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Phytochemical studies on *Lycium schweinfurthii var. schweinfurthii* (Solanaceae) and Isolation of five Flavonoids from leaves

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Abstract
Preliminary phytochemical screening in different parts (root, stem, leaves and flowers) of *Lycium schweinfurthii var. schweinfurthii* revealed the presence of alkaloids, saponines, glycosides, resins, cardiac glycosides, phenol, sterols, tannins, flavonoids and amino acids. The percentage of total (flavonoids, tannins, saponins and alkaloids) increased in plant leaves than other part. The investigation of phenolic compounds in *L. schweinfurthii var. schweinfurthii* leaves by different chromatographic methods of separation showed the presence of quercetin, kaempferol, gallic acid**,** ferulic acid, and apigenin. The structures of these flavonoids were characterized on the basis of their, UV, NMR and MS spectroscopic data.

Keywords: *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) [31], 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) [31]. The nightshade family is certainly one of most economically important plant families as food (e.g. potato and tomato genus), medicine such as Atropa belladonna, Datura stramonium, Solanum nigrum which has been extensively used traditionally to treat various ailments such as inflammation and fever (Acharya Zakaria *et al*., 2006), ornaments (e.g. Physalis Alkekengi), and several noxious weeds (Siddiqi, 1978; Hickey and King, 1981; Hussein, 1985) [32, 16, 18]. The family contains a wide range of alkaloids, saponins, coumarins, flavones, carotenoids and anthraquinones (Evans, 2002) [11]. Various compounds have been identified which are responsible for diverse biological activities (Jain *et al*., 2011) [19]. 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 idendification of plant materials was confirmed by Cairo University herbarium.

2. Materials for Chromatography
- Sheets of Whatman filter paper 1MM for paper chromatography (P.C.) (Whatman Ltd. Maidstone, Kent, England).
- Sheets of Whatman filter paper 3MM for paper chromatography (P.C.) (Whatman Ltd. Maidstone, Kent, England).
- Polyamide 6S (Riedel-De-Haen AG, Seelze 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).

Table 1: Solvents system used for paper chromatography Authentic Reference Materials

No.	Symbol	Composition	Percent by volume
1	BAW	n-Butanol/acetic acid /water	4:1:5(upper phase)
2	H_2O	Water	-
3	15% AcOH	Acetic Acid / Water	15:85
4	50% AcOH	Acetic Acid / Water	50:50
5	PhW	Phenol / Water	80:20 (w/v)
6	BBPW	Benzene/n-butanol/ Pyridine/H2O	1:5:3:3 (upper layer)

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) [27].

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) [27].

Reagents for Ultra-Violet Spectroscopic Analysis of flavonoids (Mabry *et al*., 1970) [25]

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. Preliminary Phytochemical Screening

Tests for alkaloids, glycosides, resins, Cardiac glycosides, saponins, phenol, sterols, terpenes, tannins, flavonoids, Amino acids and Volatile oil in root, stem, leaves and flower of L. schweinfurthii.

3.1. Test for Alkaloids (Woo *et al*., 1977) [34]

The alcoholic extracts for root, stem, leaves and flowers of the plant were concentrated under vacuum till dryness. The dried extracts was dissolved in 2N-hydrochloric acid on a water bath, shaken and filtered, the obtained filtrates was shacked with chloroform to remove undesirable matters.

The acidic aqueous layer was adjusted to alkaline pH with ammonia and the liberated alkaloid bases were extracted by chloroform till exhausted and then tested by Mayer's and Dragendorff's reagent. The presence of color or precipitation indicated the presence of alkaloids.

Reagents for Alkaloids (Balbaa *et al*., 1981) [7]

a) Wagner's reagent (Potassium tri-iodide)

Iodine : 1.3g

Potassium iodide : 2.0g

Water to make : 100ml

b) Dragendorrf's reagent (Potassium bismuth iodide)

- **Solution (A):** 1.7g of bismuth subnitrate and 20g tartaric acid were dissolved in 80ml water.
- **Solution (B):** 16g potassium iodide were dissolved in 40ml water
- **Stock solution:** 1:1 (v/v) mixture of (A) and (B) was freshly prepared for spraying.
- **Spray reagent:** 5ml of stock solution were added to a solution of 10g tartaric acid in 50ml water.

3.2. Test for Glycosides

a) Glycosides Test (Treare and Evans, 1985)

To small amount of extract, add 1 ml water and shake well. Then aqueous solution of NaOH was added. Yellow color appeared that indicates the presence of glycosides.

b) Modified Borntrager's Test (Treare and Evans 1985):

Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink color in the ammoniacal layer indicates the presence of anthranol glycosides.

3.3. Test for Resins (Balbaa, 1986) [6]

About 1 gm of root, stem, leaves and flower of *L. schweinfurthii var. schweinfurthii* was shacked with 10ml light petroleum ether for 1 minute, filtered and then 10ml of 10% copper acetate solution were added to 5ml of the filtrate. It was then shacked for few minutes and left to be separated into two layers. The petroleum ether layer (upper layer) acquires a bright green color in the presence of resin.

3.4. Test for Cardiac Glycosides

a) Legal's Test (Treare and Evans 1985)

Extracts were treated with sodium nitroprusside in pyridine and sodium hydroxide. Formation of pink to blood red color indicates the presence of cardiac glycosides.

3.5. Test for Saponins

a) Foam Test (Kokate *et al*., 2001) [22]:

The extract was diluted with 20 ml of distilled water and it was shaken in a graduated cylinder for 15 minutes. A 1 cm. layer of foam indicated the presence of saponins.

b) Haemolysis Tests (Kokate, 1994) [23]:

Add leaves extract to one drop of blood placed on glass slide. Hemolytic zone appears.

3.6. Test for phenols

a) Ferric Chloride Test (Ahmad *et al*., 2005) [3]:

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

3.7. Test for sterols and/ or terpens

A few mills of the alcoholic extracts were evaporated till dryness. The residue was dissolved in 2ml chloroform and filtered, where the filtrate was subjected to:

a) Salkwski Reaction's (Brieskorn & Klinger-Hand Polonius, 1961) [8]

To one ml chloroform extract, conc. sulphuric acid was added slowly down the side of the test tube, where positive reaction was indicated by the formation of yellow colored ring changing to bloody red.

b) Libermann-Burchard's test (Fieser and Fieser, 1959) [12] by adding to one ml chloroform extract, one ml anhydrous acetic acid followed by few ml of conc. sulphuric acid, poured carefully down the side of test tube, where blue, green, red, or orange colors that change with time will indicate a positive reaction.

3.8. Test for Tannins (Treare & Evan, 1985):

a) Lead Acetate Test

To 5 ml of extract, add few drops of 10% lead acetate solution were added. Formation of yellow or red precipitate indicated the presence of tannins.

b) Gelatin Test:

To the extract, gelatin (gelatin dissolves in warm water immediately) solution was added. Formation of white precipitate indicated the presence of tannins.

3.9. Test for Flavonoids

a) Schinoda's Test (Geissmann, 1962) [13]:

Half ml of hydrochloric acid was added to an aliquot of aqueous extracts followed by few mg of magnesium turnings. A pink color indicated the presence of flavonoids.

b) NaOH Test (*Khandeal*, 2008):

To 2-3 ml of extract, few drops of sodium hydroxide solution were added in a test tube. Formation of intense yellow color that became colorless on addition of few drops of dilute HCl indicated the presence of flavonoids.

3.10. Detection of proteins and amino acids

Xanthoproteic Test:

The extracts were treated with few drops of conc. Nitric acid. Formation of yellow color indicates the presence of proteins.

3.11. Steam Distillation of Volatile Oils (Balbaa *et al*., 1981) [7].

Fifty grams of fresh plant root, stem, leaves and flowers were subjected to steam distillation to extract volatile oils.

4. Investigation of Total Active Materials

4.1. Estimation of Total Flavonoids

The flavonoid content of root, stem, leaf and flowers of *Lycium schweinfurthii var. schweinfurthii* determined spectrophotometrically and calculated as quercetin (Karawya & Aboutable 1982) [21].

- **Calibration curve**
  Different aliquots of ethanolic solution of quercetin equivalent to 5-200 µgm were separately, introduced into test tubes, evaporated to dryness on a hot water both (40-50 °C). Five ml of 0.1 M aluminum chloride reagents was added. The absorbance of the color developed was measured at 445nm against a blank. Three determinations for each concentration of standard solution were carried out and recorded.
- **Estimation of flavonoid content**
  Two grams of powdered air-dried material of each plant parts were accurately weighed, defatted and then extracted with 95% ethanol until exhaustion. The ethanolic extracts were adjusted to 50ml using volumetric flask. Five ml were used for estimation of flavonoids content, treated as under calibration curve. Three determinations were carried out for each sample. With reference to the standard curve, the corresponding amounts of quercetin were found out. The mean percentages of flavonoids were calculated; as quercetin.

4.2. Estimation of Total Tannins

Gravimetric Method (Copper Acetate Method):

This method depends on quantitative precipitation of tannin with copper acetate solution, igniting the copper tannate to copper oxide and weighing the residual copper oxide (Ali *et al*., 1991) [4].

Two grams of each plant parts were separately extracted for about one hour with two successive quantities, each of 100ml of acetone-water (1:1) and then filtered. The combined extract, in each case, was separately transferred into 250ml volumetric flask and adjusted to volume with distilled water. Each extract was quantitatively transferred to a 500ml beaker and heated till boiling, then 30ml of 15% aqueous solution of copper acetate was added with stirring. The precipitate of copper tannate was collected on ashless filter paper and the precipitate was ignited in a porcelain crucible (the crucibles were previously ignited to a constant weight at the same temperature). Few drops of nitric acid were added to the residue and reignited to constant weight. The weight of copper oxide was determined and the percentage of tannin was calculated according to the following correlation: Each 1g of Cuo = 1.305g tannins.

4.3. Estimation of Total Saponins

20 g of each plant parts were dispersed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 hours with continuous stirring at about 55 °C. The mixture was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 °C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorous. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added. The combined n-butanol extract were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponin content was calculated in percentage according to Obadoni & Ochuko (2001) and Okwu & Ukanwa (2007) [29, 30].

4.4. Estimation of Total Alkaloids (Gravimetric Method)

About (10 g) of the plant powders of each plant part were extracted with 90% ethanol till exhaustion (tested with Mayer's reagent). The alcoholic extract of the plant was concentrated under reduced pressure at a temperature not exceeding 40 °C acidified with HCl (3%), and filtered; the filtrate obtained was extracted with chloroform to remove undesirable matters. The acidic aqueous layer was adjusted to alkaline media with ammonia and the liberated alkaloid bases were extracted with chloroform till exhaustion (tested by Mayer and Dragendorff's reagent). The chloroform extract was filtered over anhydrous sodium sulphate and evaporated under reduced pressure till dryness, then weighed it to calculate the percent w/w (Woo *et al*., 1977) [34].

5. Investigation of Flavonoids and Phenolic acids

5.1. Preparation of Flavonoid 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 and phytochemical screening clearly indicated that, the ethyl acetate fraction was the most one rich by phenolic constituents most of them are of flavonoids nature.

5.2. Chromatographic Investigation

5.2.1 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.

5.2.2 Column Chromatography

The separation and identification of plant extracts were carried out by column chromatographic techniques (CC) (Markham, 1982) [27]. 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) [25, 28]. 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) [20]. 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) [24].

5.3. 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) [25, 27]. 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.

5.4. 1H-NMR spectroscopy

The NMR spectroscopy is a well - established method for structure elucidation of flavonoids (Mabry *et al*., 1970) [25] 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) [15]. 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 falvone 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 anomeric 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), [25] 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 (800C) 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.

Chemical shift (ppm)	Proton type
0	Tetramethylsilan (Reference)
0 - 0.5	Trimethylsilyl ether group
c.1.0	Rhamnose C-CH_3 (broad doublet)
c.1.7	Prenyl (-CH_2-CH=C(CH_3)$_2$ methyl groups
c.2.0	Acetate (-OCOCH_2) and aromatic C-CH_2
2 – 3	H-3 of flavanones (two proton – multiplet)
3.5 - 4.0	Most sugar protons
4.2 - 6.0	H-1 of sugars (also H-2 of dihydroflavanolos, 5.0 ppm & H-2 of flavanones 5-5.5 ppm)
c 6.0	Methylenedioxy (O-CH_2-O) singlet
6.0 - 8.0	A and B- ring protons
7.5 - 8.0	H-2 of isoflavones (singlet)
12 – 14	5-OH (observed only when using DMSO- d_6

5.5. ^{13}C-NMR Spectroscopy

^{13}C-NMR provides a very sensitive method for determination of the structure of complex compounds. With the introduction of Fourier-transform methods, ^{13}C-NMR spectroscopy has developed into a powerful tool for the structure elucidation of small amounts of natural products. Over the past thirty years, ^{13}C-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) [9, 26] and only a few have dealt with C-glycosides (Chopin *et al*., 1978 and Agrawal, 1989*)* [10, 2]. 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).

5.6. 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) [5]. EI-MS, model Finnigan-Mat SSQ 7000 spectrometer and ESI-MS: LCQ Advantage Thermo Finnigan spectrometers were used.

Results and discussion

1. Preliminary Phytochemical Screening

Table (3) showed that, each of volatile oils and resin were not detected in the plant stem, while only volatile oils were not detected in plant leaves and root.

Table 3: The preliminary phytochemical screening in different parts of *Lycium schweinfurthii var. schweinfurthii* during February 2014

Group	Test	Plant parts			
		Root	Stem	Leaves	Flower
Alkaloids	Wagner's test	+ve	+ve	+ve	+ve
	Dragndorrf test	+ve	+ve	+ve	+ve
Glycosides	Glycosides test	+ve	+ve	+ve	+ve
	Modified born. test	+ve	+ve	+ve	+ve
Resin	Resin test	-ve	-ve	+ve	+ve
Cardiac glycosides	Legal's test	+ve	+ve	+ve	+ve
Saponins	Foam test	+ve	+ve	+ve	+ve
	Hemolysis test	-ve	+ve	+ve	+ve
phenol	Ferric chloride test	+ve	+ve	+ve	+ve
sterol	Salkawskis test	+ve	+ve	+ve	+ve
	Libermann burchard's test	+ve	+ve	+ve	+ve
Tannins	Lead acetate test	+ve	+ve	+ve	+ve
	Gelatin test	+ve	+ve	+ve	+ve
flavonoids	Shinoda's test	+ve	+ve	+ve	+ve
	NaOH test	+ve	+ve	+ve	+ve
Amino acids	Xanthoproteic test	+ve	+ve	+ve	+ve
Volatile oil	Stem distillation	-ve	-ve	-ve	+ve

(+ve) mean present, (-ve) mean absent.

2. Total Active Materials

Table (4) showed that, the percentage of total flavonoids, tannins, saponins and alkaloides increased in leaves other than each parts.

Table 4: Total active materials in root, stem, leaves and flowers of *Lycium schweinfurthii var. schweinfurthii* plant.

Test	Root	Stem	Leaves	Flower
Total Flavonoids (%)	0.29±0.04	0.50±0.13	2.08±0.07	1.14±0.12
Total Tannins (%)	1.81±0.08	2.65±0.26	3.07±0.09	2.19±0.1
Total Saponins (%)	1.55±0.07	5.01±0.06	5.86±0.17	2.24±0.09
Total Alkaloids (%)	0.78±0.05	1.77±0.11	2.32±0.18	1.06±0.03

3. Flavonoids and Phenolic Acids

3.1. Separation of Flavonoids and Phenolic acids

Flavonoids and phenolic compounds more concentrated in the plant leaves than in other part which was cleared from the phytochemical study so that, these active constituents were separated from the leaves of *Lycium schweinfurthii var. schweinfurthii*. Five compounds were separated from ethyle acetate fraction of the leaves.

3.2. Purification of ethyl acetate fraction

Ethyl acetate fraction was applied on the top of silica gel column chromatography, eluted firstly with hexane followed by hexane/chloroform to increase polarity until pure chloroform. It was followed by chloroform/ethyl acetate until pure ethyl acetate, then it was followed by ethyl acetate/methanol until finally pure methanol, where three main fractions I, II and III were obtained.

3.2.1 Purification and identification of fraction I

Fraction I when subjected to two dimension paper chromatography using B: A: W (4: 1:5) and AcOH-15%, two major spots were obtained. Fraction I was subjected to preparative paper chromatography using the solvent system B: A: W (4: 1:5) for 24 h. give two bands (1&2), which were cut carefully and eluted with ethanol.

Identification of compound 1

Band 1 of fraction I when subjected to preparative paper chromatography, eluted with 70% ethanol, dried under reduced pressure, purified on sephadex LH-20 column using methanol/ water as described by Jonston *et al.,* (1968) and subjected to paper chromatography using the solvent system B: A: W and AcOH-15% one major spot of flavonoid nature (compound 1) was obtained, its R_f values (0.72& 0.28) and color reaction as illustrated at Table (5) are within the range of flavonoid aglycone.

Table 5: R_f-values and color reaction of the compound 1.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.72	Untreated	—	Yellow
AcOH-15%	0.28	NH_3	—	Light yellow
		$AlCl_3$	—	Light yellow

UV spectral data, λ_{max} nm, in MeOH

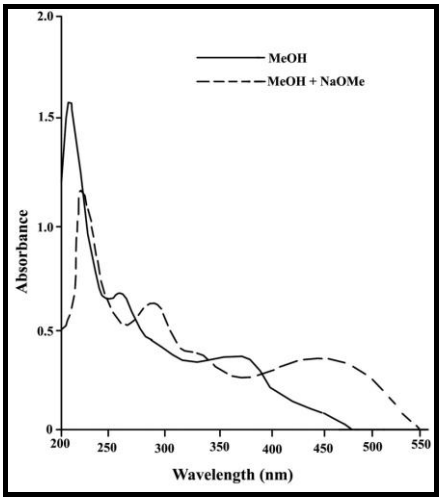


Fig 1: UV spectrum of compound 1 in MeOH and NaOMe

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

MeOH	: 260, 270 (sh.) 300 (sh), 375.
NaOMe	: 290▲, 370 (sh.), 450▲.
NaOAc	: 265, 332 (sh.), 383▲
$NaOAc + H_3BO_3$	: 260▲, 300 (sh.), 380.
$AlCl_3$	: 276▲, 318, 450▲.
$AlCl_3 + HCl$	: 273▲, 305(sh.), 355 (sh.), 427▼.

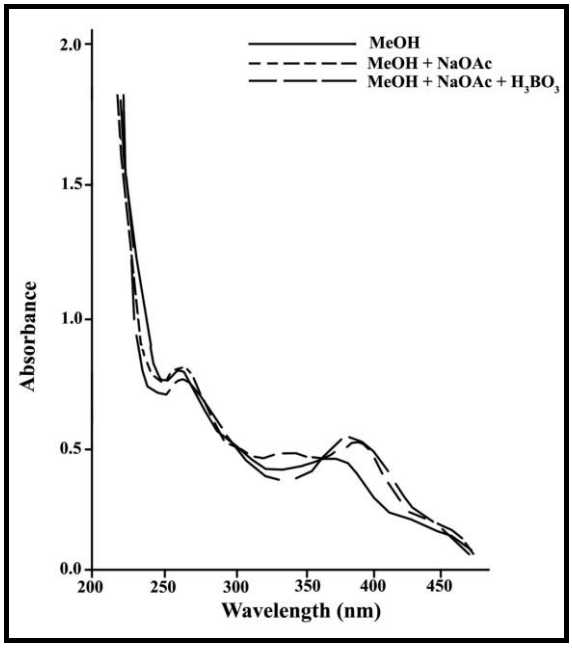


Fig 2: UV spectrum of compound 1 in NaOAc and H_3BO_3

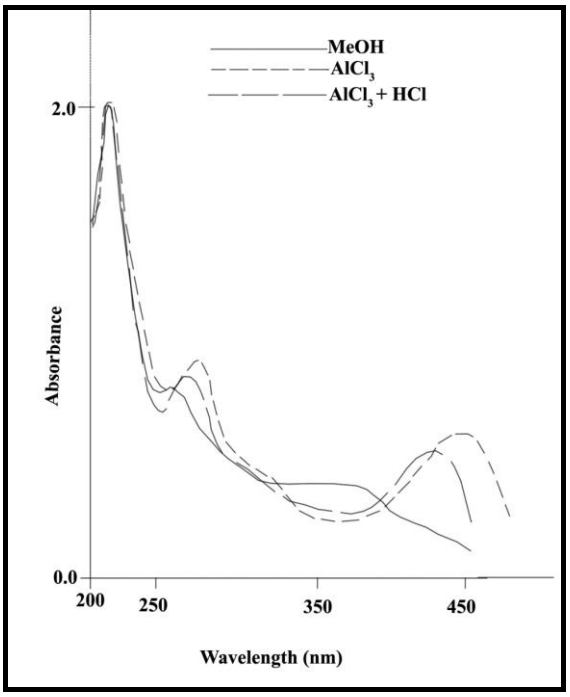


Fig 3: UV spectrum of compound 1 in $AlCl_3$ and HCl

^{1}H-NMR spectral data

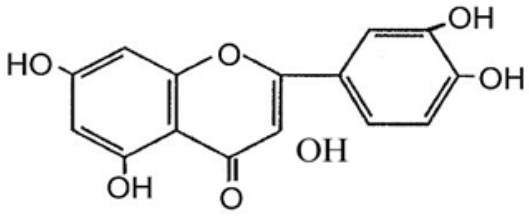
The ^{1}H-NMR spectrum of compound 1 in DMSO (Fig. 1) showed signals at δ(ppm) δ 7.6 (1H, d, J=8.5 Hz, H-2\), δ 7.5 (1H, dd, j = 8.5 Hz, H-6\), δ 6.89 (1H, d, j = 8.5 Hz, H-5\), δ 6.4 (1H, d, j = 2.5 Hz, H-8) and δ 6.2 (1H, d, j = 2.5 Hz, H-6). The change of its color from yellow to light yellow when exposed to ammonia vapor under UV light or treated with $AlCl_3$ reagent indicated that compound 1 may be a flavonol compound with free OH (Herborne, 1984 and Liu *et al*., 1989) [24].

UV spectral data of compound 1 in methanol showed maximal absorption band I at 375nm, which indicated that compound 1 is a flavonol compound with free 3-OH group. Formation of new band at 328nm. With NaOMe indicated the presence of a free 7-OH group (Fig. 1).

A bathochromic shift of band I when treated with $AlCl_3$ indicated the presence of 3 and 5-OH group. A hypsochromic shift with HCl in band I indicated the presence of O-dihydroxy group in B-ring (Fig. 3).

Bathochromic shift in band I with $NaOAc/H_3BO_3$ was an additional proof for the presence of O-dihydroxy group in B-ring (Fig. 2).

The structure of compound 1was further confirmed as quercetin by ^{1}H-NMR spectrum of (Fig. 4), which showed characteristic signals for quercetin, signal at δ 7.7(1H, d, J=8.5Hz, H-2\), 7.6 (1H, d, J=8.5Hz, H-6\), 6.89 (1H, d, J=8.5Hz, H-5\), which indicated the presence of aromatic ring with two substitution m,p- substitution δ 6.4 (1H, d, J=2.5Hz, H-8) and 6.2 (1H, d, J=2.5Hz, H-6). Thus compound 1 was identified as quercetin.



Quercetin

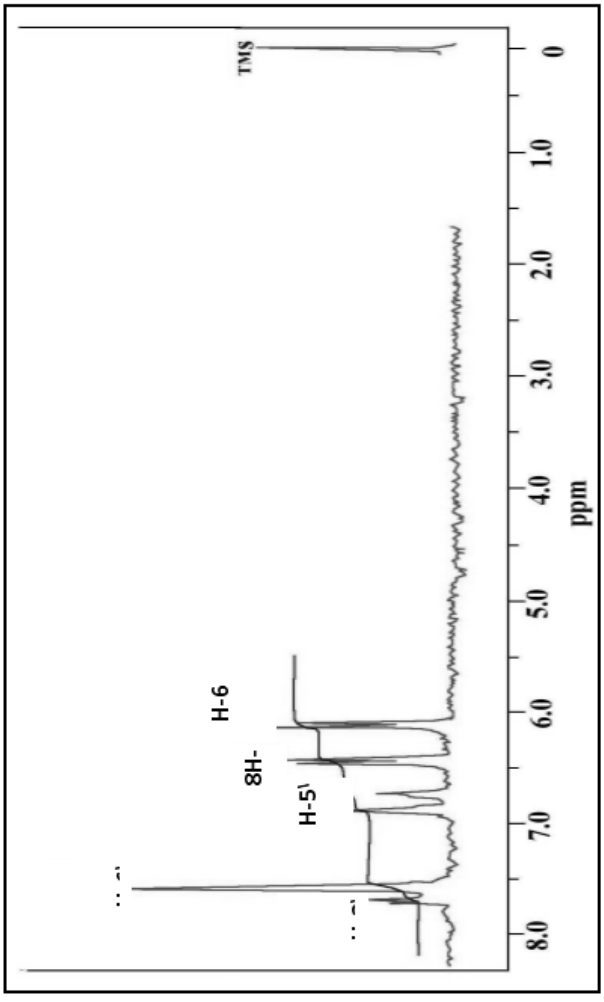


Fig. (4):^{1}H-NMR spectrum of compound 1

Identification of compound 2

Band 2 of fraction I when subjected to preparative paper chromatography, eluted with 70% ethanol, dried under reduced pressure, purified on sephadex LH-20 column using methanol/ water as described by Jonston *et al.,* (1968) and subjected to two dimensional paper chromatography using the solvent system B: A: W and AcOH-15% one major spot of flavonoid nature (compound 2) was detected, with yellow color in visible light. It gave the same color under UV, which changed to fluorescence when treated with ammonia and give light yellow when treated with $AlCl_3$. The R_f values (0.86 & 0.14) are within the range of flavonoid aglycone as illustrated at Table 6 (Herborne, 1984).

Table 6: R_f-values and color reaction of the compound 2.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.86	Untreated	yellow	Yellow
AcOH-15%	0.14	NH_3	—	yellow fluorescence
		$AlCl_3$	—	Light yellow

UV spectral data, λ_{max} nm, in MeOH:

The obtained results of UV spectral data (Figs. 5, 6 & 7) were:

MeOH : 273, 324 (sh), 370.
NaOMe : 280▲, 325(sh), 425▲.
NaOAc : 270▲, 302 (sh), 360.
NaOAc + H_3BO_3 : 277, 296 (sh), 310 (sh), 380.
$AlCl_3$: 278▲, 310 (sh), 355, 428▲
$AlCl_3$ + HCl : 278, 310 (sh), 358, 428▲

^{1}H-NMR spectral data:

The ^{1}H-NMR spectrum of compound 2 in DMSO (Fig. 8) showed signals at δ(ppm) 8.0 (2H, d, J= 8 Hz, H-2` and H-6`), 6.9 (2H, d, j= 8Hz, H-3` and H-5`), 6.4 (1H, d, j= 2.5 Hz, H-8), 6.2 (1H, d, j=2.5Hz, H-6).

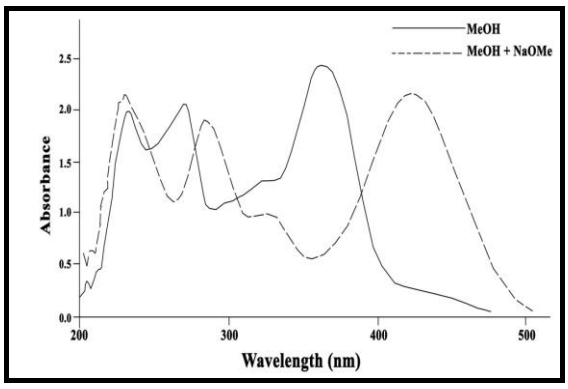


Fig 5: UV spectrum of compound 2 MeOH and NaOMe

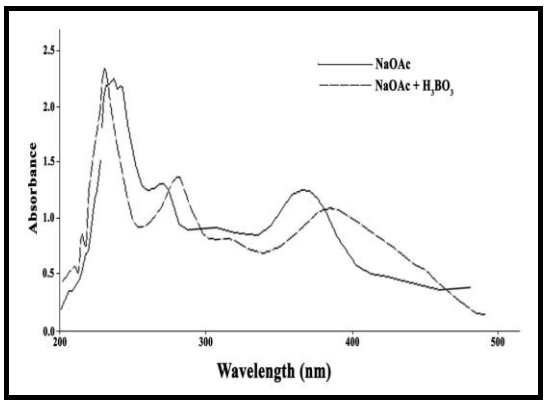


Fig 6: UV spectrum of compound 2 in NaOAc and H_3BO_3

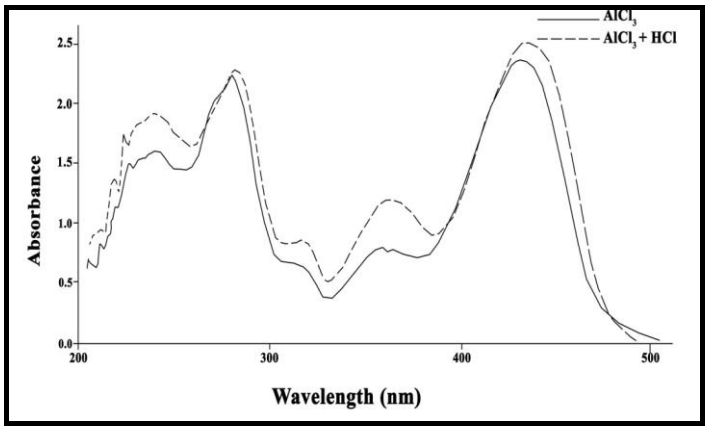


Fig 7: UV spectrum of compound 2 in $AlCl_3$ and HCl

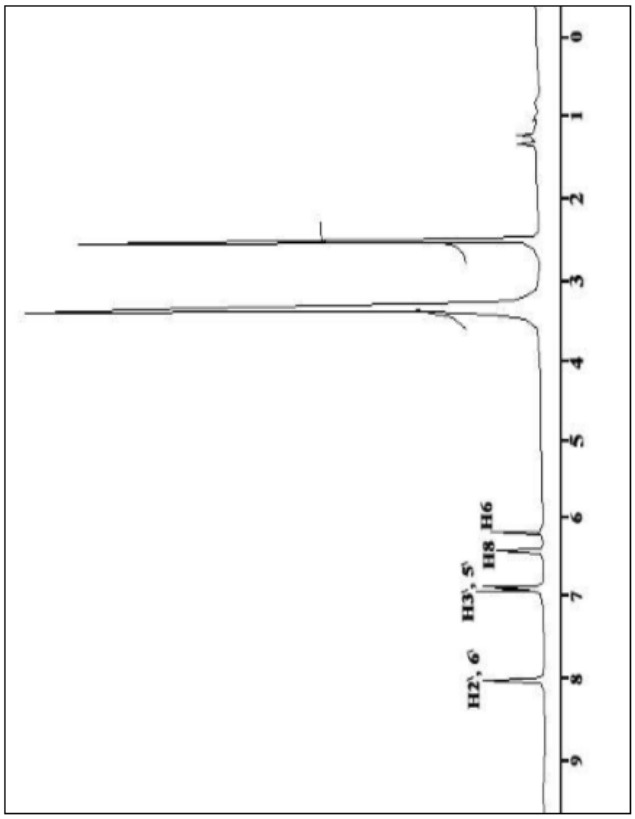


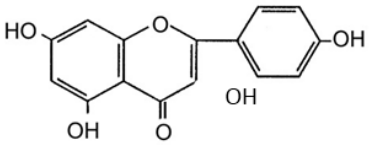
Fig. (8): ^{1}H-NMR spectrum of compound 2

The separated compound 2 in methanol showed absorption bands I at 370 and II 266 nm indicated that (Compound2) was a flavonol in nature with free OH group at position 3 (Herborne, 1984 and Liu *et al*., 1989) [24].

Addition of NaOMe induced a bathochromic shift in band I (▲53), which proved the presence of a free hydroxyl group at 4\.-position. On addition of $AlCl_3$ a bathochromic shift in band I was achieved indicated the presence of a free hydroxyl group at 3 and 5, which was unaffected with addition of HCl, which indicating the absence of free hydroxyl group at 3\ or 4\.

UV spectral data of compound 2 indicated that the compound was probably kampferol.

The structure of compound 2 was further confirmed as kampferol by ^{1}H-NMR spectrum of (Fig. 8), which showed characteristic signals for kampferol, at δ 8.0 (2H, d, J=8Hz, H-2\ and H-6\), 6.9 (2H, d, J=8.5Hz, H-3\ and 5\), 6.4 (1H, d, J=2.5Hz, H-8) and 6.2 (1H, d, J=2.5Hz, H-6). Thus compound 2 was identified as kampferol.



Kampferol

3.2.2. Purification and identification of fraction II

Fraction II when subjected to two dimensional paper chromatography using B: A: W (4: 1: 5) and AcOH-15%, two major spots of flavonoid nature were obtained. Which when subjected to preparative paper chromatography using the solvent system B: A: W (4: 1: 5) for 24h. give two bands (1&2), where they were cut carefully and eluted with 70% ethanol.

- **Identification of compound 3**

Band 1 of fraction II on preparative paper chromatography, when eluted with 70% ethanol, dried under reduced pressure, purified on sephadex LH-20 column using methanol/ water as described by Jonston *et al.,* (1968) and subjected to two dimensional paper chromatography using the solvent system B: A: W and AcOH-15%, one major spot of phenolic nature was obtained. R_f value and color reaction of the separated compound were illustrated at Table 7.

The purified compound 3 visualized as blue color under UV, which was unchanged when treated with ammonia or ferric chloride reagents.

Table 7: R_f-values and color reaction of the compound 3.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.76	Untreated	—	Blue
AcOH-15%	0.54	NH_3	—	Blue
		$FeCl_3$	Blue	-

UV spectral data, λ_{max} nm, in MeOH

The obtained results of UV spectral data (Fig. 9) were:

MeOH : 272, 335

NaOMe : 275▲, 345▲

^{1}H-NMR spectral data:

The ^{1}H-NMR spectrum of compound 3 in DMSO (Fig. 10) showed signals at 6.98 (s, H-2 and H-6).

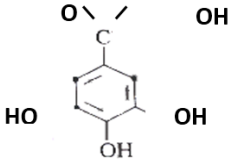
UV spectral data of compound 3 showed two absorption bands in methanol at 271 and 334 nm, addition of NaOMe gave a bathochromic shift (274 and 343 nm) which provide the presence of free OH group.

^{1}H-NMR spectral data 6.98 (s, H-2 and H-6) indicated the absence of any substitution thus the structure of compound 3 was further confirmed to be 3, 4, 5-trihydroxy benzoic acid (Gallic acid).

^{13}C-NMR Spectrum (Fig11)

^{13}C-NMR Spectrum showed that signal at δ ppm: 120.6 (C-1), 108.8 (C-2 and C-6), 145.5 (C-3 and C-5), 138.1 (C-4) and 167.7 (C-7).

The mass spectrum data of compound (1), revealed the presence of A Molecular ion peak at 170 m/z. From the above data the compound 3 is identified as gallic acid (3, 4, 5, trihydroxy benzoic acid).



Gallic acid 3, 4, 5, trihydroxy benzoic acid

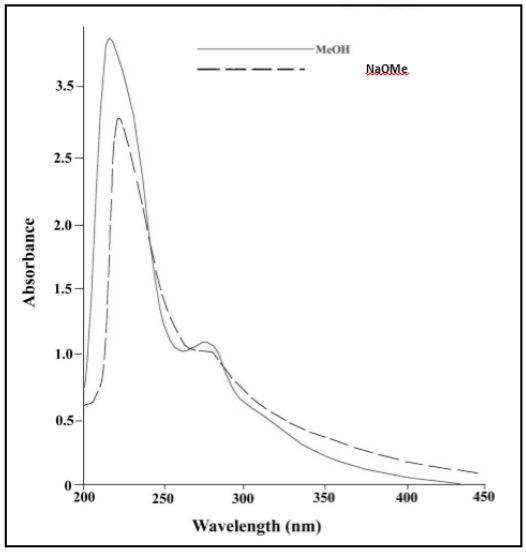


Fig 9: UV spectrum of compound 3

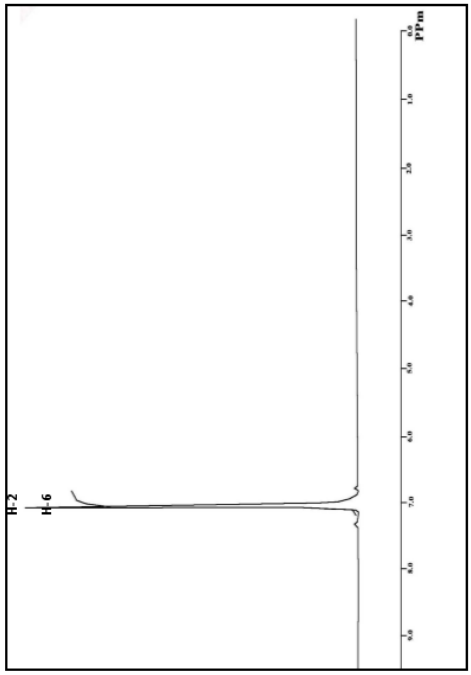


Fig. (10): ^{1}H-NMR spectrum of compound 3.

Identification of compound 4

Band 2 of fraction II when eluted with 70% ethanol, dried under reduced pressure, purified on sephadex LH-20 column using methanol/ water as described by Jonston *et al.*, (1968) and subjected to paper chromatography using the solvent system B: A: W and AcOH-15%, revealed the presence of one major spot of phenolic nature (Compound 4). The obtained. R_f values were 0.87 in B: A: W and 0.54 in AcOH-15% as illustrated at Table 8.

The purified compound 4 appear on paper chromatography as blue color under UV, which changes when subjected to ammonia to fluorescence blue.

Table 8: R_f-values and color reaction of the compound 4.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.87	Untreated	—	Blue
AcOH-15%	0.54	NH_3	—	Fluorescence Blue
		$FeCl_3$	Blue	-

UV spectral data, λ_{max} nm, in MeOH:

The obtained results of UV spectral data (Fig. 12) were:

MeOH **:** 285, 312**.**

NaOMe **:** 250 (sh), 290▲, 345▲.

^{1}H-NMR spectral data

The ^{1}H-NMR spectrum of compound 4 in DMSO (Fig.. 13) showed signals at δ(ppm) 9.15 (s, OH), 8.9 (s- OH), 7.5 (2H, d, j= 17Hz, H-2 and H-7), 7.15 (1H, d, j= 2.5Hz, H-2), 7.05 (1H, dd, j= 7.5Hz and 2.5Hz, H-6), 6.95 (1H, d, j= 7.5Hz, H-2), 6.25 (1H, d, j= 17Hz, H-8) and 3.85 (3H, s,OCH_3).

Mass Spectrum

EI-mass spectrum (Fig. 47) of the compound P_2 showed the molecular ion peak M^+ at m/e 194, M^+ -15 and C_6H_6 at m/e 77, which indicated the compound P_2 my be ferulic acid (4-hydroxy-3-methoxy cinnamic acid).

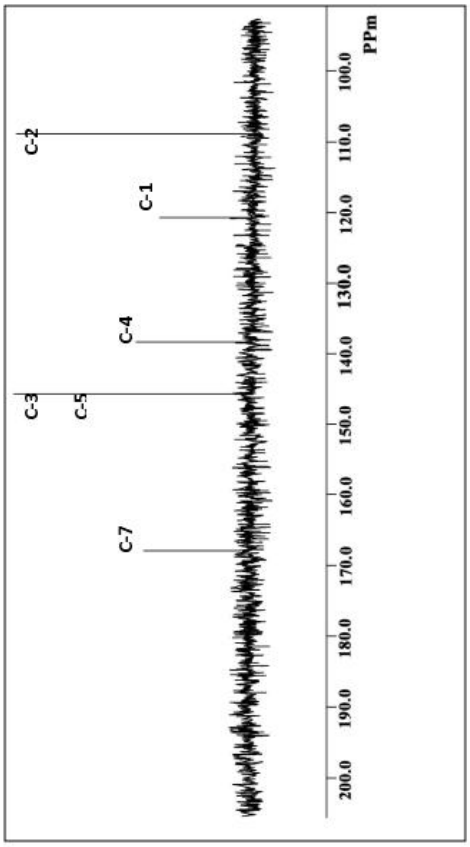


Fig. (11): ^{13}C-NMR spectrum of compound 3.

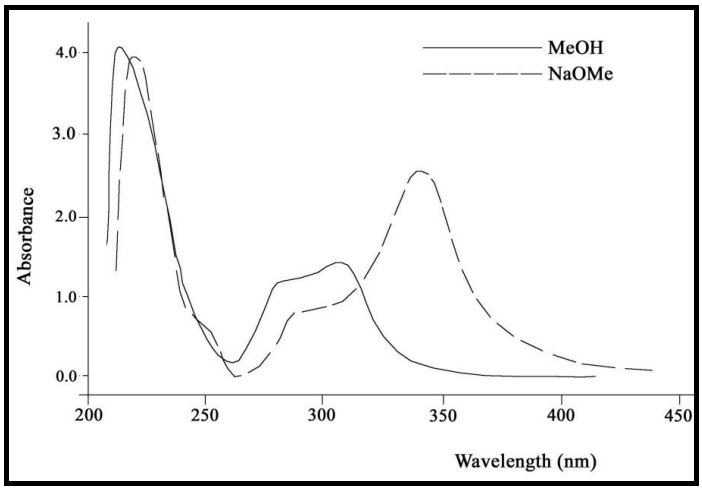


Fig 12: UV spectrum of compound 4

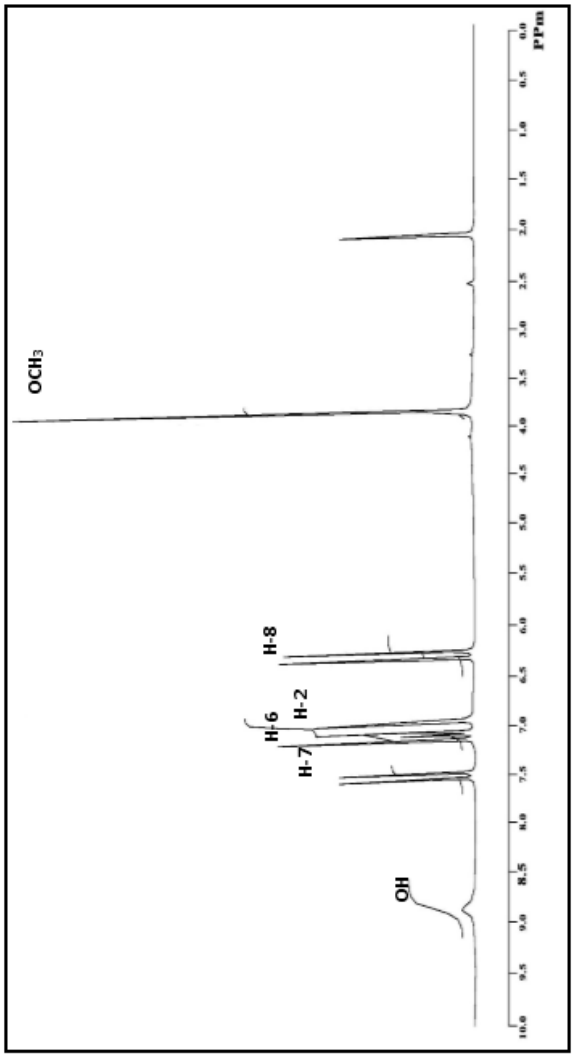


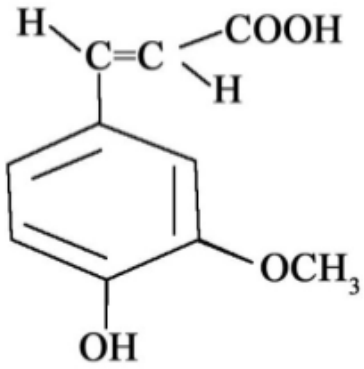
Fig. (13): ^{1}H-NMR spectrum of compound 4.

UV spectral analysis of compound 4 in methanol showed two bands at 284 and 311 nm, which were characteristic of phenyl propanoid. Addition of NaOMe gave a bathochromic shift of the two bands (291 and 345 nm) proving the presence of free OH group.

The ^{1}H-NMR spectrum of compound 4 (Fig. 12) showed two doublets at δ 7.5 and 6.25ppm, J=17Hz, characteristic for trans olefinic double band. The two doublets at δ 7.15 and 6.95 and the doublet of doublet at δ 7.05 corresponding to *ortho* and *meta* coupling which is a good evidence for the presence of trisubstituted benzene. The presence of signal at δ 8.9

confirmed the UV analysis for the presence of free OH group. The presence of a signal at δ 3.8 (s) confirmed the presence of for –OCH_3 group.

From the above data compound 4 was identification as ferulic acid (4-hydroxy-3-methoxy cinnamic acid).



Ferulic acid (4-hydroxy-3-methoxy cinnamic acid)

3.2.3. Purification and identification of fraction III

Fraction III, when applied on top of silica gel column using chloroform/methanol as separated system, then the obtained compound subjected to two dimensional paper chromatography using the solvent system B: A: W and AcOH-15% one major spot of flavonoid nature (compound 5) was obtained.

- **Identification of compound 5**

The obtained purified compound 5, which appeared on paper chromatography as deep purple color was changed to light yellow, when treated with ammonia and re-examined under UV light. R_f values (0.79 & 0.14) of the compound 5were illustrated at Table 9.

Table 9: R_f-values and color reaction of the compound 5.

Solvent	R_f-value	Reagent	Color	
			Visible	UV
B:A:W	0.79	Untreated	—	Deep purple
AcOH-15%	0.14	NH_3	—	Light yellow
		$AlCl_3$	—	Light yellow

UV spectral data, λ_{max} nm, in MeOH:

The obtained results of UV spectral data (Figs. 14, 15 & 16) were:

MeOH : 267, 300sh, 331.
NaOMe : 277▲, 325sh, 390▲
NaOAc : 274, 301(sh.), 376▲.
NaOAc + H_3BO_3 : 268, 302(sh.), 338▼
$AlCl_3$: 276, 301, 348, 384. ▲
$AlCl_3$ + HCl : 276, 299, 340, 381 ▲

^{1}H-NMR spectral data:

The ^{1}H-NMR spectrum of compound 5 in DMSO (Fig. 17) showed signals at δ(ppm) 7.7 (2H, d, J= 8.4Hz, H-2\, H-6\ ), 6.85 (2H, d, J= 8.4Hz, H-3`and H-5`), 6.7 (1H, S, H-3), 6.45 (1H, d, J= 2.5Hz, H-8), 6.20 (1H,d,J=2.5Hz, H-6).

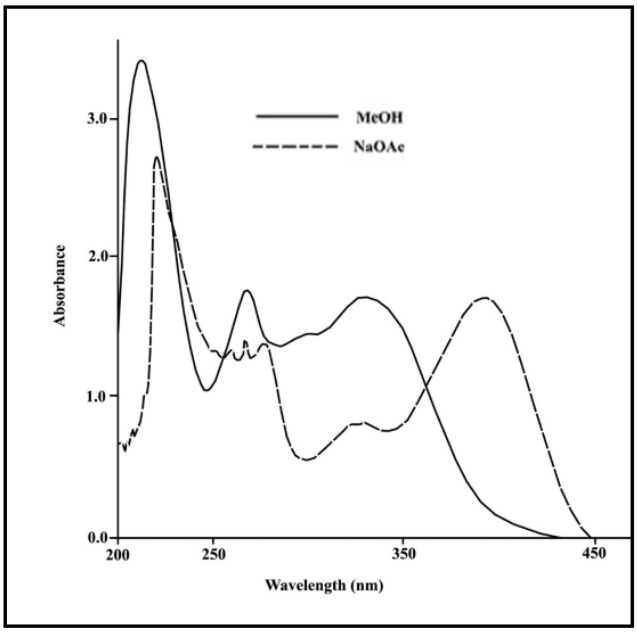


Fig 14: UV spectrum of compound 5 in MeOH and NaOMe

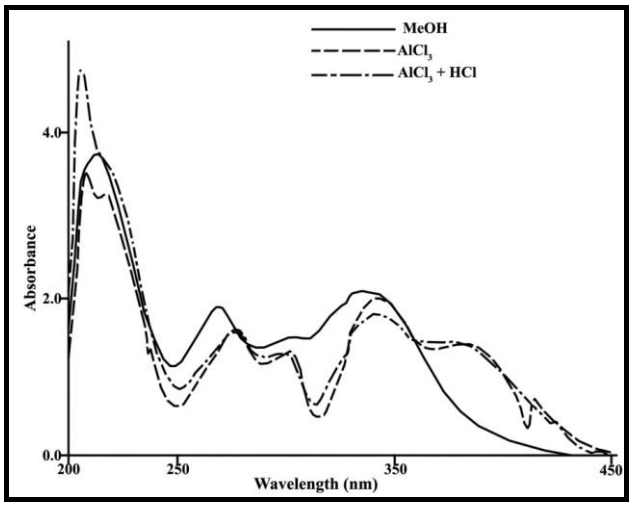


Fig 15: UV spectrum of compound 5 in $AlCl_3$ and HCl

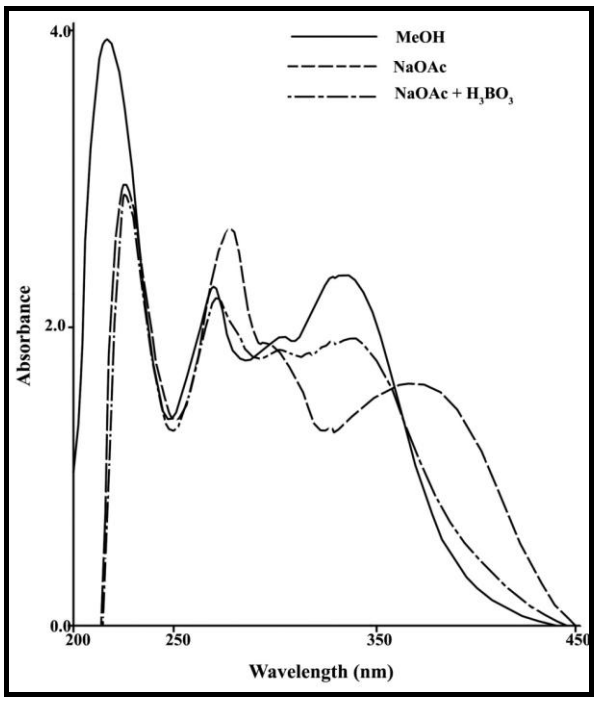


Fig 16: UV spectrum of compound 5 in NaOAc + H_3BO_3

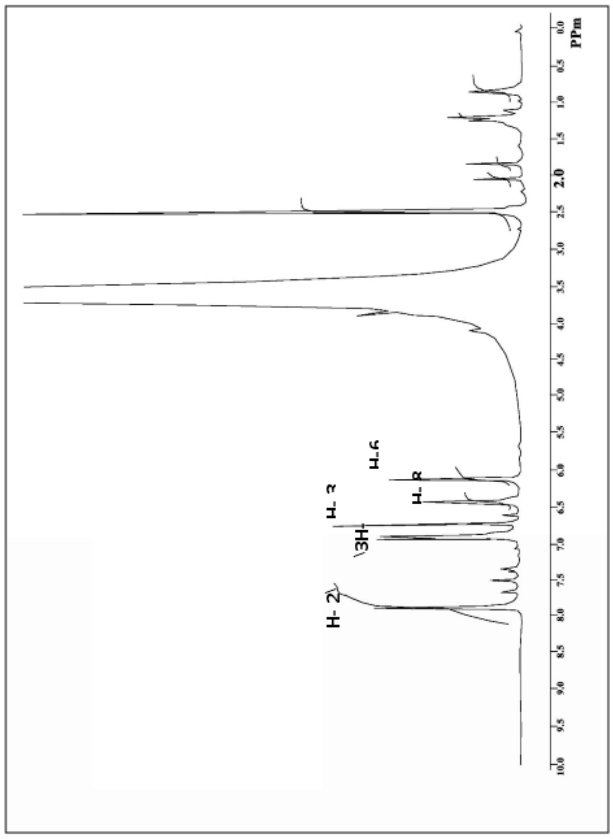
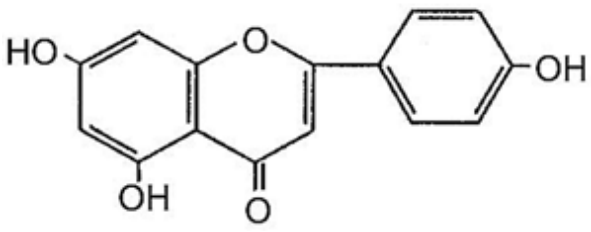


Fig. (17): ^{1}H-NMR spectrum of compound 5

UV spectral data of compound 5 in methanol showed two absorption bands at 267 and 336 nm, which were typical of flavone aglycone (Herborne, 1984 and Liu *et al*., 1989) [24]. On addition of NaOMe induced stable bathochromic shift, which indicated that 4\-position was free hydroxyl. Addition of $AlCl_3$ induced a bathochromic shift, which indicated that position 5 was free hydroxyl, which was unaffected with addition of HCl, proving the absence of catcholic hydroxyl.

Addition of NaOAc exhibit bathochromic shift in band II, which proving that 7-position was free hydroxyl, where addition of H_3BO_3 induced no shift, which indicated further confirmation for the absence of catcholic hydroxyl groups. UV spectral data of compound 5 indicated that the compound was probably apegenin.

The structure of compound 5 was further confirmed as apegenin by ^{1}H-NMR spectrum of (Fig. 16), which approved apegenin type protons at δ 7.7 (2H, d, J=8.4Hz, H-2\ and H-6\), 6.85 (2H, d, J=8.4Hz, H-3\ and 5\), 6.7 (1H, s, H-3), 6.45 (1H, d, J=2.5Hz, H-8) and 6.2 (1H, d, J=2.5Hz, H-6). Hence compound 5 was identified as apegenin.



Apegenin.

Conclusion

A detailed phytochemical study of *Lycium schweinfurthii var. schweinfurthii* leaves were carried out. From the methanolic extract of *Lycium schweinfurthii var. schweinfurthii leaves* a five flavonoids were isolated and purified using different chromatographic techniques from ethyle acetate fraction. These compounds were identified via spectroscopic tools:, UV, 1H NMR, ^{13}C-NMR and MS spectroscopy.

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