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Effect of *Passiflora incarnata* L. extract on exploratory behaviour and neurotransmitters level in structures involved in motor functions in rats

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Abstract

Introduction and Objective. The genus *Passiflora incarnata* L. is used in traditional medicine for its analgesic, anxiolytic and sedative purposes, and its flavoursome fruits have long been consumed, especially in North America. The aim of the study is to assess the effect of extract of *P. incarnata* L. on the motor activity of rats.

Materials and Method. Behavioural testing was performed in rats previously exposed to *P. incarnata* L. extract (30, 100, 300 mg/kg b.w). To explain the neurobiochemical background of behavioural changes, the concentration of neurotransmitters after extract consumption were assayed in CNS structures responsible for motor function using High Performance Liquid Chromatography.

Results. The study demonstrated that exposure to the extract resulted in dose dependent modulation of rats behaviour, and altered neurotransmission in major elements of the CNS locomotor system. The changes concerned primarily the cerebellar and spinal dopaminergic and noradrenergic systems.

Conclusions. Inhibitory effect of *P. incarnata* L. extract on the GABAergic pathway has potential as a sedative agent and may be promising alternative to benzodiazepines in controlling anxiety and hyperactivity. The significant increase in the level of spinal dopamine may be potentially useful in disorders related to a deficit in dopaminergic neurotransmission. These findings could lead to the development of specific supportive therapies for anxiety and symptoms of gradual loss or impairment of motor behaviour.

Key words

amino acids, behaviour, CNS, neurotransmission, *Passiflora incarnata* L.

INTRODUCTION

Passiflora incarnata L. (passion fruit) is an aromatic, pleasant tasting sweet fruit for culinary use. Because of its nutritional properties the fruit can be consumed fresh or in the form of preserves, as well as used as a natural flavouring agent in food manufacturing. On a global scale, passion fruit extracts have a commercial value as a source of health-promoting compounds through the pharmaceutical, nutraceutical, food and beverage industries [1–3]. In traditional herbal medicine *P. incarnata* L. has been used primarily to manage some neuropsychiatric problems. Despite the long-term use of the plant in traditional medicine, only a few good quality data of clinical importance are available. According to current knowledge, the passion flower can be considered in the treatment of anxiety, insomnia, menopause, vasomotor symptoms and depression, as well as in the treatment of opioid addiction [4–6]. Moreover, the most promising studies concerned the efficacy of the passion flower in the Attention-Deficit Hyperactivity Disorder (ADHD) in children, in the therapy of generalized anxiety disorder and pre-operative anxiety [7]. These pharmacological effects are comparable with the effectiveness of melatonin or even midazolam [8–10].

The mechanism of *P. incarnata* L. action remains unclear, yielding problems with identifying the most potent active components. Most studies show that the promising neuroactive substances are flavonoids: chrysin, vitexin, isovitexin, orientin, isoorientin, apigenin, kaempferol and indole alkaloids [11]. The most frequently repeated hypotheses indicate the participation of active plant components in the modulation of the GABA-ergic pathway and opioid systems. Indole alkaloids have structure similar to benzodiazepines – anxiolytic drugs – and like them, they have benzene

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ring. These compounds may be responsible for the central action of *P. incarnata* L. in view on the high affinity to the benzodiazepine-site of the GABA type A receptor [12]. Plant flavonoids, including apigenin and chrysin, are partial agonists of GABA A receptor and can inhibit [3H]-GABA uptake by synaptosomes in rat cortex [13]. According to some authors, such flavonoids exhibit anxiolytic activity without evident sedation and effect on muscle relaxation [14]. *In vitro* study evaluating the effect of passion fruit extract on pyramidal neurons of the hippocampus, has shown direct dose-dependent activation of GABA A receptors. This effect was inhibited by administration of gabazine – competitive antagonist of GABA A receptor [15]. Anxiolytic activity of the extract results not only from the presence of the flavonoids acting as a ligands for the GABA A receptor, but can also be caused by gamma-aminobutyric acid present in large amounts in plant. According to the authors of the study, *P. incarnata* extract can also be considered an antagonist of the GABA B receptor [13]. At the same time, some evidence suggests that oral administration of plant-derived GABA does not provide adequate concentrations in CNS tissues as GABA hardly crosses the blood-brain barrier [15].

Single studies suggest the involvement of adenosine and cannabinoid receptors or the influence of the aromatase enzyme on the mechanism of action of passion fruit extract. Nassiri-Asl et al. (2007) suggest that the anticonvulsant properties of passion flower extract are the result of an affinity for kappa opioid receptors, activation of which leads to the stimulation of the GABA-ergic system and a reduction in glutamatergic neurotransmission [16]. In a study by Aman et al. (2016), a similar hypothesis was presented about the involvement of the opioid and GABA-ergic systems in the mechanism of action of *Passiflora* [17]. The hypothesis was confirmed by the abolition of the analgesic effect of passion flower extract after administration of naloxone (strong opioid antagonist) and pentylenetetrazole (GABAA/BDZ receptor antagonist, chloride channel blocker).

Pharmacological research on the central effects of *P. incarnata* focuses mainly on the sedative, anxiolytic, anticonvulsant and analgesic properties, as well as the supportive role in the treatment of alcohol, nicotine and tetrahydrocannabinol addiction [18, 19]. To date, no detailed research or analysis has been carried out on the influence on motor functions.

OBJECTIVE

The aim of the study was to evaluate the influence of *P. incarnata* L. extract onthe exploratory activity and cognitive processes in animals. Changes at the level of neurotransmission captured in structures involved in motor functions, such as the spinal cord, medulla oblongata and cerebellum, bring us closer to clarifying the mechanisms responsible for the reduction of motor hyperactivity observed after *P. incarnata* L. administration.

MATERIALS AND METHOD

Treatment of animals. In the study, 4-week-old male Wistar Albino Glaxo rats from a registered breeding centre were used, each with an average initial body weight of 51.33±2.45 g. They received drinking water or a dry extract dissolved in water *ad libitum* for 6 weeks, as well as during all behavioural procedures (the total duration of extract administration was 47 days). All animals had free access to chaw (Labofed, Kcynia, Poland). The animals were housed in groups of 2 in standard plastic breeding cages in an air-conditioned, ventilated room at 22–24°C, and humidity about 55±10%. Behavioural experiments were conducted from 09:00–16:00. The total number of animals used in the experiment was 40. The animals were randomly divided into 4 groups: control rats receiving drinking water without restrictions (Con, n=10) and 3 groups of rats receiving the standardized extract of *P. incarnata* L. at a dose of 30 mg (P30, n=10), 100 mg (P100, n=10) and 300 mg/kg b.w./day (P300, n=10). The mean daily fluid consumption was measured to calculate the amount of plant extract corresponding to the predetermined daily doses. The solution was freshly prepared every day. In the study, a standardized extract was used of the herb *P. incarnata* L. (Preparation No. 0171319, Batch No. 12018605, certified by Dr. Volker Friese, Finzelberg, Martin Bauer Group, Andernach, Germany).

Behavioural tests. Assessment of motor and exploratory activity, anxiety and stress response, as well as the cognitive processes, was performed using behavioural tests: Novel Object Recognition (NOR) test and Hole Board test (HB). The behaviour of the animals was scored by an observers blinded to the study plan and conditions.

Novel Object Recognition (NOR) test. The recognition test of a new object was carried out in a wooden box measuring 100 cm × 100 cm × 35 cm. Before starting the test, the animals were free to acquaint themselves by exploring the apparatus for 3 minutes. On day 1 – familiarization phase, 2 identical objects, A1 and A2 (Lego bricks) – were placed in opposite corners, 10 cm from the walls of the box. On day 2 -choice phase, the objects were repositioned, and one of them was replaced with a new object, object B (a bottle – with a different shape and colour. During the study, the time of the first contact with the objects, the number of contacts and total time spent exploring the objects were measured. The Discrimination Index (DI) was calculated for the choice phase as the difference between the time (t) taken to explore the novel and known objects [DI=(tB-tA1)/(tB+tA1)]. The Global Habituation Index [GHI=(tA1+tA2)/(tB+tA1)], which compares the time spent studying 2 objects during the familiarization phase with the time spent during the choice phase, was also estimated. The Recognition Index (RI), the time spent on examining the new object in relation to the time of the overall exploration of the objects [RI = tB/(tB+tA1)], was also determined. Parameters DI, GHI and RI were calculated by using formulas presented above

Hole Board (HB) test. The Hole Board test was conducted in a similar box with dimensions of 100 cm x 100 cm x 30 cm. The floor of the apparatus was divided into 16 squares (segments). Each hole in the centre of the segments had a diameter of 3.8 cm, and depth 5.0 cm. During 3 consecutive days, the animals were placed individually inside the apparatus for 3 minutes. Parameters, such as penetration of the head into the holes, climbing, transitions between sectors of the test apparatus, transitions between adjacent squares or to the outer sector, were determined, as well as the time spent

in motion and time spent in the middle segments of the apparatus.

Data collection. All experiments were recorded on a video camera. Blinding was applied at the stages of animal treatment, analysis of animal behaviour in behavioral tests and analysis of results. All procedures were carried out according to the the directives of the National Research Council (NRC) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (8th ed., National Academic Press, 2011), and approved by the Ethical Committee for Animal Experiments at the Medical University of Warsaw. The ARRIVE guidelines 2.0 (Animal Research: Reporting of *In Vivo* Experiments) were implemented to ensure the highest quality of research standards.

Biochemistry – Sample preparation. After completion of the behavioural tests the animals were decapitated using a guillotine, and the cerebellum, medulla oblongata and spinal cord immediately sectioned on dry ice. After weighing, the tissues were placed at -80°C and stored until neurotransmitters were determined by high-performance liquid chromatography (HPLC). In the structures of the CNS, the concentration of dopamine (DA) and corresponding metabolites: 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), nor previously [2]. The final amount of neurotransmitters is expressed in ng/g of brain tissue.

The protocol for assessment of concentrations of amino acids neurotransmitters; glutamic acid (GLU), aspartate (ASP), alanine (ALA), histidine (HIS), γ-aminobutyric acid (GABA) and taurine (TAU), followed the previously described procedure [20]. The final amount of amino acids in the tissue sample is expressed as ng/mg brain tissue. All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis. Analysis of Variance (ANOVA) test was performed to determine the significance of differences between the groups. If the null hypothesis was rejected, *post-hoc* tests were conducted. Depending on the outcome of the Snedecor-Fisher F test, Duncan (D) test or Newman-Keuls (NK) was used. Results calculated with an Excel spreadsheet were presented as mean ± SEM (Mean Standard Error). The results were considered statistically significant when the level of significance (p) was less than 0.05. All statistical analyses were carried out using TIBCO Statistica® 13.3.

RESULTS

There were no differences in body weight between the groups of animals in the entire period of the study ($F_{(3,36)}$=1.01), nor in the initial ($F_{(3,36)}$=0.91) and the final body weight ($F_{(3,36)}$=2.21) (p>0.05).

Novel Object Recognition (NOR) test results – lack of important changes in cognitive skills. *Passiflora* extract did not substantially change the exploratory behaviour of rats during both the familiarization and testing phases compared to control animals (Tab. 1). There were no significant difference in the global NOR parameters, such as Discrimination Index (DI), Recognition Index (RI) and Global Habituation Index (GHI) (p>0.05) (Tab. 2).

Table 1. Results of Novel Object Recognition test during the choice phase (mean ± SEM) in rats after chronic administration *Passiflora incarnata* L. extract at doses 30, 100 and 300 mg/kg b.w./day (P30, n = 10; P100, n = 10; P300, n = 10) and in control animals (Con, n = 10). To determine the significance of differences between the groups Analysis of Variance (ANOVA) test was performed. If the null hypothesis was rejected, post-hoc tests were conducted. Depending on the outcome of the Snedecor-Fisher F test, Duncan (D) test or Newman-Keuls (NK) was used

Group	Latency to first contact with object (s)		No. of contacts with object		Total time examining objects (s)	
	Object A1	Object B (new object)	Object A1	Object B (new object)	Object A1	Object B (new object)
Con	16.5±3.12	31.5±13.35	4.6±0.5	3.3±0.4	19.9±4.16	13.1±5.96
P30	26.4±13.37	19.3±6.43	3.9±0.51	3.0±0.49	12.9±2.27	8.2±1.5
P100	18.1±3.15	19.9±8.96	4.0±0.33	3.8±0.63	13.4±2.39	8.5±1.77
P300	19.0±6.49	11.5±4.96	5.5±0.67	3.7±0.52	13.9±1.99	6.6±1.09

Table 2. Global parameters of Novel Object Recognition test (NOR) for control rats (Con, n=10) and animals received *Passiflora incarnata* L. extract (P30, n = 10; P100, n = 10; P300, n = 10). DI- Discrimination Index, GHI – Index of Global Habituation, RI – Recognition Index. To determine the significance of differences between the groups Analysis of Variance (ANOVA) test was performed and if the null hypothesis was rejected, post-hoc tests were conducted. Depending on the outcome of the Snedecor-Fisher F test, Duncan (D) test or Newman-Keuls (NK) was used

Group	NOR Global parameters			
	Total time examining objects A1A2+A1B (s)	GHI	RI	DI
Con	52.0±7.01	0.78±0.14	0.37±0.07	-0.26±0.14
P30	37.3±3.99	0.82±0.06	0.40±0.06	-0.20±0.12
P100	41.2±3.58	1.08±0.22	0.38±0.07	-0.25±0.14
P300	37.5±3.86	0.86±0.08	0.33±0.05	-0.34±0.10

Hole Board (HB) test results – lower mobility in rats treated with higher dose of extract. The analysis of variance showed a reduction in the number of crossings between segments during the 3 days of the study in animals receiving extract at doses of 300 mg, compared to the control group and P100 group (Fig. 1A). The HB test also showed a statistical reduction in the time spent in the central segments of the apparatus in the P300 group compared to the other groups (Fig. 1B). There were no statistically significant differences between the groups in the number of head dippings into the holes, nor in the number of climbs during the 3 days of the experiment (p>0.05). Motor activity, i.e. the total time spent in motion, was similar in all groups of rats studied (Tab. 3).

Level of monoamines and metabolites – important changes concerning dopaminergic and noradrenergic systems. Concentration of neurotransmitters in the cerebellum, medulla and spinal cord in rats after long-term administration of *P. incarnata* extract is presented in Table 4.

Significant changes were found in the concentration of the 5-HT metabolite 5-HIAA in the cerebellum ($F_{(3,36)}$=2.83; p<0.05). The concentration of 5-HIAA was lower in the group receiving the extract of *P. incarnata* L. compared to the control group. However, an increase in the concentration of 5-HIAA in the spinal cord was shown in the groups receiving

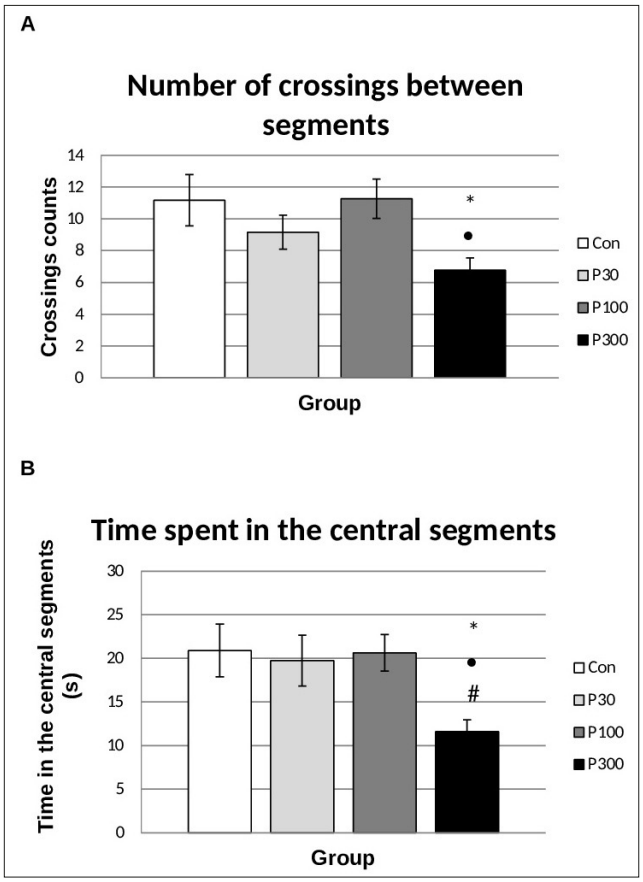

Figure 1. Hole Board test parameters for control rats (Con, n = 10) and rats treated with *Passiflora incarnata* L. extract (P30, n = 10; P100, n = 10; P300, n = 10). Rats receiving extract at doses of 300 mg/kg b.w. showed a statistically significant reduction in the number of crossings between segments (A) and exhibited reduced time spend in the central zone of the apparatus (B) during the 3 days of the study. * P300 *vs* Con, p<0.05 (NK); • P300 *vs* P100, p<0.05 (NK); # P300 *vs* P30, p<0.05 (NK); (NK) – Newman-Keuls test

the extract, compared to control animals ($F_{(3,36)}$=2.78; p<0.05). The turnover of serotonin (5-HIAA/5-HT) in the spinal cord was higher in the P30 and P300 groups compared to the control group ($F_{(3,36)}$=5.96; p<0.005). Analysis of variance showed a significant difference in the concentration of NA in the cerebellum ($F_{(3,36)}$=5.99; p<0.005). Moreover, significant differences were found in the concentration of the NA metabolite – MHPG in the spinal cord ($F_{(3,36)}$=4.88, p<0.01).

In the examined CNS structures, a higher concentration of DA was demonstrated in the groups receiving extract compared to the control group (Fig. 2A). Significant differences were found between groups of animals in the DA concentration in the spinal cord ($F_{(3,36)}$=6.99, p<0.005). Analysis of variance showed significant differences in the cerebellar concentration of the DA metabolite DOPAC ($F_{(3,36)}$=6.40; p<0.005) and HVA ($F_{(3,36)}$=9.91; p<0.005) (Fig. 2B, 2C). Moreover, significant differences were found in the concentration of HVA in the medulla oblongata ($F_{(3,36)}$=2.68; p<0.05) and the spinal cord ($F_{(3,36)}$=4.30, p<0.01) (Fig. 2D).

Reduced DA turnover was found in the cerebellum in all groups receiving extract ($F_{(3,36)}$=6.15; p<0.005). ANOVA showed significant differences in DA turnover (DOPAC/DA) in the cerebellum. In the case of HVA – another DA metabolite – significant differences were also observed in the HVA/DA turnover in the cerebellum ($F_{(3,36)}$=3.27; p<0.05) and in the spinal cord ($F_{(3,36)}$=3.98; p<0.05) (Tab. 5).

Table 3. Main Hole Board parameters for the Control rats (Con, n=10) and rats received extract of *P. incarnata* L. (P30, n = 10; P100, n = 10; P300, n = 10)

Group	Time spent in movement (s)	Time spent in the central segments (s)	No. of crossings	No. of head dips	No. of climbs
Con	58.10±2.24	20.9±3.03	11.17±1.62	14.13±0.69	4.07±0.45
P30	55.80±2.99	19.73±2.92	9.17±1.07	12.53±0.84	5.03±0.87
P100	55.57±2.63	20.63±2.10	11.27±1.23	12.93±0.68	4.40±0.43
P300	55.67±3.43	11.6±1.35*#•	6.77±0.78*•	12.36±0.61	5.23±0.68

* P300 *vs* Con, p<0.05 (NK); # P300 *vs* P30, p<0.05 (NK); • P300 *vs* P100, p<0.05 (NK); (NK) – Newman-Keuls test.

Table 4. Concentration of monoamines and their metabolites (mean ± SEM) in selected CNS structures in rats after chronic administration *Passiflora incarnata* L. extract at doses 30, 100 and 300 mg/kg b.w./day (P30, n = 10; P100, n = 10; P300, n = 10) and in control animals (Con, n = 10)

Group		Level of monoamines and metabolites in selected CNS structures (ng/g tissue)		
		Cerebellum	Medulla oblongata	Spinal cord
5-HT	Con	70.4±5.4	653.2±8.9	331.8±53.0
	P30	72.7±4.4	571.1±68.2	344.6±15.0
	P100	72.7±5.3	648.8±8.6	343.1±24.7
	P300	73.7±5.8	605.3±8.2	339.2±14.3
5-HIAA	Con	51.6±2.3	300.9±3.4	116.9±13.1
	P30	49.4±2.4	280.7±33.6	**150.8±5.9** *
	P100	**45.4±1.3** *	309.5±6.2	144.0±14.2
	P300	**44.2±2.0** *	285.8±4.9	**159.6±8.9** *
DA	Con	3.2±0.3	44.9±5.0	15.7±2.1
	P30	4.6±0.5	45.2±5.5	**27.9±2.5** ***
	P100	4.2±0.5	50.8±0.9	**26.1±2.7** ***
	P300	4.6±0.4	46.8±1.6	**28.7±1.7** ***
DOPAC	Con	3.9±0.3	27.6±0.8	0.9±0.4
	P30	3.5±0.2	30.5±4.2	2.3±2.3
	P100	3.2±0.2	35.7±1.6	0.3±0.3
	P300	**2.4±0.3** ***### •	34.5±2.4	5.7±2.4
HVA	Con	42.5±1.3	92.6±2.1	71.6±6.6
	P30	**48.5±0.8** ***	77.1±9.5	**101.2±3.9** ***
	P100	**45.7±0.8** *	81.5±2.2	87.1±8.6
	P300	**41.4±1.1••• ###**	**71.7±4.2** *	**90.0±2.7** *
3-MT	Con	n.d.	1.0±0.4	0.5±0.3
	P30	n.d.	1.0±0.3	1.3±0.8
	P100	n.d.	0.4±0.2	1.5±1.2
	P300	n.d.	0.8±0.4	0.6±0.3
NA	Con	365.4±5.2	864.9±25.6	262.6±23.8
	P30	**323.8±9.2** ***	775.7±97.3	305.1±19.1
	P100	**338.7±10.1** *	892.7±10.5	279.8±23.0
	P300	**318.2±9.1** ***	847.0±15.7	317.1±12.3
MHPG	Con	n.d.	80.9±2.3	20.7±2.4
	P30	n.d.	86.9±11.1	**36.6±4.8****
	P100	n.d.	87.8±2.6	**22.8±4.3 ##**
	P300	n.d.	79.3±1.8	**20.1±1.5 ###**

* P300, P100, P30 *vs* Con, p<0.05 (D); *** P300, P100, P30 *vs* Con, p<0.005 (D); # P300 *vs* P30, p<0.05 (D); ## P100 *vs* P30, p<0.01 (D); ### P300 *vs* P30, p<0.005 (D); • P300 *vs* P100, p<0.05 (D); ••• P300 *vs* P100, p<0.005 (D); n.d.- not detected; (D) – Duncan test

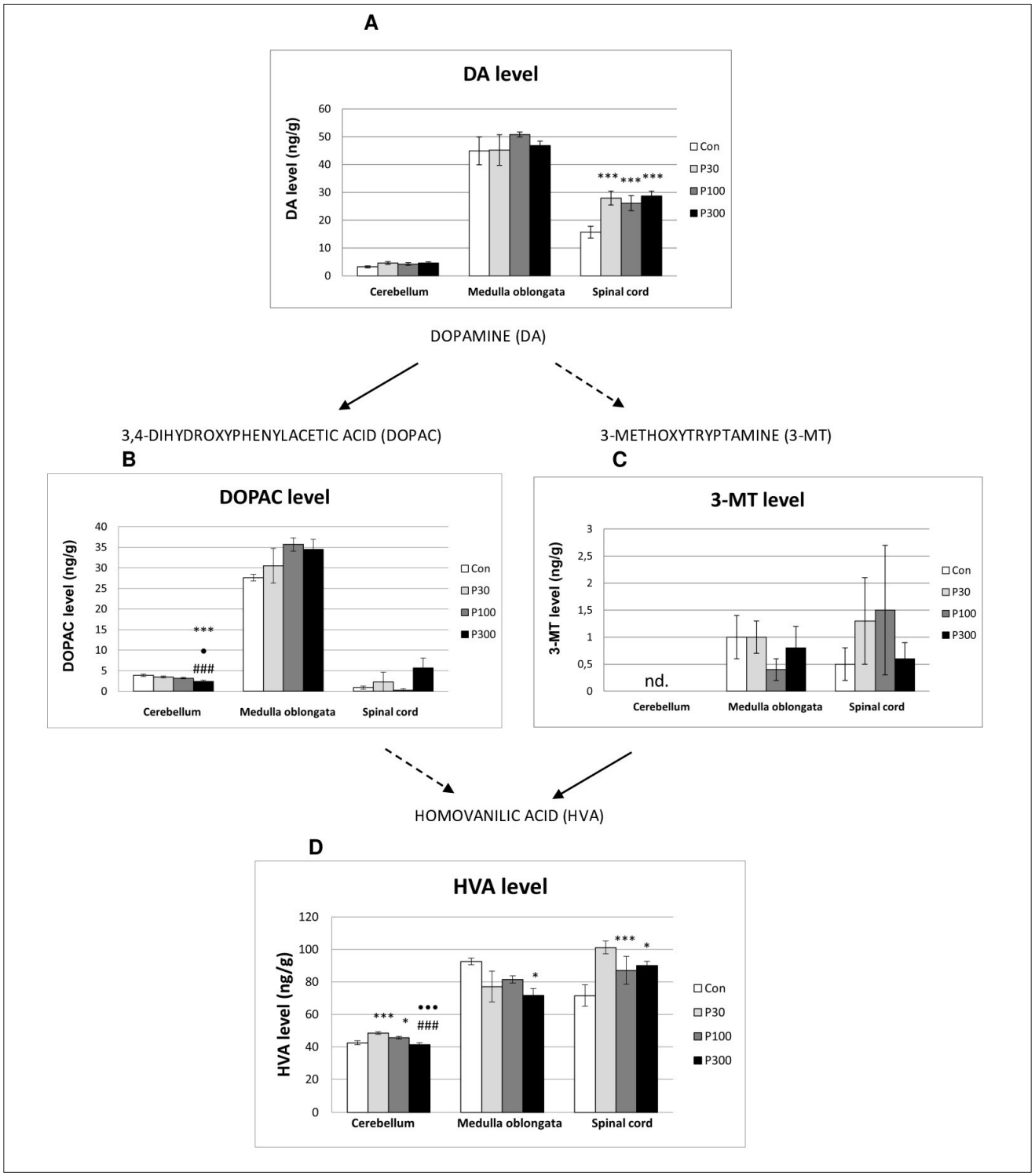

Figure 2. Effect of chronic administration of *Passiflora incarnata* L. extract at doses 30, 100 and 300 mg/kg b.w./day (P30, n = 10; P100, n = 10; P300, n = 10) on dopamine (DA) concentration (A) and level of dopamine metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) (B), 3-methoxytyramine (3-MT) (C) and homovanilic acid (HVA) (D) in selected CNS structures (mean ± SEM). Results compared to control group (Con, n = 10). Dopamine degradation is catalyzed by monoamine oxidase (reaction indicated by black arrow) and catechol-O-methyl transferase (dashed black arrow).
* **P30,** P100, P300 *vs* Con, p<0.05 (D); *** P300, P100, P30 *vs* Con, p <0.005 (D); ● P300 *vs* P100, p <0.05 (D); ●●● P300 *vs* P100, p <0.005 (D); #### P300 *vs* P30, p<0.005 (D); (D) – Duncan test; n.d. – not detected

Amino acids concentration – changes in the level of histidine, γ-aminobutyric and aspartic acid in rats that received plant extract. Amino acids concentrations in the cerebellum, medulla oblongata and spinal cord in rats after long-term – lasting a total of 47 days – administration of *P. incarnata* L. extract are presented in Table 6.

Significant differences were found in the concentration of GLU in the spinal cord ($F_{(3,36)}$=3.98; p<0.05) between the groups of tested animals. Moreover, differences between the studied groups of animals were found in the concentration of ASP acid (the second stimulating amino acid) in the cerebellum ($F_{(3,36)}$=4.15; p<0.05) and in the spinal cord

Table 5. Monoamine turnover (mean ± SEM) in selected CNS structures in rats after chronic administration of *Passiflora incarnata* L. extract at doses 30, 100 and 300 mg/kg b.w./day (P30, n = 10; P100, n = 10; P300, n = 10) and in animals from the control group (Con, n = 10)

Structure	Group	5-HIAA/5-HT	DOPAC/DA	HVA/DA
Cerebellum	Con	0.76±0.05	1.34±0.17	14.09±1.14
	P30	0.70±0.05	**0.89±0.14** *	11.99±1.34
	P100	0.65±0.04	**0.85±0.09** *	11.71±0.91
	P300	0.62±0.04	**0.56±0.08** ***	**9.46±0.70** ***
Medulla oblongata	Con	0.46±0.01	5.25±4.69	19.33±17.48
	P30	0.49±0.01	0.65±0.03	1.70±0.07
	P100	0.48±0.01	0.71±0.04	1.61±0.04
	P300	0.47±0.01	0.74±0.04	1.55±0.11
Spinal cord	Con	0.37±0.02	0.10±0.05	5.20±0.74
	P30	**0.44±0.01** *	0.07±0.07	**3.82±0.26** *
	P100	0.41±0.02	0.01±0.01	**3.48±0.31** **
	P300	**0.47±0.03** ***	0.22±0.09	**3.25±0.24** ***

P30, P100, P300 *vs* Con, p<0.05 (D); ** P100, P300 *vs* Con, p<0.01 (D); *** P300 *vs* Con, p<0.005 (D); ## P300 *vs* P30, p<0.01 (D); (D) – Duncan test

Table 6. Amino acid concentration (mean ± SEM) in selected CNS structures in rats after chronic administration of *Passiflora incarnata* L. extract at doses 30, 100 and 300 mg/kg b.w./day (P30: n = 10, P100: n = 10, P300: n = 10) and in animals from the control group (Con: n = 10)

Group		Level of amino acids in selected CNS structures (ng/mg tissue)		
		Cerebellum	Medulla oblongata	Spinal cord
TAU	Con	863.02±14.82	280.69±17.25	96.49±9.63
	P30	801.68±15.02	279.15±21.83	123.97±9.25
	P100	802.44±17.16	297.07±11.76	118.50±12.37
	P300	913.60±62.25	234.59±17.85	130.39±6.61
HIS	Con	11.54±0.38	7.58±0.62	4.39±0.73
	P30	11.63±0.34	8.84±0.72	7.37±0.63
	P100	11.21±0.44	**11.66±1.25*#●**	5.16±0.76
	P300	**13.56±0.93*#●**	7.86±1.06	8.17±2.02
ASP	Con	339.03±9.80	450.68±25.71	189.51±20.42
	P30	312.91±7.44	496.87±45.91	231.76±17.22
	P100	327.26±7.07	524.17±28.36	213.55±18.19
	P300	**382.02±25.58*#●**	458.33±30.84	**266.53±17.14***
ALA	Con	135.13±2.15	177.86±10.73	60.29±5.96
	P30	125.59±3.36	202.91±13.16	78.52±7.24
	P100	127.73±3.68	208.21±11.37	63.97±6.26
	P300	147.67±10.65	181.79±14.43	75.61±5.86
GLU	Con	2137.21±33.99	1181.23±70.61	447.20±41.43
	P30	1985.27±36.98	1270.83±139.42	578.07±38.21
	P100	1957.82±41.99	1327.16±71.56	513.26±43.40
	P300	2297.09±169.54	1164.88±94.12	**624.49±30.66***
GABA	Con	276.60±5.20	339.49±18.55	85.47±8.07
	P30	250.39±5.60	378.07±32.62	**128.04±11.30****
	P100	258.68±5.23	398.88±22.09	101.56±9.99
	P300	**308.86±25.84●#**	342.94±25.04	**141.36±9.37 ***●**

* P300, P100, P30 *vs* Con, p<0.05 (NK); ** P30 *vs* Con, p<0.01 (NK); *** P300, P100 *vs* Con, p<0.005 (NK); # P300, P100 *vs* P30, p<0.05 (NK); ● P300 *vs* P100, p<0.05 (NK); (NK) – Newman-Keuls test

($F_{(3,36)}$=3.15; p<0.05). In the cerebellum, the concentration of ASP was higher in the group receiving extract at the highest dose compared to the other group. On the other hand, an increase in the concentration of this amino acid in the spinal cord was shown in the P300 group, compared to the control animals. Significant changes in the concentration of GABA were found in the cerebellum ($F_{(3,36)}$=3.56; p<0.05) and the spinal cord ($F_{(3,36)}$=6.71; p<0.005). In the cerebellum, the concentration of HIS was higher in the group receiving *P. incarnata* L. extract at the highest dose compared to the other groups ($F_{(3,36)}$=3.44; p<0.05). However, in the medulla oblongata, an increase in the HIS concentration was shown in the P100 group compared to the animals from the other groups ($F_{(3,36)}$=3.85; p<0.05).

DISCUSSION

P. incarnata L. is a herbal remedy commonly used to reduce stress-related hyperactivity, anxiety, nervousness and depressive-like behaviour. The aim of the research presented in this study was to determine the effect of prolonged administration of the extract of *P. incarnata* L. on the motor and exploratory activity, anxiety behaviour and working memory in rats. The presented study also explains the molecular causes of the observed changes in the behaviour of animals after intake of the extract by assessing the level of neurotransmitters in the CNS structures responsible for motor function – the cerebellum, the medulla oblongata and the spinal cord.

One of the objectives of the presented study was to determine the effect of administration of *P. incarnata* L. extract on the anxiety state, as well as motor and exploratory activity in rats. In the study, the rats which received a higher dose of extract had lower mobility and fewer passages between segments than the control group, and rats treated with a lower dose showed a significantly shorter time spent in the central part of the Hole Board apparatus. This may indicate a calming or sedative effect of high doses of the extract. The results of the study are in line with data obtained by other researchers in various behavioural tests in rodents.

Analysis of the central effect of the water extract obtained from the aerial parts of the *Passiflora* plant (400 mg/kg) demonstrated a calming effect in the staircase test (reduction in the number of climbing and ascents), a reduction in motor activity in the free exploration test, and sleep elongation in animals receiving pentobarbital [21]. The behavioural effect observed in this study may be due to the action of chrysin, a flavonoid found in significant amounts in the passion fruit extract. An interesting study was presented by Zanoli et al. [22] who administered chrysin at doses of 25, 50 and 100 mg/kg b.w., and showed a dose-dependent reduction in time spent moving in the Open Field test compared to the control group. The anxiolytic and antidepressant effect of chrysin is mainly related to the interaction with the GABAergic and serotonergic systems, and the activation of neurotrophic factors, e.g. brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) [23]. The aqueous extract of *P. incarnata* L. (800 mg/kg) also proved effective in inhibiting the increased motor activity induced by nicotine administration [24].

The intraperitoneal administration of *Passiflora* extract (150, 300 and 600 mg) and diazepam (2 mg.) for 10 days

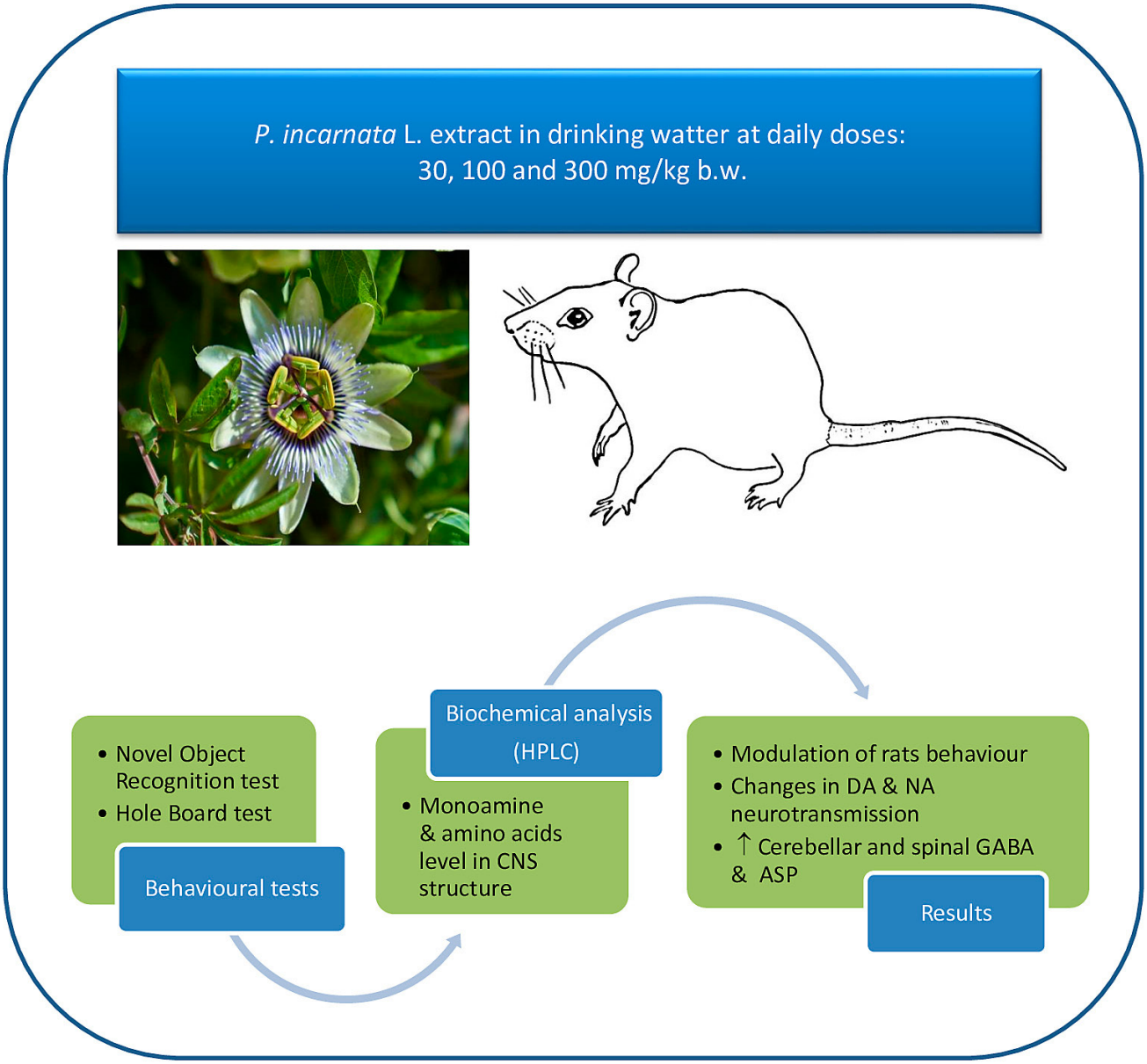

Figure 3. Graphical abstract. Passiflora photography - https://unsplash.com/photos/cGujZXgCwjM free to use under the Unsplash License

significantly reduced the motor activity of mice compared to the control group [25]. Similar results were obtained by Aman et al. [17], who observed that the extract at high doses had a sedative effect comparable to that of diazepam, and reduced the motor activity of mice in the Open Field test. The study also assessed the effect of a methanolic plant extract at doses of 200, 400 and 600 mg/kg b.w. orally administered to BALB/c strain mice for the anxiety response in the Stair test and the Open Field test. Animals receiving the extract at a dose of 200 mg climbed more stairs compared to the control group, while the mice receiving the highest dose had significantly worse results. The climbing frequency was reduced in animals at all doses of extract compared to the control group; this anxiolytic and sedative effect was reversed by administration of pentylenetetrazole. Speroni and Minghetti demonstrated a significant reduction in motor activity in rodents after administration of *P. incarnata* L. extract at a dose of 160 mg/kg b.w. [18]. Similar results were reported for an aqueous extract at doses of 400 and 800 mg/kg b.w. [21].

As in the studies presented, a lot of data show that the therapeutic and behavioural effects of *Passiflora* in animals are dependent on the dose and the solvent used to prepare the extract [21]. Sampath et al. [26] assessed the anxiolytic effect of 3 fractions obtained from the water-ethanolic extract of *P. incarnata* L. in the Elevated Plus Maze. The strongest effect was observed for the chloroform fraction, and slightly weaker for the butanol fraction at doses of 150–300 mg/kg b.w. The petroleum ether fraction showed no central effect. Neither fraction influenced the distance traveled by the animals in the Open Field test, and further dose increases did not produce a sedative effect. The anxiolytic effect is associated with an increased number of stairs climbed, while the calming effect of the compound manifests itself in a smaller number of entrances. In this context, the authors suggest that the methanolic extract of *P. incarnata* L. shows an anxiolytic effect at a dose of 200 mg and a sedative effect at a dose of 600 mg/kg b.w.

Based on the current study and other scientific data, it can

be concluded that the behavioural effects of *Passiflora* extract are largely dose-dependent. It was noticed that both in the case of *P. incarnata* L. and other species of the genus *Passiflora* (*P. actinia; P. alata; P. edulis f. flavicarpa; P. quadrangularis*), lower doses of the flower extracts have an anxiolytic effect, while a calming effect expressed by reduced motor activity occurs at the higher doses [27, 28]. The sedative properties of *P. incarnata* L. have also been confirmed in weaning piglets [29]. Dietary supplementation with an extract (1kg/t) reduces aggressive and abnormal behaviour, e.g. tail and ear biting, lowers the salivary cortisol level in piglets after weaning.

The results of animal studies have been confirmed by clinical observations, as in a study by Dantas et al. [9] which confirmed the anxiolytic effect of pre-operative administration of *P. incarnata* L. (260 mg) in patients undergoing tooth extraction. This effect was similar to midazolam, but was not pro-amnestic and did not interfere with memory formation. Similar benefits in controlling dental anxiety, such as reduced heart rate, somnolence, muscle relaxation and dizziness, were seen in patients undergoing extraction of mandibular 3rd molars [30].

The second behavioural test conducted during the presented study was the Novel Object Recognition test (NOR) – one of the tests performed to assess cognitive processes in animals. The test examines cognitive function, the ability to recognize an object, and the preferences for novelty. It is assumed that animals are more likely to explore a new object, and novelty preference means that the image of the old object still exists in the animal's memory [31]. Procedural (non-declarative) memory operates outside consciousness and concerns manual skills and habits. It is acquired through conditioning, performed by motor operations, and localized in the cerebellum, striatum and amygdala [32]. The experiment showed no significant differences in the main parameters describing the behaviour of the animals in this test. Both the control group and the rats treated with the extract of *P. incarnata* L. spent similar time examining old and new objects. It can be concluded that administration of the extract, unlike many commonly anti-anxiety drugs, does not disturb associative and cognitive functions. The only difference between the groups was greater total contacts with both objects during the choice phase in the group treated with the highest dose of the extract.

The appearance of a new object can change the behaviour of the animal – triggering a stress response/arousing interest. It is well known that exposure to stress causes the sympathetic nervous system to release NA, which plays an important role in modulating the stress response and leads in the regulation of learning and memory. Increased NA release during stress disrupts memory by altering the function of the prefrontal cortex [33]. The results indicate that stress-related learning and memory impairment are mediated by the noradrenergic system. It is also known that drugs that reduce the over-activity of the noradrenergic system may have a beneficial effect in the treatment of stress-related disorders, such as post-traumatic stress disorder (PTSD) and anxiety.

In animals receiving *P. incarnata* L. extract, a decrease in the concentration of NA in the cerebellum was observed – the structure of the CNS involved in maintaining balance and the coordination of movements, although changes in the level of this neurotransmitter did not translate into cognitive abilities: memory and objects recognition. NA, as an important neuromodulator in the cerebellum, is believed to regulate sensory information integration and synaptic transmission in the granular layer of the cerebellar cortex [34]. Sun et al. [35] found that NA inhibited decreased activity of cerebellar Purkinje cells by activating interneurons of the molecular layer in mice. The cerebellum is crucial for coordination and movement, but it also works very closely with the cerebral cortex and can affect non-motor functions. Many authors suggest that lowering NA levels counteracts the harmful effects of stress that accompany behavioural testing, and generally results in an increased release of NA [36]. This is confirmed by the current study, in which a decrease was we observed observed in NA content in cerebellum, as well as previously published data that showed a beneficial effect of *P. incarnata* L. extract on the stress response and the associated reduction of NA concentration in the prefrontal cortex [37].

Motor development is conditioned by the proper structure and function of the musculoskeletal system and the controlling activity of the nervous system. The dopaminergic system is a regulator of the spinal cord motor circuit and plays an important role in the activation and modulation of the locomotor system in both invertebrates and vertebrates [38]. Some studies emphasize the role of DA in shaping the locomotor system during development, as well as its influence in changing behaviour and motor skills from immature to more mature movement patterns [39]. Motor problems in Parkinson's Disease and restless leg syndrome are examples confirming the key role of the dopaminergic system in shaping movement patterns.

In the presented study, a strong influence was found of extract on dopaminergic neurotransmission and DA metabolism in all analyzed CNS structures. An increase was shown in the level of DA and its metabolite HVA in the spinal cord in animals receiving *P. incarnata* L. extract. The extract also caused significant changes in the level of DA metabolites in the cerebellum and medulla oblongata, and decreased DA turnover in the spine and cerebellum.

These biochemical observations confirm the studies of other scientists. Ingale and Kasture [40] acknowledged the neuroprotective properties of n-butanol extract of *P. incarnata* L. in an elevated plus maze in 2 animal models of Parkinson disease. Administration of the extract resulted in an increase in the object recognition, indicating an improvement in cognitive function in animals. At the same time, the anti-Parkinsonian and memory-enhancing effects were manifested by a significant reduction in haloperidol-induced catalepsy and tacrine-induced mandibular movement. Chrysin – the monoflavonoid present in the extract – may be responsible for the increased level of DA observed in the current study. This component is not only a specific ligand for central and peripheral benzodiazepine-binding sites, but can also activate other neurotransmitter systems involved in the physiopathology of several neuropsychiatric disorders, such as anxiety or depression. This applies in particular to the dopaminergic and serotonergic systems. Neurotransmitters, such as 5-HT, DA, GABA and GLU, interact to regulate the excitation and inhibition of motor neurons, and also contribute to the modulation of pain [41,42]. It has been shown that DA released from the neural terminals in the lumbar spinal cord of rats can affect all 5 subtypes of dopamine receptors unevenly distributed throughout the spinal cord [43]. DA stimulates the formation of new nerve cells, including both embryonic and adult neurogenesis in motor neurons of the spinal cord [44]. In the current study,

elevated levels of DA were observed in the spinal cord in animals receiving long-term *P. incarnata* L. extract. The current observations are consistent with previously published studies in which the authors showed an increase in the swimming speed in the Water Maze test, and explain the basis of changes in the motility of animals [37].

P. incarnata L. extract can also be considered a sleep inducer because its administration (500 mg/kg) reduces sleep latency and increases the amount of slow wave sleep in rats [45]. Positive modulation of circadian rhythms and wake-sleep cycle by extract is associated with an increase in DA levels and altered expression of metabolic enzymes, such as monoamine oxidase (MAO), catechol-O-methyltransferase (COMT) and glutamic acid decarboxylase in mice [46]. The water-ethanol extract did not show sedative properties in any of the doses used, while in the dose of 400 mg/kg b.w. had an anxiolytic effect.

Biochemical analysis also confirmed significant changes in the concentration of amino acids after administration of *Passiflora* extract. The GABAergic system may be responsible for the anxiolytic and sedative properties of extract, and the likely mechanism of its action is the effect on GABA A and GABA B receptors or the inhibition of GABA re-uptake. This was confirmed in the open field test, where it was observed that *P. incarnata* L. extract at doses of 200 and 600 mg/kg b.w. showed a sedative effect comparable to diazepam [17]. A significant increase was found in the content of GABA – an inhibitory neurotransmitter in the cerebellum and spinal cord after consuming the extract. It is well known that GABA blocks or inhibits certain brain signals and reduces the activity of the nervous system, which may explain the calming effect of the extract in the current study. *In vitro* studies have shown that the sleep-induced effect is due to increased mRNA expression of GABA receptors [47, 48]. In this study, *Passiflora* extract, especially in higher doses, causes an increase in GABA levels in the cerebellum and spinal cord, structures particularly involved in motor functions.

Dos Reis Izolan et al. [6] found that a commercial standardized dry extract (Sintocalmy®, Aché Laboratories, Brazil), at the dose of 50 and 100 mg *i.p.* reduced naloxone-induced jumping in mice, a morphine withdrawal model without reducing exploratory activity. At a higher dose (200 mg), a significant reduction in locomotor activity was observed in mice, compared to the vehicle and the group that received 100 mg of Sintocalmy®. Administration of the extract prevents an increase in the level of the cortical calcium binding protein S100 (S100B) and prevents DNA damage. The ethanol extract used to treat sleep disorders in rodent models modulates the inflammatory response, provides strong resistance to stressors, and improves hippocampal neurogenesis, enhancing hippocampal-dependent memory and learning in the water maze test. In DBA/2 mice, with genetic sleep defects, oral administration of the extract (50 mg/kg b.w.) caused the activation of neuroblasts and the branching of their dendrites, and an increase in serum serotonin and melatonin levels by over 30% [48]. In the current study, the administration of *P. incarnata* L. extract did not change the 5-HT level in the CNS structures; however, slight changes were detected in the level of metabolites of this neurotransmitter, and changes in 5-HT turnover to 5-HIAA in the spinal cord and cerebellum. In rats treated with the extract at a dose of 30 and 300 mg, an increase in the level of 5-HIAA in the spine and 5-HT turnover were detected. On the contrary, a significant decrease in 5-HIAA cerebellar levels was detected after a dose of 100 and 300 mg of the extract.

CONCLUSIONS

The present study shows that *P. incarnata* L. extract alters neurotransmission in major elements of the CNS locomotor system. A significant increase in the level of DA in the spinal cord may be potentially useful in disorders related to a deficit in dopaminergic neurotransmission. Due to its inhibitory effect on the GABAergic pathway, *Passiflora* extract has potential as a sedative and may be an alternative to benzodiazepines in controlling anxiety and hyperactivity. These findings could lead to the development of specific supportive therapies to support? alleviate anxiety and symptoms of gradual loss or impairment of motor behaviour. Further research is required to define the molecular mechanism of action of *P. incarnata* L. extract, and its precise impact on neurotransmitter pathways in the structures of the central nervous system and motor function.

Ethical Considerations

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures were approved by the Ethical Committee for Animal Experiments at the Medical University of Warsaw. All authors approved the final manuscript.

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