MATERIALS AND METHODS

1. Plant Material

The fresh roots, stem, leaves and flowers were collected in February, 2014 from alkom alakhdar islet, alborolos lake in Egypt. They were washed, air-dried at
lab. Temperature then dried in an oven at 50°C till constant weight, and finally ground to fine powder.

The taxonomic identification of plant materials was confirmed by Cairo University herbarium.

2. Materials for Chromatography

* Polyamide 6S (Riedel-De-Haen AG, Seelze Hanover, Germany).
* Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Solvent Systems

The solvents used are abbreviated according to the symbols given in table (1). Solvents from 1–5 were used for flavonoids, while solvents 1 and 6 were used for sugars.

<table>
<thead>
<tr>
<th>No.</th>
<th>Symbol</th>
<th>Composition</th>
<th>Percent by volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BAW</td>
<td>n-Butanol/acetic acid /water</td>
<td>4:1:5 (upper phase)</td>
</tr>
<tr>
<td>2</td>
<td>H2O</td>
<td>Water</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>15% AcOH</td>
<td>Acetic Acid / Water</td>
<td>15:85</td>
</tr>
<tr>
<td>4</td>
<td>50% AcOH</td>
<td>Acetic Acid / Water</td>
<td>50:50</td>
</tr>
<tr>
<td>5</td>
<td>PhW</td>
<td>Phenol / Water</td>
<td>80:20 (w/v)</td>
</tr>
<tr>
<td>6</td>
<td>BBPW</td>
<td>Benzene/n-butanol/Pyridine/H2O</td>
<td>1:5:3:3 (upper layer)</td>
</tr>
</tbody>
</table>

Table (1): Solvents system used for paper chromatography

Authentic Reference Materials

1- Flavonoids authentic aglycones (Fluka AG, Bucns SG, Switzerland).
2- Sugar authentic sample (E.Merck, Darmstadt, Germany).

Spray Reagents
The presence of flavonoid compounds or sugars was revealed by spraying the developed chromatograms with one of the following reagents:

Specific Spraying Reagents for Phenolic and Flavonoids

1- Ferric chloride: (1% Ferric chloride solution in ethanol) The chromatograms were sprayed with FeCl₃ then dried at 110°C for 10 min. and observed under UV light to note any changes in colour (Markham, 1982).

2- Aluminum chloride: (1% Aluminum chloride solution in methanol yields yellow - fluorescence colour in long wave UV light).

The chromatograms were sprayed with AlCl₃, then dried at 110°C and observed under UV light to note any changes in colour (Markham, 1982).

Reagents for Ultra-Violet Spectroscopic Analysis of flavonoids (Mabry et al., 1970)

1- Sodium methoxide (NaOMe): Freshly cut metallic sodium (2.5g) was dissolved in 100 ml spectroscopic methanol.

2- Aluminum chloride (AlCl₃): Anhydrous AlCl₃ (5g) were added cautiously to spectroscopic methanol (100 ml) and filtration were carried out after 24 hours to use the filtrate.

3- Hydrochloric acid (HCl): 50 ml of concentrated HCl was mixed with 100 ml distilled water.

4- Sodium acetate (NaOAc): Anhydrous reagent grade NaOAc was melted and allowed to stand for about 10 min. The material was then powdered and stored in a dry bottle.

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

3. Investigation of Flavonoids and Phenolic acids

3.1. Preparation of Flavonoidal Extract

One Kg Dry powder of *Lycium schweinfurthii var. schweinfurthii* leaves were extracted by using methyl alcohol 95%. The residue of the methanolic extract was washed with benzene to get ride of chlorophyll, then washed with successive selective organic solvents hexane, petroleum ether, ethyl acetate, chloroform, methanol 70% finally with water, where six main fractions were obtained; hexane fraction, petroleum ether fraction ethyl acetate fraction, chloroform fraction, methanol 70% fraction and water fraction.

Results of separation techniques clearly indicated that, the chloroform and methanol 70% fractions were the most rich by phenolic constituents most of them are of flavonoids nature.
3.2. Chromatographic Investigation

3.2.1 Paper Chromatography

Two dimensional paper chromatography (TDPC) was carried out on Whatman (1MM) for comparative studies of leaves extracts under investigation using BAW for the first dimension, followed by 15% AcOH for the second dimension.

For separation and purification of mixture of the flavonoid compounds, the elution techniques on Whatman paper (3MM) were used. After the material under investigation was applied to the paper chromatography, the latter was run using solvent system No: 1-4 as outlined in table (1). All glass chromatographic tanks were used applying the descending techniques. The developed chromatograms were air – dried and examined before spraying under both visible and UV light (λmax= 366 and 245nm). The chromatograms were exposed to ammonia vapours for about 2-3 seconds and immediately re-examined the chromatograms to observe any possible changes that may eventually appear in colour or fluorescence under a long wave UV lamp.

3.2.2 Column Chromatography

The separation and identification of plant extracts were carried out by column chromatographic techniques (CC) (Markham, 1982). Polyamide and Sephadex columns (glass columns 130x9cm and 100x4.5cm) were used for the separation and purification of flavonoidal mixtures. Fractions produced from a large column often yielded simple mixtures of flavononids. Further separation on small column (glass columns 50x3cm and 30x2cm) or by paper chromatography, was carried out to obtain a pure flavonoid compound.

1- Polyamide Column Chromatography

Polyamide adsorbent of the caprolactam type (MN-polyamide SC 6, Macherey Nagel, Riedal-De-Haen Ag, Sellze-Hannover, Germany) was made into slurry with water and slowly poured into a glass column (130x9cm). Water was allowed to drip through the column to make sure that the adsorbent was firmly packed. The sample was dissolved in a minimum amount of the eluting solvent (water) and applied to the top of column, then elution initiated with addition of excess water. The polarity was changed by increasing the percentage of methanol gradually untill 100% methanol which employed to the final elution of the column. Fractions were further fractionated using elution techniques on paper chromatography (Mabry et al., 1970 and Markham & Mabry, 1975). Similar fractions were combined and tested for purity of their flavonoid compounds. This column was used for separation of the flavonoid compounds.

2- Sephadex Column Chromatography

Sephadex LH-20 (Pharmacia) column was used for the final purification of each individual flavonoid compounds before being subjected to chemical and
spectral analysis (Johnston, et al., 1968). Sephadex adsorbent was soaked overnight in 100% methanol and then packed in a small column (2x50cm). Methanolic solution of a flavonoid was applied carefully on the top of the column and elution was carried out with 80% to 100% methanol, the non flavonoid impurities were eluted first and the migration of pure flavonoid was detected in UV light as dark purple or brown bands (Liu et al., 1989).

3.3. Identification Techniques of Flavonoids

The qualitative analysis of flavonoid glycosides involves three distinct methods:

1- Identification of the aglycone.
2- Identification of the sugar moiety.
3- Identification of the point of the sugar attachment.

This is usually carried out through both chemical and physical investigation as indicated below.

3.3.1. Chemical Analysis

Analysis to determine the nature of aglycones and sugars was carried out according to standard procedures performed by Mabry et al., (1970) and Harborne (1984).

3.3.1.a. Complete Acid Hydrolysis

Complete acid hydrolysis was carried out for 40-60 min. at 100°C using 5ml 2N-hydrochloric acid. The hydrolysate was then extracted with ethyl acetate, and the received extract was subjected to paper chromatographic investigation to detect the aglycones. The mother liquor was carefully neutralized, and then subject to paper chromatographic investigation to detect the sugars.

• Identification of the Aglycone

Identification of the aglycone moiety of flavonoid glycosides was based on direct co-chromatographic comparison with standard sample, UV spectroscopy followed by MS and NMR if needed.

• Identification of the Sugar

Sugars obtained by hydrolysis of the flavonoid glycosides were identified by paper chromatography. Chromatograms were developed in the upper phase of BBPW (Benzene, 1: n-butane, 5: pyridine, 3: water, 3) with a standard sugar mixture. The dried chromatograms were sprayed uniformaly with aniline phthalate. The dried chromatograms were heated at 1050°C in an oven for about 10 min., and the colored sugar spots were observed at visible light. The Rf values of unknown sugars were compared directly with those of standard sugars.
3.3.1.b. Enzymatic Hydrolysis

2mg of flavonoid glycosides were dissolved in 2 ml of 0.05N acetate buffer (pH 5) to which 1-2 mg of the appropriate enzyme for example, β-glucosidase was added. The mixture was incubated at 370C for 24 hours. Samples were taken after 5 hours and finally after 24 hours, and then chromatographed to test for any intermediates (Mabry et al., 1970).

3.3.2. Physical Analysis

Complete elucidation of the flavonoids should include physical methods such as ultraviolet (UV) and nuclear magnetic resonance (1H-NMR and 13C-NMR) measurements.

3.3.2.a. Ultraviolet Spectroscopic Analysis

Ultraviolet visible absorption spectroscopy is perhaps the most useful technique available for flavonoid structure analysis using UV-visible spectrophotometer (Shimadzu model UV-240 and 2401 PC). The technique is used to aid both identification of the oxygenation and glycosylation patterns (Mabry et al., 1970 and Markham, 1982). The flavonoid spectrum is usually determined in methanol using 2ml quartz cells. The spectrum typically consists of two absorption maxima at the ranges 240-285 nm (band II) and 300-550 nm (band I). The precise position and relative intensities of these maxima give valuable information on the nature of the flavonoid and its oxygenation pattern; the effects brought about by these changes are as follows:

1- Changes in the substitution of the A-ring tend to be reflected in the band II absorption while alternations in the substitution of the B-ring and C-ring tend to be more apparent from the band I absorption.

2- Additional oxygenation (especially hydroxylation) generally causes a shift of the appropriate band to longer wavelength, e.g. band I in 3,5,7,-tri OH flavone, 359 nm; 3,5,7,4\-tetra OH flavone, 367 nm; 3,5,7,3\,4\,-penta OH flavone, 370 nm and 3,5,7,3\,4\,5\,-hexa OH flavone, 374 nm.

3- Methylation or glycosylation (especially of 3, 5, 7, and 4\ hydroxyls) causes band shifts to shorter wavelength. The nature of the sugar in glycosides is normally of no consequence.

4- Acetylation tends to nullify the effect of a phenolic hydroxyl group on the spectrum.

5- The presence of cinnamic acid as acyl function on a flavonoid can be detected by the presence of an absorption band at 320 nm in flavonoids that themselves lack significant absorption at this region (e.g. anthocyanins).

6- In flavones and flavonols, the presence of 3\, 4\,-di-OH system is generally evidenced by a second peak (sometimes a shoulder) in band II.
3.3.2.b. 1H-NMR spectroscopy

The NMR spectroscopy is a well-established method for structure elucidation of flavonoids (Mabry et al., 1970) using a Jeol Ex-500 spectroscopy; 500MHz (1NMR), 125 MHz (13C-NMR) or Joel JNM-EX 270 spectroscopy; 270 MHz (1H-NMR), 67.5 MHz (13C-NMR).

There are widely applied techniques for obtaining 1H-NMR spectra, using DMSO-d6 (hexa deutro dimethyl sulphoxide) as a solvent for the direct 1H-NMR analysis or conversion of the flavonoids to trimethylsilyl ether (TMS ether) derivatives.

1H-NMR spectra yield three sets of information: the integrals, the coupling pattern and the chemical shifts.

1- The integrals define the number of protons represented by each signal or group of signals.

2- The coupling patterns reflect the mutual arrangement of the coupling protons. In aromatic compounds the coupling constants are 7-9 Hz between ortho protons, 1-3 Hz between meta protons and less than 1 Hz between para protons. The Para coupling, however, is usually not resolved, but causes broadening of the single only which may in turn obscure a meta coupling.

3- The chemical shift of a proton indicates its chemical environment i.e. its position relative to any other part of the molecule. Chemical shift values are nowadays almost exclusively given parts per million (ppm) downfield from the TMS signal (Harborne, 1993). i.e. 1H-NMR analysis has been used to determine the aglycone part of the isolated flavone glycosides, to decide the number of sugars in the flavone O-glycosides, and the positions of the linkages of the sugar units in the flavone C-glycosides.

The great number of published 1H-NMR spectra of flavonoids simplified the chemical shift assignments of the protons in the aromatic region of the flavonoid (Table 2). It also helps to decide the number of sugar units in the flavonoid O-glycosides, since the chemical shifts of the anemic protons are more downfield than the other sugar protons.

For structure elucidation of flavonoid C-glycoside, measurements have been made on both the TMS ether derivatives and on the parent flavonoid C-glycoside in DMSO-d6. The latter method had a few advantages, for example it is difficult to completely convert the flavone 6, 8-di-C-glycosides into the TMS ether derivatives (Mabry et al., 1970), and the same solvent could be used for both 1H and 13C-NMR spectroscopy. When only small amounts of a sample were available, the C-glycoside was dissolved in DMSO-d6 and submitted to 1H-NMR analysis followed by permethylation according to the mass spectroscopy analysis. By holding a high temperature (800°C) when recording the 1H-NMR spectrum, the signal obtained from the absorbed water was minimal.
Table (2): Approximate chemical shifts of various flavonoids proton types.

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>Proton type</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tetramethylsilan (Reference)</td>
</tr>
<tr>
<td>0 - 0.5</td>
<td>Trimethylsilyl ether group</td>
</tr>
<tr>
<td>c.1.0</td>
<td>Rhamnose C-CH3 (broad doublet)</td>
</tr>
<tr>
<td>c.1.7</td>
<td>Prenyl (-CH2-CH=C(CH3)2 methyl groups</td>
</tr>
<tr>
<td>c.2.0</td>
<td>Acetate (-OCOCH2) and aromatic C-CH2</td>
</tr>
<tr>
<td>2 – 3</td>
<td>H-3 of flavanones (two proton – multiplet)</td>
</tr>
<tr>
<td>3.5 - 4.0</td>
<td>Most sugar protons</td>
</tr>
<tr>
<td>4.2 - 6.0</td>
<td>H-1 of sugars (also H-2 of dihydroflavanolos, 5.0 ppm &amp; H-2 of flavanones 5-5.5 ppm)</td>
</tr>
<tr>
<td>c 6.0</td>
<td>Methylenedioxy (O-CH2-O) singlet</td>
</tr>
<tr>
<td>6.0 - 8.0</td>
<td>A and B- ring protons</td>
</tr>
<tr>
<td>7.5 - 8.0</td>
<td>H-2 of isoflavones (singlet)</td>
</tr>
<tr>
<td>12 – 14</td>
<td>5-OH (observed only when using DMSO- d6)</td>
</tr>
</tbody>
</table>

3.3.2.c. 13C-NMR Spectroscopy

13C-NMR provides a very sensitive method for determination of the structure of complex compounds. With the introduction of Fourier-transform methods, 13C-NMR spectroscopy has developed into a powerful tool for the structure elucidation of small amounts of natural products. Over the past thirty years, 13C-NMR spectroscopy has become well established in the structure analysis of flavonoids. Several articles have been published. Most of them have dealt with flavonoid aglycones (Chari et al., 1977; Markham et al., 1978 and Shen et al., 1993) and only a few have dealt with C-glycosides (Chopin et al., 1978 and Agrawal, 1989). The position of a signal relative to the TMS reference (0.0 ppm) is a good guide to the type of carbon represented. This position is shifted markedly by nearby substituents. Such effects are predictable in their extent and have led to the formulation of substituent effect data, which defines the expected effect (on aromatic carbon resonance) of introducing a new substituent into an aromatic ring. Using this type of substituent effect data, it is possible to calculate with great accuracy the spectrum of an unknown flavonoid form. To do this it is necessary to have available a wide range of reference spectra and a number of such complications have appeared.
In general terms sugar-related substitution include shifts are as follows:

1- O-glycosylation of a flavonoid hydroxyl produces an upfield shift of up to 2 ppm in the signal of the adjacent flavonoid carbon and downfield shifts in the signal of the ortho and especially para related carbons (1-4 ppm).

2- C-glycosylation of a flavonoid aglycone causes a 10 ppm downfield shift of the signal of the glycosylated carbon, but leaves other signals relatively unaffected.

3- Glycosylation of sugar by glucose causes an 8 ppm downfield shift in the signal of the glycosylated carbon accompanied by a 1-3 ppm upfield shift in the signal of the adjacent carbons. Glycosylation by rhamnose causes smaller shifts (3-6 ppm downfield and 1-2 ppm upfield, respectively).

3.3.3. Mass Spectrometry

Mass spectral data provide structural information on flavonoids and are used to determine molecular masses and to establish the distribution of substituents between the A- and B- rings. A careful study of fragmentation patterns can also be of particular value in the determination of the nature and site of attachment of the sugars in O- and C- glycosides (Andersen & Markham, 2006). EI-MS, model Finnigan-Mat SSQ 7000 spectrometer and ESI-MS: LCQ Advantage Thermo Finnigan spectrometers were used.

Results and discussions

1. Separation of Flavonoids and Phenolic acids

Flavonoids and phenolic compounds more concentrated in the plant leaves than in the other part which was cleared from the phytochemical study so that, these active constituents were separated from the leaves of Lycium schweinfurthii var. schweinfurthii. two compounds were separated from chloroform fraction and three compounds from methanol 70% fraction of the leaves.

Results of separation techniques clearly indicated that, the chloroform fraction and methanol 70% fraction were the most rich by phenolic constituents

Chloroform fraction when subjected to preparative paper chromatography (3MM) using system BAW (4:1:5) for 24 hours, gives two major bands. The bands were eluted separately with hot methanol / H2O (1:1v/v). to give (com1 & com2).

Fractionation of the methanol 70% extract on polyamide column using H2O/ethanol system revealed the presence of two main fractions containing flavonoids were collected from the column.

• Fraction No.(1) :

When fraction no.1 subjected to rechromatography on preparative paper chromatography (3MM) using system AcOH 15 % for 8 hours gives one major band. The band was eluted with hot methanol / H2O (1:1v/v). to give (com3).
• Fraction No.(2) :
  
  When fraction no.2 subjected to preparative paper chromatography (3MM) using system BAW (4:1:5) for 24 hours, gives two major bands. The bands were eluted separately with hot methanol / H2O (1:1v/v).to give (com4&com5).

1.1. Identification of com 1

  Band no. 1 when eluted with hot methanol / H2O (1:1v/v), dried under reduced pressure, purified on sephadex LH-20 column using methanol/water as described by Johnston et al., (1968) gave com 1. Two dimension paper chromatography of the pure compounds using system BAW (4: 1: 5) and AcOH-15% revealed the presence of one spot of flavonoid nature.

  The purified compound com1 was shown to be as amorphous powder, soluble in methanol and its $R_f$-values and color reactions were outlined in table (3).

Table (3): $R_f$-values and color reaction of the compound 1.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$R_f$-value</th>
<th>Reagent</th>
<th>Visible</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>B:A:W</td>
<td>0.87</td>
<td>Untreated</td>
<td>—</td>
<td>Deep purple</td>
</tr>
<tr>
<td>AcOH-15%</td>
<td>0.34</td>
<td>NH3</td>
<td>—</td>
<td>Greenish purple</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlCl3</td>
<td>—</td>
<td>Greenish purple</td>
</tr>
</tbody>
</table>

From $R_f$-values and color reaction of compound 1 same to be aglycone.

UV spectral data, $\lambda_{max}$ nm, in MeOH:

  The obtained results of UV spectral data (Figs. 1, 2& 3 ) were:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_{max}$ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>291, 323 (sh)</td>
</tr>
<tr>
<td>NaOMe</td>
<td>245, 266 (sh), 325</td>
</tr>
<tr>
<td>NaOAc</td>
<td>291 (sh), 328</td>
</tr>
<tr>
<td>NaOAc + H$_3$BO$_3$</td>
<td>300, 328 (sh)</td>
</tr>
<tr>
<td>AlCl$_3$</td>
<td>310 (sh), 376</td>
</tr>
<tr>
<td>AlCl$_3$ + HCl</td>
<td>310 (sh), 376</td>
</tr>
</tbody>
</table>

$^1$H-NMR spectral data (Fig4)

  The spectrum of the compound com1 in DMSO, showed signals at: $\delta$ ppm 2.6 (Cis and trans, 2H, dd, J= 17Hz, H-3), 5.2 (1H, dd, J= 5, 11Hz, H-2), 5.8 (1H, d, J= 2.5Hz, H-6), 5.8 (H, d, J= 2.5Hz, H-8), 6.8 (2H, dd, J= 8.5, 2.3Hz, H-3\ and H-5\), 7.2 (2H, dd, J= 8.5, 2.3Hz, H-7\ and H-6\).

$^{13}$C-NMR Spectrum (Fig5)

  $\delta$ (ppm) 78.5 (C-2), 42.4 (C-3), 196.4 (C-4), 164.1 (C-5), 164 (C-1), 95.9 (C-6), 167.1 (C-7), 94.7 (C-8), 153.5 (C-9), 101.8 (C-10), 128.9 (C-1\), 127.7 (C-2\, 6\), 114.9 (C-3\, 5\), 157.6 (C-4\).
**Fig. (1): UV spectrum of com1**

in MeOH and NaOMe
Fig. (2): UV spectrum of com1 in NaOAc and H$_3$BO$_3$
Fig. (3): UV spectrum of com1 in AlCl₃ and HCl
UV spectral data $\lambda_{\text{max}}$, nm of compound (1) in methanol gave a major absorption peak at $\lambda_{\text{max}}$ 291 and a minor one at $\lambda_{\text{max}}$ 323 (characteristic to dihydro flavones) (Figs. 1).

On addition of NaOMe it gave a bathochromic shift in the major absorption peak (+34nm) indicated the presence of free OH group at C-5 and C-4.

On addition of NaOAc it gave a bathochromic shift in the major absorption peak (+37nm) pointed to the presence of free OH group at position 7, which not affected after addition of H$_3$BO$_3$ indicating the absence of ortho dihydroxy group.

A bathochromic shift in the major (+19nm) with addition of AlCl$_3$ which not affected by the addition of HCl, indicated the presence of free 5 hydroxyl group and absence of ortho-dihydroxyl groups.

From data of 1H-NMR spectrum of the compound 1, the presence of one doublet of doublet signal at $\delta$ 5.2ppm ($J$= 5.11Hz) representing the proton at position 2. The presence of one doublet of doublet signal at $\delta$ 2.6ppm ($J$= 17Hz) representing proton at position 3. The presence of doublet signals at $\delta$ 6.8ppm ($J$= 8.5, 2.3Hz) representing protons at carbon 3\, 5\ and the presence of another 2 doublet of doublet signals at $\delta$ 7.2ppm representing protons at carpon 2\, 6\. The presence of 2 doublet signals at $\delta$ 5.8ppm ($J$= 2.5Hz) representing protons at position 6 and 8.

From the above data UV analysis, 1H-NMR and 13C-NMR spectrum indicated that the compound 1 is 5,7,4\-trihydroxy-2,3-dihydro flavone (Naringninin).
Fig. (4): $^1$H-NMR spectrum of com1.
Fig. (5): $^{13}$C-NMR spectrum of com 1.
1.2. Identification of com 2

Band no. 2 when eluted with hot methanol / H2O (1:1v/v), dried under reduced pressure, purified on sephadex LH-20 column using methanol/water as described by Johnston et al., (1968) gave com 2. Two dimension paper chromatography of the pure compounds using system BAW (4: 1: 5) and AcOH-15% revealed the presence of one spot of flavonoid nature.

The purified com 2 visualized as pale yellow color under UV, which was changed when treated with ammonia or ferric chloride reagents to bright yellow color.

Table (4): Rf-values and color reaction of the com2.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Rf-value</th>
<th>Reagent</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>B:A:W</td>
<td>0.79</td>
<td>Untreated</td>
<td>—</td>
</tr>
<tr>
<td>AcOH-15%</td>
<td>0.26</td>
<td>NH3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlCl3</td>
<td>—</td>
</tr>
</tbody>
</table>

UV spectral data, \(\lambda_{max}\) nm, in MeOH:

The obtained results of UV spectral data (Figs. 6, 7 &8) were:

- MeOH : 255,358.
- NaOMe : 275▲, 400▲.
- NaOAc : 265,360.
- NaOAc + H\(_3\)BO\(_3\) : 265,360.
- AlCl\(_3\) : 270▲, 305(sh.), 355, 405▲.
- AlCl\(_3\) + HCl : 270▲, 305(sh.), 355, 405▲.

\(^1\)H-NMR spectral data (Fig. 9)

The \(^1\)H-NMR spectrum of compound 2 in DMSO showed signals at 8(ppm) 7.6(1H, d, J= 8.4Hz, H-2’ ), 7.8 (1H, d, J= 8.4Hz, H-6’), 6.8 (1H, d, J= 2.3Hz, H-5’), 6.4 (1H, d, J= 2.5Hz, H-8), 6.20 (1H, d, J= 2.5Hz, H-6). 3.9 (3H, s, OCH\(_3\)) and 3.8 (3H, s, OCH\(_3\)).

The obtained purified compound 2, was colorless on visible, and pale yellow in color when treated with ammonia as well as AlCl\(_3\).

UV spectral data of compound 2 in methanol showed two absorption bands at 255 and 355 nm, which were typical of flavone aglycone (Herborne, 1984). Addition of NaOMe induced no bathochromic, which indicated the presence of substitution in 4’-position. Addition of AlCl\(_3\) induced a bathochromic shift which unaffected with addition of HCl, which suggested the absence of catcholic hydroxyl.

Addition of NaOAc induced bathochromic shift in band II, which proving that 7-position was free hydroxyl, where addition of H\(_3\)BO\(_3\) induced no shift, which indicated further confirmation for the absence of catcholic hydroxyl groups.

Hence compound 2 was probably flavone with 3-hydroxy groups and 3, 4’ position substitution.
The structure of compound 2 was further confirmed by $^1$H-NMR spectrum of (Fig. 9), which showed signals at $\delta$ 7.8 (1H, d, $J=8.5$Hz, H-2$^\parallel$), 7.6 (1H, d, $J=8.5$Hz, H-6$^\parallel$), 6.8 (1H, d, $J=8.4$Hz, H-5$^\parallel$), 6.4 (1H, d, $J=2.5$Hz, H-8), 6.2 (1H, d, $J=2.5$Hz, H-6), 3.9 (3H, s, oCH$_3$) and 3.8 (3H, s, oCH$_3$). Thus from $R_f$, Color reaction, UV, and $^1$H-NMR spectrum data compound 2 identified as 3-hydroxy-3$^\parallel$,4$^\parallel$ methoxy flavone.

![3-hydroxy-3\parallel,4\parallel methoxy flavone](image_url)
Fig. (6): UV spectrum of com2 in MeOH and NaOMe
Fig. (7): UV spectrum of com2 in NaOAc and $H_3BO_3$
Fig. (8): UV spectrum of com2 in AlCl₃ and HCl
Fig (9): $^1$H-NMR spectrum of com2.
1.3. Identification of com 3

Band resulted from sub fraction no1 when eluted with hot methanol / H2O (1:1v/v), dried under reduced pressure, purified on sephadex LH-20 column using methanol/water as described by Johnston et al., (1968) gave com 3. Two dimension paper chromatography of the pure compounds using system BAW (4:1:5) and AcOH-15% revealed the presence of one spot of flavonoid nature.

The purified com3 was showed to be as amorphous powder, soluble in methanol and its R_f-values and color reactions were outlined in table (5).

Table (5):R_f-values and color reaction of the com3.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R_f-value</th>
<th>Reagent</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>B:A:W</td>
<td>0.85</td>
<td>Untreated</td>
<td>yellow</td>
</tr>
<tr>
<td>AcOH-15%</td>
<td>0.27</td>
<td>NH_3</td>
<td>orange/yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeCl_3</td>
<td>orange/yellow</td>
</tr>
</tbody>
</table>

From R_f-values and color reaction of com3 seemed to be aglycone.

UV spectral data, λ_max, nm of com3 (Fig. 10, 11 & 12) were:

- MeOH : 250, 268 (sh), 308
- NaOMe : 265, 308 (sh), 360
- NaOAc : 268, 308 (sh), 358
- NaOAc + H_3BO_3 : 255 (sh), 271 (sh), 309
- AlCl_3 : 249, 308
- AlCl_3 + HCl : 250, 308

^1H-NMR data of com3 in DMSO (Fig.13)

^1H-NMR data of the compound 3 showed the following resonance: δ, ppm 6.9 (1H, s, H-3), 7.4 (1H, dd, J= 2.1Hz, H-6), 7.6 (1H, d, J= 2.4Hz, H-8), 7.55 (3H, m, ring B), 8 (2H, m, ring B), 8.1 (1H, d, J= 8.5Hz, H-5).

Mass spectrum (Fig. 14)

The mass spectrum of com 3 revealed the presence of a molecular ion peak at m/z 238 (100%) (aflavone bearing one hydroxyl group), other molecular ion peak at m/z at 221, two fragment at m/z 120 and 102.

UV spectral analysis in methanol gave band I at 308nm and band II at 250nm, (characteristic that flavone in nature). Bathochromic shift of band I (+50nm) with decrease intensity upon addition of NaOMe indicating the absence of 4'-hydroxyl group.

Bathochromic shift in band II (+18nm) on addition of NaOAc indicating the presence of free OH group at position 7. Addition of H_3BO_3 gave no shift indicating the absence of ortho dihydroxyl group (3', 4' positions) in B ring.
No shift occurring on addition of AlCl$_3$, which indicates the absence of free OH at position 5, also on addition of HCl gave no shift indicating the absence of ortho dihydroxyl group at B ring.

The structure of com 3 was further confirmed by $^1$H-NMR spectrum of (Fig. 13), which showed The hydroxylation at C-7 indicated by the ortho-coupling between H-5 and H-6 and the meta-coupling between H-6 and H-8, thus H-5 and H-8 appeared as 2 doublet at $\delta$ 8.1 and 7.6, respectively. While H-6 appeared as a doublet of doublet at $\delta$ 7.4. The absence of free OH at C$_4$ was indicated by the multiplet singlet at $\delta$ 7.55 and 8ppm for H-4, 3, 5 and H-2, H-6 respectively.

From the previous data the com3 was identified as 7-hydroxy flavone.
Fig. (10): UV spectrum of com3 in MeOH and NaOMe.
Fig. (11): UV spectrum of com3 in NaOAc and $\text{H}_3\text{BO}_3$
Fig. (12): UV spectrum of com3 in AlCl$_3$ and HCl
Fig. (13): $^1$H-NMR spectrum of com3.
Fig. (14): Mass spectrum of com3.
1.4. Identification of com 4

Band no 1 resulted from sub fraction no 2 when eluted with hot methanol / H2O (1:1v/v), dried under reduced pressure, purified on sephadex LH-20 column using methanol/water as described by Johnston et al., (1968) gave com 4. Two dimension paper chromatography of the pure compounds using system BAW (4: 1: 5) and AcOH-15% revealed the presence of one spot of flavonoid nature.

The purified com 4 visualized as brown color under UV, which was changed when treated with ammonia to yellow color and unchanged when treated with ferric chloride reagents.

Table (6): Rf-values and color reaction of the com4.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Rf-value</th>
<th>Reagent</th>
<th>Color</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>B:A:W</td>
<td>0.43</td>
<td>Untreated</td>
<td>—</td>
<td>Brown</td>
</tr>
<tr>
<td>AcOH-15%</td>
<td>0.29</td>
<td>NH3</td>
<td>—</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlCl3</td>
<td>—</td>
<td>Brown</td>
</tr>
</tbody>
</table>

UV spectral data, λmax nm, in MeOH (Figs. 15, 16 & 17)

The obtained results of UV spectral data were:

MeOH     : 273, 330.
NaOMe    : 285▲, 333(sh.), 400▲.
NaOAc    : 282▲, 305, 385▲.
NaOAc + H3BO3 : 272,330.
AlCl3    : 280▲, 305, 345▲, 385.
AlCl3 + HCl : 280,305,345, 385.

1H-NMR spectral data Fig (18)

The 1H-NMR spectrum of com 4 in DMSO showed signals at δ(ppm) 7.9(2H, d, J= 8Hz, H-2, H-6), 6.9 (2H, d, J= 8Hz, H-3 and H-5\'), 6.7(s, H-6) and 4.55(1H, d, J= 8Hz, H-1\glucose), 3.2-3.8(m, glucose proton).

The purified com4 appeared on paper chromatography as brown spot changed to yellow, when subjected to ammonia vapor under UV light. Rf values and color reaction was within the range of C-glycoside.

The UV spectral data of com4 showed band I at 330 and band II at 273 nm. Which indicated that com 4 was flavone (Liu et al., 1989).

Addition of NaOMe induced bathocromic shift, which indicated that 4\'-position was free hydroxyl, while addition of AlCl3 induced bathocromic shift in band I, addition of HCl case no shift, which proved the absence of any catecholic hydroxyl groups.

Addition of NaOAc induced bathocromic shift for band II, which indicated that 7-position was free hydroxyl, while addition of H3BO3 induced no positive shift for band I, which indicated the absence of any catecholic hydroxyl.
Complete acid hydrolysis of com 4 using 2N HCl at 100ºC for 4 hours, showed that com 4 resisted acid hydrolysis for 4 hours, and indicated that com4 is C-glycoside.

Further confirmation was achieved through ^1^H-NMR, which showed the absence of H-8 signal proton and presence of anomeric sugar proton at δ4.55 (^1^H, d, J=8Hz, H-1^1^ glucose) and remaining sugar protons multiple at δ3.1-3.9 (m, glucose proton).

From R_f, UV data, acid hydrolysis and ^1^H-NMR spectral data of com4 confirmed that com4 was vitexin.

vitexin.
Fig. (15): UV spectrum of com4 in MeOH and NaOMe
Fig. (16): UV spectrum of com4 in NaOAc and H$_3$BO$_3$.  

Absorbance

Wavelength (nm)

MeOH
NaOAc
NaOAc + H$_3$BO$_3$
Fig. (17): UV spectrum of com4 in AlCl₃ and HCl
Fig. (18): $^1$H-NMR spectrum of com4.
1.5. Identification of com 5

Band no 2 resulted from sub fraction no 2 when eluted with hot methanol / H2O (1:1v/v), dried under reduced pressure, purified on sephadex LH-20 column using methanol/water as described by Johnston et al., (1968) gave com 5. Two dimension paper chromatography of the pure compounds using system BAW (4: 1: 5) and AcOH-15% revealed the presence of one spot of flavonoid nature.

The purified com 5 visualized as Deep purple color under UV, which was changed when treated with ammonia to Yellowish green color and unchanged when treated with ferric chloride reagents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R_f-value</th>
<th>Reagent</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>B:A:W</td>
<td>0.50</td>
<td>Untreated</td>
<td>Deep purple</td>
</tr>
<tr>
<td>AcOH-15%</td>
<td>0.79</td>
<td>NH3</td>
<td>Yellowish green</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeCl3</td>
<td>Deep purple</td>
</tr>
</tbody>
</table>

Acid hydrolysis:

Acid hydrolysis of com5 by 0.1NHCl showed that rhamnose as sugar moiety detected by comparing its R_f values with authentic pure sugars then acid hydrolysis by 0.2NHCl showed that aglycone and two sugar residues identified as glucose and rhamnose detected by comparing its R_f-values with authentic pure sugars. This results which confirmed that the glucose moiety is directed to aglycone part and rhamnose is terminaly.

Enzymatic hydrolysis:

Enzymatic hydrolysis with β-glycosidase gave negative result which confirmed that the glucose moiety is directly linked to aglycone part and rhamnose is terminaly.

UV spectral data, λ_max, nm of com5 (Fig.19, 20 & 21) were:

The obtained results of UV spectral data were:
- MeOH : 286, 326 dec.
- NaOMe : 244 (sh), 284, 359
- NaOAc : 283, 335
- NaOAc + H_3BO_3 : 283, 328
- AlCl_3 : 308, 385
- AlCl_3 + HCl : 305, 383

^1H-NMR spectrum of com 5(Fig. 22)

The ^1H-NMR spectrum of the com 5 in DMSO showed that, signals at δ ppm 1.08 (3H-, s, CH_3 of rhamnose), 3.8 (3H, s, methoxyl protons), 4.5(1H, d, J= 6.5HZ, protons of rhamnose), 5.07 (1H, d, J= 6.6Hz, protons of glucose), 5.3 (1H,
s, H-2), 6.69 (1H, d, J= 3.4Hz, H-6), 6.7 (1H, d, J= 3.4Hz, H-8), 6.8 (1H, s, H-3), 7.1 (1H, d, J= 9Hz, H-5’), 7.45 (1H, d, J= 3.6Hz, H-6’), 7.5 (1H, s, J= 8.5Hz, H-2’).

From UV spectral data of com 5 in methanol exhibit one major band II at $\lambda_{\text{max}}$ 286 and a minor one band I at $\lambda_{\text{max}}$ 326 (Characteristic range of flavonones).

No shift occured in band II on addition of NaOMe indicated the presence of substituted OR group at position 4’ in β-ring.

No shift occured in band II on addition of NaOAc indicated the presence of substitution in position 7 which not affected by the addition of boric acid which indicating the absence of dihydroxyl group at β-ring.

Bathochromic shift (+22) in band II with addition of AlCl$_3$ which not affected by the addition of HCl indicating the presence of free hydroxyl group in position 5 and absence of ortho-dihydroxyl group.

From data of $^1$H-NMR spectrum presence of one s signal at δ 5.3ppm and δ 6.8ppm representing the proton at positions 2 and 3 respectively (Compound F$_7$ is flavonone).

The presence of 4’-methoxy group due to the presence of s signal at δ 7.5ppm representing protons at carbon 2’, doublet signals at δ 7.45ppm representing protons at 5’ and doublet signals representing protons at 6’.

The presence of 2 anomeric protons (Protons of glucose and protons for rhamnose) at 5.07ppm and 4.5ppm, also the presence of multiplet protons of disaccharide in the rejoin 3.2, 3.6ppm.

The 3 proton of methyl group characteristic to rhamnose which present at δ 3.8ppm where the glucose moiety directly attached to the aglycone. These is confirmed also from acid hydrolysis.

From the previous data the com5 was found to be hisperidine (hisperitine-7-rutinoside).
Fig. (19): UV spectrum of com5 in MeOH and NaOMe
Fig. (20): UV spectrum of com5 in NaOAc and H₃BO₃
Fig. (21): UV spectrum of com5 in AlCl$_3$ and HCl
Fig. (15): $P_1$-$R_{H-NMR}$ spectrum of compound F

Fig. (22): $^1$H-NMR spectrum of com5.
**Conclusion:**

From the methanolic extract of *Lycium schweinfurthii var. schweinfurthii leaves* a five flavonoids were isolated and purified using different chromatographic techniques. These compounds were identified via spectroscopic tools: UV, 1H NMR, $^{13}$C-NMR and MS spectroscopy.

**References**


Siddiqi, M. A. (1978) Flora of Libya . Al Faateh University, Tripoli. 62: 1