ADAMA SCIENCE AND TECHNOLOGY

UNIVERSITY

SCHOOL OFAPPLIED NATURAL SCIENCES

DEPARTEMENT OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION ON THE ROOT

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Adama, Ethiopia

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(Rhynchosiaside-2) from the root of *R. minima*

PHYTOCHEMICAL INVESTIGATION ON THE ROOT OF *RHYNCHOSIA MINIMA* LINN

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Statement of the author

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university and that any sources of materials used for this thesis have been dually acknowledged.

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Contents page

List of Tables

List of Figures

Appendix

Abbreviations

WHO: World Health Organization

mm: millimetre

cm: centimetre

TLC: Thin layer chromatography

mg**:** milligram

kg**:** kilogram

UV/Vis**:** ultraviolet visible

IR**:** infrared

NMR**:** nuclear magnetic resonance

^{1}H -NMR**:** proton nuclear magnetic resonance

^{13}C-NMR**:** carbon 13 nuclear magnetic resonance

DEPT**:** Distortionless Enhancement by Polarization Transfer

MHz**:** mega hertz

nm**:** nanometre

FT**:** Fourier transfer

KBr**:** potassium bromide

DMSO-d_6**:** deuterated Dimethyl SulfOxide

TMS**:** tetra methyl selenium

g**:** gram

RMM**:** *Rhynchosia minima* methanol extract

ppt**:** precipitate

Rf**:** retention factor

δ**:** chemical shift

Abstract

The root of the medicinal plant, *Rhynchosia minima,* was subjected to solvent extraction using methanol. The crude methanol extract was dissolved in distilled water and then subjected to solvent-solvent partitioning using hexane, chloroform, ethylacetate and methanol. The methanol extract was then subjected to phytochemical screening tests following standard methods which led to identification of alkaloids, flavonoids, Saponins, tannins and steroids in the crude extracts. After repeated CC the methanol extract afforded two tripterpene saponins, whose structures were elucidated by using IR, ^{1}H and ^{13}C NMR techniques as 2-[5,4-dihydroxy-2-hydroxymethyl-6-(4,4,6α,6β,8α,11,11,14β-octamethyl-1,2,3,4,4α,5,6,6β,7,8,8α,9,10,11,12β,13,14,14α,14β-eicosahydro-picen-3-yloxy)-tetrahydro-pyran-3-yloxy]-6-hydroxymethyl-tetrahydropyran 3,4,5-triol, (Rhynchosiaside-2) and (2, 2,6α,6β,9,9,12α-Heptamethyl-10-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydo-pyran-ylo,xy)-3,4,5,6,6α,6β,7,8,8α,9,10,11,12,12α, 12β,13,14,14α-octadecahydro-2Hpicene-4α carboxylic acid (Rhynchosiaside-1). It is reported that *R. minima* is used in folk medicine for the treatment of venereal diseases, *internal inflammation, tumorous out growths, stomach ailments and lethargy in cattle.* Therefore, antimicrobial assay of the methanol extract of root of *R. minima* was performed on three bacterial pathogens; *viz*. *Klebsiella pneumonia*, *Escherichia coli* and *Staphylococcus aurous.* The results showed that the root of *R. minima* contain some components such as saponins, tannins, steroids and triterpenoids. These compounds may not contribute for inhibiting effect against tested microorganisms.

Key words: *Rhynchosia minima*, Rhynchosiaside-1, Rhynchosiaside-2 and Antimicrobial

1. Introduction

1.1. Background of the study

Plant species present on the earth are estimated to be around 250 to 500 thousand, out of which 1 to 10% of them are used as food by human beings and other animals [1]. It is known that some plants have applications in treatment of various human ailments as well as in veterinary medicine and thus classified as medicinal. Medicinal plants are used for treating various diseases since prehistory. According to World Health Organization (WHO), 80% of the world's population still depends mainly on traditional medicines and use plant extracts in traditional treatments. This is commonly practiced in rural areas where synthetic drugs are not available or too expensive to purchase [2].

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development of the plants. These substances, referred to as secondary metabolites, often are differentially distributed among limited taxonomic groups within the plant kingdom. Their functions, many of which remain unknown, are being elucidated with increasing frequency. The primary metabolites, in contrast, such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident[3].

Secondary metabolites which are produced as defence agents against predators by plants are believed to endow the medicinal property to plants. Some of the crude drugs used in past are still in use in phytotherapeutics. *Cinchona* plant is often used in its natural and unrefined form to treat malaria [3], although some other herbal drugs are refined by isolating their pure active compounds and their active principles, helped in the advancement of scientific medicine.

In the 20^{th} century, the antibiotic era substantially has reduced the threat of infectious diseases. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. However, over the years there is a decrease in microbial susceptibility to existing antimicrobial agents, responsible for drug resistance which is exerting global problem today. In fact, the theme of World Health Day 2011 was: no action today, no cure tomorrow. There is an urgent need of new antimicrobial agents to overcome this global problem [4]. Each year, two million people are admitted with bacterial infection in US hospitals, 70% of the cases involve strains that were resistant to at least one

drug [5]. Higher plants are rich sources of antibiotics [3]. During the period (1981-2006), 109 drugs were approved in which 69% were from natural products and 21% of the antifungal drugs were refined natural derivatives [6]. The promising potentials of antimicrobial plant derived substances have attracted the attention of pharmaceutical and scientific communities [7].

The primary benefit of plant derived medicines is that they are relatively safer than their synthetic counterparts and offer profound therapeutic benefits and more affordable treatment.

Since the dawn of humanity, war against disease has been a part of everyday life and the use of plant materials to treat the sickness is as old as man [8]. Some of the common uses of the medicinal plants sold in markets include fumigation, vermifudge, pain relief, treating skin infections, etc. Antimicrobial and wound healing plants are among some of the major medicinal plants that are commonly available in markets [9].

It is customary to find medicinal plants in markets where food items and spices are sold. Fresh and dried leaves, flowers, roots, bark, seeds, etc. of medicinal plants are displayed for sale in most markets in Ethiopia along with spices such as pepper, cardamom, ginger, etc. Ethiopian plants have shown very effective medicinal value for some ailments of human and domestic animals [10]. Thus medicinal plants and knowledge of their use provide a vital contribution to human and livestock health care needs throughout the country. The major reasons why medicinal plants are demanded in Ethiopia are due to culturally linked traditions, the trust the communities have in the medicinal values of traditional medicine and relatively low cost in using them [11]. *Rhyncosia minima* is used in folk medicine for the treatment of venereal diseases, internal inflammation, tumorous out growths, stomach ailments and lethargy in cattle. To my knowledge there is no literature reports describing the chemistry and antibacterial activity of the roots of *R. minima.* Therefore, this study was aimed at isolation, structural elucidation and testing some biological activities of secondary metabolites from the roots of *R. minima*.

1.2. Significance of the study

This study provided information about the chemical constituents of root of *R.minima* and the antibacterial activity of methanol extract of root of *R. minima*

2. Literature Review

2.1 Description of *Rhynchosia minima* Linn

R. minima L (Fig. 1a) is a herb or prostrate rarely erect, twining, stems numerous, slender, much branched, pubescent when young. Leaves 3 mm foliage; petioles 6-20 mm long, more pubescent, striate; stipules 4mm long, linear- lance late. Leaflets1-2.5 cm long, as broad as long, rhomboid obviate, obtuse, a peculate, glabrous above, pubescent on the veins and conspicuously dotted beneath. Flowers reddish-yellow, in axillaries 6-12 mm, flowers lax racemes usually exceeding the leaves; pedicels very short, Calyx3-4 mm long, pubescent; teeth about twice as long as the tube, linear- sub late. Corolla, yellow, 5-6 mm long, Pods 13-16 by 6 mm, somewhat compressed, turgid, slightly re curve. Seeds 2, compressed, 3 by 2 mm, black. This plant has a worldwide distribution and in Zimbabwe it is found in most parts of the country. It grows well on sandy soil on a mixed altitude of ±1600 feet [12].

a b

Figure 1: *R. minima*, habit with inflorescence (a) and *R. minima* root (b)

2.2. Ethnobotanical Information

In India, it is reported that the people of Bhandara and Gadchiroli district in Maharashtra state apply the extract of seeds as an eye drop to cure conjunctivitis and inflammation [13]. *R. minima* is traditionally used in skin conditions and to alleviate boils in Zimbabwe. Seeds were reported to be bitter and toxic [14]. Whole plant was reported to be used for bath after delivery for body care in Pakistan [15]. The whole plant was reported to be toxic to fish. The extract of seed showed specific agglutinating activity with human red blood cells while leaves are reported to be used as abortifacient and the plant is also useful as fodder for cattle and horses [16,17].

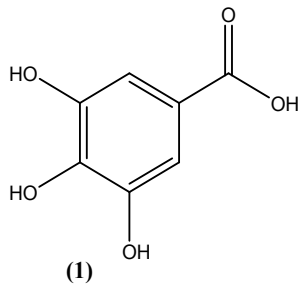
2.3. Phytochemical study in *Rhynchosia* family

Garlic acid (**1**), hydroquinone diacetate (**2**) and other phenolics were reported from seed coat of the plant [18]. Phenolics and flavonoids in the leaves were studied and it was reported that all flavonoids of the leaf extract were present in the form of C-glycosyflavones (**3**) [18]. The hydroquinone present in the seeds of *R.minima* is supposed to be involved in seed germination. Flavonoid profiles of seven species of *Rhynchosia* including *R. minima* were reported by Adinarayana et al., (1985) [19].

New flavonoids were identified in the leaf extract of *R. cyanosperma* [20]. In all these studies the medicinal uses of the phytochemical principles were not discussed [21]. However, Gundidza et al., (2009) demonstrated range of 8 essential oils that showed high antibacterial activity against several bacterial and fungal species.

The major phytochemical components of *Rhynchosia* sp. from Harare, Zimbabwe, were isopropyltoluene (**4**), o-cymene (**5**), camphene (**6**), limonene (**7**), 2-β-pinene (**8**), α-terpinolene (**9**), α-pinene (**10**) and myrcene (**11**) [22]. The *R. minima* is a popular medicinal plant in tribal medicine. There are reports of phytochemical studies carried out in the leaf and seed of this plant [18]. Aerial parts gave steroidal glycosides (**12**), stigmasterol (**13**), lupeol (**14**) and β-sitosterol (**15**). Seed extract is used as eye drops to cure conjunctivitis and inflammation [23]. The seed was shown to contain garlic acid, protocatechuic acid (**16**), prodelphinidin (**17**) [24]. The authors mentioned that pharmacological work was not reported in this plant. The TLC studies also showed phenolic compounds **1**, salicylic acid **18**, ursolic acid (**19**), betulinic acid (**20**) and olenolic acid (**21**). (1) Was potent antioxidant, antifungal, antiviral and anti- inflammatory activity [24].

Betulinic acid and ursolic acid are reported to show antitumor activities [25]. Ursolic acid is believed to be starting material for synthesis of more potent bioactive derivatives such as antitumor agents [25]. This suggestion is given on the basis of the ailment for which the root extract of *R. minima* is used as a medicine by the tribal's of Toranmal country, that is, stomach ailments and lethargy in cattle.



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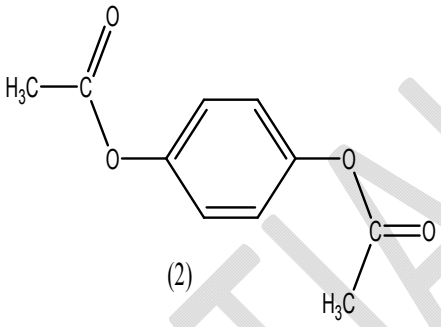

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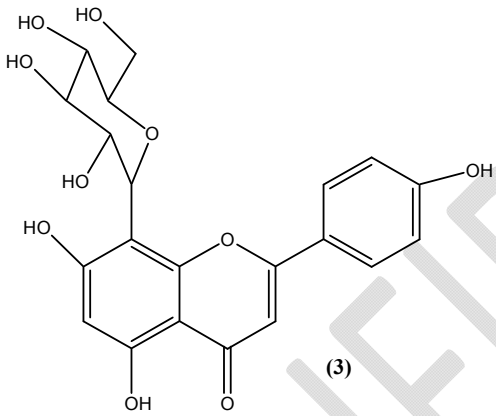

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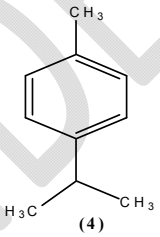

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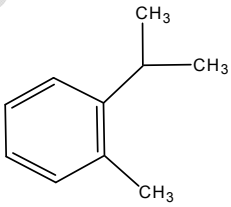

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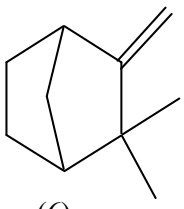

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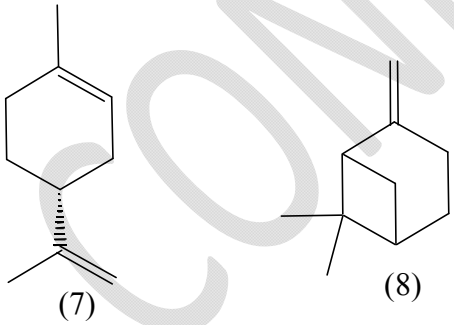

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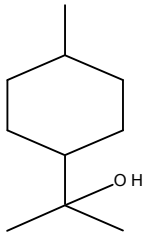

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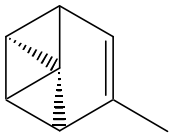

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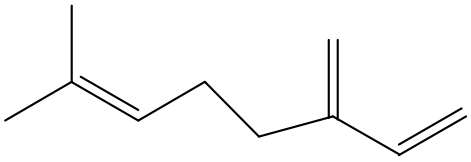

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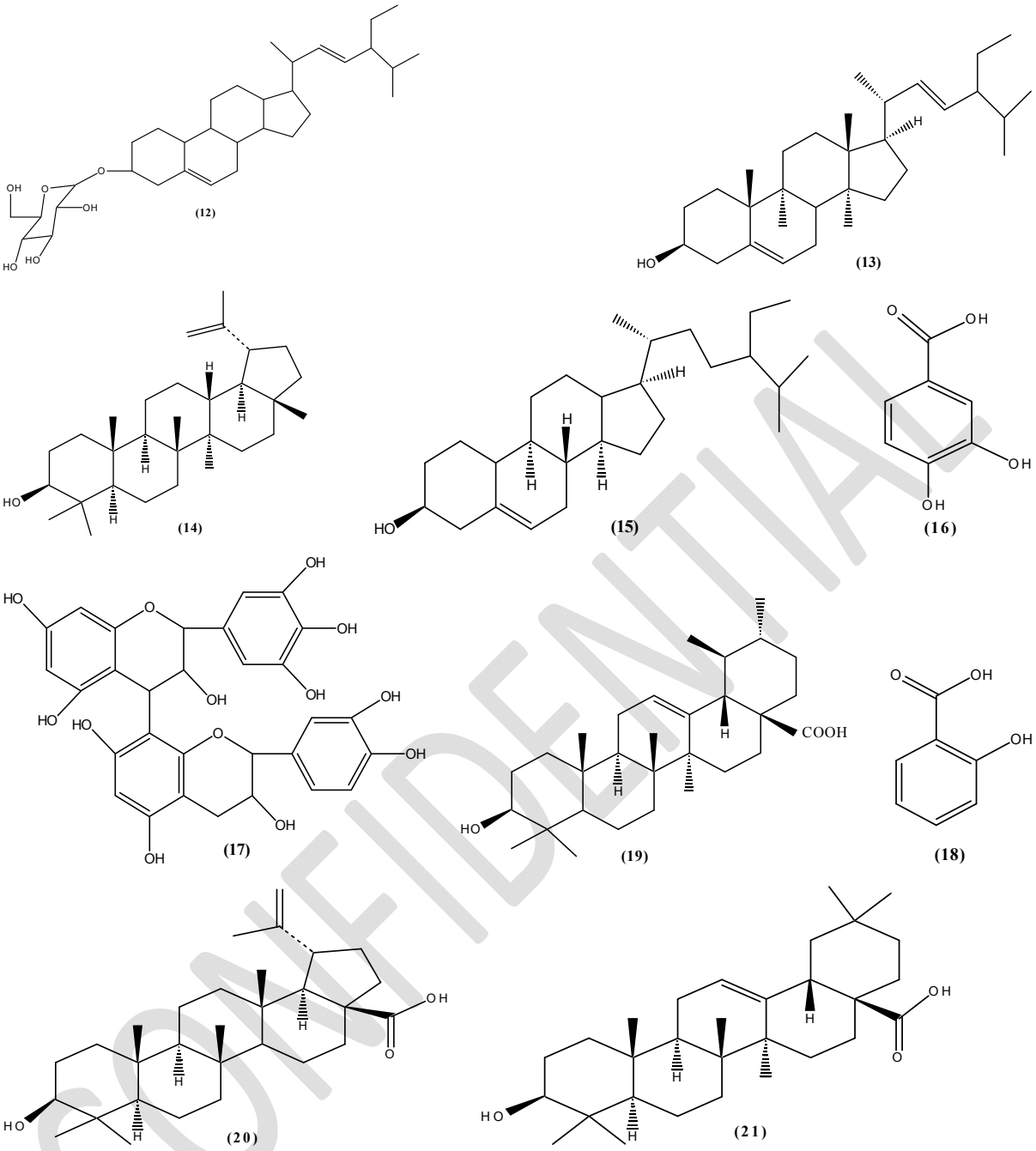


Figure 2**:** Compounds isolated from *R*. family

A 2002 study in Wistar mice found that (21) reversibly induced infertility in mice due to reduced sperm quality and motility, at a dose of 16 mg/kg for 30 days. After withdrawing this treatment, mice regained fertility and successfully impregnated female mice [25].

2.4 Bio-assay

Antimicrobial potential of some plants had been accepted long before mankind discovered the presence of microbes. The curing of plants are usually due to presence of secondary metabolites like alkaloids, steroids, tannins, phenol compounds, flavonoids, triterpenoids and the like [26].

Different literature reports indicate that medicinal plants are the backbone of traditional medicine and the antibacterial activity of plant extract is due to different chemical agent in the extract which was classified as active antimicrobial compounds. The earliest isolated pure compounds with biological activity were alkaloids because of the ease isolation as metabolic by products and have been reported as responsible for the antibacterial activity; tannins also have been form irreversible complexes with proline rich proteins, resulting in the inhibition of the cell protein synthesis [27].

Types of antimicrobial assay method like agar diffusion, micro dilution, and bio autography are used to assess the antimicrobial activity of different medicinal plant extract. Disc diffusion is selected due to its simplicity, capacity to analyze large number of samples and its ability to work well with defined inhibitors [28].

3. Objective of the study

3.1 General objective

The main objective of this research was to isolate, and characterize chemical constituents of the methanol extract of the roots of *R. minima* and carry out antibacterial activity test of the extract and isolated compounds.

3.2 Specific objectives

3.2.1. To extract the roots of *R.minima* successively using methanol.

3.2.2 To fractionate the methanol extract using n-hexane, chloroform and ethylacetate.

3.2.3. To isolate compounds from the crude methanol extract of the plant roots using column chromatography.

3.2.4. To characterize structure of isolated compounds using the UV/Vis, IR, 1D-NMR spectroscopic techniques.

3.2.4. To test the antimicrobial activity of crude extracts, fractions and isolated constituents of the plant.

4. Materials and methods

4.1. Instruments and Chemicals

^{1}H, ^{13}C and DEPT-135 NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer in DMSO-d_6 with TMS as internal standard. The ultraviolet visible (UV/Vis) spectra were taken on GENESY'S 2PC spectrometer (200-600 nm). Infrared (IR) spectra were obtained on Perkin-Elmer 65FT- infrared spectrometer (400-4000 cm^{-1}) using KBr. Melting points were recorded using digital melting point apparatus. Analytical thin layer chromatograms were run on a readymade 0.2 mm thick layer of Merck silica gel 60 F254coated on aluminium foil. Compounds on TLC were detected after dipping with iodine for a few minutes. Methanol, ethyl acetate, chloroform, n-hexane and silica gel, NaOH, $HgCl_2$, KI and H_2SO_4

4.2. Plant Material

The roots of *R. minima* were collected from Bergagy distinct, which was 365 km from south East of Addis Ababa, in Amigna Woreda, Arsi Zone, Oromia Regional State, in December 2014. Voucher specimen was deposited in the National Herbarium, Department of Biology, Addis Ababa University, Ethiopia (Voucher no.: 002). The collected roots were stored in a laboratory in Amigna preparatory school to dry at room temperature until analysis.

4.3. Apparatus

Mortar and pestle, reagent bottle, rotary evaporator, TLC plate, TLC chamber, capillary tubes, round bottom flasks, digital electronic balance, UV lamp, beakers, vials, test tubes, measuring cylinder, ruler, pencil, pipette, stirrer, cotton, Whitman no:1 filter paper, reparatory funnel and conical flasks.

4.4. Extraction and Isolation

The air dried and powdered roots of *R. minima* were stored in bottle. The air dried and powdered plant material (149g) was first soaked with 1500ml methanol for 72 hours with occasional shaking and the extract was filtered and collected and the marc was discarded. The extract was dissolved in water and partitioned successively with n-hexane, chloroform, and ethyl acetate. The organic layers were separated by using separatory funnel and then concentrated under reduced pressure using the Rotary evaporator which afforded 0.015g brown, 1.5g yellowish, and 0.03g white, for n-hexane, chloroform and EtOAc. The organic solvent insoluble fraction was found to be 10 g white solid after concentration. The methanol extract 5.25g crude powder was applied to a silica column which was packed with chloroform (100%). The column chromatography was performed as follows: Silica gel 60g was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase. In the set- up of column chromatography, the lower part of the glass column was first stocked with glass wool with the aid of glass rod. The slurry prepared by mixing 60 g of silica gel and 400 ml of chloroform was poured down carefully into the column. The tap of the glass column was left open to allow free flow of solvent into a conical flask below. The set-up was seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. The column

was allowed for some minute to stabilize, after which, the clear solvent on top of the silica gel was to drain down to the silica gel meniscus. The dry white powder methanol extract (5.52g) was applied on top of the packed column. The column tap was opened to allow elute. Elution of the extract was done with solvent systems of gradually increasing polarity using chloroform and methanol. The following ratios of solvent combinations were sequentially used in the elution process; chloroform: methanol 10:0, 9:1, 8:2, 7:3, 6:4; 5:5, 4:6, 3:7, 2:8 and 0:100 % and the following fractions were collected: chloroform (100%): fractions 1-2, chloroform: methanol (9:1): fractions 3-4, chloroform: methanol (8:2): fractions 5-8, chloroform: methanol (7:3): fractions 9-10, chloroform: methanol (6:4): fractions 11-12, chloroform: methanol(5:5): fraction 13-14, chloroform: methanol(4:6): fraction(15-16), chloroform: methanol (2:8): fraction 17-19 methanol 100%. Totally 19 fractions were collected each 50 ml. Out of 19 fractions that were collected fractions from 7-13, showed two spots on TLC developed using chloroform: methanol (8:2). The spots were visualized on TLC by dipping in iodine. Fractions 14-19 yield 600 mg of impure solid which showed characteristic coloured spots on TLC using the solvent system chloroform: methanol (8:2) upon exposure to iodine vapour. A fraction of F14-F19 (160 mg) was subjected to recrystalization which gave 45 mg white solid labelled as RMM14-19.

The second compound RMM4-8 was isolated after CC of fraction 14-19 (440 mg). In the course using $CHCl_3$: CH_3OH as eluent a total of 19 fractions each 25 ml were collected. Among these, fractions 4-8 displayed a single spot on TLC identified as RMM 4-8 (65 mg).

4.5. Thin layer chromatography (TLC) and pooling of fractions

The content of each beakers was spotted on pre coated (silica gel) aluminium plates in a small chromatographic tank to separate the different fractions based on their relative mobility's in solvent systems and colour reactions with ultra-violet light. The fractions were concentrated in a rotary evaporator at 40^0C. The mass of the different fractions was determined. A strip of the pre coated silica gel was cut out and a spot of the sample was applied on the plate about 1.0 cm from the edge. It was dried using hot air dryer. The strip was lowered into a small chromatographic jar containing the solvent system. The jar was covered with a glass lid. The solvent was allowed to ascend until the solvent front was about ¾ of the length of the strip. The strip was removed and dried by a hot air dryer and viewed under UV lamp at 365nm to identify the fluorescing spot.

The fluorescent spot was not observed and then iodine vapour used. The strip was placed in hot oven at 110^0C for 5 seconds for visibility of fluorescent bands. The colour reaction was recorded and the relative Retention factor (Rf.) value was calculated based on the formula described by Stahl

$$Rf = \frac{\text{Distance travelled by the streak from the starting point}}{\text{Distance travelled by the solvent from the starting Point to the solvent front}}$$

Table1: Characteristics of fractions from the methanol extract of *R. minima* roots.

Combined fractions	Fractions	Amount (mg)	Rf. Values	TLC coloration	UV 356nm
F1-F4	F1'	5	0.55	Brown yellow	Not observed
F5-F6	F2'	6	0.48	Brown yellow	Not observed
F7-F13	F3'	100	0.44	Brown yellow	Not observed
F14-F19	F4'	600	0.33	Brown yellow	Not observed

Elute: chloroform – methanol

4.6. Phytochemical Screening

The following chemical tests were performed for testing different phytochemical groups present in crude extract.

4.6.1 Alkaloids

To 2 ml of the crude extract 1.36gm of $HgCl_2$ & 5gm of KI in 100ml distilled water were added. Formation of a cream colour precipitate indicates presence of alkaloids.

4.6.2 Flavonoids

Alkaline reagent test: To 2 ml of the crude extract, a few drops of NaOH solution were added. Formation of intense yellow colour would indicate presence of flavonoids.

4.6.3 Saponins

Froth formation test: 2 ml of the crude extract was shaken vigorously with water in a test tube. Formation of persistent foam indicates the presence of saponins.

4.6.4 Tannins

Gelatine test: To 2 ml of the crude extract, 1% gelatine solution containing 10% NaCl was added. Formation of precipitate would suggest the presence of tannins.

4.6.5 Steroids

Salkowsiki test: 2 ml of the crude extract was treated with few drops of H_2SO_4. Formation of red colour at lower layer is likely due to the presence of steroids [29].

5. Results and Discussion

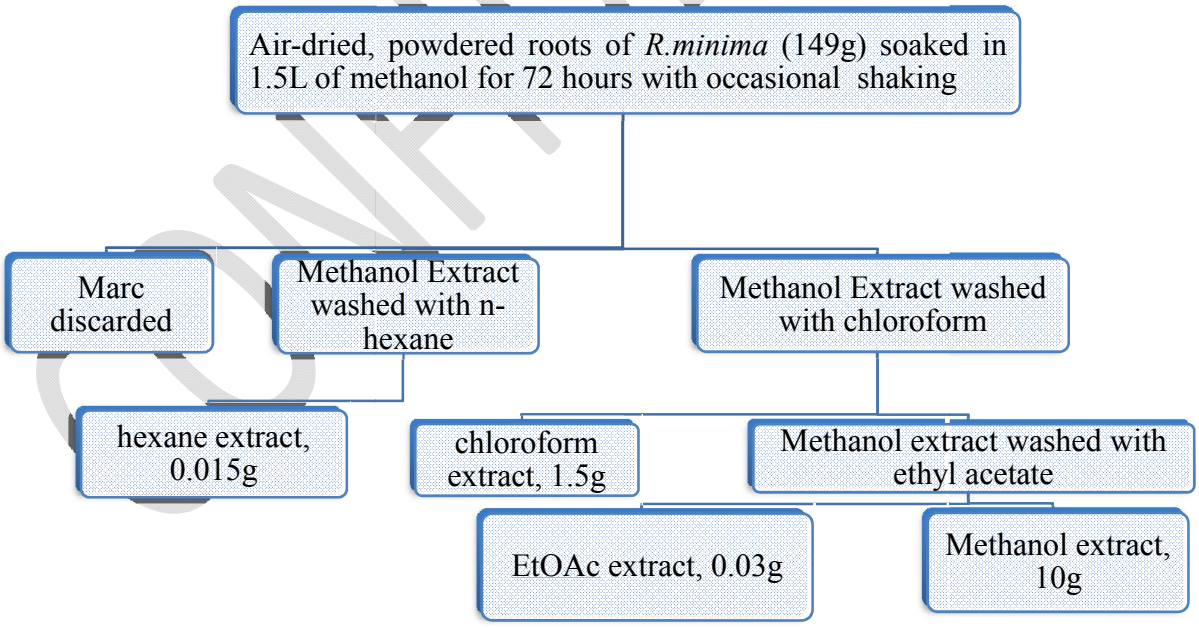
5.1. Phytochemical Screening

Following the standard methods described under methods section, phytochemical screening of the methanol extract of the roots of the plant was carried out. The results of the phytochemical screening tests are displayed in Table 2.

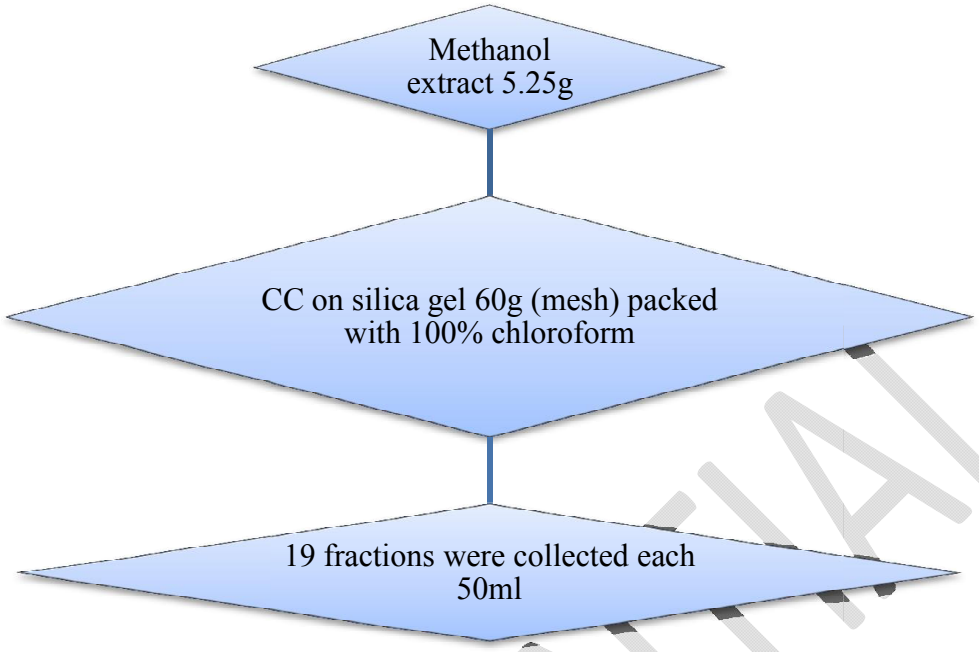
Table 2: Results of qualitative test for phytoconstituents

Phytochemical Tested	Alkaloids	Flavonoid	Saponins	Tannins	Steroids
Results	-ve	-ve	+ve	+ve	+ve
Inference	Absent	Absent	Present	Present	Present

The following (Scheme 3) shows that the path of n-hexane, chloroform, ethyl acetate and methanol extractions of powdered roots of *R. minima*.



Scheme 3: Scheme showing extraction of *R. minima*



Scheme 4: Fractionation and isolation

F1'= F1-F4 (5 mg), F2'= F5-F6 (6 mg), F3'= F7-F13 (100 mg), F4'= F14-F18 (600 mg)

Fractions 14-19 (160 mg) of the recrystalization yield (45 mg of compound RMM1418.

The other portion after silica gel CC afforded 65 mg pale yellowish solid labelled RMM 4-8.

5.2. Characterization of compound RMM1418 ((Rhynchosiaside-2))

The compound RMM1418 was obtained as a white solid that showed a characteristic colour change from colourless to brown yellow on TLC plate after dipping in iodine for a few minutes. This compound has Rf. value of 0.33 in chloroform: methanol (8:2) as a mobile phase.

In the IR spectrum of the compound RMM1418 (Appendix 1.1), the very strong broad absorption band at 3399 cm^{-1} due to the O-H stretching. The strong absorption band at 2930 cm^{-1} showed the presence of the C-H stretching for methyl groups. The absorption band at 1263 cm^{-1} showed the presence of C-C stretching. The absorption band at 1075 cm^{-1} showed the presence of the C-O bond stretching. The UV/Vis spectrum of (CH_3OH) (Appendix 1.2), showed an absorption band at 216 nm. This is likely showing the absence of conjugation in the compound.

The ^{1}H -NMR spectrum of the compound (Appendix 1.3) exhibited proton signals due to a total of eight methyl groups (all singlet), at δ 0.65, 0.71, 0.87, 1.08, 1.19 and 1.25. A singlet peak at δ 5.2 indicated an olefinic proton attached to C-19. A doublet at δ 3.29-3.93 and a triplet peak at δ 4.12-4.23 integrating for one proton assigned to C'-5 and C''-5 to the two monosaccharide sugar. The broad singlet at δ 0.87 integrating for three protons due to signal of protons attached to, C-23, C-25, C-27, C-26, C-28 and C-29 respectively. There are also different peaks from δ 0.65 to δ 5.2 which consist of CH- and CH_2- totally about 39H. A signal corresponding to the H-3 of the oxymethine proton which was appeared as a doublet of doublets at δ 3.52.

Table 3: ^{1}H-NMR (400MHz, DMSO-d_6) spectral data of compound RMM1418

Hydrogen atoms	^{1}H-NMR δ (in ppm.)
1	1.18 (*m*, 2H)
2	3.05 (*dd*, 2H)
3	3.40 (*dd*, 1H)
4	–
5	1.25 (*dd*, 1H)
6	1.45 (*m*, 2H)
7	1.23 (*m*, 2H)
8	–
9	3.11 (*dd*, 1H)
10	–
11	1.93 (*m*, 2H)
12	2.73 (*m*, 2H)
13	3.32 (*dd,* 2H)
14	–
15	0.90 (*m*, 2H)
16	1.13 (*m*, 2H)
17	–
18	–
19	5.2 (*s*, 1H)
20	–

21	3.06 (*m*, 2H)
22	3.29 (*m*, 2H)
23	0.65 (*s*, 3H)
24	0.67 (*s*, 3H)
25	0.71 (*s*, 3H)
26	0.80 (*s*, 3H)
27	0.87 (*s*, 3H)
28	1.15 (*s*, 3H)
29	1.08 (*s*, 3H)
30	1.11 (*s*, 3H)
1’	4.54 (*d*, 1H)
2’	4.14 (*d*, 1H)
3’	4.19 (*d*, 1H)
4’	3.46 (*d*, 1H)
5’	4.95 (*d*, 1H)
6’	3.95 (*t*, 2H)
1’’	-
2’’	4.19 (*d*, 1H)
3’’	3.88 (*d*, 1H)
4’’	3.87 (*d*, 1H)
5’’	4.21 (*d*, 1H)
6’’	4.12 (*d*, 2H)

The ^{13}C-NMR spectrum (Appendix 1.4) of compound RMM1418 showed a well resolved resonance of 42 carbon atoms. The DEPT-135 spectrum (Appendix 1.5) of the compound RMM1418 also indicated the presence of 6 methyl, 13 methylene, 12 methine and 8 quaternary carbons.

Table 4: Proton Decoupled ^{13}C-NMR and DEPT-135 (400 MHz, DMSO-d_6) spectral data of Compound RMM1418

Carbon atoms	^{13}C-NMR δ (in ppm)	DEPT-135	Inference
1	33.78	negative	CH_2
2	27.55	negative	CH_2
3	77.53	positive	CH-O-
4	45.90	_	C(quaternary)
5	62.54	positive	CH
6	16.50	negative	CH_2
7	41.26	negative	CH_2
8	51.36	_	C(quaternary)
9	61.36	positive	CH
10	41.89	_	C(quaternary)
11	23.46	negative	CH_2
12	30.86	negative	CH_2
13	51.76	positive	CH
14	60.94	_	C(quaternary)
15	43.88	negative	CH_2
16	47.99	negative	CH_2
17	46.11	_	C(quaternary)
18	144.24	_	C (Olefinic quaternary)
19	121.97	positive	CH (olefinic methine)
20	36.27	_	C (quaternary)
21	51.53	negative	CH_2
22	46.17	negative	CH_2
23	23.07	positive	CH_3
24	20.38	positive	CH_3
25	23.83	positive	CH_3
26	17.18	positive	CH_3
27	13.63	positive	CH_3
28	26.04	positive	CH_3
29	33.30	positive	CH_3

30	32.55	positive	CH_3
1’	103.14	positive	CH
2’	77.10	positive	CH
3’	72.09	positive	CH
4’	70.32	positive	CH
5’	74.75	positive	CH
6’	69.10	negative	CH_2
1’’	104.49	-	C(quaternary)
2’’	83.43	negative	CH_2-OH
3’’	73.27	positive	CH
4’’	83.01	positive	CH
5’’	92.20	positive	CH
6’’	74.06	negative	CH_2

The compound RMM1418 has been classified under triterpenoid saponins that have glycoside as substituent group. The proposed structure of compound RMM1418 is in Figure 5 but more data including mass spectrum is required for confirmation:

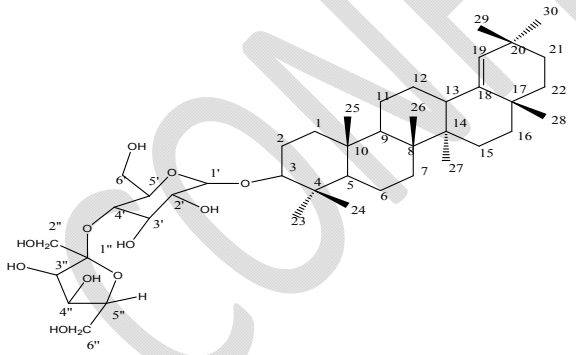


Figure 5: chemical structure of compound RMM1418 **(**Rhynchosiaside-2) was elucidated as2-[5,4-Dihydroxy-2-hydroxymethyl-6-(4,4,6α,6β,8α,11,11,14β-octamethyl 1,2,3,4,4α, 56, 6β,7,8,8α,9,10,11,12β,13,14,14α,14β-eicosahydro-picen-3-yloxy)-tetrahydro-pyran-3-yloxy]-6-hydrox ymethyl-tetrahydro-pyran-3,4,5-triol

5.3. Characterization of Compound RMM48 ((Rhynchosiaside-1))

The compound RMM48 was obtained as a pale yellow solid that showed the characteristic coloured change to brown yellow on TLC plate dipping in iodine vapour followed by heating for a few minutes. This compound has RF value 0.38 using chloroform: methanol (8:2) as a mobile phase.

In the IR spectrum of the compound RMM48 (Appendix 2.1), the absorption band at 3414 $cm^{-1}$is due to O-H stretching. The strong absorption band at 2928 cm^{-1} showed the presence of the C-H stretching for methyl groups. The absorption band at 1698 cm^{-1} showed the presence of the C=O stretching. The absorption band at 1075 cm^{-1}showed the presence of the C-O bond stretching. The absorption band at 1263 cm^{-1}showed the presence of the C-C bond stretching. The UV/Vis spectrum of compound (CH_3OH) (Appendix 2.2), showed the absorption band at 231 nm indicated that the molecule has no conjugation.

The ^{1}H -NMR spectrum (Appendix 2.3) showed a broad singlet peaks at δ 3.87 indicate that the presence of hydroxy proton. A singlet peak at δ 5.2 an olefinic proton attached to C-19 methine which were the terminal olefinic carbon at δ 121.97. A broad singlet peak at δ 0.84, integrating for three protons, corresponded to the C-27 which is attached to quaternary carbon at δ 13.25. The ^{1}H- NMR spectrum also exhibited signals due to 8 quaternary methyl protons with their peaks at δ (0.67, 0.84, 1.044, 1.15, and 1.228) integrating for 6 protons, corresponded to the methyl protons attached with C-23, C-27, C-25, C-26 and C-24 each integrating for three protons, corresponded to carbon atoms respectively. There are also different peaks from δ 0.90 to δ 5.2.00 which consist of different CH- and CH_2-groups. These interpretations showed the structure of compound RMM48.

Table 5: ^{1}H-NMR (400MHz, DMSO-d_6) spectral data of compound RMM48

Hydrogen atom	1H NMR δ (in ppm)
1	1.26 (*m*, 2H)
2	1.59 (*dd*, 2H)
3	2.68 (*dd*, 1H)
4	_
5	1.38 (*dd*, 1H)
6	1.51 (*m*, 2H)

7	1.27 (*m*, 2H)
8	–
9	1.41 (*dd*, 1H)
10	–
11	1.46 (*m*, 2H)
12	1.54 (*m*, 2H)
13	1.89 (*dd*, 1H)
14	–
15	1.15 (*m*, 2H)
16	1.56 (*m*, 2H)
17	–
18	–
19	5.2 (*s*, 1H)
20	–
21	1.57 (*m*, 2H)
22	1.83 (*m*, 2H)
23	0.65 (*s*, 1H)
24	0.67 (*s*, 1H)
25	1.04 (*s*, 1H)
26	0.99 (*s*, 1H)
27	0.84 (*s,* 1H)
28	–
29	1.10 (*s*, 1H)
30	1.07 (*s*, 1H)
1’	4.03 (*d*, 1H)
2’	3.4 (*d*, 1H)
3’	3.00 (*d*, 1H)
4’	3.09 (*d*, 1H)
5’	3.66 (*d*, 1H)
6’	2.99 (*d*, 1H)

The ^{13}C-NMR spectrum of the compound RMM48 (Appendix 2.4) showed well resolved resonance of the 36 carbon atoms. The DEPT-135 spectrum (Appendix 2.5) indicated the presence of 11 methylene, 10 mithine, 8 quaternary and 7 methyl carbons. The appearance of a carbonyl group resonating at δ 179.32 in the ^{13}C-NMR spectral data of compound RMM48 suggested the presence of carboxylic acid functional group in skeleton structure. Table 6 which showed the ^{13}C- NMR and DEPT-135 spectral data of the compound RMM48.

Table 6: Proton Decoupled ^{13}C-NMR and DEPT-135 (400 MHz, DMSO-d_6) spectral data of compound RMM48.

Carbon No.	^{13}C- NMR δ (in ppm.)	DEPT-135	Inference
1	33.24	negative	CH_2
2	23.74	negative	CH_2
3	76.77	positive	CH-O-
4	45.92	_	C(Quaternary)
5	69.60	positive	CH
6	16.37	negative	CH_2
7	41.22	negative	CH_2
8	46.1	_	C(Quaternary)
9	61.2	positive	CH
10	41.82	_	C(Quaternary)
11	22.97	negative	CH_2
12	25.98	negative	CH_2
13	51.57	positive	CH
14	51.75	_	C(Quaternary)
15	43.71	negative	CH_2
16	36.17	negative	CH_2
17	69.1		C(Quaternary)
18	144.17	_	C(Quaternary)
19	121.97	positive	CH olefinic
20	33.7	_	C(Quaternary)
21	47.98	negative	CH_2
22	13.46	negative	CH_2

23	20.38	positive	CH_3
24	17.12	positive	CH_3
25	23.40	positive	CH_3
26	16.44	positive	CH_3
27	32.51	positive	CH_3
28	179.32	_	C(Quaternary)
29	30.77	positive	CH_3
30	27.47	positive	CH_3
1’	103.07	positive	CH-O-
2’	83.31	positive	CH-OH
3’	73.87	positive	CH-OH
4’	73.87	positive	CH-OH
5’	76.87	positive	CH-O-
6’	70.18	negative	CH_2-OH

Based on the above spectroscopic data the following structure was suggested for the compound.

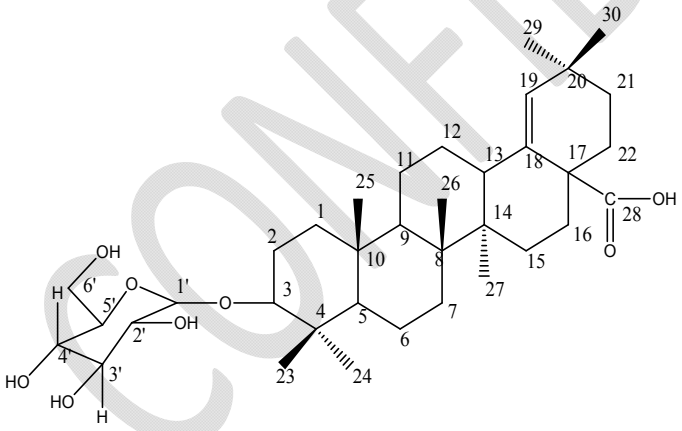


Figure 6: Chemical structure of RMM48 ((Rhynchosiaside-1))

2,2,6α,6β,9,9,12α-Heptamethyl-10-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydo-pyran-2-ylo,xy)-3,4,5,6,6α,6β,7.8,8α,9,10,11,12,12α,12β,13,14,14α-octadecahydro-2H-picene-4α-carboxylic acid.

6. Spectral Data

Compound RMM1418: white solid, 237-241^{0}cMp. IR ν_{max}KBr (400-4000) cm^{-1}, 1074, 1263, 1694, 2930, 3399, UV/Vis λmax (CH_3OH) 216nm.

^{1}H-NMR (400MHz, DMSO-d_6):5.2 (1H, *s*, H-19), 4.95 (1H, *s*, H-5'), 4.54 (1H, *s*, H-1'), 4.82 (1H, *s*, H-4''), 4.21 (1H, *s*, H-5''), 4.19 (2H, *s*, H-2''), 4.18 (1H, *s*, H-3'), 4.14 (1H, *s*, H-2'), 4.12 (1H, *s*, H-6''), 3.92 (1H, *s*, H-6'), 3.88 (1H, *s*, H-3'), 3.87 (1H, *s*, H-4''), 3.39 (1H, *dd*, H-3), 3.31 (1H, *dd,* H-13), 3.29 (2H, *m*, H-22), 3.06 (2H, *m*, H-21), 3.04 (2H, *dd*, H-2), 2.73 (2H, *m*, H-12), 1.93 (2H, *m*, H-11), 1.45 (2H, *m*, H-6), 1.25 (1H, *dd*, H-5), 1.23 (2H, *m*, H-7), 1.18 (2H, *m*, H-1), 1.15 (3H, *s*, H-28), 1.12 (2H, *m*, H-16), 1.1 (3H, *s*, H-30), 1.08 (3H, *s*,H-29), 0.9 (2H, *m*, H-15), 0.87 (3H, *s*, H-27), 0.8 (3H, *s*, H-26), 0.71 (3H, *s*, H-25), 0.67 (3H, *s*, H-24), and 0.65 (3H, *s*, H-23).

^{13}C- NMR (400MHz, DMSO-d_6): 144.24 (C-18), 121.97 (C-19), 103.14 (C-1'), 92.20 (C-5''), 74.75 (C-5'), 77.10 (C-2'), 77.53 (C-2''), 72.09 (C-3'), 83.01 (4''), 70.32 (C-4'), 73.27 (C-3''), 69.10 (C-6'), 74.06 (C-6''), 62.54 (C-5), 61.36 (C-9), 51.53 (C-21), 51.36 (C-8), 47.99 (C-16), 46.17 (C-22), 46.11 (C-17), 45.90 (C-4), 43.88 (C-15), 41.89 (C-10), 41.26 (C-7), 36.27 (C-20), 33.78 (C-1), 77.53 (C-3), 32.55 (C-30), 30.86 (C-12), 27.55 (C-2), 33.30 (C-29), 23.46 (C-11), 23.07 (C-23), 20.38 (C-24), 17.18 (C-26), 16.50 (C-6), and 13.63 (C-27).

Compound RMM48: Pale yellow solid, 166-169^{0}c Mp. IR ν_{max} KBr (400-4000) cm^{-1}, 1075, 1263, 1698, 2928, 3414, UV/Vis λmax (CH_3OH) 231 nm.

^{1}H-NMR(400MHz, DMSO-d_6): 5.2 (1H, *s*, H-19), 4.03 (1H, *s*, H-1'), 3.66 (1H, *s*, H-1'), 3.00 (1H, *s*, H-3'), 3.4 (1H, *s*, H-2'), 2.99 (1H, *s*, H-6'), 3.09 (1H, *s*, H-4'), 2.68 (1H, *s*, H-3), 1.89 (1H, *s*, H-13), 1.83 (2H, *m*, H-22), 1.57 (2H, *m*, H-21), 1.59 (2H, *dd*, H-2), 1.54 (2H, *m*, H-12), 1.46 (2H, *m*, H-11), 1.51 (2H, *m*, H-6), 1.38 (1H, *dd*, H-5), 1.23 (2H, *m*, H-7), 1.26 (2H, *m*, H-1), 1.56 (2H, *m*, H-16), 1.07 (3H, *s*, H-30), 1.1 (3H, *s*, H-29), 1.15 (2H, *m*, H-15), 0.84 (3H, *s*, H-27), 0.99 (3H, *s*, H-26), 1.04 (3H, *s*, H-25), 0.65 (3H, *s*, H-24), and 0.67 (3H, *s*, H-23).

^{13}C- NMR (400MHz, DMSO-d_6): 33.24 (C-1), 23.74 (C-2), 77.76 (C-3), 45.92 (C-4), 69.6 (C-5), 16.37 (C-6), 41.22 (C-7), 46.1 (C-8), 61.2 (C-9), 41.82 (C-10), 22.97 (C-11), 25.98 (C-12), 51.57 (C-13), 51.75 (C-14), 43.71 (C-15), 36.17 (C-16), 69.18 (C-17), 144.17 (C-18),

121.97 (C-19), 33.7 (C-20), 47.98 (C-21), 13.46 (C-22), 20.38 (C-23), 23.4 (C-25), 16.44 (C-26), 32.51 (C-27), 179.32 (C-28), 30.77 (C-29), 27.47 (C-30), 103.07 (C-1'), 76.77 (C-2'), 76.87 (C-3'), 73.39 (C-4'), 83.01 (C-5'), 70.18 (C-6').

7. Antimicrobial activities

The crude and pure fraction isolated from *R. minima* was tested against three bacteria stains using the disc diffusion method. The organisms used in the present study included**:** *Klebsiella pneumonia (K. pneumonia), Escherichia coli (E.coli) and Staphylococcus aurous (S. aurous).*

Table 7: Antimicrobial activity of crude methanol extract and pure fraction extract from *R. minima* at different concentrations.

Crude=450mg						
	Organism	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
		180 mg/ml	1/2	1/4	1/8	1/16
1	*S. aurous*	6mm(R)	6mm(R)	6mm(R)	6mm(R)	6mm(R)
2	*E. coli*	6mm(R)	6mm(R)	6mm(R)	6mm(R)	6mm(R)
3	*K. pneumonia*	6mm(R)	6mm(R)	6mm(R)	6mm(R)	6mm(R)

Pure fraction=15mg						
	Organism	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
		12mg/ml	1/2	1/4	1/8	1/16
1	*S. aurous*	6mm(R)	6mm(R)	6mm(R)	6mm(R)	6mm(R)
2	*E. coli*	6mm(R)	6mm(R)	6mm(R)	6mm(R)	6mm(R)
3	*K. pneumonia*	6mm(R)	6mm(R)	6mm(R)	6mm(R)	6mm(R)

Key: >6mm= sensitive =S, =6mm=Resistance=R

The mean value of minimum (-3 mm) of inhibition zone was reported for *R. minima.* Normal the local resident used the root of *R. minima* for anti rabies treatments, abdominal dissection, food of cattle and as soap. This plant may not have inhibiting components. Our study showed that the root of *R. minima* contain some components such as saponins, tannins, steroids and triterpenoids.

8. Conclusions

The dried powder of roots of *R.minima* was extracted with methanol. This was fractionated to n-hexane, chloroform and ethyl acetate. The methanol crude extract was subjected to column chromatography which led to the isolation of two compounds. These two compounds were isolated for the first time from this plant. The chemical structures were characterized on the basis of NMR spectral analysis, ^{1}H-NMR and ^{13}C-NMR.

The antimicrobial activity of the methanol crude extracts and the pure fractions are not sensitive on *Klebsiella pneumonia (K.pneumonia*), *Escherichia coli* (*E.coli*) and *Staphylococcus aurous (S. aurous*). In general, the compounds were not UV sensitive using different solvent systems. They only showed spots on TLC upon dipping iodine vapour for a few minutes.

9. Recommendations for further work

Based on the present study, the following are areas to be considered for further work:

The structure elucidation of compound RMM1418 and RMM48 were conducted by IR, UV, ^{1}H- NMR and ^{13}C-NMR. It is recommended to carry out full characterization by using HPLC-MS and MS.

It is recommended to carry out antimicrobial tests by other methods such as the dilution method for all the extracts to check if there are differences in activity.

To enable identification of the active constituent(s) for the demonstrated pharmacological activities, whether beneficial or adverse, it is highly recommended to carry out bioassay guided fractionation.

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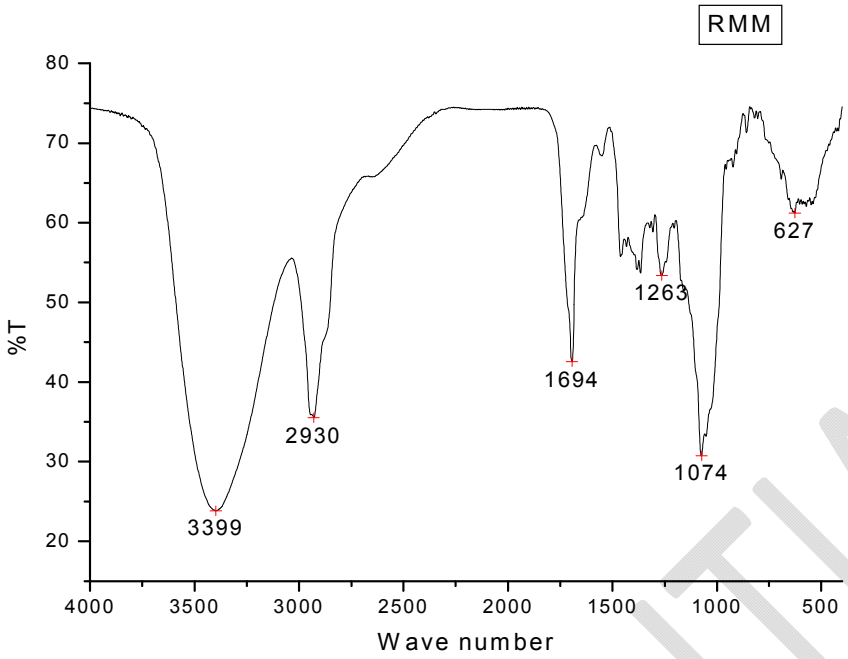
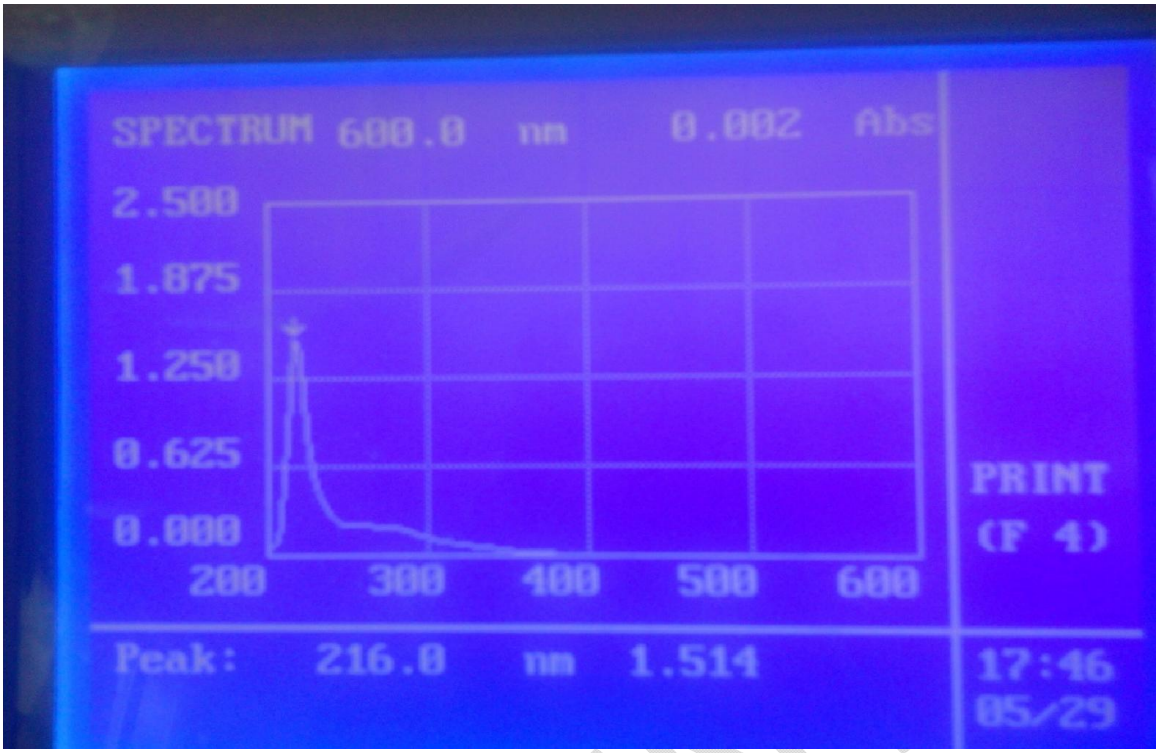
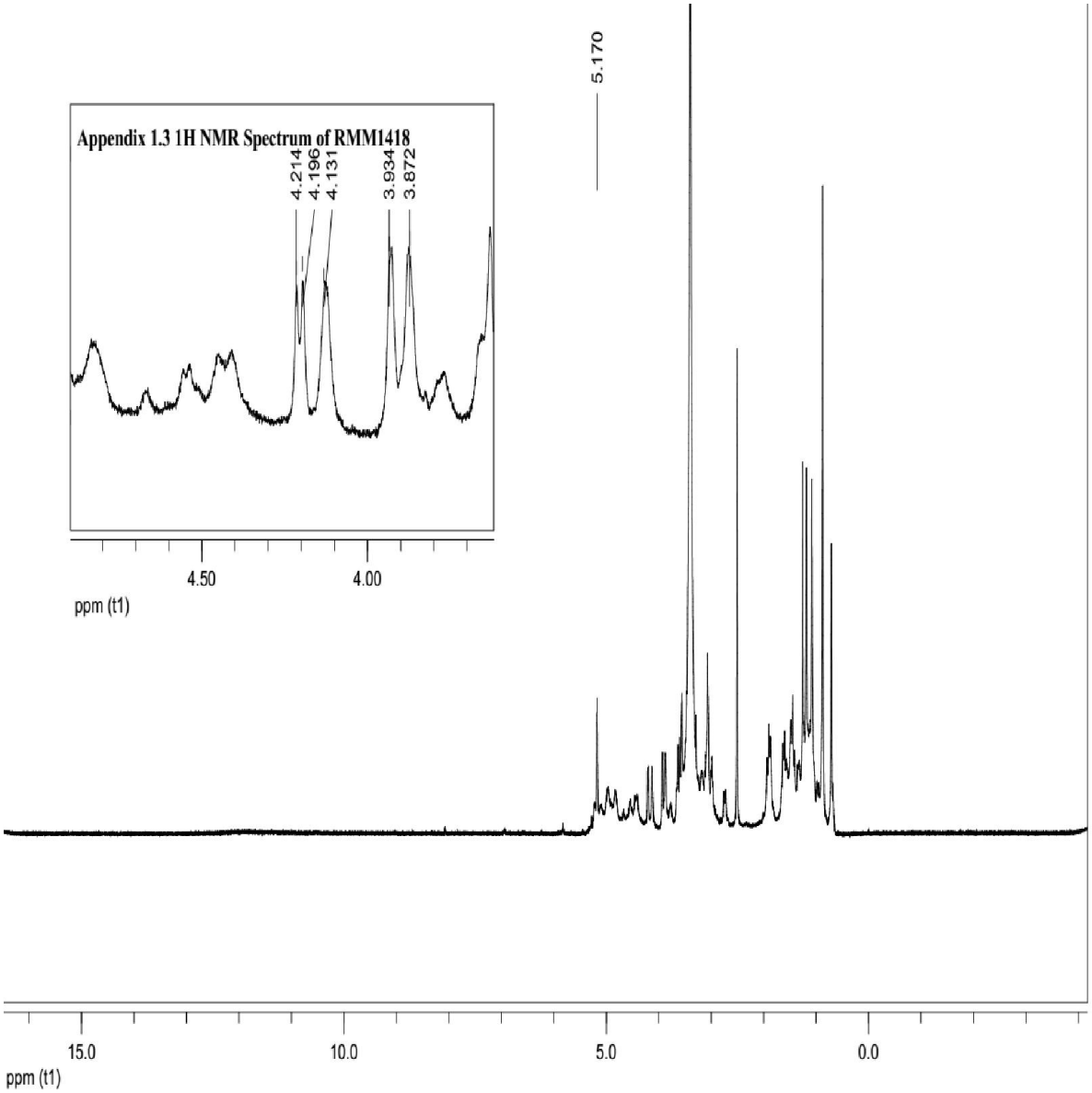


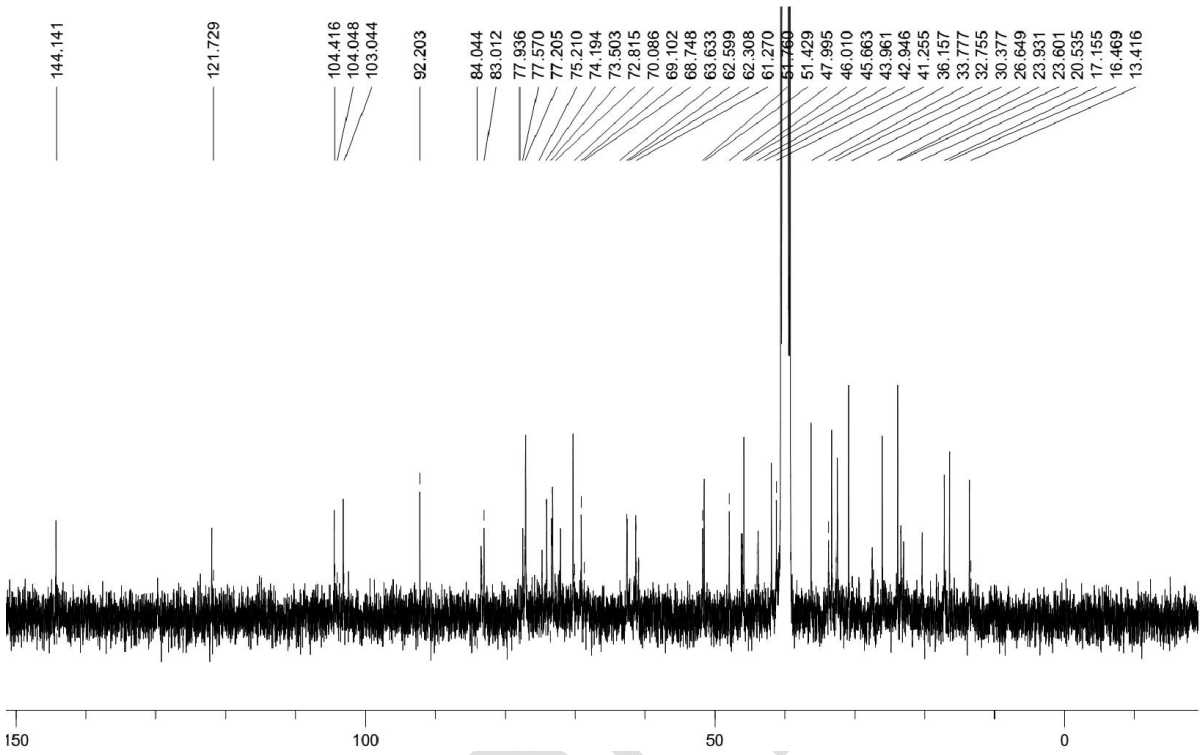
Appendix 1.1 IR spectrum of RMM1418 (Rhynchosiaside-2)



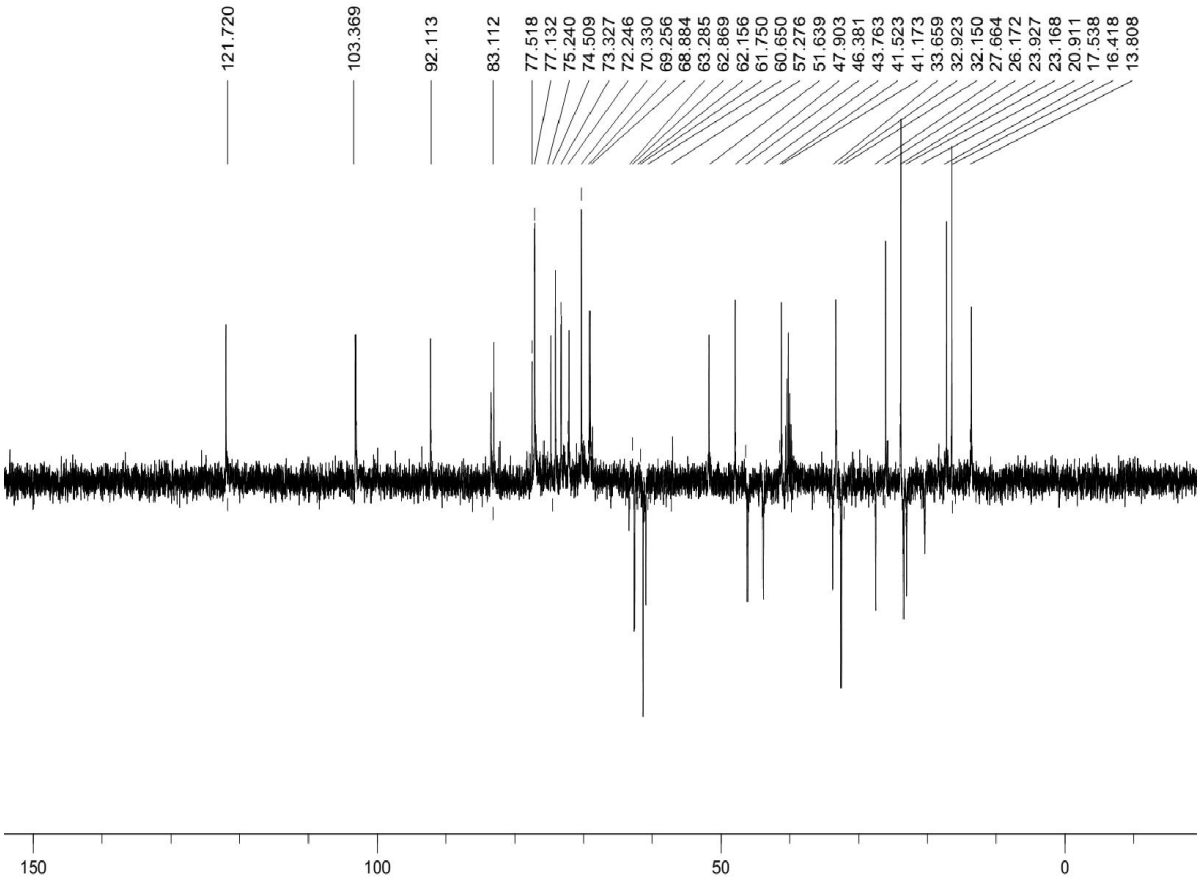
Appendix 1.2 UV/vis. Spectrum of RMM1418 (Rhynchosiaside-2) in methanol



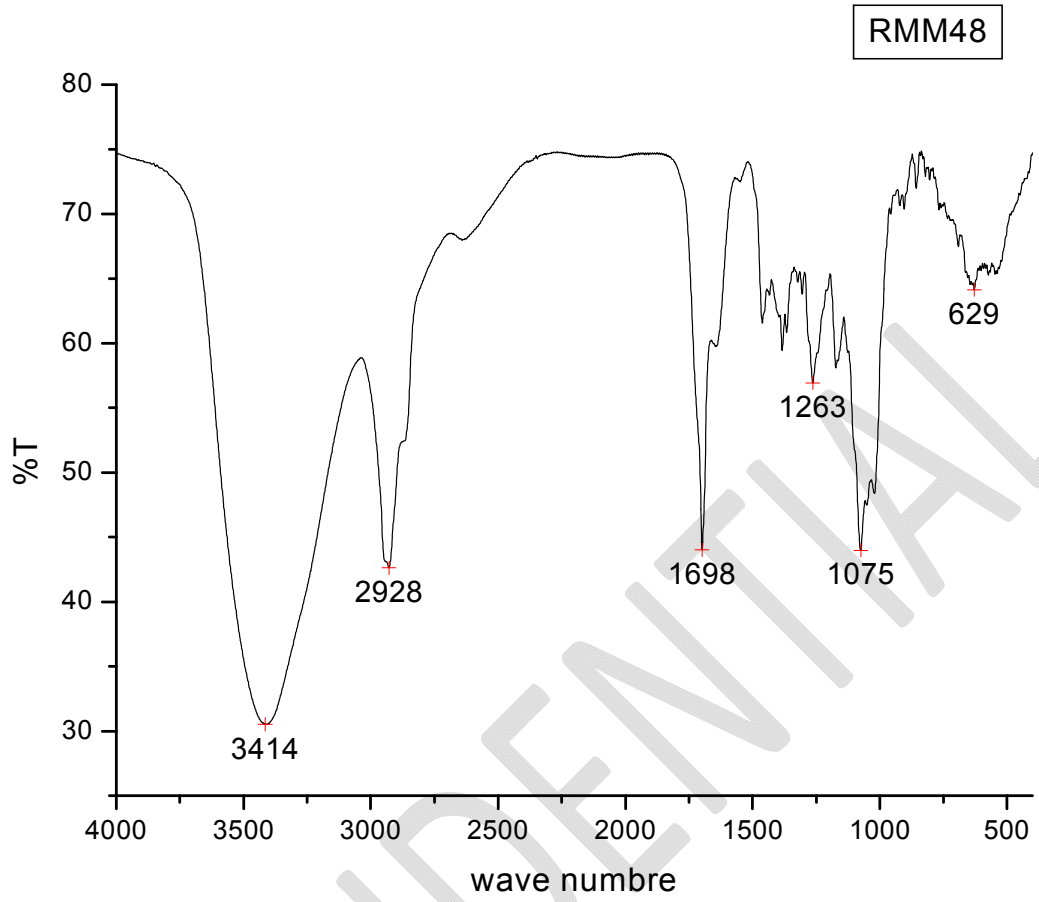
Appendix 1.3 ^{1}H-NMR spectrum of RMM1418 (Rhynchosiaside-2) in DMSO-d_6



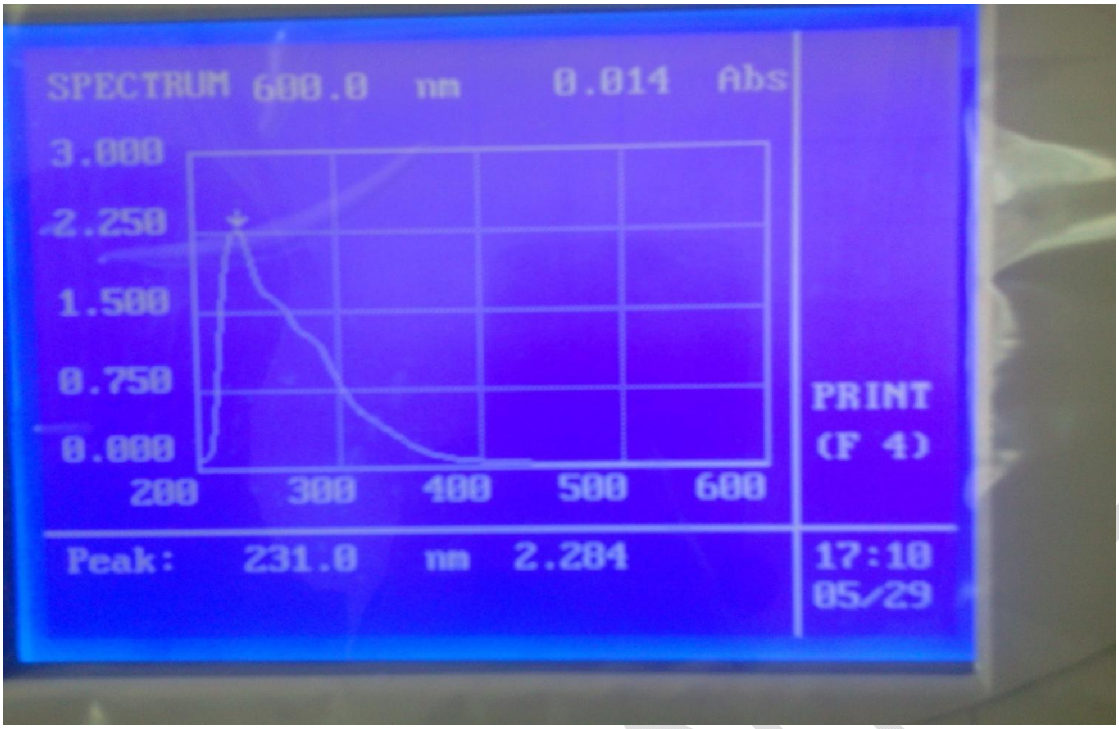
Appendix1.4 ^{13}C-NMR spectrum of RMM1418 (Rhynchosiaside-2) in DMSO-d_6



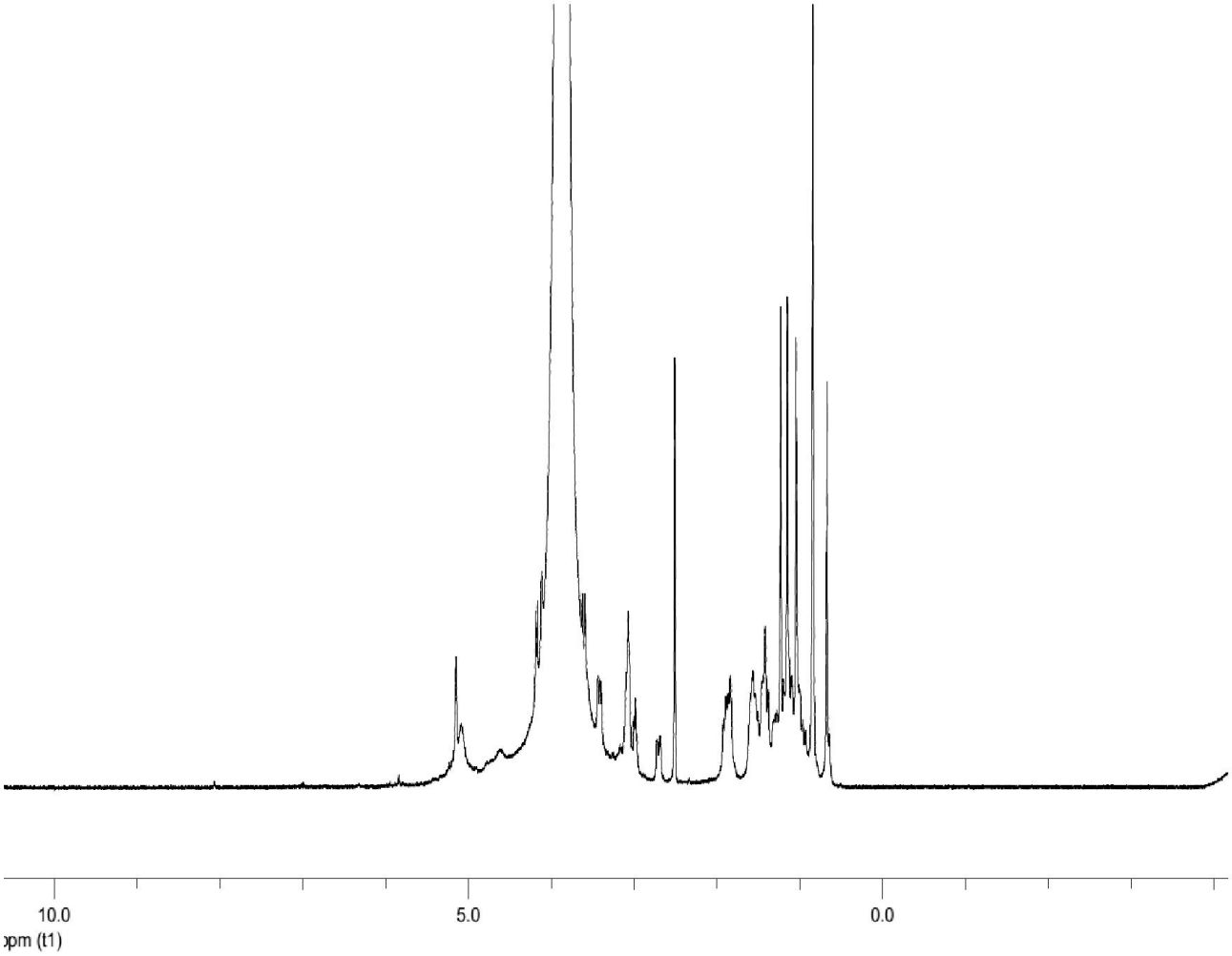
Appendix1.5 DEPT spectrum of RMM1418 (Rhynchosiaside-2) in DMSO-d_6



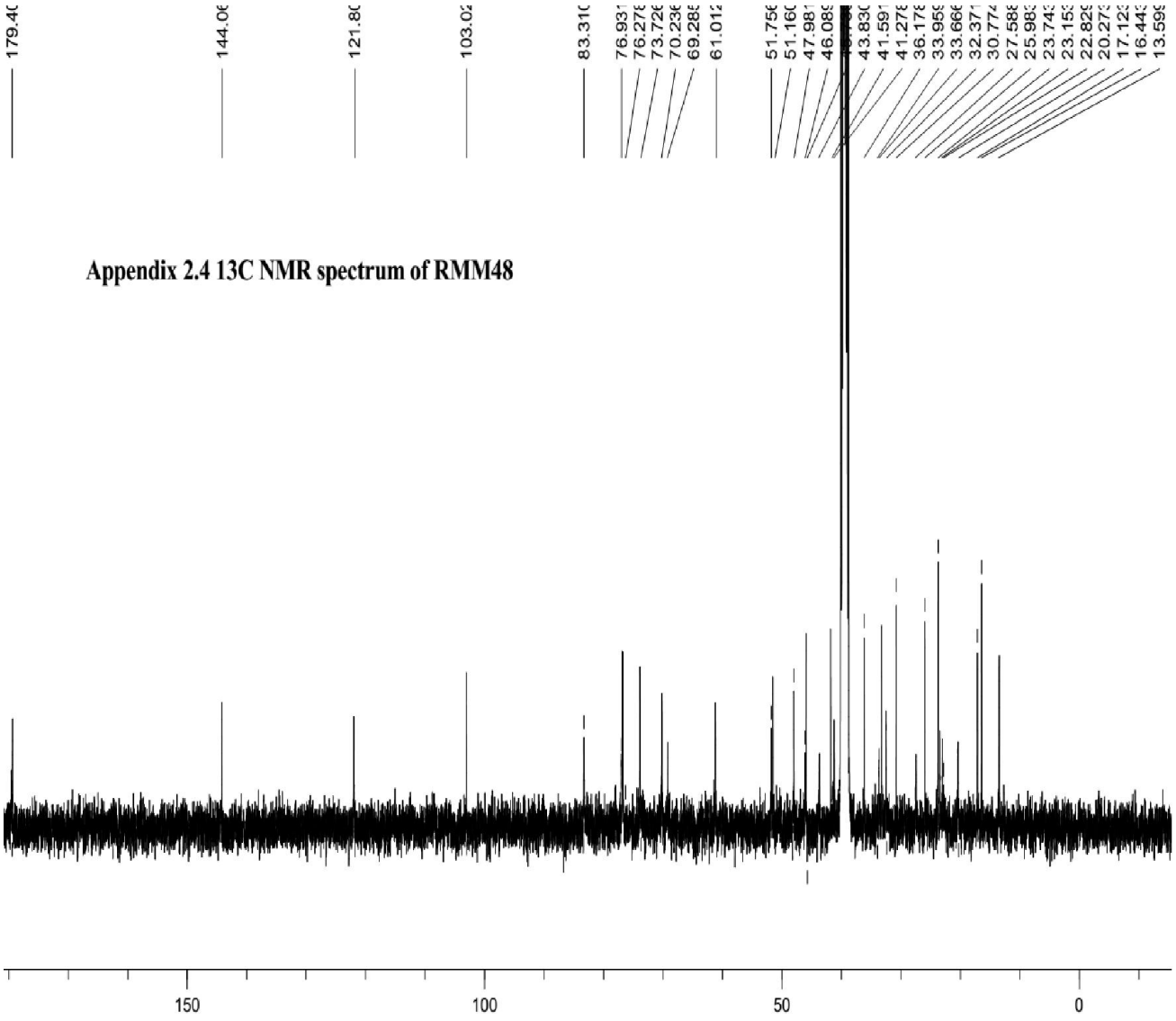
Appendix 2.1 IR Spectrum of RMM48 (Rhynchosiaside-1)



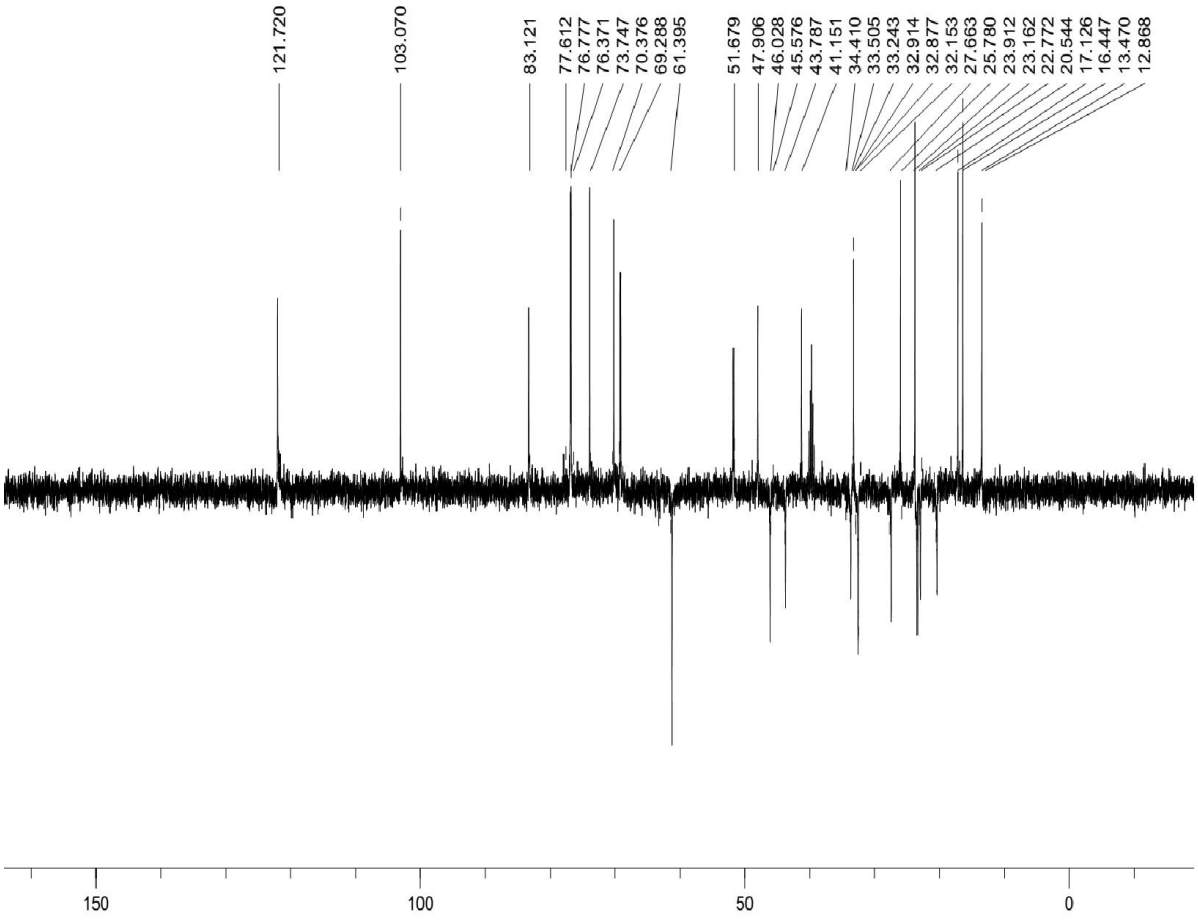
Appendix 2.2 UV/vis. Spectrum of RMM48 (Rhynchosiaside-1) in methanol



Appendix 2.3 ^{1}H-NMR spectrum of RMM48 (Rhynchosiaside-1) in DMSO-d_6



Appendix 2.4 ^{13}C-NMR spectrum of RMM48 (Rhynchosiaside-1) in DMSO-d_6



Appendix 2.5 DEPT spectrum of RMM48 (Rhynchosiaside-1) in DMSO-d_6