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Phytochemical studies of *Ziziphus xylopyrus* wild

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ABSTRACT

Traditional medicine is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. Traditional medicine that has been adopted by other population (outside its indigenous culture) is often termed alternative or complementary medicine. In India, out of the 17,000 species of higher plants, 7500 are known for medicinal uses. This proportion of medicinal plants is the highest proportion of plants, known for their medical purposes in any country of the world for the existing flora of that respective country. In our study we have collected different extracts of stem parts of Ziziphus xyloporus wild using different solvents like ethyl alcohol, pet. ether, water etc. using different techniques. .Different parametes of extractive values were performed. After the extraction of the compounds different phytochemical investigations were performed for the identification of the compounds group. After phytochemical investigation purification of compounds using TLC and column chromatography. The Rf value of the compound was found to be 0.460. The structural study of the extracted compound was carried out by using chemical tests, FT-IR, N.M.R., Mass spectroscopy. Mol. Wt of the compound was found 925.

Keywords: *Ziziphus xyloporus, Powder, Extraction, Phytochemical investigation, Structure Elucidation*

INTRODUCTION

Ayurveda remains one of the most ancient and yet living traditions practised widely in India, Sri Lanka and other countries and has a sound philosophical and experiential basis. *Atharvaveda* (around 1200 BC), *Charak Sanhita* and *Sushrut Sanhita* (1000–500 BC) are the main classics that give detailed descriptions of over 700 herbs. Ayurveda, the oldest medical system in Indian sub-continent, has alone reported approximately 2000 medicinal plant species, followed by Siddha and Unani (Table 1). approximately 25% of drugs are derived from plants, and many others are synthetic analogues built on prototype compounds isolated from plant species in modern pharmacopoeia.

Table- 1: Distribution of medicinal plants

Country or region	Total number of native species in flora	No. of medicinal plant species reported	% of medicinal plants	Source
World	297000	52885	10	Schippmann et al., 2002
India	17000	7500	44	Shiva, 1996
Indian Himalays	800	1748	22	Samant

Both China and India share 38% in marketing of medicinal plants worldwide. While China's turnover in medicinal plants has been Rs.22,000crore, India's business is hardly about Rs.450 crore. In fact the country has a rich collection of medicinal plant in Uttaranchal, Himalayas, Kerala and North Eastern States, but hardly some medicinal plants have been marketed here.

- As against a demand of 35,000 tonnes of medicinal plants, the supply is around 5000 tonnes. While 98% of herbal plants depending upon forest production.
- In some Asian and African countries, 80% of the population depends on traditional medicine for primary health care.
- Herbal medicines are the most lucrative form of traditional medicine, generating billions of dollars in revenue.
- Traditional medicine can treat various infectious and chronic conditions: new antimalarial drugs were developed from the discovery and isolation of artemisinin from *Artemisia annua* L., a plant used in China for almost 2000 years.
- Counterfeit, poor quality or adulterated herbal products in international markets are serious patient safety threats.
- More than 100 countries have regulations for herbal medicines.

There are over 750,000 plants on earth relatively speaking, only a very few of the healing herbs have been studied scientifically. And because modern pharmacology looks for one active ingredients and seeks to isolate it to the exclusion of all the others most of the research that is done on plants continuously focus on identifying and isolating active ingredients, rather than studying the medicinal properties of the whole plants.

MATERIALS AND METHODS

Collection, authentication and preparation of plant material

Ziziphus xyloporus wild plants were cultivated in medicinal plant garden of S.V. College, Aligarh and S.S. I. T.M, Aligarh. The whole plant at flowering stage was dug out and after removal of mud it is kept for herbarium preparation after that herbarium was poisoned with mercuric chloride and deposited for authentication. Authentication is done by Dr.Tarique Husain, Head and Scientist Biodiversity and Angiosperm Taxonomy N.B.R.I Lucknow, Uttar Pradesh and a herbarium of both plants were kept and stored. After authentication, around 1 kg of twigs and leaves were collected; after collection , aerial plant`s parts and dust was removed and plant`s parts were kept for shade drying, after complete drying the drug was stored in a well closed container away from sun light. Drying bark was totally peeled off from branches and dried stem part was converted into pieces manually after that these pieces were exposed to grinding and coarse powder was stored in well closed container away from sun light.

Procedures of Different Parameters:

- **Determination of Alcohol-Soluble Extractive**

Macerated 5 g of the air-dried drug, coarsely powdered, with 100 ml of alcohol in a closed flask for twenty-four hours, shaked frequently during six hours and allowed to stand for eighteen hours. Filtered rapidly taking precaution against loss of alcohol; evaporated 25 ml of the filtrate to dryness in a tared flat-bottomed shallow dish, dried at 105ºC,and weighed Calculated the percentage of alcohol-soluble extractive with reference to the air-dried drug.

- **Determination of Water-Soluble Extractive:**

Proceeded as directed for the determination of alcohol-soluble extractive, used chloroform water I.P. instead of alcohol.

- **Determination of Chloroform Soluble Extractive:**

Proceeded as directed for the determination of alcohol-soluble extractive, used chloroform instead of alcohol.

- **Determination of ethyl acetate extractive:**

Proceeded as directed for the determination of alcohol-soluble extractive, used benzene instead of alcohol.

- **Determination of Petroleum Ether soluble (40-60^{0}c) extractive:**

Proceeded as directed for the determination of alcohol-soluble extractive, used petroleum ether (40^0-60^{0}C) instead of alcohol.

Loss on Drying

About 5-6 gm of powdered drug was accurately weighed in a Petri dish and kept in a hot-air oven maintained at 110^{0}C. After cooling in a desiccator, the loss in weight was recorded.

Extraction:
About 2 kg of air dried and standardized root powder of *Ziziphus ziziphus (L) Karst* and *Ziziphus xyloporus* wild was taken and subjected to extraction with various solvents such as petroleum ether (40-60^{0}C), alcohol and aqueous using soxhlet assembly the procedure follows:-

Preparation of Petroleum Ether (40^0-60^{0}C) Extract:
About 200 gm of fresh air dried and standardized leaves powder and stem powder of a *Ziziphus xyloporus* wild. was subjected to hot continuous extraction with petroleum ether (40^0-60^{0}C) in eight soxhlet extractor (four for each drug- 200$_*$ 4= 800$_*$2) at a temperature of 40^0-50^{0}C to about 40 cycles per batch for 4 batches. The extraction was continued until the solvent in thimble became clear when few drops of solvent was collected in a test tube during the completion of the cycle (during siphoning) and tested for exhaustive chemical tests.

Preparation of Alcohol Extract:
The marc obtained from pet ether extract, was dried for half and hour; the dried marc was charged into soxhlet extractor for continuous extraction with alcohol solvent at a temperature 40^0-50^{0}C to about 40 cycles per batch to 4 batches. The extraction was continued until the solvent in thimble became clear when few drops of solvent was collected in a test tube during the completion of the cycle (during siphoning) and tested for exhaustive chemical tests.

Preparation of Aqueous Extract:
The fresh dried coarse powdered drug about 500 gm was taken in two conical flaks (500 gm for each- 500$_*$ 2= 1 kg $_*$ 2 for each drug)and sufficient quantity of chloroform water I.P. was added into a conical flask and was subjected to cold maceration for about 15 days at room temperature. The flask was securely plugged with absorbent cotton. The conical flask was stirred every 2 hours to 18 hours and occasionally shacked constantly till the maceration process completed. After maceration, the marc was pressed in muslin cloth and the filtrate was concentrated under vacuum in rotary flash evaporator. It was filtered and concentrated the residue to dryness on water bath at low temperature.

Preliminary Phytochemical Investigation :
Qualitative chemical tests were conducted for all the extracts to identify the various Phytoconstituents. The various tests and reagents used are given below and observation are recorded.

1. Tests for phytosterols:
Salkowaski test: To the test extract solution added few drops of conc. H_2SO_4 shaken and allowed to stand, lower layer turn red indicating the presence of sterols.

2. Test for steroidal glycosides:
Prepared extract solution with water and added 1 volume of 10 % v/v solution of sulphuric acid solution. Heated on the water bath for half an hour and extracted the hydrolysed extract with the chloroform as the solvent. Separated the chloroform fraction and concentrated. For concentrated fraction, carried out the test for steroids or phytosterols.

3. Test for Triterpenoids:
Preparation of test extract solution*:* The test extract solution was prepared by dissolving extracts in the chloroform.
Salkowski test: Few drops of concentrated sulphuric acid were added to the test solution of the extract, shaken and on standing lower layer turn golden yellow.

4. Test for Triterpenoid Glycoside:
Prepared extract solution with water and added 1 volume of 10 % v/v solution of sulphuric acid solution. Heated on the water bath for half an hour and extracted the hydrolysed extract with the chloroform as the solvent. Separated the chloroform fraction and concentrated. For concentrated fraction, carried out the test for Triterpenoids.

5. Tests for Glycosides:
Preparation of test solution: The test solution was prepared by dissolving extract in the alcohol or aqua alcoholic solution.

Baljet's test: The test solution treated with sodium picrate, gives yellow to orange colour.

6. Test for Anthraquinones:
Borntrager's test: Boiled test extract solution with 5 ml of 10% sulphuric acid for 5 mins. Filtered while hot, cooled the filterate shaked gently with equal volume of benzene. Separated the benzene layer and when treated with half of its volume solution of ammonia (10%). Allowed to separate ammoniacal layer aquires rose pink colour due to the presence of Anthraquinones.

7. Cyanogenetic glycosides:
Grignard's test: Strips of sodium picrate filter paper were inserted between split cork stopper which was fitted in to the neck of the test tube containing a small amount of residue in water. Care was exercised not to touch the inner side of the test tube with paper strips. The content was warmed for half an hour. The red colour of the strips indicates the presence of cyanogenetic glycosides.

8. Tests for saponins
Preparation of test solution: The test solution was prepared by dissolving extract in the water.

***a*) Foam test:** Test solution and shaken shows the formation of foam, which is stable at least for 15 mins.

9. Tests for Carbohydrates
Preparation of test solution: The test solution was prepared by dissolving test extract with water then hydrolysed with 2N HCl and subjected to following tests.

***a)*Molisch's test:** Test solution with few drops of Molisch's reagent and 2 ml of concentration H_2SO_4 added slowly from the sides of the test tube shows a purple ring at the junction of two liquids.

10. Tests for Alkaloids
Preparation of test solution: The test solution was prepared by dissolving extracts in the dilute Hydrochloric acid solution.

***a)*Mayer's test:** Test solution with Mayer's reagent (Potassium Mercuric iodide) gives cream coloured precipitate.

11. Tests for Flavonoids
Preparation of test solution:
i. A small amount of extract and added equal vol of 2M HCl and heated the test tube for 30-40 min at 100^0C.
ii. The cold extract is then filtered and extracted with ethyl acetate.
iii. The ethyl acetate extract was concentrated to dryness, and followed the test for flavonoids to ethyl acetate fraction by dissolving the residue with ethyl acetate.
***a)*Shinoda test:** Test solution with few fragments of magnesium ribbon and conc. HCl shows pink to magenta red colour.

Purification of compound:
A. By TLC Method
The plates were prepared using silica-gel-G and activated at 120°C for half an hour and the extracted compound was spotted using fine capillary and different solvent components were used as mobile phase given as follows :-

- Ethanol : water : chloroform (40:40:20)
- n-butanol : acetic acid : water (2:1:1)
- Chloroform : methanol (5:95)
- Methanol : chloroform (10:90)

First of all different ratio of mobile phase were prepared and kept in TLC Chamber, after adding mobile phase TLC chamber were closed and allow to saturate for some time.

The plates consisting of stationary phase were kept in the TLC chamber containing mobile phase and the chamber was closed and allow running the mobile phase for sufficient time until it does not reaches up to ¾ of the plate height. After reaching the solvent ¾ height the plates are taken outside and air dried and observed in U.V. chamber.

B. By Column Chromatography

- Columns were taken and used for purification as same solvent taken in T.L.C.

Analysis of the isolated anti-microbial compounds

The physical and chemical analysis of the compound was performed.

Spectral analysis of isolated compounds

- Calculation of λ max of the isolated compounds.
- I.R. SPECTROSCOPY.
- MASS SPECTROSCOPY.
- NMR SPECTROSCOPY.

Calculation of λ max of the isolated compounds :

The isolated antibacterial compound was taken and diluted upto 5µg/ml using $CHCl_3$ and Spectrum was taken using U.V-Spectrometer for the λ max calculation of the compound.

Using FT-IR Spectroscopy :

Procedure:

The spectrum was scanned using KBr pellet technique. The spectra were plotted against Wave number cm^{-1} Vs Transmittance (%) which is shown in results and discussion.

Using NMR Spectroscopy :

Procedure:

The NMR spectrum was taken using $CDCl_3$ as a solvent at 300 MHz. The graph was plotted.

Using Mass Spectroscopy

RESULTS AND DISCUSSION

As the plants Ziziphus xyloporus wild are common plant and used for food purposes from ancient time, so the toxicity is very less.

Table 2: Name of the Plant: Ziziphus xyloporus wild, Part of the Plant: Stem powder

Sl. No.	Parameters	I.P./B.P/As per literature	Observation
I	**Physical Tests**		
	Nature	Fine powder	Fine powder
	Colour	Light Brown	Light Brown
	Odour	No	No
	Taste	No	No
II	**Extractive Value**		
	Aqueous	---	12.634% w/w
	Alcoholic	---	19.321%w/w
	Chloroform	---	8.321%w/w
	Ethyl acetate	---	13.041%w/w
	Pet. ether (40-60^{o}C)	---	6.206%w/w
III	**Loss on drying**	**NMT 10%w/w	6.204%w/w
IV	**Ash values**		
	Total ash	---	5.734%w/w
	Acid insoluble ash	---	0.55%w/w
	Water soluble ash value	---	19.13%w/w
V	**Fluorescence analysis**	---	Blue fluorescence (366 nm)
VI	**Major chemical constituents**		
	Total Glycosides	--	5.280 %w/w
	Total Flavanoids	--	18.234%w/w
	Total Tannins	--	6.321 % w/w
VII	**TLC Profiles**		Enclosed in table no.7 & 8

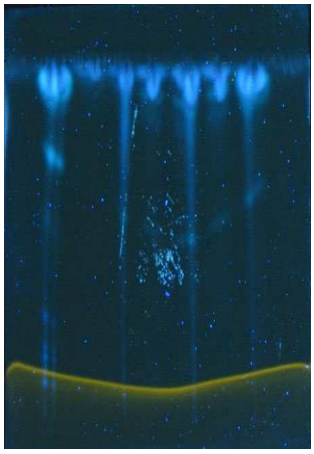
** Not less than* *** Not more than*

Table-3: Results of Phytochemical analysis of Ziziphus xyloporus wild

Chemical Tests	Extracts		
	Petroleum Ether	Alcohol	Aqueous
1. Test for Sterols			
a. Salkowaski test	-	-	-
b. Liebermann-Burchard test	-	-	-
2. Test for Steroidal glycosides	-	+	-
3. A) Test for Triterpenoids			
a. Salkowaski test	-	+	-
b. Liebermann-Burchard test	-	+	-
c. Briekorn and Brinar test	-	+	-
B) Test for Triterpenoid Glycosides (Hydrolysis)			
a. Salkowaski test	-	+	+
b. Liebermann-Burchard test	-	+	+
c. Tschugajew test	-	+	+
d. Briekorn and Brinar test	-	+	+
4. Test for Glycosides			
i. Cardiac glycosides			
a. Baljet's test	-	+	-
b. Legal's test	-	+	-
c. Killar-Killiani test	-	+	-
ii. Anthraquinone Glycosides			
a) Borntrager's test	-	-	-
b) Modified Borntrager's test	-	-	-
5. Test for Saponins			
a. Foam test	-	-	+
b. Haemolysis test	-	-	+
6. Test for Flavonoids/ Flavonoid Glycosides			
a. Shinoda test	-	+	-
b. Zinc Hydrochloric acid reduction test	-	+	-
7. Test for Carbohydrates			
a. Molisch's test	-	+	+
b. Fehling's test	-	+	+
c. Barfoed's test	-	+	+
d. Benedict's test	-	+	+
e. Selvinoff's test	-	+	+
f. Test for pentoses	-	+	+
8. Test for Alkoloids			
a. Mayer's test	-	+	+
b. Wagner's test	-	+	+
c. Hager's test	-	+	+
d. Dragendorff's test	-	+	+
9. Test for Phenolics and Tannins			
a. Lead acetate test	-	-	+
b. Ferric Chloride test	-	-	+
c. Gelatin test	-	-	+
d. Dilute Nitric acid	-	-	+
e. Potassium dichromate	-	-	+
f. Dilute Iodine solution	-	-	+

Extraction of compound:
At last after extraction of powder (using ethyl alcohol) the brown gummy like substance obtained.

Purification of compound:
After Purification of the compound using TLC and Column chromatography { n-butanol: acetic acid : water (2:1:1) mobile phase or eluent.},whitish brown.



Photograph No.1. Shows TLC of Isolated flavonoid from Ziziphus xylopyrus wild stem At UV 366 nm

Photograph No.2. Shows the Purified Extracted Diagram

USING NMR SPECTROSCOPY:
As per spectrum result antimicrobial compound- A shows the following important bands. It may be defined as follow:

A. 1.603 ppm (1-5.5 ppm : Alcohol hydroxyl protons)
1.625 ppm
1.687 ppm
B. 0.828 ppm (0.9-1.5 ppm : Protons not attached to a Heteroatom).
0.855 ppm
0.880 ppm
0.901 ppm
C. 1.254 ppm (0.9-1.5 ppm : Protons not attached to a Heteroatom).
D. 1.433 ppm (0.9-1.5 ppm : Protons not attached to a Heteroatom).
E. 1.994 ppm (1-5.5 ppm : Alcohol hydroxyl protons)
F. 2.324 ppm(1-5.5 ppm : Alcohol hydroxyl protons)
2.349 ppm
2.372 ppm
G. 3.428 ppm (1-5.5 ppm : Alcohol hydroxyl protons)
H. 4.128 ppm (1-5.5 ppm : Alcohol hydroxyl protons)
I. 7.288 ppm (6-8 ppm :Aromatic proton)

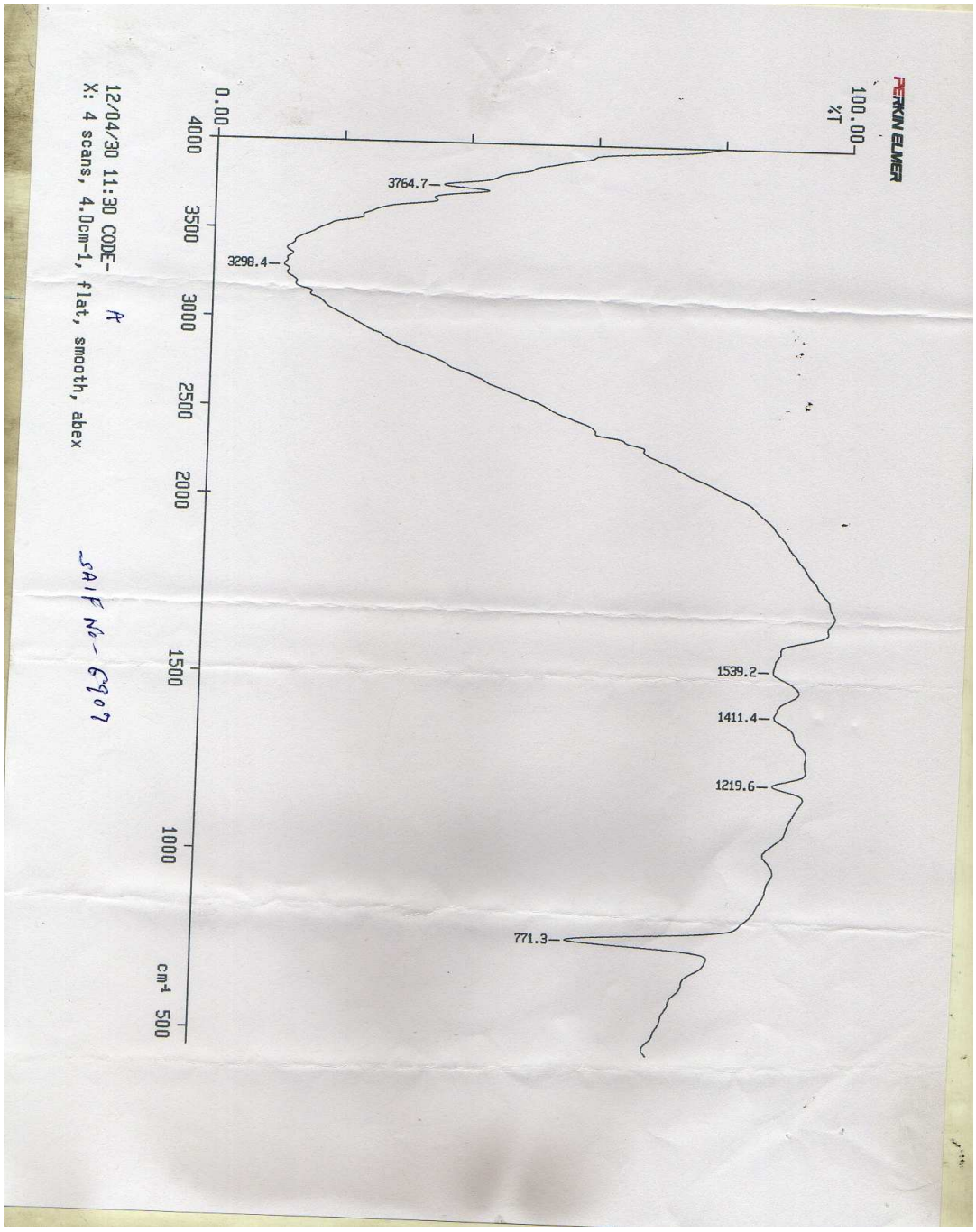


Figure 1: Shows I.R. spectra of extracted compound

A. USING I.R. SPECTROSCOPY

As per spectrum result antimicrobial compound A and B shows the following important bands. It may be defined as follow:

1.Compound A

A. 3764.70 cm^{-1}

B. 3298.40 cm^{-1} (3300-2500 cm^{-1} O-H stretching of a free carboxyl group)

C. 1539.20 cm^{-1}

D. 1411.40 cm^{-1}

E. 1219.60 cm^{-1}
F. 771.30 cm^{-1} (700-850 cm^{-1} C-H bending aromatic)

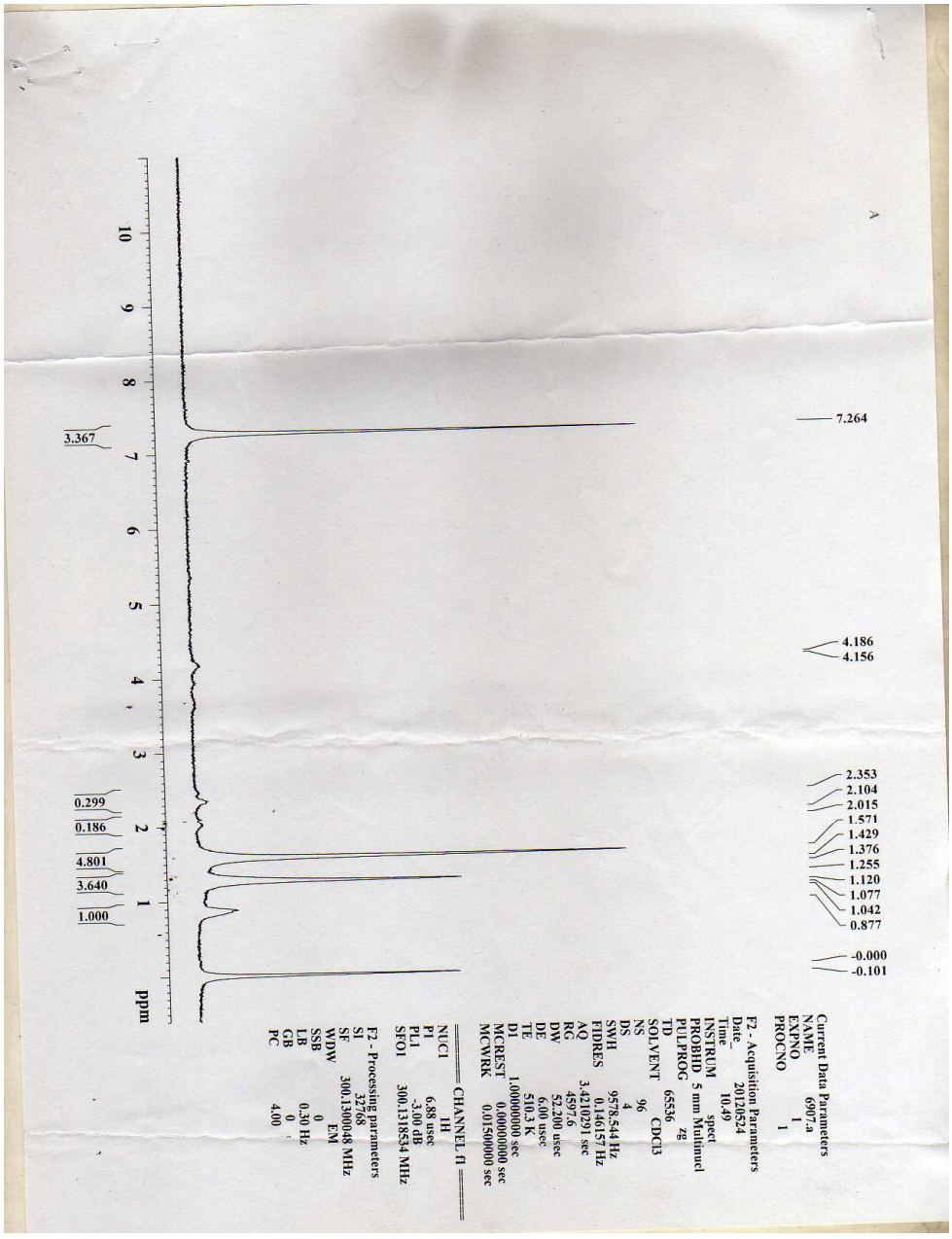


Figure 2: Shows N.M.R. spectra of extracted compound

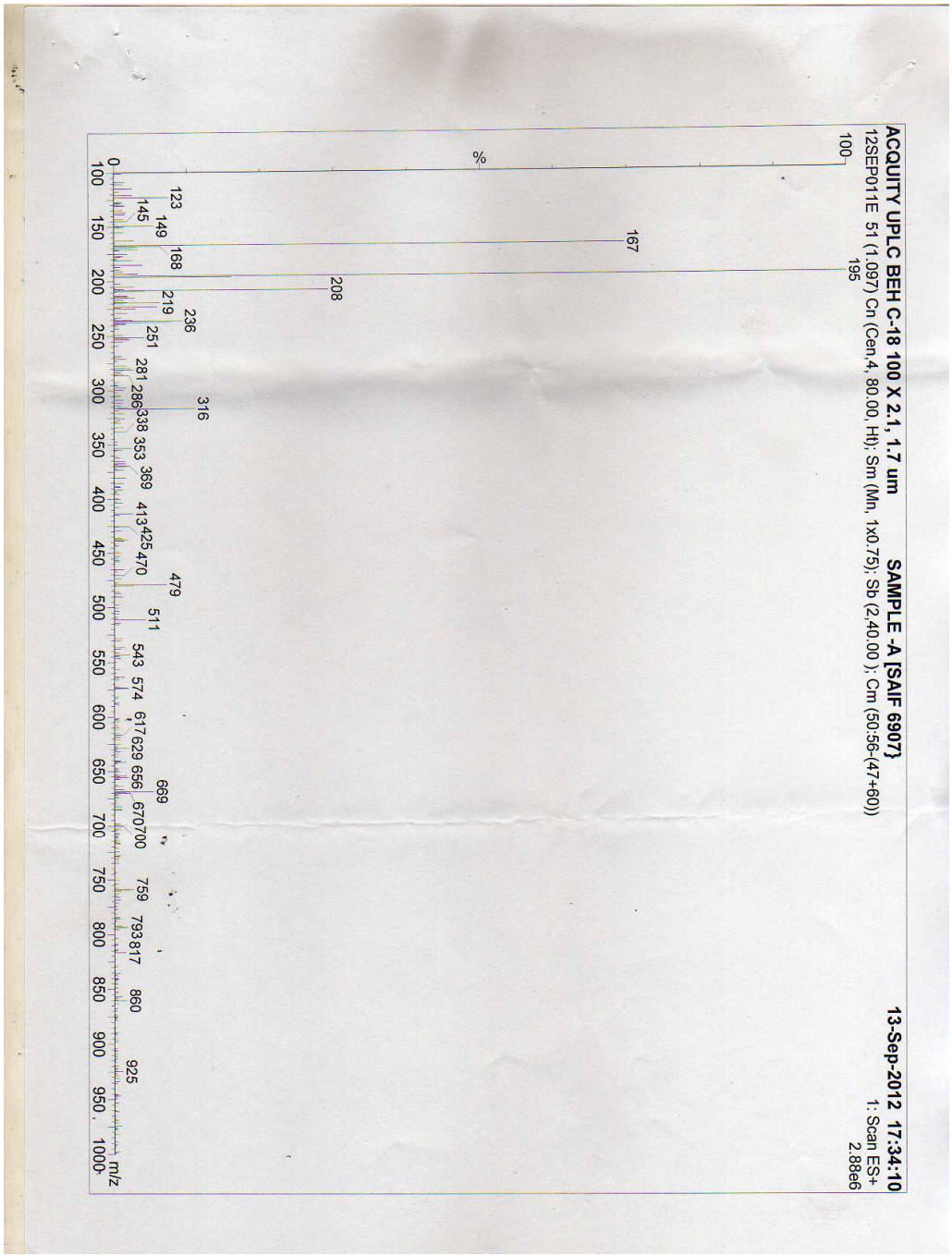


Figure 3: Shows Mass spectra of extracted compound

The chemical structures of the isolated compounds were elucidated through after observing all the data of I.R. Spectra, N.M.R. Spectra, Mass Spectra and Anti-bacterial study of both of the compound i.e. compound A and compound B. There were two structures were draw for the compound named as:-

Compound A: The extracted compound A showed the structure similarity to Sennoside D.
As per our study reveals that *Ziziphus ziziphus xyloporus* wild plant will be better for human use.

Acknowledgement
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