To investigate the anti-inflammatory activity of sub-fraction F3.3 and sub-fraction F3.3.0 derived from the sub-fraction F3.3 that was derived from Peltophorum africanum leaf.

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First isolation of glutinol and a bioactive fraction with good anti-inflammatory activity from n-hexane fraction of Peltophorum africanum leaf

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ABSTRACT

Objective: To investigate the anti-inflammatory activity of different fractions and glutinol (isolated compound), using nitric oxide synthase and cyclooxygenase (COX) inhibition as an indication of anti-inflammatory activity.

Methods: Anti-inflammatory activity was evaluated using an in vitro assay determining the inhibition of the activity of pro-inflammatory enzyme model. Cyclooxygenases and inducible nitric oxide synthase are crucial enzymes involved in the pathogenesis of many chronic inflammatory conditions.

Results: Sub-fraction F3.3 that was derived from n-hexane fraction of PA leaves significantly inhibited (P = 0.01) the catalytic activity of COX-2 (IC50 = 0.67 μg/mL) better than isolated compound, glutinol (IC50 = 1.22 μg/mL), compound 2 (CP2) (IC50 = 1.71 μg/mL) and sub-fraction F3.3.0 (IC50 = 1.30 μg/mL). A similar trend was observed in investigation of the inhibition of nitric oxide synthesis in RAW 264.7 cells by F3.3, glutinol, CP2 and F3.3.0. Inducible COX-2 and inducible nitric oxide synthase are among potent signalling enzymes that exacerbate inflammation.

Conclusions: Bioactive sub-fractions (F3.3 and F3.3.0) derived from the n-hexane fraction of PA had good anti-inflammatory activity, and the isolated compound, and glutinol may be useful as a template for the development of new anti-inflammatory drugs.

1. Introduction

Pro-inflammatory enzymes such as inducible cyclooxygenases 2 (COX-2) and nitric oxide synthase (iNOS) are important mediators of inflammation. Cyclooxygenases promote inflammation through the synthesis of prostaglandins, which, in turn, propagate inflammation. Nitric oxide (NO) is a signalling molecule produced by iNOS, and has a key role in the pathogenesis of inflammation [1]. Under normal physiological conditions, NO has anti-inflammatory effects. However, overproduction and release of NO by endothelia and immune-competent cells is implicated in the pathogenesis of inflammatory diseases involving the joints, lung and gut [2].

The painful symptoms associated with chronic inflammatory diseases are treated with non-steroidal agents targeting COX-1/COX-2. However, these modern single compound drugs are associated with undesirable adverse effects such as gastric ulceration, renal toxicity, joint destruction and cardiovascular disorders [3]. The potential for plant-derived remedies to alleviate painful conditions associated with inflammatory diseases has been described in some studies [4,5]. Furthermore, the therapeutic use of medicinal plants continues to gain popularity in developed countries, where it was previously considered of little value due to a lack of empirical proof of their efficacy of herbal remedies.

Peltophorum africanum (PA) is a medicinal plant species used widely in southern Africa to treat pain associated with many chronic conditions such as arthritis. Extracts of the leaves, root and bark are used in traditional medicine to treat infections, pain and aches [6]. Some biologically active compounds have been isolated from PA, but to our knowledge, this is the first

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report describing the isolation of glutinol, a compound with known anti-inflammatory activity [7-9], from the n-hexane fraction of PA. Compounds with anti-inflammatory activity are believed to mediate the activities of pro-inflammatory mediators such as COX-2, or at least act as scavengers of free radicals such as NO.

2. Materials and methods

2.1. Plant materials

*Peltophorum africanum* is known with several local names as weeping wattle (English), dopperkiaat (Afrikaans), umsehla (Ndebele), muzeze (Shona), mosëthlha (Tswana), isiKhamba-mkhombe (Zulu). Fresh leaves of PA were collected into open mesh orange bags from a tree in the garden of the Faculty of Veterinary Sciences, University of Pretoria, Onderstepoort in September 2013, authenticated by a botanist, Dr. Lita Pauw. Leaves were allowed to dry at room temperature for two weeks in a well-ventilated room. After proper drying, the plant material was ground into fine powder using a MacSalab model 200 grinder and stored in closed and labelled honey jars. The honey jars were kept in a dark cupboard for storage until used. A herbarium specimen was prepared and deposited at HGWJ Schweickerdt Herbarium, University of Pretoria (PRU number 120792).

2.2. Column chromatography

The sub-fraction (F3.3) yield was 0.878 g from 220.56 g of n-hexane fraction of ground dried leaf of PA (1000 g). This represents a yield of 0.4% from the n-hexane fraction. Column chromatography is a widely used technique for the separation of compounds in mixtures such as plant extracts.

The column was packed with 87.8 g of silica gel (1:100) made in suspension with n-hexane, and poured into a separation column for good compound separation. Compound separation was initiated with 100% n-hexane, with polarity increased stepwise with addition of ethyl acetate (95:5, 90:10). The column was washed with absolute methanol after collecting the separated compounds into clean test-tubes.

2.3. Thin layer chromatography (TLC) techniques

The TLC is a method for analysis of mixtures through adsorption. This method can be used to determine the diversity of compounds in mixtures such as plant extracts. The fractions were collected into clean vials and the solvent was evaporated at room temperature to determine the yield. The TLC fingerprinting methods was used as a guide for further purification of F3.3 and isolation of compounds from F3.3.2. Fraction F3.3 contained several compounds that were separated into F3.3.1–F3.3.8 (Figure 1). It was observed from the TLC plate that F3.3.2 contained at least two compounds in significant amounts and this led to further purification of F3.3.2 to yield two compounds, glutinol and CP2 (Figure 2). The remaining fractions (F3.3.1, F3.3.3, F3.3.4, F3.3.5, F3.3.6, F3.3.7 and F3.3.8) were combined as F3.3.0 and were tested along with the isolated compounds in subsequent bioassays.

The compound yield from 60 mg of F3.3.2 was 21 mg for glutinol and 24 mg for CP2, respectively. The Rf value for each of the compounds was calculated by dividing the distance travelled by the compound by the solvent front (distance travelled by the solvent). The Rf value for the compounds were 0.31 (glutinol) and 0.44 (CP2) in n-hexane/ethyl acetate (9:1) solvent system respectively (Figure 2).

After complete solvent evaporation F3.3.2, glutinol and CP2 were reconstituted to 10 mg/mL with acetone and spotted onto a silica gel plate (Aluminium sheet 20 cm × 20 cm, TLC Silica gel 60 F254, Merck, Germany) at 10 μL using a micropipette. The silica gel plate was developed in n-hexane/ethyl acetate (9:1). The dried silica gel plate was placed under UV at 254 and 366 nm to visualize any fluorescing compounds. None of the isolated compounds fluoresced at those wavelengths. The plate was sprayed with vanillin (0.1 mg vanillin dissolved in 28 mL methanol, with 1 mL concentrated sulphuric acid slowly added) in order to visualize the compounds by gently heating the silica gel plate to 110 °C. Glutinol appeared bluish on the TLC plate while CP2 appeared purplish, with different intensities (Figure 2). There appeared to be a higher concentration of CP2 than glutinol based on the intensities of the colours of the compounds on the TLC plate.

![Figure 1. Thin-layer chromatography profile for F3.3 and F3.3.1-F3.3.8 in BEA solvent system (90:10:1).](image-url)
2.4. COX-1/COX-2 inhibition by fraction and isolated compounds from the leaf of PA

The Cayman COX colorimetric inhibitor screening assay kit (Cayman, item No. 701050) was used for the assessments of the inhibitory activity of F3.3, F3.3.0, glutinol and CP2 (dissolved in 100% DMSO) on COX-1 and COX-2 by following the manufacturer's guidelines. In brief, 160 μL of assay buffer, 10 μL of heme and 10 μL of enzyme (COX-1/COX-2) was added to appropriate well in a 96-well microtitre plate. Thereafter, 10 μL of inhibitor (F3.3, F3.3.0, glutinol and CP2) were added to appropriate wells, DMSO was used for negative controls. Indomethacin (1 mg/mL) dissolved in 100% DMSO was used for positive controls and the samples were tested at 10 mg/mL (F3.3 and F3.3.0) and 1 mg/mL (glutinol and CP2) dissolved in 100% DMSO respectively. The microtitre plate was shaken carefully and incubated at 25°C for 5 min. After the incubation period, 20 μL of colorimetric substrate solution and 20 μL of arachidonic acid (substrate) were added to appropriate wells. The microtitre plate was carefully shaken and incubated at 25°C for exactly 2 min. After the incubation period, the absorbance was read at 590 nm using MutiskanGO (Thermo scientific, Finland). The experiment was conducted in triplicate and repeated so that each value represents the average of six determinations. The percentage inhibition was plotted against the concentrations of the inhibitors (samples) to determine the IC50 (concentrations that resulted in 50% inhibition of enzyme activity) for each sample.

2.5. Nitric oxide inhibitory assays

The procedure for NO inhibitory assay has been described elsewhere [10].

2.6. Isolation and characterization of compounds

Isolation of glutinol, CP2 from F3.3.2 (60 mg) was carried out using a small column packed with silica gel with 100% n-hexane. In brief, compounds were eluted with 100% n-hexane with increasing polarity with 5% ethyl acetate resulting in sequential elution of glutinol (21 mg) and CP2 (24 mg), respectively. The initial nuclear-magnetic resonance (NMR) spectral data for CP2 indicated the presence of impurities, and further attempt at purification did not yield enough material for structural elucidation. Glutinol had a clearer NMR-spectral probably due to the purity of the compound isolated. The spectral data for glutinol was compared with data reported by Sule et al. [8] and was found to be similar (Table 1).

2.7. 13C and 1H NMR-procedures and structural elucidation of isolated compounds

The NMR analysis was conducted on glutinol and CP2 at the Chemistry department of the Tshwane University of Technology (TUT), Pretoria by Dr. Khosi Gamedze. The experiments were performed on an NMR spectrometer operating at 100.52 MHz for 13C and 399.76 MHz for 1H respectively using deuterated chloroform (CDCl 3) as solvents. Structure elucidation and identification of isolated compounds was done by Prof Ladislaus Mdee of the Department of Pharmacy, University of Limpopo, South Africa.

3. Results

The results indicated that glutinol, compound 2 (CP2), fractions F3.3.0 and F3.3 inhibited the activity of COX albeit non-selectively, and NO synthesis in RAW 264.7 cells in a similar manner.
3.1. COX-1/COX-2 inhibition by F3.3, F3.3.0, glutinol and CP2

Fraction F3.3 significantly inhibited COX-2 (IC50 = 0.67 µg/mL) better than glutinol (IC50 = 1.22 µg/mL), and two-fold better than CP2 (IC50 = 1.71 µg/mL), F3.3.0 (IC50 = 1.30 µg/mL) and indomethacin, the positive control (IC50 = 1.33 µg/mL). In addition, F3.3 also inhibited the activity of COX-1 with an IC50 of 0.70 µg/mL.

Inhibitory activity (expressed as IC50 values) of F3.3, F3.3.0, glutinol and CP2 on COX-1 and COX-2 were as follows, INDO: 1.27 µg/mL and 1.33 µg/mL, respectively; F3.3: 0.70 µg/mL and 0.67 µg/mL, respectively; Glutinol: 1.25 µg/mL and 1.22 µg/mL, respectively; CP2: 0.93 µg/mL and 1.71 µg/mL, respectively; F3.3.0: 0.88 µg/mL and 1.30 µg/mL, respectively. The bioactive F3.3 significantly inhibited COX-1 (P = 0.008) and COX-2 (P = 0.002) better than indomethacin positive controls, respectively.

3.2. Nitric oxide inhibitory assay results

The results indicated that CP2, F3.3 and F3.3.0 significantly inhibited NO production in a dose-dependent manner (Figure 3). In particular, F3.3, CP2 and F3.3.0 significantly inhibited NO synthesis by RAW 264.7 cells at 100 µg/mL (Figure 3). The results further indicated that the combined compounds in F3.3 inhibited NO production in RAW 264.7 macrophage cell lines better than individual compound in isolation, a similar trend observed with COX inhibition.

3.3. Physical properties and reported biological activities of glutinol

Glutinol was obtained as a white crystalline solid, highly non-polar with molecular formula of C30H50O [8]. The structure of glutinol (Figure 4) has been published in some literature reports [8,9] including its anti-inflammatory and analgesic activities [7]. The mechanism of anti-inflammatory action of glutinol is thought to be similar to the action of NSAIDs such as indomethacin [7]. Such mechanism of action is characterised by the inhibition of the synthesis of prostaglandin, hence a peripheral mechanism of pain inhibition [11].

4. Discussion

Cyclooxygenases (COX-1/COX-2) are responsible for the synthesis of prostaglandins from arachidonic acid, a prominent molecule responsible for the exacerbation of inflammation [10–12]. The resolution of uncontrolled inflammatory response requires anti-inflammatory agents to inhibit the activity of these mediators. However, because F3.3 inhibited both enzymes non-selectively, this is an indication that it is a non-specific inhibitor of COX. Inhibition of COX-2 is more desirable because inhibition of COX-1 in the gastric mucosa is associated with gastric problems [13,14]. A similar trend was observed in the activity of glutinol, CP2 and F3.3.0, which were all non-specific inhibitors of COX. Indomethacin was also non-specific in its inhibition of
COX in this study. The COX inhibitory activity of F3.3 (more active than glutinol and CP2) could be attributed to a synergistic effect of glutinol and CP2, because they were all derived from fractionation of F3.3. The additive or synergistic effect of naturally occurring compounds has been reported previously [15]. Fraction F3.3 was a better inhibitor of COX than glutinol, CP2 and F3.3.0 in isolation. Fractions or compounds with specific inhibition of COX-2 are considered as promising candidate for anti-inflammatory drug development [14].

The synthesis of NO by the cells was increased as the concentrations of the test compound reduced from 30 µg/mL to 2 µg/mL in a dose response manner. Although, glutinol inhibited NO release at 100 µg/mL, it was not as active as F3.3, CP2 and F3.3.0 at the same concentration. Nitric oxide is a signalling molecule and a potent mediator of the inflammatory process. Furthermore, NO also mediates a variety of biological actions ranging from vasodilatation, neurotransmission, inhibition of platelet adherence and aggregation, as well as the macrophage- and neutrophil-mediated killing of pathogens [2,16]. Various inflammatory mediators, such as bradykinin and histamine, produce vasodilation through stimulation of endothelial release of NO [17].

The study results led to the following conclusions: Fraction F3.3 significantly inhibited COX-2 synthesis in RAW 264.7 cells better than indomethacin that is used widely to treat pain associated with inflammation. The results also indicated that F3.3 had the best NO inhibitory activity among the samples tested. This suggests that, the bioactive compounds present in F3.3 act synergistically to exert their anti-NO synthesis activity. The mechanism of action was presumed to involve the modulation of the activity of iNOS, the inducible enzyme responsible for NO synthesis during inflammation. Because F3.3 inhibited COX synthesis non-selectively, work is on-going to determine the compound(s) responsible for COX-2 inhibition in F3.3.

Conflict of interest statement

We declare that we have no conflict of interest.

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