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Evaluation of anti-inflammatory and analgesic activities of extracts from herb of *Chelidonium majus* L.

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Abstract

The aim of the study was to evaluate analgesic activity ("hot plate" test), anti-inflammatory activity (carrageenan-induced paw edema) and locomotor activity in rats under the influence of three fractions of Chelidonium majus herb extract: full water extract (FWE), protein enriched fraction (PEF), and non-protein fraction (NPF). Effects of the fractions on the level of chosen cytokines and their mRNA levels were also assessed using lipopolysaccharide (LPS) administration as a proinflammatory cue. All fractions and diclofenac did not affect the locomotor activity of rats in comparison with the control group. FWE and PEF three hours after administration showed statistically significant analgesic activities comparable to morphine ($p < 0.05$). A slight reduction in rat paw edema was observed after three (comparable with diclofenac) and six hours in the NPF group. FWE revealed a statistically significant pro-inflammatory effect after three hours in comparison with the control group. Peripheral IL-1 and IL-4 cytokine concentrations were reduced under FWE and NPF, PEF fractions. The combination of FWE, PEF and NPF together with LPS showed only the effects of LPS. We suggest that protein enriched fraction (PEF) produced centrally mediated (morphine-like) analgesic action, whereas the anti-inflammatory potential was shown only after LPS-induced inflammation. The precise mechanisms involved in the production of anti-nociceptive and anti-inflammatory responses of studied fractions are not completely understood, but they may be caused rather by the presence of protein more than alkaloids-enriched fraction. This fraction of the extract could be used as an alternative therapy for the prevention of inflammatory-related diseases in the future, but further studies are needed.

Key words: *LPS–induced rats, analgesic, anti-inflammatory, Chelidonium majus aqueous extract, non-protein fraction, protein enriched fraction, proinflammatory cytokines, mRNA.*

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Introduction

Pain occurring in acute or chronic diseases is a very common challenge in medical care. Thus, pharmacotherapy of pain is one of the top priorities. This is even more important, because due to the fact that opioids and nonsteroidal anti-inflammatory drugs are among the most commonly used drugs in clinical practice, their use subsequently can induce unexpected drug interactions and/or several types of medicines often produce several adverse reactions [1, 2]. A very interesting option in this field may be a plant extract from *Chelidonium majus* (CM) which has been traditionally used

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in the treatment of skin diseases such as eczema, ringworm, oral infection, pains and nervous disorders and gastrointestinal diseases [3, 4]. It has been shown that CM extracts have anti-inflammatory, choleretic, antimicrobial, antiviral, antitumor, analgesic, anti-spasmodic and hepatoprotective properties [5-7]. *Chelidonium majus* (greater celandine) belongs to *Papaveraceae* and it is distributed throughout the world, including Europe, Asia, Northwest Africa and North America [5]. The aerial part of this plant contains isoquinoline alkaloids, such as chelidonine, chelerythrine, sanguinarine, berberine, coptisine and stylopine [4, 7, 8]. Moreover, this herb includes organic acids, carotenoids, flavonoids and proteins [9-11]. Studies showed that methanolic extract from the herb of CM significantly suppressed the progression of collagen-induced arthritis (mice model) and that this action was characterized by the decreased production of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interferon γ (IFN-γ), B cells, γδ T cells (in spleen) and increased proportion of CD4+CD25+ regulatory T cells *in vivo*. Moreover, it was observed that in the serum of mice, the levels of IgG and IgM RA factor were decreased [12]. Furthermore, recent studies showed that hydroalcoholic extracts and its alkaloids, especially berberine, chelidonine and sanguinarine have a significant hERG potassium channel blocking effect [5]. Other results revealed that the aqueous extract of *Ch. majus* suppressed glycine and gamma-aminobutyric acid (GABA), activated ion currents and elevated glutamate-activated ion currents in rat periaqueductal gray neurons, which represent a key structure of the descending pain control system [13, 14]. Other studies showed that berberine, one of isoquinoline alkaloids from CM, possesses neuroprotective action in an animal model of various CNS diseases [3, 15] and showed an antinociceptive effect on visceral hypersensitivity in rats [16]. Berberine was found to completely block both morphine-induced locomotor sensitization and analgesic tolerance, and reduce D(1) and NMDA receptor bindings in the cortex of mice [17]. According to Yoo *et al.* [17], berberine should be viewed as a potential novel means of attenuating morphine-induced sensitization and analgesic tolerance. However there are no data on the analgesic effect of fractions of CM extract. Thus, the aim of the present study was to evaluate: (1) analgesic activity using "hot plate" test, (2) anti-inflammatory activity in a model of carrageenan-induced paw edema, (3) locomotor activity of three fractions of the extract: full water extract (FWE), protein enriched fraction (PEF), and non-protein fraction (NPF) in rats. Moreover, effects of the fractions on level of some cytokines (IL-1, IL-4, IL-6) and their mRNA levels were also assessed.

Material and methods

Plant material

Plant material, aerial parts of *Ch. majus* L. cvar. Cynober, were collected from a controlled cultivation at the Institute of Natural Fibers and Medicinal Plants in Poznan, Poland. The field cultivation was established in spring by sowing seeds directly into ground (lessivé soil with granulometric composition of light loamy sands). The herb was harvested during beginning of flowering of plants. The raw material was dried at 55-60°C. The material was identified at the Department of Medicinal and Cosmetic Natural Products (Faculty of Pharmacy, Poznan University of Medical Sciences).

Phytochemical study

Extract preparation

Full water extract (FWE)

10 kg of raw plant material was extracted with water by percolation (3 h, 25 l/min). The extract was concentrated under vacuum to 1/3 volume and fractionated with acetone (4 : 1).

Non-protein fraction (NPF) and protein enriched fraction (PEF)

10 kg of raw plant material was powdered and extracted with water (90 l, 90°C) by liquid-solid extraction (FT-29, Armfield, England). The extract was concentrated under vacuum to 1/3 volume. In the concentrated and clear extract, proteins were precipitated with cold acetone (4 : 1; –20°C; 30 min). The protein suspension was centrifuged and the purified protein fraction was suspended in distilled water and then it was concentrated under vacuum. The supernatant, which was obtained after precipitation of proteins, followed the same procedure. The fractions were freeze-dried and frozen at –50°C (36 hours).

Determination of alkaloids by HPLC-DAD

Alkaloids were analyzed by means of modified methods: European Pharmacopea 6 (monography of greater celandine) and Sárközi *et al.* [18]. Sample: 0.5-1.0 g of dry greater celandine was weighted in a 250 ml round-bottomed flask and heated under reflux condenser for 30 min in 150 ml of 12% V/V glacial acetic acid. The sample was cooled down and diluted with the same solution to 200 ml. 6.0 ml of concentrated ammonium and 200.0 ml of methylene chloride was added to 60.0 ml of filtrate. The solution was shaken for 30 min. The organic fraction was evaporated to dryness in vacuum at a temperature not exceeding 40°C. The residue was dissolved in 5.0 ml of methanol. HPLC-DAD analysis was performed on an Agilent 1100. Separation of the methanolic sample was prepared on ZORBAX Poroshell 120 SB-C18 (Agilent) 3 × 100 nm (2.7 µm). The column temperature was: 40°C. The volume of injection was 50 µl. A gradient mixture of: phase A: 30 mM ammonium formate (pH = 2.8) and phase B: acetonitrile: methanol 14.7 : 18.0 (V : V) were used as eluent, starting from 20% phase B to 60% phase B in 16 min. The flow-rate was: 0.50 ml/min. Peaks were identified by the addition of standard solutions and by

UV-VIS spectra. Quantitative determination for coptisine and chelidonine was performed at 240 nm, for sanguinarine and chelerythrine – 280 nm and for berberine – 345 nm by an external standard method. Standard solutions were prepared in methanol.

Pharmacological study

Rats

Experiments with rats were performed in accordance with the Polish governmental regulations (Journal of Laws of 2005, no. 33, item 289). The study was conducted in accordance with the ethical guidelines for investigations in conscious animals and the study protocol was approved by the Local Ethics Committee (61/2010). The experiments were performed on six week-old male Wistar rats, housed in controlled room temperature (20 ±0.2°C) and humidity (65-75%) under a 12 h : 12 h light-dark cycle (lights on: 7 a.m.). Animals were kept in groups (8-10 rats/group) in light plastic cages (60 × 40 × 40 cm) and given *ad libitum* access to standard laboratory chow (pellets-Labofeed B) and tap water.

Treatments and groups

Experiments were performed on male Wistar rats housed in controlled conditions. The extracts were administered intragastrically (p.o.) at a dose of 200 mg/kg b.w. (groups FEW, PEF, NPF); morphine (5 mg/kg b.w., s.c.) and diclofenac (50 mg/kg b.w., i.p.) were used as standard analgesic drugs, control animals were treated with *vehiculum* (water) (p.o.). In order to measure locomotor activity ($n = 53$) and to perform the hot plate test ($n = 40$) 6 groups of rats were used (FEW, PEF, NPF, morphine, diclofenac 50, *vehiculum* (water) (p.o.). Anti-inflammatory activity was measured in a model of carrageenan-induced raw paw edema using 5 groups of rats (FWE, PEF, NPF, diclofenac, *vehiculum* (water)). Lipopolysaccharide (LPS) from *Escherichia coli* serotype 026:B6, (Sigma Chemicals Co.) was dissolved in an aqua pro injectione and administered in a single dose of 100 µg/kg intraperitoneally (i.p.) according to Manikowska *et al.* [19].

Measurement of locomotor activity

Locomotor activity assessment was performed with a licensed activity meter (Activity Cage, Ugo Basile, Italy) by placing the animals in the center of the apparatus and recording their horizontal activity. The data obtained were expressed as signals corresponding to animal movements for 5 min. The locomotor activity was measured 60 min after the administration of a single dose of extracts, or reference drugs or *vehiculum*. Any distracting factors were reduced to the minimum (noise, presence of people, presence of other rats).

Hot plate test

Rats were brought to the testing room and allowed to acclimate for 10 minutes before the test. Pain reflexes in response to a thermal stimulus were measured using a Hot-Plate Analgesia Meter. Each animal was tested only once and was not habituated to the apparatus prior to testing. The hot plate test was carried out in groups of rats using a hot plate apparatus (20 × 20 cm surrounded by a clear acrylic cage) maintained at 48 ±2°C. The extracts or reference drugs were given and the effects were measured at 3.0 and 6.0 h following the application. Using timer, the latency to respond was observed with a hind paw lick, hind paw flick or jump which was measured to the nearest 0.1 second. Subsequently, the mouse was immediately removed from the hot plate and returned to its home cage. The maximal time of the response was established as 30 seconds. Morphine (30 min before the test) treated animal group was included as positive controls. The cut-off time was 60 s in order to minimize skin damage.

Carrageenan-induced paw edema

Skin inflammation was induced in the right hind paw of rats by the topical application of 2 mg/paw of carrageenan dissolved in 0.2 ml of 0.9% saline solution. The rear left paw of the rats, which was used as the control, received the same volume of 0.9% saline solution. Single doses of the extract (dissolved in water) 60 min after carrageenan injection. For comparison (positive control), one group of rats was treated with the acute diclofenac injection 60 min after carrageenan administration. The rate of edema of the two paws was measured at 3.0, 6.0 h after carrageenan injection using a plethysmometer (Hugo Sachs Electronic, Germany).

Change of rat's paw thickness was evaluated using the following equation:

$$\Delta G = (L_c - L_w) - (R_c - R_w),$$

ΔG – value expressing the change in paw's thickness against baseline (before inflammation),
L_w – left paw's thickness before carrageenan injection,
R_w – right paw's thickness before carrageenan injection,
L_c – left paw's thickness 3.0 or 6.0 h after carrageenan injection,
R_c – right paw's thickness 3.0 or 6.0 h after carrageenan injection.

Measurement of cytokine levels

On the next day after analgesic and anti-inflammatory activity tests, the extracts were given again and 60 min later, each group (treated with extracts or diclofenac) was divided into two subgroups, where one subgroup was administered a single dose of LPS (100 µg/kg, i.p.) and the remaining rats were injected with vehicle (aqua pro injectione). After next 60 min rats were sacrificed, the blood was collected and centrifuged at 4000 rpm for 15 minutes and the serum was separated and stored at –80°C for further

measurements. Biochemical measurements were done by means of the ELISA method using commercially available kits (RayBiotech Inc.). These tests comprised recombinant cytokines from *Escherichia coli* and antibodies against rats IL-1, IL-4, and IL-6. The results were calculated based on the absorbance of complex cytokines-antibodies and concentrations were obtained from model curves.

Influence of protein and non-protein fraction extracts from *Chelidonium majus* L. on mRNA levels of studied genes

From the peripheral blood of rats, mononuclear cells (MNCs) were isolated via a gradient centrifugation in Ficoll. From the resulting cell pellets a total RNA was isolated using TriPure Isolation Reagent (Roche) according to the manufacturer's protocol. The integrity of RNA was visually assessed electrophoretically and spectrophotometrically (BioPhotometer Eppendorf). 1 µg of total RNA from all samples was used for reverse transcription into cDNA using Transcriptor First Strand Synthesis Kit (Roche), according to the manufacturer's protocol, then they were stored at –20°C or used directly for quantitative real-time PCR (qRT-PCR).

The resulting cDNA will be a template for PCR reaction in real time, to be conducted in the capillaries in the LightCycler® using the reagent kit of the LightCycler® FastStart DNA Master SYBR Green I (Roche). IL-1, IL-6, TLR-1 mRNAs levels were analyzed by two-step quantitative real-time PCR (qRT-PCR), in a volume of 10 µl reaction mixture, using relative quantification methodology with a LightCycler TM Instrument (Roche, Germany) and a LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science), according to the manufacturer's instructions. All primer sequences were self-designed using Oligo 6.0 software (National Biosciences) and verified by the electrophoretic assessment and by a single temperature dissociation peak (melting curve analysis) of each cDNA amplification product. A GAPDH gene was used as a housekeeping gene (endogenous internal standard). Standard curves were prepared from dilution of cDNA and generated from a minimum of four data points for each quantified gene. All quantitative PCR reactions were repeated twice. Data were evaluated using LightCycler Run 4.5 software (Roche Applied Science). Each PCR run included a non-template control to detect potential contamination of reagents.

Statistical analysis

All values were expressed as means ± SEM. Statistical comparison of the results was carried out using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference post hoc test for detailed data analysis. A p value of < 0.05 was considered as statistically significant.

Results

Phytochemical study

Phytochemical analysis showed coptisine, chelidonine, berberine and chelerythrine in all fractions, but sanguinarine was observed only in PEF (Table 1). The HPLC analysis revealed that the highest concentration of berberine (23.4 mg/100 g) and coptisine (628.9 mg/100 g) was identified in FWE, chelidonine (134.3 mg/100 g) and chelerythrine (1.16 mg/100 g) – in NPF.

Measurement of locomotor activity

A one-way ANOVA ($F(5,47) = 4.51$; $p < 0.002$) analysis revealed that only morphine resulted in a significant, inhibitory effect against locomotor activity of rats expressed as their horizontal spontaneous activity ($p < 0.05$). All fractions (FWE, PEF, NPF) and diclofenac did not affect the locomotor activity of rats in comparison with the control group (Table 2). Therefore, it can be stated that they exclude the effect of sedation to the results obtained in further studies. Sedative effects were observed only for morphine, which is consistent with previous knowledge.

Hot plate test

A one-way ANOVA revealed significant differences in analgesic activity of all substances ($F(5,31) = 3.62$; $p < 0.01$). However, no effect of time on the analgesic effects was noted ($F(1,31) = 2.20$; $p < 0.16$). At the same time, analysis of the interaction of both factors in this test indicated the significance of the action of both (F(5,31)

Table 1. HPLC quantification of alkaloids in extracts of *Ch. majus*

Rf	Compound	FWE (mg/100 g)	NPF (mg/100 g)	PEF (mg/100 g)
8.8 (9)	coptisine	628.9	509.2	404.3
8.4	chelidonine	109.7	59.3	134.3
10.4	sanguinarine	–	–	19.0
10.7	berberine	23.4	17.1	17.7
11.1	chelerythrine	0.67	1.16	0.29

FWE – full water extract; PEF – protein enriched fraction; NPF – non-protein fraction

Table 2. Analgesic and anti-inflammatory activities of fractions of water extract of *Ch. majus*

Group	Locomotor activity test	Hot plate test		Carrageenan-induced paw edema	
	[number of impulses/5 min]	time [s]		ΔG [ml]	
		t = 3 h	t = 6 h	t = 3 h	t = 6 h
control	646 ±92	31.9 ±2.3	39.9 ±2.9	1.62 ±0.09	1.51 ±0.10
morphine	325 ±63**	44.2 ±1.2**	53.2 ±2.6**	–	–
diclofenac	703 ±64	39.7 ±6.4	47.4 ±2.3	1.25 ±0.10	0.75 ±0.10**
FWE	499 ±63	51.7 ±2.7**	48.9 ±1.5*	2.15 ±0.08**	1.73 ±0.08
PEF	662 ±66	50.1 ±3.1**	42.8 ±3.0	1.94 ±0.13	1.90 ±0.10
NPF	714 ±82	40.7 ±6.1*	45.2 ±5.7	1.50 ±0.19	1.21 ±0.18

values are means ± SEM; n = 6-7 in each group
FWE – full water extract; PEF – protein enriched fraction; NPF – non-protein fraction; t – time after administration of the substance (3 h and 6 h); ΔG – value expressing change in paw's thickness against baseline (before inflammation) after 3/6 h
***, * significant difference vs. control group; $p < 0.05$ or $p < 0.1$, respectively*

= 3.00; $p < 0.02$). Detailed post-hoc analysis of the time of the pain response in animals (licking hind paw) after application of each substance compared to the control group shows that morphine has a statistically significant analgesic effect ($p < 0.05$), both after 3 and 6 hours after application. However, diclofenac did not show significant analgesic activity in any of the tested time intervals. It was found out that three hours after administration of the FWE and PEF, a statistically significant analgesic effect was observed ($p < 0.05$), whereas the NPF showed only a tendency to the analgesic effect ($p < 0.1$). It was also noted that these substances showed activity comparable to morphine, because there were no specific differences in relation to the opioid. After 6 hours, the PEF and the NPF, in addition to a low tendency of the FWE ($p < 0.1$), did not show significant analgesic activity compared to the control (Table 2).

Carrageenan-induced paw edema

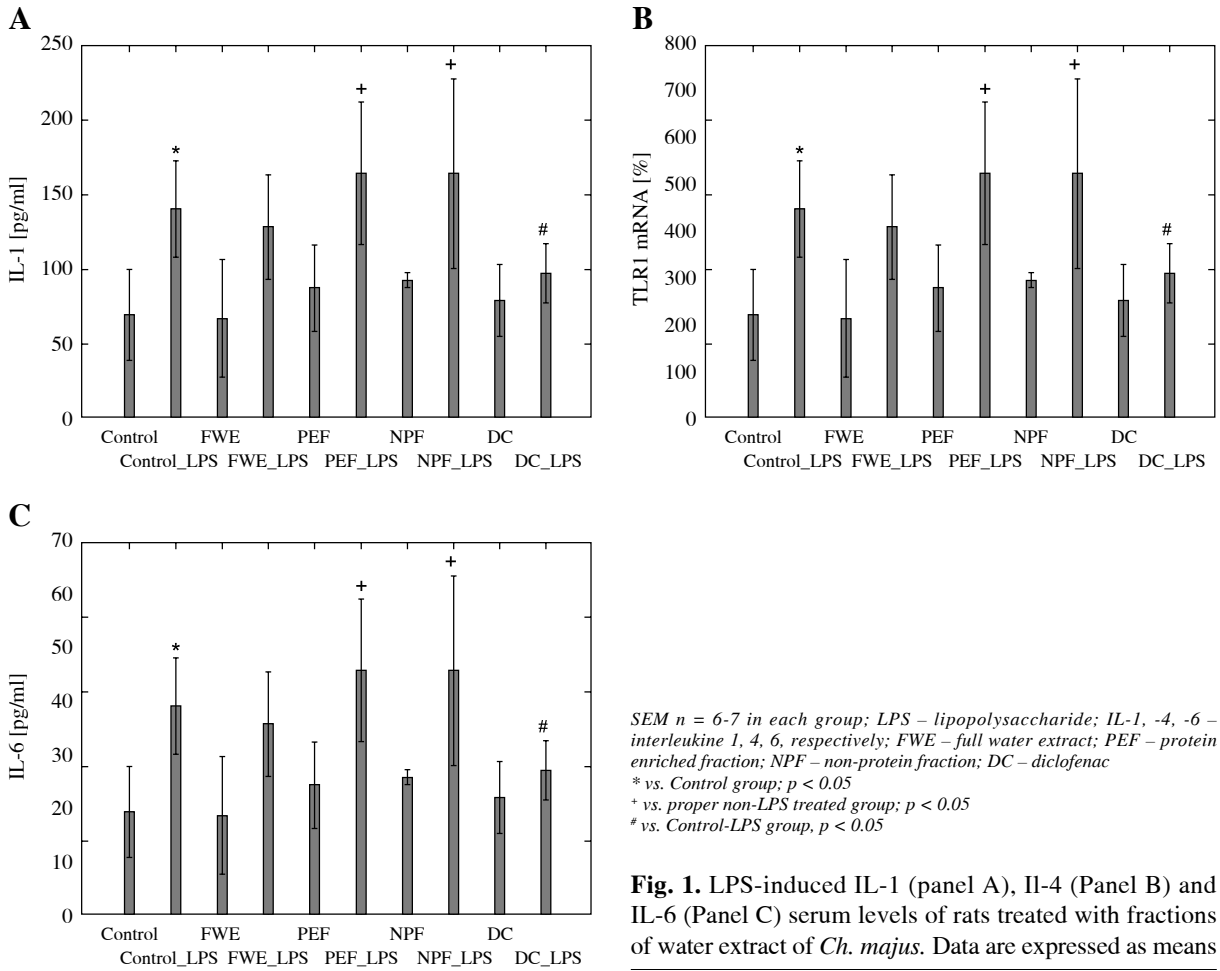
A one-way ANOVA revealed significant differences in anti-inflammatory activity of all substances ($F(4,55) = 11.6$; $p < 0.001$). The influence of time on the anti-inflammatory activity ($F(1,55) = 72.9$; $p < 0.001$) was also observed. At the same time, analysis of the interaction of both factors in this test indicated the significance of the action of both $F(4,55) = 7.94$; $p < 0.001$). The detailed post-hoc analysis revealed a statistically significant anti-inflammatory activity of diclofenac after six hours, because a statistically significant diminishing of the values compared to control ($p < 0.05$) was shown. The reduction of rat paw edema was observed after three hours for diclofenac, and after three and six hours for NPF, but differences were statistically non-significant ($p > 0.05$). Moreover, it was noted that FWE after three hours resulted in a statistically significant pro-inflammatory effect in comparison with the control group ($p < 0.05$). Other substances did not show a significantly differences when compared with the control group (Table 2).

Peripheral cytokine concentration changes

A one-way ANOVA revealed significant differences between the groups in the levels of IL-1 ($F(9,50) = 7.01$, $p < 0.001$) (Fig. 1, Panel A). LPS treatment led to a significant increase in the cytokine concentration in rats when compared with the control group ($p < 0.05$). Diclofenac (DC) showed a significant reduction in IL-1 concentration only in LPS-induced rats in comparison to control-LPS-treated animals ($p < 0.05$). Moreover, the combination of FWE, PEF and NPF together with LPS showed only the effects of LPS since the differences were significant only when compared with the proper extract of non-LPS treated group ($p < 0.05$), whereas there were no statistical significant differences in IL-1 level in comparison to control LPS-treated animals ($p > 0.05$).

Similarly, the one-way ANOVA revealed significant differences between the groups in the levels of IL-4 ($F(9,50) = 3.01$, $p < 0.01$) (Fig. 1, Panel B). All the effects were the same as observed for the above mentioned concentrations of IL-1.

On the contrary, the effects of extracts and DC administration on IL-6 level differed when compared with IL-1 and IL-4 concentrations. It was found that a one-way ANOVA revealed significant differences between the groups in the levels of IL-6 ($F(9,50) = 4.05$, $p < 0.001$) (Fig. 1, Panel C). However, detailed statistical analysis showed that both FWE and PEF produced anti-inflammatory activities similarly as DC, since FWE-LPS and PEF-LPS groups had lower IL-6 levels when compared with control-LPS group ($p < 0.05$). The effect is probably produced mainly by proteins presented in the extracts, because NPF-LPS did not show such activity when combined with LPS-treated rats ($p > 0.05$).



SEM n = 6-7 in each group; LPS – lipopolysaccharide; IL-1, -4, -6 – interleukine 1, 4, 6, respectively; FWE – full water extract; PEF – protein enriched fraction; NPF – non-protein fraction; DC – diclofenac
** vs. Control group; p < 0.05*
+ vs. proper non-LPS treated group; p < 0.05
 # vs. Control-LPS group, p < 0.05

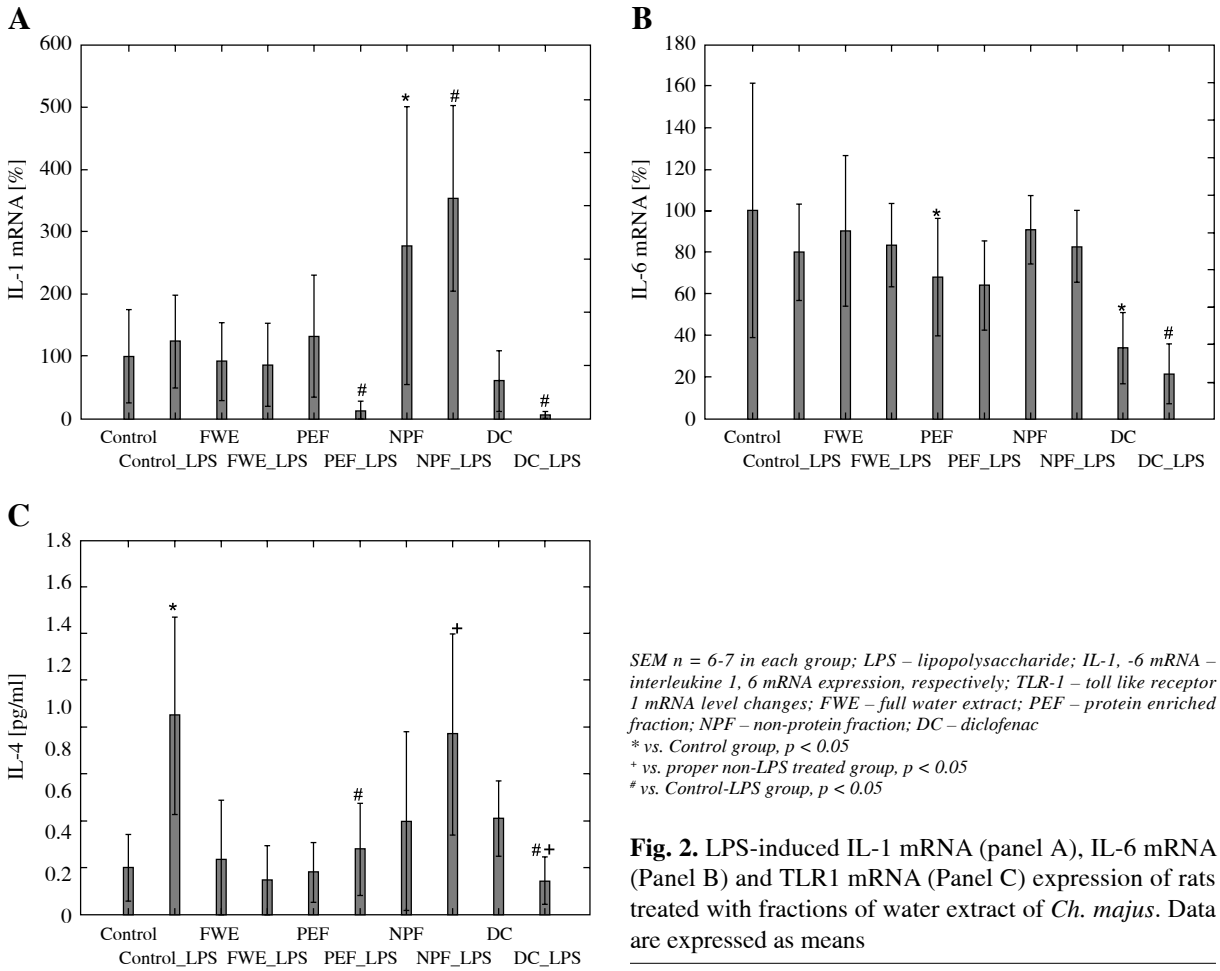
Fig. 1. LPS-induced IL-1 (panel A), Il-4 (Panel B) and IL-6 (Panel C) serum levels of rats treated with fractions of water extract of *Ch. majus.* Data are expressed as means

Cytokine (IL1B, IL6, TLR1) mRNA level changes

A one-way ANOVA revealed significant differences between the groups in the expression of interleukin 1B (IL1B) mRNA ($F(9,48) = 7.40$, $p < 0.001$) (Fig. 2, Panel A). In control rats the LPS administration (Control-LPS group) caused a slight, insignificant elevation of IL-1B mRNA level ($p > 0.05$) vs. non-LPS treated control rats (Control). Diclofenac (DC) showed a significant reduction in IL-1B mRNA expression only in LPS-induced rats in comparison to control LPS-treated animals (Control-LPS) ($p < 0.05$). The whole aqueous extract of *Ch. majus* herb (FWE) did not cause any statistically significant changes in the level of IL1B transcripts both in LPS-treated and LPS-non-treated rats On the contrary, PEF combined with LPS significantly lowered (by 86%) transcription of IL1B encoding gene ($p < 0.05$), whereas in non-LPS-treated rats, no such effect was found. However, both a combination of NPF with or without LPS produced a very strong, statistically significant induction of IL-1B mRNA transcription (by 250 and 178%, respectively) when compared with proper control groups ($p < 0.05$).

A one-way ANOVA revealed significant differences between the groups in the expression of interleukin 6 (IL6) mRNA ($F(9,50) = 4.68$, $p < 0.001$) (Fig. 2, Panel B). Detailed post-hoc analysis showed only PEF a statistically significant decrease of IL 6 mRNA expression when compared with the control group ($p < 0.05$). Moreover, DC both in LPs-treated and non-LPS-treated rats lowered significantly the transcription of IL6 encoding gene ($p < 0.05$). The rest of the group did not produce statistically significant effects ($p > 0.05$).

A one-way ANOVA revealed significant differences between the groups in the expression of toll-like receptor 1 (TLR-1) mRNA ($F(9,50) = 5.66$, $p < 0.0001$) (Fig. 2, Panel C). It was found that LPS induction produced a very strong and statistically significant elevation of TLR1 mRNA (by 338%) when compared with control rats ($p < 0.05$). On the contrary, PEF and DC administration in LPS-treated animals showed a strong decreasing effect in comparison to Control-LPS group ($p < 0.05$). Moreover, the administration of NPF in LPS receiving rats caused a very strong elevation of TLR1 mRNA level (by 300%) when compared with the Control-LPS group ($p < 0.05$).



SEM n = 6-7 in each group; LPS – lipopolysaccharide; IL-1, -6 mRNA – interleukine 1, 6 mRNA expression, respectively; TLR-1 – toll like receptor 1 mRNA level changes; FWE – full water extract; PEF – protein enriched fraction; NPF – non-protein fraction; DC – diclofenac
** vs. Control group, p < 0.05*
+ vs. proper non-LPS treated group, p < 0.05
 # vs. Control-LPS group, p < 0.05

Fig. 2. LPS-induced IL-1 mRNA (panel A), IL-6 mRNA (Panel B) and TLR1 mRNA (Panel C) expression of rats treated with fractions of water extract of *Ch. majus*. Data are expressed as means

Discussion

Pain and inflammation occur in many diseases at different stages and are among the most important therapeutic goals of modern medicine. In addition to medications used for a long time, we are still looking for new drugs that are more effective in use, but also exhibit insignificant side effects. Several models of nociception and inflammation induced in animals are known and can be used to evaluate the analgesic and anti-inflammatory activities of new compounds and crude extracts from plants [20-22]. Thus, the aim of the present study was to evaluate analgesic and anti-inflammatory activities in various rat's models and assessment of the impact of full water extract (FWE), protein enriched fraction (PEF), non-protein fraction (NPF) of *Ch. majus* on the level and gene expression for selected mediators of inflammation.

In the present study, it was observed that single application of FEW, NPF and PEF did not affect the locomotor activity of rats, similarly as diclofenac, only morphine resulted in a significant, inhibitory effect against locomotor activity of rats expressed as their horizontal spontaneous activity. The sedative effect of morphine is consistent with the pharmacological profile of this opioid drug [23, 24].

In our study, to investigate the pain response the hot plate test was used. This test is a good model for the study of drugs acting through the central and supramedullary mechanisms of nociception [25, 26]. It was observed that after three hours the FEW and PEF exerted a pronounced analgesic effect, which strength was comparable to that of morphine. We found that the FEW contains many alkaloids, among which berberine is the major component (23.4 mg/100 g). Recent studies have shown that water extract of *Ch. majus* at 200 mg/kg dose, produced higher analgesic activity than aspirin at 90th minute (tail-flick test on mice) [27]. Moreover, it was observed that berberine have antiallodynic effects after streptozotocin (STZ)-induced diabetes using a rat model [28], and could mitigate allodynia induced by chronic constriction injury of the sciatic nerve in rats (a neuropathic pain model) [29]. Furthermore, Citoglu *et al.* [27] showed that chelidonine (other major alkaloid in CM) produced higher analgesic activity than aspirin (tail-flick test on mice). Thus, it can be concluded that berberine and chelidonine may be responsible for the analgesic effect

in our study. On the other hand, it was observed that PEF also showed an analgesic effect, although the mechanism of action of these proteins is not known.

Most authors using different experimental models of inflammation *in vitro*, demonstrated anti-inflammatory activity of *Ch. majus* extracts [12, 30]. It was found that mainly alkaloids contained in extracts may be responsible for these anti-inflammatory effects [31, 32].

In our study, in a carrageenan-induced rat paw edema model, it was demonstrated that only NPF fraction caused a reduction in rat paw edema after three and six hours of the experiment duration (and after three hours for diclofenac), but differences were statistically non-significant. Moreover, FEW revealed some statistically significant pro-inflammatory effect after three hours (Table 2). Analysis of changes in concentrations of peripheral cytokines indicates some anti-inflammatory properties of FWE and NPF, PEF fractions in the case of IL-1 and IL-4 concentrations. We have observed that the combination of FWE, PEF and NPF together with LPS showed only the effects of LPS, since the differences were significant only when compared with the proper extract of the non-LPS treated group (Fig. 1, Panel A, B). In the case of IL-6 the anti-inflammatory potential was especially visible in both, FWE-LPS and PEF-LPS groups of animals resulting in its level reduction when compared with the Control-LPS group, similar to the effect of DC shown in LPS-treated rats. We assume that this effect could probably be produced mainly by proteins presented in the extracts, because NPF-LPS did not show such activity when combined with LPS-treated rats (Fig. 1, Panel C). It should be mentioned that the standard drug – diclofenac showed a strong anti-inflammatory effect lowering the cytokine levels after previous stimulation inflammation using LPS (Fig. 1, Panel A-C).

A transcription profile of genes encoding studied cytokines shows variabilities in the mRNA levels between particular LPS or non-LPS treated groups, regardless of whether the animals received FWE or its fractions (PEF, NPF) (Fig. 2, Panel A, B, C). For example, a statistically insignificant influence of FEW on the level of IL1B transcripts in both, LPS-treated and LPS-non-treated rats, a very strong statistically significant induction of transcription in the NPF group with or without LPS was observed, in contrast to the decreasing effect of PEF when combined with LPS. On the contrary, in the case of IL-6 mRNA a slight and generally insignificant reduction of transcription in FEW, PEF or NPF groups treated with or without LPS was observed. It is difficult to clearly explain the modifications in the cytokine mRNA expression profile in the fraction of lymphocytes and changes in their peripheral concentrations. Also an evident correlation between the degree of change in the TLR1 gene expression profile and the all studied genes encoding cytokines cannot be clearly seen. For example, a similar degree of induction of TLR mRNA transcription can also be seen in the case of a FEW in non-LPS animals for IL-1B encoding gene, while suppression of IL-1 and IL-6 mRNA levels occurred in the case of FEW-LPS animals and of IL-6 mRNA itself in the PEF-non-LPS-treated group. We have observed that many of analyzed groups of animals had, however, an altered state of TLR1 gene mRNA transcription in relation to other cytokines depending on whether the animals receiving the extract fractions were stimulated with LPS or not (Fig. 2). This may prove a diversified affinity of individual metabolites in studied fractions of the plant extract on the enzymatic machinery regulating transcription of the gene encoding the TLR1 receptor and above mentioned cytokines.

The observed changes in the transcriptional profile in non-LPS-treated and LPS-treated groups for each fraction of the extract may be the cause, at least in part, of different affinity components of studied plant extract fractions to the cellular machinery regulating the transcription of examined genes and the process of secretion of cytokines. In addition, it should be noted that molecular studies presented in this paper were conducted on the material being a fraction of various types of rats' white cell.

Only a few *in vivo* experiments were carried out attempting to determine anti-inflammatory potential of the extract from *Ch. majus* [12, 33] or several isochinoline alkaloids [34-37], but they were made in a different experimental scheme. For example, in a study performed by Lenfeld *et al*., sanguinarine revealed a higher anti-inflammatory activity than chelerythrine, which was explained by the authors with the different oxygen electron donating substituents [34]. Chung *et al*. revealed that a water extract from *Ch. majus* significantly induced the production of TNF-α and NO in peritoneal macrophages in mice via nuclear factor-κB (NF-κB) dependent pathway. This effect was more significant when co-administered this extract (1 mg/ml) with recombinant IFN-γ (rIFN-γ, 10 U/ml). The increased production of NO and TNF-α was almost completely inhibited by pyrrolidone dithiocarbamate (100 μm) – a NF-κB inhibitor, which suggests that NF-κB may play a crucial role in the anti-inflammatory action of this *Ch. majus* extract [33]. Le *et al.* reported that the methanolic extract from *Ch. majus* dose dependently [4, 40, 400 mg/kg/day] suppressed the progression of collagen-induced arthritis in mice causing the reduction in secretion of TNF-α, IL-6, IFN-γ, B cells, γδ T cells (in spleen), increased proportion of CD4+CD25+ regulatory T cells *in vivo* as well as a decrease in the immunoglobulins IgG and IgM (rheumatoid arthritis factors) in the serum [12]. Results from these studies were, at least, partially different than ours.

The reason for this discrepancy in above-mentioned results and results from our study may be due to different experimental conditions, applied in our research, different doses and the nature of the extract of *Ch. majus*. Furthermore, the above-mentioned studies have not evaluated the changes in the peripheral concentrations of the cytokines.

Several studies showed that berberine, one of the most studied alkaloid occurring in *Chelidonium majus* and many other plants (i.e. *Berberis vulgaris*, *Hydrastis canadensis*, *Coptis chinensis*), exerted blocking the induction of edema on mouse ear, inhibition of cyclooxygenase-2 (COX-2) transcriptional activity, immunomodulation of adjuvant-induced arthritis [38]. Previous studies have shown that berberine reduced prostaglandin E2 production *in vitro* (in the oral cancer cell line and KB cells) and *in vivo* (in carrageenan induced air pouch in rats), moreover berberine inhibited activator protein 1, a key transcriptional factor in inflammation and carcinogenesis [39]. It was shown that a stylopine concentration-dependently reduced, among others, IL-6 production as well as IL-1B, prostaglandin E2 (PGE2) and TNF-α cytokine concentrations in LPS stimulated RAW 264.7 cells (macrophages) [40]. It was observed that chelerythrine showed pronounced inhibition of the acetic acid-induced writhing response and has a significant anti-inflammatory action, which may be relevant to the inhibition of the release/production of exudates and prostaglandin E(2) mediated through cyclooxygenase-2 regulation [36]. Li *et al.* observed that this alkaloid inhibits LPS-induced TNF-α level and NO production in LPS-induced murine peritoneal macrophages through selective inhibition of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) activation [35]. Another alkaloid, sanguinarine, occurring in *Ch. majus*, greatly inhibited the production of PGE2, and also decreased COX-2 protein expression, without affecting COX-1 expression, in LPS-stimulated peritoneal macrophages [37]. However, in none of the above experiments the gene expression profile of selected pro- and anti-inflammatory cytokines under specific alkaloids has not been examined. A screening analysis was also conducted in LPS stimulated RAW 264.7 macrophages for the anti-inflammatory potential of several alkaloids, such as stylopine, methyl 2'-(7,8-dihydrosanguinarine-8-yl)acetate, protopine, norchelidonine, chelidonine, berberine and 8-hydroxydihydrosanguinarine, isolated from *Ch. majus* in which chelidonine and 8-hydroxydihydrosanguinarine showed strong inhibitory activities toward the LPS-induced NO production in RAW264.7 cells with IC(50) values of 7.3 and 4.5 μM, respectively. A transcription profile of COX-2 and iNOS mRNA in dose-dependent manner under these two compounds was also declined, indicating that these compounds attenuated the syntheses of these transcripts at the transcriptional level [32].

On the other hand, there were also many reports on the immunomodulating activity of milk proteins (caseins), and whey proteins (i.e. α-lactalbumin, β-lactoglobulin, lactoferrin, osteopontin, immunoglobulins) [41, 42]. A recent study showed that protein hydrolysate from *Pisum sativum* (seeds) showed significant inhibition of NO production by activated macrophages, significantly inhibited their secretion of pro-inflammatory cytokines. Moreover, oral administration of pea protein hydrolysate enhanced the phagocytic activity of their peritoneal macrophages and stimulated the gut mucosa immune response [43]. In other study, it was shown that albumin fraction from *Pisum sativum* seed extract ameliorated the colonic mRNA expression of different proinflammatory markers: cytokines, inducible enzymes, metalloproteinases, adhesion molecules, and toll-like receptors, as well as proteins involved in maintaining the epithelial barrier functioning [44]. Also, soy proteins showed immunomodulating activity on proliferation of murine splenic lymphocytes and phagocytic effect of peritoneal macrophages [45].

According to our conviction presented in this paper study is the first attempt to clarify the molecular mechanism of action (analysis of changes in mRNA transcription profile of selected cytokines, assessment of changes in the concentration of cytokines in peripheral blood of animals) of different fractions from the *Chelidonium majus* aqueous extract in experimental animals undergoing induction of LPS.

Conclusions

In conclusion, the results presented in this study suggest that protein enriched fraction produced centrally mediated (morphine-like) analgesic action and anti-inflammatory activity in LPS-induced inflammation. The precise mechanisms involved in the production of the anti-nociceptive and anti-inflammatory responses of fractions of *Ch. majus* extract are not completely understood, but they may be caused by the presence of protein more than alkaloids. Thus, this fraction of extract could be used as an alternative therapy for the prevention of inflammatory-related diseases in the future, but further studies are needed.

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