

## ANTINOCICEPTIVE AND ANTIANXIETY ACTIVITY OF HYDROETHANOLIC EXTRACTS OF THREE *IMPATIENS* SPECIES IN MICE

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**Abstract:** The plants of the *Impatiens* L. (Balsaminaceae) have been used for a long time in folk medicine in different painful conditions, and to treat rheumatism, isthmus and crural aches, fractures, superficial infections, fingernail inflammation. This study was undertaken to determine the pharmacological profile of hydroethanolic extracts from *Impatiens glandulifera*, *I. noli-tangere* and *I. parviflora*. A range of behavioral assessments was applied to evaluate the effects of obtained extracts i.e. measurement of body temperature, tests of locomotor activity and motor coordination, nociceptive reaction and anxiety-like behavior. Hydroethanolic extracts were analyzed for total polyphenol (TPC), flavonoid (TFC), flavones/flavonols (TFFC), and flavonones/dihydroflavonols (TFDC) content. Our results show that the extracts from *Impatiens* species contain high levels of TPC, TFC, TFFC, and TFDC. Oral (i.e., by gavage) administration of *Impatiens* L. extracts (except for *I. noli-tangere*) presented an antinociceptive or/and anti-inflammatory activity in the writhing test. The antinociceptive effect of *I. parviflora* leaves (100 mg/kg) and *I. glandulifera* flowers (100 mg/kg) was reversed by naloxone. *I. glandulifera* flowers and roots extracts (100 mg/kg) increased the reaction time to the thermal stimulus in the hot-plate test. All extracts from *I. glandulifera* (100 mg/kg) showed antianxiety effect in the elevated plus-maze test. It is worth noting that none of the extracts, at the highest used dose – 0.1 ED<sub>50</sub> (200 mg/kg), caused coordination impairments or myorelaxation as measured in the rota-rod and chimney tests. These results seem to suggest that the tested extracts are not neurotoxic. These findings show the potential use of hydroethanolic extracts from different parts of *I. glandulifera* as phytomedicine.

**Keywords:** CNS screening; antinociception; antianxiety; mice; *Impatiens*; Balsaminaceae

In accordance with the International Association for the Study of Pain (IASP), pain is defined as an unpleasant sensory and emotional experience caused by existing or potential tissue or organ damages or describes in terms of such damage (1). Many patients, who suffer for intense, especially chronic pain, have to depend on opioid analgesics, despite their well-known side effects, such as respiratory depression, constipation, and addiction (2). Therefore, the search for new compounds with a large spectrum of biological activities is very important. Plants are one of the potential sources for new drugs. It is estimated that about 50% of available drugs are derived from compounds first identified/isolated from plants, including also insects and animals, as active ingredients (3).

The *Impatiens* L. species occur in tropical and subtropical climate zones, mainly in parts of the Old

World such as tropical Africa, India, and the southwestern part of Asia, southern China. Some species were also found in Japan, Northern Europe, Russia, and North America (4). *Impatiens glandulifera* Royle (Himalayan balsam), *I. noli-tangere* L. (touch-me-not balsam) and *I. parviflora* DC. (small balsam), occur especially in the North-Western and Central Europe (5), North America (4) and New Zealand (6). They are annual herbaceous plants of the Balsaminaceae family (7). *I. glandulifera* and *I. parviflora* are among the invasive plants originally native to Asia that is rapidly spreading across Europe. In Poland, these are two of the top 20 invasive alien plants (8).

Among the members of the genus *Impatiens* L., some species have been used since a very long time in Asian and American medicine (9-11). The majority of phytochemical studies on the genus

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*Impatiens* focused on *I. balsamina*, because it has been applied in Chinese traditional medicine to treat many medicinal problems, i.e. rheumatism, fractures, swelling, contusions and beriberi disease (12). It has been also used to alleviate parturient and puerperal pain (13). Moreover, there is an *in vivo* study which has proven antinociceptive activity of methanol extract of its flowers (9). *I. parviflora* has been used in the treatment of warts (14). Flowers of *I. glandulifera* are used in Bach flower remedies, which cause sedation, relax and help to balance the emotional state, and they are recommended for psychological problems and pain (15). The roots and leaves of the plant are traditionally used in India as cooling agents, and the leaves decoction is used as a tonic in stress and mental tension (10). *I. noli-tangere* L. has been used in the Beskid in Poland to treat *susto*, a folk illness whose symptoms are: sleep disturbance, irritability, weakness, depression and muscle tension (16, 17). In our previous studies, we confirmed that the extracts from species of *Impatiens* contained significant amounts of phenolic acids and flavonoids, and have interesting multidirectional biological activity, such as antimicrobial and antioxidant abilities (18, 19). The LC-ESI-MS/MS analyses allowed identifying phenolic acids in extracts from the leaves, flowers, and roots of *I. glandulifera*, and from the leaves of *I. noli-tangere* and *I. parviflora* (20). Moreover, the results of UHPLC-DAD-MS<sup>3</sup> analyses revealed the presence of flavonoids in these species, especially kaempferol and quercetin derivatives with proven antinociceptive properties (18). We also showed the chemical composition of the essential oils obtained from four *Impatiens* species, *Impatiens glandulifera* Royle, *I. parviflora* DC., *I. balsamina* L. and *I. noli-tangere* L. and their antioxidant activities (21).

Based on the above phytochemical analyses, the use in folk medicine and *in vitro* studies indicating a putative biological action of different extracts from *Impatiens glandulifera*, *I. noli-tangere* and *I. parviflora*, the aim of the present study was to evaluate their pharmacological profile. Thus, a range of behavioral assessments was applied to evaluate the effects of extracts from flowers, leaves, and roots of *I. glandulifera*, and for comparison leaves extracts of *I. noli-tangere* and *I. parviflora*. Body temperature, locomotor activity, motor coordination, anxiety-like behavior and nociceptive reaction were examined. The nociceptive reaction is the time lapse between the beginning of the application of a stimulus, e.g. heat and the evoked response, e.g. to lift the hind paws. These measurements provide generally-accepted assessments of behavior in investigations

of bioactivity of new compounds (22). It is essential to characterize pharmacological activity of unknown substances in animal models because it is not possible to replicate the complexity of the organ systems, especially, the central nervous system *in vitro*. Such efforts are expected to provide meaningful data about the response of a biological system to new compounds.

## MATERIALS AND METHODS

### Plant materials

The plants were collected during July – August 2014. *Impatiens glandulifera* Royle (no. IG-0814) and *I. noli-tangere* L. (no. INT-0814) were gathered in Józefów near Biłgoraj (Poland) at an altitude of 240 m a.m.s.l. (coordinates N 50°29'06''; E 23°02'12'' and N 52°57'58''; E 23°04'46'', respectively). *Impatiens parviflora* DC. (no. IP-0814) was collected in Lublin (Poland) at an altitude of 210 m a.m.s.l. (coordinates N 51°16'24''; E 22°30'2''). Voucher specimens were deposited in the Department of Pharmaceutical Botany, Faculty of Pharmacy, Medical University of Lublin. Plants were identified by Prof. Tadeusz Krzaczek.

### Extraction method

Air-dried leaves, flowers and roots of *I. glandulifera*, and leaves of *I. noli-tangere* and *I. parviflora*, were ground to a fine powder using a laboratory mill, and sieved. 100 g of each powdered samples was weighted accurately and extracted with sonicated with a mixture of ethanol/water (8/2, v/v; 3 x 1000 mL) at a controlled temperature (40 ± 2°C) for 30 min. Supernatants were filtered and concentrated to dryness under vacuum at controlled temperature, and then subjected to lyophilization using vacuum concentrator until constant weights were obtained. Dry extracts were weighted and stored in a freezer at -20°C. The obtained yields: *I. glandulifera* flowers – 21.90 g; *I. glandulifera* leaves – 20.91 g; *I. glandulifera* roots – 11.53 g; *I. noli-tangere* – 10.75 g; *I. parviflora* – 11.68 g. The obtained samples were redissolved in the appropriate solvents for each determination.

### Total polyphenol content (TPC)

The amount of total phenolics was determined using the colorimetric method with some modifications (23). The absorbance was measured at 660 nm (Spectrophotometer UV-VIS, Evolution 300, Thermo-Finnigan, Italy). The results were expressed as mg of gallic acid equivalent (GAE) per 1 g of dry weight (DW).

**Total flavonoid content (TFC)**

Total flavonoids were evaluated according to the method described by Lamaison and Carret (24). The absorbance was measured at 394 nm. Finally, the total flavonoid content was expressed as mg of quercetin equivalent (QE) per 1 g of DW.

**Total phenolic acids content (TPAC)**

Total phenolic acids content was determined by the spectrophotometric method with Arnov's reagent according to the procedure described in Polish Pharmacopoeia IX (official translation of PhEur) (25). The absorbance was measured at 490 nm, and the percentage of phenolic acids, expressed as caffeic acid equivalents (CAE) on DW, was calculated according to the formula:  $A \times 1.7544/m$ , where A – the absorbance of the test solution at 490 nm, m – mass of the powdered plant material, in grams.

**Total flavones and flavonols content (TFFC)**

The total flavone and flavonol contents of the *Impatiens* samples were determined using aluminum chloride, according to the method described previously (26, 27). The absorbance was measured at 394 nm. The results were expressed as mg of quercetin equivalent (QE) per 1 g of DW.

**Total flavonones and dihydroflavonols content (TFDC)**

The flavonone and dihydroflavonol contents were determined using dinitrophenylhydrazine method with some modifications (27, 28). The absorbance of the supernatant solution was measured at 486 nm and the results were expressed as mg of eriodictyol equivalent (EE) per 1 g of DW.

**Animals**

The experiments were performed on male Albino Swiss mice (20-25 g), where 8 animals were kept in a cage, at room temperature of  $22 \pm 1^\circ\text{C}$ , with free access to food and water. All behavioral experiments were carried out according to the European Community Council Directive for Care and Use of Laboratory Animals (2010/63/EU) (29) and approved by the Local Ethics Committee for Animal Experimentation.

**Drug administration**

All extracts were administered intragastrically (i.g.), 60 min before the tests, dissolved in DMSO (its final concentration of 0.1%), suspended in 0.5% Tween-80 (1-2 drops) and then diluted with saline solution (0.9% NaCl). Naloxone (Nx), purchased

from Sigma Chemicals (St. Louis, USA) was administered subcutaneously (s.c.). Morphine was obtained from Sigma Chemicals (St. Louis, USA) and administered intraperitoneally (i.p.). In 'writhing' procedure, the acetic acid (0.6% solution; Avantor Performance Materials Poland S.A.; formerly POCH S.A.) and metamizole (Pyralgin®, Polpharma, Poland) were administered i.p. All solutions were given in a volume of 0.1 mL per 10 g body mass. Control animals were administered a corresponding vehicle.

**Oral acute toxicity test**

Acute toxicity assay was performed as per Organization of Economic and Corporation Development (OECD) guidelines 425 (Up-and-Down Procedure) with some modification (30). First animal received extracts orally at a dose of 500 mg/kg, i.e. at the level of the best estimation of the median effective dose ( $ED_{50}$ ), calculated as "the loss of righting reflex" after 24 h. The righting reflex is considered lost when a mouse, placed on its back, cannot move the head or body. Depending on the outcome for the previous animal, the dose for the next animal was adjusted up or down. If an animal did not show any signs of the loss of righting reflex, the dose for the next animal was increased (1000 mg/kg); if it lost the righting reflex, the dose for the next animal was decreased (250 mg/kg). The  $ED_{50}$  is calculated using the method of maximum likelihood. Doses should not exceed 2000 mg/kg which is considered the upper limit dose.

**Spontaneous locomotor activity**

Spontaneous locomotor activity was measured using an animal actometer Opto-Varimex-4 Auto-Track (Columbus Instruments, OH, USA). The device consists of four transparent cages with a lid ( $43 \times 43 \times 32$  cm), a set of four infrared emitters (each emitter has 16 laser beams), and four detectors monitoring animal movements. Each mouse was placed individually into the cage for 30 min. A number of break beams of a tested mouse was measured after 2, 4, 6, 10, 20 and 30 min to characterize dynamics of changes. The cages were cleaned up with 70% ethanol after each mouse (31).

**Motor coordination**

The effects of *Impatiens* L. extracts on motor coordination was evaluated in the rota-rod and chimney tests (22, 31). In the first test, motor impairments were measured, defined as the inability to keep balance on a rotating rod (at constant speed of 18 rpm) for 1 min. In the second test, motor

impairments were assessed by mouse inability to climb up the tube backwards (3 cm in inner diameter, 25 cm long) within 60 s. Before the tests, the animals were trained once a day for 3 days. The animals, able to stay on the rotating rod or to leave the chimney for 60 s, were approved for experiments.

#### Effects on body temperature

Body temperature in normothermic mice was measured in animal's rectum with a thermistor thermometer during a total period of 180 min (60 min before and 120 min after tested compound injection). The mean value from the first two measurements (60 and 30 min before drug administration) was assumed as initial temperature ( $t_i$ ). The final temperature ( $t_f$ ) was measured 30, 60, 90 and 120 min after the administration of tested extracts. Body temperature changes ( $\Delta t$ ) were calculated according to the formula:  $\Delta t = t_f - t_i$  (32).

#### Acetic acid-induced writhing test

Nociceptive reactions were studied in the acetic acid-induced writhing test (22). The mice were administered with extracts or metamizole and then the writhing was induced by 0.6% acetic acid solution. The number of writhing episodes was measured for 10 min, starting 5 min after i.p. administration of 0.6% acetic acid solution. The contraction of the abdomen, elongation of the body, twisting of the trunk and/or pelvis ending with the extension of the limbs were considered as complete writhing. The influence of Nx (5 mg/kg, s.c.; 30 min before the test) on the antinociceptive effect of the extracts was assessed according to the same procedure.

#### Hot-plate test

In this test, mice were individually placed on a hot plate (Ugo Basile, Italy) with adjustable temperature (to  $55 \pm 1^\circ\text{C}$ ). The response in the form of lifting either of the hind paws or a jumping with all four feet off of the hot-plate was recorded at 30 min time intervals up to 120 min after drugs injection. The baseline latency response before administration of a compound was first measured. The cut off time for the hot plate latencies was set at 20 s. Animals were administered with extracts or morphine, according to the experimental paradigm. The antinociceptive effects of morphine and extracts were expressed as a percent maximum possible effect (%MPE), which was calculated according to the following equation:  $[(T_1 - T_0)/(20 - T_0)] \times 100$ , where  $T_0$  and  $T_1$  are the pre-drug and post-drug latencies for hot-plate response, respectively (33).

#### Elevated plus-maze test (EPM)

The EPM studies were carried out on mice according to the method of Lister (34). The EPM apparatus was made of Plexiglas and consisted of two open ( $30 \times 5$  cm) and two enclosed ( $30 \times 5 \times 15$  cm) arms. The arms extended from a central platform of  $5 \times 5$  cm. The apparatus was mounted on a Plexiglas base, raising it 38.5 cm above the floor, and illuminated by a red light. The test consisted of placing a mouse in the center of the apparatus (facing an open arm) and allowing it to freely explore. The number of entries into the open arms and the time spent in these arms were scored for a 5 min test period. An entry was defined as placing all four paws within the boundaries of the arm. The following measures were obtained from the test: the total number of arm entries; the percentage of arm entries into the open arms; and the time spent in the open arms expressed as a percentage of the time spent in both the open and closed arms. Anxiolytic activity was indicated by an increase in the time spent in open arms and in the number of open arm entries. The total number of entries into either type of arm was used additionally as a measure of overall motor activity.

#### Statistical analysis

The results were calculated by the two-way analysis of variance (ANOVA) (body temperature and hot-plate test) and one-way ANOVA (other tests), followed by the Dunnett's or Bonferroni's *post hoc* test as appropriate. The results are presented as means  $\pm$  standard errors of means (S.E.M). The level of  $p < 0.05$  was considered statistically significant. All figures were prepared by the GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, California, USA), [www.graphpad.com](http://www.graphpad.com).

## RESULTS

#### Polyphenols content

The total phenolic contents expressed as gallic acid equivalents for *Impatiens* extracts were in the range from  $13.26 \pm 1.68$  to  $24.72 \pm 1.91$  mg GAE/g DW. The highest TPC content was found in extract from *I. glandulifera* flowers ( $24.72 \pm 1.91$ ), *I. glandulifera* leaves ( $22.84 \pm 2.12$ ), and *I. parviflora* leaves ( $19.34 \pm 0.57$ ). The highest content of phenolic acids was found in samples of *I. noli-tangere* leaves ( $4.67 \pm 1.28$  mg CAE/g DW) and *I. glandulifera* flowers ( $4.39 \pm 1.91$  mg CAE/g DW). The content of flavones and flavonols was higher than flavanones and dihydroflavonols content in all

examined samples. High amounts of TFFC and TFDC were observed in *I. glandulifera* leaves ( $5.34 \pm 0.29$  mg QE/g DW and  $4.72 \pm 1.83$  mg EE/g DW, respectively) (Table 1).

#### Acute toxicity of *Impatiens L.* extracts

Administration of *Impatiens L.* extracts at the doses 500-2000 mg/kg, i.g., did not cause any behavioral changes. Therefore, it has been calculated that the ED<sub>50</sub> for *Impatiens L.* extracts is 2000 mg/kg and the starting dose for behavioral tests was equivalent to 0.1 ED<sub>50</sub>.

#### Effects of *Impatiens L.* extracts on the motor activity of mice

One-way ANOVA showed no significant changes in the locomotor activity of mice in all tested points. Whereas, Dunnett's *post hoc* test indicat-

ed a significant decrease in locomotion of mice after the administration of the extract from *I. glandulifera* roots at the dose of 200 mg/kg in 30 min of measurement as compared to the control group ( $p < 0.05$ ) (Fig. 1).

#### Effects of *Impatiens L.* extracts on the motor coordination of mice

One-way ANOVA showed no significant changes in motor coordination of mice in both tests – the rota-rod and chimney test (Figs. 2A and 2B).

#### Effects of *Impatiens L.* extracts on the body temperature of mice

Two-way ANOVA showed statistically significant effects of the extracts ( $F_{(5,158)} = 18.95$ ;  $p < 0.0001$ ) and time ( $F_{(3,158)} = 4.04$ ;  $p < 0.01$ ). Bonferroni's *post hoc* test revealed a significant

Table 1. Total phenolic (TPC), phenolic acids (TPAC), flavonoids (TFC), flavones/flavonols (TFFC) and flavonones/dihydroflavonols content (TFDC) in examined plants expressed as mg GAE, mg CAE, mg QE or mg EE per 1 g of dry plant material, respectively. Results are means  $\pm$  SD of three different experiments.

Sample	Total phenolic content [mg GAE/g DW]	Total phenolic acids [mg CAE/g DW]	Total flavonoid content [mg QE/g DW]	Total flavones and flavonols content [mg QE/g DW]	Total flavonones and dihydroflavonols dihy [mg EE/g DW]
<i>I. glandulifera</i> flowers	$24.72 \pm 1.91$	$4.39 \pm 0.81$	$9.74 \pm 0.93$	$4.61 \pm 1.34$	$2.86 \pm 0.73$
<i>I. glandulifera</i> leaves	$22.84 \pm 2.12$	$3.84 \pm 0.98$	$10.06 \pm 1.13$	$5.34 \pm 0.29$	$4.72 \pm 1.83$
<i>I. glandulifera</i> roots	$13.26 \pm 1.68$	$1.38 \pm 0.11$	$3.27 \pm 0.48$	$1.06 \pm 0.48$	$0.24 \pm 1.17$
<i>I. noli-tangere</i>	$16.53 \pm 0.36$	$4.67 \pm 1.28$	$5.13 \pm 0.71$	$3.20 \pm 0.03$	$0.87 \pm 0.46$
<i>I. parviflora</i>	$19.34 \pm 0.57$	$2.09 \pm 0.38$	$5.82 \pm 0.17$	$5.17 \pm 1.29$	$0.42 \pm 0.79$

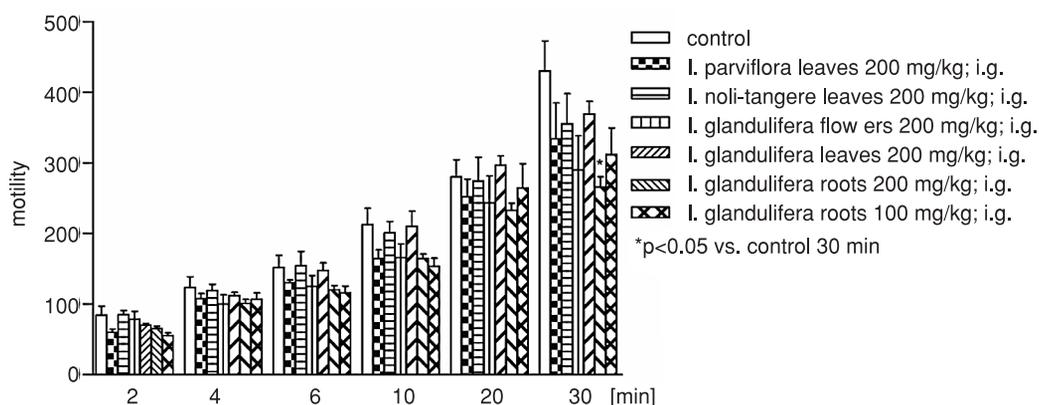


Figure 1. The influence of *Impatiens L.* extracts on the spontaneous locomotor activity of mice. Extracts were administered i.g. 60 min before the test. Locomotor activity was noted after 2, 4, 6, 10, 20 and 30 min. Data are expressed as mean  $\pm$  SEM values. \*  $p < 0.05$  vs. control group 30 min (Dunnett's test)

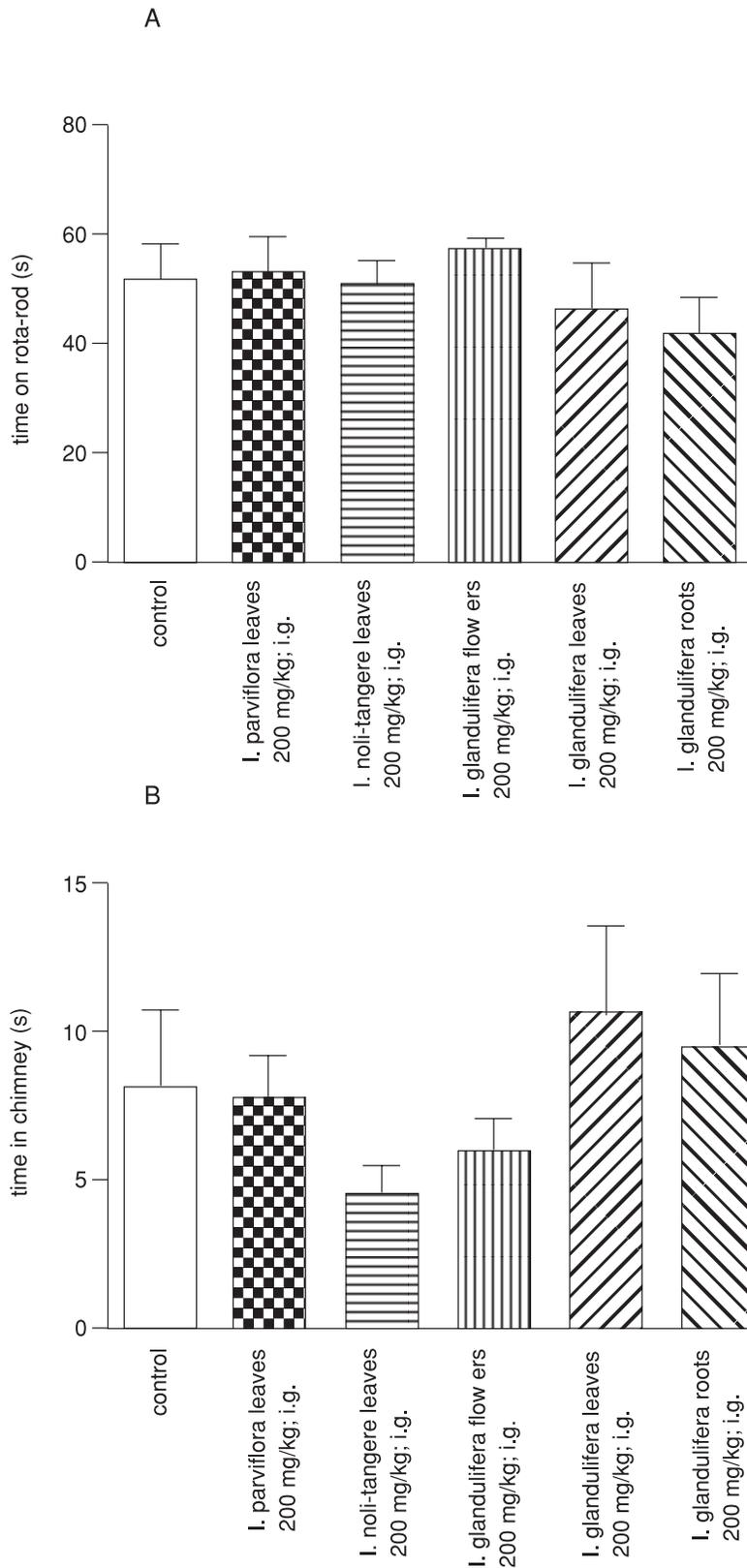


Figure 2. The influence of *Impatiens* L. extracts on motor coordination in mice evaluated in rota-rod (A) and chimney (B) tests. Extracts were administered i.g. 60 min before the test. Data are expressed as mean  $\pm$ SEM values

decrease in mice body temperature after administration of the extract from *I. glandulifera* roots in 60 ( $p < 0.001$ ), in 90 and 120 min ( $p < 0.01$ ) and the extract from *I. noli-tangere* leaves in 60 ( $p < 0.05$ ), in 90 ( $p < 0.01$ ) and in 120 min ( $p < 0.05$ ) (Fig. 3).

**Effects of *Impatiens L.* extracts on nociceptive reactions and the influence of naloxone on the antinociceptive activity of *Impatiens L.* extracts in the 'writhing' test in mice**

One-way ANOVA showed significant changes in the number of writhing episodes of mice after the administration of tested extracts ( $F_{(17, 138)} = 6.437$ ;  $p < 0.0001$ ). Dunnett's *post hoc* test revealed a significant reduction in the writhing episodes of mice after the administration of the extract from *I. parviflora* leaves at doses of 200 and 100 mg/kg ( $p < 0.05$ ), the extract from *I. glandulifera* flowers at doses of 200 ( $p < 0.001$ ) and 100 mg/kg ( $p < 0.01$ ), the extract from *I. glandulifera* leaves at dose of 200 ( $p < 0.01$ ), 100 ( $p < 0.01$ ) and 50 mg/kg ( $p < 0.05$ ), the extract from *I. glandulifera* roots at doses of 200 ( $p < 0.001$ ), 100 ( $p < 0.01$ ), 50 ( $p < 0.01$ ) and 25 mg/kg ( $p < 0.05$ ) and a classical non-steroidal analgesic drug, metamizole (250 mg/kg; i.p.,  $p < 0.001$ ) (Figure 4A). The medium doses of *I. parviflora* and *I. glandulifera* extracts (i.e., 100 and 50 mg/kg, respectively) have been chosen for the subsequent experiments. One-way ANOVA showed significant changes in the number of writhing episodes of mice after the administration of the extracts from *I. parviflora* leaves, *I. glandulifera* flowers, *I. glandulifera* leaves and *I. glandulifera* roots and co-administration of naloxone (5 mg/kg) with the above-men-

tioned extracts ( $F_{(8,65)} = 5.785$ ;  $p < 0.0001$ ). Bonferroni's *post hoc* test revealed that naloxone reversed antinociceptive effect only of the extracts from *I. parviflora* leaves and *I. glandulifera* flowers ( $p < 0.05$  and  $p < 0.001$ , respectively) administered at the dose of 100 mg/kg (Fig. 4B).

**Effects of *Impatiens L.* extracts on nociceptive reactions in the hot-plate test in mice**

Two-way ANOVA showed statistically significant effects of treatment ( $F_{(5,236)} = 11.72$ ;  $p < 0.0001$ ), time ( $F_{(4,236)} = 9.67$ ;  $p < 0.0001$ ) and interactions ( $F_{(20,236)} = 2.09$ ;  $p = 0.0051$ ). Bonferroni's *post hoc* test confirmed that a classical opioid analgesic, morphine (3.3 mg/kg, i.p.) produced a significant effect on the latency to respond to noxious stimulus as compared to control group at 30 ( $p < 0.01$ ), 60 ( $p < 0.05$ ), 90 ( $p < 0.01$ ) and 120 ( $p < 0.01$ ) min of measurement. Extract from *I. parviflora* leaves (100 mg/kg) significantly increased the reaction time to the thermal stimulus in 60 ( $p < 0.01$ ); 90 ( $p < 0.05$ ) and 120 ( $p < 0.001$ ) min. Extract from *I. glandulifera* flowers significantly increased the reaction time to the thermal stimulus in 90 ( $p < 0.05$ ) and 120 ( $p < 0.01$ ) min. Extract from *I. glandulifera* roots significantly increased the reaction time to the thermal stimulus in 60 ( $p < 0.001$ ); 90 ( $p < 0.01$ ) and 120 ( $p < 0.001$ ) min (Fig. 5).

**Effects of *Impatiens L.* extracts on anxiety-like performance of mice estimated in the EPM**

One-way ANOVA showed significant changes in time spent in the open arms of the EPM ( $F_{(5,36)} = 3.731$ ;  $p < 0.01$ ). Dunnett's *post hoc* test revealed

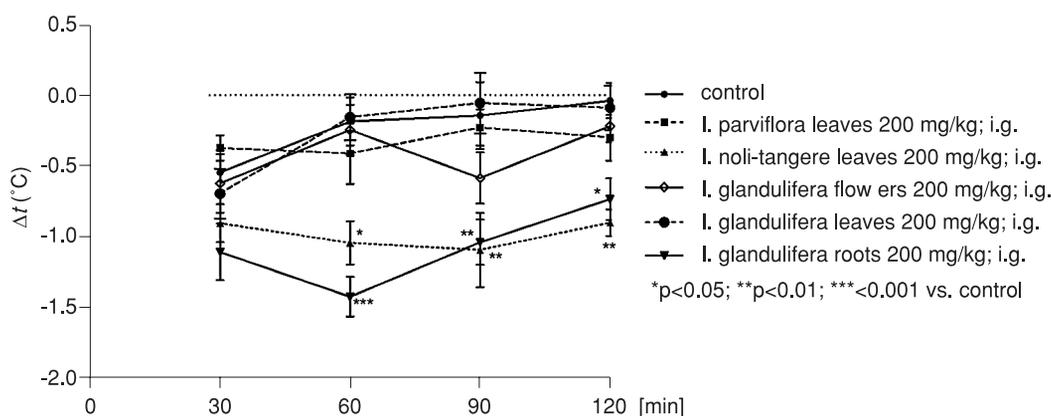


Figure 3. The influence of *Impatiens L.* extracts on the body temperature in mice. Body temperature was measured during the total period of 180 min (60 and 30 min before and 30, 60, 90 and 120 min after the extracts administration). Data are expressed as mean  $\pm$  SEM values. \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ; vs. control group (Bonferroni's test)

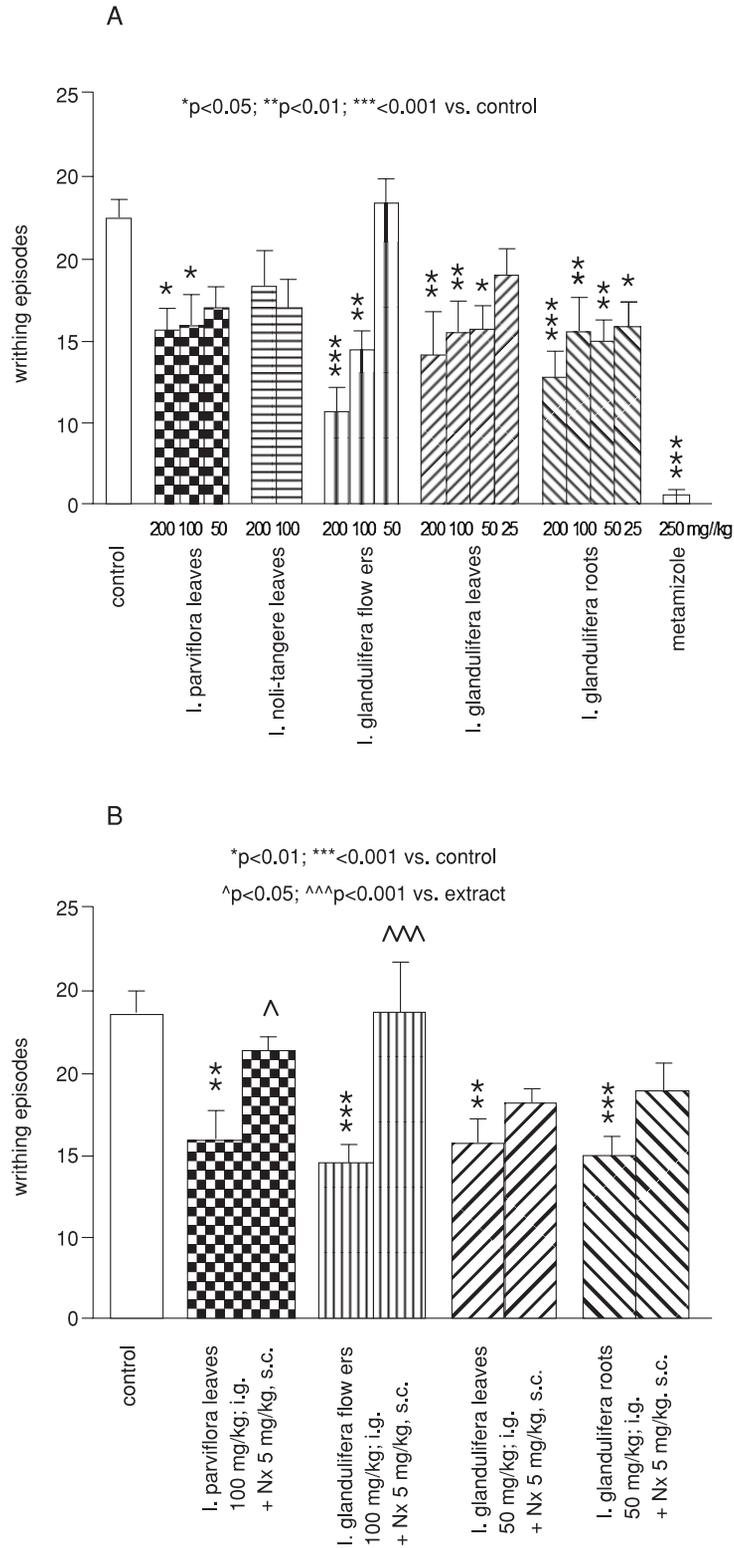


Figure 4. The influence of *Impatiens* L. extracts and metamizole on nociceptive reactions (A) and the effect of naloxone (Nx; 5 mg/kg, s.c.) on the antinociceptive effects of *Impatiens* L. extracts (B) assessed in the 'writhing' test in mice. Extracts were administered 60 min, Nx and metamizole (250 mg/kg; i.p.) 30 min and acetic acid (0.6 % solution) 5 min before the test. Data are expressed as mean ±SEM values. \*p < 0.05; \*\*p < 0.01 and \*\*\*p < 0.001 vs. control group; ^p < 0.05 and ^^p < 0.001 vs. appropriate extract (Bonferroni's test)

that extracts from *I. glandulifera* flowers, leaves and roots at a dose of 100 mg/kg produced a substantial increase in time spent in the open arms ( $p < 0.05$ ) (Fig. 6A).

One-way ANOVA showed significant changes in number of entries into the open arms ( $F_{(5,37)} = 3.284$ ;  $p < 0.05$ ). Dunnett's *post hoc* test revealed that extracts from *I. glandulifera* leaves and roots at a dose of 100 mg/kg produced a substantial increase in the number of entries into the open arms ( $p < 0.01$  and  $p < 0.05$ , respectively) (Fig. 6B).

One-way ANOVA showed no significant changes in the total number of entries into both arms (Fig. 6C).

## DISCUSSION

The major findings of the present work are that (1) the  $ED_{50}$  of all tested *Impatiens* L. extracts is more than 2 g/kg in mice; (2) oral (i.g.) administration of *Impatiens* L. extracts at the dose of 0.1  $ED_{50}$  (200 mg/kg) did not disturb motility or motor coordination of mice; (3) the extracts from *I. noli-tangere* leaves and *I. glandulifera* roots, both at 200 mg/kg, i.g., decreased body temperature of mice; (4) oral administration of *Impatiens* L. extracts (except from *I. noli-tangere*) significantly inhibited the acetic acid-induced abdominal constriction; (5) the antinociceptive effect of *I. parviflora* leaves and *I. glandulifera* flowers had been effectively antagonized by the use of naloxone suggesting involvement of opioid receptors; (6) oral administration of extracts from *I. parviflora*, *I. glandulifera* flowers and roots increased the reaction time to the thermal

stimulus in the hot plate test; (7) oral administration of all extracts from *I. glandulifera* showed anxiolytic effects in the EPM test.

At the beginning of the experiment, the acute toxicity of the extracts was calculated as the  $ED_{50}$ , based on the loss of the righting reflex within 24 h, using the up-and-down methods. The results showed that the dose of 2000 mg/kg of tested extracts did not cause any behavioral changes. Therefore, it can be assumed that *Impatiens* L. extracts possess low toxicity profile. This value of  $ED_{50}$  was adopted and the regressive doses of  $ED_{50}$  were used for further studies.

Further results of the pharmacological investigations showed that the examined extracts (except from *I. glandulifera* roots) did not exert any influence on locomotor activity of animals, at the dose of 0.1  $ED_{50}$ , i.e., the starting dose. Whereas, oral administration of *I. glandulifera* roots extract (200 mg/kg) showed statistically significant decrease in motility of mice, 30 min after the beginning of the measurement. Thus, at the lower dose of 100 mg/kg, this extract did not affect locomotion of animals. It can be supposed that *I. glandulifera* roots extract has a weak depressive effect on the CNS. The measurement of motor activity is a standard behavioral assay for testing the sedative effects of drugs (22). A drug-induced decrease in the animal's spontaneous activity is defined by Katzung et al. (35) as sedation.

It is worth noting that none of the extracts, at the highest used dose – 0.1  $ED_{50}$  (200 mg/kg), caused coordination impairments or myorelaxation as measured in the rota-rod and chimney tests. These results seem to suggest that the tested extracts are not neurotoxic, thus prompting further studies to

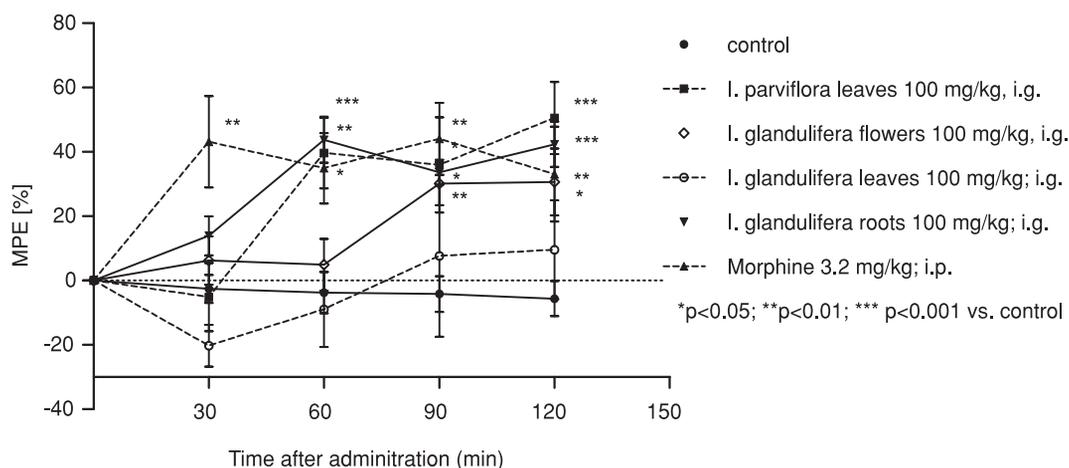


Figure 5. The influence of *Impatiens* L. extracts and morphine (3.2 mg/kg, i.p.) on nociceptive reactions assessed in the hot plate test in mice. Nociceptive reactions were measured during the total period of 120 min (30, 60, 90 and 120 min after the extracts and morphine administration). Data are expressed as mean  $\pm$ SEM values. \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. control group (Bonferroni's test)

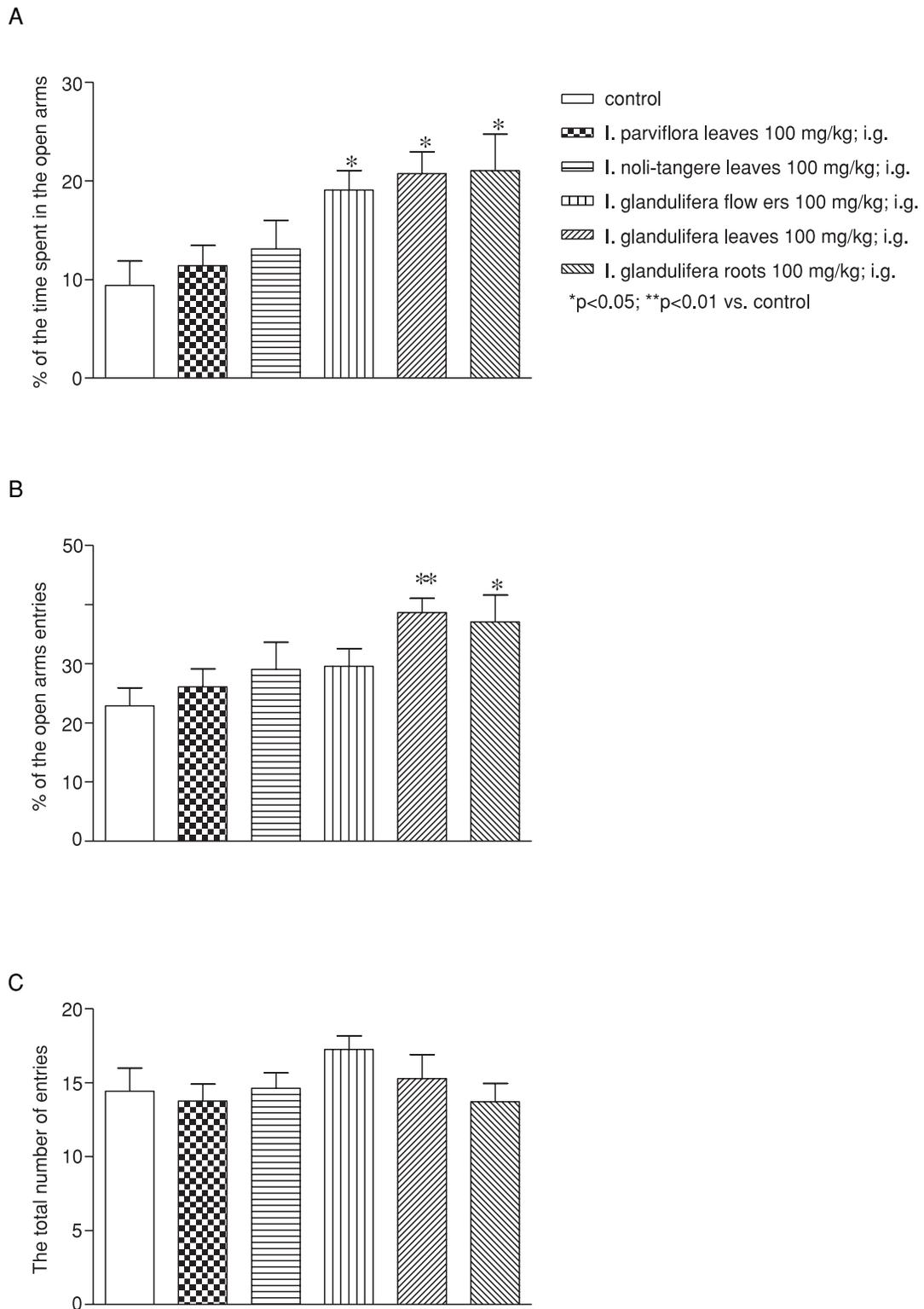


Figure 6. The influence of *Impatiens L.* extracts on anxiety-like performance of mice estimated in the EPM. Data are expressed as mean  $\pm$  SEM values. \* $p < 0.05$  and \*\* $p < 0.01$  vs. control group (Dunnett's test)

confirm their safety and therapeutic efficacy. What is more, the lack of coordination disturbances and muscle relaxant potency are important features, because their presence can affect reliability of the other behavioral performances (22).

In the present experiments, the body temperature of normothermic mice was also tested after the oral administration of the extracts, every half an hour up to 2 h. This measurement is a part of preliminary behavioral investigations (22). The extracts from *I. noli-tangere* leaves and *I. glandulifera* roots, both at a dose of 200 mg/kg, caused a significant decrease in the body temperature of mice up to 120 min of the experiment. The most important role in the regulation of body temperature plays hypothalamic receptors whose action mainly depends on serotonin. According to the literature data, the hypothermic effect may be caused by substances which are agonists of serotonin 5-HT<sub>1A</sub> receptor as well as 5-HT<sub>2A</sub> receptor antagonists (36, 37), and we can suggest that these mechanisms of action can be also involved in the effects of extract under study.

An important observation of the presented study was the antinociception and/or anti-inflammation induced by the tested extracts, excluding *I. noli-tangere*. Obtained results demonstrated that an oral administration of *Impatiens* L. extracts elicited a potent and dose-dependent antinociceptive and/or anti-inflammatory effect in acetic acid-induced writhing test. The writhing procedure is a chemical-induced nociception method, useful for sifting compounds the pharmacological features of which are unknown. This test is based on chemical stimulation, induced by an i.p. administration of agents that irritate serous membranes and provoke characteristic abdominal contractions. The writhing test lacks specificity but is very sensitive, predictive and the closest in its nature to clinical pain. It is a model of visceral or peritoneal pain, although it does not preclude central mechanisms of antinociception (32). The specificity of the writhing test can be improved by undertaking a preliminary rota-rod test to detect and eliminate substances that alter the motor performance of animals. In our study, it was estimated that none of the examined extracts impaired motor coordination in mice, as already mentioned. A strong impact on mice behavior in that test was observed for *I. glandulifera* leaves and roots extracts at a wide range of doses, up to 0.025 (for leaves) and up to 0.0125 ED<sub>50</sub> (for roots). At the highest doses, i.e., 0.1 ED<sub>50</sub> (200 mg/kg) of *I. glandulifera* flowers and roots extracts showed a significant reduction in writhing episodes which were comparable to the action of the reference drug – metamizole (250

mg/kg), a non-steroidal analgesic agent. It is known that i.p. administration of the acetic acid causes an increase in eicosanoids biosynthesis i.e., cyclooxygenase, lipoxygenase, and prostaglandins activity, which are associated with the development of inflammatory pain and abdominal constriction (38). It has been reported that the phytochemicals, particularly the phenolics and flavonoids could be responsible for the anti-inflammatory activity which was shown in the writhing test (38, 39). An important anti-inflammatory feature of flavonoids is the capability to inhibit eicosanoids biosynthesis (40). This data is consistent with our previous results showing that members of the genus *Impatiens*, in particular *Impatiens glandulifera*, and *Impatiens parviflora*, contain significant amounts of phenolic acids and flavonoids (18). Our results revealed the presence of eriodictyol-di-O-hexoside, eriodictyol-O-hexoside, quercetin-3-O-galactoside (hyperoside), quercetin-3-O-glucoside (isoquercitrin), kaempferol-3-O-glucoside (astragaline), eriodictyol, and kaempferol in methanolic extracts from the leaves of *I. glandulifera*, and hyperoside, isoquercitrin, quercetin-O-malonylhexoside, astragaline, and kaempferol-O-malonylhexoside in the leaves of *I. parviflora*, and hyperoside, isoquercitrin, quercetin-O-malonylhexoside, and astragaline in the leaves of *I. noli-tangere*. Moreover, a great amount of protocatechuic, vanillic, *trans*-p-coumaric and a 3-hydroxycinnamic acid was observed in these three species (20). Some of the identified in *Impatiens* species flavonoids, such as kaempferol and quercetin, and its glycosides, possesses proven anxiolytic activities. Grundmann et al. (41) tested kaempferol for its potential anxiolytic activity in mice. Kaempferol exhibited significant activities in the percentage time spent on open arms after oral administration in concentrations above 0.01 mg/kg, and the observed activity decreased at higher doses. An anxiolytic-like effect was also reported for pure quercetin by oral and intranasal way in rats (42, 43).

Because the writhing test is not a specific method and it cannot be precluded the central mechanism of tested compounds which are active in this test, naloxone was used to antagonize the effects. This nonselective opioid antagonist reversed the action only of *I. parviflora* leaves and *I. glandulifera* flowers observed in the writhing test. These results seem to indicate a possible interaction with the opioid system (44). Additionally, an oral administration of extracts from *I. glandulifera* flowers and roots at a dose of 100 mg/kg significantly increased the latency period to the thermal stimuli in the hot-plate test suggesting the central antinociceptive activity of

these extracts. The hot-plate procedure demonstrates the supraspinal reflex mediated by opioid receptors (33). The antinociceptive activity and the direct involvement of the activation of opioid receptors in this effect have been already reported for flowers extracts from another *Impatiens* species – *I. balsamina* (9).

Whereas the reduction in the writhing episodes caused by *I. glandulifera* leaves and roots extracts was not antagonized by naloxone, it can be suggested rather a peripheral mechanism involved in these effects. On the other hand, it was shown that the antinociceptive effect of polyphenolic compounds, e.g., vanillic acid occurs by a mechanism partly dependent upon the opioid system, while the anti-inflammatory action was manifested in inflammatory processes dependent on the vanillic acid inhibition of cytokines (45).

In the present study, the potential anxiolytic profile of the investigated extracts was assessed in the EPM. All extracts from *I. glandulifera* showed anxiolytic-like activity, increasing, in a statistically significant manner, the time spent in the open arms of the EPM and the number of entries into open arms. It is worth to note that these extracts did not influence the total number of entries which means that they did not change locomotor activity of mice. Regulation of anxiolytic activity depends on different neurotransmitters (e.g., the gamma-aminobutyric acid, dopamine or serotonin) (31). Thus, further chemical studies are required to elucidate the precise molecular mechanisms responsible for the activity of the *I. glandulifera* extracts.

Taking together, it seems that the extracts obtained from *I. glandulifera* (leaves, flowers or roots) are more biologically active than extracts from leaves of *I. parviflora* and *I. noli-tangere*. All these extracts possess low toxicity and have insignificant influence on the based neuropharmacological activities, i.e., locomotion and motor coordination.

The presented results provide some preliminary estimations, thus, need to be further extended for closer identification and deeper understanding of the entire pharmacological profile of the examined extracts.

## CONCLUSIONS

In conclusion, the results of the present study indicated that *I. glandulifera* extracts caused strong reduction in the number of writhing episodes. The effects of hydroethanolic extracts from flowers, leaves and roots of *I. glandulifera* seem to be medi-

ated by the peripheral inflammatory mediators such as cyclooxygenase-2, while the effect of *I. glandulifera* flowers can be also associated with the opioid receptor. These results justify the use of the plant in folk medicine in the treatment of mental tension and different painful conditions. It will be of considerable interest for the future to isolate the bioactive compound(s) and elucidate the precise molecular mechanisms responsible for the pharmacological activities of the plant.

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## Conflicts of interest

The authors declare no conflict of interest.

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