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Corrigendum: *Tecoma stans* (Bignoniaceae), an invasive species, fractions and isolated compound has promising activity against fungal phytopathogens

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In the version of this article initially published, Moraba M. Meela's surname was misspelled as 'Meelah'. The error has been corrected in the PDF version of the article. The editor apologises for any inconvenience this error may have caused.

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

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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 gloeosporoides, 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 LC_{50} of 0.413 mg/mL and 0.129 mg/mL respectively, when compared with berberine, a toxic compound with LC_{50} of 15.48 µg/mL. Oleanolic 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.

Research correlation: This article is the translated version, made available to provide access to a larger readership, of which the original English article is available here: https://doi.org/10.4102/satnt.v36i1.1496

Fraksies en geïsoleerde verbinding uit *Tecoma stans* (Bignoniaceae), 'n indringerplant, het belowende aktiwiteit teen fungus fitopatogene. Fungi wat plante aanval lei tot groot verliese in plantproduktiwiteit en ook tot verliese in opbrengs nadat die produkte geoes is. Die beheer van hierdie fungi deur chemiese fungisiede lewer komplikasies vanweë menslike en omgewingstoksisiteit Die koste en die ontwikkeling van weerstand deur plant patogeniese fungi teen fungisiede lewer ook probleme. Sekondêre plantmetaboliete het 'n goeie potensiaal as antifungusverbindings. Die doel van die studie was om die aktiwiteit van *Tecoma stans* ekstrakte en fraksies te bepaal en om die aktiewe verbinding te isoleer deur die bioaktiwiteit van fraksies gedurende die fraksionering te bepaal. Die dichlorometaanfraksie het die hoogste aktiwiteit gehad en die geïsoleerde verbinding se struktuur is bepaal as oleanoliese suur. Die antifungus aktiwiteit is bepaal teen tien belangrike plant fungus patogene (*Aspergillus niger, Aspergillus parasiticus, Collectotrichum gloeosporoides, Fusarium oxysporium, Penicillum expansum, Penicillum janthinellum, Pythium ultimum, Phytophthora nicotiana, Trichoderma harzianum* en *Rhizoctonia solani*). Die gemiddelde minimum inhiberende konsentrasie was 130 µg/mL. Die DCM ekstrak en oleanoliese suur was minder toksies as die positiewe kontrole berberien teen Vero selle met LC_{50} waardes van 0.413 mg/mL, 0.129 mg/mL en 15.48 µg/mL respektiewelik. Die selektiwiteitindeks van 20 met verskeie fungi dui op moontlike relatiewe veiligheid om onder gekontroleerde toestande selfs vir eetbare produkte te gebruik. Die groot massa plantmateriaal wat beskikbaar is van hierdie indringerplant mag tot 'n kommersieel bruikbare produk lei in die bekamping van fitopatogeniese fungi.

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

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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 monoterpene 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 1.0 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

permanent record. R_f values of active compounds were calculated using the following formula:

R_f = distance 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 oxysporium, Penicillum janthinellum, Penicillum expansum, Aspergillus parasiticus, Aspergillus niger, Collectotrichum gloeosporoides, Trichoderma harzianum, Pythium ultimum* and *Phytophthora nicotiana*. This method was applied as follows.

Dried residues of the *Tecoma stans* DCM extract, fractions and oleanolic 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 0.5×10^3 cells into each well of a 96-well microtitre plate. After overnight incubation at 37° C in a 5% CO_2 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).

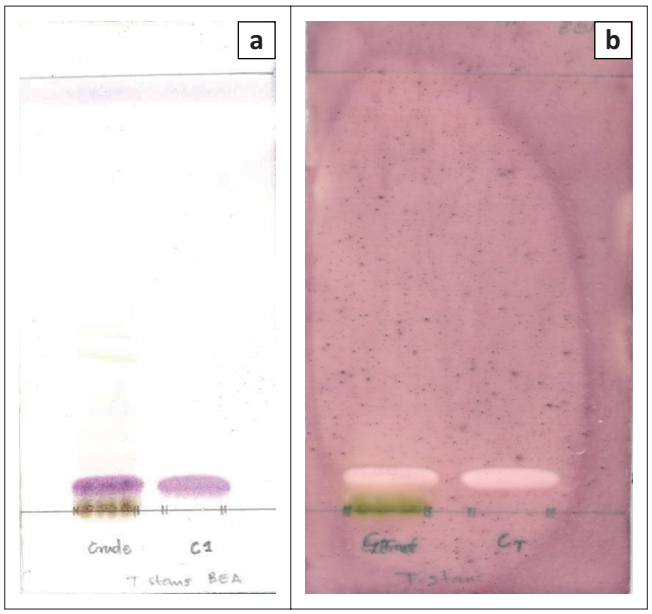

FIGURE 1: Chromatogram and bioautogram, loaded with dichloromethane fraction, and eluted with benzene/ethanol/ammonium hydroxide 90/9/1 (1) and compound isolated from the extract (2), sprayed with vanillin sulphuric acid spray reagent (a) and *Penicillum expansum* cell suspension, followed by p-iodonitrotetrazolium violet (b).

R_f values of active compounds may help in dereplicating isolation of antifungal compounds from other plant extracts. The R_f 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.

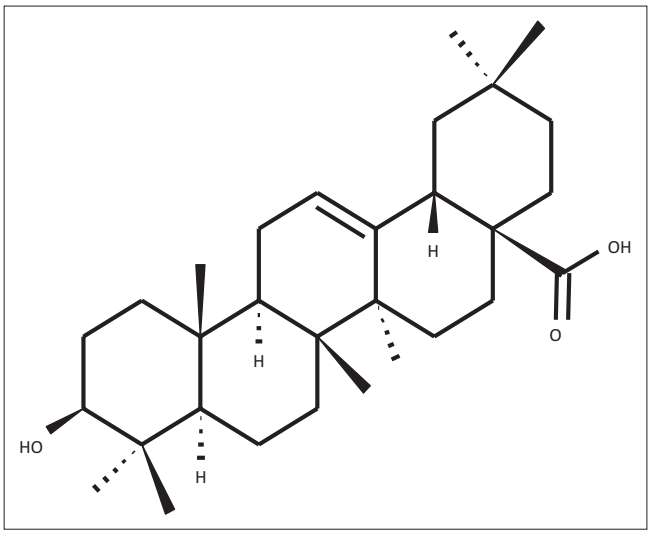

FIGURE 2: Structure of oleanolic acid, isolated from *Tecoma stans* dichloromethane extract.

The bioautography results (Figure 1) indicate inhibition of fungal growth by the extract and oleanolic acid by compounds with the same R_f 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 R_f 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

TABLE 1: MIC values in µg/ml of *Tecoma stans* dichloromethane crude fraction and oleanolic acid tested against 10 plant pathological fungi.

Microorganisms	*Tecoma stans* crude	Oleanolic acid	Amphotericin B
Aspergillus parasiticus	160	250	0.78
Aspergillus niger	120	250	0.31
Phytophthora nicotiana	120	190	7.5
Penicillum janthinellum	470	250	40
Trichoderma harzianum	20	8	7.5
Rhizoctonia solani	20	2	25
Fusarium oxysporium	20	50	20
Penicillum expansum	20	20	40
Pythium ultimum	60	16	40
Average	130	130	18.1

TABLE 2: Total activity of the *Tecoma stans* dichloromethane fraction and oleanolic acid against the 10 fungal pathogens.

Samples	Mass in mg	Average MIC in mg/mL	Total activity in mL
DCM extract	7820	0.13	60 154
Oleanolic acid	1204	0.13	9 262

DCM, dichloromethane.

compounds may have been present in the crude extract and that these evaporated during the long period of removing the solvent from the chromatograms before spraying with the fungi. Alternatively, some antifungal compounds may have been destroyed during the isolation process.

Amphotericin B was active with an average MIC value of 18 µg/mL, but in some cases against certain fungi the crude extract had a higher activity than amphotericin B. This points to the possible use of crude extracts in protecting plants against fungi.

If the conclusion reached in the previous paragraph is correct, that there must be substantial activity residing in compounds outside oleanolic acid, an analysis of the total activity of the crude extract and the isolated compound can be made (Eloff 2004) (Table 2). This takes into account not only the antimicrobial activity but also the quantity. If all the activity is presented by the isolated compound, the two values would be the same. The value for the extract was 6.5 times higher than that of the isolated compound. Even if only 50% of the active compound was isolated in the end with the rest residing in impure fractions, this only represents a third of the activity present in the crude. There may be two explanations. The other active compounds were decomposed or inactivated during the isolation procedure. Alternatively, and more likely, because there was only one antifungal compound present in the crude, based on bioautography, more than one compound played a role in determining the antifungal activity through synergism.

Toxicity testing

The motivation for determining the cytotoxicity of the extract and oleanolic acid is that if the cytotoxicity is low, there is a possibility that it could be used to treat fungal pathogens on edible plants. If it has a high toxicity, it could still be useful in the horticultural industry (Eloff, Angeh & McGaw 2017).

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 LC_{50} values, calculated from the regression curve. *T. stans* DCM extract had an LC_{50} 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 LC_{50} 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

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

Microorganisms	Crude extract LC_{50} (µg/mL)	Crude extract MIC (µg/mL)	Selectivity index (crude extract)	Oleanolic acid LC_{50} (µg/mL)	Oleanolic acid MIC (µg/mL)	Selectivity index (oleanolic acid)
Collectotrichum gloeosporoides	413	310	1.0	129	250	0.5
Aspergillus parasiticus	413	160	2.5	129	250	0.5
Aspergillus niger	413	120	3.0	129	250	0.5
Phytophthora nicotiana	413	120	3.0	129	190	0.7
Penicillum janthinellum	413	470	0.9	129	250	0.5
Trichoderma harzianum	413	20	21.0	129	8	16.0
Rhizoctonia solani	413	20	21.0	129	2	64.0
Fusarium oxysporium	413	20	21.0	129	50	2.6
Penicillum expansum	413	20	21.0	129	20	6.5
Pythium ultimum	413	60	7.0	129	16	8.0

selectivity index. Under controlled conditions the crude extract and oleanolic acid may be used against 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.

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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.

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Appendix 1:

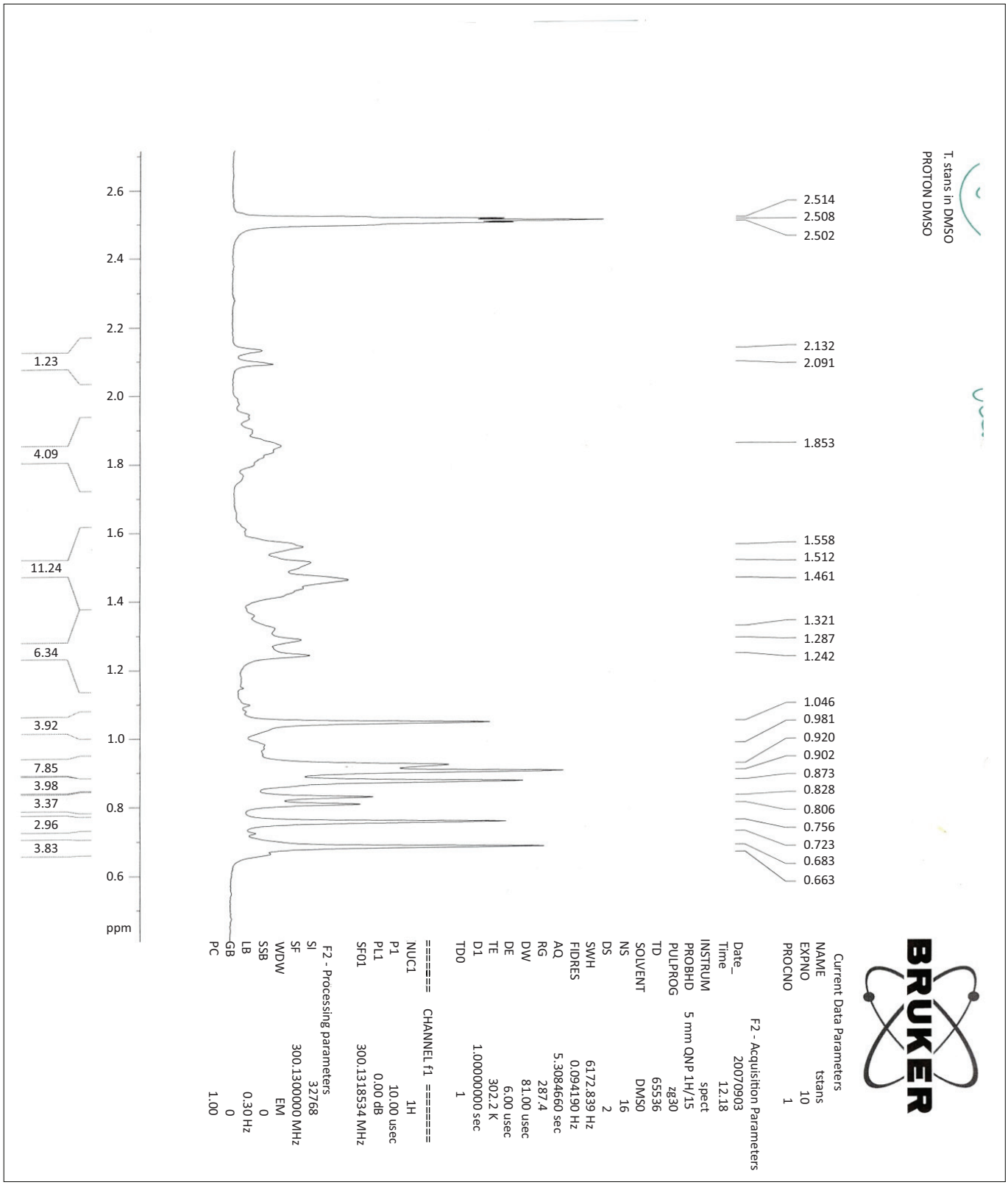

FIGURE 1-A1: Nuclear Magnetic Resonance spectrum of isolated compound.