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In-vitro and *in-vivo* anti-inflammatory properties of extracts and isolates of Pangdahai

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ABSTRACT

Background: **:** Pangdahai (matured, ripened, and dried seeds of *Scaphium affine* (Mast.) Pierre) is widely used in managing several diseases in countries like China, Vietnam, Japan, and India. This study evaluated the anti-inflammatory effects of the crude extracts (ethanol and aqueous) and isolated compounds of Pangdahai.

Methods: **:** Xylene-induced ear edema in mice, carrageenan-induced paw edema in rats, and nitric oxide (NO) assay were used to evaluate and screen the crude extracts and isolated compounds from the ethanolic extracts of Pangdahai. TNF-α and IL-1β levels in the tissues of rat foot and ear were determined by ELISA. The cytotoxicity of the isolated compounds was also determined by MTT assay. Molecular docking studies using targets involved in the inflammatory process were also used to further evaluate the compounds.

Results: **:** Both aqueous and ethanol extracts demonstrated significant anti-inflammatory effect and markedly attenuated vascular permeability in mice induced by acetic acid in a dose-independent manner. The ethanol extract also significantly inhibited levels of IL-1β and TNF-α. Four (4) compounds exhibited significant inhibitory effects on NO release without cytotoxicity on RAW 264.7 macrophage. These compounds also showed good binding affinities for COX-2, PLA2, IRAK-4 and NIK.

Conclusions: This study validates, provides scientific evidence and justification for the use of the aqueous decoctions of Pangdahai in pharyngitis traditionally. (+) – Pinoresinol, tiliroside, *Z*-caffeic acid, and 3,4-dihydroxybenzoic acid (protocatechuic acid) isolated from Pangdahai showed anti-inflammatory activities, which might be responsible for the actions of Pangdahai. Tiliroside showed high binding affinity comparable to the native ligands of inflammatory mediators.

List of abbreviations

COX-2	cyclooxygenase-2
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's modified eagle medium
ELISA	enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
IL	Interleukin
iNOS	inducible nitric oxide synthase
IRAK-4	Interleukin-1 Receptor-Associated Kinase-4
LPS	Lipopolysaccharide
NIK	NF-κB–Inducing Kinase
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory agents
NTF	Tumor necrosis factor
OD	Optical density
PBS	Phosphate buffered saline
PDH	Pangdahai

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PGE	Prostaglandin E2
PMSF	phenylmethylsulfonyl fluoride
PLA2	Phospholipase A2
TCH	Traditional Chinese Medicine
WHO	World Health Organization.

1. Introduction

Inflammation can be said to be one of the body's reaction to tissue damage or infection and is typically characterized by redness, swelling, heat, and pain (Megha et al., 2021). In inflammation, there can be activation of inflammatory mediators like chemokines and cytokines (Zhu et al., 2018). There are three main phases of inflammation. Phase 1is marked by an increase in vascular permeability leading to the exudation of fluids from the blood into the interstitial space; phase 2 involves the leukocytes infiltration from the blood into the tissues, and phase 3 is distinctively shown by granuloma formation and tissue repair (Mukhopadhyay et al., 2019). Edema is also a characteristic feature of the acute inflammation (Li et al., 2021; Tian et al., 2021). Inflammation is a common symptom for most disease conditions, some of which include pharyngitis, bowel diseases, arthritis, allergic rhinitis, and atopic dermatitis (Zhu et al., 2018). Therefore, regulating mediators of inflammation could decrease disease severity or progression.

Conventionally, inflammatory conditions are managed with anti-inflammatory agents. These agents could be steroidal or non-steroidal in nature. The steroidal agents include glucocorticoids, such as prednisone, prednisolone, triamcinolone, methylprednisolone, and dexamethasone. Glucocorticoids can cause side effects like high blood sugar, difficulty responding to insulin, high blood pressure, muscle weakness, vulnerability to infections, Cushing's syndrome, stomach ulcers, and mental health issues (Yang and Yu, 2021). The non-steroidal anti-inflammatory agents (NSAIDs), such as piroxicam, aspirin, aceclofenac, ibuprofen, diclofenac, naproxen, indomethacin, and celecoxib, mainly inhibit the synthesis of prostaglandin or cyclooxygenase. Despite their clinical utility, NSAIDs are also known to cause gastric ulcers, liver and kidney damage (Olry de Labry et al., 2021). NSAIDs elevate blood pressure and increase the risk of myocardial infarction (Patrono, 2016).

The contribution of natural products to maintaining health and wellbeing is underestimated. Natural products are the engine behind the successes of traditional medicine and/or herbal medicine practices. Medicinal plants are good sources of secondary metabolites which form the basis for most commercially produced pharmaceuticals and herbal remedies (Li et al., 2020). The use of medicinal plants in preventing and treating/curing human diseases dates back to antiquity. The analgesic and antipyretic properties of the bark of the willow tree have long been documented by the Greeks and Romans (Montinari et al., 2019). Empirical knowledge of these medicinal plants and their potential toxic effects were passed on by oral tradition and sometimes recorded in texts (Jansen et al., 2021). Monographs on specific herbs are accessible from several sources, for example, the European Scientific Cooperative on Phytotherapy and the World Health Organization (WHO, 2019). Furthermore, Traditional Chinese Medicine (TCM) has attracted interest, acceptability, and significance in many countries. TCM continues to play a major role in the management of diseases and is also an excellent source in the discovery of natural bioactive compounds or lead compounds (Wang et al., 2018).

Pangdahai (PDH) is the dried seeds of *Scaphium affine* (Mast.) Pierre, of the family Malvaceae (Medicinal Plant Name Services, 2021). In the Chinese Pharmacopoeia, it is recorded as *Sterculia lychnophora* Hance Pierre (scientific synonym) (Chinese Pharmacopoeia Commission, 2015). PDH is famously used in traditional/folk medicine in Asia (China, Japan, Vietnam, Thailand, and India). Decoctions of PDH are used for treating pharyngitis, laryngitis, constipation, cough, menorrhagia, and pain. The crude extracts and isolates of PDH have shown diverse pharmacologic effects, including anti-inflammatory, neuroprotective, anti-microbial, anti-hypertensive, analgesic, antipyretic, anti-ulcer, and anti-oxidative effects (Oppong et al., 2018). Clinically, PDH is notable for treating chronic pharyngitis in China (Oppong et al., 2018). Data also suggest that it contains many secondary metabolites such as lignans, phenylpropanoids, flavonoids, nitrogenous bases, phenolic acids, heterocyclic aromatic acids, phytosteroids, glycosides, sesquiterpenoids, and nucleosides (Oppong et al., 2020). Indeed, continuous investigations must be done to ascertain and validate the traditional or folkloric uses of plants and their extract. This work reports, the anti-inflammatory properties of the aqueous and ethanol extracts and some isolated secondary metabolites of PDH for the first time.

2. Methods

2.1. Chemicals and reagents

Dexamethasone acetate was purchased from Zhejiang Xianjun Pharma Ltd., China. The water used was purified with Millipore Milli Q plus purification system (Thermo Fisher Scientific, USA) Carrageenan, xylene, physiological normal saline solution, 0.6 $\%^{v}/_{v}$ acetic acid solution, 0.5 $\%^{w}/_{v}$ Evans blue solution, Griess reagent were purchased from Sigma, USA. Bacterial lipopolysaccharide (LPS) was purchased from Sigma, USA. RAW 264.7 murine macrophage cell line was bought from the American Type Culture Collection (USA). Fetal Bovine Serum (FBS) was obtained from Hyclone (USA), Dimetylsulfoxide (DMSO) from Solarbio (China), and Dulbecco's modified eagle medium (DMEM) from Thermo Fisher Scientific (USA). All other reagents used were of analytical grade and commercially available.

2.2. Preparation of PDH extracts

The PDH was obtained in March 2016 from the Guangxi province (China). A voucher specimen (No.: 20161205SL) was kept at the Tianjin State Key Laboratory of Modern Chinese Medicine at Tianjin University of Tradition Chinese Medicine, China.

Briefly, 1 kg each of PDH was extracted separately with water and 95 $\%^{v}/v$ ethanol, concentrated and dried *in vacuo* to yield 20.80 and 6.50 $\%^{w}/_{w}$ of aqueous and ethanol extracts, respectively as previously described in our work Oppong et al., 2020.

2.3. Acquisition of animals

Male Sprague–Dawley rats, SPF grade (200–220 g) were obtained from Shandong Yantai Raphael Biotechnology Co. (China). Male Kunming mice (20 ± 2 g) were obtained from Shandong Yantai Raphael Biotechnology Co. (China). The experimental animals were kept in a temperature- and humidity-controlled room (23 °C, 60 % air humidity). They had unrestricted access to standard diet and water. They were kept in separate metabolic cages with no food but unrestricted access to water for 12 h before the experiment. All procedures conformed to the Guidelines associated with Care and Use of Laboratory Animals (National Institutes of Health).

Xylene–induced ear edema in mice

In brief, 35 male Kunming mice, weighing averagely 20 ± 2 g, were randomly grouped into 5: the model (negative control), dexamethasone (positive control), low dose of PDH, medium dose of PDH and high dose of PDH groups. With the aqueous extract of PDH, the low, medium, and high dose groups were treated with 200, 400, and 800 mg/kg.d (bw) of the extract. With the ethanol extract, the low, medium, and high dose groups were treated with 20, 40, and 80 mg/kg.d (bw) of the extract. For both extracts, 6 mg/kg.d (bw) of dexamethasone was used as a positive control. The test agents (extracts and dexamethasone) were administered directly into the stomach by oral gavage at a volume of 0.2 mL/10 g using normal saline as the vehicle.

The negative control group was given 2 mL of normal saline once daily. After the fifth day of treatment, 0.1 mL of xylene was evenly smeared on both the inner and outer sides of the right ear of each of the mice (to induce edema), and the left ear was left as the control. The mice were sacrificed after 4 h. The left and right ears were cut along the ear line. Ear discs were cut from both ears from the same part of each ear of the same mouse with a stainless-steel perforator (diameter: 6 mm). The ear discs were then weighed with an analytical balance (Zhao et al., 2018). The degree of edema was evaluated by the difference in weight between the right and left ear discs of the same mice. The degree of edema inhibition was used as an index of the anti-inflammatory activity of the extracts.

$$\text{Ear edema}(\text{mg}) = \text{weight of left ear disc} - -\text{weight of right ear disc}$$

The ear tissues were stored in a refrigerator at -80 ℃.

2.5. Carrageenan-induced paw edema in rats

Male Sprague Dawley rats (SPF grade), weighing an average of 200 ± 20 g, were randomly put into 5 groups of 7 animals. One set of the animals were assessed using the aqueous extract of PDH, and another set of animals were assessed using the ethanol extract of PDH. For the first set (aqueous extract), the grouping included 7 rats put in 5 group: negative control (2 mL normal saline), dexamethasone (positive control - 6 mg/kg·day bw), low dose of aqueous extract of PDH (200 mg/kg·day bw), medium dose PDH (400 mg/kg·day bw), and high dose PDH (800 mg/kg·day bw). The extracts were administered directly into the stomach by oral gavage at a volume of 0.2 mL/10 g using normal saline as the vehicle.

The ethanol extract of PDH followed the same procedure (7 rats in 5 groups) as done for the aqueous extract: low, medium, and high dose groups received 20, 40, and 80 mg/kg·day (bw). Dexamethasone was used as a positive control at 6 mg/kg·day (bw). The negative control group received 2 mL of normal saline once daily.

Two (2) hours after the last treatment, the volumes of both hind-paws up to the ankle joint of the rats were measured with a plethysmometer. Afterwards, the rats were injected with 1 $\%^{w}/_{v}$ carrageenan solution (0.1 mL each) into the distal end of their left hind limbs. The paw volumes were measured again after 1, 2, and 4 h. Each measurement was done in triplicate (Rezq et al., 2021). The rats were then sacrificed by injecting 3 mL of 10 $\%^{v}/_{v}$ chloral hydrate solution into their abdominal cavity. The paw tissues were removed and stored in a refrigerator at -80 ℃.

The degree of edema in the rats was calculated as the difference in weight between the paw volumes measured before carrageenan injection (basal volumes (V_B)) and after carrageenan injection (pathological volumes (V_A)). Edema inhibition, relative to the percentage increase in paw volume, was used as an index of the anti-inflammatory activity of the extracts.

$$\text{Percentage increase in paw volume} = \{(V_A - V_B) / V_B\} \times 100$$

Where

V_A: Rat paw volume after carrageenan injection

V_B: Rat paw volume before carrageenan injection

2.6. Acetic acid-induced vascular permeability in mice

Thirty-five male Kunming mice, weighing averagely 20 ± 2 g, were randomly grouped into 5: the negative control, dexamethasone, low, medium, and high groups. For the aqueous extract, mice in the low, medium, and high dose groups were given 200, 400, and 800 mg/kg·day (bw) of the extract. The extracts were administered directly into the stomach by oral gavage at a volume of 0.2 mL/10 g using normal saline as the vehicle. For the ethanolic extract, mice in the low, medium, and high dose groups were given 20, 40, and 80 mg/kg·day (bw) of extracts. The positive control was Dexamethasone at 6 mg/kg·day (bw) in both cases. After the fifth day of treatment, the tails of the mice were injected with 0.5 $\%^{w}/_{v}$ Evans blue saline solution (0.2 mL), followed by an injection of 0.2 mL 0.6 $\%^{v}/_{v}$ acetic acid 0.2 mL intraperitoneally. After sacrificing the mice, 10 mL saline solution was to wash their peritoneal cavities (3x). The saline washings were pooled, filtered and centrifuged (3000 rpm, 10 min) to obtain the supernatant (5 mL). The optical density (OD) values of the supernatant were measured at 590 nm with a UV–Vis spectrophotometer (Rezq et al., 2021).

The intraperitoneal injection of dilute acetic acid causes an increase in capillary permeability, and this can cause Evans blue to extrude into the abdominal cavity. The amount of Evans blue represented the capillary permeability, which was estimated by measuring the optical density values of the supernatant.

2.7. Histo-pathological study of sections of mice edematous ear induced by xylene

The ear tissues of the negative control group, dexamethasone group, and PDH ethanol extract (low – high dose) groups were kept in 10 $\%^{v}/_{v}$ formaldehyde solution for 24 h to prepare paraffin sections. The paraffin sections were dewaxed and then stained with hematoxylin and eosin. The pathological changes of the local tissues of the mice auricles were observed under a light microscope to ascertain the degree of inflammation (Huang et al., 2011).

2.8. Determination of the levels of TNF-α and IL-1β in the rat foot tissue

The rat foot tissues stored at -80 °C were obtained and crushed into centrifuge tubes. Afterwards, 500 μL PBS and 5 μL PMSF (100 mM) were added and placed on an ice water bath for 30 s and then centrifuged at 4 °C, 13,000 r/min for 6 min. The supernatant was collected, and the amount of protein was estimated with Bradford method kit. The TNF-α and IL-1β levels contained in 1 mg protein of rat foot tissue were measured using enzyme-linked immunosorbent assay (ELISA) (Huang et al., 2011). This was repeated for samples obtained from rats treated with the ethanolic extract of PDH (showed the highest activity).

2.9. Determination of the levels of TNF-α and IL-1β in mouse ear tissue

The ear tissues of the mice stored at -80 °C were removed, crushed and placed in centrifuge tubes. Afterwards, 500 μL PBS and 5 μL PMSF (100 mM) were added and placed on an ice water bath for 30 s and then centrifuged at 4 °C, 13,000 r/min for 6 min. The supernatant was collected, and the amount of protein was estimated with Bradford method kit. TNF-α and IL-1β levels in 1 mg protein of mice ear tissue were measured using enzyme-linked immunosorbent assay (ELISA), according to manufacturer's protocol (Huang et al., 2011). This was repeated for samples obtained from mice treated with the ethanolic extract of PDH that showed the highest activity.

2.10. Isolation and characterization of compounds from Pangdahai

Compounds from PDH extracts were isolated and characterized using various chromatographic and spectroscopic techniques described in our previous work (Oppong et al., 2020).

2.11. In vitro anti-inflammatory screening of isolated compounds

2.11.1. Cell culture and MTT assay

Complete DMEM media containing 10 $\%^{v}/_{v}$ FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin was used to culture RAW 264.7 macrophage cells. The culture was incubated in a humidified incubator set at 5 % CO_2 and 37 ℃ with daily replacement of the culture media. The cells were then seeded at 1 × 106 cells/well in a 96-well microtiter plate. After overnight incubation, LPS (1 μg/mL) with or without the isolated compounds from PDH (Uridine, Ethyl-3,4-dihydroxy benzoate, (+) – Pinoresinol, Daucosterol, Vomifoliol, 2-(Hydroxymethyl)–

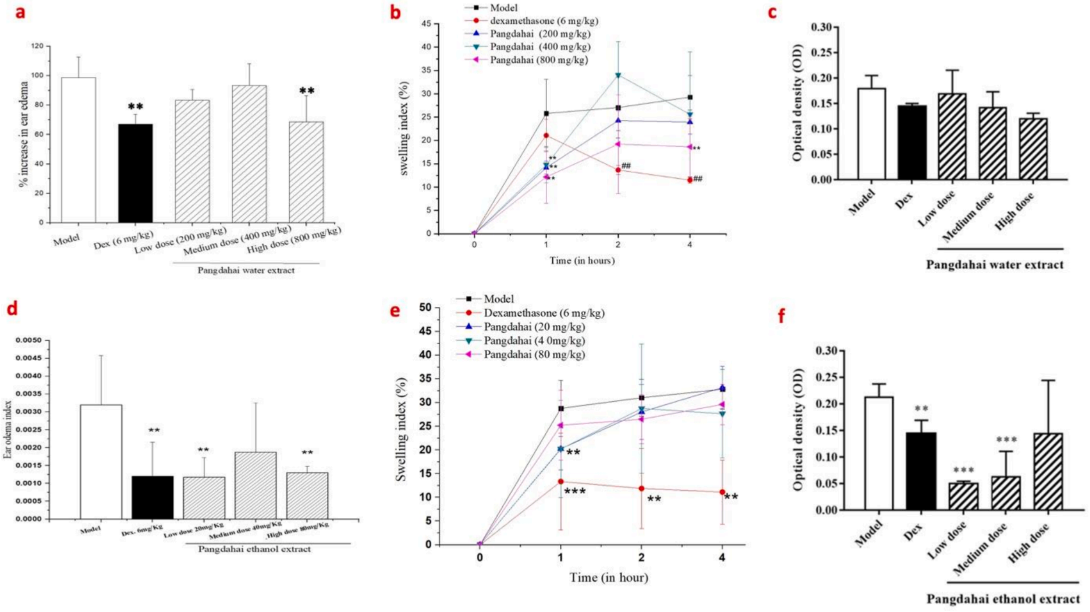


Fig. 1. : a. Effect of dexamethasone and aqueous extracts of PDH on xylene-induced ear edema in mice. b. Effect of Pangdahai aqueous extract on carrageenan-induced paw edema in rats. c. Effect of aqueous extracts of Pangdahai on vascular permeability induced by acetic acid. d. Effect of dexamethasone and ethanol extracts of Pangdahai on xylene-induced ear edema in mice. e. Effect of Pangdahai ethanol extract on carrageenan-induced paw edema in rats. f. Effect of ethanol extracts of Pangdahai on vascular permeability induced by acetic acid. ** and $^{\#\#}$ Statistically significant at *$P < 0.01$,* *** statistically significant at *$P < 0.001$.*

5-hydroxy pyridine, *E* – Caffeic acid, 1-*O*-Caffeoyl-*β*-*d*-glucopyranoside, 1-(*β*-*d*-Ribofuranosyl)−1*H*-1,2,4,-triazole, Tiliroside (Kaempherol-3-*O*-*β*−6''-*p*-hydroxycoumaroylglucose), 3-Cinnamoyltribuloside, *β*-Adenosine, 3,4-Dihydroxybenzoic acid (Protocatechuic acid), Falandin B, *Z*-Caffeic acid, Murratetra C, Uracil, *p*-hydroxy benzoic acid, 5-hydroxymethyl-3-furoic acid, *β*-Sitosterol, 2-Furoic acid) serially diluted from 0 to 100 μM were then added and incubated further for 24 h. MTT reagent was then added to each well and incubated at 37 °C for further for 2.5 h. The formazan crystals formed in each well were sonicated for 15 min in 150 μL DMSO. Finally, a microplate reader was used to estimate absorbance at 490 nm (Huang et al., 2011). Dexamethasone was used as the control drug. The cell inhibition percentage was estimated using the formula;

$$\text{Percentage cell inhibition} = 100 - \{(A_t - A_b) / (A_c - A_b)\} \times 100$$

Where
A_t = Absorbance of test compound
A_b = Absorbance of blank
A_c = Absorbance of control

2.11.2. Nitric oxide assay

RAW 264.7 macrophage cells were treated with test compounds or dexamethasone as described in Section 2.11.1. 100 μL of Griess reagent was added to 100 μL of the cell culture supernatant. The mixture was incubated at room temperature for 10 min, and the absorbance measured at 540 nm. $NaNO_2$ solutions with concentrations of 10, 20, 40, 60, 80, and 100 μM were prepared, and their corresponding absorbance values at 540 nm were measured. A standard calibration curve of concentration *vs* absorbance was plotted. The concentrations of nitrite in the treated RAW 264.7 cells were calculated using the standard calibration curve (Huang et al., 2011).

2.12. Data processing, statistical analysis and molecular docking

Origin Lab software (2018) (OriginLab, USA) was used to analyze the data. Experimental data were reported as the mean value ± SD. Differences between groups (One-way ANOVA) at a $P<0.05$ were considered significant.

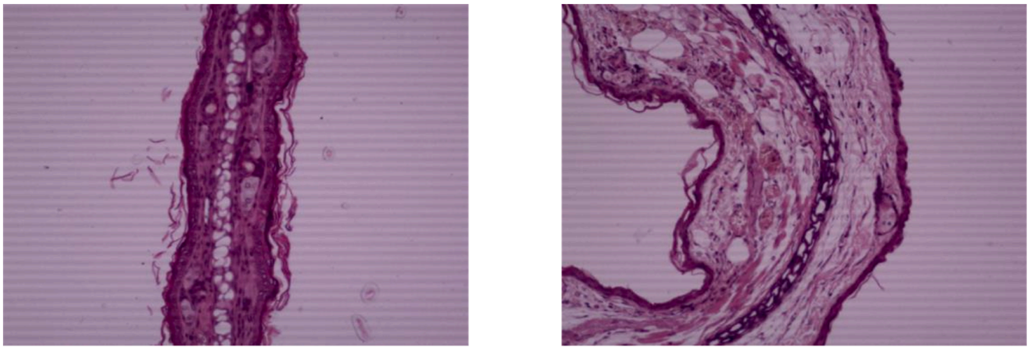
Four inflammatory targets namely, Cyclooxygenase-2 (COX-2, UniProtID: Q05769), Phospholipase A2 (PLA2, UniProtID: P00624), Interleukin-1 receptor-associated kinase-4 (IRAK-4, UniProtID: Q9NWZ3) and NF-κB–inducing kinase (NIK, UniProtID: Q99558) were obtained from RSCB PDB. Where the targets were ligand bound, the coordinates of the ligand were removed. The structures were cleaned using Discovery Studio version 21.1.0 (BIOVIA, San Diego). The following compounds showing high anti-inflammatory activity, namely, (+) – pinoresinol, tiliroside, *Z*-caffeic acid, and 3,4-dihydroxybenzoic acid (protocatechuic acid) were virtually screened using Python Prescription Virtual Screening tool (PyRx 0.8, AutoDock Vina module). The interactions between the protein-ligand were analyzed using Discovery Studio version 21.1.0 (BIOVIA, San Diego). As a positive control, the binding interactions of known ligands for each of the four targets were also evaluated.

3. Results

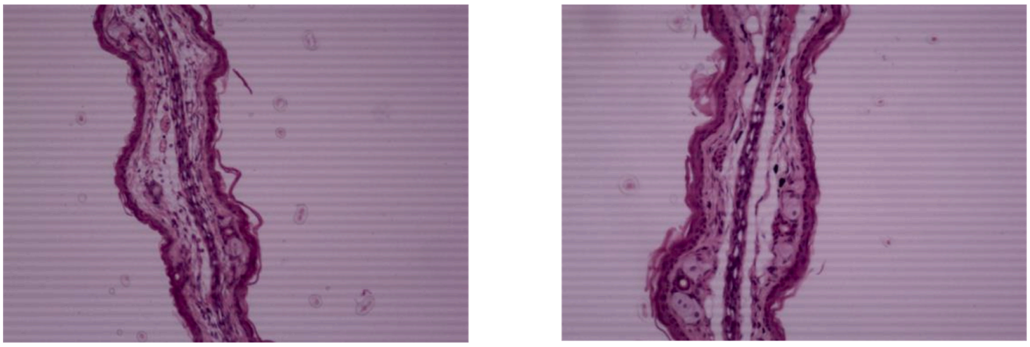
3.1. Effect of aqueous and ethanol extracts of PDH on xylene–induced ear edema in mice

The mice in the negative control group showed significant edema after xylene application for the aqueous extracts. Compared with the negative control group, the dexamethasone treated group significantly inhibited ear edema induced by xylene ($^{\#\#}P < 0.01$), which showed that the experimental model was appropriately designed. The low and medium doses of PDH extract had no significant inhibitory effect. However, the high dose had a significant inhibitory effect equivalent to dexamethasone ($^{**}P < 0.01$). The effects of the aqueous extracts are presented in Fig. 1a.

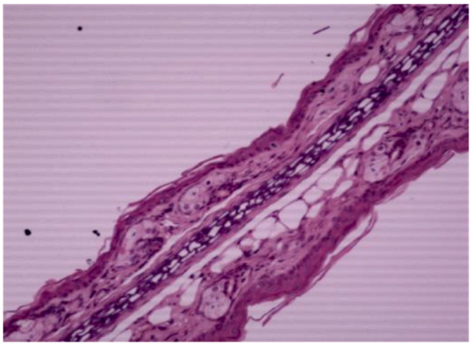
The ethanolic extracts also exhibited similar results as the aqueous extract. All the tested doses of the ethanol extract inhibited ear edema in mice induced by xylene. The low dose and high dose exhibited significant inhibition of edema comparable to the dexamethasone group ($^{**}P < 0.01$). The medium dose, however, did not show significant inhibition. The effects of the ethanol extracts are presented in Fig. 1d.



Normal group **Model group**



Dexamethasone group **Medium dose group**



High dose group

Fig. 2. Pathological changes in mice ears treated with Pangdahai ethanol extract (HE staining, ×100).

3.2. *Effect of aqueous and ethanol extracts of PDH on carrageenan-induced rat paw edema*

With the aqueous extract, the rats in the negative control group showed increasing paw edema at 1, 2, and 4 h, while the positive control (Dexamethasone) group significantly inhibited rat paw edema induced by carrageenan ($^{\#\#}P < 0.01$). This indicated the appropriateness of the experimental design. All tested doses of PDH aqueous extract significantly inhibited rat paw edema induced by carrageenan at 1 h after compared to the negative control group ($^{**}P < 0.01$), but the effect was not obvious at 2 h. However, the high dose demonstrated a significant ($^{**}P < 0.01$) inhibitory effect compared with the negative control group, and the effect was most obvious at 4 h. Nevertheless, the degree of this inhibitory effect was lower than that of the dexamethasone group. The paw edema/swelling index of the experimental groups is shown in Fig. 1b.

The ethanolic extracts also exhibited similar results as the aqueous extract. For all the tested doses, the ethanol extract exhibited some degree of inhibition of rat paw edema induced by carrageenan. The low and medium doses exhibited significant inhibition of edema comparable to the dexamethasone group ($^{**}P < 0.01$) at 1 h. The high dose, however, showed no significant inhibitory effects. The paw edema/swelling index of the experimental groups is shown in Fig. 1e.

3.3. *Effect of aqueous and ethanol extract of PDH on acetic acid-induced mice vascular permeability*

The vascular permeability was measured by the OD which represented the amount of Evans blue exuded into the peritoneal cavity. In both aqueous - and ethanol-treated groups, the OD values of the negative control groups significantly increased following treatment with acetic acid. Compared with the model group, the aqueous extract exhibited a non-significant reduction in the OD values at all tested doses (Fig. 1c). The ethanol extracts, however, demonstrated a significant ($^{**}P < 0.01$) but dose-independent reduction of the OD values (Fig. 1f).

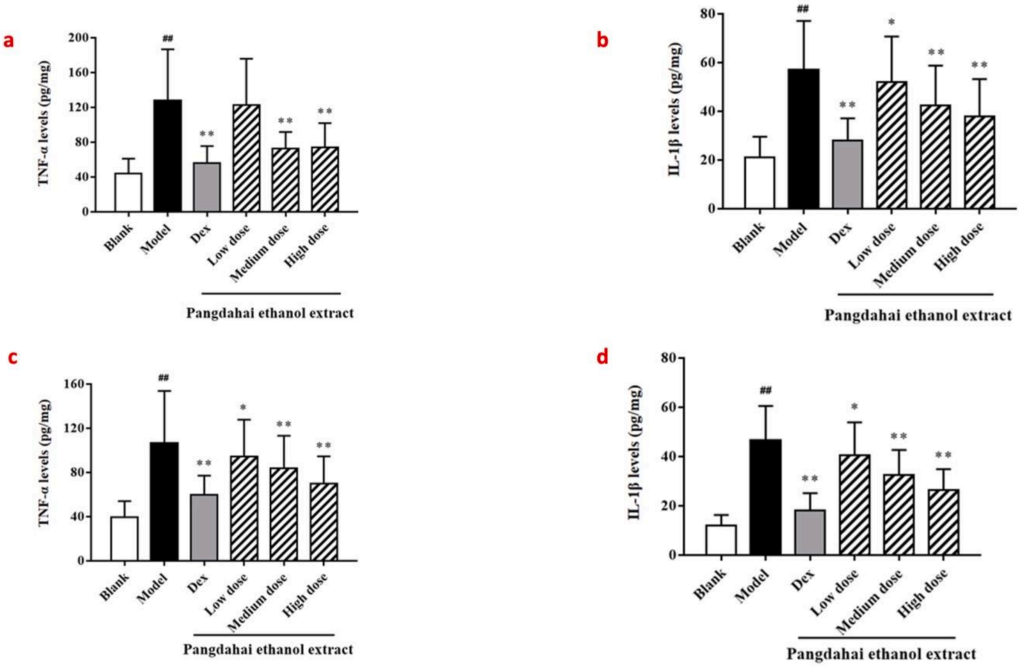


Fig. 3. a. Effect of Pangdahai ethanol extract on the levels of TNF-α in paw tissues of rats induced by carrageenan. b. Effect of Pangdahai ethanol extract on the levels of IL-β in paw tissues of rats induced by carrageenan. c. Effect of Pangdahai ethanol extract on the levels of TNF-α in ear tissues of rats induced by xylene. d. Effect of Pangdahai ethanol extract on the levels of IL-1β in ear tissues of rats induced by xylene. $^{\#\#}$Compared with blank group $p < 0.01$; *Compared with model group $p < 0.05$; **Compared with model group $p < 0.01$.

3.4. Histo-pathological study of sections of mice edematous ear induced by xylene

HE staining was used to observe and confirm the changes in the inflamed cells induced by xylene. The normal group showed normal tissues. Compared with the normal group, the negative control group exhibited high degree of swelling which was marked by blistering of the epithelial and conjunctival tissues, red-stained mesh-like collagen fibers, and significant infiltrated inflammatory cells. Contrary to the xylene groups previously given dexamethasone (positive control group) or PDH extracts (low and medium dose groups), there was a reduction in edema (slight edema), amount of red-stained mesh-like collagen fibers and infiltration of inflammatory cells (Fig. 2). These results collectively indicate that ethanol extracts of PDH inhibited xylene-induced ear edema and infiltration of inflammatory cells.

3.5. Effect of PDH ethanol extract on the levels of inflammatory cytokines

The levels of TNF-α and IL-1β were significantly ($p < 0.01$) inhibited by dexamethasone in both *in vivo* acute inflammation models. The ethanol extracts of PDH exhibited significant ($p < 0.01$) dose -dependent inhibition of the expression of TNF-α and IL-1β in both models (as shown in Fig. 3a–d). These results indicate that the anti-inflammatory properties of PDH ethanol extract were cognate to the inhibition of TNF-α and IL-1β.

3.6. In-vitro anti-inflammatory effects of some isolated compounds from PDH

The results show that all tested compounds showed inhibition of NO production with no obvious cytotoxicity at 100 μM. Among these compounds, (+) - pinoresinol, tiliroside, 3-cinnamoyltribuloside, 3,4-dihydroxybenzoic acid, *Z*-caffeic acid, and 2-furoic acid showed significant inhibition ($P < 0.05$) of NO-production in LPS stimulated RAW cells with percentage inhibitions greater than 70 %. The percentage inhibitions and IC_{50} are shown in Table 1.

3.7. Binding affinities of selected compounds

The binding interactions between the four COX-2, PLA2, IRAK-4 and NIK, which are known mediators of anti-inflammatory process and four of the isolated compounds were evaluated. As a positive control, known ligands of these targets were used positive control. COX-2 and PLA2 were evaluated with celecoxib and Niflumic acid, respectively. IRAK-4 and NIK on the other hand were evaluated using 1-(3-Hydroxypropyl)-2-[(3-N*itrobenzoyl*) *amino*]-1h-Benzimidazol-5-Yl Pivalate and Cdk1/2 Inhibitor III, respectively. The results of these studies are shown in Table 2.

4. Discussion

The current study ascertained and validated the traditional or folkloric use of PDH as an anti-inflammatory agent. Thus, we report the anti-inflammatory properties of the aqueous and ethanol extracts and some isolated secondary metabolites of PDH.

Data from this study showed that extracts of PDH (ethanol and aqueous) exhibited an inhibitory effect on xylene-induced ear edema in mice. The aqueous extracts demonstrated a dose–independent inhibition, while the low and high doses of ethanol extracts significantly inhibited ear edema in mice induced by xylene. Xylene induces acute neurogenic edema (Singsai et al., 2020) and cause swelling by increasing vasodilation and vascular permeability when applied (Zhao et al., 2018).

Furthermore, this study showed that the extracts of PDH inhibited paw edema in rats induced by carrageenan. Carrageenan induces edema in two phases with several mediators including histamine, serotonin, 5-hydroxytryptamine, prostaglandins, bradykinin, cyclooxygenase, TNF-α, IL-1 and IL-6 involved (Umare et al., 2014; Karim et al., 2019; Patil et al., 2019).

This study suggests that the ethanol extracts markedly attenuated acetic acid-induced vascular permeability in a dose-independent manner (Fig. 1c and f). Acute inflammation is characterized by vasodilatation, exudation of plasma, increase in vascular permeability, and

Table 1
Inhibitory activity of the compounds from Pangdahai on LPS-induced NO release in RAW 264.7 cells.

Compound	Concentration/ μM	NO inhibition (%)	IC_{50}/ μM
Uridine	100	46.53	>100
Ethyl-3,4-dihydroxy benzoate	100	32.29	>100
*(+) – Pinoresinol	100	84.68	16.36 ± 0.79
	50	83.06	
	25	79.26	
	12.5	36.94	
Daucosterol	100	36.84	>100
Vomifoliol	100	46.65	>100
2-(Hydroxymethyl)−5-hydroxy pyridine	100	43.61	>100
E – Caffeic acid	100	34.86	>100
1-*O*-Caffeoyl-*β*-*d*-glucopyranoside	100	14.49	>100
1-(*β*-*d*-Ribofuranosyl)−1*H*-1,2,4,-triazole	100	47.11	>100
* Tiliroside (Kaempherol-3-*O*-*β*−6′’-*p*-hydroxycoumaroylglucose)	100	89.47	17.58 ± 1.05
	50	85.55	
	25	77.69	
	12.5	31.04	
*#3-Cinnamoyltribuloside	100	102.65	27.78 ± 1.58
	50	77.13	
	25	46.63	
	12.5	0.66	
β-Adenosine	100	65.55	56.57 ± 2.55
	50	48.27	
	25	23.24	
	12.5	18.98	
*3,4-Dihydroxybenzoic acid (Protocatechuic acid)	100	100.79	18.55 ± 1.35
	50	75.40	
	25	62.88	
	12.5	37.92	
3,6-Dihydroxy-5,11-epoxy-7*E*-magastimaen-9-one (Falandin B)	100	43.34	>100
**Z*-Caffeic acid	100	57.03	30.34 ± 2.00
	50	56.46	
	25	48.27	
	12.5	26.98	
2-Methoxy-benzoyl-*β*-*d*-glucopyranoside (Murratetra C)	100	33.34	>100
Uracil	100	51.20	94.85 ± 4.13
	50	40.61	
	25	33.46	
	12.5	14.73	
p-hydroxy benzoic acid	100	40.96	>100
5-hydroxymethyl-3-furoic acid	100	25.64	>100
β-Sitosterol	100	19.46	>100
*#2-Furoic acid	100	101.25	25.49 ± 1.73
	50	99.58	
	25	50.99	
	12.5	22.09	

*Significant inhibitory effect on NO release at ($P < 0.05$).
#Some degree of cytotoxicity.

Table 2
Binding affinities of selected compounds with mediators of inflammation.

Ligands	Binding Affinities (kcal/mol)			
	COX-2	PLA2	IRAK-4	NIK
3,4-dihydroxybenzoic acid	-5.1	-5.5	-6.3	-5.4
Caffeic acid	-5.8	-6.6	-6.6	-6.6
(+) – Pinoresinol	-7.7	-5.8	-7.4	-6.5
Tiliroside	-8.2	-9.1	-7.8	-9.6
Native Ligand	-8.4[a]	-8.4[b]	-9.4[c]	-9[d]

Native Ligands: [a]Celecoxib; [b]Niflumic acid; [c]1-(3-Hydroxypropyl)-2-[(3-N*itrobenzoyl*) *amino*]-1h-Benzimidazol-5-Yl Pivalate; [d]Cdk1/2 Inhibitor III.
Targets: COX-2 - Cyclooxygenase-2; PLA2 - Phospholipase A2; IRAK-4 - Interleukin-1 Receptor-Associated Kinase-4; NIK - NF-κB–Inducing Kinase.

neutrophil migration into the site of inflammation (Chen et al., 2018). Exudation is a direct consequence of increased vascular permeability. In acetic acid-induced vascular permeability assay, acetic acid causes the level of mediators such as prostaglandins, serotonin, and histamine in peritoneal fluids to increase consequently, resulting in dilation of the capillary vessels and an increase in vascular permeability (Dantas et al., 2020; Rezq et al., 2021).

Data from this study showed that the anti-inflammatory effects of PDH ethanol extract could be related to TNF-α and IL-1β inhibition. Though several cytokines are involved in inflammation (Delgado et al., 2003), TNF-α is the most significant cytokine associated with local and/or systemic inflammation (Cuzzocrea et al., 1999). TNF-α stimulates T cells and macrophages. It elevates levels of kinins and leukotrienes (Yun et al., 2008; Huang et al., 2011).

One of the of aims of this work was to assess the anti-inflammatory potentials of some isolated compounds from PDH. This was achieved by measuring the degree of inhibition of nitric oxide (NO) in RAW 264.7 macrophages treated with Lipopolysaccharides (LPS). LPS activates macrophages to release cytokines and inflammatory mediators. These include NO, cyclooxygenase-2, TNF-α and IL-6 (Saadat et al., 2019). NO plays a significant role in regulating physiological responses including inflammation (Doulias and Tenopoulou, 2020), and is used as a biomarker of inflammation in many biological samples (Rana, 2020). Therefore, the ability of a compound to inhibit the production of nitric oxide in LPS-stimulated RAW cells is indicative of its anti-inflammatory potentials (Rana, 2020).

In addition, molecular docking studies performed using known mediators of the inflammatory process, *i.e.* COX-2, PLA2, IRAK-4 and NIK demonstrated that (+) – pinoresinol, tiliroside, Z-caffeic acid and 3,4-dihydroxybenzoic acid demonstrated good binding affinities. This further supports our assertion that these agents could be the components responsible for the observed anti-inflammatory activity. It is interesting to note that amongst the top four compounds, tiliroside demonstrated binding affinities comparable and in some cases higher (PLA2 and NIK) that those of the positive control as shown in Table 2.

Previous anti-inflammatory studies on some of the compounds isolated from PDH have shown that these compounds have anti-inflammatory properties. Tiliroside is reported to significantly inhibit mouse paw edema induced by phospholipase A2 and mouse ear edema inflammation induced by TPA (Sala et al., 2023). The work of Correa et al., 2018 and Luhata et al., 2017 have also demonstrated the anti-inflammatory effects of tiliroside. Several studies (both *in vitro* and *in vivo*) have established that protocatechuic acid possess anti-inflammatory effects (Semaming et al., 2015; Kakkar and Bais, 2014; Song et al., 2020; Hu et al., 2020). Anti-inflammatory effects of caffeic acid is also well demonstrated. Caffeic Acid is reported to significantly inhibit pro-inflammatory cytokines, downregulated mRNA expression of *IL-1β*, *IL-6*, and *TNF-α* (Gamaro et al., 2011; Wan et al., 2021; Ehtiati et al., 2023), lymphocytes, polymorphonuclear neutrophils and, macrophages (Morones et al., 2016). Pinoresinol is reported to exert potent anti-inflammatory effects (Jang et al., 2022). Studies conducted by Jung et al., 2010 and During et al., 2012 have demonstrated that pinoresinol significantly inhibits NO, PGE(2), *TNF- α, IL-1β and IL-6* and attenuates mRNA and protein levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and proinflammatory cytokines in LPS-activation. It is believed to exhibit the strongest anti-inflammatory properties by acting on the NF-κB signaling pathway (During et al., 2012).

5. Conclusions

The aqueous and ethanol extracts of PDH have significant anti-inflammatory effects in the animals used. This study validates and provides scientific evidence for the traditional use of the aqueous decoctions of PDH in the treatment of inflammatory-related conditions such as pharyngitis. Additionally, (+) – pinoresinol, tiliroside, 3-

cinnamoyltribuloside and 3,4-dihydroxybenzoic acid (protocatechuic acid) isolated from PDH showed *in vitro* anti-inflammatory activities supported by molecular docking studies, which could be the anti-inflammatory constituents of PDH.

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CRediT authorship contribution statement

Mahmood B. Oppong: Investigation, Formal analysis, Data curation, Writing – original draft. **Shijie Cao:** Investigation, Formal analysis, Data curation. **Shi-Ming Fang:** Supervision. **Seth K. Amponsah:** Writing – review & editing, Formal analysis, Data curation. **Paul O. Donkor:** Writing – original draft, Formal analysis, Data curation. **Michael Lartey:** Writing – review & editing, Formal analysis, Data curation. **Lawrence A. Adutwum:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Kwabena F.M. Opuni:** Writing – review & editing, Formal analysis, Data curation. **Feng Zhao:** Writing – review & editing, Validation, Supervision, Methodology. **Qiu Feng:** Supervision, Conceptualization, Project administration, Funding acquisition.

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.

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