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Natural Product Communications
Volume 14(9): 1–7
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DOI: 10.1177/1934578X19872927
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Antibacterial and Antimycobacterial Activity of Crude Extracts, Fractions, and Isolated Compounds From Leaves of Sneezewood, *Ptaeroxylon obliquum* (Rutaceae)

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Abstract

Ptaeroxylon obliquum (Thunb.) Radlk. (Rutaceae) is traditionally used to treat human and animal diseases in South Africa. In this study, the activity of leaf extracts, fractions, and isolated compounds was determined against nonpathogenic mycobacterial species and nosocomial bacterial pathogens. An acetone leaf extract was partitioned by liquid-liquid fractionation, and obliquumol, a mixture of lupeol and β-amyrin, and eranthin were isolated. Antimicrobial activity was determined using a serial microdilution assay against *Mycobacterium smegmatis* (American Type Culture Collection [ATCC] 1441), *M. bovis* (BCG P1172), *M. aurum* (NCTC 10437), *M. fortuitum* (ATCC 6841), *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 25922), and *Escherichia coli* (ATCC 27853). The *n*-hexane fraction had minimal inhibitory concentration (MIC) values as low as 20 and 40 μg/mL against *M. fortuitum* and *S. aureus,* respectively. The chloroform fraction also had promising activity with an MIC value of 80 μg/mL against both *P. aeruginosa* and *M. fortuitum*. Obliquumol had excellent activity (MIC 8 μg/mL) against *M. fortuitum*. Fractionation of the crude extract potentiated the antimicrobial activity of the nonpolar fractions. The isolated compound, obliquumol, had good antimicrobial and excellent antimycobacterial activities. The antimicrobial activity provides some scientific rationale for the use of *P. obliquum* against infectious diseases and related symptoms. This is the first report on the antibacterial activity of obliquumol.

Keywords

Ptaeroxylon obliquum, mycobacteria, nosocomial pathogens, obliquumol, eranthin, minimum inhibitory concentration

Received: March 11th, 2019; Accepted: June 26th, 2019.

Tuberculosis (TB) is a chronic bacterial infection caused mainly by *Mycobacterium tuberculosis* and to a lesser degree by *M. bovis* and *M. africanum*.[1] This disease is of global importance and is transmitted from person to person through air droplets released into the air via coughs and sneezes.[2] TB is one of the most prevalent diseases mainly in developing countries because of the lack of adequate health systems to provide the required expensive and lengthy treatment, as well as late presentation of illness which leads to poor prognosis.[3] The increasing emergence of multidrug-resistant TB (resistance to isoniazid and rifampicin) and extensively drug-resistant TB (additional resistance to fluoroquinolone, kanamycin, amikacin, and capreomycin) continues to exacerbate the situation.[4]

According to the World Health Organization, South Africa is among the 4 countries with the highest number of multidrug-resistant TB and extensively drug-resistant TB cases.[5] The increase may be attributed to the acquired immune deficiency syndrome pandemic and inadequate healthcare systems in sub-Saharan Africa.[6] *Mycobacterium bovis*, which is closely related to *M. tuberculosis* in terms of genetic composition, is an important zoonotic agent that can spread through ingestion of raw milk and by inhalation of infectious droplet nuclei and reservoirs in wildlife.[7-9] Different assay systems and mycobacterial test organisms have been used to screen plant extracts and isolated compounds for antimycobacterial activity in the search for new antimycobacterial drugs.[3,10] Many scientists are either reluctant

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or not allowed to work with *M. tuberculosis* because of its virulence and slow growth rate. As a result, working on *Mycobacterium* species such as *M. smegmatis*, *M. fortuitum,* and *M. aurum* is often preferred because of their rapid growth and avirulent and saprophytic attributes.[11,12] The sensitivity of *M. smegmatis* and *M. tuberculosis* to drugs may be closely related. Therefore, screening against these species may provide leads for indicating activity against virulent forms.[13]

The spread of HIV infection has also increased the risk of other opportunistic and common bacterial infections. *Staphylococcus aureus* is considered among the most common nosocomial pathogens and causes bacterial infection in HIV-positive patients.[14] The antibiotic resistance of methicillin-resistant *S. aureus* and multiresistant Gram-negative organisms is the major contributing factor to the problem of nosocomial pathogens.[15,16] Other nosocomial pathogens which are of great health concern for immunocompromised patients include *Pseudomonas aeruginosa, Escherichia coli*, and *Enterococcus faecalis*.[17]

Ptaeroxylon obliquum (sneezewood), a member of the Rutaceae (previously Ptaeroxylaceae) family, is the only species in the genus and occurs only in South Africa, Mozambique, Zimbabwe, Namibia, and Zambia.[18] Sneezewood is traditionally used to treat human and animal diseases.[19] The bark is taken to combat rheumatism, arthritis, and fevers in Portugal.[20,21] The powdered bark is used as a snuff for recreational purposes and medicinally to relieve headaches by the Xhosa people.[20] The wood of the plant is used to treat anthrax infections, rheumatism, heart diseases, lupus, warts, sinusitis, fits and for ticks in cattle and ritual sacrifices made to ancestral spirits.[22] Pegs of wood are commonly used as protection against lightning.[21] Pieces of the wood are still placed in cupboards to repel moths and other insects. This insect-repelling property made it a popular wood for bedsteads.[21]

Previous studies on *P. obliquum* leaf extracts led to the isolation and characterization of a novel compound, obliquumol, which had good anticandidal activity and was not toxic to fibroblast cells (Figure 1).[23] The compound had a novel structure that could possibly be developed as a new framework antifungal compound and was patented in the United States and Europe. The activity of obliquumol was not determined against other microorganisms because only a small quantity of the compound was isolated. There was a need to determine if there are also other antimicrobial compounds present in the extract and to develop a method to isolate larger quantities of bioactive compounds.

Also isolated from the bark of *P. obliquum* are saptaeroxylon, volatile oil, pyrogallol-type tannins, resins, fat, a glycoside, ptaeroxylon, an alkaloid with cardiac depressant properties and, from the heartwood, a variety of aromatic compounds.[24,25] Methylalloptaeroxylin, an aromatic compound isolated from the leaves, has antihypertensive activity in rats.[26] The antiparasitic activity of a crude extract has been reported[27,28] and, more recently, a new meroterpenoid, ptaerobliquol, together with other known compounds were isolated from the roots of *P. obliquum*.[29]

This study was undertaken to determine the antibacterial and antimycobacterial activity of crude extracts, fractions, and isolated bioactive compounds from *P. obliquum* leaf extracts. The leaves were collected from different localities within South Africa in order to investigate the possibility of geographical or

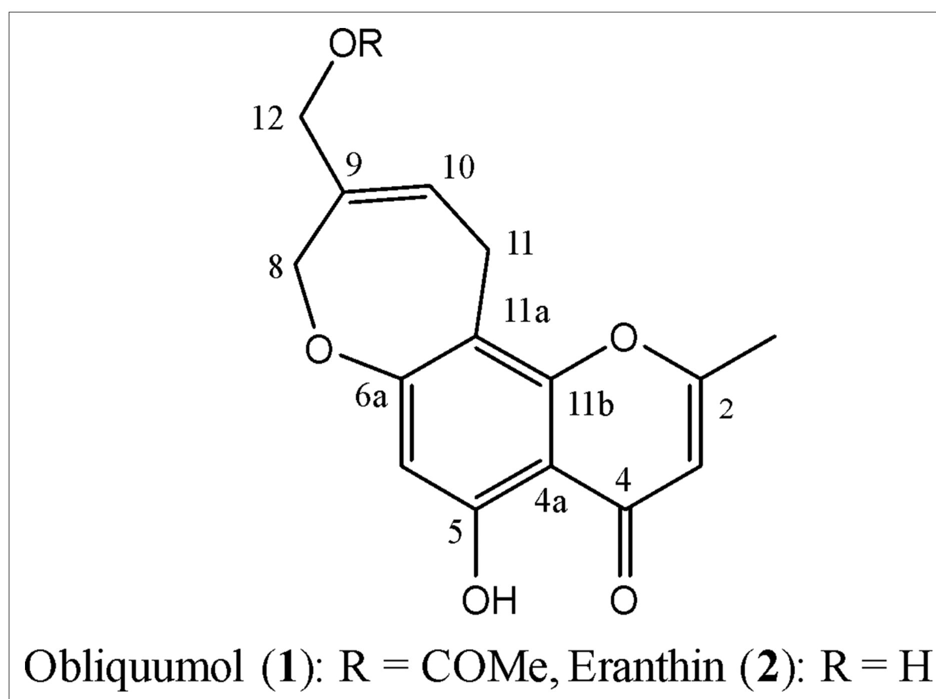

Figure 1. Structures of obliquumol (**1**) and eranthin (**2**) from *Ptaeroxylon obliquum* leaves.

genetic variation that may affect the use of herbal products prepared from this plant. Herein we report the antibacterial and antimycobacterial activities of the fractions and isolated compounds from the leaves.

The highest percentage yield (14%) of the 3 acetone leaf extracts of *P. obliquum* collected from different localities was obtained from the KwaZulu-Natal (KZN) material. There was very little difference between the material collected from the University of Pretoria Onderstepoort (UP-OP) campus (10%) and South African National Botanical Gardens (SANBI) (9%). Thin layer chromatography (TLC) indicated that all three crude extracts contained a high percentage of nonpolar secondary metabolites. The chromatograms also indicated that there was very little variation between the plants since similar compounds were present in the 3 acetone extracts. The only noticeable difference was that some compounds were more pronounced in the inland samples (UP-OP campus and SANBI compared with the sample from the coastal area, KZN).

The amount of crude extract collected after bulk extraction of 500 g powdered leaf with acetone was 44.6 g, which was further fractionated using solvent-solvent fractionation into 5 fractions based on increasing polarity. The highest masses were obtained for nonpolar fractions, chloroform ($CHCl_3$) (26.5 g) and *n*-hexane (8.3 g), compared with 30% water in methanol (H_2O-MeOH) (1.8 g), *n*-butanol (*n*-BuOH) (1.2 g), and water (H_2O) (1.3 g). With 21% in the nonpolar (*n*-hexane) and 68% in the intermediate polarity ($CHCl_3$) range, the results show low level of polar compounds in the acetone extracts of *P. obliquum* leaves. The sum total mass of the 5 fractions was 39.1 g compared with the initial amount of 44.6 g which indicated a reasonable recovery of 88%. The 12% loss was probably through the formation of a pellicle during solvent-solvent fractionation.

The TLCs of the crude extracts of *P. obliquum* collected from different localities showed antimycobacterial activity with clear zones of inhibition when sprayed with a culture of *M. smegmatis*. The same bioactive compounds were responsible for the activity in all the crude extracts. The *n*-hexane and $CHCl_3$ fractions showed clear zones of inhibition compared with the red-colored background indicating the growth of *M. smegmatis*. The 30% H_2O-MeOH fraction also had some antimycobacterial activity, while the *n*-BuOH and H_2O fractions were not active. The bioautograms also showed that the antimycobacterial compounds responsible for the activity in both *n*-hexane and $CHCl_3$ fractions were probably the same. Based on R_f values, the same compounds were present in trees collected from different areas. None of the isolated compounds had activity against *M. aureum*.

The acetone crude extracts of *P. obliquum* collected from different localities had reasonable antibacterial activity inhibiting the growth of the 4 nosocomial pathogens at concentrations ranging from 100 to 320 μg/mL (Figure 2). The variation in activity based on geographical location was minimal against all the tested bacteria. The crude extracts from all 3 locations had good activity mainly against *S. aureus* American Type Culture Collection (ATCC) 29213 with minimal inhibitory concentrations (MICs) ranging from 100 to 120 μg/mL, which is pharmacologically significant.[30] The KZN extract was more active than the other 2 against all the tested mycobacteria. An MIC value as low as 20 μg/mL was obtained for the KZN crude extract against *M. fortuitum*. This difference may be associated with either the growth stage or age of the leaves collected. Because the MIC is determined based on a 2-fold serial dilution, a difference of 1 well in the assay leads to a doubling or halving of the MIC, which may not be significant. The MIC values of the UP-OP campus and SANBI samples were equal when tested against all the mycobacteria chosen for the study with only the exception of *M. aurum*. The MICs of the fractions and isolated compounds are presented in Table 1.

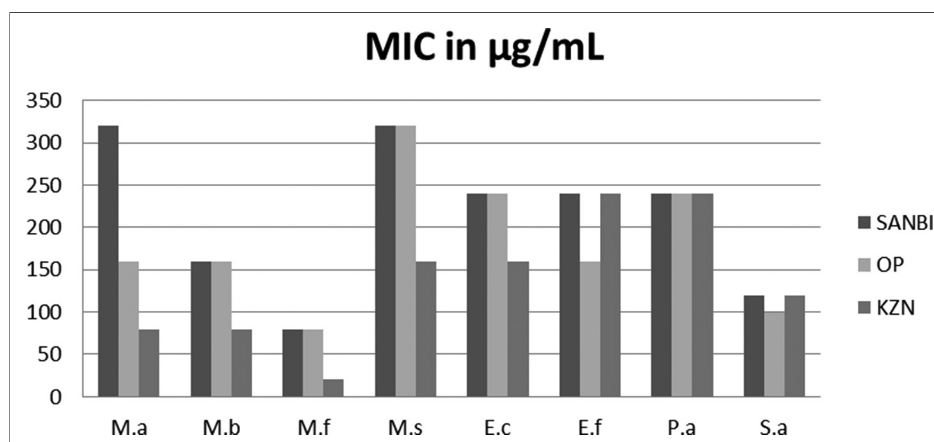

Figure 2. Minimal inhibitory concentrations (MIC in μg/mL) of *Ptaeroxylon obliquum* leaf extracts collected from different localities. Key: South African National Botanical Gardens (SANBI), KwaZulu-Natal (KZN) Botanical Gardens, and Onderstepoort campus (OP), *Mycobacterium aurum* (M.a), *M. bovis* BCG (M.b), *M. fortuitum* (M.f), *M. smegmatis* (M.s), *Escherichia coli* (E.c), *Enterococcus faecalis* (E.f), *Pseudomonas aeruginosa* (P.a), and *Staphylococcus aureus* (S.a).

Table 1. Mass Obtained From 500 g Powdered Leaves and Minimal Inhibitory Concentration (MIC) Values in μg/mL of the 5 Fractions (*n*-Hexane, $CHCl_3$, 30% H_2O-MeOH, *n*-BuOH, and H_2O) and Isolated Compounds From *Ptaeroxylon obliquum* Tested Against *Escherichia coli* (E.c), *Enterococcus faecalis* (E.f), *Pseudomonas aeruginosa* (P.a), *Staphylococcus aureus* (S.a), *Mycobacterium aurum* (M.a), *M. bovis* (M.b), *M. fortuitum* (M.f), and *M. smegmatis* (M.s).

	Mass (g)	**E.c**	E.f	**P.a**	**S.a**	**M.a**	**M.b**	**M.f**	**M.s**
n-Hexane	8.3	80	80	160	40	80	80	20	80
$CHCl_3$	26.5	240	320	80	240	1250	630	80	630
30% H_2O-MeOH	1.8	630	320	630	320	630	320	40	320
n-BuOH	1.2	1250	1250	1250	630	1250	1250	320	1250
H_2O	1.3	2500	2500	630	630	2500	2500	630	2500
Obliquumol	0.21	80	>250	31.5	31.5	125	31.5	8	16
Lupeol and β-amyrin	0.2	80	>250	62.5	62.5	>250	250	62.5	62.5
Eranthin	0.013	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Gentamicin	-	8.0	1.6	0.2	0.2	Not tested	Not tested	Not tested	Not tested
Ciprofloxacin	-	-	-	-	-	0.9	0.9	0.048	0.19

All the fractions had antimicrobial activities with MIC values ranging from 20 to 2500 μg/mL. Fractionation of the crude extracts potentiated the nonpolar fractions with MIC values of <100 μg/mL, particularly against *M. fortuitum* and *S. aureus*. The *n*-hexane fraction was the most active with MIC values ranging from 20 to 160 μg/mL, and of 20 and 40 μg/mL against *M. fortuitum* and *S. aureus,* respectively. The $CHCl_3$ fraction also had promising activity with an MIC value of 80 μg/mL against both *P. aeruginosa* and *M. fortuitum*. The polar fractions were not active against any of the tested bacteria. The antibacterial activity of obliquumol and the lupeol and β-amyrin mixture was also determined. Insufficient eranthin was available to test (Table 1). Obliquumol was more active against *M. fortuitum* and *M. smegmatis* with MIC values of 8 and 16 μg/mL, respectively. It was also active against *M. bovis* BCG with an MIC of 31.5 μg/mL. The lupeol and β-amyrin mixture had moderate activity against most of the bacteria with the lowest MIC of 62.5 μg/mL against *P. aeruginosa*, *S. aureus*, *M. fortuitum*, and *M. smegmatis*.

Lupeol and β-amyrin are found in abundance in many medicinal plants and have a spectrum of pharmacological activities. The potential of lupeol has been tested in various in vitro and in some cases in vivo models as antimicrobial, anti-inflammatory, antiarthritic, antiprotozoal, antidiabetic, anticancer, cardioprotective, skin protective, hepatoprotective, and nephroprotective agents.[31–37] In another study, lupeol had moderate activity against *E. faecalis* (63 μg/mL), but does not inhibit the growth of *E. coli*, *P. aeruginosa*, and *S. aureus* at a concentration of 250 μg/mL.[33]

Experimental

Plant Material

Ptaeroxylon obliquum leaves were collected from trees growing on the UP-OP campus, Faculty of Veterinary Science, SANBI in Pretoria, and KZN Botanical gardens in the summer of 2011. The leaves were collected in open woven 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 were prepared and kept at the HGW Schweikert Herbarium of the University of Pretoria.

Extraction and Isolation

For TLC and MICs powdered leaves (1 g) were extracted with 10 mL of technical grade acetone (Merck). The extracts were shaken on a Labotec model 20.2 shaker for 30 to 60 minutes and centrifuged at 3500 rpm for 5 minutes. Extraction and centrifugation were repeated 3 times on the pellet. The 3 supernatants were then combined and dried in preweighed vials under a stream of cold air. Fractions were dried and dissolved in acetone to a concentration of 10 mg/mL for bioassays and phytochemical analysis.

All the plant extracts or fractions were reconstituted in acetone to a concentration of 10 mg/mL. Samples of the plant extracts (10 μL) were loaded onto TLC plates (Merck silica gel 60 F254) in 1 cm thin bands using a micropipette. Mobile phases of varying polarities were used to develop the chromatograms, namely *n*-hexane/ethyl acetate (H:EtOAC) (6:4) and $CHCl_3$/EtOAc/HCOOH (CEF) (5:4:1).[23] Development took place in closed tanks saturated with the mobile phase. The developed plates were then visualized under ultraviolet light (254 and 360 nm, Camac universal UV lamp TL-600), sprayed with vanillin-sulfuric acid (0.1 g vanillin, 28 mL MeOH, and 1 mL sulfuric acid), and heated at 105°C to optimal color development of the separated compounds.[38]

Specific ATCC strains, that is, 2 Gram-positive (*S. aureus* [ATCC 29213] and *E. faecalis* [ATCC 29212]) and 2 Gram-negative (*P. aeruginosa* [ATCC 25922] and *E. coli* [ATCC 27853]) bacteria were chosen for the study. The selection of test bacteria was based on the recommendation of the National Committee for Clinical Laboratory Standards that these bacterial strains should be used as clinical laboratory standards,[39] now known as the Clinical Laboratory Standards Institute. All

the bacteria were maintained in Mueller-Hilton agar at 4°C until needed.

Four nonpathogenic mycobacterial species (*M. smegmatis* ATCC 1441, *M. bovis* BCG P1172, *M. aurum* NCTC 10437, and *M. fortuitum* ATCC 6841) were used in the study to determine the antimycobacterial activity of the extracts. The mycobacteria were grown at 37°C in Middlebrook 7H9 broth supplemented with 10% oleic albumin dextrose catalase and maintained on Middlebrook 7H10 agar supplemented with either glycerol or Tween 20 at 4°C until needed.

A serial microplate dilution method with *p*-iodonitrotetrazolium violet (INT) as growth indicator was used to determine the MICs.[40] Two-fold serial dilutions were dispensed into 96-well microplates as follows: distilled H_2O (100 μL) was placed in each well using a micropipette and 100 μL of a 10 mg/mL plant extract was placed in each of the first wells of the relevant series of dilutions, and thereby the extracts were diluted to 50%; 100 μL 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. To each well, 100 μL of overnight cultured bacteria was added. To measure growth, INT (0.2 mg/mL in H_2O) was added (40 μL) to each well. The plates were incubated for 18 hours at 37°C in a humidified atmosphere. The MIC was recorded as the lowest concentration of the extract that inhibits growth based on color intensity. The reduction of INT to respective red formazan indicates bacterial growth. Gentamicin and ciprofloxacin were used as positive controls and the acetone dilutions as negative control.

Qualitative analyses of the number of antimicrobial compounds were performed by the bioautography method.[41] Plant extracts, 10 μL, were loaded onto the TLC plates (10 × 10 cm) and dried before development with the selected eluent. The chromatograms were allowed to dry at room temperature under a stream of cold air in order to evaporate all traces of the eluents. *Mycobacterium smegmatis* cultured in Middlebrook 7H9 broth was sprayed on the chromatogram and incubated overnight in 100% relative humidity to allow bacterial growth. The following day, a solution of 2 mg/mL INT was sprayed and further incubated at 37°C for 1 hour. Clear zones on the plates indicated inhibition of bacterial growth.

Powdered leaf material (500 g) of *P. obliquum* was extracted with 5 L of acetone and shaken vigorously for 8 hours on a Labotec shaking machine. The supernatant was filtered through Whatman No.1 filter paper using a Büchner funnel and evaporated using a Büchi rotavaporator R-114 (Labotec). The concentrated extract was then poured into a preweighed beaker. The same procedure was repeated 2 times on the pellet. The extract was then left to dry under a stream of cold air and the extracted mass was determined.

Solvent-solvent fractionation was used to fractionate the acetone extract based on polarity of the compounds.[42] The acetone extract was reconstituted in 500 mL of $CHCl_3$:H_2O (1:1) in a separatory funnel to give the preliminary $CHCl_3$ and H_2O fractions. The H_2O fraction was then mixed with an equal volume of *n*-BuOH to yield the H_2O and *n*-BuOH fractions. The preliminary $CHCl_3$ fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of *n*-hexane and 10% H_2O-methanol mixture, to yield the *n*-hexane fraction. The 10% H_2O-MeOH fraction was then further diluted to 30% H_2O-MeOH and mixed with $CHCl_3$ to yield the 30% H_2O-MeOH fraction and the $CHCl_3$ fraction. Five fractions were therefore separated, namely, H_2O, *n*-BuOH, 30% H_2O-MeOH, $CHCl_3$ and *n*-hexane fractions.

Isolation of Active Compounds

The isolation of the bioactive compounds was carried out on the $CHCl_3$ fraction using column chromatography and silica gel as the stationary phase. About 1000 g of silica gel (Merck) was mixed with *n*-hexane to form a homogenous slurry, which was transferred into a glass column (40 cm height, 4.5 cm diameter). The $CHCl_3$ fraction (26.5 g) was dissolved in 100 mL of acetone and mixed with 50 g of silica gel and the mixture was then allowed to dry under cold air for about 2 hours. The dried $CHCl_3$ fraction was then layered on the column bed. *n*-Hexane with an increasing amount of EtOAc at intervals of 5% until 100% EtOAc was used to gradually elute the sample loaded in the columns to yield different fractions, which were collected. All the fractions were left overnight to dry under cold air.

Compound **1** was obtained as a white solid (210 mg) from the 70% to 60% *n*-hexane fraction by continuously purifying the fractions with acetone. The compound was identified as obliquumol by comparison of its m.pt, UV, ^{1}H NMR, and ^{13}C NMR spectroscopic, and HR ESIMS data with that in the literature.[43] Lupeol and β-amyrin mixture (200 mg) was obtained from fractions collected at 95% to 85% *n*-hexane by continuously purifying the fractions with EtOAc. Fractions collected from 50% to 30% hexane were combined since they contained similar compounds. The 5.6 g yield of the combined fractions was dissolved in acetone and mixed with 5 g of silica gel and allowed to dry under cold air for about 1 hour. The sample was then loaded into a silica gel bed (40 cm height, 2.5 cm diameter) and eluted with 90% *n*-hexane in EtOAc until 100% EtOAc. From the 50% to 40% *n*-hexane fraction, about 13 mg of compound **3** was collected. It was identified as eranthin by comparison of its m.pt, UV, ^{1}H NMR, and ^{13}C NMR spectroscopic, and HR ESIMS data with that in the literature.[44]

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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