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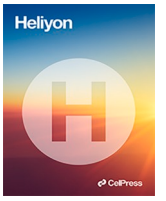

Research article

The activity of leaf extracts, fractions, and isolated compounds from *Ptaeroxylon obliquum* against nine phytopathogenic fungi and the nematode *Meloidogyne incognita*

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ABSTRACT

Phytopathogenic fungi and nematodes cause great losses in economically important crops and food production especially in developing countries. To minimize the use of fungicides and nematicides, researchers have concentrated on the use of natural products for crop disease prevention or control. The aim of the study was to investigate the antifungal activity of *Ptaeroxylon obliquum* leaf extracts, fractions, and isolated compounds (obliquumol and a mixture of lupeol and β-amyrin) and nematocidal activity of fractions (hexane, chloroform and 30% water in methanol and the isolated compounds) on *Meloidogyne incognita*. Nine phytopathogenic fungi (*Aspergillus niger, A. parasiticus, Colletotrichum gloeosporioides, Fusarium oxysporum, Penicillium digitatum, P. expansum, P. italicum, P. janthinellum,* and *Rhizoctonia solani*) were used for testing and nematocidal activity was determined on motility of plant parasitic nematode *Meloidogyne incognita* race 2 juveniles. Serial microdilution test was utilized to determine the minimum inhibitory concentration (MIC) of each sample against the fungus. Motility tests was done on the second-stage juveniles (J2s) of *M. incognita*. The most susceptible phytopathogenic fungal species to the acetone crude leaf extracts were *A. niger, C. gloeosporioides* and *P. digitatum* with MIC of 80 μg/ml which is considered pharmacological significant. *Rhizoctonia solani* was the most susceptible fungus against obliquumol and, lupeol and β-amyrin mixture with MIC values of 8 μg/ml and 16 μg/ml respectively. Lupeol & β-amyrin mixture had good activity on juvenile motility at high concentrations used which was significantly high ($p \leq 0.05$) after 24 h, further incubation resulted in temporary paralysis at lower concentrations. Fractions and obliquumol showed good activity after 48 h, stable paralysis was observed up to 72 h. The extracts and isolated compounds may be useful as fungicides if the *in vitro* results can be confirmed under field conditions at levels not toxic to beneficial soil organisms.

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1. Introduction

Plant pests such as parasitic nematodes, viruses, bacteria, fungus, and weeds are responsible for a significant portion of the global yield reduction in crop production, which can reach 16–34% [1]. It is reported that the crop harvest losses caused by plant disease is estimated to 12% worldwide and possibly higher in developing countries [2,3]. The presence of plant pathogenic fungi causes severe losses to economically important crops and food supply [4]. The available fungicides have been found to be resistant to fungal diseases caused by plant pathogens [5]. Approximately 50% of global postharvest losses are attributed to bacterial and fungal diseases [6].

Blue mould is a prevalent postharvest disease brought on by *Penicillium italicum* that develops during storage and transportation while *P. digitatum* (green mould) causes significant fruit losses after export because of decay [7,8]. *Penicillium expansum* also causes serious postharvest losses especially in fruit such as apples, peaches, and cherries [5]. *Aspergillus niger* and *A. flavus* are typical causes of peanut crown rot diseases in different countries across the globe [9]. *Rhizoctonia solani* is associated with diseases that affect the roots and tubers of various crops such as tomato, maize, rice, cucumbers, cassava and sweet potato amongst others [10]. *Fusarium oxysporum* is the cause of vascular wilt disease in tomatoes while Fusarium head is a widespread fungal disease that affects wheat and barley globally [5]. Apart from crop harvest losses and food decay attributed to pathogenic fungi, many of them cause a serious risk to consumers because the dangerous secondary metabolites they produce [11]. Numerous toxigenic species of *Aspergillus*, *Penicillium, Fusarium*, and that grow on seeds, grains, and feed in the field and/or in storage and they produce chemicals known as mycotoxins. Aflatoxin B1 and ochratoxin A are the two most toxic mycotoxins with a range of effects including liver toxicity, renal toxicity, immunotoxicity, mutagenicity, and carcinogenicity ones [12,13].

Root-knot nematodes (*Meloidogyne* spp.) are economically significant global plant parasites that severely harm various crops, such as cucumbers, tomatoes, and rice [14]. Root-knot nematodes reproduce and feed on modified living plant cells within plant roots, where they induce galls or root-knots. Infections due to root-knot nematodes impact the root system's ability to transport water and nutrients, and some phytopathogens like bacteria and fungus may penetrate the xylem and interfere with the flow of water, severely harming the crop [15]. *Meloidogyne* has the potential to result in approximately 5–43% vegetable yield loss in tropical and subtropical regions and as much as $100 billion every year worldwide [16,17]. *Meloidogyne incognita* is a significant species of root-knot nematode in the world [18]. Additional damage is caused by root rots and wilts that arise from disease complexes with opportunistic root pathogens such *Fusarium solani*, *Verticillium* spp., *R. solani*, etc [19]. Agbenin and Marley [20] reported that fusarium wilt caused by *F. oxysporum* has a synergistic relationship with *M. incognita* and can cause high yield loss. The above-ground symptoms such as leaf chlorosis, stunting and patchy growth which are the most common symptoms often resemble those associated with nutrient deficiencies [21] and they are usually not linked to root-knot nematodes infection.

Chemicals such as antimicrobials and nematicides have been successfully used to protect plants from pest diseases. Antimicrobials such as benzimidazoles, aromatic hydrocarbons and sterol biosynthesis inhibitors and more recently pyrimethanil and fludioxonil have become important in the market [22]. To overcome developing fungal resistance, higher concentrations of the chemicals have been used. Many fungicidal agents in the market negatively affect other organisms in the environment. Some residues from the application of nematicides, fertilizers, herbicides, insecticides, and fungicides to farmland remain in the soil after plant absorption and can reach to surface water by adsorbing sediment or dissolving in runoff [23]. Humans can be affected by pathogen transmission through food chain as some synthetic fungicides that are utilized are not biodegradable and can accumulate in the soil, plants, and water [24]. In the past, methyl bromide was used as a multifunctional pre-plant broad-spectrum soil fumigant to manage, nematodes, insects, weeds and other soil-borne diseases in important crops [25]. However, its usage was suspended worldwide after 2005 due to its adverse effects on humans and the environment, which also included beneficial organisms [26]. In developing and underdeveloped nations, improper chemical usage such as overdosing, applying pesticides too frequently, applying them after their expiration date, and applicators lacking literacy are prevalent problems [27]. This has detrimental effects on the environment and crop production, and it has encouraged the use to find safe and cost-effective options. Researchers have placed emphasis on using natural ingredients either alone or alongside synthetic chemicals to protect crops from variety of these diseases [28–31].

Plant extracts or secondary metabolites constitute a major source of bioactive substances against pathogenic fungi. Many studies from the Phytomedicine Programme at the University of Pretoria have reported on the antifungal and antiparasitic activities of plant extracts and isolated compounds against dangerous fungi and parasites of plant, humans and animals [24,32–40]. Because plants contain different classes of secondary metabolites these compounds may have a novel mechanism of action that may minimize the development of resistance by the pathogens [22].

Ptaeroxylon obliquum (Thunb.) Radlk, is a medicinal plant widely used in South Africa to treat various diseases including fungal infections, parasite infections, and associated symptoms. Extracts, fractions, and isolated compounds had excellent activity against fungi attacking animals and humans and good activity against nematodes attacking production animals [38]. *Ptaeroxylon obliquum* tree usually commonly referred to as sneezewood, is indigenous to southern Africa, primarily to South Africa, Mozambique, and Zimbabwe [41,42]. Previous research studies on *P. obliquum* leaf extracts have led to the discovery of obliquumol, a chemical compound that was effective *in vitro* against *Candida albicans* and less toxic to a number of cell lines [43,44]. Other *in vitro* pharmacological activities of the obliquumol were also studied, and the information was patented [43]. Much work has been done on *P. obliquum* and led to isolation of many compounds mainly from chromones and terpenoids classes [44–49]. Several studies have reported the antimicrobial, antimycobacterial, antiparasitic, anti-inflammatory, antiproliferative, and antioxidant activity on extracts, fractions and isolated compounds of *P. obliquum* [50–54]. Recently, the plant has been reported to be used to treat microbial phytopathogens on crops. Subsistence farmers in the Eastern Cape Province, South Africa apply a decoction of *P. obliquum* leaves to their crops to prevent spinach and cabbage diseases [55]. In this study we investigated the activity of products from leaves of *P. obliquum* against nine phytopathogenic fungi and a nematode that cause production losses in plant crops.

2. Material and methods

2.1. Plant selection, collection, and storage

The *P. obliquum* plant leaves used in this section of the study were harvested from trees that were growing in three separate locations, i.e., location 1: Onderstepoort campus (OP), Faculty of Veterinary Science, University of Pretoria; location 2: Pretoria National Botanical Garden of the South African National Biodiversity Institute (SANBI) in Pretoria; and location 3: KwaZulu Natal Botanical gardens (KZN) in the summer of 2011. Collected leaves were allowed to dry at room temperature (±25 °C) in the shade, and then milled into a finer powder using a grinder. The powders were then kept until they were needed in tight and dark containers. Voucher specimens (PRU130509, PRU130510 and PRU130628) were prepared and kept at the HGWJ Schweickerdt Herbarium, University of Pretoria.

2.2. Preparations of the fractions and isolated compounds from the P. obliquum acetone leaf extracts

The dried *P. obliquum* acetone leaf extract (44.6 g) from SANBI was fractionated using solvent-solvent fractionation into five fractions based on polarity (chloroform: 26.5 g, hexane: 8.3 g, 30% water in methanol: 1.8 g, butanol: 1.2 g, and water: 1.3 g fractions). As previously reported, the pure obliquumol and a combination of lupeol and β-amyrin compounds were separated from the chloroform fraction using silica gel chromatography [51].

2.3. Antifungal assays

2.3.1. Fungal strains and inoculum quantification

The plant pathogenic fungi namely, *A. niger, A. parasiticus, C. gloeosporioides, F. oxysporum, P. digitatum, P. expansum, P. italicum, P. janthinellum,* and *R. solani* which are among the most important fungi of economic significance to plant were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria. All the plant fungi were maintained in Potato dextrose agar (Oxoid, Basingstoke, United Kingdom) at 4 °C. Prior to usage, the fungus inoculums were prepared in potatoes dextrose broth. Plant fungi cultures were sub-cultured in the broth at 35 °C for two to four hours before being used [35].

2.3.2. Minimum inhibitory concentration (MIC) evaluation of the acetone crude leaf extracts, fractions, and isolated compounds against the phytopathogenic fungi

A serial microdilution assay of Eloff [56] and slightly modified by Masoko et al. [57] was used to determine the MIC of the acetone crude leaf extracts, fractions and the isolated compounds using *p*-iodonitrotetrazolium violet (INT) reduction as an indicator of growth. The MICs were determined against each of the plant fungus selected for the study. The samples were tested in triplicate in every experiment and the experiments were repeated to validate the results.

The crude leaf extracts and fractions were dissolved in acetone to final concentrations of 10 mg/ml. Obliquumol was dissolved in 50% dimethyl sulfoxide (DMSO) and lupeol and β-amyrin mixture in acetone to final concentrations of 1 mg/ml. Exactly 100 μL of the samples were serially diluted with 50% water in 96-well microtitre plates and 100 μL of fungal culture was transferred to each well. Amphotericin B (1000 μg/ml) was used as the positive control while diluted 100% acetone and 50% DMSO were the negative controls. The microplate wells were filled with 40 μL of 0.2 mg/ml INT dissolved in water, which functioned as growth indicators. To reduce fungal contamination, the covered microplates were sealed in a plastic bag and incubated for 24 and 48 h at 35 °C and 100% relative humidity. The MICs were recorded as the lowest concentration of the samples that suppressed fungal growth. Biologically active organisms use the colourless tetrazolium salt as an electron acceptor, reducing it to a red formazan product [58]. After incubation with INT, the solution in the well either remains clear or exhibits a noticeable decrease in colour intensity, indicating that fungal growth is suppressed.

2.3.3. Total activity (TA)

To determine which acetone crude leaf extract or fractions have the highest potential for further development, not only the MIC value is important, but also the quantity extracted from the plant material. The TA, which represents the volume to which an active component in one gram (g) of plant material may be diluted and still prevent the growth of the test organism, is determined by dividing the quantity extracted in milligram (mg) from one gram (g) of plant material by the minimum inhibitory concentration (MIC) in mg/ml [59]. It can also be used to evaluate losses or increases of biological activity during the isolation of active compounds and the presence of synergism [60].

2.4. Root-knot nematode assays

2.4.1. Preparation of nematode inoculum

Meloidogyne incognita race 2 population was obtained from the University of Mpumalanga. The infested roots of susceptible tomato 'Rodade' plants with *M. incognita* were extracted with a solution of 1% Sodium hypochlorite (NaOCl) to release the eggs as well as the second-stage juveniles (J2s) from the plant tissues following the method of Hussey and Barker [61]. The suspension was added through a set of sieves with apertures sizes from 150, 63, 38 to 25 μm. Eggs contained in the 38 and 25 μm aperture sieve were combined and was washed with distilled water and incubated at 25 °C for 7 days. Following incubation period, the hatched J2 were washed with

distilled water and collected in the 25 μm aperture sieve.

2.4.2. Meloidogyne incognita (J2) motility assay

Second-stage juvenile motility assay was carried out following the method explained by Khosa et al. [62]. Briefly, total volume of 100 μL was attained by adding 10 μL of fractions (30% water in methanol, hexane and chloroform) and compounds (β-amyrin and lupeol mixture, and obliquumol) and 90 μL containing 100 ± 20 J2s. Concentrations ranged from 0.2 to 1 mg/ml for treatments. For proper mixing, plates were agitated and incubated at 22 °C in the dark. Using an inverted compound microscope at 400× magnification, the total number of motile and immotile J2 in each well was counted after the incubation periods of 24, 48, and 72 h. The measurements were done in triplicate and was repeated twice. Distilled water was used as the negative control for water extracts while 10% DMSO (used to solubilise the organic extracts) was used as the control for the organic solvents and compounds. Salicylic acid was used as a positive control (20 mg/ml). Inhibition motility percentage was calculated using Abbott's formula [63] as follows:

Immotility (%) = [(non-motile percentage in treatment – non motile percentage in untreated control)/(100-non motile percentage in untreated control)] × 100%

2.4.3. Data analysis

Percentage inhibitions for *M. incognita* J2s' motility was calculated using excel on Microsoft office. The data obtained was analyzed using analysis of variance (ANOVA) through Statistix 10.0 software. Shapiro-Wilk normality test was used to determine the normality of motility data before ANOVA. The p value of less than 0.05 led to rejection of normality hypothesis. Log transformation Log (x+10) of data was carried out on data which was not normally distributed. The mean separation was achieved using Fisher's least significance different at a 5% probability level.

3. Results and discussion

3.1. Antifungal activity of the acetone crude leaf extracts

Several factors influence the antimicrobial activity of acetone crude leaf extracts, including the phytochemical content, the extractant utilized, the solubility and miscibility of the active principles in the test medium, the vulnerability of the used microorganisms, and the evaluation method [64]. The extractant of choice was acetone because it was miscible with both polar and non-polar phytochemicals and had minimal toxicity at the concentration utilized in the assay against fungal diseases that affect plants and animals [57,65]. As illustrated in Fig. 1, the acetone crude leaf extracts of *P. obliquum* obtained from various locations exhibited wide minimal inhibitory concentrations against the nine plant fungal pathogens, ranging from 80 to 1250 μg/ml. The results of evaluating the acetone crude leaf extracts of *P. obliquum* collected from different geographical locations in same season indicated that at least in this case, environmental factors do not play a major role in antifungal activity as there was very little variation in the biological activity based on geographical location. The similar activity could also mean that the phytochemicals present in the plant material collected in summer months were related and had similar quantity. Ramadwa et al. [66] quantified obliquumol in the acetone extract of *P. obliquum* collected from various geographic locations using ultra-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (UPLC-qTof-MS). Their findings showed that there were no significant variations in the distribution of secondary metabolites in acetone extracts collected from SANBI and Onderstepoort. However, concentration of obliquumol was high in the acetone extract collected from SANBI.

According to literature antimicrobial activities of crude extracts is considered significant and promising for further investigation if the MIC is less than 100 μg/ml [60,67]. Based on the determination of the MICs of 714 acetone tree leaf extracts from 537 distinct tree species against four nosocomial pathogenic bacteria and two yeasts. Eloff [68] classified the antimicrobial activity as follows: Excellent

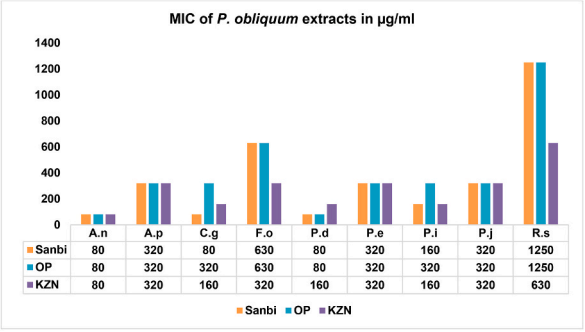

Fig. 1. Minimal inhibition concentrations (MIC in μg/ml) of different *P. obliquum* acetone leaf extracts collected from South African National Biodiversity Institute (SANBI) in Pretoria, Onderstepoort campus (OP), Faculty of Veterinary Science, University of Pretoria and KwaZulu Natal Botanical gardens (KZN) against nine phytopathogenic fungi. (**A.s**: *Aspergillus niger,* **A.p**: *Aspergillus parasiticus,* **C.g**: *Colletotrichum gloeosporioides,* **F.o**: *Fusarium oxysporum,* **P.d**: *Penicillium digitatum,* **P.e:** *Penicillium expansum,* **P.i**: *Penicillium italicum,* **P.j**: *Penicillium janthinellum,* **R.s**: *Rhizoctonia solani).* Acetone (negative control) did not show any inhibitory effects.

activity 0.021–0.04 mg/ml (top 3%), very good activity 0.041–0.08 mg/ml (top 9%), good activity 0.081–0.16 mg/ml (top 25%), average activity 0.161–0.32 mg/ml (top 50%), and weak activity >0.32 mg/ml. Compounds having MIC of 10 μg/ml are deemed pharmacologically significant [68].

The most susceptible plant fungal species to the acetone crude leaf extracts were *A. niger, C. gloeosporioides* and *P. digitatum* with MICs as low as 80 μg/ml which is considered very good activity. The acetone leaf extracts of *P. obliquum* collected from KwaZulu Natal and SANBI also had a noteworthy MIC value of 160 μg/ml (good activity) against *P. italicum.* The least susceptible plant fungal species to the acetone crude leaf extracts was *R. solani* with MICs ranging from 630 to 1250 μg/ml.

3.2. MIC values of the five fractions and isolated compounds

The MIC results of the five fractions and isolated compounds (a mixture of lupeol and β-amyrin and obliquumol) from the *P. obliquum* leaf extracts against important phytopathogenic fungi are presented in Table 1. The fractions obtained from non-polar solvents were more active than those obtained from polar solvents. The highest activity was shown mostly on the non-polar fractions of hexane and/or chloroform with the lowest activity in the water and butanol fractions. This clearly indicates that the antifungal compounds were mainly non-polar. There was substantial selectivity of the fractions to the different fungi even between different species of the same genus. The MIC value lower than 100 μg/ml was obtained on hexane fraction against *A. niger* and *P. digitatum. Colletotrichum gloeosporioides* also had an MIC of 160 μg/ml against hexane, chloroform and 30% water in methanol fractions. The two polar fractions, butanol and water had practically no activity against most of the phytopathogenic fungi with MIC values of 1250 μg/ml and greater >2500 μg/ml which was the highest concentration used in the study. Fractionation of the acetone crude leaf extract therefore might have concentrated the active antifungal metabolites in the non-polar fractions. *Rhizoctonia solani* was the most susceptible fungi against obliquumol and, lupeol and β-amyrin mixture with MIC values of 8 μg/ml and 16 μg/ml respectively. Previous studies have indicated that obliquumol and a combination of β-amyrin and lupeol had also been reported to have antifungal activity against both *Candida albicans* and *Cryptococcus neoformans* [39]. The chemical structures of obliquumol and, lupeol and β-amyrin mixture are presented in Fig. 2. The two compounds had MIC value of 32 μg/ml against *A. niger* and *P. digitatum.*

3.3. The total activity of the acetone crude leaf extracts and the fractions against the phytopathogenic fungi

The amount of 44.6 g of the acetone crude leaf extract from SANBI was obtained after bulk extraction of 500 g powdered leaf material, which was further fractionated using solvent-solvent fractionation into five fractions of different polarity. The highest masses were attained on non-polar fractions, chloroform (26.5 g) and hexane (8.3 g) while the lowest were from 30% water in methanol (1.8 g), H_2O (1.3 g) and butanol (1.2 g) [51]. With 21% in the non-polar (hexane) and 68% in the intermediate polarity (chloroform) range.

The total activity considers not only the MIC of the different fractions but also the mass of the different fractions. The biological activity of the crude extract is a result of the sum of the activities of the individual phytochemicals. However, on some occasions the pharmacological activity of the total crude extract may be lower or higher than the activities of the major constituents [69]. The total activity of the acetone crude leaf extracts and fractions was determined. It is possible to determine if activity increased or decreased during fractionation by calculating the total activity [60]. An increase in biological activity may indicate the removal of antagonistic phytochemicals and a decrease in activity may indicate disruption of synergistic effects or inactivation of biological active compounds [60].

The total activity results of the acetone crude leaf extract and the five fractions (water, butanol, 30% water in methanol, chloroform and hexane, fractions) on antifungal activity are presented in Table 2. The highest total activity was obtained in the acetone crude extract, chloroform and hexane fractions against all the used phytopathogenic fungi. Water fraction attained the lowest total activity. Notably, the chloroform fraction exhibited the highest total activity against all of the used fungi as compared to other fractions. Comparison of the total activity of the acetone crude leaf extract with the total activities of the fractions assist to determine if there was

Table 1
Minimum inhibitory concentration (MIC) expressed in μg/ml after 24 h incubation of the fractions and the isolated compounds against phytopathogenic fungi. The highest activity is indicated in bold font.

Fungi	MIC in μg/ml of the fractions and isolated compounds							
	HEX	$CHCl_3$	30% H_2O	BuOH	H_2O	Obliquumol	Lupeol & β-amyrin	Amphotericin B
A. n	**80**	**160**	**160**	630	2500	32	32	125
A. p	320	630	630	1250	>2500	250	>250	2.5
C. g	**160**	**160**	**160**	320	2500	32	63	0.16
F. o	630	320	320	1250	2500	63	63	2.5
P. d	**80**	320	320	1250	>2500	32	32	125
P. e	320	320	320	1250	>2500	32	125	<0.02
P. i	**160**	630	630	1250	>2500	125	>250	32
P. j	320	320	320	1250	>2500	63	63	<0.02
R. s	1250	630	630	1250	>2500	**8**	**16**	<0.02

NB: Negative controls did not show any inhibitory effects. HEX: Hexane, $CHCl_3$: Chloroform, H_2O: Water, BuOH: Butanol, 30% H_2O: 30% H_2O in methanol: **A.n**: *Aspergillus niger;* **A.p**: *Aspergillus parasiticus,* **C.g**: *Colletotrichum gloeosporioides,* **F.o**: *Fusarium oxysporum,* **P.d**: *Penicillium digitatum,* **P.e**: *Penicillium expansum,* **P.i**: *Penicillium italicum,* **P.j**: *Penicillium janthinellum*, **R.s**: *Rhizoctonia solani*.

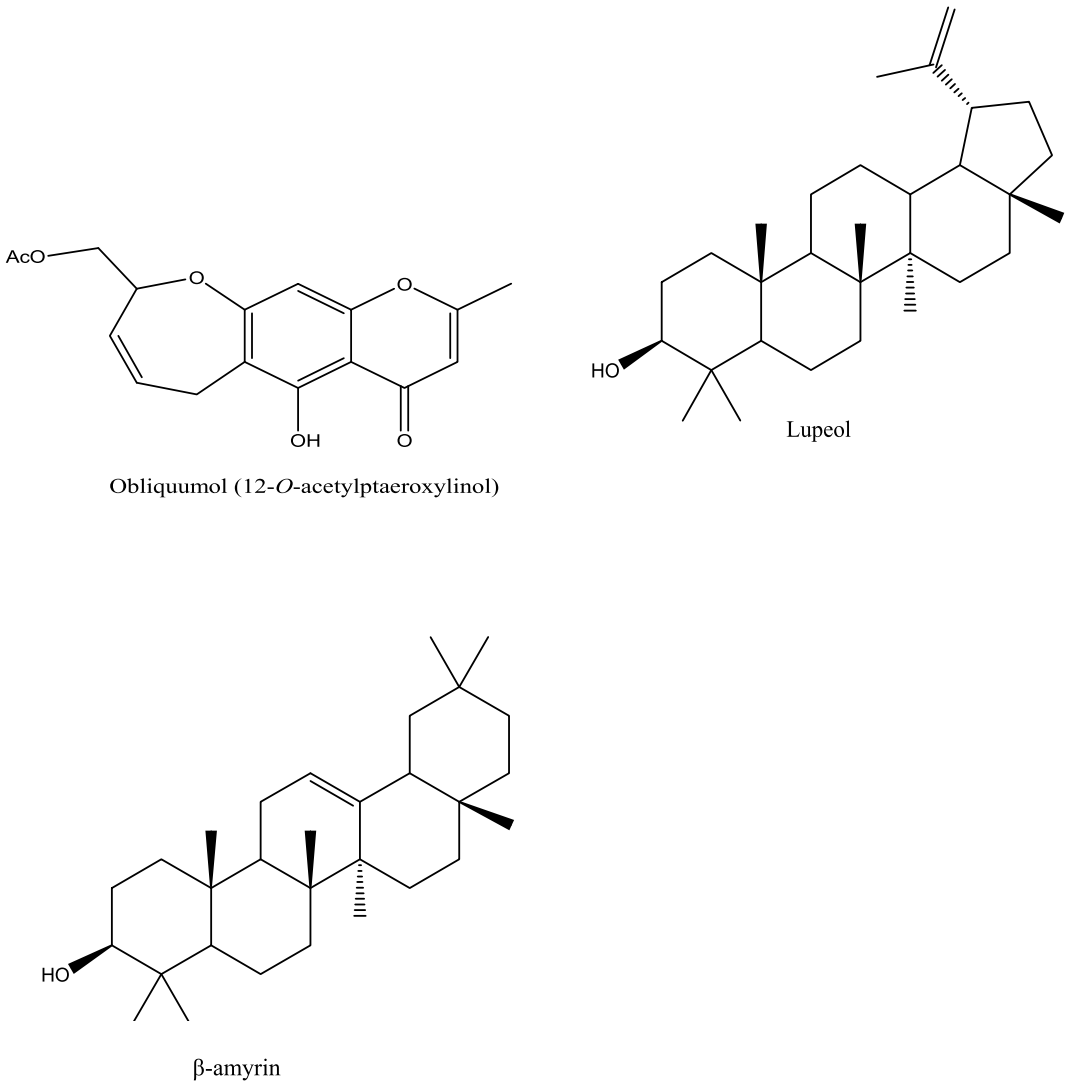

Fig. 2. Structures of obliquumol, and lupeol and β-amyrin mixture isolated from *P. obliquum* leaves [51].

Table 2
Total activity (ml) of the acetone crude leaf extract (ml/crude) from SANBI and five fractions (ml/fraction) of *P. obliquum* against phytopathogenic fungi.

Fungi	Total activity in ml of the crude extract (ml/crude) and fractions (ml/fraction)							
	Crude	HEX	$CHCl_3$	30% H_2O	BuOH	H_2O	SF	CE (%)
A.n	557,500	103,750	165,625	11,250	1904	520	283,049	51
A.p	139,375	25,937	42,063	2857	960	520	72,337	52
C.g	557,500	51,875	165,625	11,250	3750	520	233,020	42
F.o	70,793	13,174	82,812	5625	960	520	103,091	146
P.d	557,500	103,750	82,812	5625	960	520	193,667	35
P.e	139,375	25,937	82,812	5625	960	520	115,854	83
P.i	278,750	51,875	42,063	2857	960	520	98,275	35
P.j	139,375	25,937	82,812	5625	960	520	115,854	83
R.s	35,680	6640	42,063	2857	960	520	53,040	149

NB: HEX: Hexane, $CHCl_3$: Chloroform, BuOH: Butanol, H_2O: Water, **A.n**: *Aspergillus niger,* **A.p**: *Aspergillus parasiticus,* **C.g**: *Colletotrichum gloeosporioides,* **F.o**: *Fusarium oxysporum,* **P.d**: *Penicillium digitatum,* **P.e**: *Penicillium expansum,* **P.i**: *Penicillium italicum,* **P.j**: *Penicillium janthinellum,* **R.s**: *Rhizoctonia solani,* **SF**: Sum of all fractions, **CE**: % of Crude extract..

any decrease or increase in the activity. There was increased activity of fractions by 146% and 149% obtained against the two plant fungi, *F. oxysporum* and *R. solani* respectively. The highest loss of activity of the fractions of 65% was obtained against both *P. digitatum* and *P. italicum.* The reduction in activity may occur because of disruption of synergism among different phytochemicals present in the acetone crude leaf extracts, not present in all the fractions. The mass of the crude leaf extract after bulk extraction with acetone was 44.6 g while the total mass of the fractions was 39.1 g. This indicates that about 12% was not recovered after solvent-solvent fractionation possibly due to the formation of a pellicle between different phases in the solvent-solvent fractionation. It is also possible that some of the compounds that were present in acetone crude leaf extracts were volatile and had evaporated throughout the drying process after solvent-solvent fractionation. In some instances, decomposition of the active compounds may also contribute to the loss of activity. Some of these factors may have been involved in the loss of activity as an alternative explanation to synergism dissolution. Because the method for determining activity depends on two-fold serial extraction a doubling or halving of the activity means only one well of differentiation. Although the final figure is the result of nine analyses, the differences may not be significant.

When not only the MIC but also the mass in the different fractions were considered, highest activity was in the chloroform fraction against 8 of the 9 pathogens compared to when only MIC is considered (Table 1). It is again abundantly clear that the non-polar compounds were mostly responsible for the effectiveness of the antifungal activity. The magnitude of this effect is clear when the total activity against all the pathogens were added and calculated as a percentage of the total. The hexane fraction had 29%, the chloroform fraction 66%, the 30% water in methanol 4% and the butanol and water fractions combined 1% of the total activity for all fractions.

3.4. *Meloidogyne incognita (J2) motility*

The J2s hatch from the egg and represent the infective stage. Nematodes invade root elongation zone and eventually becoming sedentary [70]. Any voluntary movement throughout a 5-s period is considered as the motility of hatched juveniles [71] and they were counted after 24, 48 and 72 h. When nematodes moved from the head or tail, they were regarded as motile; if they did not move within five seconds of observation, they were considered non-motile. Only the fractions (30% water in methanol, hexane, and chloroform) and the isolated chemical compounds were subjected to the motility assay on hatching juveniles. Obliquumol was more active than the acetone leaf extract in our prior study on *Haemonchus contortus* (a sheep parasite) [39]. As a result, acetone crude leaf extract was not tested in the current study. The three most active fractions were chosen based on the antifungal activity. Data presented in the tables was for the combined experiment as there was no significant difference $p > 0.05$ between experiment 1 and 2, and in some results where p was less than 0.05, the high significant experiment was reported. After 24 h exposure (Table 3), only lupeol & β-amyrin mixture showed a significant ($p < 0.05$) inhibition at 0.8 and 1.0 mg/ml and lower concentrations exhibited dose-dependent effect. Further incubation on lupeol & β-amyrin mixture (Tables 4 and 5) continued to exhibit good inhibitions at high concentrations. However, poor inhibitions were observed at low concentrations of 0.2–0.6 mg/ml and this could imply temporary paralysis. Inhibitions increased with exposure times for fractions as well as obliquumol. Although the interaction between concentrations, fractions and obliquumol after 48 and 72 h was not statistically significant ($p \geq 0.05$). The ability of the lupeol & β-amyrin mixture to maintain similar activity at all 3-day exposure period in high concentrations could have a potential to assist in nematode reduction. The promising activity of this mixture is supported by the study conducted by Shai et al. [72] on *Caenorhabditis elegans* (model anthelmintic organism) where lupeol produced over 80 % inhibition of larval motility at concentration of 40 μg/ml. In the same study, lupeol was active at high concentrations against other animal parasites, *Haemonchus contortus* and *Trichostrongylus colubriformi*. Though in the present study lupeol was tested as a mixture with β-amyrin, it can be a potential nematocidal agent. The two studied compounds had excellent activity which were comparable to salicylic acid at 1 mg/ml (Table 6). Salicylic acid inhibited J2s in all hours, and the inhibitions were significantly high ($p < 0.05$) at the highest concentration used for testing (2.5–10 mg/ml). Obliquumol also had good activity after 48 and 72 h, though it was only active from 48 h paralysis on nematodes prolonged as second stage juveniles were not able to recuperate after 72 h. Obliquumol was effective against *H. contortus* ova and larvae, exhibiting an LC_{50} of 95 μg/ml against the larvae [39], which supports the potential use of the compound against nematodes. The good activity was observed in other fractions which could mean that longer exposure might results in permanent paralysis on juveniles and *P. obliquum* can assist in the delaying the feeding of J2s thus reproductive activity.

Table 3
Effect of *P. obliquum* fractions and compounds against motility of *M. incognita* J2 exposed at 24 h.

Concentration (mg/ml)	30% Water	Chloroform	Hexane	Lupeol & β-amyrin mixture	Obliquumol
0.2	20.21^{b}	32.31^{a}	1.01^{b}	1.47^{c}	25.48^{a}
0.4	17.69^{b}	34.81^{a}	1.12^{ab}	1.64^{b}	25.46^{a}
0.6	23.10^{b}	36.43^{a}	0.91^{b}	1.75^{b}	28.69^{a}
0.8	31.77^{a}	41.19^{a}	1.28^{a}	2.00^{a}	26.54^{a}
1.0	20.57^{b}	31.00^{a}	1.01^{b}	2.00^{a}	23.19^{a}
P value	0.018	0.3051	0.0587	0.0000	0.8371
F value	5.84	1.28	3.27	22.41	0.45
$LSD_{0.05}$	2.060	2.060	2.228	2.060	2.228

NB: Nematodes were motile in negative controls. Letters sharing the same alphabet in column are not significantly different at $p \leq 0.05$ according to Fisher's least significance. Transformed values were calculated using ([Log(x+1)].

Table 4
Effect of *P. obliquum* fractions and compounds against motility of *M. incognita* J2 exposed at 48 h.

Concentration (mg/ml)	30% Water	Chloroform	Hexane	Lupeol & β-amyrin mixture	Obliquumol
0.2	1.84^{ab}	55.89^{a}	1.27^{a}	1.18^{b}	1.93^{a}
0.4	1.88^{a}	59.34^{a}	1.29^{a}	1.28^{b}	1.93^{a}
0.6	1.73^{b}	62.17^{a}	1.25^{a}	1.12^{b}	1.81^{a}
0.8	1.84^{ab}	55.52^{a}	1.44^{a}	1.98^{a}	1.86^{a}
1.0	1.84^{ab}	64.31^{a}	1.31^{a}	1.99^{a}	1.85^{a}
P value	0.1470	0.7492	0.0617	0.0000	0.2880
F value	1.87	0.48	3.20	40.16	1.45
$LSD_{0.05}$	2.060	2.060	2.228	2.060	2.228

NB: Nematodes were motile in negative control. Letters sharing the same alphabet in column are not significantly different at $p \leq 0.05$ according to Fisher's least significance. Transformed values were calculated using ([Log(x+1)].

Table 5
Effect of *P. obliquum* fractions and compounds against motility of *M. incognita* J2 exposed at 72 h.

Concentration (mg/ml)	30% Water	Chloroform	Hexane	Lupeol & β-amyrin mixture	Obliquumol
0.2	1.91^{a}	62.51^{a}	10.04^{a}	1.40^{b}	1.92^{a}
0.4	1.90^{a}	92.95^{a}	10.04^{a}	1.41^{b}	1.89^{a}
0.6	1.81^{b}	69.23^{a}	2.15^{b}	1.52^{b}	1.85^{a}
0.8	1.90^{a}	62.31^{a}	7.89^{ab}	1.98^{a}	1.90^{a}
1.0	1.90^{a}	73.52^{a}	10.04^{a}	2.00^{a}	1.90^{a}
P value	0.0619	0.3491	0.5103	0.0000	0.7693
F value	2.58	1.17	0.88	29.80	0.45
$LSD_{0.05}$	2.060	2.060	2.228	2.060	2.228

NB: Nematodes were motile in negative control. Letters sharing the same alphabet in column are not significantly different at $p \leq 0.05$ according to Fisher's least significance. Transformed values were calculated using ([Log(x+1)].

Table 6
Effect of Salicylic acid against motility of *M. incognita* J2 exposed at 24, 48 and 72 h.

Concentration (mg/ml)	24 Hours	48 Hours	72 Hours
0.63	1.49^{c}	1.76^{c}	1.88^{c}
1.25	1.60^{d}	1.78^{c}	1.86^{c}
2.50	1.76^{b}	1.88^{b}	1.94^{b}
5.00	1.95^{a}	1.99^{a}	2.00^{a}
10.0	2.00^{a}	2.00^{a}	2.00^{a}
P value	0.0000	0.0000	0.0000
F value	43.22	51.07	58.52
$LSD_{0.05}$	2.060	2.060	2.060

NB: Nematodes were motile in negative control. Letters sharing the same alphabet in column are not significantly different at $p \leq 0.05$ according to Fisher's least significance.

4. Conclusions

The most susceptible plant fungal pathogen to the acetone crude leaf extracts were *A. niger, C. gloeosporioides* and *P. digitatum.* Chloroform, hexane and 30% water in methanol fractions as well the isolated obliquumol and, lupeol & β-amyrin mixture compounds from *P. obliquum* had a broad spectrum of antifungal activity against plant fungal pathogens used. Chloroform fraction had the highest total activity compared to other fractions. Combining the chloroform and hexane fractions should yield 95% of the activity. Forty-eight- and 72-h exposure might result in permanent paralysis on juveniles in tested fractions, obliquumol and lupeol & β-amyrin mixture with the latter at the highest concentration used which can assist in the delaying the feeding of J2s. These findings could be helpful in accelerating the development of an inexpensive plant-based nematocidal and/or fungicidal pesticides derived from *P. obliquum* leaves. A critically important aspect to investigate is the safety of the treatments to soil beneficial organisms. The selectivity ratio will determine whether higher concentration could be used. A shortcut to separate the non-polar fractions from the polar fractions at a much lower cost could be extracting the leaves with a chloroform or a chloroform water mixture and then using the non-polar part. The fractions and isolated compounds had great potential in the studied fungal pathogens and the plant parasitic nematode and may be developed as fungicides and nematocidal agents if the *in vitro* results can be confirmed under field conditions.

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Data availability statement

Data associated with the study has been deposited at the University of Pretoria and will be made available on request.

CRediT authorship contribution statement

Thanyani Emelton Ramadwa: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Fikile Nelly Makhubu:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Jacobus Nicolaas Eloff:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Thanyani Ramadwa reports financial support, equipment, drugs, or supplies, and travel were provided by National Research Foundation. Thanyani Ramadwa reports a relationship with National Research Foundation that includes: funding grants. Candice van Wyk, Francien Botha, Jacobus Nicolaas Eloff, Thanyani Ramadwa, has patent PLANT EXTRACTS OF PTAEROXYLON OBLIQUUM NAND COMPOUNDS HAVING ANTMICROBAL AND ANTHELMINTHC ACTIVITY licensed to University of Pretoria. None If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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