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Full Length Research Paper

A novel antifungal phenolic compound from *Parrotia persica*

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Aqueous and methanol extracts of *Parrotia persica* leaves were assayed for antifungal activity against phytopathogenic *Fusarium oxysporum* and human pathogenic *Candida albicans* by poisoned food technique. Both the aqueous and methanol extracts demonstrated significant antifungal activity. Further fractionation of methanol extract guided by antifungal activity resulted in the isolation of an active principle and it is identified as phenolic compound. The structure of the active principle was elucidated by mass spectroscopy, ^{1}H NMR and ^{13}C NMR spectroscopy. These results revealed that the compound is 6-(ethoxymethyl)-tetrahydro-2H-pyran-2, 3, 4, 5-tetraol compound with 1- isopropyl-4-methoxybenzene, the compound was found responsible for antifungal activity against both *F. oxysporum* and *C. albicans*.

Key words: *Parrotia persica*, phenolic compound, antifungal activity.

INTRODUCTION

The spread of multi-drug resistant (MDR) strains of microbes necessitates the discovery of new class of antibiotics and compounds that inhibits these resistant pathogens. Thus, there is an increased pressure to find novel antimicrobial agents (Gibbons, 2005). Such compounds would play a significant role in the development of new drugs in medicine (Verma and Dubey, 1999; Cragg et al., 1999).

Plants are reservoir of several phytochemicals which are known to play important role in plant metabolism and defense against several diseases. Phytochemicals, as the word implies, are the individual chemicals from which plants are made. These phytochemicals from plants have countless benefits to humans, they are exploited as natural pesticides, flavorings, fragrances, medicinal compounds, fibers, beverages, and food metabolites. Grapes for example contain several antifungal compounds, such as caffeic acid, chlorogenic acid, pterostilbene, resveratrol, and viniferin. These compounds confer protection against bacteria, fungi, herbivores, insects, and viruses that plague the plant (Duke and Bogenschutz-Godwi, 1999). Scientific techniques have now been developed to isolate such natural constituents with desired biological activity and exploit them in the management of both phytopathogenic and human pathogenic microbes. In this study therefore, both human and phytopathogenic fungi were selected for the study.

A large number of plant based preparations are utilized for treating various diseases and disorders in Iran, not much work has been done on the scientific validation of the utility of these plants in traditional medicine. Further, the biological activities of these plants have not been scientifically demonstrated. Thus, there is a need to conduct systematic and scientific investigations to justify the use of these plants in the traditional medicine of Iran.

One of the successful strategies for investigation of medicinal agents from higher plants includes the pharmacological screening of plant extracts followed by bioassay - guided fractionation of crude extracts leading to the isolation of the pure active constituents (Hostettmann and Lea, 1987). Though many possible sources of extractions and synthesis of antibiotics have been elaborately worked out, the search for a better, safer and cost effective source is however, a necessity. Consequently, in the present investigation, an attempt has been made to isolate and characterize the bioactive principle from leaves of *Parrotia persica* (DC.) C.A. Mey, which

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exhibited antifungal activity during regular screening in our laboratory.

P. persica (Persian Ironwood) is a deciduous tree (Hamamlidaceae), it is cultivated as an ornamental tree for its stunning autumn color and the smooth patterned bark. It is native to northern Iran, where it is endemic in the Alborz mountains (Rechinger, 1999; Mozaffarian, 2006). Many herbalists use this plant in the treatment various fevers and respiratory infections and this plant is known to posse's antibacterial activity reported from our laboratory (Ahanjan et al., 2007). Recently leaves of this are known to posse's antioxidant activity (Nabavi et al., 2008).

MATERIALS AND METHODS

Plant material

Healthy disease free, mature leaves of *P. persica* were collected from Mazandaran region of Iran and used for the preparation of aqueous and different solvent extracts. A voucher specimen of the plant has been deposited in the herbarium of the Department of Studies in Botany, University of Mysore, Mysore.

Preparation of plant extracts

Samples (50 g) of shade dried powderd leaves of *P. persica* was macerated with 100 ml of sterile distilled water in a waring blender (Waring International, new Hartford, CT, USA) for 10 min. The macerate was first filtered through double layer muslin cloth and then centrifuged at 4000 x g for 30 min. The supernatant was filtered through Whatman No. 1 filter paper and heat sterilization at 120°C for 30 min. The extract was preserved aseptically in a brown bottle at 5°C until further use (Satish et al., 1999).

For solvent extraction, 25 g of powdered leaves were loaded to thimble and extracted successively with petroleum ether, chloroform, ethanol and methanol solvents using a Soxhlet extractor for 48 h. All the extracts were then concentrated using a rotary flash evaporator and later preserved at 5°C in airtight bottles until further use.

Separation of active fractions from methanol extract

The methanol extract which showed significant activity was subjected to active fraction separation as described by Becknett and Stenlake (1986). The 4 fractions viz., acidic (fraction i), basic (fraction ii), phenolic (fraction iii) and neutral (fraction iv), obtained were dried under reduced pressure and subjected to antifungal activity assay at 500 ppm concentration.

Test fungi

Human pathogenic *C. albicans* (MTCC 183) was obtained from the institute of microbial technique, Chandigrah, India. Phytopathogenic *F. oxysporum* was isolated from sorghum seeds, pure cultured and identified using standard manual (Booth, 1971; Booth, 1977).

Determination of antifungal activity of extracts

To test aqueous extract, Czapek Dox Agar (CDA) medium with 500 ppm concentration of the aqueous extract was prepared. About 15 ml of the medium was poured into each petriplate, allowed to cool and solidify. To test solvent extracts, 5 mg each of the dried evaporated solvent extract was dissolved in 100 ml of respective solvents. Each of the solvent extracts was then amended with 15 ml of CDA medium per plate before solidification of the medium and the same procedure was followed for isolated TLC spots. A 5 mm disc of 7 day-old culture of the test fungi was placed at the center of the each petriplate and incubated at 25 ± 2°C and 37°C for *F. oxysporum* and *C. albicans* respectively for 7 days. After incubation the colony diameter was measured in mm. For each treatment, 3 replicates were maintained. CDA medium without the aqueous extract or with methanol alone were served as control. Thus the percent inhibition of mycelial growth, if any, was determined.

The fungi toxicity of the extracts and compound in terms of percentage inhibition of mycelial growth was calculated by using the formula

Percent inhibition = DC - DT×100 / DC
Where, DC = Average increase in mycelial growth in control
DT = Average increase in mycelial growth in treatment (Singh and Tripathi, 1999).

The MIC of the pure compound was determined following procedures of Zehavi et al. (1986).

Separation of active compound from phenolic fraction by TLC

Preparation of TLC

The TLC plates were prepared as described by Harborne (1998). 25 g of silica gel-G (Hi Media, Manufactured, India) was mixed with 50 ml of distilled water and the slurry formed was uniformly spread over TLC plates with a thickness of 0.25 mm using the spreader. The plates were allowed to dry at room temperature and heated in an oven at 100°C for 2 h.

Isolation of active principle

The phenolic fraction which showed significant antifungal activity in poisoned food technique (Singh and Tripathi, 1999) was subjected to TLC separation with chloroform as an elutant (Harborne, 1998). The separated bands were identified under UV fluorescence at 365 nm wavelength and relative front (Rf) values of the spots were determined. The respective bands were scraped out separately along with silica dissolved in methanol and filtered through Whatmann No.1 filter paper. The filtrate was collected in chromic acid washed glass vials and allowed to dry. After complete evaporation of methanol all the bands were once again subjected to antifungal activity assay to identify the band responsible for the activity. TLC plates were sprayed with dilute Folin-Ciocalteau reagent (1:1 with water) (Harborne, 1998) to confirm the nature of the active principle.

Determination of melting point of active principle

The melting point of the active principle was determined following the procedures of Roberts et al. (1981). The melting point was determined on SELCO- 650 hot stage apparatus and was uncorrected.

Identification of active compound by nuclear magnetic resonance (NMR) spectroscopy and mass spectral analysis

The pure active principle was dissolved in $CDCl_3$ and subjected to ^{1}H NMR at 400.1359 MHz and ^{13}C NMR at 100.6141 MHz. The mass spectrometer used was an electrospray ionization- quadruple (ESI-Ms) Hewlett Packward series 1100 MSD. The sample was

Table 1. Antifungal activity of different extracts of *Parrotia persica* against test fungi.

Fungi	Percent mycelial inhibition	
	Aqueous extract (500 ppm)	Methanol extract (500 ppm)
F. oxysporum	40.00 ± 0.33	42.38 ± 0.55
C. albicans	30.39 ± 0.46	35.58 ± 0.35

Data given are mean of three replicates ±standard error.
Analysis of variance (ANOVA), $p < 0.005$.

Table 2. Antifungal activity of different fractions of methanol extract of *P. persica* at 500 ppm.

Fungi	Percent mycelial inhibition			
	Acidic (Fraction I)	Basic (Fraction II)	Phenolic Fraction III)	Neutral Fraction IV)
F. oxysporum	36.27 ± 0.45	0.00 ± 0.00	43.33 ± 0.27	0.00 ± 0.00
C. albicans	34.20 ± 0.66	0.00 ± 0.00	40.45 ± 0.33	0.00 ± 0.00

Data given are mean of three replicates ±standard error.
Analysis of variance (ANOVA), $p < 0.005$.

Table 3. Antifungal activity assay of different bands separated from Phenolic fraction at 500 ppm concentration.

Fungi	Percent mycelial inhibition			
	Band I (Rf 0.10)	Band II (Rf 0.60)	Band III (Rf 0.82)	Band IV (Rf 0.92)
F. oxysporum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	48.42
C. albicans	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	46.15

Values presented are means of three replicates ± Standard error.
When subjected to analysis of variance (ANOVA), $p<0.005$.

dissolved in 1:1 (ACN: H_2O) directly infused at a constant flow rate of 20 µl/min into the ion spray source using an integrated syringe pump. The instrument was operated in positive ionization mode with the following setting: High Vaccum 6.4 E-0.06 Torr; Nebulizer gas (N_2) 19 Psig; Quadruple temperature 99°C; Drying gas (N_2) 2.9 L/min; Fragmentor 90; Rough vaccum 2.10 Torr. Full-scan data acquisition was performed, scanning from *m/z* 100 to *m/z* 2000 in the profile mode and using a cycle time of 1 s to identify the compound (Meyers, 2000).

Determination of (MIC) of active principle

100, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 ppm concentration of the active principle was prepared as described by Rai and Linganna, (2000). Sterilized Czapek Dox Agar (CDA) media with different concentration of active principle was transferred to sterilize petri plate. The concentration at which complete inhibition of the mycelial growth was observed was recorded as MIC.

The data was subjected to statistical analysis using SPSS 2006 for windows software.

RESULTS

Antifungal activity of the aqueous extract

Aqueous extract recorded significant antifungal activity against both the test fungi. The results are shown in Table 1.

Antifungal activity of the different solvent extract

Among the different solvent extracts tested viz., petroleum ether, chloroform, methanol and ethanol, only methanol recorded significant antifungal activity against both tested fungi. The antifungal activity varied among the pathogens, *F. oxysporum* inhibited up to 43% and *C. albicans* up to 35% (Table 1).

Separation of active fraction from methanol extract of leaves of *P. persica*

4 fractions viz., acidic, basic, phenolic and neutral fractions were obtained from the methanolic extract following the standard procedures. The antifungal activity of all 4 fractions against test fungi was presented in Table 2. Phenolic and acidic fractions showed antifungal activity against tested fungi, basic and neutral fractions did not show any activity. Both *C. albicans* and *F. oxysporum* were found highly sensitive to the presence phenolic fraction than acidic fraction.

Separation of active compound from phenolic fraction by TLC

4 bands were developed in chromatogram. The Rf values

Table 4. Assignment of NMR chemical shifts, with reference to the proposed active principle structure.

Position	^{1}H ppm	^{13}C ppm	Predicted ^{1} H	Prediction ^{13}C	Splitting
1	1.29	23.3	1.23	21.40	1:1
2	1.29	23.3	1.23	21.40	1:1
3	3.12	36.3	2.87	29.30	overlapping
4		140.8		145.50	overlapping
5	6.93	114.8	6.90	126.10	1:1
7	6.93	114.8	6.90	126.10	1:1
6	7.39	127.1	7.54	125.10	1:1
8	7.39	127.1	7.54	125.10	1:1
9		157.8		148.20	1:2:1
11	1.11	15.2	1.10	17.20	1:1
12	3.72	55.8	3.73	48.80	1:1
13	3.41	69.7	3.45	70.10	1:2:1
14	3.40	71.8	3.45	70.90	1:2:1
15	3.49	73.1	3.43	80.10	1:3:3:1
16	3.49	75.2	3.43	80.50	1:1
17	4.00	76.00	3.94	78.10	1:1
18	5.41	94.70	5.35	90.70	1:1

observed are as follows: Band i (0.10), Band ii (0.60), Band iii (0.82) and band iv (0.92). The antifungal activity assay of all the 4 bands revealed that only band iv was active, while, band I - iii did not show any activity (Table 3). TLC plates sprayed with Folin-Ciocalteau reagent (1:1 with water) showed blue color spots indicating that the isolated bioactive compound is a phenolic compound.

Identification of active compound *P. persica* by nuclear magnetic resonance (NMR) and mass spectral analysis

The compound was obtained as light green semisolid in 10 mg/50 g leaves, melting point; 150°C. $C_{18}H_{30}O_7$: C, 60.32%; H, 8.44%; O, 31.25% for C, 60.30%; H, 8.43%; O, 31.30%. The characteristic parameters observed for the active principle are; ^{1}H NMR: δ, M,1.29 C1,C2., 3.12 C3., 6.93 C5, C7., 7.39 C6, C8., 1.11 C11., 3.72 C12., 3.41 C13., 3.40 C14., 3.49 C15, C16., 4.00 C17., 5.41 C18]. ^{13}C NMR: δ, 23.3 (1-C, 2-C aliphatic), 39.3 (3-C), 140.8 (14-C), 114.8 (5-C, 7-C), 127.1 (6-C, 8-C), 157.8 (9-C), 15.2 (11-C), 69.7 (13-C), 7.18 (14-C), 73.1 (15-C), 75.2 (16-C), 76.00 (17-C), 94.7 (18-C) and its identity conformed by the mass spectral analysis.

The spectral data obtained from mass spectra (Figure 1), ^{1}H NMR (Figure 2) and ^{13}C NMR (Figure 3) (Table 4) showed that the active principle was aromatic glucose the compound is identified as 6-(ethoxymethyl)-tetrahydro-2H-pyran-2, 3, 4, 5-tetraol with 1- isopropyl-4-methoxyben-zene (Figure 4).

Determination of MIC of the active principle

The active principle showed complete inhibition of the mycelial growth (MIC) of *F. oxysporum* at 2000 ppm and *C. albicans* at 2500 ppm (Table 5).

DISCUSSION

Plants are an important part of traditional medicine world over and have been used extensively for thousands of years in China (Chang and But, 1986) and India (Kapoor, 1990). The use of plants in the traditional system of health care of many other countries such as China and India and other cultures has also been reported (Schultes and Raffauf, 1990). Plant extracts and plant based preparations are extensively used in the traditional medicine of Iran (Mosaddegh and Naghibi, 2005). More than 8,000 species of plants are reported from Iran, out of which more than 13% are reported to possess medicinal properties (Koocheki and Nadjafi, 2003). Scientific validation of the herbal drug always begins with the preparation of aqueous extract and its screening for desired biological activity ending with the isolation and characterization of the bioactive principle. The present study is successful in reporting the antifungal activity of aqueous extract of *P. persica* for the first time and also the active principle responsible for it.

The work has identified the novel antifungal phenolic compound 6-(ethoxymethyl)-tetrahydro-2H-pyran-2, 3, 4, 5-tetraol compound with 1- isopropyl-4-methoxybenzene. There is however the need to consider the possibility of using this compound to prepare a molecular mimics as a Novel Chemical Entity (NCE) for chemical synthesis. There is also need to assess the side effect of this compound by investigating its toxicological properties and its mode of action with a view to develop antifungal agents

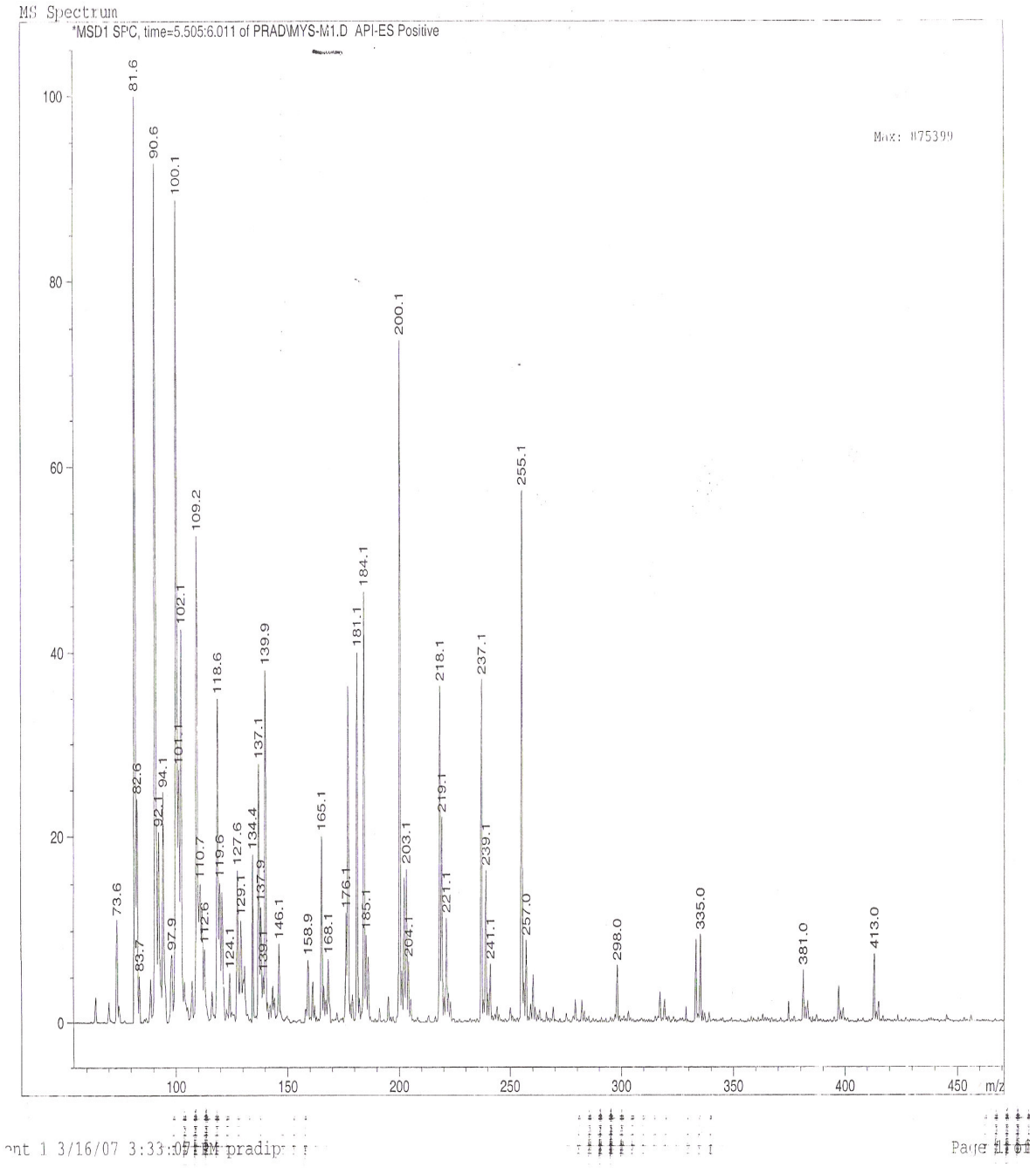

Figure 1. Mass Spectroscopy of active principle.

that will be safe for human and anima consumption.

The present study is also successful in identifying the compound with antifungal activity both against human and phytopathogenic fungi; this directly reveals that the compound has broad spectrum of fungicidal activity, which could be exploited in both human disease management and control of phytopathogenic fungal pathogens.

C. albicans has recorded resistance to many of the available antifungal agents (Krishnamurthy et al., 1998; Borowski and Milewski, 2007) and is also true with many phytopathogenic fungi including *F. oxysporum* (Reis et al., 2008), hence novel antifungal compounds are promising candidate molecules against these multi-drug resistant pathogens.

ACKNOWLEDGEMENTS

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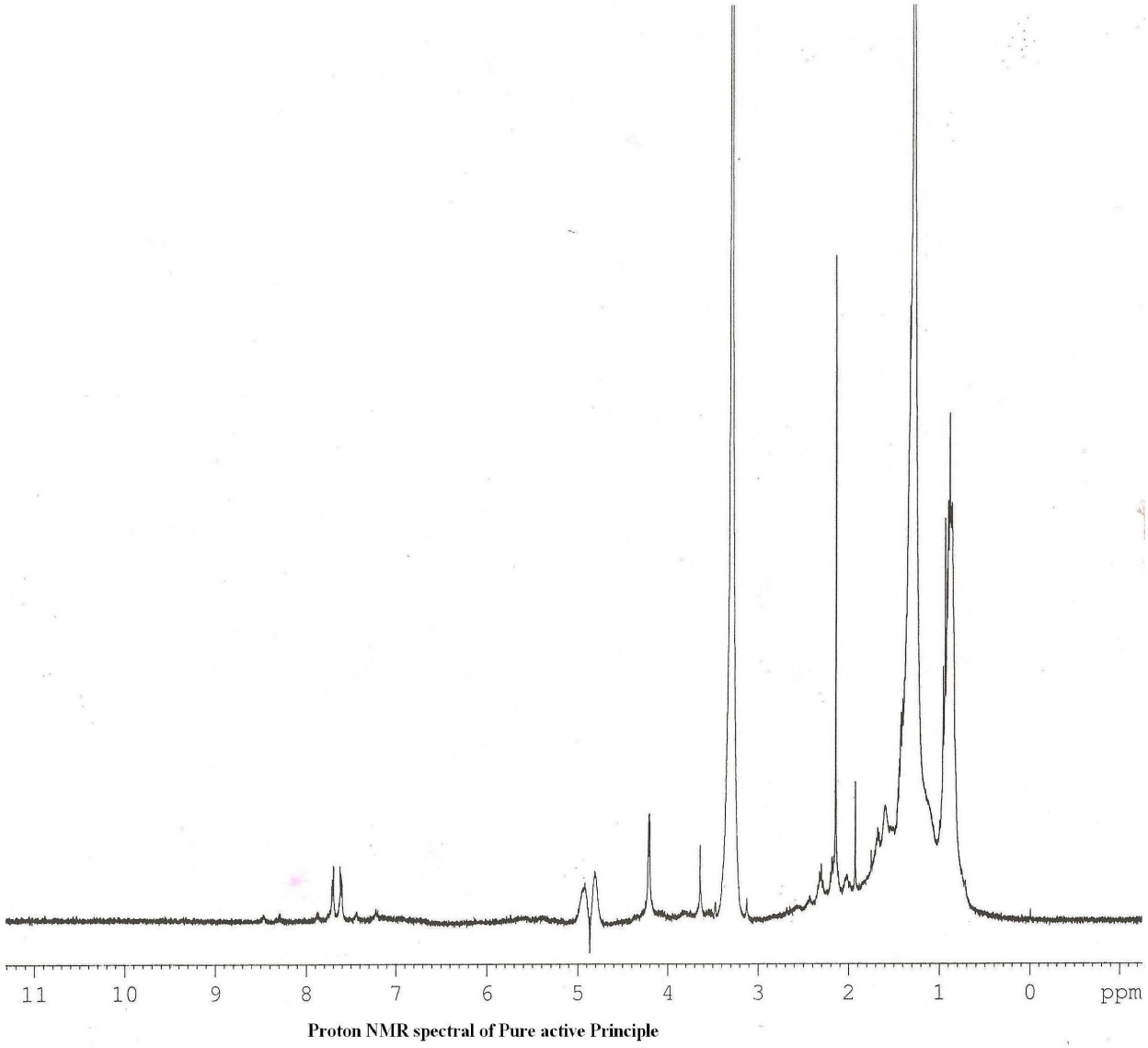

Figure 2. ^{1}H NMR spectroscopy of the active principle.

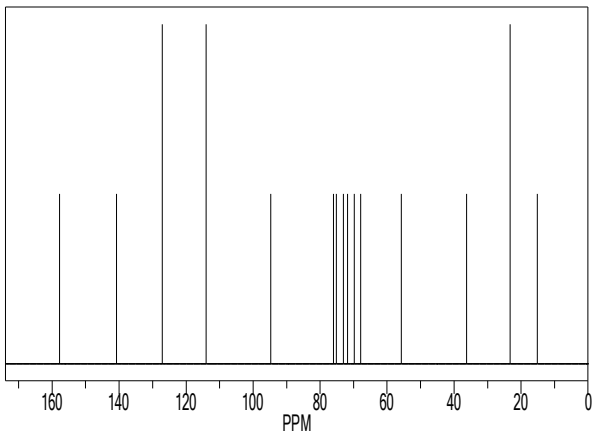

Figure 3. ^{13}C NMR spectroscopy of the active principle.

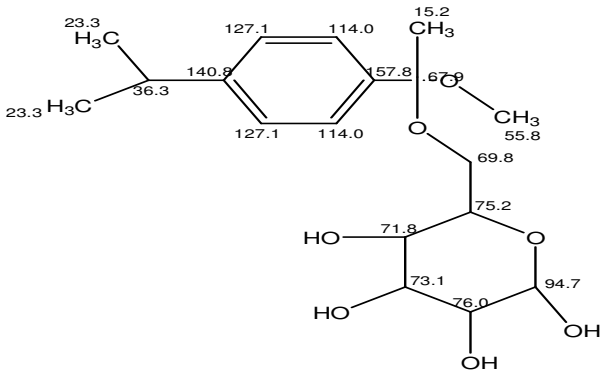

Figure 4. Structure of the active principle [6-(ethoxymethyl)-tetrahydro-2H-pyran-2, 3, 4, 5-tetraol compound with 1-isopropyl-4-methoxybenzene].

Table 5. Minimal Inhibitory Concentration (MIC) of the active principle isolated from *Parrotia persica* against test fungi.

Fungi	Percent inhibition concentration (ppm)										
	100	250	500	750	1000	1500	2000	2500	3000	3500	4000
F. oxysporum	10.52 ± 0.26	26.52 ± 0.47	48.61 ± 0.21	59.36 ± 0.38	70.36 ± 0.38	87.89 ± 0.59	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
C. albicans	10.39 ± 0.55	29.26 ± 0.45	46.25 ± 0.36	57.44 ± 0.25	65.18 ± 0.71	80.22 ± 0.64	90.89 ± 0.35	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Data given are mean of three replicates ±standard error Analysis of variance (ANOVA). $p<0.005$.

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