Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.

Pharmacological activity of subcritical CO_2 extract of *Plantago major*

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Article info
Received 01.04.2025
Received in revised form 06.05.2025
Accepted 29.05.2025

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Alimova, U., Ustenova, G., Kozhanova, K., Zhandabayeva, M., Kantureyeva, A., Mombekov, S., Mukanova, A., Alimzhanova, M., Yudina, Y., Hrubnyk, I., Maloshtan, L., & Botabayeva, R. (2025). Pharmacological activity of subcritical CO_2 extract of Plantago major. Regulatory Mechanisms in Biosystems, 16(2), e25075. doi:10.15421/0225075

This study comprehensively investigates the anti-inflammatory, wound-healing, and toxicological properties of *Plantago major* CO_2 extract using a series of *in vivo* experimental models in rats and mice. The anti-inflammatory activity was assessed in two models of acute inflammation. In the carrageenan-induced paw edema model, which reflects prostaglandin-mediated inflammation, the extract demonstrated a dose-dependent effect. At the highest dose of 50 mg/kg, paw swelling was reduced by up to 43% during peak inflammation, though the effect remained inferior to that of the reference drug, sodium diclofenac. In the zymosan-induced paw edema model, which mimics leukotriene-driven inflammation via the lipoxygenase pathway, the extract at a dose of 50 mg/kg significantly inhibited edema formation throughout the experiment and showed comparable efficacy to quercetin, suggesting potential lipoxygenase inhibition. The reparative effects of the extract were examined in a linear skin incision model. Administration of the extract at 50 mg/kg significantly increased the tensile strength of the wound (578.4 ± 4.2 vs. 416.6 ± 6.0 g in the control group), accelerated granulation tissue formation, and normalized serum protein levels, indicating improved wound healing capacity. In acute toxicity studies, no signs of behavioral abnormalities or mortality were observed in rats or mice following a single oral dose of up to 5000 mg/kg. According to standard toxicological classifications, the extract can be considered practically non-toxic. Taken together, the results indicate that *P. major* CO_2 extract possesses moderate but stable anti-inflammatory activity, notable wound-healing potential, and excellent safety. These findings support the further pharmacological development of the extract and highlight its promise as a plant-derived therapeutic agent for treating inflammatory conditions and promoting tissue repair.

Keywords: CO_2 extract; *Plantago major*; acute toxicity; anti-inflammatory activity; wound healing activity.

Introduction

The unique medicinal properties of broadleaf plantain (*Plantago major*), a perennial plant belonging to the plantain family (Plantaginaceae), have been well known for centuries (Munawer, 2024). This plant is widespread and can be found in lawns, fields and ruderal areas, such as roadsides, in temperate regions around the world. Species of the genus *Plantago* are used in both folk and official medicine, as well as in functional foods (Samuelsen, 2000; Nazarizadeh, 2013; Haddadian, 2014). The main raw materials are the seeds and leaves which contain significant amounts of polysaccharides: xylose (39.7%), arabinose (13.1%), galacturonic acid (17.2%), glucuronic acid (15.5%), and in the leaves – arabinose (9.9%), glucose (21.5%), galactose (8.02%), xylose (2.1%), and galacturonic acid (55.4%) (Adom et al., 2017; Turgumbayeva, 2022; Zhang et al., 2022).

Among other groups of substances, flavonoids have been identified, including apigenin, apigenin-7-glucoside, rutin, quercetin, luteolin, luteolin-7-glucoside, and their derivatives (baicalein, baicalin, cynaroside), as well as derivatives of scutellarin (plantaginin), baicalein, and plantaginin (Beara, 2009). Hydroxycinnamic acids are represented by chlorogenic, neochlorogenic, caffeic, chicoric, ferulic, vanillic, and p-coumaric acids, which exhibit anti-inflammatory, antioxidant, immunostimulatory, and antimicrobial effects (Jamilah, 2012; Janković, 2012; Zhang et al., 2022).

Iridoid glycosides (aucubin, asperuloside, and catalpol) and triterpenic acids (ursolic and oleanolic acids) have been found in *P. major*, along with alkaloids such as indicain and plantagonin. Fatty acids in *P. major* include palmitic, stearic, oleic, and linolenic acids. Free organic acids (citric, malic, tartaric, succinic, and ascorbic acid) and vitamin C have also been identified in the leaves of species like *P. major* and *P. lanceolata* (Tarvainen, 2011; Rahamouz-Haghighi, 2022; Laanet et al., 2024).

Phytosterols (PSs) biologically active compounds with various pharmacological properties, including anti-inflammatory, cytotoxic, and antioxidant activities have also been found in plants of the *Plantago* genus (Albadri et al., 2023).

This rich composition of biologically active substances determines the wide range of pharmacological effects of plantain preparations. Polysaccharide preparations from the *Plantago* species are known for their expectorant, wound-healing, emollient, analgesic, and laxative properties, improving intestinal function and exhibiting hypoglycemic and immunomodulatory effects (Ji, 2019; Fierascu, 2021; Amin et al., 2024). A dry extract from the *P. major* leaves shows immunomodulatory, hypoglycemic and antiviral effects, while a liquid extract demonstrates hepatoprotective and anti-inflammatory activityes (Velasco-Lezama, 2006; Adom et al., 2017). The antimicrobial activity of extracts against strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus* sp. has been proven. Antioxidant, anti-inflammatory, and cytotoxic effects are associated with the presence of hydroxycinnamic acids (both chlorogenic and non-chlorogenic) and flavonoids. Due to iridoids, phytopreparations from plantain are used as anti-inflammatory and wound-healing agents in the treatment of acute pancreatitis, cystitis, pyelonephritis, as well as bactericidal agents in infectious processes of the stomach, gastrointestinal tract, kidneys, and bladder. Thanks to aucubin, the plantain extract demonstrates a neuroprotective mechanism of hypoglycemic action in a rat model of diabetic encephalopathy at doses of 1, 5, and 10 mg/kg (Tafazoli, 2022; Zhakipbekov, 2023).

The chemical composition of plantain preparations depends on the method of extracting biologically active substances from the raw material. Water extraction is the most common method for extracting polysaccharides (the main group of biologically active substances) from *P. major*. Other extraction methods (decoction, maceration, green extraction) using various solvents (petroleum ether, methanol, ethyl acetate, n-butanol, and water) allow the production of extracts with different chemical profiles and the isolation of individual active substances from *P. major* (Palavicini, 2022).

Subcritical CO_2-assisted ($SubCO_2$) extraction has several advantages over traditional extraction methods (liquid extraction with organic solvents, steam distillation, and others). It is an innovative, environmentally friendly, and highly efficient method that ensures high purity of extracts, preservation of active compounds, and reduced environmental impact. This method has found widespread application in the food, pharmaceutical, and cosmetic industries as an alternative to traditional extraction methods due to its benefits (Zhandabayeva et al., 2021; Afraz et al., 2023).

Environmental friendliness and safety. Traditional extraction methods using liquid solvents require large amounts of organic solvents to isolate biologically active compounds from natural sources. Additionally, the use of high temperatures during extraction and solvent evaporation can lead to the degradation of thermosensitive functional compounds. $SubCO_2$ extraction does not require the use of organic solvents (such as hexane, acetone, methanol, etc.), which can be harmful to human health and the environment. Supercritical CO_2, which is used as an extractant for this method, is a safe, inert gas that leaves no toxic residues in the final product. It can be regenerated and reused multiple times, reducing costs and minimizing waste (Afraz et al., 2023).

High extraction efficiency. $SubCO_2$ allows the extraction of biologically active compounds in a shorter time and with a higher yield. It has several advantages over liquid solvents, such as:

a) the solubility of substances depends on the density of the supercritical fluid, which can be easily adjusted by changing temperature and pressure;

b) mass transfer is significantly enhanced due to the higher diffusion coefficient and lower viscosity of the supercritical fluid compared to liquid solvents (Zhandabayeva et al., 2021).

Due to the high solvating power of CO_2, a wide range of nonpolar substances (lipids and fat-soluble compounds) can be extracted. The use of co-solvents (such as ethanol) enables the extraction of polar compounds that are poorly soluble in pure CO_2. By adjusting extraction parameters (pressure and temperature), specific compounds (e.g., phytosterols, polyphenols, carotenoids) can be selectively extracted. Additionally, this method allows the extraction of thermosensitive compounds (essential oils, antioxidants, vitamins) without degradation, unlike traditional methods that require high temperatures (Zhandabayeva et al., 2021).

Purity and quality of the product. $SubCO_2$ extracts are pure and safe for food, cosmetic, and pharmaceutical applications. Many natural compounds (such as phytosterols and flavonoids) lose their activity when exposed to chemical solvents or heat. However, $SubCO_2$ preserves their structure and properties. The process occurs under anaerobic conditions, preventing the oxidation of lipids and other sensitive components (Reverchon & Donsì, 2006; de Melo, 2014).

Economic benefits. The absence of organic solvents simplifies extract purification and reduces costs associated with impurity removal. The final product has a longer shelf life due to the lack of oxidation and degradation of bioactive compounds. The ability to reuse CO_2 lowers operational costs, making the process more cost-effective in the long term (Zhandabayeva et al., 2021; Afraz et al., 2023).

In the given paper we present the results of investigations of anti-inflammatory and wound-healing activities of *P. major* CO_2 extract obtained under subcritical conditions, as well as determining the acute toxicity of the extract.

Materials and methods

The research was carried out in accordance with the general ethical principles concerning animals (Guidelines for ethical conduct in the care and use of nonhuman animals in research, 2012), which are in line with the provisions of the "Guide for the care and use of laboratory animals". All personnel who worked with the animals underwent training in ethical principles, animal handling methods, and the recognition of signs of pain and suffering. The experiment was designed to minimize the number of animals required, while maintaining the statistical significance of the results, and received approval from an independent ethics committee (protocol No. 387). We used 109 albino male and female rats (210–240 g) and twenty five mice (18–20 g) obtained from the National Pharmaceutical University, Kharkov, Ukraine. All animals were provided with comfortable housing conditions with optimal temperature (22 ± 2 °C), humidity (60–70%), lighting (12-h light/dark cycle), ventilation and libitum access to food and water to minimize stress.

$SubCO_2$ extraction of *P. major* leaves was used in this study. Ground *P. major* raw material (3.5 kg) was charged into the extractor with carbon dioxide flow-through extraction (working volume 5 L), designed for obtaining small volumes of plant extracts of lipophilic character for applied research and development work by means of liquid carbon dioxide extraction. The laboratory unit of CO_2 extraction consists of extractor, evaporator, condenser, accumulator, shut-off and control equipment. The entire system is mounted on a single, vertically standing, mounting rack. The operating mode is cyclic, extraction is carried out by liquid carbon dioxide flow at pressure (5.7–6.5 MPa) and temperature (18–27 °C).

Extraction procedure was performed according to the method described by Zhandabaeva et al. (2021). Extraction is carried out using carbon dioxide at the parameters set for this technological process – pressure (6.3 MPa), temperature (23 °C) and extraction time (11 hours). Liquefied carbon dioxide is supplied through the drive from a high-pressure pump (6 MPa) and enters the extractor vessel. From the extractor, the stream with dissolved substances in carbon dioxide enters the manifolds, where the solvent and dissolved substance are separated by successive depressurization. In this case, the carbon dioxide is converted to gas and the extract settled in the collector and then flows out as a finished product (13 g of CO_2 extract of *P. major*) (Ursia, 2015).

The extract obtained is a thick mass of light brown to dark brown color with a green tinge, with a faint characteristic odor.

The effect of the extract on the cyclooxygenase system in albino male rats weighing 210–240 g was studied in a model of acute inflammatory paw edema in rats caused by carrageenan, which was injected under the aponeurosis of the rat hind limb (0.1 mL of 1% carrageenan solution). Within 2–5 hours, the volume of the healthy and injured paws was measured using a mechanical oncometer. The volume of paw edema in rats was calculated by the difference between the volume of a healthy and edematous (inflamed) limb. The antiinflammatory activity of the extract was determined by the degree of reduction of edema in the experimental animals compared to control animals and expressed as a percentage. The animals were divided into 4 experimental groups. Group 1 consisted of control animals (CP). Animals in groups 2–4 received CO_2 extract of *P. major* in doses of 10, 25, 50 mg/kg respectively administrated perorally 1 hour before injection of carrageenan. The study was conducted in comparison with the sodium diclofenac drug in form of tablets (PJSC "Chervona Zvezda Chemical Plant") in an equivalent conditionally therapeutic dose (group 5). The anti-inflammatory activity was calculated as (1):

$$A=\frac{Pc-Pex}{Pc}*100\% \quad (1)$$

where, Pc – is the average difference in the volume of affected and healthy limbs in the control group; Pex – is the average difference in the volume of affected and healthy limbs in the experimental group.

The effect of the studied extracts on the course of leukotriene-induced inflammation was studied in the model of zymosan foot edema in rats. The studied extract was administered orally one hour before to the edema modeling. The study was conducted in comparison with

granules of the herbal drug quercetin (PJSC STC Borshchyagovsky Chemical Pharmaceutical Plant) in an equivalent conditionally therapeutic dose.

Zymozan was administered subplantarly at the rate of 0.1 mL per animal as a 2% suspension. The volume of the feet was measured before the administration of the phlogogen (inflammatory agent) and after 0.5, 1, 2, and 3 hours and calculated by formula 1 according to the method described above.

Given that the studied *P. major* CO_2 extract at a dose of 50 mg/kg showed a pronounced anti-inflammatory effect, this dose was chosen to study its wound healing activity. To study the incised wounds, rats weighing 220–240 g were anesthetized with barbamyl and incisions 5.0 cm long were made on a depilated area of 5x3 cm^2 on the back. The wounds were sutured immediately for a distance of 1.0 cm. The animals were divided into groups: control pathology; animals treated with plantain extract. The study was conducted in comparison with granules of the herbal drug quercetin (PJSC STC Borshchyagovsky Chemical Pharmaceutical Plant) in an equivalent conditionally therapeutic dose.Treatment with the extract began the next day and was carried out for 5 days. The healing of the linear incised wounds on the backs of rats the ends with an epithelialized connective tissue scar, which is formed due to an increase in the speed and strength of the fusion of the wound edges. On the 6th day of the experiment, the animals were tested for the strength of wound edges' fusion using a tensiometer. The reparative activity (Ar, %) was calculated as ratio of the difference (ΔM_{ex} is the load leading to opening of the seam in the joint study group of animals – ΔM_{cont} is the load leading to opening of the seam in the control animals) / ΔM_{cont}.

One of the determining factors to evaluate reparative activity is the speed of protein regeneration.

To verify the content of total protein in blood serum, the biuret method was used with application of the reagent kit company "Lachema" (Czech Republic).

Acute toxicity of the *P. major* CO_2 – extract was determined in order to obtain security information for the health of the substance in terms of short-term actions and the establishment of the mean dose (LD_{50}). To assess the degree of safety, we studied the extract of *P. major* using a rapid method. Acute toxicity thick extract *P. major* was studied in two species of animals: albino mice and albino rats at intragastric administration.

The animals were divided into groups depending on the dose of the administered drug: 500, 1000, 2000, 3000, 4000, and 5000 mg/kg for rats, and 500, 700, 1000, 1500, 2000 and 2500 mg/kg for mice. Number of animal in one group – 5. After substance administration, the animals were observed for 24–48 hours (main period) and additionally for up to 14 days. At the end of the experiment, the LD_{50} is calculated by method described by Mironov et al. (2012). All experimental data were statistically processed using GraphPad Prism 9.0 software (GraphPad Software, USA, 2020). Results are expressed as the mean ± standard error of the mean ($\bar{x} \pm SE$). The significance of differences between experimental groups was determined using one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons with Bonferroni's correction (for the carrageenan model) or Tukey's test (for the zymosan, wound healing, and protein concentration models). A P-value of less than 0.05 was considered statistically significant. The anti-inflammatory activity (AIA) was calculated as the percentage reduction in edema relative to the control pathology group using the formula (2):

$$AIA\ (\%) = [(CP - EG) / CP] \times 100, \quad (2)$$

where CP is the mean value in the control pathology group and EG is the mean value in the experimental group.

Each experiment was conducted in triplicate, with five animals per group (n = 5), and all results reflect data from repeated independent trials to ensure reproducibility and reliability.

Results

Anti-inflammatory activity of the of P. major CO_2 – extract. According to the results presented in Table 1, *P. major* CO_2 extract exhibited a dose-dependent anti-inflammatory effect in the carrageenan-induced paw edema model in rats. At all tested doses (10, 25, and 50 mg/kg), the extract significantly reduced paw swelling compared to the control pathology group (CP) ($P < 0.05$, ANOVA with Tukey's post hoc test). The anti-inflammatory activity (AIA) increased with the dose: 19.4% at 10 mg/kg, 30.6% at 25 mg/kg, and 35.0% at 50 mg/kg. At a dose of 10 mg/kg, the extract produced a modest reduction in edema at each hour point, with the effect becoming more pronounced between 2 and 4 hours post-administration. The 25 mg/kg dose showed a significant decrease in inflammation beginning as early as the first hour, with consistent effects observed through the 5-hour period. The highest activity was observed with the 50 mg/kg dose, which resulted in a marked reduction of paw edema from the first hour, reaching a 43% reduction at 2 hours.

While the extract demonstrated a clear dose-dependent effect, its activity was inferior to that of the reference drug diclofenac sodium (8 mg/kg), which exhibited a much stronger anti-inflammatory effect across all time points (66.8% AIA). These findings suggest that *P. major* CO_2 extract primarily exerts its effect through mechanisms associated with cyclooxygenase-2 (COX-2) inhibition, albeit with moderate potency compared to standard NSAIDs. Because the 50 mg/kg dose exhibited the most pronounced activity, it was selected for further investigation in a second model of inflammation.

Table 1

Effect of *Plantago major* CO_2 extract on carrageenan-induced inflammation in rats (n = 5, mean ± SE)

Experimental group	1 h	2 h	3 h	4 h	5 h	AIA, %
Control pathology (CP)	12.6 ± 0.73	21.0 ± 0.94	25.4 ± 0.89	28.4 ± 0.69	27.4 ± 1.15	–
P. major extract 10 mg/kg	10.0 ± 0.60**	17.2 ± 1.26**	19.8 ± 0.59*†	20.6 ± 0.50**	25.0 ± 0.62**	19.4
P. major extract 25 mg/kg	9.4 ± 0.34*	15.0 ± 1.33*	15.5 ± 0.96*/**	18.5 ± 0.70**	20.0 ± 0.62**	30.6
P. major extract 50 mg/kg	8.4 ± 0.64*	12.0 ± 1.31*	15.0 ± 0.76*/**	17.5 ± 1.34*	21.8 ± 1.35*	35.0
Sodium diclofenac 8 mg/kg	5.4 ± 0.70*	7.4 ± 0.88*	6.6 ± 0.90*	7.8 ± 0.66*	9.4 ± 0.61*	66.8

Note: AIA – anti-inflammatory activity calculated as % reduction vs. control pathology; statistical analysis was performed using one-way ANOVA with Bonferroni post hoc correction; * – $P < 0.05$ vs. control pathology; ** – $P < 0.05$ vs. sodium diclofenac.

In the zymosan-induced paw edema model (Table 2), which predominantly reflects leukotriene-mediated inflammation via the lipoxygenase (LOX) pathway, *P. major* CO_2 extract (50 mg/kg) significantly reduced edema at all time points compared to the control group ($P < 0.05$). The average anti-inflammatory activity of the extract was 28.0%, which was comparable to that of the reference compound quercetin (29.1%).

These results suggest that *P. major* CO_2 extract has a broader anti-inflammatory spectrum, affecting both the COX- and LOX-mediated inflammatory pathways. The extract's comparable activity to quercetin – a known LOX inhibitor – supports the hypothesis that it may interfere with leukotriene biosynthesis or signaling, thereby reducing exudate formation.

Reparative activity of P. major CO_2 – extract. Given the pronounced anti-inflammatory effect of *P. major* CO_2 extract at a dose of 50 mg/kg, this dose was used to assess its reparative potential in a model of linear incised wounds in rats. Table 3 shows that the extract significantly enhanced wound healing, as evidenced by a 38.9% increase in tensile strength of the wound edges compared to the control pathology group ($P < 0.05$). This suggests an acceleration of connective tissue regeneration and enhanced wound closure. In parallel, the total protein level in the blood serum – a key indicator of protein metabolism and tissue repair – also increased significantly in the group treated with the extract (81.57 ± 3.15 g/L), approaching values of the intact control. These findings reflect a systemic reparative effect in addition to local wound healing. Quercetin, used as a reference

herbal preparation, also improved wound strength and increased protein levels compared to CP, but the reparative activity (24.6%) was notably lower than that of *P. major* extract. The plantain CO_2 extract outperformed quercetin by 1.60 times in reparative efficacy, confirming its potential as a more effective topical healing agent.

Table 2
The effect of CO_2 extract of *Plantago major* and reference preparation on the course of zymosan edema in rats (x ± SE, n = 5)

Experimental group	0.5 h	1 h	2 h	3 h	AIA, %
Control pathology (CP)	26.0 ± 0.63	43.0 ± 1.39	55.3 ± 1.56	27.5 ± 0.85	–
P. major, 50 mg/kg	18.1 ± 0.42*	34.3 ± 0.67*	35.8 ± 1.25*	20.5 ± 0.71*	28.0
Quercetin, 50 mg/kg	17.9 ± 0.67*	34.8 ± 0.81*	33.9 ± 0.85*	19.8 ± 0.98*	29.1

Note: statistically significant differences versus control pathology group (CP) are marked with * – $P < 0.05$ (ANOVA with Tukey's post hoc test); AIA – anti-inflammatory activity (% reduction in edema).

Thus, based on both biomechanical (tensile strength) and biochemical (protein synthesis) criteria, *P. major* CO_2 extract demonstrates significant reparative activity and holds promise for wound healing applications.

Table 3
Reparative activity of *Plantago major* CO_2 extract
in the model of linear incised wounds in rats (mean ± SE, n = 5)

Experimental group	Tensile strength, g	Reparative activity, %	Total protein, g/L
Intact control	–	–	85.14 ± 3.46
Control pathology (CP)	416.6 ± 6.0	–	69.42 ± 4.07*
P. major CO_2 extract, 50 mg/kg	578.4 ± 4.2*	38.9	81.57 ± 3.15*
Quercetin, 50 mg/kg	518.9 ± 4.6*	24.6	73.42 ± 4.01*

Note: statistical analysis was performed using one-way ANOVA with Tukey's post hoc test; $P < 0.05$ vs. control pathology (CP); reparative activity was calculated as % increase in tensile strength vs. CP.

Acute toxicity studies of CO_2 – extract of P. major. The acute toxicity assessment of the *P. major* CO_2 extract revealed complete safety in both albino rats and mice following single oral administration at doses ranging from 500 to 5000 mg/kg. No mortality was recorded in any group during the 14-day observation period (Table 4). Moreover, no signs of systemic or behavioral toxicity were observed: all animals remained active, exhibited normal grooming, retained good appetite, and displayed no autonomic disorders.

Based on the absence of adverse effects and deaths even at the maximum tested dose (5000 mg/kg in rats), the extract can be classified as non-toxic, in accordance with the Mironov classification system. These findings indicate a high margin of safety and support the potential for further pharmacological development of the extract.

Table 4
Acute toxicity of *Plantago major* CO_2 extract
in albino rats and mice after single oral administration (n = 5)

Species	Dose, mg/kg	Mortality, n/N	Toxic effects observed
Rats	500	0/5	none
	1000	0/5	none
	2000	0/5	none
	3000	0/5	none
	4000	0/5	none
	5000	0/5	none
Mice	500	0/5	none
	700	0/5	none
	1000	0/5	none
	1500	0/5	none
	2000	0/5	none
	2500	0/5	none

Note: no signs of toxicity or behavioral abnormalities were observed during the 14-day observation period; statistical analysis was not applied due to absence of mortality; according to Mironov's classification, the extract is considered non-toxic ($LD_{50} > 5000$ mg/kg).

Discussion

Only a few studies of CO_2 extraction of *Plantago* sp. are known. Conducted by Mazzutti et al. (2017), comparative analysis of *P. major* extracts obtained by supercritical fluid extraction (SFE) with CO_2 pure and with ethanol as co-solvent, conducted at temperatures from 40 to 60 °C and pressures from 10 to 30 MPa, and by Soxhlet (SOX) and ultrasound-assisted extraction (UE) with different solvents, has shown that the moderate-polar and polar solvents presented the best TPC values and antioxidant performance, included the supercritical extraction of *P. major* with 5.0% of ethanol as co-solvent (EC_{50} 276 ± 1). SC-CO_2 extract presented the best antibacterial activity, being more effective against the Gram-positive bacteria (*Bacillus cereus*). Conventional Soxhlet extraction using a variety of solvents and with pilot scale supercritical fluid (CO_2) extraction (SFE-CO_2) was applied by Mazzutti et al. (2017) for isolation of hydroxy pentacyclic triterpene acids (HPTAs), oleanolic acid and ursolic acid, but SFE-CO_2 extraction without polar modifier was found not to be a suitable technology for the leaf extraction due to the low content of lipophilic and volatile compounds. Extraction of geniposidic acid from plantain seeds using SFE was conducted by Wang et al. (2014) and found to be promising.

Our previous study has shown that *P. major* CO_2 – extract contains a wide range of phytochemical constituents – 32 substances were identified, including terpenes and terpenoids of various structures, phytosterols, sterols, steroids, vitamin E, and essential fatty acids. Five main phytocompounds identified in the extract include phytosterols β-sitosterol (44.1%) and stigmasterol (4.7%), triterpenoids lupeol (10.9%) and β-amyrin (4.2%), and the diterpenoid compound phytol (8.59%, Fig. 1) (Alimova et al., 2016).

The antimicrobial activity of the *P. major* CO_2 extract has been studied, demonstrating activity against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, and *Aspergillus brasiliensis* ATCC 16404, with growth inhibition zones ranging from 16 to 22 mm (Urzia, 2015, 2016).

We did not find any data on the study of anti-inflammatory and reparative activity of CO_2 extract of *Plantago*. However, the anti-inflammatory and wound-healing and antimicrobial activity of plants of the *Plantago* genus is proven and well studied in various animal models, and it is attributed to a greater extent to phenolic and polysaccharide compounds, which are the main group of bilologically active substances of this plant (Zhakipbekov, 2023).

Our research has proven that *P. major* CO_2 extract is an effective agent that exhibits moderate anti-inflammatory activity in the carrageenan edema model and expressive activity in the zymosan edema model by affecting the prostaglandin and leukotriene phases of inflammation due to terpene compounds that are part of the plantain CO_2 extract.

It is known that carrageenan, as a phlogogen, leads to activation of the prostaglandin-kinin system, which activates COX-2 synthesis (Khakimiv et al., 2021). Zymosan is able to disorient and translocate membrane phospholipids, which provokes Ca^{+2} flux into the cell and hydrolysis of membrane phospholipids. As a result, this leads to the release of arachidonic acid via the lipoxygenase pathway of metabolism with the formation of leukotrienes and other products (Wu et al., 2022).

According to the reparative effect, it can be concluded that on the model of stencil wounds plantain CO_2 extract exceeds quercetin in terms of regenerative effect. CO_2 extract activates proliferative processes at the cellular level by increasing the level of total protein in the wound. In our opinion, the reparative activity of plantain CO_2 extract is provided by phytosterols, which are part of the extract.

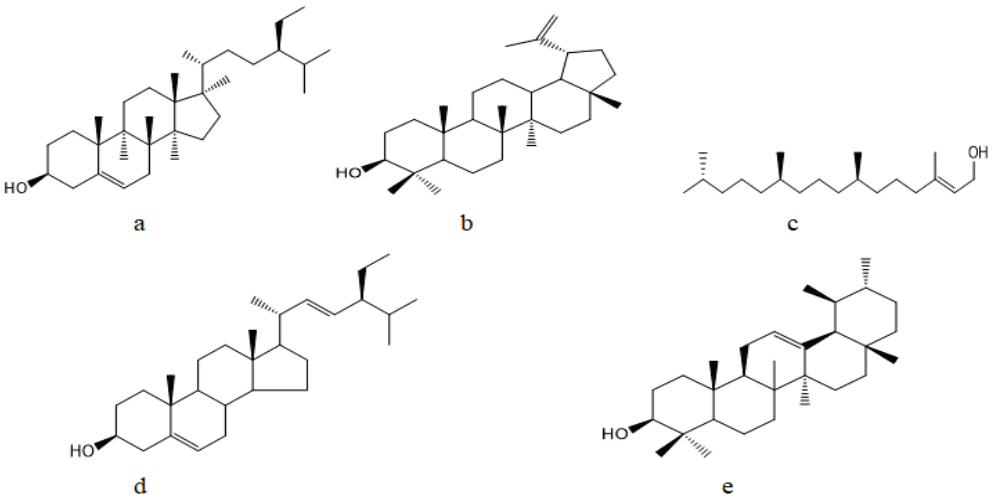

Fig. 1. Chemical structure of main constituents of *P. major* CO_2: *a* – β-sitosterol, *b* – lupeol, *c* – phytol, *d* – stigmasterol, *e* – β-amyrin

The anti-inflammatory effects induced by plant sterols/stanols (PSs) have been demonstrated in *in vitro* studies and in experimental animal models (Vilahur et al., 2019). Our studies correlate with the studies conducted by Arivarasu & Gunam (2022), which demonstrated the pronounced anti-inflammatory activity of sigmasterol in the carrageenan-induced inflammation model at a dose of BS (10 and 20 mg/kg, i.p.). The anti-inflammatory effect of PSs is due to their ability to modulate various mechanisms of the inflammatory response including suppression of pro-inflammatory cytokines, reduction of oxidative stress, modulation of lipid metabolism and stabilization of cell membranes (Li et al., 2022).

PSs have found widespread use in clinical practice, primarily for the treatment and prevention of a wide range of cardiovascular diseases. Numerous clinical and preclinical studies have demonstrated their ability to regulate blood cholesterol levels. They exhibit hypocholesterolemic effects (reducing total cholesterol levels and low-density lipoprotein cholesterol concentration), hypolipidemic, and hepatoprotective actions (Li et al., 2022).

A wide number of studies have reported remarkable pharmacological effects of PSs including antioxidant, anxiolytic, analgesic, antidiabetic, antimicrobial, antiinflammatory, and immunomodulatory properties (Nattagh-Eshtivani et al., 2022). There is also evidence of the effectiveness of PSs in obesity treatment. A particularly promising area of research is their potential efficacy in neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, etc. (Sharma et al., 2021)), particularly their neuroprotective role.

PSs are widely used in cosmetic products. Since their structure and properties are similar to cholesterol, which is essential for the production of vitamin D and a range of hormones, as well as for maintaining the skin's barrier functions, PSs in cosmetics primarily help restore and strengthen the skin's hydrolipid barrier. PSs are commonly used in the production of anti-aging creams and sunscreen lotions, with their concentration in such products typically ranging from 0.5% to 2.0%, while the content of sterol esters can be as high as 5%. The permissible concentration is mainly limited by solubility constraints. The addition of PSs to creams significantly enhances protection against UV radiation (Xinyue, 2024).

Moreover, the anti-inflammatory effects of PSs can be utilized in products designed for the treatment of a wide range of dermatological conditions, including atopic dermatitis, eczema, infectious skin diseases, and as protective agents.

Despite promising results from *in vitro* and animal model studies, clinical data remain inconclusive, and further research is needed to accurately determine their efficacy in various inflammatory diseases.

Conclusion

The results of this study confirm that the subcritical CO_2 extract of *P. major* possesses multifaceted pharmacological properties, including anti-inflammatory, reparative, and non-toxic effects. In the carrageenan-induced inflammation model, the extract demonstrated moderate but dose-dependent anti-inflammatory activity, with the highest dose (50 mg/kg) reducing paw edema by up to 43% and showing a clear inhibitory effect on the cyclooxygenase pathway of inflammation. In the zymosan-induced edema model, the extract showed a comparable efficacy to quercetin, suggesting its potential role as a lipoxygenase pathway modulator. This dual-pathway action may explain its consistent anti-exudative effects across different models of inflammation.

Furthermore, the extract significantly improved wound healing outcomes, as evidenced by increased tensile strength of healed skin and normalized total protein levels in serum, supporting its reparative properties. Importantly, the acute toxicity assessment revealed no signs of toxicity or mortality at doses up to 5000 mg/kg in both rats and mice, indicating a high safety margin and classifying the extract as practically non-toxic.

Taken together, these findings highlight *P. major* CO_2 extract as a promising phytotherapeutic agent with anti-inflammatory and wound-healing activity, supported by a favorable toxicological profile. These properties make it a potential candidate for further preclinical and clinical studies aimed at developing safe and effective anti-inflammatory or wound care formulations.

We would like to express gratitude to B. Proskurin for the assistance with carrying out of subcritical CO_2 – extraction of *Plantago major* at the "Herbal – Aroma" Ltd. (Almaty, Kazakhstan).

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