ADAMA SCIENCE AND TECHNOLOGY UNVERISTY

SCHOOL OF APPLIED NATURAL SCIENCE

PROGRAM OF CHEMISTRY

PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITIES OF LEAVES OF *WITHANIA SOMNIFERA*

M.SC.THESIS RESEARCH

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BY Almaz Geremew

Approved by the Examining Boar Sign and date

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Acronyms

DEPT	Distortion less Enhancement by Polarization Transfer
TMS	Tetra Methyl Silate
IR	Infra red
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
UV	Ultraviolet
CC	Coloumn Chromatography
EtOAc	Ethyl Acetate
MeOH	Methanol
$CHCl_3$	Chloroform
W.S	*Withania somniforia*

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Abstract

In this research, a phytochemical study has been undertaken on the leaves of *Withania somnifera* and the solvent extract of the plant has been conducted. *Withania somnifera* is indigenous to Eastern and Southern Africa and is occasionally cultivated for its medicinal uses, including antibacterial, anticancer, and for the treatment of ulcers, wounds, stomachache, toothache, asthma, throat and chest infection. The methanol extract of the leaves of *Withania somnifera* lead to the isolation of two compounds *W.S* -24 and *W.S*-36 The structure of compound *W.S*-24 and *W.S*-36 was determined by means of spectroscopic methods (IR, ^{1}H-NMR, ^{13}C NMR, and DEPT). The methanol extract which also checked for its antibacterial activity. It exhibited modest activity.

Key words: *Withania somnifera*, *WS*-24, *WS*-36, NMR.

1. INTRODUCTION

Some Plants are used medicinally in different countries and are the source of potential and powerful drugs. According to World Health Organization (WHO) more than 60% of the world's population and over 80% of the people in developing countries depend upon traditional medicine for their primary health care which has compounds derived from medicinal plants. However, such plants should be investigated to better understand their properties, safety, and efficiency [1].

Natural products are important sources for biologically active drugs. There has been an increasing interest in the medicinal plants as natural products in different parts of the world [2].

The medicinal value of these plants depends on bioactive phytochemical constituent's action in the human body. Phytochemicals are bioactive chemicals of plant origin and they are regarded as secondary metabolites because the plants that manufacture them may have little need for them. They are naturally synthesized from all parts of the plant body; bark, leaves, stem, root, flower, fruits, seeds, etc. i.e. any part of the plant body may contain active components [3].

Phytochemicals are basically divided into two groups with primary and secondary metabolites based on the function in plant metabolism. The major constituents of phytochemical are carbohydrates, amino acids, proteins and chlorophylls while secondary metabolites consist of alkaloids, saponins, steroids, flavonoids, tannins glycosides, anthraquinones, and terpenoids and among others Secondary metabolites are non-nutritive plant chemicals that have protective or disease preventive properties [4].

1.1. Justification of the study

The plant selected for this study is endemic to northern Ethiopia, and important in traditional medicine for the treatment of various disease, such as diarrhea, cough, common cold, and general muscular pain, internal wound, loss of appetite malaria, syphilis, gonorrhea stomachache, toothache, asthma, throat and chest infection [4].

2. OBJECTIVE

2.1. General Objective

The general objective of this study is to study the chemistry and antibacterial activities of the leaves of *Withnia somnifera*.

2.2. Specific Objectives

The specific objectives of this study are:

- ✓ To isolate secondary compounds from the leaves of *Withania somnifera.*
- ✓ To elucidate the structures of the isolated compounds using a combination of spectroscopic methods, such as ^{1}H NMR, ^{13}C NMR, IR and UV.
- ✓ To evaluate antibacterial activity of leaf extracts of *Withania somnifera*

3. LITERATURE REVIEW

3.1. Genus of *Withania*

Withania somnifera, commonly known as Ashwagandha in India, is an important medicinal plant that has been used in Ayurvedic and indigenous medicine for over 3,000 years [5].

Withania is one of the sharp plants classified in the family Solenacea and the species name *somnifera* means sleep bearing in lain indicating its potent action as a sedative [6, 7]. The Solanacea family is comprised of 84 genera that include about 3,000 species, scattered throughout the world. Members of this family are generally annual shrubs. The genera *Withania* and *Physalis* play an important role in the indigenous medicine of South East Asia. The twenty-three known *Withania* species are widely distributed in the drier parts of tropical and subtropical zones, ranging from the Canary Islands, the Mediterranean region and northern Africa to Southwest Asia [8-11]. Among them, only two species, *Withania somnifera* and *Withania coagulans*, are economically and medicinally significant, being used and cultivated in several regions [12, 13].

3.2. Withania somnifera

Withania somnifera is a small shrub growing to 2m high and to 1m across. Almost the whole plant is covered with short, fine, silver-grey, branched hairs. The stems are brownish and prostrate to erect, sometimes leafless below. The leaves are alternate (opposite on flowering shoots), simple, margins entire to slightly wavy, broadly ovate, obovate or oblong, 30–80mm long and 20–50mm broad, narrowed into the 5–20mm long petioles, almost hairless and green above, densely hairy below.

In Ethiopia the flowering time is mostly from October to June, while the fruiting time is mostly from October to July. It mainly occurs in Amhara (Gojjam), in Tigray, Oromia (Shewa) and southern Ethiopia regions [14].

Figure.1 *Withania somnifera* from *Gohatsion [picture taken by Almaz Geremew, 12/7/2007ETC*

The species *Withania* is named with various vernacular name as shown in Table.1. This indicates the wide popularity of the plant.

Table 1. Various vernacular name of Withania

Laungage	**vernacular name**
English	Winter cherry
Arabic	Kaknaj –e-Hindi
Afan Oromo	Kumo
Amharic	Gizewa

3.2.1. Ethnobotanical Background

Withania is known to be used against coughs and asthma, as a narcotic and anti-epileptic in Ethiopia. Research notes other traditional uses for headache (as a dressing), paludism or malaria, ague fever stomachache and as a diuretic. The smoke of the burning root is commonly inhaled for Satan beshifa or devil diseases [16]. Gizewa is also found to have anti-fertility properties and traditionally used as vaginal douche (aqua extract) for its utero- tonic and anti-implantation activity on butanol fraction extract [17].

Western herbalists are familiar with *Withania (*gizewa) and it is used as tonic, in western herbal medicine decoction or extract made from the root is a popular remedy from the Ayurvedic tradition, as an 'adaptogen' remedy and for the treatment of debility and nervous exhaustion, for convalescence and as a general tonic [18].

3.2.2. Medicinal uses of *Withania somnifera*

Withania root drug used for the treatment of rheumatic pain, inflammation of joints, nervous disorders and epilepsy. Dried roots are used as tonic for hiccup, cold, cough, female disorders, as a sedative, in care of senile debility, ulcers, etc. Leaves are applied for carbuncles, inflammation and swellings. Leaves juice is useful in conjunctivitis. *Withania* has anti-inflammatory, anti-tumor, anti-stress, antioxidant, mind-boosting, immune-enhancing, and rejuvenating properties. *Withania* root has also been noted to have sex-enhancing properties [20, 21]. *Withania somnifera* plants are rich in bioactive compounds which can resist health hazards and in recent time's antibacterial activity of *Withania somnifer*a and species have been reported [22, 23].

3.3. Phytochemical investigation of *Withania somnifera*

Phytochemical reports showed the presence of various types of bioactive secondary metabolites such as alkaloids, steroids, tannins, flavonoids, glycosides, terpenoids and saponins. Below is a brief description of some of their metabolites

3.3.1. Phenols

This group of secondary metabolites is characterized by the presence of one or several hydroxyl (OH) groups attached to an aromatic ring. Phenolic compounds are very diverse and occur either as simple compounds with one aromatic ring or as complex polymers (poly phenols) with different functional groups attached to their ring. The classification is based on the numbers of carbon atoms in the basic skeleton and major groups include the flavonoids, phenolic acids and tannins.

Table 2 show some examples of the different classes of ‘phenols with their basic carbon skeleton and the number of carbons in the skeleton.

Table.2 Examples of different class of phenolic compound

No of carbons	Basic carbon skeleton	Class of phenols
6	C6	Simple phenols and benzoquinones
10	C6-C4	Naphathquinones
14	C6-C2-C6	Antraquinones
15	C6-C3-C6	Flavonoids
N	(C6-C3-C6)n	Tannins

Phenolic compounds are widely distributed in the plant kingdom and accumulate in plants mainly as pigment responsible for the flavour of color and different plant parts. For instance most floral pigments and the compounds responsible for the flavour of species like ginger and cinnamon are phenolic.

3.3.2. Flavonoids

Flavonoids represent one of the largest classes of naturally occurring phenolic compounds. Chemically flavonoids are phenolic glycosides, and their glycones consists of two aromatic rings joined by a 3-C unit in other words they are phenyl propane but these fall into a much smaller number of groups which are classified according to the oxidation level of the 3-C unit of the flavone nucleus [24].

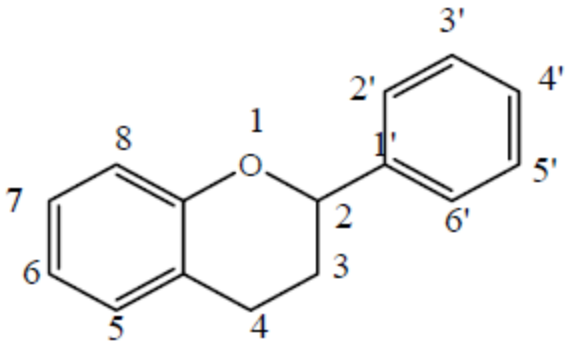

Figure.2 Flavonoid skeleton

The flavonoids include many of the most pigments and occur throughout the entire plant kingdom from the fungi to the angiosperms. About 150 a glycones are known in higher plants, they are found both in vegetative parts and in flowers, as pigments, they have a well-known role in attracting pollinating birds and insects. Flavonoids can also be powerful inhibitor of oxidative phosphorylation [24].

3.3.2.1. Biological activities of flavonoids

Flavonoids can be considered as derivatives of the 2-phenylchromone parent compound composed of two phenyl rings, and a heterocyclic ring referred to as the A, B and C- rings [25]. Flavonoids are secondary metabolites characterized by flavan nucleus [26] and a C6-C3-C6 carbon skeleton [27]. These are group of structurally related compounds with a chromate-type skeleton having phenyl substituent in C-2-C-3 position [28].

The flavonoids belong to one of the most bioactive compounds which naturally exist in the plant kingdom. Different naturally occurring flavonoids have been described and subcategorized into flavones, flavans, flavanones, isoflavonoids, chalcones, aurones and anthocyanidines [29, 30]. These flavonoids have remarkable biological activities, including inhibitory effects on enzymes, modulatory effect on some cell types, protection against allergies, antiviral, anti-malarial, antioxidant, anti-inflammatory and anti-carcinogenic properties [29, 30]. A number of flavones, flavonols, flavanones, and isoflavones, as well as some of their methoxy, isoprenyl, and acylated derivatives, show antibacterial activity [31].

3.3.3. Tannins

Tannins are biologically active compounds having diverse antimicrobial actions. Tannins have wide applications in food, pharmaceutical and leather industries. It is extracted from the roots and fruits of *Rhustrilobata.* Tannic acid has antibacterial, antidermatotic, antihemorrhoidal, antiseptic, astringent, antiulcer and antiviral properties. It also has well-described antimutagenic and antioxidant activities.

3.3.4. Terpenes

Terpenes are a large and varied class of hydrocarbons, produced primarily by a wide variety of plants and also by some insects such as swallowtail butterflies, which emit terpene from their osmeterium. They are the major components of resin, and of turpentine produced from resin. The name “terpene” is derived from the word “turpentine”, the suffix “ene” indicating the presence of olefinic bonds. Terpenoids are oxygen- containing analogues of terpenes. They are thoroughly distributed in the plant kingdom, especially in those plants that have abundant chlorophyll. 4
Among these are compounds which fall in the general class of terpenes, compounds made of 5-carbon unit, often called isoprene units, put together in a regular pattern, usually head-to-tail in terpenes up to 25 carbons. Terpenes containing 30 carbons or more are usually formed by the fusion of two smaller terpene precursors such that the head to tail “rule” appears to be violated. In overall, terpenes hold potential interest practical applications especially in the fragrance and flavors industries, as well as in the pharmaceutical and chemical industries.

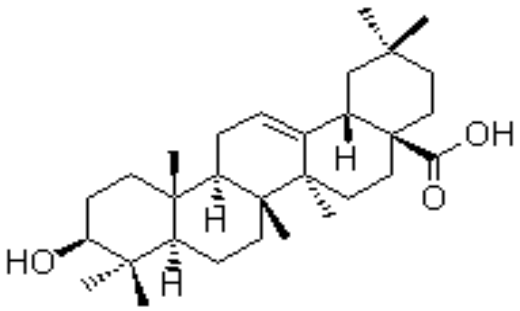

Figure.3 Chemical structure of Oleanolic acid

3.3.5. Saponins

Saponins are steroids and triterpene glycosides. They are called as saponins due to their soap like properties. Saponins possess anti-ulcer, anti-tumor and anti-diabetic properties. Four saponins, bugbanosides have been isolated from *Cimifuga simplex*.

3.4. Some compounds isolated from the genus *Withania*

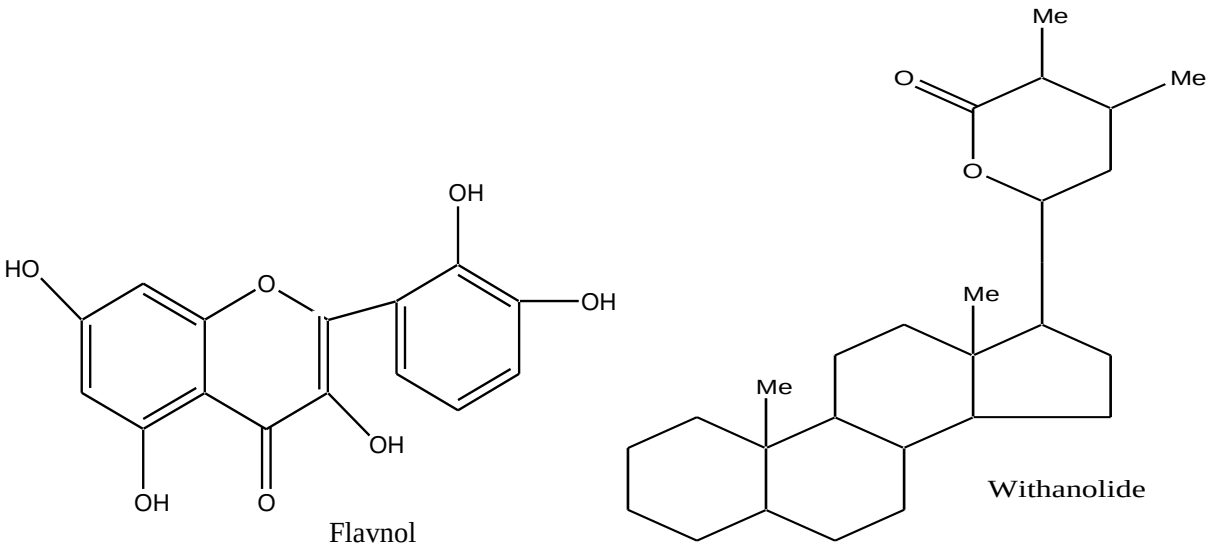

Figure.4: Some compounds reported from the genus *Withania*

4. MATERIALS AND METHODS

4.1. Plant material

Leaves of *Withania somnifera* were collected from Gohatsion, North Shewa Oromia, Ethiopia approximately 185km from Addis Ababa and 285km from Adama in October 2014. The plant was identified by a botanist and specimens stored in National Herbarium, Biology Department of Science Faculty, Addis Ababa University .

4.2. Chemicals and Equipment

Chemical employed in this research are ethanol, chloroform, methanol, *n*-hexane, acetone, deuterated chloroform ($CDCl_3$) thin layer chromatography (TLC), TLC chamber, column chromatography, Filter paper, Rotary evaporator, Shaker, suction filtration, Ultraviolet lamp, UV, Nutrient agar media (N.A.M), Petri dish .

UV spectrum was measured with genesis' spectrometer (200-400) in CH_3OH at room temperature. IR spectrum was recorded as KBr pellets on Perk-Elmer BX Infrared Spectrometer in the range 4000-400 cm^{-1}.

^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra were recorded on a Bruker avance 400 MHz NMR spectrometer with TMS as internal standard.

4.3. Extraction and isolation

The powdered dried leaves of *Withania somnifera* 200gm were successively extracted with hexane followed by chloroform, and then the mark after chloroform was extracted with methanol at room temperature for 72hr. It was then filtered and concentrated using rotary evaporator with the whole results depicted in Table 3.

Table.3 Different solvent extracts of the leavs of *Withania somnifera*

Solvent	**Amount in ml**	**Crude extraction**	**The dried marc**
n-Hexane	1000ml	7.56gm	200gm
Chloroform	800ml	10.8gm	189.4gm
Methanol	600ml	25gm	180.4g m

Five gram (5gm) of the methanol extract was applied on a column chromatography packed with 160(gm) of silica gel. Isolation was carried out using the solvent chloroform and methanol with increasing polarity. A total of 31 fractions were obtained.

Fraction that showed the same Rf value and the same characteristics color on TLC was combined.

Table.4 Column Chromatographic fractionate of the MeOH extract

Solvent system	Ratio	Fraction	Volume	Code
Chloroform	1	1-2	150ml	*W.S*-1
Chloroform/ Methanol	9:1	2-4	150ml	*W.S*-24
Chloroform/ Methanol	8:2	5-9	150ml	*W.S*-3
Chloroform/Methanol	7:3	10-12	150ml	W.S-36
Chloroform/methanol	6:4	13-17	15oml	*W.S*-5
Chloroform/methanol	5:5	18-24	150ml	*W.S*-6
Methanol	1	25-31	150ml	*W.S*-7

Based on TLC analysis, fraction 2-4 (*W.S*-24) and 10-12 (*W.S*-36) showed a single spot.

The solvent system used for the TLC examination of fraction 2-4 (*W.S*-24) was chloroform/methanol 9:1 and for fraction 10-12 (*W.S*- 36) was chloroform/methanol 7:3.

Fraction 2-4 was labeled as *W.S* -24 (10mg) while fraction 10-12 was labeled *W.S*-36 (13mg) and *W.S*-36 were recorded and characterized.

4.4. Preliminary phytochemical screening of the *Withania somnifera*

The methanolic crude extracts were tested for the presence of secondary metabolities, such as flavonoid, alkaloids, Phenol and terpenoids. The whole procedure was duplicated in phytochemical test.

Procedure of phytochemical test

- **Alkaloid**

Mayer reagent -Solution; - dissolve 1.36gm of $HgCl_2$ in 60ml and 5gm of KI dissolve in 10ml distill water add a few drops to an acidified extract solution H_2SO_4. Alkaloids are present, a white to yellowish precipitate.

- **Flavonoids**

Alkaline reagent test; -the extract were treated with few drops of NaOH solution the formation of intense yellow color indicate the presence of flavonoids.

- **Phenol**

The extract was treated with 3-4 drop of Ferric chloride solution for the formation of blush color indicated the presence of phenol.

- **Terpenoids**

5ml of each extract was mixed in 2ml of chloroform, and concentrated H_2S0_4 (3ml) was carefully added to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoid.

4.5. Antibacterial study

The bacterial SPP used for the test were *Escherichiacol*i (*E.coli*), *Klebsiella pneumonia* (K. pneumonia) and Staphylococcus *aureus* (*S. aureus*). All the stock cultures were obtained from micro lab, Institute of Research and Technology, the microorganisms were grown overnight at 37°C in Mueller-Hinton Broth at pH 7.4 [6, 7].

4.5.1. Culture media and inoculums preparation

Nutrient agar broths were used as the media for the culturing of bacterial strains. Loops full of all the bacterial cultures were inoculated in the nutrient broth and incubated at 37° C for 72hrs.

4.5.2. Testing for antibacterial activity

The extracts obtained above were screened for their antibacterial activity in comparison with standard antibiotic Ciprofloxacin (10µg/ml) *in-vitro* by well diffusion method [7, 8, 9]. The cup-plate agar diffusion method was employed to assess the antibacterial activity of the prepared extracts 20ml of the inoculated nutrient agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates, 5mm in diameter, were cut using a sterile cork borer No. 4 and the agar discs were removed using sterilized micropipettes 25µl of different solvents with selected *Withania somnifera* extracts was added in to the well, allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37ºC for 18 hours. The respective solvents were used as controls. The diameters of the growth inhibition zones were measured at 24hours of incubation averaged and the mean values were tabulated.

5. RESULTS AND DISCUSSION

5.1. Bacterial Tested

- ✓ The antibacterial activity of the extract of *Withania somnifera* were determined against three pathogens, namely, *Staphylococcus aureus ,Escherichia coli, Klebsiella pneumonia.*
- ✓ These were obtained from Adama Oromia regional laboratory, found in Adama. All test bacteria were grown in Mueler Hinton Agar at 37^0C for 24hr.

5.2. Antibacterial activity

5.2.1. Preparation of extract solution

The extracts were dissolved in methanol to a final concentration of 10 mg/ml for disc diffusion assay.

5.2.2. Bacterial Cultures

All the bacterial strains were grown and maintained on nutrient agar slants.

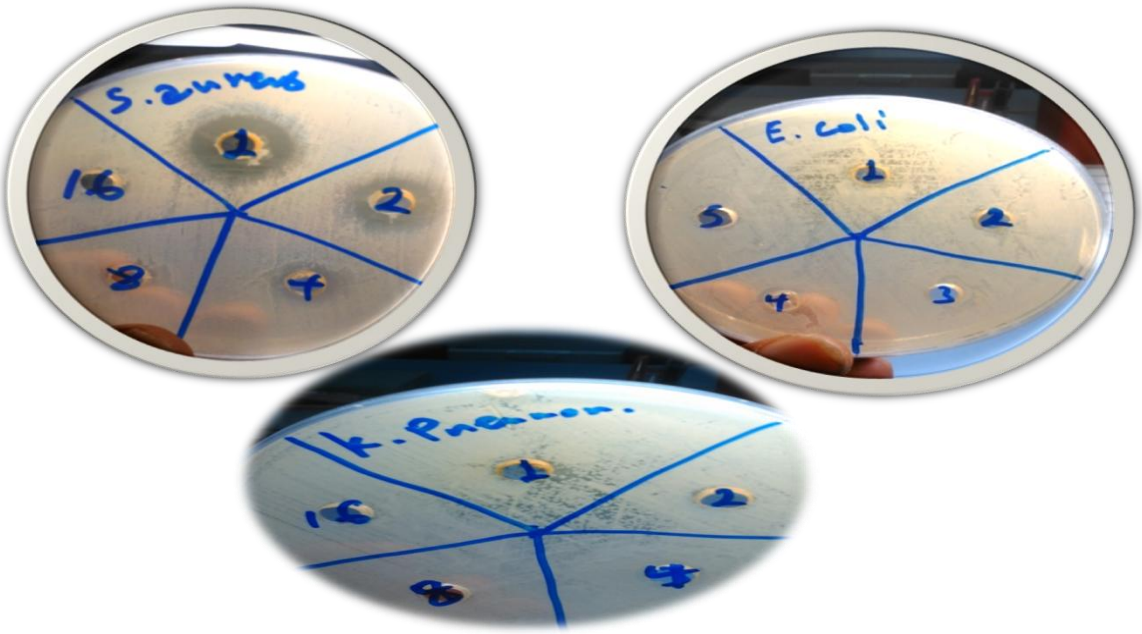

Figure.5 The antibacterial activity of the methanol extract of *Withania somnifera* against *Staphylococcus aureus, Escherichia coli* bacteria and *K. pneumonia*

Table.5 Antibacterial activity of methanol extract determined by disk diffusion assay

S.NO	**Organism**	**Tube 1**	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**
		X gm/ml	½	¼	**1/8**	**1/16**
1	*S. aurous*	17mm(S)	13mm(S)	9mm(S)	6mm(R)	6mm(R)
2	*E. coli*	15mm(S)	12mm(S)	8mm(S)	7mm(S)	6mm(R)
3	*K. pneumonia*	10mm(S)	8mm(S)	7mm(S)	6mm(R)	6mm(R)

Key

>6mm= sensitive =S

<6mm=Resistance=R

Table 5 and Figure 4 showed the results of the gram positive bacteria used in the test (*Staphylococcus aureus)* and the gram negative bacteria (*Escherichia coli*, and klebsiella pneumonia). The methanol extract of the leavs of Withania somnifera were found sensitive at higher doses against the three bacterial pathogens.

Furthermore, it was observed that methanol extract of *Withania somnifera* showed more activity against *E.coli* and *S. aureus* compared to *K. pneumonia.*

5.3. Phytochemical screening of *Withania somnifera*

The methanol extract of the leavs of this plant was assessed for the presence of various secondary metabolities using standard procedures. The results were briefly shown in Table 6.

Table. 6 Phytochemical screening of *Withania somnifera*

S. No	Phytochemical	Reagents	Methanol extract M(+means present –means absent)
1	Alkaloids	Wagner test	+
2	Phenols	Ferric chloride test	+
3	Flavonoids	Alkaline reagent test	+
4	Terpenoids	Salkowski test	+
5	Saponins	Chloroform and H_2SO_4test	_

As show in Table 6, the methanol extract of the leavs of *Withania somnifera* were found to be rich in alkaloids, phenols, flavonoids, and terpenoids. However, saponins was not detected.

5.4. Characterization of *W.S*-24

The UV spectrum of *W.S*-24 [Appendix.1] showed absorption maximum at 274nm which indicates the presence of conjugation in the structure.

The IR [Appendix 2] showed absorption maximum at 2923cm^{-1} due to C-H stretching of aliphatic group, absorption at 1080cm-^{1} due to of ester C-O stretching and absorption at 802cm^{-1} due to trisubstituted double bond. The ^{1}H-NMR spectrum (Appendix 3) showed peaks at δ 0.99, 0.99 and 1.14 due to three aliphatic methyl protons. δ 5.0 (1H, m) due to olefinic proton & peaks at δ 1.59-1.21 showed methylene protons. The peaks at δ 7.5 -7.75 indicate protons of aromatic ring .

Table.7 ^{1}H-NMR, ^{13}C NMR and DEPT spectral data of *W.S*-24

Carbon Number	**C^{13} NMR**	**DEPT- 135**	**H NMR δ**	**Number of hydrogen**
1'	147.07	Quaternary	-	-
2'&6'	119.10	CH	6.95	1
3'	130.91	CH	7.18	1
4'	132.39	Quaternary	-	-
5'	123.98	CH	5.27	1
7'	124.46	CH	7.10	1
8'	128.85	CH	7.11	1
9'	139.28	CH	6.49	1
10'	114.07	CH_2	5.20	2
1	167.68	Quaternary	-	-
2	31.95	CH	2.26	1
3	31.44	CH_2	1.59	2
4	34.53	Quaternary	-	-
5	33.83	CH_2	1.36	1
6	28.96	CH	1.40	1
7	30.20	CH	2.08	1
8	30.04	CH_2	1.25	2
9	27.73	CH_2	1.33	2
10	19.17	CH_3	0.99	3
11	37.10	CH_2	1.21	2
12&22	30.45	CH_2	1.21	2
13	34.87	Quaternary	-	-
14	29.96	CH_2	1.25	2
15	14.12	CH_3	0.96	3

16	29.71	CH_2	1.36	2
17	29.37	CH_2	1.59	2
18	71.80	CH	3.16	1
19	31.63	CH	1.51	1
20	29.52	CH_2	1.25	2
21	27.10	CH_2	1.33	2
23	22.70	CH_3	1.16	3

The ^{13}C NMR and DEPT-135 spectrum (Appendix 4, & 5 Table. 6) indicated that W.S-24 has 31 carbon atoms. The spectra showed the presence of three methyls, eleven methine, twelve methylene, and five quaternary carbon atoms, indicating 44 proton connected with 31 carbon atom. Since, IR and NMR spectrum showed the presence of ester and alcohol functionalities there should exist at least three oxygen atoms.

The ^{13}C NMR spectrum of *W.S*-24 displayed signals characteristic of five double bonds at δ 147.07 & 119.10,130 .91 & 123.98, 132.39 & 119.10 and 139.28 & 114.07, 130.91 and 124.46. It showed the carbon attached with hetroatom (O), which is methine at δ 71.80. Based on the above spectral data the following structure was proposed *W.S*-24.

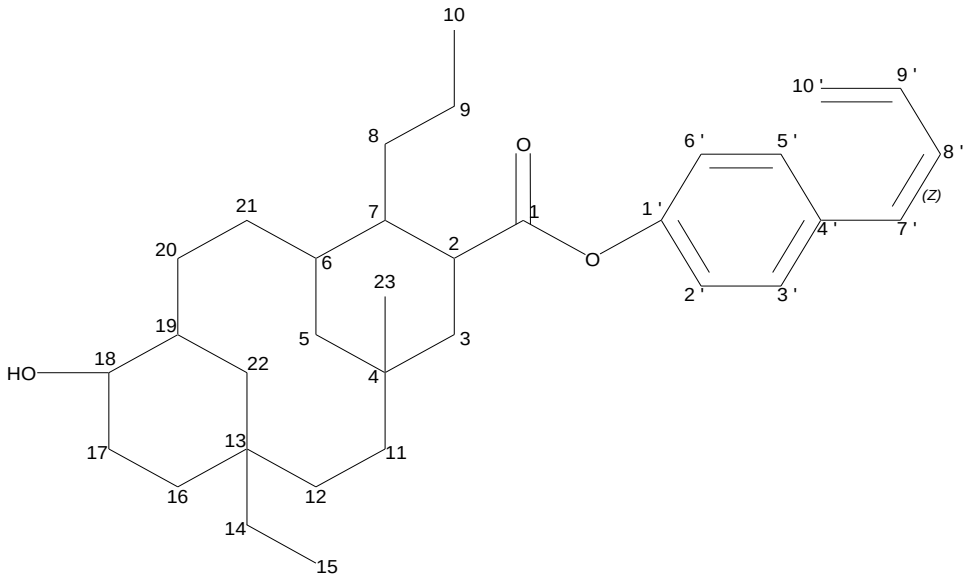

Figure.6 Proposed structure of Compound *W.S*-24

4-[13-(13-Ethyl-18-hydroxy-19-propyl-cyclohexy)-ethyl]-4-methyl-7-propyl cyclohexane carboxylic acid 5'-buta-1' ester.

To fully characterize compound *W.S*-24, more data including MS is required.

5.5. Characterization of *W.S*-36

The UV spectrum of *W.S*-36 (Appendix-6) showed maximum absorption at 273 and 289nm which indicate the presence of aromatic ring. The IRμcm^{-1} (KBr disk) spectrum (Appendix7) showed absorption bands assigned to hydroxyl group (3436 broad), to olefnic carbons (1624), to saturated groups (2925), The presence of absorption bands between 1300-1000cm^{-1} illustrated C-O stretches of the ester functional group. A band at 700 cm^{-1} showed the presence of a tri substituted double bond.

The ^{1}H-NMR spectrum (Appendix 8) showed peaks at δ 0.96, 0.96, 1.16, and 1.16 indicate three aliphatic methyl protons. δ 5.0 multiplet and integrated for one proton indicate olefinic proton. δ 1.59-1.21 showed methylene protons. The peaks at δ 7.45 -7.75 indicate protons of aromatic ring.

Table.8 ^{13}C, ^{1}H-NMR and DEPT spectral data of *W.S*-36

Number of carbon	**C13 NMR for compound W.S-24**	**DEPT- 135**	**H NMR δ**	**Number of hydrogen**
1'	147.07	Quaternary	-	-
2'	115.90	CH	7.48	1
3'	132.38	Quaternary	-	-
4'	123.98	CH	5.27	1
5'&7'	130.91	CH	7.68	1
6'	119.11	CH	7.18	1
8'	128.85	CH	7.73	1
9'	139.27	CH	6.49	1
10'	114.07	CH_2	7.43	2
1	167.68	Quaternary	-	-
2	33.8	CH	2.47	1
3	29.70	CH_2	2.19	2
4	138.45	Quaternary	-	-
5	34.5	CH_2	2.09	2
6	71.80	CH	2.98	1
7	79.54	CH	2.77	1
8	27.73	CH_2	1.57	2
9	29.63	CH_2	1.36	2
10	27.74	Quaternary	-	-
11	38.87	CH_2	1.72	2
12	69.84	CH	3.26	1
13	33.83	CH_2	1.36	2

14	31.44	CH_2	1.59	2
15	34.87	CH	2.08	1
16	34.73	Quaternary	-	-
17	31.93	CH_2	1.44	2
18	34.53	Quaternary	-	-
19	29.52	CH_2	1.29	2
20	124.05	Quaternary	-	-
21	31.44	CH_2	1.92	2
22	30.70	CH	2.27	1
23	30.45	CH_2	1.25	2
24	24.75	CH_2	1.33	2
25	21.71	CH_3	0.96	3
26	123.49	CH	5.27	1
27	124.46	CH	7.0	1
28	28.29	CH_3	1.33	2
29	19.26	CH_3	0.96	3
30	22.70	CH_3	1.16	3

The ^{13}C NMR andDEPT-135 spectrum (Appendix 9,&10 Table 7) indicated that *W.S*-36 had 39 carbon atoms. The spectrum showed the presence of four methyl, fourteen methine, thirteen methylene, and eight quaternary carbon atoms, indicating 52 proton connected with 39 carbon atoms. The ^{13}C NMR spectrum of *W.S*-36 displayed signals characteristic seven double bonds at δ 147.07 & 114.07, 132.38 & 124.63, 130.91 & 119.11 & 128.85 & 123.49, 128.85 & 124.46, 138.45 & 138.45, 139.27 & 139.27. It showed the carbon attached with hetroatom (O), which is methine at δ 69.84. Based on the above spectral data evidences the following structure was proposed for the compound *W.S*-36.

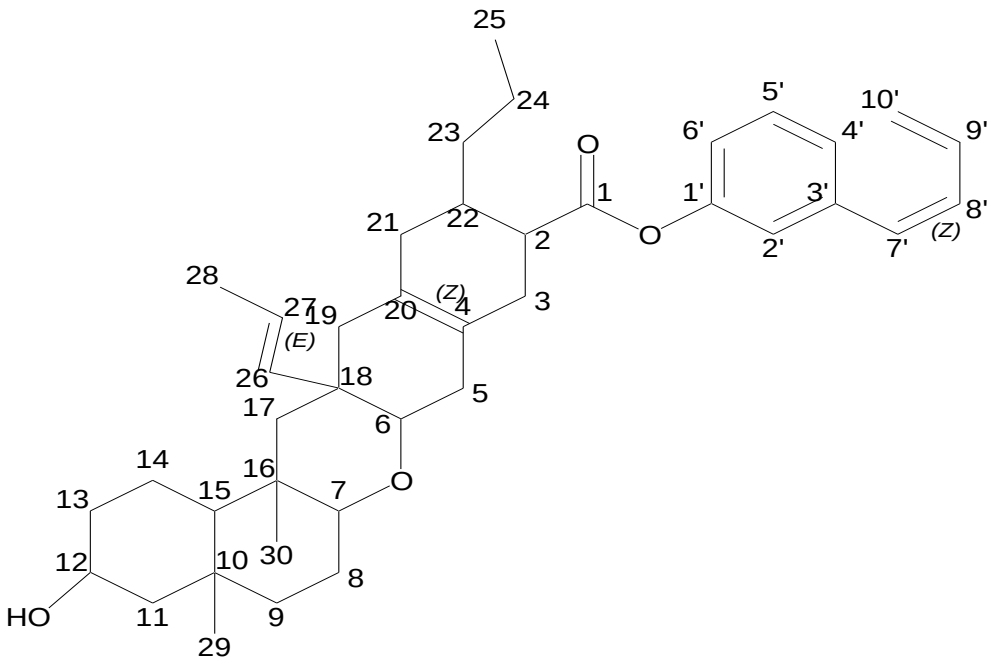

Figure.7 Proposed structure of Compound*W.S*-36

18-Butyl-12-hydroxy-10,16-dimethyl-22-propyl-sixten-hehydro-6-7-oxa-benzo[a]naphthacene-10-carboxylicacid naphthalene-2-yl ester.

To fully characterize the structure of compound W.S-36 more NMR and MS data is mandatory.

6. CONCLUSION AND RECOMMENDATION

This study has indicated that the extraction and isolation of *Withania* plants using *n*-hexane, chloroform, and methanol. Methanol extracts was subjected to phytochemical screening for identification of possible classes of secondary metabolites present in the extract. The presence of phenolics, flavonoids, alkaloids and, terpenoids were confirmed while saponins was absent. After TLC analysis of the crude extracts, the methanol extract was subjected to chromatographic separations on silica gel columns eluted with chloroform and methanol of increasing polarity. Two compounds *W.S*-24 and *W.S*-36 was isolated from the methanol extract of the leavs of *Withania somniferia*. The structure *W.S*-24 and *W.S*-36 has been elucidated using the data from its ^{1}H-NMR, ^{13}C-NMR, DEPT experiments. The crude extract and pure compounds exhibited moderate activity against the human pathogens: *Escherichia coli*, *Klebsiella pneumonia*, and *staphylococcus aures.*

Further studies are needed to isolate and characterize the bioactive principles which many aid to get antibacterial drugs. To the end more advanced chromatographic technique, for separation and MS for characterization is necessarily in the future.

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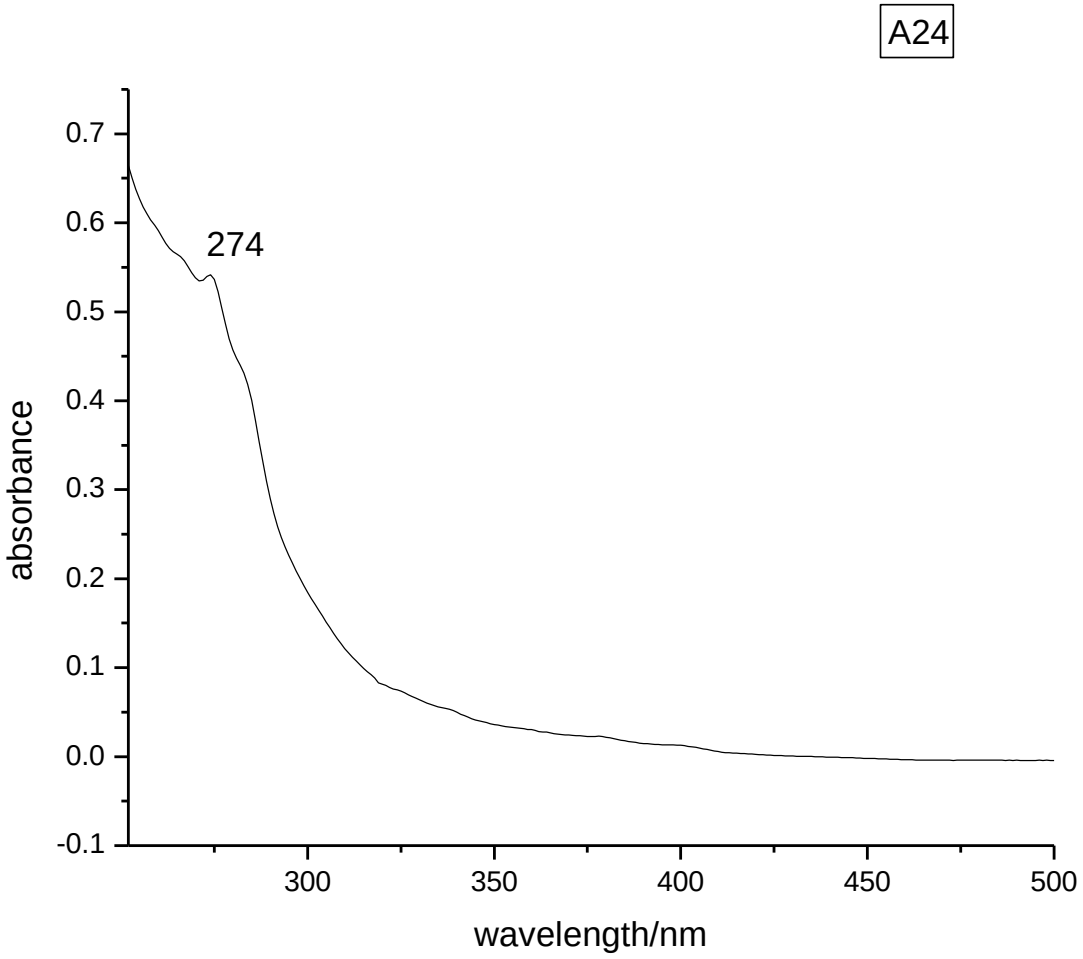
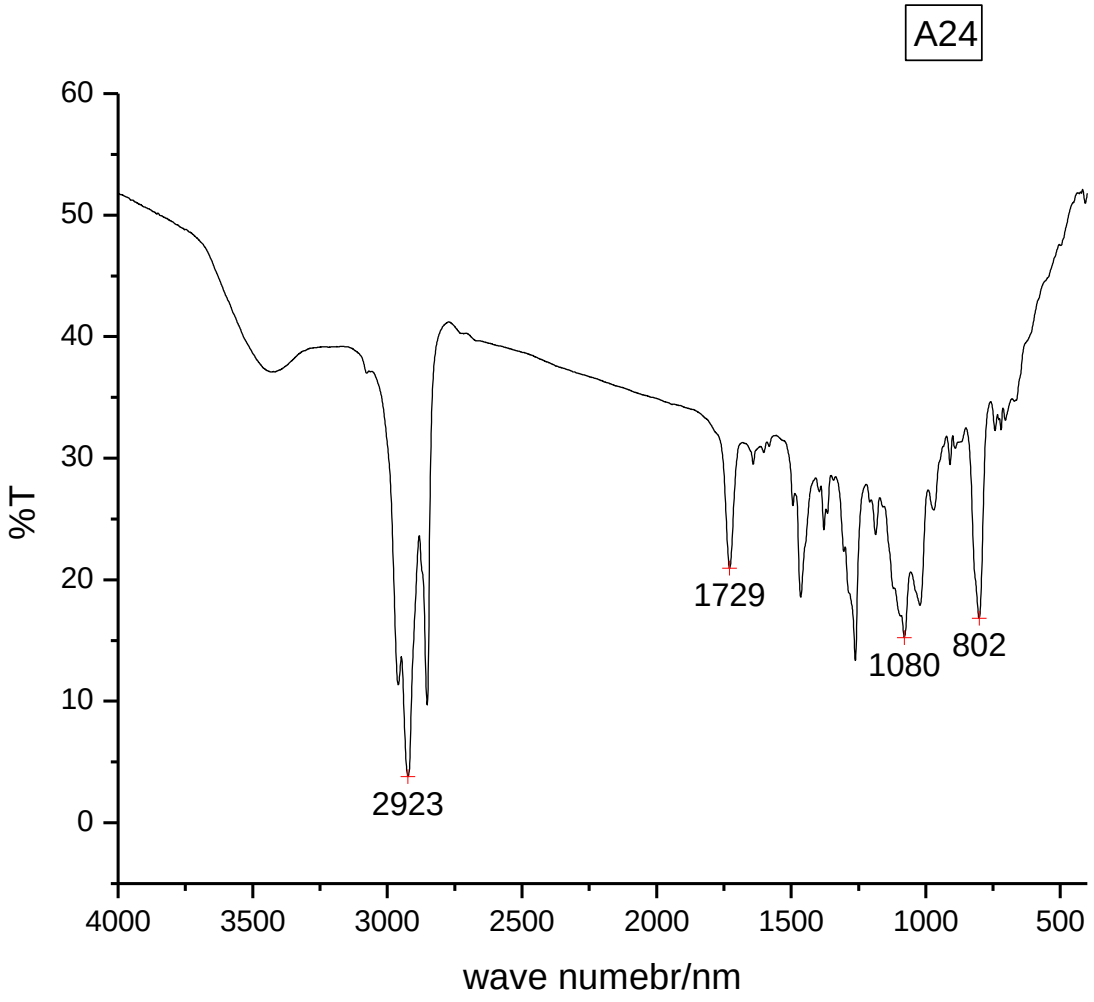
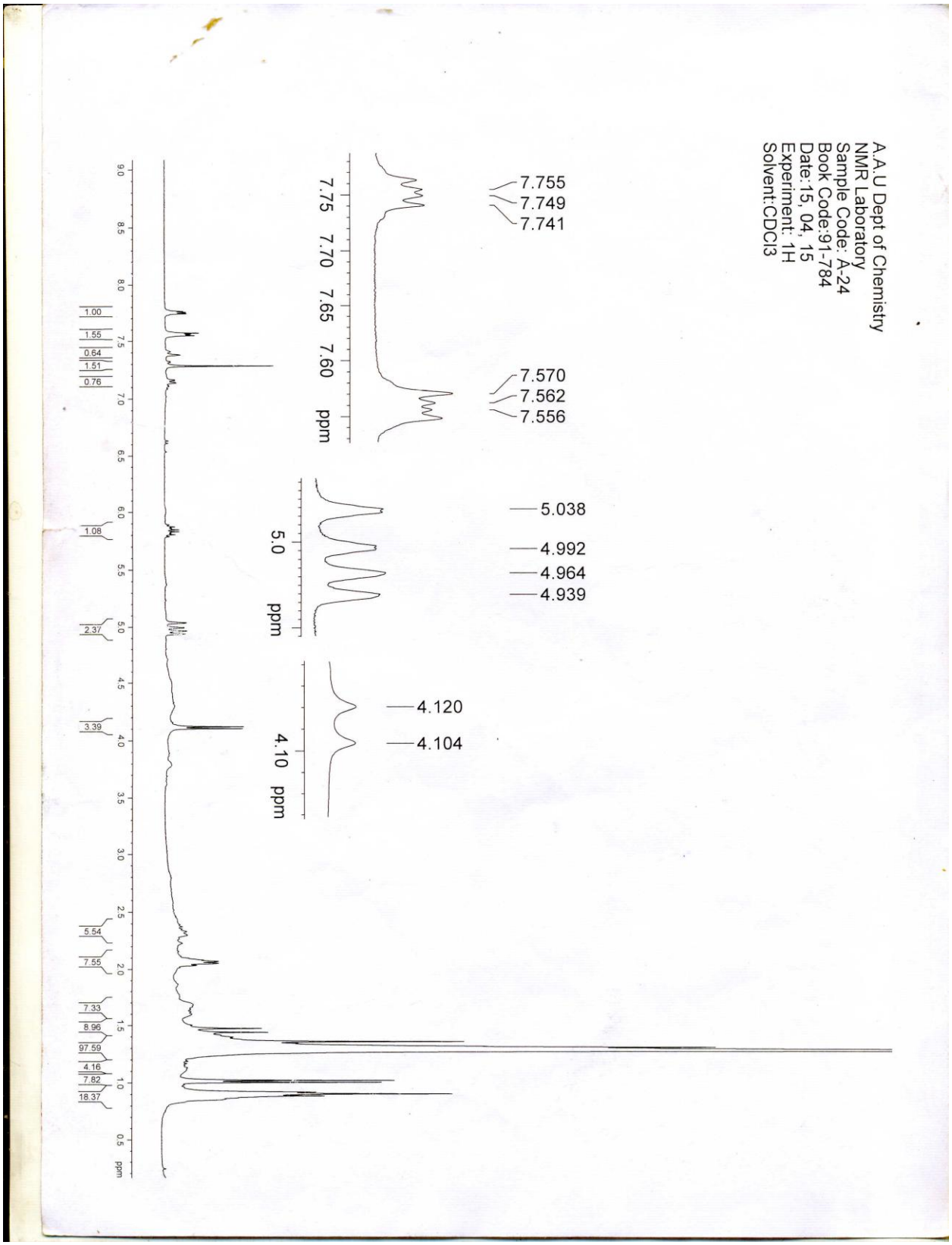


Appendix 1 UV spectrum of *W.S 24*

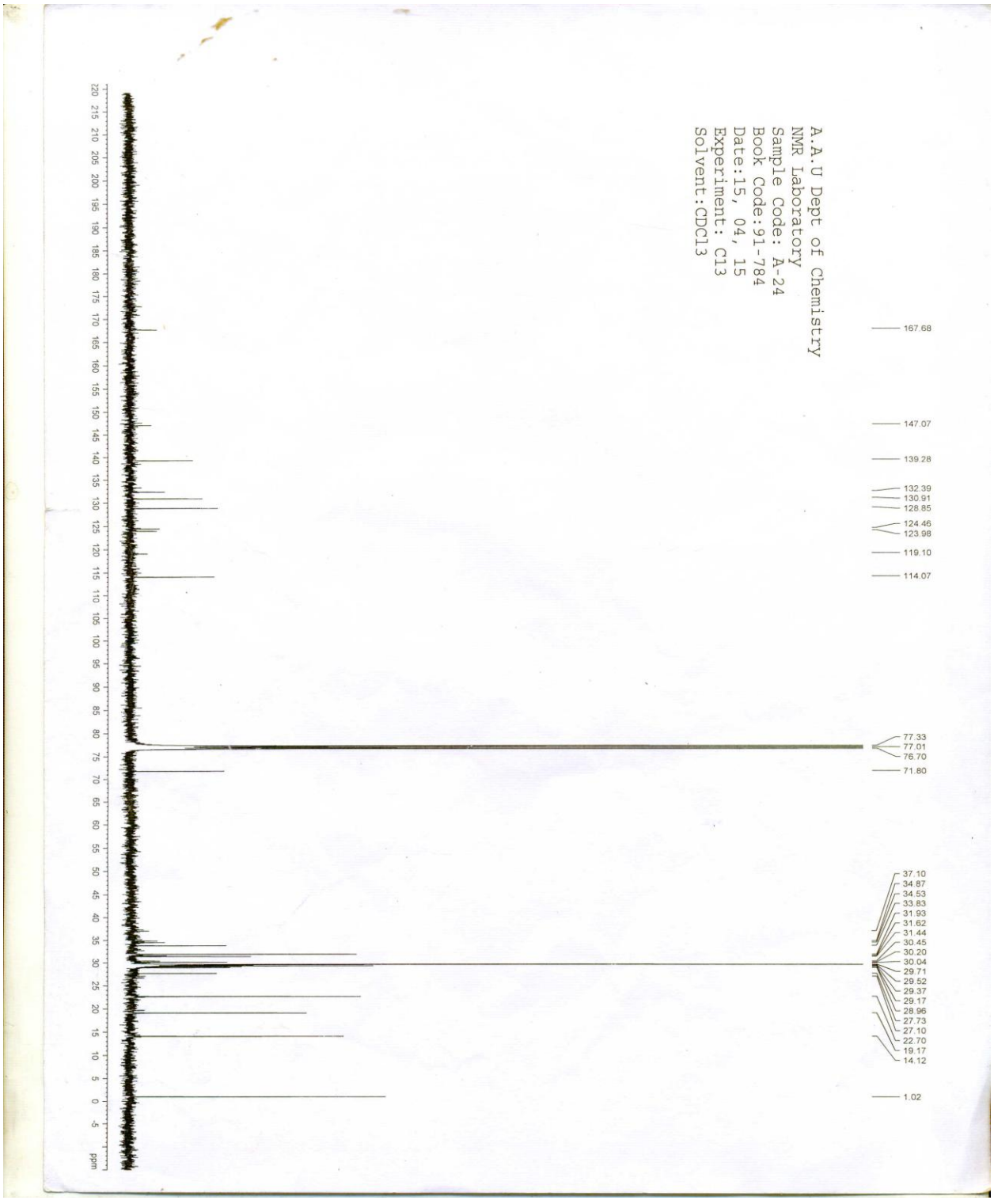
APPENDICES



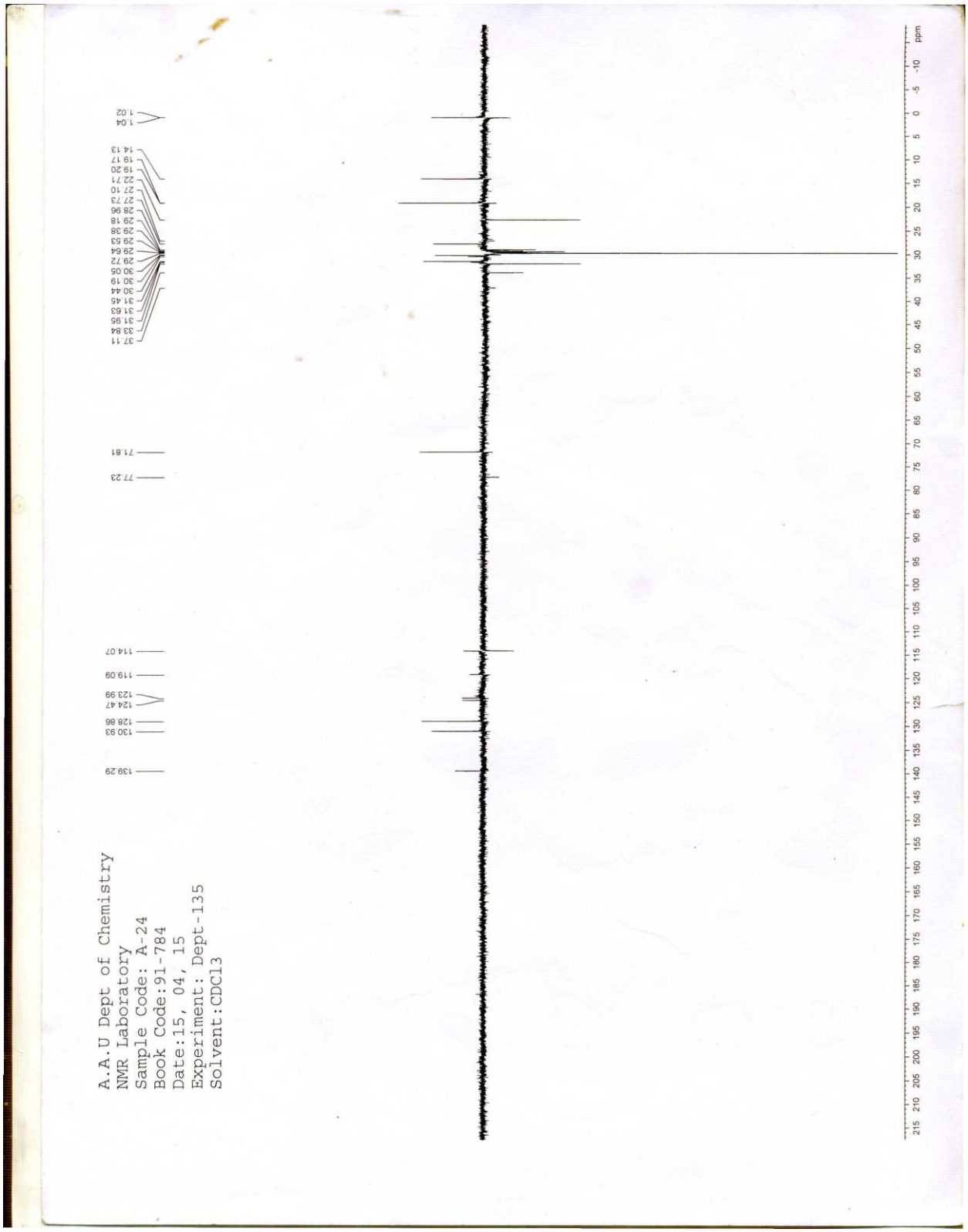
Appendix2IR spectrum of *W.S*-24



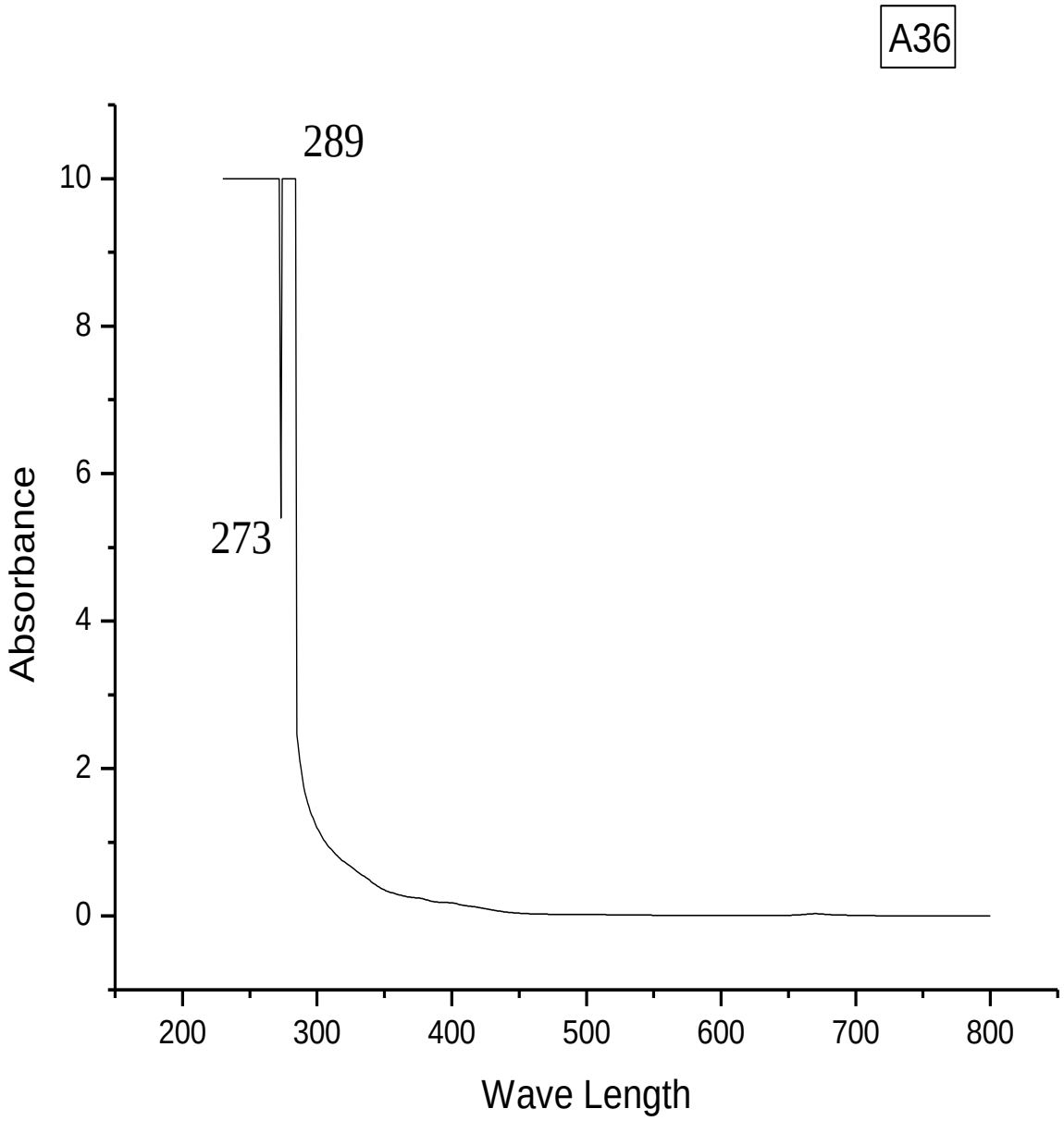
Appendix 3 ^{1}H spectrum of compound *W.S* 24



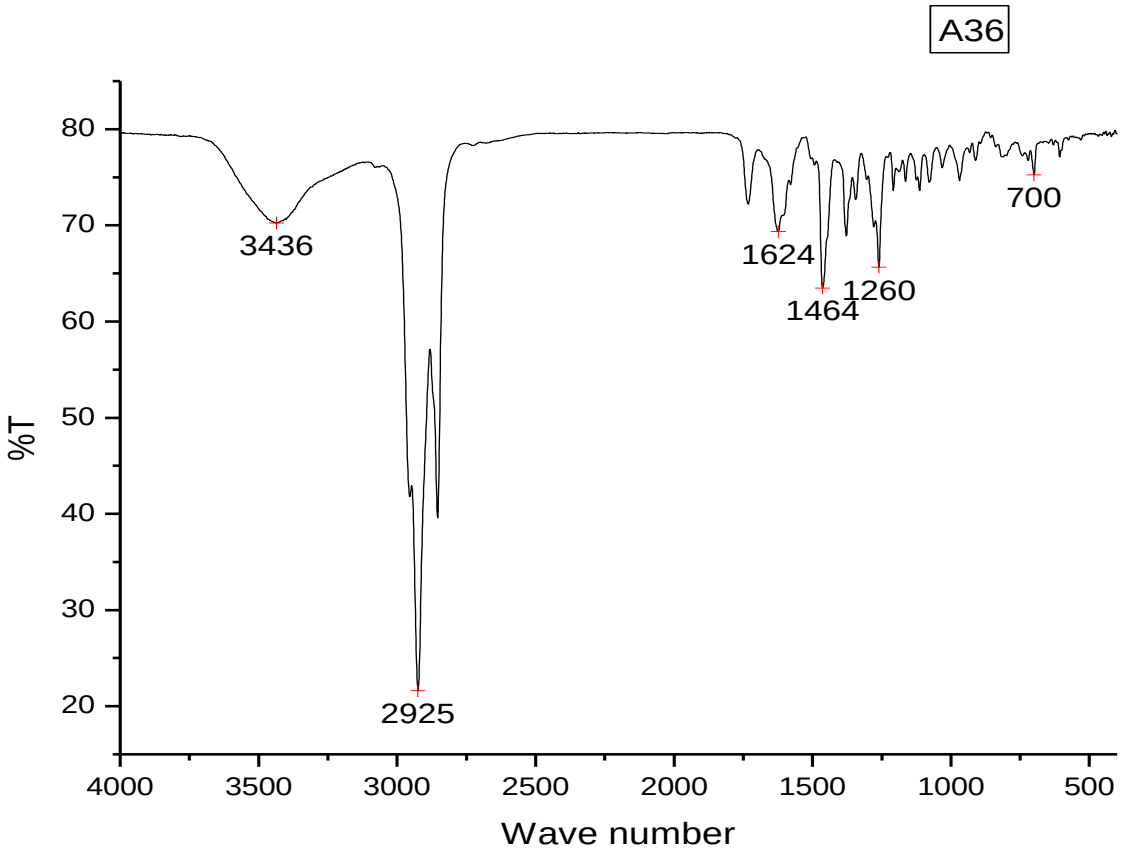
Appendix 4.^{13}C NMR spectrum of *W.S* 24



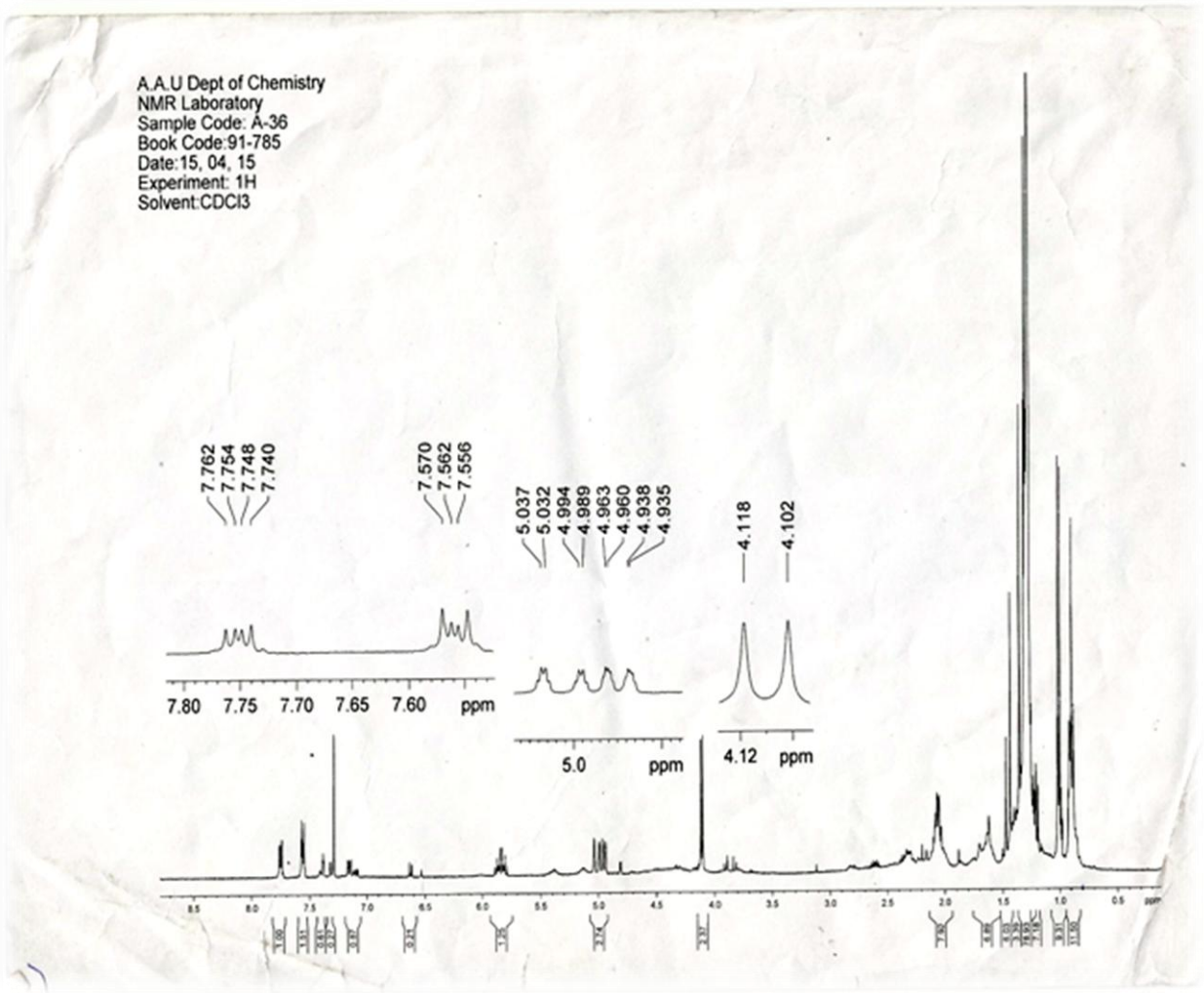
Appendix 5 DEPT 135 spectrum of *W.S*24



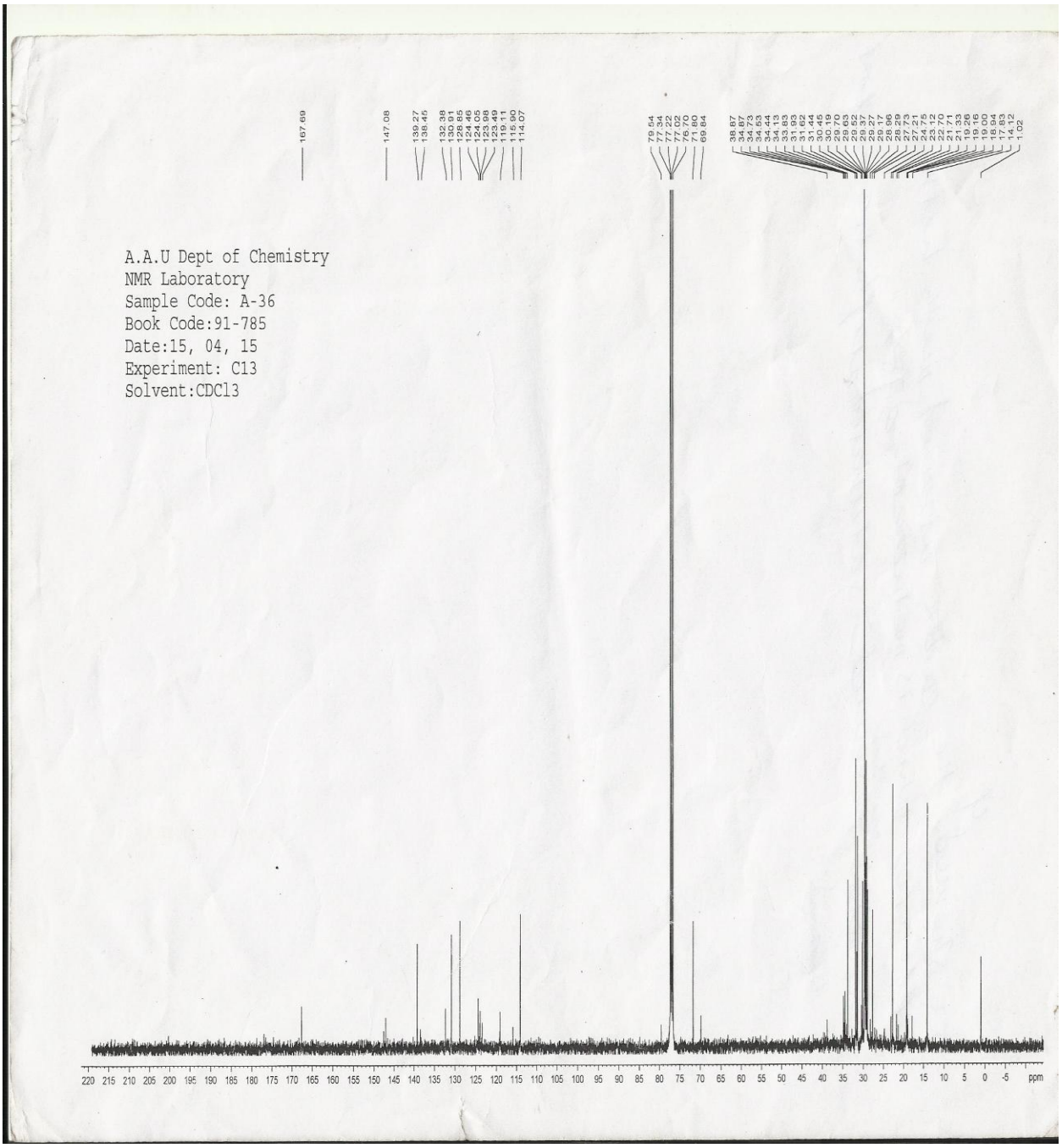
Appendix 6 UV spectrum *W.S* 36



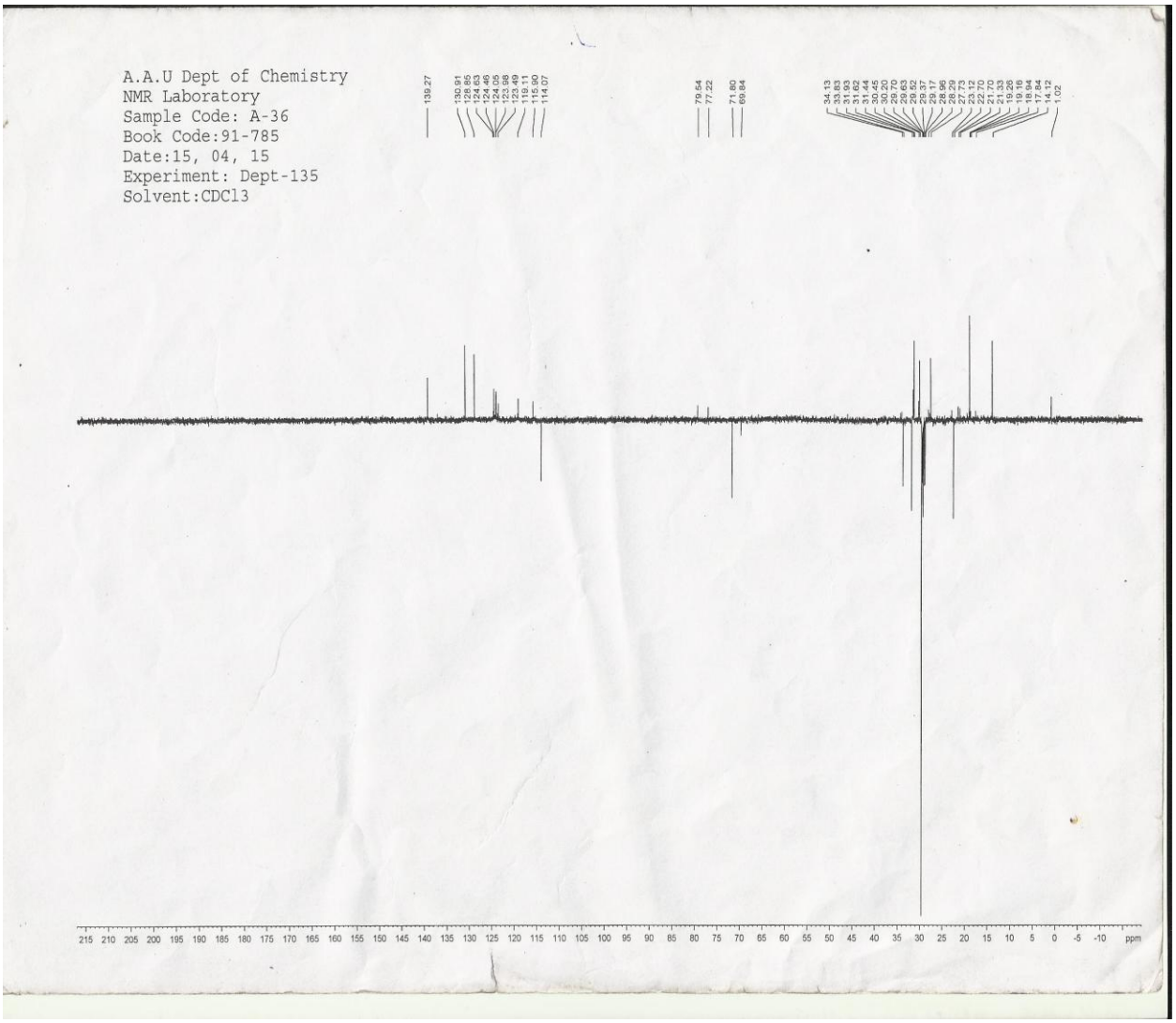
Appendix 7 IR spectrum of *W.S* 36



Appendix .8 ^{1}H spectrum of *W.S* 36 CCl_3



Appendix 9 13Cspectrum of *W.S* 36



Appendix.10 DEPT -135 spectrum of *W.S* 36