Quantitative UPLC-MS/MS analysis of obliquumol from *Ptaeroxylon obliquum* (Thunb.) Radlk. extracts and biological activities of its semi-synthesised derivative ptaeroxylinol

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Highlights

- UPLC-MS/MS method developed to quantify obliquumol concentration in *P. obliquum* acetone extracts.
- Ptaeroxylinol had MIC values as low as 8 µg/mL and 16 µg/mL against *C. albicans* ATCC 10,231 and *cryptococcus neoformans* respectively.
- Ptaeroxylinol had some antimycobacterial activity with MIC value of 62.5 µg/mL against both *M. bovis* BCG and *M. fortuitum*.
- Ptaeroxylinol had low toxicity against both vero and human liver (C3A) cells with IC_{50} = 85.7 and 126.51 µg/mL respectively.

Abstract

Quantification of compounds in plant extracts is rarely conducted to determine variation in concentrations of bioactive constituents. The aim of the study was to develop a method using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) to identify and quantify obliquumol (12-*O*-acetylptaeroxylinol) in *Ptaeroxylon obliquum* leaves collected from different localities in South Africa. Additionally, biological activity of a semi-synthesized derivative, ptaeroxylinol was investigated. Column chromatography was used to isolate obliquumol from *P. obliquum* leaves, and thereafter it was saponified to ptaeroxylinol. Ultra-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (UPLC-qTof-MS) was carried out on the different *P. obliquum* extracts to quantify obliquumol. A serial microdilution method was used to determine the minimum inhibitory concentration (MIC) against non-pathogenic mycobacteria and fungi. The cytotoxicity was determined using Vero monkey kidney and human liver (C3A) cells. A method was developed to isolate large quantities of obliquumol (0.14%) from dried *P. obliquum* leaves. The different *P. obliquum* acetone extracts had variable obliquumol concentrations between 0.1–38.5 µg/mg. Ptaeroxylinol had an MIC as low as 8 µg/mL and 16 µg/mL against *Candida albicans* ATCC 10,231 and *Cryptococcus neoformans,*

respectively. With an IC_{50} of 85.7 µg/mL for Vero cells and 126.51 µg/mL for C3A cells, respectively, ptaeroxylinol had low cytotoxicity to the cells tested. A UPLC-MS/MS method was developed to quantify obliquumol content in the *P. obliquum* acetone extracts. Ptaeroxylinol had good activity against *C. albicans* (MIC = 8 µg/mL) and it appears that the cleavage of the acetoxy to alcohol group played a role in the antimicrobial activity.

Keywords: Antifungal; Antimycobacterial; Cytotoxicity; *C. neoformans; C. albicans*

Abbreviations

HPLC-High-performance liquid chromatography; GC-Gas Chromatography; MS-Mass spectrometry; LC-Liquid Chromatography; UPLC-Ultra-performance liquid chromatography; UPLC–MS/MS-Ultra-performance liquid chromatography–tandem mass spectrometry; OP-Onderstepoort campus; SANBI-South African National Botanical Gardens; TLC-Dwarf *P. obliquum* tree from University of Pretoria,Hatfield campus: Dwarf,Thin layer chromatography; DCM-Dichloromethane; *n*-H: EtOAc-*n*-hexane/ethyl acetate; CEF-Chloroform/ethyl acetate/ formic acid; EtOAc-Ethyl acetate; NMR-Nuclear Magnetic Resonance; NaOH-Sodium hydroxide; MeOH-Methanol; HCl-Hydrochloric acid; Na_2SO_4-Sodium sulphate; ESI-Electrospray ionisation; MIC-Minimum inhibitory concentration; INT *p*-iodonitrotetrazolium violet; OADC-Oleic albumin dextrose catalase; C3A-Human liver; DMSO-Dimethyl sulfoxide; IC_{50}-Lethal concentration; SI-Selectivity index; MeCN-Acetonitrile

1. Introduction

There is a great deal of interest in probing the structural features responsible for the pharmacological effects of many biologically active compounds isolated from medicinal plants to further optimize their bioactivity profile while reducing their cytotoxic effects, enhancing their solubility and enhancing the structure-activity relationship (Kwon et al., 2009). Medicinal plants have inspired many developments in medicinal chemistry, leading to advances in synthetic methodologies and to the possibility of making analogues of the original compounds with improved pharmacological and pharmaceutical properties (Kopp and Marahiel, 2007; Newman, 2008). Thousands of compounds are isolated from medicinal plants, but very few of them have been tested *in vivo* due to low yield of the isolated compounds from plants, poor or moderate biological activities or exorbitant costs to conduct animal experiments. Chemical synthesis or semi-synthesis (chemical modification of a compound isolated from natural products) of bioactive compounds from plants may be possible and commercially feasible, especially for bioactive compounds with relatively simple chemical structures which can be modified to potentially enhance their biological activities (Wink et al., 2005).

It is widely accepted that the biological activity of medicinal plants is related to the chemical composition of individual compounds, which is dependant on genetic and a variety of other factors, such as geographic variation, harvest time, environmental and agronomic conditions, the botanical parts of plants, and extraction methods (Fan et al., 2011; Xi et al., 2014; Bessada et al., 2016; Liu et al., 2016). Compared with the long history of traditional use of *P. obliquum* (Thunb.) Radlk. in South Africa and in other countries (Watt and Breyer-Brandwijk, 1962; Pujol, 1990; Hutchings et al., 1996), phytochemical analysis and development of quantification methods for secondary metabolites from this medicinal plant are rather limited. Development of quantification methods for secondary metabolites from

different populations of medicinal plants is rarely done to determine variation in concentrations of bioactive constituents responsible for biological activity. Therefore, there is a lack of systematic investigation on the phytochemical profiling of *P. obliquum* to quantify the concentration of the bioactive and potentially commercially useful compounds from medicinal plants.

Over the years, several techniques have been explored for analysing and characterising secondary metabolites in plants. These include chromatographic and spectroscopic techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and many more. The hybridisation of HPLC with tandem mass spectrometry (MS/MS) is an effective tool for identifying compounds at low concentration levels in the analysis of complex matrices (Cooper et al., 2007; Ortega 2017). There have been improvements in liquid chromatography (LC) techniques with the development of ultra-performance liquid chromatography (UPLC), performing separations using columns packed with smaller particles (1.7 mm) and/or at higher pressure. This has led to a shorter analysis time, higher peak efficiency and improved resolution (Cooper et al., 2007). As a result, the UPLC–MS/MS technique is now most preferred technique for the analysis of biological samples due to its high sensitivity, selectivity and improved separation properties with minimum consumption of solvents (Iqbal et al., 2019). The aim of the study was to develop a method using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) to identify and quantify bioactive obliquumol in populations of *P. obliquum* leaves collected from different localities in South Africa. Additionally, the biological activities of the compounds ptaeroxylinol, semi-synthesised from obliquumol, were investigated.

Previous studies on *P. obliquum* leaf extracts resulted in the discovery of a compound, obliquumol, which was more effective against *Candida albicans* than amphotericin B, the gold standard medication and was less toxic to several cell lines (Van Wyk et al., 2018; Ramadwa et al., 2021). This information was patented, and other *in vitro* biological activities of the obliquumol were also investigated (Van Wyk et al., 2017; Ramadwa et al., 2022). For additional work on obliquumol such as determining efficacy and safety in animal studies, larger quantities of obliquumol were required. For this purpose, there was a need to develop a method to isolate larger quantities of obliquumol based on experience gained.

2. Materials and methods

2.1. Plant collection and extraction procedure

2.1.1. Plant selection, collection, and storage

The plant leaves of *P. obliquum* used in this part of the study were collected from trees growing on the Onderstepoort campus (OP), Faculty of Veterinary Science, University of Pretoria, South African National Botanical Gardens (SANBI) in Pretoria and a dwarf *P. obliquum* tree from University of Pretoria, Hatfield campus (Dwarf) in the summer. The *P. obliquum* dwarf tree was included to determine if secondary metabolites concentrations are affected by developmental age of the plant when leaves harvested. The leaves were collected in open woven orange bags, dried at room temperature in the shade and powdered using a mill. The powders were then stored in closed containers in the dark until needed. Voucher specimens (PRU130509, PRU130510, PRU130628) were prepared and kept at the HGWJ Schweickerdt Herbarium of the University of Pretoria.

2.1.2. Extraction procedure for preliminary bioassay

Powdered leaves (1 g) were extracted with 10 mL of technical graded acetone (Merck) (Eloff JN, 1998a). The extracts were shaken on a Labotec model 20.2 shaker for 30–60 min, and centrifuged at 3500 x g for 5 min. Extraction and centrifugation was repeated 3 times on the pellet. The three supernatants were then combined and dried in pre-weighed vials under a stream of cold air.

2.2. Thin layer chromatography (TLC)

2.2.1. Phytochemical analysis

All the plant extracts were reconstituted in acetone to a concentration of 10 mg/mL. Acetone has been demonstrated to be non-toxic to fungi at the final dose to which the fungi were exposed (Eloff et al., 2007). Samples of the plant extracts (10 µL) were loaded on thin-layer chromatography TLC plates (Merck silica gel 60 F_{254}) in 1 cm thin bands using a micropipette. Mobile phases of varying polarities were used to develop the plates, namely *n*-hexane/ethyl acetate (*n*-H/EtOAc) [6:4] and chloroform/ethyl acetate/ formic acid (CEF) [5:4:1] (Kotze and Eloff, 2002). Development of the chromatograms took place in closed tanks saturated with the mobile phase. The developed plates were then visualised under ultraviolet light (254 and 360 nm, Camac universal UV lamp TL-600) and sprayed with vanillin-sulphuric acid (0.1 g vanillin, 28 mL methanol (MeOH) and 1 mL sulphuric acid) and heated at 105 ºC to optimal colour development of the separated compounds (Kotze and Eloff, 2002).

2.3. Isolation of large quantities of obliquumol from *P. obliquum* leaf extracts

For additional work on obliquumol such as determining efficacy and safety in animal studies, synthesising derivatives and determining activities on other pathogens and other biological activities, larger quantities of obliquumol were required. Bioassay-guided fractionation has been used for many years to isolate bioactive compounds from medicinal plants. The approach involves repetitive fractionation of extracts and determining the biological activity of all the fractions (Brusotti et al., 2014). The method provides a rational means to isolate bioactive compounds from a complex mixture, but it is time consuming and labour intensive (Bero et al., 2011; Michel et al., 2011; Manvar et al., 2012). Previous studies on *P. obliquum* leaves led to isolation of obliquumol in small quantities to conduct further *in vivo* studies despite excellent *in vitro* biological activity (Ramadwa et al., 2019). Solvent-solvent fractionation was used to fractionate the acetone extract based on polarity of the compounds (Suffness and Douros, 1979). A total of five fractions were separated and isolation of obliquumol and other bioactive compounds was carried out on the chloroform fraction using silica gel column chromatography. Efforts were therefore made to simplify the isolation of obliquumol from *P. obliquum* leaves in larger quantities based on the experience gained from the previous studies (Ramadwa et al., 2019). Slight modifications were then made to the solvent-solvent fractionation method used to isolate larger quantities of obliquumol.

The dried acetone extract (48.6 g, obtained from 500 g of dried ground leaves) was dissolved in 500 mL of chloroform: water (1:1) in a separatory funnel to give the chloroform and water fractions. In the original method the chloroform fraction was dried in a vacuum rotary evaporator before fractionation. To isolate a large quantity of obliquumol the chloroform fraction (45.5 g) was not further fractionated by liquid-liquid fractionation. It was dried and

dissolved in a small volume of acetone and 15 g of silica gel was added. The acetone was allowed to evaporate at room temperature.

A homogenous slurry of 1000 g of silica gel in *n*-hexane was transferred into a glass column 40 cm high and 4.5 cm in diameter, and the excess *n*-hexane was allowed to pass through the column. The dried chloroform and silica mixture was layered on the column bed, covered with a 4.5 cm diameter filter paper circle and washed cotton wool. The compounds were separated using gradient elution, starting with about 3.5 L of 90% *n*-hexane, 10% EtOAc, followed by 3.5 L increasing in increments of 5% EtOAc to 70% *n*-hexane. The separation was followed by TLC of fractions, which was made easier by the blue colour of obliquumol after spraying with vanillin-sulphuric acid. Obliquumol started eluting out after about 50 honey jars and was collected into different honey jars. The column was then eluted isocratically with 70% hexane in ethyl acetate until obliquumol was exhaustibly collected. Obliquumol started crystallising as white crystals in the honey jars. The crystals were then washed with acetone to remove impurities and yielded white crystals. About 500 mg of obliquumol was isolated using this technique from 48.6 g of acetone crude extract. It may be possible to streamline the process even more by extracting the leaves with chloroform and removing water soluble molecules by solvent-solvent fractionation. The purity of the isolated obliquumol was confirmed by TLC. The structure of obliquumol was confirmed by NMR and LC-MS.

2.4. Semi-synthesis of ptaeroxylinol from obliquumol

Ptaeroxylinol, a derivative of obliquumol, has been previously isolated from *P. obliquum* (Dean et al., al.,1967). Obliquumol had excellent antifungal activity and was not toxic to several cell lines (Ramadwa et al., 2021). Consequently, ptaeroxylinol was prepared from the isolated obliquumol to determine its antimycobacterial, antifungal and cytotoxicity which has not been investigated yet. Furthermore, the semi-synthesis of ptaeroxylinol will assist in determining the structure-activity relationship with obliquumol.

2.4.1. Procedure for ptaeroxylinol synthesis

Aqueous sodium hydroxide (NaOH) (5 M, 1 mL, 5.00 mmol) was added to a solution of obliquumol (50.0 mg, 0.158 mmol) in tetrahydrofuran (THF) (5 mL) at 0 °C. The cooling bath was removed and the mixture was stirred at room temperature for 4 h. Then, THF was removed under vacuum and the residue was diluted with water (5 mL), acidified with 1.0 M hydrochloric acid (HCl) and extracted with EtOAc (3 × 5 mL). The resulting organic layers were combined, washed with water, brine (saturated solution of sodium chloride), dried over sodium sulphate (Na_2SO_4) and removed in a rotary evaporator to afford the expected product (35 mg, 81%). The structure of the product was confirmed by NMR and LC-MS analysis.

2.5. Quantification of secondary metabolites from different populations of *P. obliquum* leaves collected from different localities of South Africa using UPLC-MS analysis

2.5.1. Sample and analytical standard preparation

The crude *P. obliquum* leaf extracts from different geographical locations and the reference compound obliquumol (5 mg) were dissolved in 3 mL acetonitrile (MeCN): water (1:1), sonicated in an ultrasonic bath for 5 min and syringe filtered using 0.22 µm PVDF filters. The crude extracts and obliquumol standard were further diluted in volumetric flasks to give

a total volume of 5 mL. Dilutions of high (50.0 µg/mL) and low (0.5 µg/mL) concentration were prepared from the obliquumol stock solution in order to ensure that the concentrations of obliquumol in the samples fell within the respective concentration response figures of the standard.

2.5.2. UPLC-MS conditions

The samples were analysed by ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-qTof-MS) (Waters, Milford, MA, USA). The system was driven by MassLynx software. The UPLC analysis was performed using an Acquity HSS T3 column (100 mm x 2.1 mm i.d., 1.8 µm particle size) with a binary mobile phase at a flow rate of 0.4 mL/min. The eluent was water with 0.1% formic acid (A) and MeCN with 0.1% formic acid (B). The column and sample temperatures were maintained at 40 °C and 4 °C, respectively. An injection volume of 5 µL was used. The gradient elution was carried out as follows: 0.0–0.1 min, 15% B; 0.1–0.5 min, 15–35% B; 0.5–3.0 min, 35% B; 3.0–8.0 min, 35–80% B; 8.0–8.5 min, 80–95% B, 8.5–11.0 min, 95% B; 11–12 min, 95–15% B; 12–14 min, 15% B. The peaks eluting from the UPLC system were analysed by qTof-MS using an electrospray ionisation (ESI) in positive mode. MS data acquisition was performed in resolution mode from 50 to 1200 Da. The MS working conditions were as follows: capillary voltage, 2.6 kV; source temperature, 120 °C; sampling cone voltage 30 V; extraction cone voltage 4.0 V; desolvation temperature, 350 °C; cone gas flow rate, 10 L/h and desolvation gas flow rate, 600 L/h. Data acquisition and processing was carried out by MassLynx v4.1 software.

2.5.3. Quality parameters

2.5.3.1. Linearity

The quality parameters of the UPLC-MS/MS chromatographic method were studied by using a serial dilution of obliquumol. The parameters that were considered to determine the calibration curve were the slope, intercept and correlation coefficient of linear regression equation. The calibration curves (based on the UPLC-MS integrated peak area) were calculated using eight points at different concentrations from 0.5 to 50 µg/mL and injections were made in triplicate for each concentration. A linear response curve with correlation coefficient (r^2) of 0.995 was obtained.

2.5.3.2. Recovery

Recovery experiments were performed using OP crude extracts. OP crude extracts (3 mg) were spiked with three different concentration levels of obliquumol standard (5, 10 and 20 µg/mL) in MeCN:Water (1:1). The spiked and non-spiked samples were extracted by ultrasonication followed by filtration through 0.22 µm PVDF filters. The total volume was adjusted to 5 mL with MeCN:Water (1:1). The percentage recovery was calculated by subtracting the amount of obliquumol that was found in non-spiked samples (control) from the amount of obliquumol found in spiked samples.

2.5.3.3. Intraday and interday precision

A fixed concentration of obliquumol sample was analysed repeatedly on the same day (n = 5) and on five consecutive days (n = 5), followed by calculations of the relative standard deviation (RSD) for each set of measurements.

2.6. Biological activities of the semi-synthesised derivative (ptaeroxylinol)

2.6.1. Antifungal activity

The antifungal activity of ptaeroxylinol was determined against the three clinical isolates, namely *Aspergillus fumigatus, Candida albicans* and *Crytococcus neoformans,* and *Candida albicans* ATCC 10,231. The fungal strains were obtained from the Department of Tropical Diseases, Faculty of Veterinary Science at the University of Pretoria. *C. albicans* was isolated from a Gouldian finch, *C. neoformans* from a cheetah and *A. fumigatus* from a chicken, all of which suffered from systematic mycosis. All the animal and plant fungi were maintained in Potato Dextrose (PD) agar (Oxoid, Basingstoke, United Kingdom) at 4 ºC under anaerobic conditions. The fungal inoculums were prepared in PD broth before use.

A serial microdilution assay (Eloff JN, 1998b) with slight modification (Masoko et al., 2005) was used to determine the minimum inhibitory concentration (MIC) value of ptaeroxylinol using *p*-iodonitrotetrazolium violet reduction as an indicator. The sample was tested in triplicate in the assay. Ptaeroxylinol was dissolved in acetone to final concentrations of 1 mg/mL. Exactly 100 µL of the compound was serially diluted with 50% water in 96-well microtitre plates and 100 mL of fungal culture was added to each well. Amphotericin B was used as the positive control while 100% acetone was the negative control. As an indicator of growth, 40 µL of 0.2 mg/mL of *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells. The covered microplates were incubated for 24 and 48 h at 35 ºC at 100% relative humidity after sealing in a plastic bag to minimize fungal contamination in the laboratory. The MIC was recorded as the lowest concentration of the compound that inhibited antifungal growth. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (Eloff JN, 1998b). Where fungal growth is inhibited, the solution in the well remains clear or shows a marked reduction in intensity of colour after incubation with INT.

2.6.2. Antimycobacterial activity

Four non-pathogenic mycobacterial species (*Mycobacterium smegmatis* ATCC 1441, *Mycobacterium bovis* BCG P1172, *Mycobacterium aurum* NCTC 10,437 and *Mycobacterium fortuitum* ATCC 6841) were used in the study to determine the antimycobacterial activity of ptaeroxylinol. The mycobacteria were grown at 37 ºC in Middlebrook 7H9 broth supplemented with 10% oleic albumin dextrose catalase (OADC) and maintained on Middlebrook 7H10 agar supplemented with glycerol or Tween 20 at 4 ºC until needed.

A serial microplate dilution method was used to determine the minimal inhibitory concentrations (MIC) (Eloff, 1998a). Two-fold serial dilutions were dispensed into 96-well microplates as follows; distilled water (100 µL) was placed in each well using a micropipette and 100 µL of a 1 mg/mL ptaeroxylinol was placed in each of the first well of the relevant series of dilution, and thereby diluting the extracts to 50%. One hundred microlitres was removed from it and placed into the next well. The process was repeated all the way to the

bottom of the plates with 100 µL from the last rows being discarded to ensure that all wells contained the same volume of extract. Exactly 100 µL of overnight cultured mycobacteria was added in each well. To measure growth *p*-iodonitrotetrazolium violet (INT) (0.2 mg/mL in water) was added (40 µL) in each well. The plates were incubated for 18 h at 37 °C in a humidified atmosphere. The MIC was recorded as the lowest concentration of obliquumol that inhibits growth based on colour intensity. The reduction of INT to respective red formazan indicated of mycobacterial growth. The gentamicin and ciprofloxacin were used as positive controls and the solvent dilutions as negative control.

2.6.3. Cytotoxicity determination

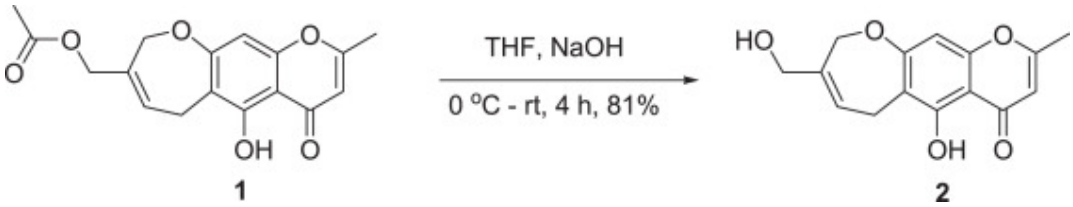
The cytotoxicity of the ptaeroxylinol was determined using Vero monkey kidney and human liver (C3A) cells lines using the MTT (3-[4.5-*dimethylthiazol*-2-*yl*]−2.5 diphenyltetrazolium bromide) reduction assay as previously described (Mosmann, 1983) with slight modifications (McGaw et al., 2007). The semi-synthesised ptaeroxylinol was prepared to a final concentration of 2 mg/mL in dimethyl sulfoxide (DMSO). Cells were seeded at a density of 1×10^5 cells/mL (100 mL) in 96-well microtitre plates and incubated at 37 ºC and 5% CO_2 in a humidified environment. After 24 h incubation, test samples (100 µL) at varying final concentrations were added to the wells containing cells. Doxorubicin was used as a positive control. Suitable blank controls with equivalent concentrations of DMSO were also included and the plates were further incubated for 48 h in a CO_2 incubator. Thereafter, the medium in each well was aspirated from the cells, which were then washed with PBS, and finally fresh medium (200 µL) was added to each well. Then, 30 mL of MTT (5 mg/mL in PBS) was added to each well and the plates were incubated at 37 ºC for 4 h. The medium was aspirated from the wells and DMSO was added to solubilise the formed formazan crystals. The lack of purple formazan colour or clear appearance in the wells indicated cytotoxicity of the tested samples on the cells. The absorbence was measured on a BioTek Synergy microplate reader at 570 nm. The percentage of cell growth inhibition was calculated based on a comparison with untreated cells and linear regression equation was used to calculate the cytotoxicity of the ptaeroxylinol sample which was expressed as 50% lethal concentration (IC_{50}) inhibiting cell growth by 50%. The selectivity index values was calculated by dividing cytotoxicity IC_{50} values by the MIC values in the same units ($SI=IC_{50}/MIC$).

3. Results and discussion

3.1. Structure elucidation of the synthesised derivative

Only a small quantity of obliquumol was isolated in the previous studies (Ramadwa et al., 2019). To evaluate the biological activity of bioactive compounds against different pathogens and for *in vivo* studies, a larger quantity is required. As result, we decided to isolate larger quantities of obliquumol from leaves of the tree growing at SANBI in Pretoria. A method was developed to isolate large quantities of obliquumol for determining activities of other pathogens and other biological studies. The compound could be isolated with a yield of about 0.14% from dried leaves, which was more than double the amount of obliquumol isolated from our previous studies (Ramadwa et al., 2019). The increased yield of obliquumol may be attributed to the slight modification of the liquid-liquid fractionation method used in the current study. We also succeeded in synthesising ptaeroxylinol (35 mg) from 50 mg of isolated obliquumol as shown in Scheme 1. Ptaeroxylinol was obtained as cream white crystals. Its structure was confirmed on the basis of NMR spectroscopic and mass spectrometry data. The ^1H NMR spectrum displayed signals at δ_H 13.08 (1 H, s), 6.53 (1 H,

s), 6.05 (1 H, s), 6.00 (1 H, tt, J = 5.7 and 1.1 Hz), 4.75 (2 H, d, J = 1.1 Hz), 4.04 (2 H, s), 3.58 (2 H, d, J = 5.7 Hz), 2.36 (3H, s). The ^{13}C NMR spectrum showed signals at δ_c 182.8, 167.2, 164.6, 158.2, 155.9, 138.0, 125.4, 115.9, 108.7, 106.7, 99.3, 71.1, 65.7, 21.1, and 20.5. The HRMS-ESI gave a protonated molecular ion at *m/z* 275.0918 $[M + H]^+$; calculated for $C_{15}H_{15}O_5$ 275.0919. The ^{1}H NMR, ^{13}C NMR, and HRMS data matched with the one of ptaeroxylinol which has been previously isolated from the plant (Dean et al., 1967; Malefo et al., 2020). Several other compounds have been previously isolated from *P. obliquum* including an acid saptaeroxylon, volatile oil, pyrogallol type tannins, resins fat and the glycoside, ptaeroxylon, methylalloptaeroxylin, meroterpenoid, ptaerobliquol and an alkaloid with cardiac depressant properties (Murray and Ballantyne, 1970; Pachler and Roux, 1967; Dean and Robinson, 1971; Langenhoven et al., 1988; Agostinho et al., 2013).



Scheme 1. Schematic representation of the synthesis reaction of obliquumol (**1**) to ptaeroxylinol (**2**).

3.2. Solubility test of obliquumol in different solvents suited for UPLC-MS

Previous studies on obliquumol indicated that the compound dissolves in chloroform and DMSO (Ramadwa et al., 2019). The compound was then dissolved in number of solvents used in UPLC-MS and it was then observed that MeCN and water mixtures can be used for dissolving obliquumol for LC-MS analysis as shown in Fig. 1. The other solvents that dissolved the compound were MeCN/Water, MeCN/dichloromethane (DCM), and 100% MeCN.

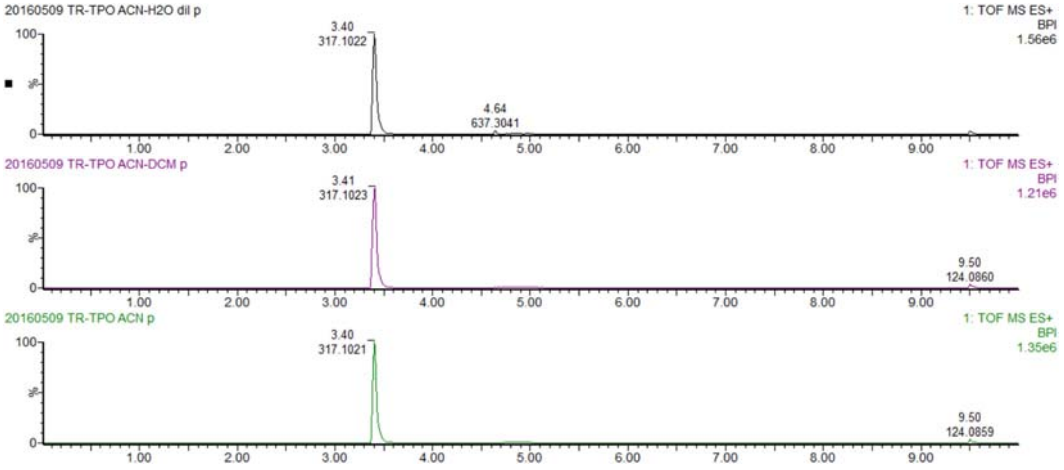


Fig. 1. UPLC-MS chromatograms of obliquumol solubilised in different solvents in ESI positive ionisation mode. MeCN (bottom), MeCN:DCM (middle), MeCN:Water (top).

3.3. MS/MS conditions

MS spectra were studied in both negative and positive ion modes. Compared with the negative ion mode, obliquumol had higher sensitivity and intensity in the positive ion mode (Fig. 2). As a result, the positive MS ion mode was selected for method development. The MS showed a protonated molecular ion peak at *m/z* 317.1021 $[M + H]^+$ (calculated for $C_{17}H_{17}O_6$, 317.1025 $[M + H]^+$), thereby confirming the molecular formula of obliquumol to be $C_{17}H_{16}O_6$. Several fragment ions were observed at *m/z* 257, 229, 214, 177, 155, and 127.

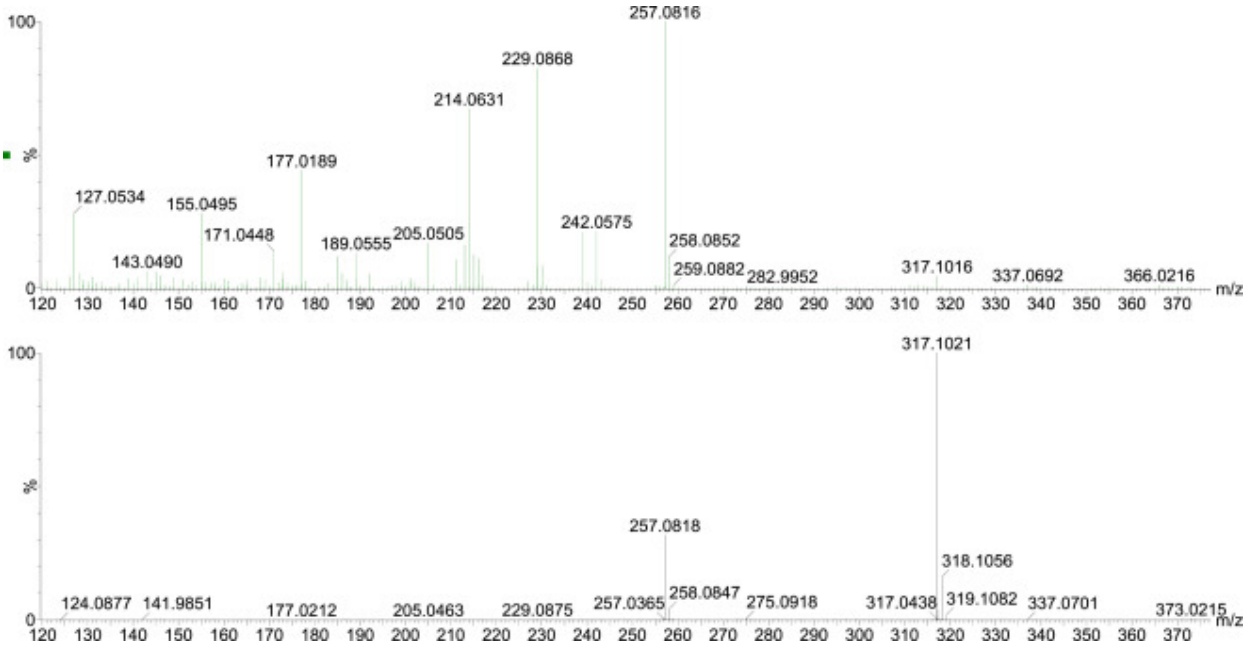


Fig. 2. MS (bottom) and MSMS (top) spectra of obliquumol.

The phytochemical composition of plants may be affected by several environmental factors including altitude, soil type, geographical location and change of season (Nchabeleng et al., 2012). It has however been shown that water and temperature stress did not affect the biological activity extracts of some South African medicinal plants (Netshiluvhi and Eloff, 2016; Netshiluvhi, 2019).

The changing temperatures and wind patterns associated with climate change may cause noticeable effects on the life cycle, distribution and phytochemical composition of the world's vegetation, including medicinal and aromatic plants (Dweck, 2009). In this study, there were no major differences observed between *P. obliquum* acetone leaf extracts of Onderstepoort (C2) and SANBI (C202) chromatograms, beside the compounds eluting at 6.37 and 6.60 min with *m/z* of 317.102 (equivalent to that of obliquumol) as shown in Fig. 3. The two chromatograms showed that both samples contained the same classes of compounds that ranged from polar to non-polar compounds. However, the Dwarf (C303) *P. obliquum* leaf extracts collected from University of Pretoria Hatfield campus had mostly non-polar compounds eluting at higher retention times and the intensity of the compound of interest obliquumol was greatly suppressed. These secondary metabolites are synthesised by various metabolic pathways for a multitude of reasons including protection against microorganisms, resistance to insect attacks and even as protection against ultraviolet and drought conditions (Aryal, 2015). The composition of secondary metabolites may also vary depending on age of

the plant. It is possible that certain phytochemicals like obliquumol is only synthesised in higher concentration when *P. obliquum* tree has fully grown which may explain its suppression and of other secondary metabolites from the Dwarf tree collected from University of Pretoria, Hatfield campus. It is also possible that this dwarf tree had a different genetic composition. Number of other factors such as climate, altitude, rainfall and other conditions may affect growth of plants which in turn affect the number and/or concentration of phytochemicals present in a particular species even when it is produced in the same country, region or season.

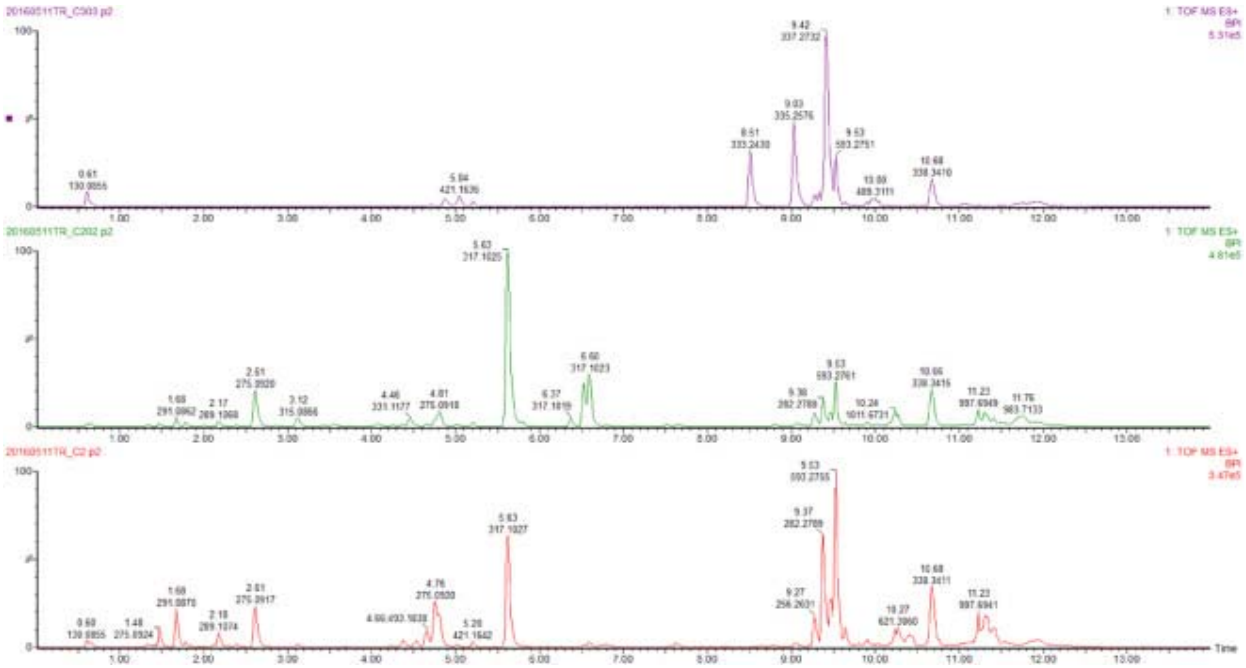


Fig. 3. UPLC-MS stacked chromatograms of *P. obliquum* leaf extracts collected from different localities Dwarf (C303, top), SANBI (C202, middle) and Onderstepoort (C2, bottom). Obliquumol eluted at 5.63 with *m/z* of 317.102.

3.5. Quality parameters

The calibration curve was plotted using 8 points with concentration ranging from 0.5 µg/mL to 50 µg/mL and provided excellent linearity with a correlation coefficient of $r^2 = 0.9954$. The linear regression equation obtained for obliquumol was $y = 361.925x + (-21.3068)$. The intraday and interday precision of the method was calculated from multiple measurements of a fixed concentration of obliquumol sample on the same day ($n = 5$) and on five consecutive days ($n = 5$). The intraday RSD was determined to be 2.25% and interday RSD 2.89%. The recovery rate was determined by spiking three different concentrations of obliquumol sample to the fixed amount of OP extract in replicates. The overall recovery rate was determined to be 98.1%. The *P. obliquum* leaf extracts collected from different localities were variable in obliquumol concentrations ranging: 0.15 µg/mg (Dwarf), 14.8 µg/mg (OP) and 38.5 µg/mg (SANBI). Therefore, the SANBI acetone leave extracts were ideal for the isolation of larger quantities of obliquumol.

3.6. Biological activities of semi-synthesised derivative ptaeroxylinol

The minimal inhibitory concentration results of ptaeroxylinol against fungal and mycobacterial test organisms, cytotoxicity and the selectivity index (SI) are presented in Table 1. The MIC of ptaeroxylinol ranged between 62.5–8 µg/mL against the opportunistic fungal pathogens and 250–62.5 µg/mL against non-pathogenic mycobacterial strains. Ptaeroxylinol had good antifungal activity against the *C. albicans* ATCC 10,231 and *C. neoformans* with MIC values of 8 µg/mL and 16 µg/mL, respectively. The derivative had poor antimycobacterial activities with the lowest MIC value of 62.5 µg/mL against both *M. bovis* BCG and *M. fortuitum.* When comparing the antimicrobial activity of obliquumol (Ramadwa et al., 2019; Ramadwa et al., 2021) and ptaeroxylinol, the results suggest that the cleavage of the acetyl group of obliquumol to ptaeroxylinol reduced the biological activity against both fungi and mycobacteria (Scheme 1). The only exception was against the clinical isolate of *C. albicans* where antifungal activity was slightly higher than that of obliquumol. It was very interesting to note that ptaeroxylinol had very poor antimycobacterial activity against *M. fortuitum* while obliquumol had MIC <10 µg/mL against the same mycobacterium (Ramadwa et al., 2021). Ptaeroxylinol had low toxicity against both Vero cells and C3A cells with IC_{50} of 85.7 µg/mL and 126.51 µg/mL respectively. The selectivity index (SI) was calculated by dividing the IC_{50} by the MIC. The SI helps to determine the relatively safety of the ptaeroxylinol in relation to its antimicrobial activity. A higher number indicates selective activity against fungal pathogens while a number below one represents relatively high toxicity. The SI of 10.7 and 15.8 on *C. albicans* ATCC 10,231 against Vero cells and human liver (C3A) respectively and SI of 5.6 and 7.9 on *C. neoformans* against the two cells tested cell lines confirm that the good antifungal activity of ptaeroxylinol was not due its toxicity.

Table 1. Cytotoxicity of ptaeroxylinol on Vero monkey kidney and human liver (C3A) cells expressed as IC_{50} in µg/mL, minimal inhibitory concentration (µg/mL) against fungal and mycobacterial test organisms and the selectivity index (SI.

Test organisms	**MIC (µg/mL)**			**Cytotoxicity**	
Empty Cell	**Ptaeroxylinol**	**Ciprofloxacin**	**Amphotericin B**	**Vero cells (SI)**	**C3A cells (SI)**
A. fumigatus	62.5	Nd	4	1.4	2
C. albicans	31.5	Nd	8	2.7	4
C. albicans ATCC 10,231	**8**	Nd	**4**	**10.7**	**15.8**
C. neoformans	**16**	Nd	**4**	**5.6**	**7.9**
M. aurum NCTC 10,437	250	0.97	Nd	0.3	0.5
M. bovis BCG P1172	62.5	0.97	Nd	1.8	2
M. fortuitum ATCC 6841	250	0.048	Nd	0.3	0.5
M. smegmatis ATCC 1441	62.5	0.19	Nd	1.4	2
Ptaeroxylinol IC_{50} in µg/mL	Nd	Nd	Nd	**85.7**	**126.51**
Doxorubicin (µM)	Nd	Nd	Nd	**2.51**	**0.12**

Nd: Not done.

4. Conclusion

The main aim of this study was to develop a UPLC–MS/MS method for identification and quantification of bioactive obliquumol in *P. obliquum* leaves collected from different

geographical locations in South Africa and to isolate a large quantity of obliquumol and subject it to ester saponification in order to obtain ptaeroxylinol, which has been previously isolated from *P. obliquum.* A UPLC-MS/MS method was developed for separation of components in the *P. obliquum* acetone crude extracts collected from different localities and quantified obliquumol concentrations in the extracts used in the study. Quantitative secondary metabolites analysis revealed that the three *P. obliquum* acetone leaf extracts from different locations had different amounts of obliquumol. The acetone extracts of leaves collected from SANBI had the highest concentration of obliquumol. To obtain a large quantity of obliquumol, we developed a rapid isolation technique from the crude acetone extract that yielded more than 1% of obliquumol from the crude acetone extract which was more than double the amount from previous studies. Obliquumol was therefore present in a concentration of about 0.1% in the dried leaves of *P. obliquum*. Furthermore, ptaeroxylinol was synthesised from the isolated obliquumol and this made it possible to determine the antifungal and antimycobacterial activities and cytotoxicity. Although ptaeroxylinol was less active than obliquumol, and had moderate cytotoxicity, it had better activity against *C. albicans*. It appears that the acetoxy and alcohol groups play a role in the antifungal, antimycobacterial and cytotoxicity of the two compounds. The derivative had MIC values as low as 8 µg/mL and 16 µg/mL against *C. albicans* ATCC 10,231 and *C. neoformans* respectively. The SI indicated that ptaeroxylinol also had selective activity against the microbial pathogens used in the study. The synthesis of other obliquumol derivatives may lead to the analogues with improved pharmacological activity and/or lower toxicity. It will also be important to determine *in vivo* animal toxicity of the bioactive compounds.

Authors' contributions

TER carried out the experiments, analysed the data and wrote the first draft as part of a PhD thesis supervised by JNE. MAS assisted with UPLC-MS work. MSS helped with the structural elucidation of the compounds and LJM assisted with the cytotoxicity work. JNE supervised the whole study and edited the final version and submitted it for publication. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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