**Tecoma stans** (Bignoniaceae), leaf extracts, fractions and isolated compound have promising activity against fungal phytopathogens

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This article is partially based on the author’s dissertation to the Phytomedicine Programme in the Department of Paraclinical Sciences, for the degree of Magister Scientiae at the University of Pretoria, South Africa, with supervisors Prof. J.N. Eloff and L.K. Mdee, received December 2008, available here: https://repository.up.ac.za/bitstream/handle/2263/23195/dissertation.pdf?sequence=1

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Plant pathogenic fungi are a major cause of reduced plant production and post-harvest losses of plant produce. The control of these fungi by some synthetic fungicides is complicated by human and environmental toxicity, the development of resistance by some fungi and high costs, thus prompting the investigation of other means of fungal control. Plant secondary metabolites have a good potential as antifungal agents. The aim of this study is to investigate the potential use of *Tecoma stans* as a plant-derived fungicide by determining the antifungal activity of extracts, isolating the bioactive compound and testing the toxicity of both the extract and the isolated compound. In bioassay-guided fractionation of the leaves of the *Tecoma stans* dichloromethane (DCM) extract contained one major compound that was isolated and characterised as oleanolic acid. The DCM extract and oleanolic acid were active against 10 tested plant fungal pathogens (*Aspergillus niger*, *Aspergillus parasiticus*, *Collectotrichum gloeosporioides*, *Fusarium oxysporium*, *Penicillum expansum*, *Penicillum janthinellum*, *Pythium ultimum*, *Phytophthora nicotiana*, *Trichoderma harzianum* and *Rhizoctonia solani*) with an average minimal inhibitory concentration of 130 µg/mL. The DCM extract and oleanolic acid were toxic to Vero cells with an LC50 of 0.413 mg/mL and 0.129 mg/mL respectively, when compared with berberine, a toxic compound with LC50 of 15.48 µg/mL. Oleoanolic acid was more toxic than the crude extract, supporting the potential use of plant extracts for controlling plant fungal pathogens. The selectivity indices of 20 with several fungi indicated that extracts could possibly be used under controlled conditions against infections of certain fungal pathogens, even on edible plants. The large quantities available of this invasive plant species could lead to a commercially useful product in controlling plant fungal pathogens.

**Frasies en geïsoleerde verbinding uit *Tecoma stans* (Bignoniaceae), ’n indringerplant, het beloweende aktiviteit teen fungus fitopatogene. Fungi wat plante aanval lei tot groot verlies in plantproduktiwiteit en ook tot verlies in opbrengst nadat die produkte geoes is. Die beheer van hierdie fungis deur chemiese fungisiede lewer komplikasies vanwee menslike en omgewingsstoksititeit. Die koste en die ontwikkeling van weerstand deur plant patogene fungis teen fungisiede lewer ook probleme. Sekondêre plantmetaboliete het ’n goeie potensiaal as antifungusverbindings. Die doel van die studie was om die aktiviteit van *Tecoma stans* ekstrakte en fraksies te bepaal en om die aktiewe verbinding te isoleer deur die bioaktiviteit van fraksies gedurende die fraksionering te bepaal. Dit is gevind dat die dichlorometaanfraksie het die hoogste activiteit teen *Fusarium oxysporium*, *Penicillum expansum*, *Penicillum janthinellum*, *Pythium ultimum*, *Phytophthora nicotiana*, *Trichoderma harzianum* en *Rhizoctonia solani*. Dit is gevind dat *Tecoma stans* een beloweende herbicide is met een gemiddelde minimum inhiberende koncentrasie van 130 µg/mL. Die DCM ekstrakte en oleanolise suur was minder toksies as die berberine met een LC50 van 0.413 mg/mL en 0.129 mg/mL. Oleoanolic acid was more toxic than the crude extract, supporting the potential use of plant extracts in controlling plant fungal pathogens. The selectivity indices of 20 with several fungi indicated that extracts could possibly be used under controlled conditions against infections of certain fungal pathogens, even on edible plants. The large quantities available of this invasive plant species could lead to a commercially useful product in controlling plant fungal pathogens.

**Navorsing korrelasie:** Hierdie artikel is die vertaalde weergawe en is beskikbaar gestel om ’n breër leersersgroep te bereik. Die oorspronklike Engelse artikel is beskikbaar hier: https://doi.org/10.4102/satnt.v36i1.1496
Introduction

Plant fungal pathogens threaten food security worldwide as more than 800 million people have inadequate food supplies and at least 10% of food production is lost to plant diseases (Strange & Scott 2005). One major cause of plant disease is pathogenic fungi (Prescott, Harley & Klein 1996). When many agricultural crops are infected by certain fungal pathogens, they produce mycotoxins that are harmful to both humans and livestock (Awuah & Kpodo 1996).

Synthetic fungicides are the primary means of controlling plant pathogens. However, safety risks, high costs, side effects and development of resistance towards the use of these fungicides are raising serious concerns (Tripathi & Dubey 2004). These drawbacks prompt the investigation of other means of fungal control.

Plants are good candidates in the search for fungicidal compounds, since they have to exist under difficult conditions and are attacked by all manner of parasites, especially fungi (Hostettman et al. 2000). Their use as natural additives emerged from an increasing tendency to replace synthetic antimicrobial agents with natural ones (Sharma & Tripathi 2006). Therefore, plant extracts or plant secondary metabolites that are not toxic and specific in their action could be considered as an alternative to synthetic fungicides. Based on the availability of material we decided to investigate Tecoma stans.

_Tecoma stans_, also known as yellow elder, is a member of the family Bignoniaceae. This tropical tree is found predominantly in Central and South America, although its native environment extends from the southern states of the United States to north of Argentina. However, _T. stans_ has also become established in other tropical and subtropical areas in Africa, Asia, Pacific islands and Australia. _T. stans_ is a drought-tolerant shrub and is relatively resistant to pests (http://cloudbridge.org/trees/tecomastans.html).

_Tecoma stans_ leaves, bark and roots have been used for various medicinal purposes in herbal medicine (http://cloudbridge.org/trees/tecomastans.html). The control of diabetes is the most mentioned traditional use of the plant in Mexico. The plant leaf extract reduces blood sugar of fasting rabbits (Hammouda, Rashid & Amer 1964). The plant contains monoterpenoid alkaloids and two of them (tecomine and tecostanine) are responsible for the hypoglycaemic activity of the plant (Hammouda & Amer 1966). No information in the literature was found on the antifungal activity of the plant, therefore leaving scope for this study to provide information regarding possible antifungal uses.

In the study we isolated and characterised the main antifungal compound present in the plant extracts and determined the antifungal activity against important plant fungal pathogens and its cytotoxicity.

Materials and methods

Plant collection

Leaves of _T. stans_ were collected from the Lowveld National Botanical Garden, Nelspruit, South Africa, in summer. The leaves were stored hanging in open mesh bags, used for selling oranges, in the shade with good ventilation to dry. Voucher specimens in the garden herbarium verified the identity of the species.

Extraction

Powdered dried leaves of _T. stans_ were exhaustively extracted with acetone at a ratio of 10:1, dried under a stream of air in a fume cupboard at room temperature. The extract was stored in preweighed labelled containers to quantify extraction. Acetone was used as an extractant, based on its efficacy with reference to quantity and diversity of compounds extracted, ease of removal, safety and toxicity to assay organisms in the case of bioassay work (Eloff 1998a).

Solvent-solvent fractionation of crude extract

Solvent-solvent extraction is one of the popular techniques used in the preparation of samples for qualitative and quantitative analysis. It is a process of separating one constituent from a mixture by dissolving it into a solvent, in which it is soluble, while the other constituents of the mixture are not, or are at least less soluble (Holden 1999). The acetone crude extract of _T. stans_ was separately suspended in 20% methanol in distilled water in a separatory funnel (2000 mL) and successively partitioned with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH) respectively, in this order. These solvents have varying polarities to extract different constituents of the plant with different polarities. The chemical composition (by thin layer chromatography), the number of antifungal compounds (by bioautography), antifungal activity (by serial microplate dilution) and cytotoxicity against Vero cells of the four partition fractions were determined.

Bioautography

A bioautographic method, developed in our laboratory (Masoko & Eloff 2005), was used to determine activity. Thin-layer chromatography (TLC) plates were prepared and loaded with extract and oleanolic acid dissolved to a final concentration of 10 mg/mL and 1 mg/mL respectively. Ten microlitres was applied and the plates were developed in mobile phase, dried and sprayed with conidia of the fungal organisms until they were just wet, incubated overnight and then sprayed with a 2 mg/mL solution of p-iodonitrotetrazolium violet (Sigma®) (INT), and further incubated overnight or longer at 35 °C in a clean chamber, at 100% relative humidity in the dark. White areas indicated where reduction of INT to the coloured formazan did not take place, due to the presence of compounds that inhibited the growth of tested fungi. To minimise fungal spreading and infection in our laboratory, the bioautograms were sealed in clear plastic envelopes before scanning in for
permanently moved by compound/distance moved by solvent front

**Determining antifungal activity by serial microdilution assay**

The microdilution method developed by Eloff (1998b), and modified by Masoko, Picard and Eloff (2005), was used to determine the minimum inhibitory concentration (MIC) values of *T. stans* extracts, fractions and isolated compound against 10 plant pathogenic fungi viz. *Rhizoctonia solani*, *Fusarium oxysporum*, *Penicillium janthinellum*, *Penicillium expansum*, *Aspergillus parasiticus*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Trichoderma harzianum*, *Pythium ultimum* and *Phytophthora nicotiana*. This method was applied as follows.

Dried residues of the *Tecoma stans* DCM extract, fractions andoleanolic acid were dissolved in acetone to a concentration of 10 mg/mL and 1 mg/mL respectively. The *Tecoma stans* DCM extract and oleanolic acid (100 µL) were serially diluted 50% with water in a 96-well microtitre plate (Eloff 1998b). Fungal cultures were transferred into a fresh potato dextrose broth, and 100 µL of this was added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included. As indicator of growth, 40 µL of 0.2 mg/mL of INT dissolved in water was added to each of the microplate wells. The covered microplates were incubated for 1 day at 35 °C and 100% relative humidity. The experiment was performed in triplicate and repeated three times. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 h of incubation. Acetone was used as a solvent and negative control, because fungi are not affected by acetone concentrations lower than 50% (Eloff, Picard & Masoko 2007). The highest acetone concentration the fungi was subjected to in this procedure was 25% in the first well.

**Cytotoxicity assay**

The samples were tested for cytotoxicity against the Vero monkey kidney cell line (obtained from the Department of Veterinary Tropical Disease, University of Pretoria). The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). To prepare the cells for the assay, cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5 × 10^5 cells into each well of a 96-well microtitre plate. After overnight incubation at 37°C in a 5% CO2 incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extract were prepared in growth medium (1 µg/mL–1000 µg/mL). The viable cell growth after 120 h incubation with plant extracts/samples was determined, using the tetrazolium based colorimetric assay (MTT assay) described by Mosmann (1983).

The absorbance was measured on a Titertek Multiscan MCC/340 microplate reader at 540 nm test wavelength and reference wavelength of 690 nm. Berberine chloride (Sigma Chemical Company) was used as a positive control. Tests were carried out in quadruplicate and each experiment was repeated three times.

**Isolation of bioactive compound**

The DCM extract obtained from solvent-solvent extraction was subjected to column chromatography on silica gel 60. The column was eluted with 100% DCM and, subsequently, the polarity of the eluting solvent was increased with methanol (MeOH). A volume of 1000 mL of 100% DCM was initially used, followed by solvent mixture of 10% MeOH, 20% MeOH, 30% MeOH, 40% MeOH, 50% MeOH, 60% MeOH, 80% MeOH, all in DCM, and finally the column was eluted with 100% MeOH. A total of nine fractions, each 1000 mL, were collected. Fractions were pooled according to their similarity in behaviour on TLC. The fractions were concentrated and chromatographed to remove impurities to finally obtain the compound. To confirm that the isolation of the antifungal compound present in the extract is not an artefact of the isolation, bioautography was carried out on the isolated compound and the DCM crude extract.

**Nuclear Magnetic Resonance Spectroscopy**

Nuclear Magnetic Resonance Spectroscopy (1H NMR and 13C NMR) was carried out to determine the structure of the compound, using a Varian Inva 500 MHz spectrometer. The isolated compound from *T. stans* was dried, weighed (15 mg–20 mg) and dissolved in 2 mL of deuterated chloroform into NMR tubes, using a clean Pasteur pipette and analysed in the Department of Chemistry, University of Botswana.

**Results and discussion**

**Quantity of *Tecoma stans* extracted using solvent-solvent extraction**

A quantity of 46.67 g of serially extracted acetone extracts of *T. stans* leaves powder was subjected to solvent-solvent extraction leading to four fractions of increasing polarity viz. hexane, DCM, EtOAc and BuOH. Hexane was the best extractant in terms of quantity extracted with 46.23% (21.58 g) extracted, followed by DCM with 16.75% (7.82 g), EtOAc with 1.79% (0.84 g) and BuOH with 0.64% (0.3 g) respectively. This indicated that these leaves are very rich in non-polar compounds.

The DCM fraction was selected to isolate active compounds by bioassay guided fractionation, based on its activity. Fractions eluted from the DCM extract in open column silica gel chromatography were pooled according to their TLC composition, concentrated and chromatographed to remove impurities which yielded a white precipitate. To examine the activity of the antifungal compounds, bioautography was undertaken of the combined fractions by TLC and the DCM extract that was fractionated and sprayed with *P. janthelium* suspension (Figure 1).
Rf values of active compounds may help in dereplicating isolation of antifungal compounds from other plant extracts. The Rf value of the extract and isolated compound was 0.08 with BEA (benzene/ethanol/ammonium hydroxide 90/9/1) (Kotze & Eloff 2002) as eluant (Figure 1). This confirms that the compound isolated was probably the antifungal compound present in the crude extract and not an artefact of the isolation process.

Identification of compound

The compound was isolated as a white precipitate. NMR spectroscopy identified the compound as oleanolic acid (Figures 2 and 1-A1). Oleanolic acid is a triterpenoid compound present in the leaves and bark of many plant species. This isomer of ursolic acid has been recognised to have hepatoprotective, anti-inflammatory, and antihyperlipidemic properties and has recently been noted for antitumor promotion effects. It has been marketed as an oral drug in China for human liver disorders (www.alibaba.com/product-gs/203129384/Oleanolicacid.html). The compound is well known but literature reveals little about its antifungal activity. It has not been isolated from Tecoma stans before.

Antifungal activity and toxicity testing

Bioautograms

In isolating bioactive compounds from plant extracts, it is always possible that the major biologically active compounds may be inactivated during the isolation process and that a minor compound may be isolated in the end. To examine this possibility, bioautography was carried out on the crude extract and the isolated oleanolic acid.

The bioautography results (Figure 1) indicate inhibition of fungal growth by the extract and oleanolic acid by compounds with the same Rf value, by showing a clear zone, which resulted from INT that was not reduced to the red product by actively growing organisms. The two identical bands observed in the chromatography of the crude extract and oleanolic acid show that the active compound present in the extract was isolated because the active bands had the same Rf value of 0.08 in BEA, confirming the isolation of the main active compound present in the crude extract.

Microdilution assay

To evaluate the degree to which the activity has increased by isolating oleanolic acid and the sensitivity towards different fungal pathogens, microdilution assays were carried out against several pathogens.

In several cases the T. stans crude extract had similar MICs or even lower MICs than oleanolic acid (Table 1). The microorganisms responded differently to the extract and the isolated compound. P. janthinellum and C. gloeosporoides were relatively resistant, while T. harzianum, R. solani, F. oxysporium and P. expansum were relatively sensitive. The extract had good activity against some fungi with MIC values of 20 µg/mL and 60 µg/mL. In the case of P. expansum the extract had better activity than the positive control amphotericin B. The difference in activity to different fungi may be significant and indicate selectivity, because different targets are addressed.

Because the activity of the extract was comparable with the activity of the compound, it means that despite removing approximately 99% of ‘supposedly inactives’ from the crude extract, based on bioautography on a mass basis, the activity did not increase 100 fold. The most probable explanation for this is that there must be a large degree of synergism taking place. The other compounds acting synergistically were not active after they were separated from other compounds during bioautography. An alternative explanation is that other volatile compounds...
TABLE 1: MIC values in µg/mL of Tecoma stans dichloromethane crude fraction and oleanolic acid tested against 10 plant pathological fungi.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Tecoma stans crude</th>
<th>Oleanolic acid</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus parasiticus</td>
<td>160</td>
<td>250</td>
<td>0.78</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>120</td>
<td>250</td>
<td>0.31</td>
</tr>
<tr>
<td>Phytophthora nicotiana</td>
<td>120</td>
<td>190</td>
<td>7.5</td>
</tr>
<tr>
<td>Penicillium janthinellum</td>
<td>470</td>
<td>250</td>
<td>40</td>
</tr>
<tr>
<td>Trichoderma harzianum</td>
<td>20</td>
<td>8</td>
<td>7.5</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>20</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Fusarium oxysporium</td>
<td>20</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Penicillium expansum</td>
<td>20</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Pythium ultimum</td>
<td>60</td>
<td>16</td>
<td>40</td>
</tr>
<tr>
<td>Average</td>
<td>130</td>
<td>130</td>
<td>18.1</td>
</tr>
</tbody>
</table>

DCM extract, dichloromethane.

Cellular cytotoxicity of the T. stans DCM extract and compound were determined with the toxic alkaloid berberine as positive control. The percentage cell viability increased with a decrease in concentrations of DCM extract, compound and berberine. The highest concentration of both DCM extract and compound killed all the Vero cells at concentrations higher than 150 µg/mL. Berberine was toxic at concentrations higher than 10 µg/mL.

Cellular toxicity can also be expressed by the LC₅₀ values, calculated from the regression curve. T. stans DCM extract had an LC₅₀ 0.413 mg/mL, the compound 0.129 mg/mL and berberine 15.48 µg/mL, which means that the crude extract and oleanolic acid were several-fold less toxic than berberine. Oleanolic acid was more toxic to Vero cells than the extract.

Potential use of Tecoma stans in plant production

Many antifungal compounds are toxic, because several more similar metabolic pathways exist between fungi and mammals than between bacteria and mammals. Therefore there are fewer specific targets that might be addressed. The important question is how the toxicity to the target organism relates to the cellular toxicity. The selectivity index can be defined here as the LC₅₀ in (µg/mL)/MIC in (µg/mL). The higher this value is, the safer the product is to use under controlled conditions (Table 3).

The crude extract had a high selectivity index value of 21 against microorganisms T. harzianum, R. solani, F. oxysporium and P. expansum which means crude extracts can be used against infection of these fungi and pose little toxicity if used under controlled conditions. It is interesting to note that P. expansum, which is a major problem in fruit spoilage, can be controlled by the crude extract at a dosage level with low toxicity. Fusarium spp. which can be a big problem in maize contamination and damping off of seedlings, can also be controlled with little toxicity.

The high selectivity index of 16 and 64 of oleanolic acid against T. harzianum and R. solani, respectively, stood out as the most promising for the use of oleanolic acid against infections, caused by these two fungi, at the correct dose. With many other phytopathogens the extract or oleanolic acid were more toxic to Vero cells than to the fungus.

Conclusion

It appears that there may be greater potential in using plant extracts than the isolated oleanolic acid. The crude plant extract would cost much less to produce. There were major differences in the sensitivity of the different pathogens to the crude extract. This may be valuable because it indicates that the activity is on a basic metabolic target present in all the fungi.

Because many antifungal agents are also highly toxic, it was important to determine the cellular toxicity and the...
selectivity index. Under controlled conditions the crude extract and oleanolic acid may be used to test infections caused by some pathogens with a reasonably good selectivity index value; therefore posing relatively low toxicity threats. In the case of other pathogens, neither the crude extract nor oleanolic acid would be of any potential use, due to its toxicity. One should, however, keep in mind that cellular toxicity does not necessarily correspond to toxicity via other routes. If the toxic compound is ingested and it is destroyed at the low gut pH, or not taken up from the gut or rapidly detoxified, the toxicity may be much lower. It is also possible that a more toxic compound could be produced after uptake of the extract, stressing the importance of in vivo animal experiments on toxicity. However, it is beyond the scope of this work.

Acknowledgements

We are grateful to the curator of the Lowveld National Botanical Garden for allowing us to collect plant material. Prof. Lise Korsten (Department of Plant Pathology, University of Pretoria), who provided cultures of the phytopathogens used. This research was supported by the National Research Foundation (NRF) and the University of Pretoria.

Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors’ contributions

M.M. did the work as part of a M.Sc. study. L.M. helped in isolating the active compound and elucidated the structure. J.N.E. identified the project, supervised the study and revised the manuscript and submitted it.

References


Oleanolic acid was viewed 04 December 2008, from www.alibaba.com/product s/203129384/Oleanolic_acid.html


TABLE 3: Selectivity index of crude and oleanolic acid against 10 fungi.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Crude extract LC₅₀ (μg/mL)</th>
<th>Crude extract MIC (μg/mL)</th>
<th>Selectivity index (crude extract)</th>
<th>Oleanolic acid LC₅₀ (μg/mL)</th>
<th>Oleanolic acid MIC (μg/mL)</th>
<th>Selectivity index (oleanolic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collectotrichum gloesporioides</td>
<td>413</td>
<td>310</td>
<td>1.0</td>
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<tr>
<td>Aspergillus parasticus</td>
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<td>250</td>
<td>0.5</td>
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<tr>
<td>Aspergillus niger</td>
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</tr>
<tr>
<td>Phytophthora nicotiana</td>
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<td>3.0</td>
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<td>250</td>
<td>0.5</td>
</tr>
<tr>
<td>Penicillium janthinellum</td>
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<td>470</td>
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<td>Trichoderma harzianum</td>
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<td>21.0</td>
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<tr>
<td>Rhizoctonia solani</td>
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<td>21.0</td>
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<td>64.0</td>
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<td>Fusarium oxysporium</td>
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<td>Pythium ultimum</td>
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<td>7.0</td>
<td>129</td>
<td>16</td>
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</tr>
</tbody>
</table>
Appendix 1:

**FIGURE 1-A1:** Nuclear Magnetic Resonance spectrum of isolated compound.