

BIOACTIVE EXTRACTS FROM CULTIVATED *AJUGA GENEVENSIS* L. AND *A. REPTANS* L.: *IN VITRO/IN VIVO* PHARMACOLOGICAL EFFECTS

ANDREI FLORIN PĂDURARU¹, OANA CIOANĂ^{1*}, CORNELIA MIRCEA¹, ADRIANA TRIFAN¹, ANA CLARA APROTOSOAIE¹, ANCA MIRON¹, ELVIRA GILLE², LUCIAN HRITCU³, MONICA HÂNCIANU¹

¹Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania

²National Institute of R&D for Biological Sciences, "Stejarul" Biological Research Centre, Piatra Neamț, Romania

³Department of Biology, Faculty of Biology, "Alexandru Ioan Cuza" University, Iași, Romania

*corresponding author: oana.cioanca@gmail.com

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Abstract

Ajuga genevensis and *Ajuga reptans* are medicinal plants often used as remedies in the Romanian traditional medicine for their benefic properties. They are also credited with astringent, tonic and diuretic activities. Although these species are common for the wild flora in our country, our investigation was conducted on specimens harvested from the experimental fields of "Stejarul" Biological Research Centre, Piatra Neamț, Romania. Their introduction in culture as a source of bioactive compounds is intended for pharmaceutical purposes. Their chemical profile was assessed on hydro-alcoholic extracts by spectrophotometry and RP-UPLC techniques. The *in vitro* biological effects of the samples were investigated against lipoxygenase, butyryl- and acetylcholinesterase. For the *in vivo* biological activities, the extracts (25 and 75 mg/kg bw) were administered in a 6-hydroxidopamine Parkinsonian rat model. The results confirmed the taxonomic and chemical variability between the investigated samples. In terms of compound profile, the polyphenols (flavonoid and polyphenolic acids) were in a higher amount in *Ajuga genevensis* (168.3 mg % and 230.4 mg % respectively), whereas *Ajuga reptans* contained higher quantities of iridoids (1860 mg % as compared to 1250 mg %). Harpagide and its acetylated derivative were the main iridoids identified in both extracts. The biological activity confirmed the antioxidant and protective properties of both extracts. Moreover, the anti-amnesic activity was significantly increased for *Ajuga reptans* as compared to *Ajuga genevensis*. Such results confirm a direct correlation between the concentration of iridoids and neuroprotective capacity of the extract obtained from *A. reptans*.

Rezumat

Ajuga genevensis și *Ajuga reptans* sunt plante medicinale utilizate adesea ca remedii în medicina tradițională românească pentru proprietățile lor benefice. Acestea li se atribuie proprietăți astringente, tonice și diuretice. Deși aceste specii sunt comune pentru flora spontană din țara noastră, cercetarea noastră a fost efectuată pe specimene recoltate din câmpurile experimentale ale Centrului de Cercetări Biologice "Stejarul", Piatra Neamț, România. Introducerea lor în cultură ca sursă de compuși bioactivi este destinată scopurilor farmaceutice. Profilul lor chimic a fost evaluat pe extracte hidro-alcoolice prin spectrofotometrie și tehnici RP-UPLC. Efectele biologice *in vitro* ale probelor au fost investigate față de o serie de enzime precum lipoxigenaza, butiril- și acetilcolinesteraza. Pentru activitățile biologice *in vivo*, extractele uscate (25 și 75 mg/kg greutate corporală) au fost administrate într-un model animal de Parkinson indus cu 6-hidroxidopamină. Rezultatele au confirmat variabilitatea taxonomică și chimică între probele investigate. În ceea ce privește profilul chimic, polifenolii (flavone și acizi polifenolici) se regăsesc în concentrații mai mari în *Ajuga genevensis* (168,3 mg %, respectiv 230,4 mg %), în timp ce *Ajuga reptans* conține cantități mai mari de iridoide (1860 mg % față de 1250 mg %). Harpagozida și derivatul său acetilat au fost principalele iridoide identificate în ambele extracte. Activitatea biologică a confirmat proprietățile antioxidante și protectoare ale ambelor extracte. Mai mult, activitatea antiamezică a fost semnificativ crescută pentru *Ajuga reptans* comparativ cu *Ajuga genevensis*. Astfel de rezultate confirmă o corelație directă între concentrația în compuși iridoidici și capacitatea neuroprotectoare a extractului obținut din *A. reptans*.

Keywords: *Ajuga species*, antioxidant, enzymes, Parkinson, memory

Introduction

Folk medicine has always represented a rich source for knowledge regarding old therapies and natural medicines. *Ajuga genevensis* and *Ajuga reptans* are two medicinal plants used in the Romanian traditional

medicine for their anti-inflammatory properties for rheumatism and gout. The most important effects for which the species are included in the folk medicine of various European countries are: astringent, tonic and diuretic activities. Recent data sustains the antioxidant, anti-inflammatory, antimicrobial and anti-

fungal properties of different *Ajuga* species [4, 13-16]. These species are common for the wild flora in our country [3, 14, 15]. Their introduction in culture as a source of bioactive compounds is intended for pharmaceutical purposes. Therefore, the investigation of their characteristics and benefits is of extreme importance for future use in pharmaceutical preparations and/or food supplements.

The brain is one of the organs most exposed and sensitive to the action of reactive oxygen species (ROS) due to the large amount of poly-unsaturated fatty acids found in the phospholipid bilayer of which the neuronal membrane is constituted, but also due to the reduced number of brain antioxidant systems. On the other hand, increased amounts of hydroxyl radicals (HO) are formed in the basal ganglia, especially due to the presence of a small amount of iron in the tissue [1]. In a series of neurodegenerative diseases, including Parkinson's disease, in addition to an increase in the level of reactive oxygen species, it is noted a decrease in the activity of enzymes catalysing the transformation of superoxide anion into oxygen peroxide and free oxygen [7-9]. But in these patients, the antioxidant levels (vitamin C, A, E) are also low. The existence of modern civilization diseases (diabetes, atherosclerosis, heart disease) in such patients also comes with an additional cerebral oxidative stress, so lately it has become clear that an inflammatory component is also involved in the Parkinson disease [10, 15, 17, 18]. Inflammation is a complex response of the body to physical, chemical, bacterial or viral factors, being itself a source of ROS.

Assuming that the neuroprotective effect of extracts may be due to both anti-inflammatory action and antioxidant qualities, we have investigated the biological qualities of the extracts obtained from the two *Ajuga* species. The antioxidant potential was assessed *in vitro* against three enzymes (lipoxygenase, butyryl- and acetylcholinesterase) implicated in inflammation and neurodegeneration. The neuroprotective effect *in vivo* testing was observed in laboratory animals with experimental induced Parkinson. Oxidopamine (syn. 6-hydroxydopamine or 6-OHDA) is a dopamine analogue which induces oxidative stress in dopaminergic neurons by blocking the complex I of the cell respiratory chain. The modifications in the dopamine transporters lead to neurodegeneration and its administration mimics Parkinson symptomatology in animal models [10].

Materials and Methods

Plant material and selective extracts preparation

Our investigation was conducted on specimens harvested in May 2016 from the experimental fields of "Stejarul" Biological Research Centre, Piatra Neamț, Romania. The aerial parts characteristic features were observed with an optic microscope in several surface sections for each species.

5 g of plant product either from *Ajuga reptans* or *Ajuga genevensis* were treated with 30 mL of 70% ethanol, the extraction mixture being refluxed for 15 minutes. After cooling, the mixture was filtered, and the filter and the vegetable residue were taken up with another 70% alcohol portion (30 mL). Again, the mixture was boiled in a water bath at reflux for 15 minutes, after which the extract was separated and the filter and the vegetable residue were resumed for the third time with 30 mL of 70% ethanol at reflux. The three hydroalcoholic extracts were combined in a 100 mL volumetric flask and the filters and the vegetable residue were washed with about 15 mL of 70% ethanol. After cooling, the extract was levelled to the mark in a volumetric flask with the filtrate obtained by washing the residues. These were the initial stock extracts, which were then brought into a porcelain chunk. Each extract was subjected to evaporation, finally obtaining two soft, dark brown, sticky, moderately bitter taste extracts that were weighed and transferred into two sealed vials. After coating with wax, they were stored in the refrigerator until either the chemical analysis or testing on animals with experimentally 6-OHDA-induced Parkinson's were performed.

Total phenolic content

The extracts (corresponding to 200 mg of plant product *per* mL DMSO) were mixed with ultra-pure water and Folin-Ciocalteu reagent for phenols at a final volume of 3.4 mL. After 5 min 0.6 mL of 20% sodium carbonate were added, and after vigorous stirring, the solutions were kept at room temperature in the dark for 2 hours. The absorbance of the samples was measured at 765 nm and the results were expressed in mg gallic acid % [3, 8]. Total polyphenols concentration was calculated using a calibration curve of gallic acid established under the same conditions with the extracts.

Flavonoid content

Flavonoids were quantified as previously described [3, 8] and their concentration was expressed as rutin equivalents (mg/g). 5 mL of extract sample was mixed with 5.0 mL of 100 g/L sodium acetate and 3.0 mL of 25 g/L aluminium chloride. Methanol was added up to 25 mL in a calibrated flask. The absorbance corresponding for the yellow colour of the complex was photometered at 430 nm.

Iridoid quantification (Trim-Hill reaction)

The total iridoid content was calculated and expressed as aucubin equivalents, following the method employed by Toiu *et al.* [13, 14]. Briefly, 0.4 mL of sample was vortexed with 4 mL of Trim-Hill reagent (acetic acid – 0.2 % CuSO₄ - conc. HCl, mixed in 10:1:0.5 ratio). The absorbance of the blue colour was measured at 609 nm, and the concentration of iridoids was calculated using an aucubin (0.1 - 1 mg/mL) calibration curve ($R^2 = 0.998$).

RP-UPLC analysis

Quantitative determination of the polyphenol-carboxylic acids and flavonoids was made by reverse-phase liquid chromatography in linear gradient of acetonitrile and phosphoric acid aqueous solution [3]. A Thermo Ultimate 3000 system with Luna PFP (2) column (150 x 4, 6 x 4) and the following parameters were used for iridoids identification: detection wavelength - 245 nm, flow rate - 0.8 mL/min, linear gradient (1% phosphoric acid: acetonitrile) from 38% to 55% in 20 minutes, and then 55% to 100% in 15 minutes; injection volume - 2.5 µL. Harpagide, catalpol, aucubin and 8-O-acetyl-harpagide (Sigma Chemical Co.) were used as standards. Polyphenols were identified by scanning absorbance from 240 nm to 520 nm with the same system, using a gradient of acetonitrile (A) and 0.1% acetic acid (B) (10% - 23% (A) in 5 min; 23% (A) isocratic for 10 min and then 23% - 35% (A) in 12 min; 35% - 70% (A) for 5 min). Authentic standards of HPLC grade (Sigma Chemical Co.) were used to obtain the calibration curves. Samples UV spectra were automatically compared by Chromeleon 7.2 software and the concentration was expressed as % of the standard's area/concentration. All chromatographic experiments were performed at 28°C in triplicate and average values were used for calibration curves and quantification of the identified compounds. NIST and Wiley libraries were used for confirmation.

In vitro enzyme inhibitory/neuroprotective activity

First, the enzymatic activities of the samples were investigated against lipoxygenase, butyryl- and acetylcholinesterase.

Lipoxygenase inhibition assay (Maltreud modified method). The polyphenolic compounds present in the extract have the ability to block the activity of lipoxygenase in buffer solution (pH = 9) that catalyses oxidation of linoleic acid, thus inducing a decrease in the absorbance at 234 nm [7, 12, 17].

Butyrylcholinesterase inhibition assay. The ability of the investigated extracts to inhibit butyrylcholinesterase was assessed by measuring the absorbance variations at 412 nm for 5 minutes at 25°C, using acetylthiocholine iodide (0.2 M) as substrate [2, 11]. The difference between the initial and last value represented intensity of the inhibitory effect.

Acetylcholinesterase inhibition assay (Ellman's method). Acetylcholinesterase from *Electrophorus electricus* (0.2 U/mL in phosphate buffer), acetylthiocholine iodide (0.2 M) and diluted samples in DMSO (0.625 mg - 20 mg/mL) were used to evaluate the inhibitory capacity of *Ajuga* extracts [2, 11]. The activity value was calculated as a percentage of the difference between the absorbance of the enzyme solution with inhibitor at 412 nm after 5 minutes and the absorbance of the same solution at the initial time.

In vivo neuroprotective activity

For the *in vivo* biological activity, the extracts (25 and 75 mg/kg bw) were administered in a 6-hydroxy-

dopamine (6-OHDA) Parkinsonian rat model (5 animals/group). For the investigation of the biological activity, 1.25 mg of soft extract of *A. reptans* and *A. genevensis* respectively, were suspended in 5 mL of physiological saline so that 6.25 mL suspension A was obtained. The equivalent to 25 mg (1.25 mL of suspension A) were administered intraperitoneally (groups 3 and 5). For the second concentration, 3.75 mg of the investigated extract were dissolved in 5 mL of physiological saline (suspension B). This was injected to the treated rats (experimental groups 4 and 6) as the equivalent to 75 mg/kg bw (1.075 mL/kg bw). All surgical procedures were performed under aseptic conditions, using Nembutal 9[®] anaesthesia by injection at a dose of Nembutal[®] 50 mg/kg bw.

The pharmacological tests included: Y-maze spontaneous locomotor activity and rotation, radial - arm maze test of working memory and the reference memory, and the working memory and reference memory errors were calculated/group/day. All tests were previously approved by the Ethics Committee, and respected the European guideline of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare from Romania, and all procedures were in compliance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 [1, 2].

Statistical analysis

As statistical tools, we used one-way ANOVA test and multiple variance ANOVA test for intergroup comparisons, and statistical analysis software GraphPad Prism version 6.00 for Windows, La Jolla California USA. All reported values represent the mean of triplicate determination, for both *in vitro/in vivo* testing.

Results and Discussion

After initial observation of the microscopic features (glandular and surface hairs, epidermis structure, the number of and type of stomata) we confirmed their identification: *Ajuga genevensis* L. (blue bugle) and *Ajuga reptans* L. (common bugle or bugleweed). The two soft extracts obtained from *A. reptans* and *A. genevensis* according to the above methodology, weighed 0.96 g for bugleweed (which means a 19.2% yield) and 1.00 g respectively for blue bugle, representing a yield of 20%. The analysis of the compounds spectra from the investigated samples showed chemical similarities and differences at the same time, thus indicating an interspecific variability between which is present although the plant material has been grown in identical pedoclimatic conditions. In terms of the chemical profile, the polyphenols (flavonoid and polyphenolic acids) were richer in *Ajuga genevensis* (168.3 ± 0.13 mg % and 230.4 ± 0.42 mg % respectively), whereas *Ajuga reptans* contained higher quantities of iridoids (1860 ± 0.21 mg % as compared to 1250 ± 0.03 mg %) as shown in Figure 1.

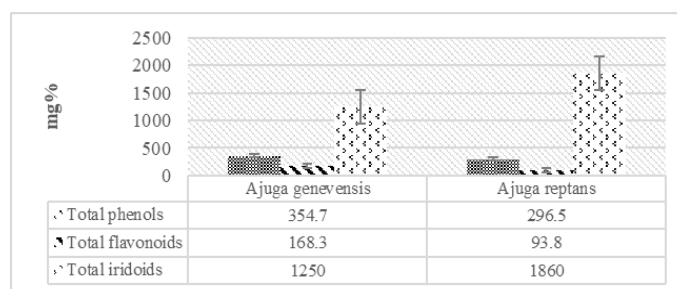


Figure 1.

Quantitative results for iridoids, total polyphenols and flavonoids in the two *Ajuga* samples

The obtained results are comparable only to some extent to the data from literature [3, 14, 15] but one need mentioning that the source of the analysed plant material is different from the data previously published. In the other researches, it is mainly used the raw material from the wild flora, whereas in this investigation the plants were cultivated in controlled conditions.

Still, it is notable that *A. reptans* is richer in iridoids than *A. genevensis*.

The identification and quantification of the most important classes of secondary metabolites indicated the presence of polyphenolic acids, flavonoid and iridoid glycosides, as indicated in the Table I.

Table I

UPLC identification of the most important compounds found in the analysed samples

Sample	Compound ($\mu\text{g/g dw}$)					
	Caffeic acid	p-Coumaric acid	Rosmarinic acid	Luteolin-O-glucoside	Luteolin	Apigenin
<i>Ajuga reptans</i>	27.412 ± 0.2	13.232 ± 0.3	20.209 ± 0.1	83.255 ± 0.1	13.600 ± 0.3	25.227 ± 0.1
<i>Ajuga genevensis</i>	11.822 ± 0.1	12.205 ± 0.2	9.461 ± 0.1	63.212 ± 0.4	27.15 ± 0.2	17.469 ± 0.2

As indicated in Table I the proportion between the polyphenolic compounds is different from one sample to another. But there are situations in which *A. genevensis* is richer than *A. reptans*. Similar values have been registered for the wild flora samples, but there is no general pattern other than the fact the flavonoid fraction is usually found in higher amounts in *A. genevensis* extracts [14].

According to our standards we identified four iridoids (Table II), out of which harpagide and its acetylated derivate amounted to approx. 45% from the total iridoid content investigated earlier. On the other hand, low amounts of aucubin and catalpol were also determined.

Table II

Quantification and identification of the iridoids found in the analysed samples

Sample	Compound ($\mu\text{g/g dw}$)			
	Harpagide	Aucubin	Catalpol	8-O-acetyl-harpagoside
<i>Ajuga reptans</i>	203.899 ± 0.210	17.011 ± 0.150	11.211 ± 0.171	610.862 ± 0.112
<i>Ajuga genevensis</i>	178.61 ± 0.022	8.205 ± 0.130	10.431 ± 0.401	420.112 ± 0.220

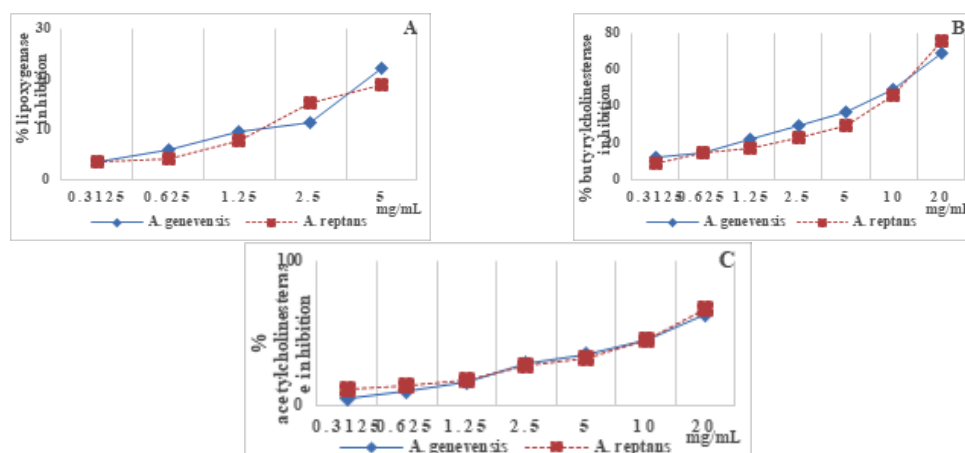


Figure 2.

The antioxidant activity of the investigated samples:

A) lipoxygenase inhibition; B) butyrylcholinesterase inhibition; C) acetylcholinesterase inhibition

It is notable the quantitative difference between *A. reptans* and *A. genevensis*. Although, *A. genevensis* extract contains flavonoids in higher amounts, iridoids are less in this sample. This is certified also by scientific data [3, 4, 14-16].

The enzyme inhibitory effects were moderate and varied within the same limits, depending on the assay and on the sample. The results are included in Figure 2 (A, B and C).

Analysing the results, we see that the inhibitory activity of both extracts follows a similar trendline. Nevertheless, the intensity of the effect varies mostly at lower concentrations (1.25 - 5 mg/mL).

15-Lipoxygenase (LOX) is a non-heme iron containing enzyme that catalyses the oxidation process of various polyunsaturated fatty acids. Its inhibition leads to cellular membrane protection and a decrease in oxidative parameters [7, 12, 17, 18]. Therefore, our results suggest that *Ajuga genevensis* extract is a better inhibitor against LOX than *Ajuga reptans*.

On the other hand, the activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is better for *Ajuga reptans* extract. Both cholinesterases are structurally related enzymes known to be involved in different physiological processes. While AChE is mainly implicated in cholinergic signalling, BChE is expressed through the body and less in the brain [2, 11]. Noteworthy is that the intensity of the antioxidant effect against lipid peroxidation (LOX – inhibition) is directly correlated with the concentration of total phenols and flavonoids, and less related to the quantity of iridoids. Similar results were obtained by other researchers, but once again the employed tests involved mainly free radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) [14, 15]. Therefore, a perfect comparison cannot be made between our results and the literature data.

Starting from the idea that surgery and injection of 6-OHDA in black matter, besides the neuronal apoptosis induces also an inflammation doubled by high oxidative stress, we considered that administering the two *Ajuga sp.* extracts could improve the cognitive deficits associated with the Parkinson's disease induced experimentally [1, 10]. Knowing that iridoids and polyphenols possess antiinflammatory and antioxidant effects, we chose the *Ajuga species* as the main focus of our study.

The obtained results during the testing confirmed that the *Ajuga sp.* extracts have a positive impact on the affected brain, diminishing the error rate. The Y-maze test used in this study quantifies short-term memory and reward search behaviour, relying on rodents' tendency to explore any new environment in which they are located. Moreover, it is notable that in the group treated with 6-OHDA (group 2) the locomotor activity decreased significantly ($p < 0.01$)

as compared to the false group operated (group 1). This indicates that 6-OHDA administration affects the nigrostriatal pathway of dopamine, but does not induce a severe debilitation of locomotor activity to prevent animals from moving through the labyrinth (Figure 3).

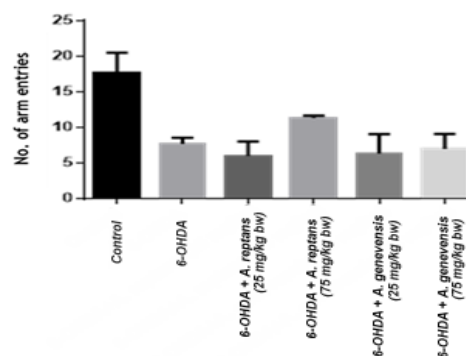


Figure 3.

Locomotor activity (assessed by the number of arm entries) for the groups treated with *Ajuga reptans* extract (25 mg/kg or 75 mg/kg) and *Ajuga genevensis* (25 mg/kg or 75 mg/kg)

In this situation, comparing the locomotor activity of all the animals enrolled in the experiment, we note that it was reduced due to injection of 6-OHDA into *substantia nigra*. Also, such a reduction is outlined in three of the *Ajuga*-treated groups except group 4, in which animals were treated with 75 mg/kg bw common bugle extract. This means that only larger doses of extract obtained from *A. reptans* can counteract the 6-OHDA-induced degenerative effect on the black matter.

Regarding the percentage of spontaneous alternation which evaluates short-term memory, the results showed that the group treated with 6-OHDA had significant decreases ($p < 0.0001$) from the control group (group 1). Administration of 6-OHDA resulted in marked decrease in short-term memory.

Group 3 treated 14 days later by intraperitoneal administration of *Ajuga reptans* extract at a dose of 25 mg/kg bw, showed less spontaneous alternation than control, but slightly better than group 2 - the untreated animals with induced Parkinson's disease (Figure 4). For the 75 mg/kg bw extract, the spontaneous alternation is almost as good as control, which proves that at this dose the active principles in the extract are very effective.

A similar potential is observed for the *A. genevensis* extract, but the intensity of the effect is lower than the dose of 75 mg/kg bw *A. reptans* extract. The obtained results suggest that the locomotor activity was apparently less influenced, whereas the percentage of improvement of the spontaneous alternation significantly increased.

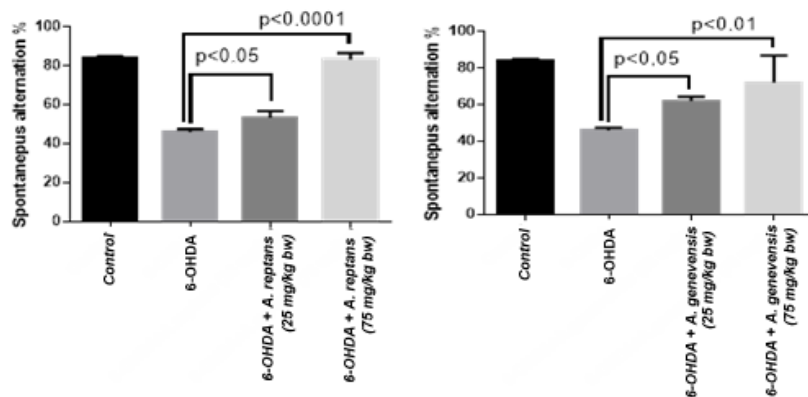


Figure 4.

Comparative view on spontaneous alternation for the treated and untreated animals with 6-OHDA-induced Parkinson's

Moreover, the working memory, which is a form of short-term memory, and the reference memory as a form of long-term memory, have been evaluated. It is well known that the working memory depends on training, while the reference memory remains unchanged during the experiments [1, 5]. Following daily the number of working memory errors for *Ajuga reptans* injected batches, we find that the both treated groups the start the experiment with a small number of errors

which are kept down till the end, but the better answer is given by the rats treated with a high dose of common bugle extract, even better than control (Figure 5). As in the case of the bugle extract, the administration of *Ajuga genevensis* extract in two different doses (group 5 = 25 mg/kg bw and group 6 = 75 mg/kg bw), the reference memory errors are lower than both witnesses (Figure 5), both control (Group 1) and untreated (Group 2).

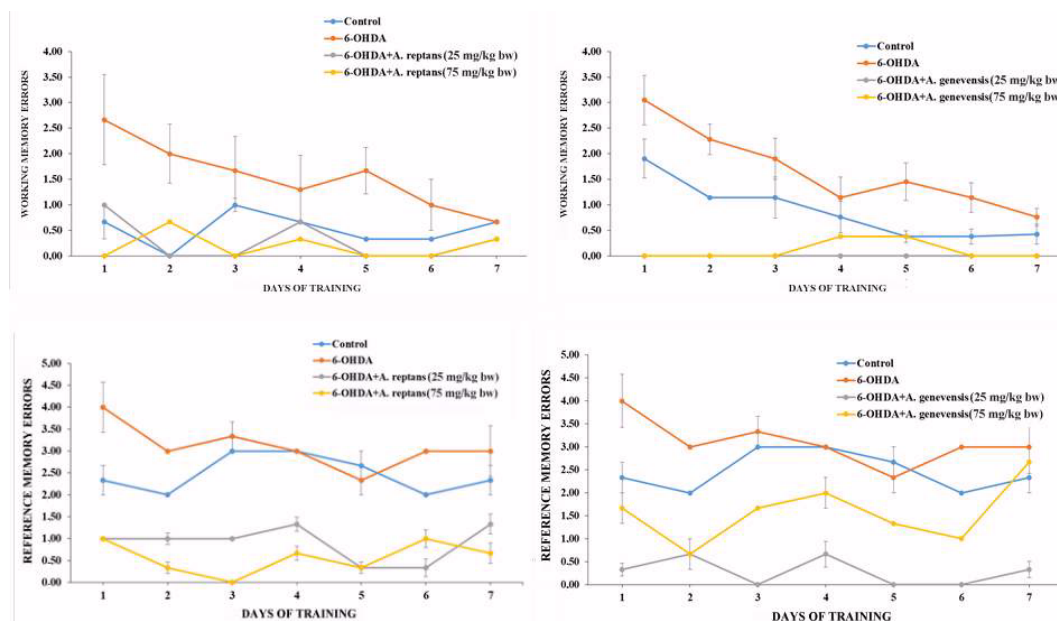


Figure 5.

Comparative view on working memory errors and reference errors for the investigated *Ajuga* samples

There is, however, a major difference in the mean of errors in the animals treated with blue bugle extract, namely: the low dose (25 mg/kg bw) significantly improves memory (lowering the error rate) compared to 75 mg/kg bw. We note that if for *Ajuga reptans* extract the memory quality improves with the increase in the administered dose, in the case of blue bugle this dose/action ratio is reversed, a lower dose (25 mg/kg

bw) being more effective than a high dose 75 mg/kg bw). Such results can only be explained by the composition of the phytocomplex, in the sense that it varies between the two *Ajuga* species due to the different ratio between bioactive substances (e.g. higher luteolin glycoside and harpagide derivatives concentrations in *A. reptans* extract). Further studies are necessary for a comprehensive view of this aspect

which is most probably related to the pharmacokinetic distribution of iridoid and flavonoid metabolites, especially those that could pass the brain-blood barrier.

The study suggests that the degenerative process induced by 6-OHDA injection in the experimental groups 3, 4, 5 and 6 has been partially offset by the treatment. If for *Ajuga reptans* extract the dose of 75 mg/kg bw was more effective, for blue bugle the lower dose of 25 mg/kg bw seems to be more active in terms of neuroprotection. Both working memory and reference memory improved under the treatment with plant extracts

Conclusions

The obtained results illustrate the differences between the two species considered in this study. The investigations regarding the quantitative analysis show that *Ajuga genevensis* and *Ajuga reptans* represent important sources for secondary metabolites with potential biologic activities. The *in vitro* antioxidant activity was moderate for both extracts, but depending on enzyme and test an important potential was shown by *A. genevensis*. Furthermore, all *in vivo* tests indicated that *A. reptans* significantly improves cognitive deficits induced by 6-OHDA, more than the extracts obtained from *A. genevensis*. All these correlates mostly with the higher content of iridoids which was quantified in *A. reptans* extract. Therefore, further research is undergoing to establish which of the compounds passes the blood-brain barrier and has the greatest impact on the cognitive behaviour of the animals. All in all, *Ajuga reptans* proves to be a promising source for new therapeutics for the prevention of neuronal degeneration.

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