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Anti-inflammatory activities of *Ptaeroxylon obliquum* leaf extracts, fractions and bioactive compounds

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ABSTRACT

Ptaeroxylon obliquum (Thunb.) Radlk is traditionally used to treat inflammation and related symptoms such as arthritis, rheumatism, fever, pain and headache. The aim of this study was to determine the anti-inflammatory activities by investigating different potential mechanisms of action of the crude extracts, fractions and isolated compounds from *P. obliquum* leaves from different localities in South Africa. The anti-inflammatory activity of *P. obliquum* acetone leaf extracts, aqueous extracts (hot and cold), fractions and isolated compounds (obliquumol, *O*-methylalloptaeroxylin and a mixture of lupeol and β-amyrin) was determined by investigating the following: the nitric oxide (NO) inhibition in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages, soybean 15-lipoxygenase (15-LOX) inhibition, as well as cyclooxygenase (COX)-2 enzyme inhibition using an ELISA method. The effects of the extracts on the pro-inflammatory cytokines, interleukin (IL)-1β and TNF-α, as well as the anti-inflammatory cytokine (IL-10) production, were also determined using ELISA assays. All acetone extracts demonstrated greater 15-LOX inhibitory activity than aqueous extracts and the positive control, with IC_{50} values ranging from 5.7 μg/mL to 10.4 μg/mL. Obliquumol had the highest level of 15-LOX inhibition with an IC_{50} of 3.66 g/mL. The isolated compounds, fractions, and extracts from *P. obliquum* all inhibited NO production in a dose-dependent manner. The acetone extracts reduced NO with percentages as high as 95 % and 102 % with extracts prepared from plants collected from Walter Sisulu Botanic Gardens and Nelspruit, respectively, at the lowest tested concentration (1.6 μg/mL). The high proportion of NO was not caused by toxicity of the acetone extracts to the cells, as was demonstrated by cell viability of >100 % RAW 264.7 macrophages. The extracts and the compounds reversed the LPS-induced COX-2 significantly ($p < 0.001$) in a dose dependent manner in comparison with the positive control, quercetin. The LPS-induced pro-inflammatory cytokines that were assessed in the study were reduced by the extracts and compounds. The acetone extract prepared from plant material collected at Walter Sisulu Botanic Gardens and obliquumol significantly inhibited the LPS-induced TNF-α levels ($p < 0.027$ and $p < 0.008$, respectively). The acetone extracts, fractions and isolated compounds of *P. obliquum* had good anti-inflammatory activity, thus supporting its medicinal use against inflammation.

1. Introduction

Inflammation is a defence mechanism of the body's immune system against tissue damage, undesired external objects, or pathogens that enter tissue cells (Nkadimeng et al., 2020; Ribaldone et al., 2018). Prolonged or unregulated inflammation is associated with acute and chronic diseases such as rheumatoid arthritis, cancer, heart disease and inflammatory bowel disease (Ramadwa et al., 2022; O'Byrne and Dalgleish, 2001). When foreign pathogens invade host tissue, native tissue cells produce a number of inflammatory mediators that activate different signalling pathways to release and attract

List of abbreviations: ANOVIA, analysis of variance; ATCC, American Type Culture Collection; COX, cyclooxygenase; COX-2, cyclooxygenase-2; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; Il-1β, interleukin one beta; Il-10, interleukin ten; iNOS, inducible nitric oxide synthase; LOX, lipoxygenase; 15-LOX, 15-lipoxygenase; LPS, lipopolysaccharides; MTT, 3- (4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NF-ϰB, nuclear factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate buffered saline; PSF, penicillin/streptomycin/fungizone; RNS, reactive nitrogen species; ROS, reactive oxygen species; TNF-a, tumor necrosis factor alpha

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leukocytes (Chen et al., 2018). The response is accompanied by an increase in the level of pro-inflammatory mediators including nitric oxide (NO), nitric oxide synthase (iNOS), prostaglandins, cytokines including interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) and enzymes such as cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) (Muniandy et al., 2018). Anti-inflammatory cytokines including IL-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, IL-11, and IL-13 are released to prevent the process by regulating pro-inflammatory cytokine responses (Summer et al., 2024; Boshtam et al., 2017). As one of the key cytokines that promotes inflammation, TNF-α plays a critical role in the pathophysiology of inflammatory diseases such as rheumatoid arthritis (Nazakat et al., 2025; Li et al., 2017).

COX-2 is an important pro-inflammatory mediator that promotes inflammation via the production of prostaglandins, which are known as molecules that play a key role in the propagation of inflammation (Riaz et al., 2024; Nanjundaiah et al., 2016). Pro-inflammatory COX-2 is inducible by cytokines (IL-1, IL-6 and TNF-α) expressed in the vascular endothelium, rheumatoid synovial endothelial cells, monocytes and macrophages (Brune and Patrignani, 2015; Vane et al., 1998).

Lipoxygenases (LOX) are dioxygenases that also produce appropriate hydroperoxides like the polyunsaturated fatty acids linoleic acid and arachidonic acid. LOX enzymes are expressed in immunological, epithelial, and tumour cells that exhibit a range of physiological functions, such as inflammation, rashes and carcinogenesis. Depending on where oxygen is inserted into the arachidonic acid, the lipoxygenase enzymes present in mammals are divided into three groups: 5, 12, and 15 (Schneider and Bucar, 2005). Humans express the enzymes in a tissue-specific manner, with 15-LOX being mostly expressed in reticulocytes, eosinophils and macrophages (Klil-Drori and Ariel, 2013). The role of 15-LOX has been implicated in various inflammation-related diseases. Increasing evidence highlights the nature of 15-LOX in inflammation, as its metabolites have been shown to have both pro- and anti-inflammatory properties (Ondua et al., 2019; Adebayo et al., 2015).

Numerous inflammatory mediators, such as prostaglandin and nitric oxide (NO), are produced by macrophages. NO is a crucial cellular signalling molecule that plays a role in several physiological processes in mammals, such as vasodilation, smooth muscle relaxation, neurotransmission and the immunological response (Ramadwa et al., 2022; Keibel et al., 2009). Overproduction of NO may be a factor in immune-pathology of macrophage-dependent inflammation and degenerative illnesses, such as cancer. NO, a free radical, is created by a group of enzymes called nitric oxide synthases (NOSs). Inhibition of NO production is a promising therapeutic target in the development of potential anti-inflammatory agents (Ramadwa et al., 2022).

Various nonsteroidal anti-inflammatory drugs have been shown to reduce pain and inflammation by blocking the metabolism of arachidonic acid by isoforms of the cyclooxygenase enzyme, thereby reducing the production of prostaglandin. However, there are many side effects associated with the administration of nonsteroidal anti-inflammatory drugs. The African continent is richly endowed with diverse medicinal plants with anti-inflammatory activities that have been shown to be effective in the treatment of inflammatory conditions in traditional medicine. There are at least 555 medicinal plants that are used traditionally for the treatment of pain and inflammatory disorders in southern Africa (Khumalo et al., 2022). Disorders treated include inflammation and pain associated with inflammation, including toothache, headache, backache, rheumatism, oedema or swellings, general body pains, earache, abdominal pains, arthritis, chest pains, internal body pains, haemorrhoids, labour pains and rheumatic fever (Khumalo et al., 2022). The medicinal plants possess phytochemicals that are very effective, considerably safer, and exhibit less side effects (Riaz et al., 2024). Phytochemicals can reduce inflammation by inhibiting NF-kB and neutralizing free radicals. Moreover, phytochemicals affect additional signaling pathways such as transforming growth factor-ß (TGF-ß) and the mitogen-activated protein kinase pathway (Riaz et al., 2024).

Ptaeroxylon obliquum (Thunb). Radlk (Rutaceae), also known as sneezewood, is used traditionally to treat a wide range of diseases including inflammation-related diseases and symptoms such as arthritis, rheumatism, fever and headache (Iwalewa et al., 2007; Moyo and Masika, 2009; Ribeiro et al., 2010; Ramadwa et al., 2021). Medicinal plants yield secondary metabolites that may serve as templates for the synthesis of novel anti-inflammatory agents with high pharmacological activity and fewer side effects. The aim of the study was to determine the anti-inflammatory activities in terms of different mechanisms of action of the crude extracts, fractions and isolated compounds from *P. obliquum* leaves from different localities in South Africa.

2. Materials and methods

2.1. Plant collection

Ptaeroxylon obliquum leaves used in this study were collected during the summer of 2019 from trees growing at the Hatfield Campus (University of Pretoria), National Botanical Gardens of the South African National Biodiversity Institute (SANBI) in Pretoria, the Lowveld Botanic Gardens (Nelspruit, Mpumalanga) and Walter Sisulu National Botanical Garden (Roodepoort, Johannesburg). The leaves were gathered in nylon open weave bags, allowed to dry at room temperature in the shade, and then ground into a powder. The plant material was kept until needed in sealed containers in the dark. Voucher specimens (PRU130509, PRU130510, PRU130511 and PRU130512 respectively) were prepared and kept at the HGWJ Schweickerdt Herbarium of the University of Pretoria. The specimens were identified by Magda Nel and Dr Kenneth Oberlander (Curator).

2.2. Fractionation and isolation of bioactive compounds from P. obliquum

The plant fractionation procedure and isolation of bioactive compounds were carried out as described previously, leading to the isolation of obliquumol, *O*- methylalloptaeroxylin and a mixture of lupeol and β-amyrin from *P. obliquum* acetone leaf extract from Hatfield, Pretoria (Khunoana et al., 2022). Solvent-solvent fractionation was used to separate the *P. obliquum* acetone leaf extract into five fractions based on the polarity of the secondary metabolites. The chloroform fraction was then subjected to silica gel chromatography to separate the pure compounds, which were reconstituted in dimethyl sulphoxide (DMSO) for the anti-inflammatory assays.

2.3. The 15-lipoxygenase (15-LOX) inhibitory assay

The anti-inflammatory activity of extracts was evaluated *in vitro* via the soybean 15-lipoxygenase (15-LOX) (obtained from Sigma-Aldrich) inhibitory assay according to the method of (Ondua et al., 2019). This was done in the presence of linoleic acid based on the formation of the complex Fe3+/xylenol orange (Sigma-Aldrich) with absorption at 560 nm. Percentage inhibition of 15-lipoxygenase activity was determined and the 50 % inhibition concentrations (IC_{50}) were determined using the non-linear regression curve of the 15-LOX percentage inhibition against the logarithm of concentrations tested (Ondua et al., 2019). LOX activity was determined using a spectrophotometric assay according to (Del Carmen Pinto et al., 2007) with slight modifications. The substrate linoleic acid (Sigma-Aldrich, Burlington, MA, United States) (final concentration, 140 μM) was prepared in Tris–HCl buffer (50 mM, pH 7.4). All crude extracts and fractions (10 mg/mL) were prepared in 100 % DMSO and further diluted to 2 mg/mL in Tris–HCl buffer, except the water extracts which were directly prepared at 2 mg/mL in the Tris–HCl buffer.

Except for the water extracts, which were prepared directly at 2 mg/mL in the Tris–HCl buffer, all crude extracts and fractions (10 mg/mL) were prepared in 100 % DMSO and further diluted to 2 mg/mL in Tris–HCl buffer. Exactly 20 μL of various concentrations (100–0.78 μg/mL) of test samples or quercetin (positive control) were combined with 40 μL of 15-LOX, diluted in ice-cold Tris–HCl buffer (final concentration, 0.2 U/mL), at 25 °C for 5 min. Linoleic acid (40 μL) was added to the mixture and was further incubated at 25 °C for 20 min in the dark. In order to terminate the experiment, 100 L of freshly made FOX reagent [iron (II) sulphate (100 M) in methanol/water (9:1), sulphuric acid (30 mM), and xylenol orange (100 M)] were added. The enzyme 15-LOX solution, Tris–HCl buffer, substrate, and FOX reagent made up the negative control, whereas the enzyme 15-LOX and buffer were present in the blanks, but the substrate was introduced after the FOX reagent. As shown in formula (2) below, the lipoxygenase inhibitory activity was calculated by calculating the percentage of hydroperoxide generation inhibition from changes in absorbance values at 560 nm after 30 min at 25 °C.

Percentage 15–LOX inhibition (%) = [Absorbance (control) – Absorbance (sample)] / [Absorbance (control)] *x* 100

The 50 % inhibitory concentrations (IC_{50}) were determined using the non-linear regression curve of the percentage 15-LOX inhibition against the logarithm of concentrations tested.

2.4. Inhibition of nitric oxide (NO) production

RAW 264.7 macrophages cells obtained from the American Type Culture Collection (ATCC TIB-71™) (Rockville, MD, USA) were used. The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) at 37 °C with 5 % CO_2, 4.5 g/L of glucose, and 4 mM of l-glutamine (HycloneTM), with 10 % foetal calf serum (FCS) (Capricorn Scientific Gmbh, South America) and 1 % penicillin/streptomycin/fungizone (PSF) added as supplements (PSF). RAW 264.7 cells were seeded in a 96-well microtitre plate using a cell suspension in the amount of 100 μl (2×10^6 cells/mL) and were then incubated overnight at 37 °C with 5 % CO_2 to facilitate adhesion. The RAW 264.7 cells were stimulated by incubation in a medium containing 5 μg/mL of lipopolysaccharide (LPS), treated simultaneously with various doses of the samples dissolved in DMSO, and then further diluted in culture media.

Nitrite oxide concentration in supernatant was measured using the Griess reagent after RAW 264.7 macrophages had been incubated for 24 h at 37 °C and 5 % CO_2. In a new 96-well microtitre plate, 100 μL of cell supernatant from each well was transferred, and the same volume of Griess reagent (Sigma-Aldrich, Burlington, MA, United States) was added (Adebayo et al., 2015). Serial dilutions of sodium nitrite (0–50 μM) were used to compute the concentrations of the nitrites from the standard curve. Based on how well extracts, fractions or chemicals prevented RAW 264.7 macrophages from producing nitric oxide in comparison to the control (cells treated with LPS without samples), the percentage of NO inhibition was calculated.

2.5. Cell viability

The cytotoxicity of crude extracts, fractions and isolated compounds was determined using the 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983).

After removing the culture media from the wells, 200 μL of phosphate buffered saline (PBS) was used to wash the wells; then 200 μL of fresh culture medium, and 30 μL of MTT solution (5 mg/mL in PBS) were added to each well. The plates were then incubated at 37 °C with 5 % CO_2 for four hours.

A suction pump (Integra, USA) was used to carefully aspirate the culture media after incubation, and 50 μL of dimethylsulfoxide (DMSO) was then added to each well. By comparing the absorbance of the samples to the negative control (cells treated exclusively with LPS were regarded as 100 % viable), the percentage of cell viability was estimated.

2.6. Treatment of the cells for cyclooxygenase and cytokine detection

The cells were prepared and treated with the extracts and compounds according to (Nkadimeng et al., 2020) to determine their effects on the levels of cytokine and cyclooxygenase-2 activity. Briefly, the RAW 264.7 macrophages were plated at 1×10^6 cells per 25 cm^2 tissue culture flask (NEST, Whitehead Scientific, Johannesburg, South Africa) and incubated for 24 h. Thereafter, the medium was removed, and fresh medium was added. The cells were stimulated with LPS (1 μg/mL) and treated with 50 and 100 μg/mL concentrations of the extracts and compounds. Quercetin was used as a positive control. The LPS-control cells were cells that were stimulated with LPS but not treated and were referred to as LPS cells in the study. Control cells were cells that were neither stimulated with LPS nor treated with extracts. The cells were exposed to LPS and treated over 24 h. After 24 h, medium was removed and stored at −80 °C until the day of analysis.

2.7. COX-2 activity measurements

The human PTGS2/COX-2 Prostaglandin endoperoxide synthase 2 (PGE2) E-EL-H1846 ELISA kit (Elabscience, Biocom Africa, Johannesburg South Africa) was used to evaluate the effects of extracts on COX-2 levels. The methods were conducted in accordance with the manufacturer's protocol. The absorbance correlated closely with the levels of PGE2/COX-2 in the sample medium. From the standard curve, percentage inhibition of human COX-2 in the cell culture media samples were determined.

2.8. Cytokine activity measurements

Using the human ELISA kits E-EL-M0049, E-EL-M0037 and E-EL-M0046 (Elabscience, Biocom Africa), the effects of the extracts, fractions and isolated compounds on TNF-α, IL-1β, IL-6 and IL-10 concentrations were identified and quantified. The assay was done according to manufacturer's instructions. Standard curves were used to calculate the concentration.

2.9. Statistical analysis

Results are expressed as mean ± standard deviations, and statistically significant values were compared using one-way analysis of variance (ANOVA) using an interactive statistical program (Sigmastat, SPSS version 26, San Jose, *CA*, USA) and pairwise multiple comparison procedures using Holm—Sidak method. Normality testing was done using Shapiro—Wilk and equal variance test using Brown—Forsythe. The p-value of $\leq$0.050 was considered statistically significant.

3. Results and discussion

3.1. Lipoxygenase inhibitory activity of the acetone and aqueous extracts, fractions and isolated compounds from P. obliquum

Lipoxygenase enzymes have recently garnered significant attention due to their pivotal role in the production of leukotrienes, which are crucial in the pathophysiology of various inflammation-related illnesses (Lončarić et al., 2021). There is an urgent necessity to produce targeted inhibitors of the 15-lipoxygenase enzyme to address inflammation-related illnesses. Soybean lipoxygenase is utilized in many investigations to identify possible inhibitors of mammalian lipoxygenase, as it catalyzes the oxidation of linoleic acid, arachidonic acid, and other unsaturated fatty acids (Roussaki et al., 2010). In this

Table 1
Inhibition of 15-LOX (IC_{50} in μg/mL) by crude extracts, fractions and isolated compounds from *P. obliquum*.

Samples	15-LOX IC_{50} (μg/ml)
WALTER SISULU	
Acetone	**9.71 ± 0.99**
H_2O Cold	189.78 ± 5.42
H_2O Hot	226.15 ± 9.7
UP HATFIELD	
Acetone	**8.60 ± 0.89**
H_2O Cold	127.61 ± 4.04
H_2O Hot	259.06 ± 10.14
SANBI	
Acetone	**5.70 ± 1.12**
H_2O Cold	114.01 ± 6.65
H_2O Hot	238.61 ± 18.74
$CHCl_3$ fraction	**14.35 ± 2.33**
Hexane fraction	**21.57 ± 1.63**
NELSPRUIT	
Acetone	**10.44 ± 1.92**
H_2O Cold	185.74 ± 11.13
H_2O Hot	328.78 ± 12.48
$CHCl_3$ fraction	**10.87 ± 1.35**
Hexane fraction	22.72 ± 0,44
Obliquumol	**3.66 ± 0.15**
Lupeol & β-amyrin	**11.30 ± 3.85**
O-Methylalloptaeroxylin	**18.30 ± 0.66**
Quercetin	**10.08 ± 0.64**

study, the potential inhibition of 15-LOX activity by *P. obliquum* extracts was investigated. All acetone extracts had good 15-LOX inhibitory activity compared to aqueous extracts and positive control, with IC_{50} values ranging from 5.7–10.4 μg/mL (Table 1). Acetone extracts from SANBI (Pretoria) had the best 15-LOX inhibitory activity of the extracts collected from different localities with IC_{50} of 5.4 μg/mL. Similar results were observed in our previous study where acetone crude extracts had better activity than aqueous extracts and fractions with the lowest IC_{50} of 1.61 mg/mL respectively (Ramadwa et al., 2022). However, the acetone extracts in the current study had better activity than those in the previous study.

Although all the water extracts had poor activity, it was interesting to note that cold water extracts had better anti-inflammatory activity than hot water extracts. Both hot and cold water extraction methods were used since traditional healers use water as an extractant. The two non-polar fractions tested had IC_{50} values ranging from 10.87–22.72 μg/mL which were generally comparable with activity of acetone extracts. Furthermore, the chloroform and hexane fractions indicated that the compounds responsible for the activity of the crude acetone extract are probably the non-polar compounds. In our prior investigation, obliquumol, lupeol and β-amyrin mixture, and eranthin, had interesting 15-LOX inhibitory effects with IC_{50} values as low as 7.4 μg/mL for obliquumol and 13.9 μg/mL for lupeol and β-amyrin mixture. In the current study, obliquumol exhibited superior activity compared to those we had previously identified with IC_{50} of 3.66 μg/mL, which was two times lower than the positive control quercetin. Other tested compounds, *O*-methylalloptaeroxylin and a mixture of the triterpenoids lupeol and β-amyrin had promising anti-inflammatory activity against LOX-15 with IC_{50} of 18.30 μg/mL and 11.30 μg/mL, respectively. Additionally, as far as our review of the literature could determine, this is the first report on the anti-inflammatory effect against 15-LOX of *O*-methylalloptaeroxylin. The enzyme 15-lipoxygenase (15-LOX) contributes to the development of rheumatoid arthritis through the NF-kB pathway. The NF-kB pathway is active during the initial phase of joint inflammation, and NF-kB, an intracellular mediator and transcriptional regulator in the inflammatory process, exhibits enhanced DNA binding activity in individuals with rheumatoid arthritis (Nazakat et al., 2025). The 15-LOX inhibitory activity of the isolated compounds may explain the traditional uses of *P. obliquum* to treat various inflammatory diseases..

3.2. Inhibition of nitric oxide production from LPS-induced RAW 264.7 cells by the crude extracts, fractions and isolated compounds from P. obliquum

In the LPS-activated RAW 264.7 macrophage cell line, the ability of *P. obliquum* crude extracts, fractions and isolated compounds to suppress NO generation was tested (Table 2). Untreated cells were used as the negative control, LPS-stimulated cells as the positive control, and additionally, a cell group was used as the reduction control group with LPS-stimulated cells, co-incubated with quercetin, which is used as an inhibitor of NO, to demonstrate the validity of the assays.

Nitric oxide serves as a principal inflammatory mediator in the immuno-inflammatory response. Optimal levels of nitric oxide facilitate a robust defense against invading pathogens, while the failure to produce NO leads to significant and possibly lethal vulnerability to infections (Ondua et al., 2019). Moreover, excessive production of NO has been associated with the development of various diseases, including atherosclerosis, neurological diseases, autoimmune inflammatory diseases, and cancers. Consequently, NO possesses the capacity to function as a "double-edged" biological sword depending on its level within the physiological system (Karpuzoglu and Ahmed, 2006).

The inhibitory effect of iNOS or NOS by plant-derived compounds or crude extracts may account for their anti-inflammatory properties, with numerous natural compounds from plants identified as iNOS inhibitors in LPS-activated macrophages (Ondua et al., 2019). LPS-stimulated macrophages lead to the accumulation of NO and superoxide anion. The excessive accumulation of nitric oxide and superoxide anion may enhance the production of peroxynitrite (OONO-), leading to inflammatory disorders. The inhibition of any molecular target involved in the NO pathway of inflammation such as ROS, iNOS,and NO itself could have potential to inhibit inflammatory responses (Ondua et al., 2019).

All *P. obliquum* isolated compounds, fractions and extracts had dose dependent inhibition of NO production at the range of concentrations tested. At the lowest tested concentration (1.6 μg/mL), the crude extracts were able to suppress NO with percentages as high as 95 % and 102 % for Walter Sisulu and Nelspruit acetone extracts, respectively. The high proportion of NO was not caused by acetone extracts being toxic to the cells, as was demonstrated by the excellent cell viability of >100 % on RAW 264.7 macrophage cells. Aqueous extracts from all the different localities had low NO inhibition with an average of 59 % NO inhibition. Both hexane and chloroform fractions were able to inhibit NO production at the lowest concentration tested with percentage of inhibition ranging from 64.7–79 % and cell viability of >100 %. In our previous study the *P. obliquum* crude extracts, fractions, and compounds exhibited a significant proportion of inhibition on NO production in stimulated cells. The compound eranthin had the best activity with a NO inhibition of 71.1 % (Ramadwa et al., 2022). However, more pronounced anti-inflammatory activity was noted in the present investigations with obliquumol inhibiting almost 80 % of NO generation at 1.6 μg/ml, the lowest concentration tested. The mixture of lupeol and β -amyrin had notable NO inhibition of >70 % with cell viability of above 100 %. Blocking or lowering iNOS is significant in anti-inflammatory research since it is a critical macrophage-derived inflammatory mediator involved in the emergence of several inflammation disorders (Muniandy et al., 2018). By reducing iNOS generation, the extracts, fractions, and isolated substances have considerable potential therapeutic benefit in pathological inflammation.

Table 2
Inhibitory effects of the acetone and aqueous extracts, fractions and isolated compounds from *P. obliquum* on NO production in LPS-induced RAW 264.7 cells and their viability against macrophage RAW 264.7 cells.

Samples	Conc (μg/mL)	% NO inhibition	Cell viability (%)
WALTER SISULU			
Acetone	1.6	**95.5 ± 3.1**	**135.4 ± 1.9**
	12.5	99.6 ± 3.3	124.4 ± 0.1
	50	108.9 ± 5.6	114.5 ± 1.5
	100	111.1 ± 6.3	105 ± 2.4
H_2O (hot)	1.6	29.6 ± 1.8	139.4 ± 6.2
	12.5	35.3 ± 2.9	130.4 ± 5.6
	50	46.7 ± 5.2	119 ± 5.1
	100	65.4 ± 7.8	108.3 ± 4.4
UP HATFIELD			
Acetone	1.6	**62.7 ± 2.3**	**149.2 ± 6.1**
	12.5	100 ± 5.4	132.5 ± 2.9
	50	107 ± 5.2	112.2 ± 6.6
	100	112.2 ± 11.2	90.2 ± 5
H_2O (hot)	1.6	20.7 ± 2.1	105.2 ± 3.7
	12.5	29.4 ± 5.5	92.9 ± 6.1
	50	36.3 ± 6.4	92.9 ± 13.4
	100	51.5 ± 9.1	83.9 ± 12.7
SANBI			
Acetone	1.6	**78.9 ± 25.1**	**125 ± 5.9**
	12.5	89.5 ± 12	112.2 ± 2.6
	50	109.8 ± 2.2	96 ± 15.1
	100	110 ± 4.0	84 ± 15.6
H_2O (hot)	1.6	23.4 ± 2.9	119.9 ± 8.6
	12.5	44.5 ± 4.0	113.3 ± 1.7
	50	51 ± 4.8	100.7 ± 13
	100	72.1 ± 7.7	86.1 ± 4.6
$CHCl_3$ fraction	1.6	79.1 ± 4.3	121.4 ± 2.22
	12.5	89.7 ± 4.8	108.9 ± 15.2
	50	101.5 ± 0.5	93.9 ± 15.4
	100	104.7 ± 1.9	70.8 ± 8.4
Hexane fraction	1.6	76 ± 3.8	115.2 ± 3.7
	12.5	89.8 ± 5.7	106.1 ± 2.7
	50	98.3 ± 12.9	99.5 ± 13.7
	100	104.7 ± 3	69 ± 4.6
NELSPRUIT			
Acetone	1.6	**102.8 ± 1.5**	**115.2 ± 3.7**
	12.5	109.4 ± 7.4	106.1 ± 2.7
	50	117.3 ± 13.6	99.5 ± 13.7
	100	105.6 ± 3.8	63.3 ± 10.6
H_2O (hot)	1.6	35.1 ± 10.9	117.1 ± 11.7
	12.5	46.2 ± 4.8	108.8 ± 5.5
	50	47.2 ± 6.5	106.1 ± 3.8
	100	51.7 ± 4.8	99.5 ± 0.9
$CHCl_3$ fraction	1.6	77.3 ± 4.4	126.3 ± 2.2
	12.5	80.2 ± 0.4	114.4 ± 0.1
	50	98.3 ± 2.3	101.1 ± 7.1
	100	101.6 ± 3.3	60.5 ± 10.1
Hexane fraction	1.6	64.7 ± 13.1	136.2 ± 9.2
	12.5	87.6 ± 6.7	136.1 ± 3.3
	50	102.8 ± 12.7	121.7 ± 5.9
	100	111.5 ± 3.6	78.9 ± 10.1
Obliquumol	1.6	82.1 ± 7.4	136.6 ± 11.2
	12.5	86.9 ± 1.7	127.9 ± 6.4
	50	93.5 ± 4.8	118.2 ± 1.0
	100	101.4 ± 1.6	98.6 ± 12.8
O-Methylalloptaeroxylin	1.6	18.8 ± 2.6	121.9 ± 4.4
	12.5	50.9 ± 3.2	111 ± 8.3
	50	94.2 ± 4.9	103.2 ± 3.3
	100	97.6 ± 16.8	93.3 ± 4.7
Lupeol & β-amyrin	1.6	70.3 ± 22.5	126.7 ± 12.2
	12.5	92.0 ± 4.7	121.4 ± 11.9
	50	103.7 ± 18.5	116.3 ± 8.7
	100	118.2 ± 36,8	98.4 ± 11,6
Quercetin	1.6	53.84 ± 6.37	99.82 ± 5.42
	12.5	89.18 ± 7.32	83.35 ± 7.66
	50	93.46 ± 1.71	63.97 ± 3.77
	100	95.68 ± 2.89	39.54 ± 4.31

Conc: Concentration.

3.3. *Effects of* P. obliquum *acetone extracts and isolated compounds on LPS-induced IL-1β production in RAW 264.7 macrophages*

Cytokines play a critical role in maintaining our body's optimal performance and protecting it from dangerous agents. IL-1β is one of the cytokines that is regarded as a pro-inflammatory mediator since it helps to initiate an inflammatory cascade (Nazakat et al., 2025; Zhang and An, 2007). However, IL-1β should be controlled and kept at tolerable levels because an accumulation of this pro-inflammatory cytokine at excessive levels worsens inflammation. TNF and IL-6 are induced by IL-1β (Dinarello, 2000). A dependent cytokine may therefore be reduced if IL-1β production is suppressed. In this study, exposure of the cells to LPS increased the amount of IL-1β production in comparison to the non-stimulated controls (Fig. 1). However, the increase was not significant. Treatment with *P. obliquum* acetone leaf extracts and two tested compounds suppressed the production of the IL-1β levels non-significantly similar to the positive control quercetin (Fig. 1).

3.4. *Effects of* P. obliquum *acetone extracts and isolated compounds on LPS-induced TNF-α production in RAW 264.7 macrophages*

The results of the effects of acetone leaf extracts and the two isolated compounds as potential stimulants of TNF-α production are presented in Fig. 2. Stimulation with LPS induced a significant increase in TNF-α levels of the cells in comparison to the non-stimulated control cells. Quercetin lowered the LPS-induced TNF-α levels and the decrease was significant ($p < 0.001$) with the highest concentration. Treatment with the extracts and compounds also reduced the LPS-induced TNF-α levels and the inhibition was significant with the lower 50 ug/mL concentration of the Walter Sisulu acetone extract ($p < 0.027$) and the highest obliquumol concentration ($p < 0.008$), Fig. 2.

TNF-α is a member of the peptide mediator family. This cytokine acts as an intercellular chemical messenger in inflammatory processes (Riaz et al., 2024; Idriss and Naismith, 2000). Monocytes and macrophages are the major sources of TNF production, but other cells such as T and B lymphocytes, natural killers, mast and endothelial cells, neutrophils, and smooth and cardiac muscle cells can also release this cytokine (Boshtam et al., 2017).

3.5. *Effects of* P. obliquum *acetone extracts and isolated compounds on LPS-induced IL-10 production in RAW 264.7 macrophages*

Interleukin-10 is an effective anti-inflammatory cytokine that is often essential in preventing inflammatory and autoimmune disorders. The IL-10 family of cytokines, which consists of nine members, emerged before the adaptive immune response and has indispensable functions in many infectious and inflammatory diseases (Iyer and Cheng, 2012). IL-10 is mainly produced by CD4 + Th2 cells, monocytes and B cells, with two tightly packed 160 amino acid proteins as a homodimer (Boshtam et al., 2017). The most potent anti-inflammatory cytokine, IL-10 can suppress pro-inflammatory reactions and reduce tissue changes brought on by inflammation (Iyer and Cheng, 2012). The major way that IL-10 exerts its anti-inflammatory effects is by blocking the production of the inflammatory cytokines TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, macrophage inflammatory protein (MIP)-1a, and MIP-2a (Oft, 2014). As demonstrated in Fig. 3, stimulation with LPS triggered a significant ($p < 0.001$) increase in IL-10 concentrations compared to the non-stimulated control cells. Treatment with the extracts and compounds did not increase the IL-10 levels higher than LPS similar to

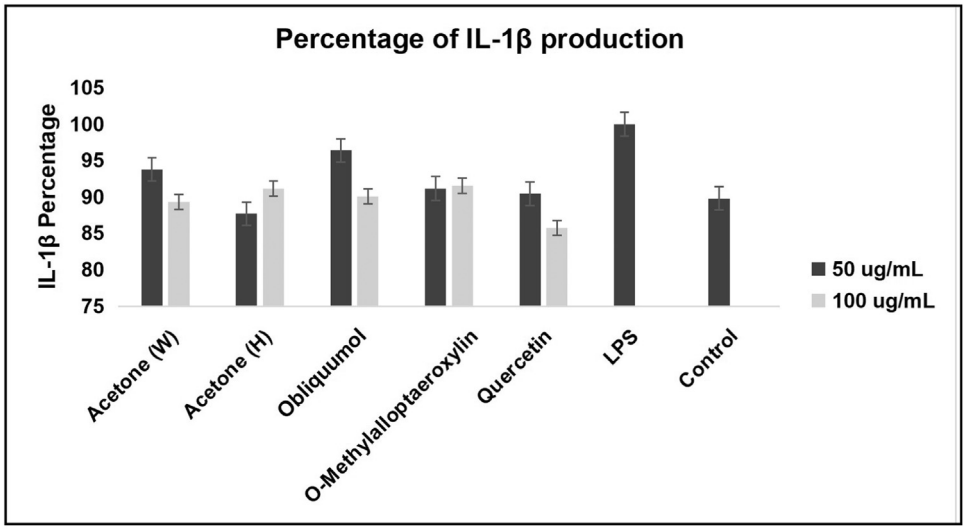

Fig. 1. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and *O*-methylalloptaeroxylin on LPS-induced IL-1β production in RAW 264.7 macrophages treated with different concentrations (50 and 100 μg/mL), and positive controls quercetin and control (No LPS) over 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield), Statistically significant (*).

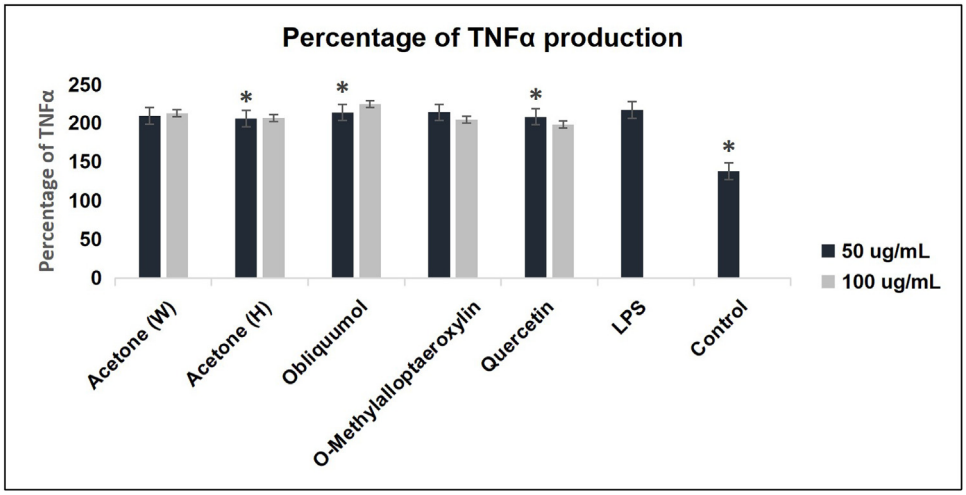

Fig. 2. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and *O*-methylalloptaeroxylin on LPS-induced Tumor Necrosis Factor (TNF-α) production in RAW 264.7 macrophages treated with different concentrations (50 and 100 μg/mL), and positive controls quercetin and control (no LPS) for 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield), Statistically significant (*).

quercetin indicating that they did not have a direct activity on the IL-10 anti-inflammatory cytokine.

3.6. *Effects of P. obliquum acetone extracts and isolated compounds on LPS-induced COX-2 production in RAW 264.7 macrophages*

Steroids and non-steroidal medications have been effectively utilized as anti-inflammatory agents to address numerous inflammatory illnesses over the years. However, numerous drugs have significant adverse side effects, and managing chronic inflammation sometimes necessitates prolonged use. Non-steroidal anti-inflammatory drugs (NSAIDs) can function as COX-2 inhibitors, hence producing anti-inflammatory effects. The suppression of COX-1 correlates with the adverse effects of these medications. Consequently, researchers are seeking bioactive compounds that selectively inhibit COX-2 (Zarghi and Arfaei, 2011). Ibuprofen, paracetamol and aspirin are popularly marketed as anti-inflammatory medication, however, they non-selectively inhibit COX enzymes, leading to various side effects caused by inhibition of COX- 1 regulated functions in the body such as ulceration and gastrointestinal bleeding (Theoduloz et al., 2019; Park et al., 2018; Ho et al., 2018). Celecoxib and rofecoxib are examples of selective COX-2 inhibitors that have been developed. These drugs were more effective at suppressing COX-2 than COX-1 and specifically suppressed the biosynthesis of pro-inflammatory prostaglandins that COX-2 catalyzes (Ho et al., 2018). However, these selective COX-2 inhibitors exhibited severe skin-related and cardiovascular toxicities, including myocardial infarction, leading to partial withdrawal of this class of drugs from the market (Ho et al., 2018).

The inhibition of inducible COX-2 enzymes is crucial in pathological inflammation, as these enzymes contribute to tissue damage, pain, and edema associated with the disease process. This work sought to develop treatment capable of suppressing COX-2 expression or concentrations, serving as effective tools in the prevention or treatment of pathological inflammatory illnesses (Riaz et al., 2024; Baraf, 2007). As shown in Fig. 4, LPS induced a significant increase in COX-2 ($p < 0.001$) compared to non-stimulated control cells. Treatment with quercetin reversed these effects significantly ($p < 0.001$) in a dose dependent manner (Fig. 4). The extracts and the compounds

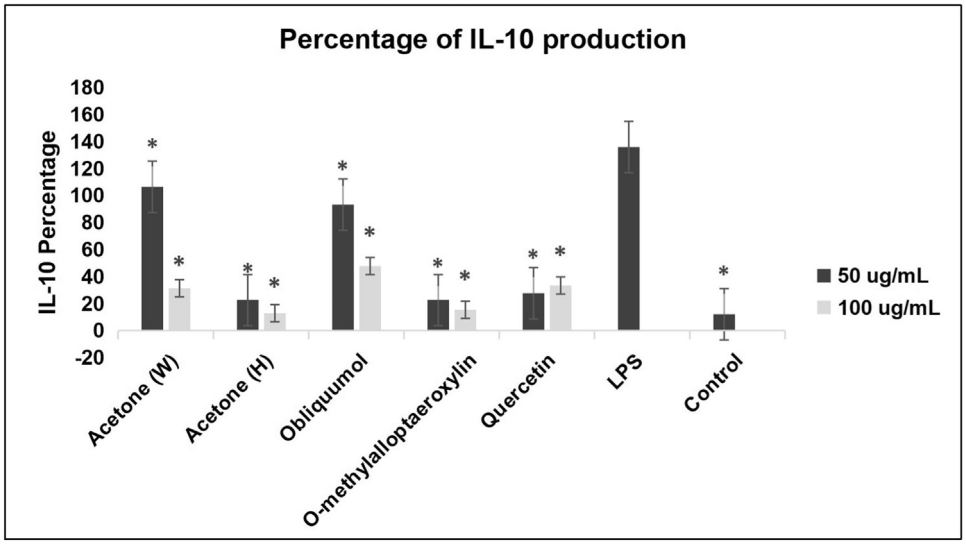

Fig. 3. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and *O*-methylalloptaeroxylin on LPS-induced IL-10 production in RAW 264.7 macrophages treated with different concentrations (50 and 100 μg/mL), and positive controls quercetin and control (no LPS) for 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield), Statistically significant (*).

also reversed LPS-induced COX-2 significantly ($p < 0.001$) in a dose dependent fashion similar to the quercetin treatment. These inhibition effects were more pronounced with the Walter Sisulu acetone extract and obliquumol treatment with COX-2 levels that were even lower than that of the positive control, quercetin. These results demonstrated the significant inhibitory effects of the plant extracts and compounds on LPS-induced COX-2, which is one of the most potent mediators of inflammation, indicating their potential ability to alleviate inflammation in a pathological inflammation (Nkadimeng et al., 2020; Baraf, 2007).

The anti-inflammatory activity of the isolated compounds may explain the traditional uses of *P. obliquum* to treat various inflammatory diseases including rheumatoid arthritis. We believe that *P. obliquum* might be using mechanisms that are like those used by *Curcuma longa* and *Tripterygium wilfordii* to target rheumatoid arthritis. *Curcuma longa* generates a compound known as curcumin, which mitigates inflammation by decreasing oxidative stress, modifying NF-kB transcriptional activity, and blocking PGE2 formation, thus alleviating symptoms of rheumatoid arthritis (Dou et al. 2018). This possible treatment for rheumatoid arthritis also downregulates pro-inflammatory cytokines, including TGF β, TNF-α, IL-1β, and protease (Wang et al. 2019a, b), hence mitigating joint inflammation (Nazakat et al., 2025). Similarly, it functions by inducing macrophage apoptosis via the inhibition of the inflammatory response and attenuation of the NF-kB signaling pathway in arthritic joints (He et al., 2023). COX-2 is an essential enzyme that amplifies inflammation in rheumatoid arthritis, while NF-κB activity regulates COX-2 expression. By inhibiting NF-kB, *P. obliquum* and *Curcuma longa* can suppress COX-2 expression and impede inflammation. The plants exhibit several biological activities, regulating intracellular signaling pathways and

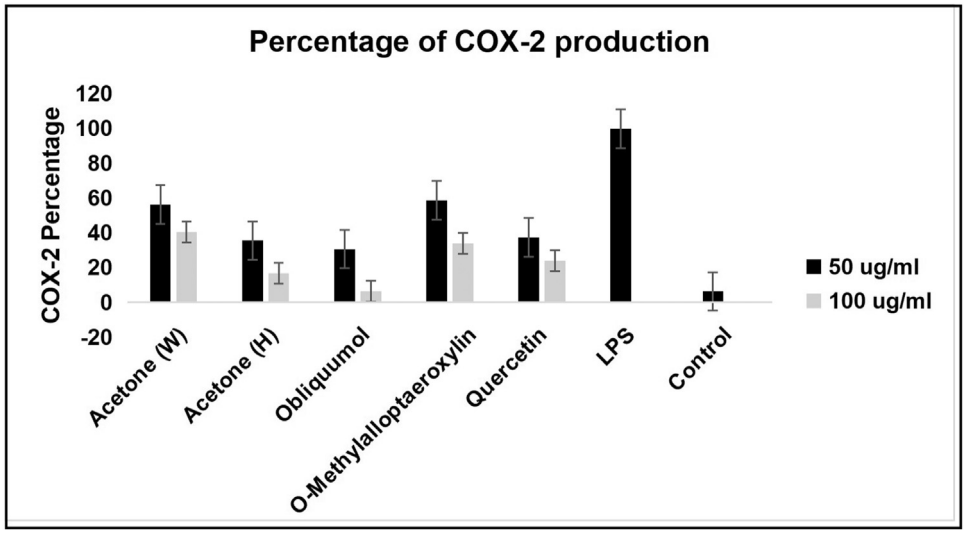

Fig. 4. Effects of *P. obliquum* acetone extracts from different locations, obliquumol and *O*-methylalloptaeroxylin on COX-2 production in RAW 264.7 macrophages treated with different concentrations (50 and 100 μg/mL), and positive controls quercetin (50 μg/mL) and control (no LPS) for 24 h. Key: Acetone (W); Acetone (Walter Sisulu), Acetone (H); Acetone (University of Pretoria, Hatfield), Statistically significant (*).

altering multiple transcription factors (Kou et al., 2023). Similarly, *Tripterygium wilfordii* extracts prevent fibroblasts from producing inflammatory molecules such as prostaglandin E2 (PGE2) synthesis by inhibiting the activity of COX-2. It also suppresses the transcription of the iNOS gene, hence reducing nitric oxide levels (Luo et al., 2023). Consequently, it lowers NF-kB activity by inhibiting its binding to DNA, ultimately further decreasing the transcription of COX-2 and iNOS. These mechanisms may elucidate the conventional applications of *P. obliquum* in the treatment of numerous inflammatory disorders (Nazakat et al., 2025).

4. Conclusion

The purpose of the current study was to investigate using *in vitro* studies the potential effectiveness of *P. obliquum* in treating pain and inflammation. Isolated compounds and acetone extracts of plant material collected from several locations had anti-inflammatory effects by reducing the expression of the pro-inflammatory cytokine IL-1 in macrophages stimulated by LPS, and also by inhibiting the production of 15-LOX, NO and COX-2. *P. obliquum* acetone extract, fractions and isolated compounds may reduce inflammation by inhibiting pro-inflammatory cytokines, activating IL-10, and inhibiting NO and 15-LOX. However, in order to properly understand the mechanism of action, it is necessary to continue investigations on the effect of the extracts and compounds on COX-2 and iNOS. The historic usage of *P. obliquum* to treat inflammatory-related illnesses and symptoms is supported by the *in vitro* anti-inflammatory efficacy in various bioassays by its acetone extracts, fractions and isolated components, providing motivation for additional *in vivo* studies. The study advocates for additional *in vivo* investigations of the isolated compounds, as it is crucial to assess their safety and efficacy, given that *in vitro* activity may not correspond to *in vivo* effects. The results indicate the anti-inflammatory activity of obliquumol in addition to other beneficial properties such as antifungal, antiparasitic and anticancer activities (Khunoana et al., 2022; Ramadwa et al., 2021).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The corresponding author is an Editorial Board Member of South African Journal of Botany.

CRediT authorship contribution statement

Edward Thato Khunoana: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Jacobus Nicolaas Eloff:** Writing – review & editing, Supervision, Conceptualization. **Thanyani Emelton Ramadwa:** Writing – review & editing, Supervision, Conceptualization. **Sanah Malomile Nkadimeng:** Validation, Formal analysis, Data curation. **Lyndy Joy McGaw:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Appendix

Fig. A1.
Additional data

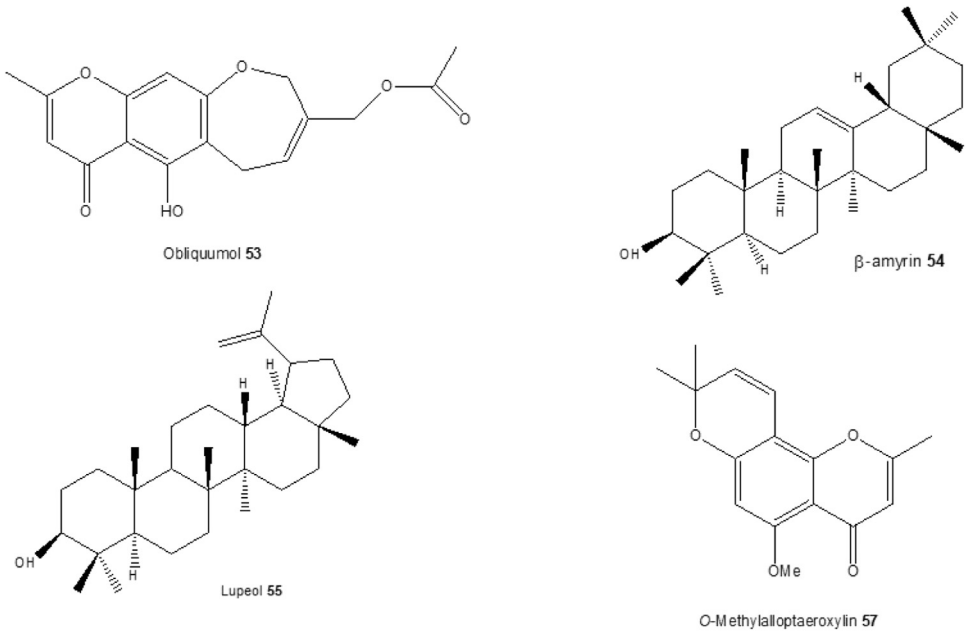

Fig. A1. Structures of isolated compounds from *P. obliquum* leaves (Khunoana et al., 2022).

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