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NEUROTROPHIC EFFECTS OF IRIDOID GLYCOSIDES FROM *LAMIUM ALBUM* L. AS NATURAL GLP1-R MODULATORS IN REPEATED RESTRAINT STRESS

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ABSTRACT. The pleiotropic effects of *Lamium* species are extensively utilized for treating urinary bladder injuries and infections and addressing blood hypertension or liver toxicities. Despite widespread *Lamium* consumption in animals and humans, its impact on brain biochemical parameters remains unexplored. In our study, we demonstrated the regenerative effect of *L. album* L. in a rat model of restraint stress, commonly employed to investigate neuropsychological stress. Following extract administration, there was a reduction in stress hormones (corticosterone, adrenaline), inflammation (TNFα), and oxidative stress. These neuroregenerative effects may be attributed to *Lamium*'s phytochemical composition, particularly its iridoids and luteolin content, which appear to mimic the action of glucagon-like peptide 1 (GLP-1). These findings suggest the potential use of *L. album* as a neuroregenerative adjuvant.

Keywords: *iridoids, Lamium album, GLP1*

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INTRODUCTION

Lamium species are utilized in traditional medicine for treating infections, hypertension, as well as uterine and vaginal bleeding. Extracts of these species also exhibit anti-hypoxic, anti-inflammatory, and antioxidant activities [1-3] owing to their biologically active compounds, including flavonoids, iridoids, phenolic acids, amino acids, carotenoids, sterol derivatives, trigonelline, ursolic, and oleanolic acids [4-6].

The primary compounds found in *L. album* herbs include verbascoside, isoverbascoside, and various derivatives of isoscutelareine. Minor compounds such as apigenin-7-O-glucoside, luteolin-7-O-glucoside, apigenin-7-O-rutoside, and naringenin-7-O-rutinoside have also been identified [3,7]. These plant extracts demonstrate antioxidant effects due to their phytochemicals. Furthermore, certain genera-specific compounds from the iridoids class exhibit a wide range of beneficial properties, including anticancer, antioxidant, antibacterial, antiviral, anti-inflammatory, antiarthritic, immunomodulatory, neuroprotective, and wound healing properties [8]. Among these, 6-shanzhiside methyl ester and 8-acetylshanzhiside methyl ester have been identified as the main effective iridoid glycosides (IGs) in the *Lamiaceae* group [9]. They have been shown to block TNF-α-induced nuclear factor-κB (NF-κB) and IkB-α phosphorylation while increasing Akt phosphorylation in hypoxic neuronal environments [10-12].

These features of *L. album* highlight its therapeutic potential in inflammatory and oxidative stress disorders such as toxic hepatitis, xenobiotic-induced kidney failure, and brain hypoxic impairments. Brain hypoxia is associated with neuropsychological stress, brain traumatic injuries, aging, or disturbances in oxygen delivery (e.g., anemia, hemoglobin dysfunctions, -SH depletion, hypoxic environments) [13-16]. Various experimental studies have utilized repeated restraint stress, which correlates with physical and psychological stress, to investigate brain hypoxia and its effects in rodents [17].

Restraint stress induces hypoxia and imbalance in the HPA axis (activation of the hippocampus-hypothalamus-adrenal axis (HHPA) stimulus), leading to increased neuronal damage and elevation of stress hormone concentrations, including corticotropin-releasing hormone, ACTH, adrenaline, noradrenaline, and glucocorticoids [17, 18, 19-29]. Instress conditions, the CA3 field of the hippocampus plays a specific role in coping mechanisms, as noticed in our previously published work [30], by developing a balancing function in the input-output relationship of stress signals according to large literature shreds of evidence [24, 25, 31-34]. Repeated restraint stress profoundly affects the nervous system endocrine glands, digestive system, and kidneys [23, 24, 35-38]. Both acute (3-7 days) and chronic HHPA stimulation (14-22 days) lead to increased glucocorticoid levels [32, 35, 38], which act synergistically with

excitatory amino acids to cause concentration-dependent hippocampal neuronal damage [37-41] during prolonged exposure to repeated restraint stress (> 5 days, 3 hours of stress per day).

Iridoids, as GLP-1R agonists [42], determine endogenous regulation of the HPA axis by their stimulatory action on GLP-1 receptors. This stimulatory effect of the shanzhiside-methyl esters is accompanied by decreases in corticosterone and adrenaline and structural improvement of the dentate gyrus by modulating granular and subgranular cell regeneration and distribution.

On the other hand, the same authors [42] mentioned that GLP-1 agonists enhanced stress-induced corticosterone release. These studies have shown that recruitment of central GLP-1 receptors potently activates the HPA axis in both humans and rodents, resulting in increased ACTH and corticosterone concentrations in the blood. Importantly, central administration of exendin-4, a GLP-1 agonist, leads to an increase in corticosterone concentration in rodents, suggesting the involvement of central GLP-1 receptors possibly expressed on CRH-expressing neurons in the hypothalamus. Central blockade of CRH receptors blocks exendin-4-induced increases in ACTH and corticosterone, establishing a role for central GLP-1 receptors in HPA axis regulation. For example, in a study focusing on the role of GLP-1 in cocaine addiction, GLP-1 neurons were found to be activated by an injection of corticosterone into the fourth ventricle. Fourth ventricle corticosterone administration reduced cocaine self-administration, and this reduction was blocked by GLP-1 receptor antagonists in the ventral tegmental area. These data suggest that not only does central GLP-1 activate the HPA axis, but corticosterone in turn activates the central GLP-1 system.

Our study aimed to investigate whether shanzhiside derivatives modulate the activity and plasticity of the hippocampus in response to repeated restraint stress and induce cellular regeneration associated with reducing oxidative stress and corticosterone concentration. This research hypothesis was based on the anti-hypoxic, anti-inflammatory, and GLP-1R regulatory properties of iridoids found in *L. album*, such as 6-shanzhiside methyl ester and 8-acetyl shanzhiside methyl ester.

RESULTS AND DISCUSSION

Phytochemistry of the *L. album* extract

The content of phytochemical compounds, namely iridoids, flavonoids, and phenolics in the *L. album* extract, was determined using spectrophotometric assays. The results showed that phenolics are present in the highest amount

(1.082 ± 0.055 mg/mL gallic acid equivalents), followed by iridoids (158.84 ± 1.92 µg/mL aucubin equivalents), with flavonoids being present in the smallest amount (70.13 ± 0.02 µg/mL rutin equivalents). The *in vitro* antioxidant capacity of the *L. album* extract was determined using two widely used assays: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid radical cation (ABTS•+) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•). The antioxidant capacity determined using the DPPH assay (2.40 ± 0.14 µmol/mL Trolox equivalents) was higher than that determined using the ABTS method (0.84 ± 0.01 µmol/mL Trolox equivalents). The High-Performance Thin-Layer Chromatography (HPTLC) method was optimized and employed for the screening of phytochemical compounds, particularly iridoids, which are of particular interest in the extract. HPTLC screening of the *L. album* extract confirmed the presence of iridoids through derivatization with a specific reagent (p-dimethylamine benzaldehyde), as well as certain specific phenolic compounds (**Figure 1A-B**). The presence of iridoids is confirmed by the blue spots on a cream/beige background.

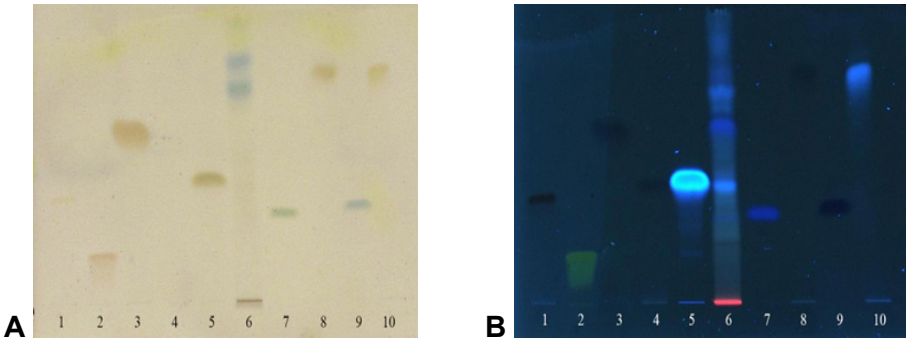


Figure 1. HPTLC separation on RP-18 F254s with acetonitrile – 0.1% formic acid (5:5, v/v) as the mobile phase. (**A**) Visible light after derivatization with p-dimethylaminobenzaldehyde. (**B**) UV 366 nm. Tracks: 1 – rutin; 2 – quercetin; 3 – procatechuic; 4 – vanillic acid; 5 – caffeic acid; 6 – *L. album* exact; 7 – ferulic acid; 8 – gallic acid; 9 – p-coumaric acid; 10 – chlorogenic acid.

Biochemical analyses

Brain biochemical spectrum reactions (**Figure 2A - F**) demonstrated a particular biochemical behavior in the hippocampus under stress conditions. Cholesterol concentration in the C group was 4.34 ± 0.19 mg/g, and stress exposure exhibited a significant increase (17.78 ± 0.33 mg/g, $p < 0.001$) in hippocampal cholesterol levels, while the administration of *L. album* significantly decreased the cholesterol concentration (10.5 ± 0.28 mg/g, $p < 0.001$, **Figure 2A**).

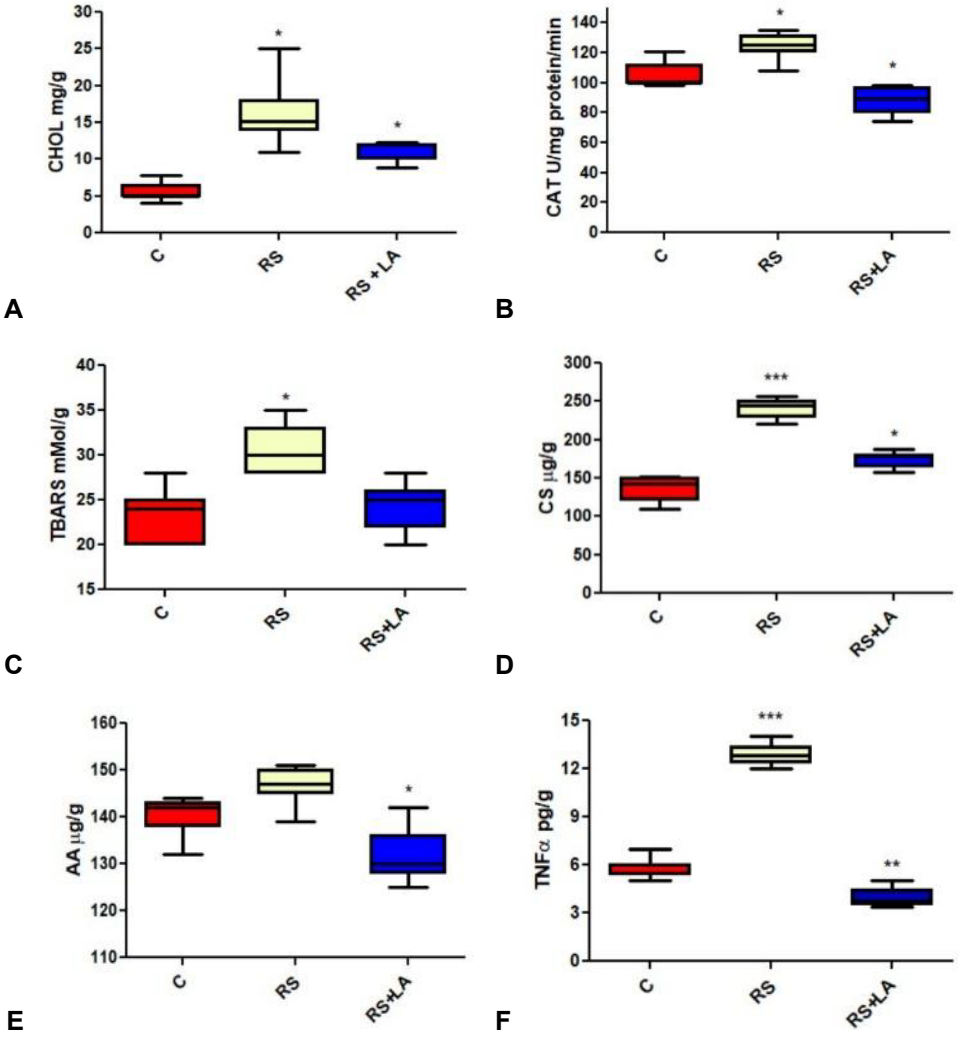


Figure 2. Biochemical markers in brain homogenates. An increase in cholesterol (**A**) was noted to be correlated with reactions in the oxidative microenvironment, based on levels of catalase (**B**) and TBARS (**C**). Administration of *L. album* resulted in a decrease or normalization of oxidative stress indicators ($p < 0.05$). Stress-related hormones, corticosterone (**D**) and adrenaline (**E**), increased the RS group. At the same time, the extract significantly reduced these hormone levels ($p < 0.05$), similar to the variations in TNFα (**F**) levels in hippocampal homogenates. The values are expressed as mean in the Q1-Q3 quartile range and the error bars show the non-outlier range.

Repeated restraint stress determined a prominent increase in oxidative stress markers in the hippocampus. In stressed animals, CAT (**Figure 2B**) was 115.8 ± 2.3 U/mL ($p < 0.01$), and TBARS (**Figure 2C**) was 33.0 ± 0.6 nMol/g ($p < 0.01$), suggesting the activation of the HPA axis associated with redox imbalance. Additionally, such an imbalance generates ROS/NOS, commonly related to oxidative stress-associated pathologies (e.g., hypertension, diabetes, mental disorders). Treatment with *L. album* extract significantly counteracted the oxidative stress level compared with stressed animals (CAT, 81.1 ± 17.9 U/mg of protein/min, and $p < 0.05$; TBARS, 24.9 ± 0.8 nMol/g, $p < 0.05$). However, the increased oxidative stress markers are not sufficient to confirm the activation of the HPA axis; therefore, a strong link with hormones (corticosterone, testosterone, adrenaline, renin, etc.) and cytokines (IL-8, IL-9, IL-12, TNFα) signaling is mandatory. Stressed rats expressed a high level of corticosterone (263 ± 3.64 µg/g, **Figure 2D**) and adrenaline (162 ± 2.41 µg/g, **Figure 2E**) in the hippocampus as well as in the blood, where CS was 164.5 ± 3.63 µg/dL (Figure 3A) and AA was 55 ± 0.96 µg/mL (**Figure 3B**). Consequently, stressed rats that expressed a high hippocampal level of CS (Figure 2D) marked a decreased corticosterone concentration after *L. album* administration (RS + LA, 168 ± 1.75 µg/dL, $p < 0.001$, Figure 2D). In addition, blood CS was slightly decreased (**Figure 2A**) in RS + LA (112.4 ± 2.63 µg/dL), which approaches the C value (152.3 ± 3.11 µg/dL, $p < 0.001$).

Alongside, repeated restraint stress determined an increase in TNFα concentration in the hippocampus (**Figure 2F**) (12.28 pg/mg protein, $p < 0.01$) after stress exposure versus the C group (6.26 pg/mg protein), and *L. album* administration significantly decreased hippocampal TNFα concentration (4.25 pg/mg protein, $p < 0.001$, **Figure 2F**). In addition, hippocampal adrenaline (**Figure 2E**) follows the same dynamics as corticosterone variations. During stress conditions, AA plays a key role in stress habituation [31], the reason why slight increases were noticed in stressed groups (171 ± 11.2 µg/g, $p > 0.05$). Nevertheless, the hippocampal AA concentration in group C (161.6 ± 1.6 µg/g) closely resembled that of the RS group, underscoring the absence of a connection between long-term exposure to restraint stress and stimulation of the sympathetic nervous system. In this context, the actions of an *L. album*-antioxidant-rich diet on stressed animals seemed to lead to high autonomic nervous system resistance reflected by a low concentration of hippocampal AA (121.4 ± 16.5, µg/g, $p < 0.05$) compared to C and RS groups. Concerning the blood biochemistry, the cholesterol concentrations (**Figure 3C**), and oxidative stress parameters (CAT, TBARS - **Figure 3D** and **Figure 3E** respectively) in the Control and experimental groups suggest that restraint stress generates hypercholesterolemia and blood redox imbalance. Our study points out that the serum level of cholesterol (**Figure 3C**) is significantly increased (190.7 ± 0.7 mg/dL, $p < 0.001$) in the RS group, which was a specific alteration during acute restraint stress conditions.

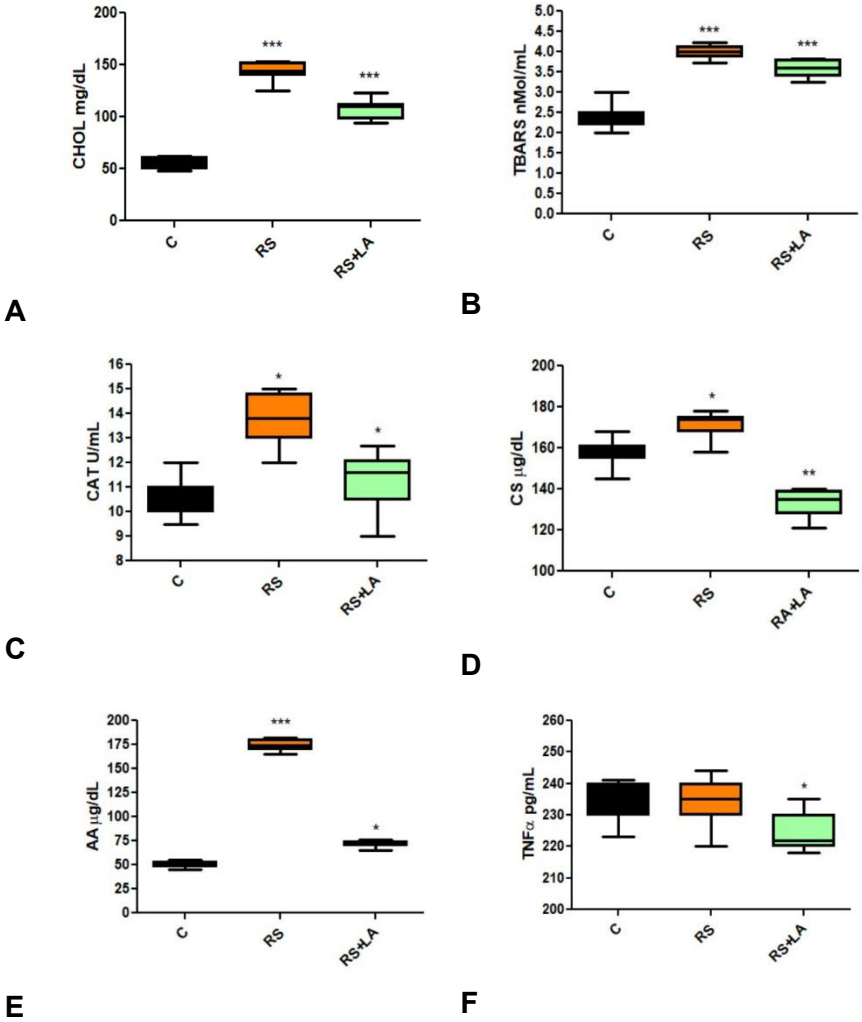


Figure 3. Serum biochemical markers variations. An increase in serum concentrations of cholesterol (**A**) was noted, along with changes in oxidative status indicated by TBARS (**B**) and catalase (**C**) levels. Plant extract administration led to a slight decrease in catalase whereas cholesterol and TBARS were unchanged. The stress effects were more pronounced in the blood compared to the hippocampus. Following extract treatment, corticosterone (**D**) and adrenaline (**E**) levels decreased significantly ($p < 0.05$), as did TNFα (**F**) levels ($p < 0.05$). The values are expressed as mean in the Q1-Q3 quartile range and the error bars show the non-outlier range.

On the other hand, *L. album* extract protects the metabolism by an emphatic decrease in cholesterol concentration, as seen in the RS + LA group (67.7 ± 8.6 mg/dL, $p < 0.001$, **Figure 3C**). In the context of restraint stress-induced redox unbalancing, the increased TBARS level is closely related to the high CAT activity during repeated stress conditions, which marks an elevated oxidative status in the blood. Blood oxidative stress in the RS + LA group did not present remarkable changes. Therefore, in the RS + LA group, CAT activity (12.8 ± 1.23 U/mL) and TBARS concentration (3.4 ± 0.16 nMol/mL) were decreased compared to the RS group which had CAT: 15.0 ± 1.56 U/mL and TBARS: 4.0 ± 0.18 nMol/mL ($p < 0.05$ for CAT and $p > 0.05$ for TBARS) but remained elevated compared with the Control group.

Molecular Docking of GLP1-Like Iridoids with GLP1 Receptor

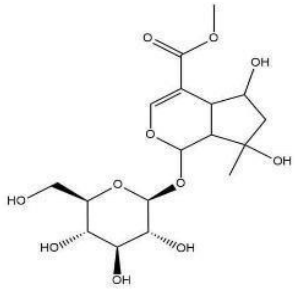
The interactions between the 6-O-acetyl shanzhiside methyl ester and 8-O-acetyl shanzhiside methyl ester found in *L. album* and their GLP1 mimetic actions were evaluated through molecular docking tests using the HDOCK server using exendin-4, a GLP1-R agonist with 39 amino acids and an incretin analog(PDB doi: https://doi.org/10.2210/pdb1JRJ/pdb) as positive control andGLP-1R Antagonist 1 (7-(4-chlorophenyl)-1,3-dimethyl-5,5-bis(trifluoromethyl)-8H-pyrimido[4,5-d]-pyrimidine-2,4-dione) as negative control molecules (HDOCK server, Huang Laboratory, School of Physics, Huazhong University of Science and Technology, 1037 Luoyu Rd, Wuhan, Hubei 430074, P.R. China).

Given that the protein-protein/RNA/DNA complexes in the Protein Data Bank typically have a docking score of around -200 or better, the docking was empirically related to a docking confidence score to indicate the likelihood of binding between two molecules as follows:

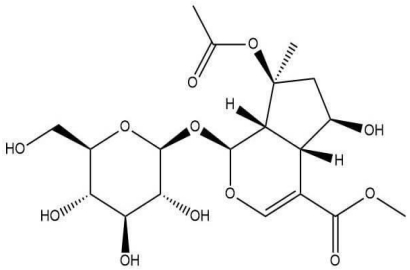
$$\text{Confidence score} = 1.0/\ [1.0+e^{0.02x\ (\text{Docking\_Score}+150)}]$$

Roughly, when the confidence score was above 0.7, the two molecules would be very likely to bind; when the confidence score was between 0.5 and 0.7, the two molecules would be possibly binding, anda confidence score below 0.5, indicated that the molecules would be unlikely to bind. Nevertheless, the confidence score here should be used carefully due to its empirical nature.

The docking scores, as indicated by the confidence score, show that 8-ASME have a confidence score slightly above 0.7, suggesting that 8-ASME can bind to GLP1-R more probable than 6-ASME with a potentiation effect as was described by GLP1-R-ligands geometry. Exendin-4 and GLP1-R antagonist 1 were in opposite docking values which confirm their agonistic and antagonistic behavior regarding GLP1-R.

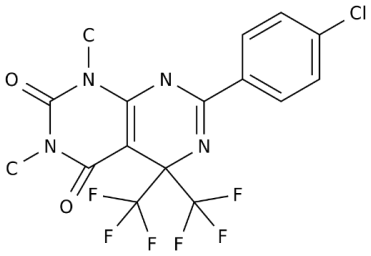


(a)6-O-acetyl shanzhiside methyl ester



(b)8-O-acetyl shanzhiside methyl ester

(c) Exendin-4



d)GLP-1R Antagonist 1 (7-(4-chlorophenyl)-1,3-dimethyl-5,5-bis(trifluoromethyl)-8H-pyrimido[4,5-d]-pyrimidine-2,4-dione)

Figure 4. Perspective formulas of (**a**) 6-O-acetyl shanzhiside methyl ester and (**b**) 8-O-acetyl shanzhiside methyl ester found in *Lamium* species. The structures depicted in (**c**) Exendin-4 and (**d**) GLI-1R antagonists represent positive and negative docking controls.

Table 1. Docking scores of the two interaction models between GLP1-R and iridoid glycosides 6-ASME (6-O-acetyl shanzhiside methyl ester), 8-ASME (8-O-acetyl shanzhiside methyl ester), GLP1-R agonist exendin-4 and antagonist molecule as GLP1-R antagonist 1.

Molecular interactions		Model 1	Model 2
GLP1-R + Exendin-4	DK Score	-270.18	-260.71
	Conf. Score	0.91	0.90
GLP1-R + 6-ASME	DK Score	-186.89	-176.29
	Conf. Score	0.67	0.62
GLP1-R + 8-ASME	DK Score	-214.78	-212.38
	Conf. Score	0.78	0.77
GLP1-R+GLP-1R Antagonist 1	DK Score	-186.89	-176.29
	Conf. Score	0.67	0.62

The interaction modeling was simulated between ligands: exendin-4 as standard GLP1-R agonist, 6-O-acetyl shanzhiside methyl ester, 8-O-acetyl shanzhiside methyl ester, and the GLP1 receptor (GLP1-R) as was depicted in **Figure 5**.

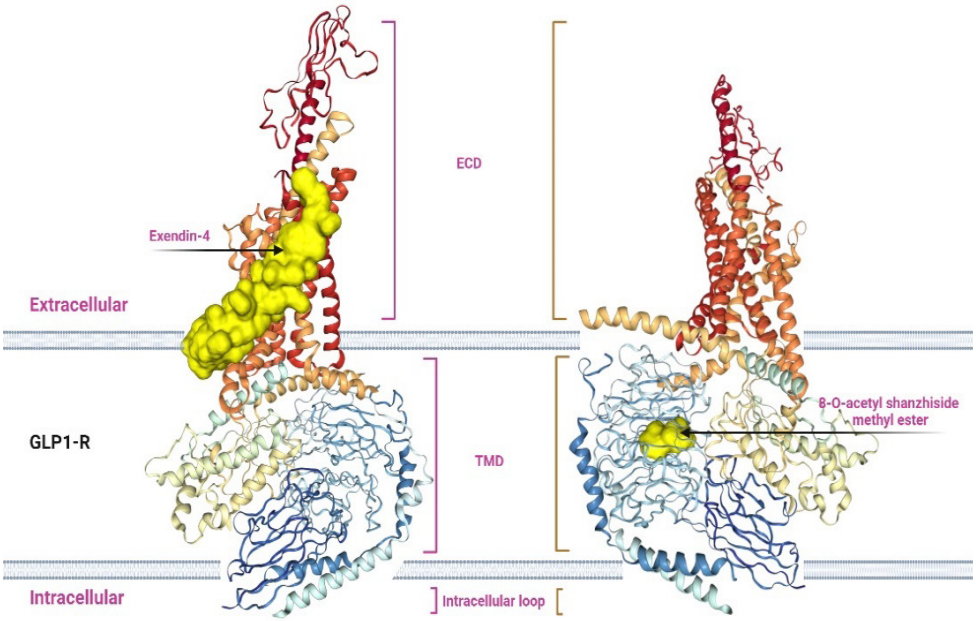


Figure 5. GLP1-R geometry and interaction models with exendin-4 and 8-O-acetyl shanzhiside methyl ester (8-ASME). The tested compound was selected based on the docking and the confidence scores given by HDOCK simulations. Compared to 6-ASME, 8-ASME was described as the compound with the highest probability of docking with GLP1-R.

Figure 5 depicts new aspects of 8-ASME, a representative iridoid glycoside from *Lamium*, and its interaction with the receptor's binding pocket compared to exendin-4. The agonist binds to the ECD of the receptor, while 8-ASME binds to the TMD, based on steric constraints and probably its membrane solubility. If the receptor's binding pocket is not occupied by 8-ASME, these findings suggest that the receptor can be stimulated by exendin-4. Based on these interactions, the iridoid glycoside 8-ASME acts as a GLP1-R modulator in the presence of the agonist, and iridoid glycosides can function as potent receptor molecules, not only as agonists. Further studies with multimolecular systems will elucidate these assumptions. However, our experimental data strongly support these processes. An interesting interaction

between GLP1-R and GLP1-R antagonist 1 was noticed after HDOCK simulation. The antagonist also binds to the TMD like 8-ASME, but the docking score was very low, as noted in **Table 1**. The docking scores (DK) were calculated using the knowledge-based iterative scoring functions IT Score PP or IT Score PR. A more negative docking score indicates a more probable binding model. However, the score should not be regarded as the true binding affinity of the two molecules since it has not been calibrated to experimental data (**Table 1**).

Following previous studies [3-5, 7], we also report the complex composition of the *L. album* extract. Phytochemical analysis confirmed the presence of polyphenols, iridoids, and flavonoids, which justify the observed biological effects of this plant's extracts on various diseases. The composition of the extract and the ratio of its bioactive compounds determines its biological particularities. Jiang et al. [10] found that 8-ASME increased the expression of VEGF, Ang 1, and the phosphorylation of Tie2 and Akt in an *in vitro* model. They also noted a decrease in TNFα-induced nuclear factor κβ (NF-κβ) and IκB-α phosphorylation due to 8-O-acetyl shanzhiside methyl ester, confirming our experimental data. Furthermore, in 2010, the same author [11] demonstrated that 6-ASME protected the brain against injury in a rat model of ischemia and reperfusion.

Phytochemical analysis revealed that iridoids are among the major bioactive compounds in *L. album*. The iridoids are characteristic chemical features of the *Lamiaceae* family compared to other plant families [1, 3, 12]. These bioactive compounds provide a brain-protective effect in the context of experimental brain hypoxia or inflammatory events induced by restraint stress [13]. Moreover, hippocampal cellular changes are a response to hypoxia as well as corticosteroid signaling, which negatively affect neuronal function according to McEwen [33], marked by lower connectivity, axonal edema, IL-1 upregulation, and behavioral changes. Previously published studies indicate a tandem stress reaction of these structures related to corticosterone signaling as well as excitatory mediation [32, 34, 37]. Consistent with our results, increasing concentrations of corticosterone induced by restraint stress are linked to ROS generation [43]. Additionally, Richard-Jane et al. [44] reported that chronic stress, but not acute stress, significantly increased plasma cholesterol concentration. We have noticed that the serum level of cholesterol significantly increased, and this elevation is associated with acute restraint stress. These data demonstrate that both acute and chronic restraint stress induces high cholesterol concentration in the blood and brain. Oxidative stress, as a side effect of restraining, is known to cause inactivation of membrane receptors, impairments of membrane permeability, and disruption of membrane structure [45]. As shown by our results, the increased TBARS levels in the blood and hippocampus are closely related to high CAT activity, reflecting pressure on the antioxidant defense system according to Novozhilov et al. (2013) [46].

In search of a relationship between corticosterone via ROS/NOS, cytokine generation [34], and the neurotrophic actions of *Lamium* iridoids, we found that GLP-1 signaling impairments act as a mediator between the detrimental effects of restraint stress and the neurotrophic actions of 6- and 8-ASME [46]. GLP-1, somatostatin, substance P, neuropeptide Y, and enkephalin are molecules that modulate the HHPA axis in stress response areas such as the hypothalamic nuclei, the pituitary gland, hippocampal formation, and the frontal cortex [45]. GLP-1+ neurons project to multiple brain areas, including regions critical for the regulation of the HHPA axis stress response. Furthermore, GLP-1R is widely distributed throughout the CNS, with GLP-1R mRNA expression observed in the olfactory bulb, the basal portion of the frontal cortex, the nucleus accumbens, the amygdala, and the dorsal portion of the hippocampus [43].

We hypothesize that the administration of *L. album* in animals exposed to restraint stress contributes to hippocampal regeneration due to the effect of shanzhiside-methyl esters, which are recognized as natural agonists for GLP-1R. Our data suggested that shanzhiside-methyl esters serve as agonists according to the literature [12], or based on our new findings, these compounds can be agonist-like or GLP-1R potentiator molecules for glucagon-like peptide 1 (GLP-1) receptors. Other results indicated that shanzhiside-methyl esters inhibit the release of TNFα, NFκβ, Iκβ, Ang1, and VEGF, and microglial β-endorphin, while also acting as agonistic molecules on GLP-1R [47,48].

Restraint stress disrupts the HPA axis, cholesterol metabolism, adrenaline levels, and oxidative stress parameters. However, administration of *L. album* hydroalcoholic extract normalizes and improves the biochemical steady-state in the blood and hippocampus. The presence of other antioxidants, such as polyphenols and flavonoids, contributes to the pleiotropic effects of the *L. album* extract, marked by decreased lipid peroxidation, reduced catalase activity, and lower cholesterol concentration. Iridoids, acting as GLP-1R agonists or GLP-1R agonist analogs, can regulate the HPA axis endogenously through their stimulatory action on GLP-1 receptors. Previously published research [49] demonstrated that HPA axis activation and variations in corticosterone levels are time-dependent processes. Our data demonstrate that GLP-1R agonists/agonist analogs from *L. album* reduce HPA axis activation and corticosterone concentration after five days of repeated stress exposure.

Some studies have mentioned that GLP-1 agonist (exenatide-4) injection decreased corticosterone concentration after two weeks of exposure. Exenatide-4 at 10 μg/kg and liraglutide at 1200 μg/kg were effective [50-52]. Our doses of 6-shanzhiside methyl ester were 1807 μg/kg and 8-acetyl shanzhiside methyl ester were 2226 μg/kg. Daily oral administration of these doses also exerted an inhibitory effect on corticosterone release after five days of repeated exposure to restraint stress, demonstrating a possible plant-based modulated response of GLP-1R in restraint stress.

CONCLUSIONS

The results of the current study suggest that bioactive compounds from *L. album* have protective and regenerative actions on the hippocampus exposed to repeated restraint stress. This is achieved by modulating the oxidative-inflammatory status and GLP1-R stimulation to regulate the stress response via HPA axis modulation through the GLP1 agonist or more probably, GLP1-R potentiator effects of *L. album* iridoids. These actions could serve as a link between stress effects on the nervous system and digestive function, which are often impaired by various stressors. Given the obtained results, this encourages further studies in this direction, specifically regarding the role of iridoids such as 8-acetylshanzhiside methyl ester in neuroprotection and brain-digestion relationship improvement.

EXPERIMENTAL SECTION

Plant material and extract preparation

A hydro-alcoholic extract of *L. album* leaves and flowers was prepared. *L. album* was collected from the Brasov depression area, Romania, in June 2021. The extract was prepared using a 70% v/v ethanol solution. The plants were dried at room temperature (+22°C) and ground into a powder. 2.5 g of *L. album* powder was mixed with 100 mL of 70% v/v ethanol in a water bath at +80°C for 90 minutes, followed by filtration. This resulted in the creation of the stock extract.

Phytochemical analysis

Phytochemical characterization of the *Lamium album* extract involved determining the levels of iridoid, flavonoid, and phenolic compounds and assessing its antioxidant capacity. The total iridoid content (TIC) was determined using the Trim-Hill reaction. To achieve this, 0.4 mL of the extract was mixed with 4 mL of Trim-Hill reagent (consisting of acetic acid, 0.2% $CuSO_4$, and concentrated HCl in a ratio of 10:1:0.5). Subsequently, absorbance was measured at 609 nm, with the presence of iridoids indicated by blue color. The iridoid amount was quantified using a calibration curve with aucubin (ranging from 0.1 to 1 mg/mL). Results were averaged from three replicates [42]. Total flavonoid content (TFC) was estimated using the aluminum chloride reagent. Initially, 1.2 mL of the extract was mixed with 0.6 mL of $NaNO_2$ (5%), followed by adding 1.2 mL of $AlCl_3$ (10%) after 5 minutes. Subsequently, 2 mL of NaOH

0.1 M was added, and absorbance was measured at 430 nm after another 10 minutes. A calibration curve with a standard solution of rutin (ranging from 10 to 125 µg/mL) was used, and results were expressed in µg of rutin/mL of *Lamium album* extract [53]. The total phenolic content (TPC) was determined using the Folin-Ciocâlteu method. Initially, 0.3 mL of the extract was mixed with 1.5 mL of Folin-Ciocalteu reagent, followed by adding 1.2 mL of sodium carbonate 0.7 M after 5 minutes. The mixture was then incubated at room temperature in a dark place for 2 hours, and absorbance was measured at 760 nm. Results were expressed in µg of gallic acid/mL of *L. album* extract based on a calibration curve obtained using a standard solution of gallic acid (ranging from 10 to 250 µg/mL) [53].

The antioxidant capacity of the extract was evaluated using the DPPH and ABTS assays [53,54]. Chromatographic analysis was performed on RP-18 F254s HPTLC plates developed with acetonitrile – 0.1% formic acid (in a ratio of 5:5, v/v) as the mobile phase. Aliquots of 10 µl of the plant extract and 5 µl of the standard solutions were applied to the plate as 8 mm bands using a Linomat 5 device (Camag, Muttenz, Switzerland). Polyphenols and flavonoids were visualized under UV light at 366 nm, while iridoids were detected as blue/red-violet spots after derivatization with p-dimethylamine benzaldehyde.

Chemicals for biological assays

Malondialdehyde standard solution and thiobarbituric acid were obtained from Sigma Chemical Company, St. Louis, MO, USA. For the determination of plasma catalase activity, a Catalase Assay Kit from Abcam, Cambridge, UK, was employed. Adrenaline, corticosterone, and TNFα were assayed using ELISA kits provided by Bio Vision Inc., Milpitas, CA 95035, USA. The Folin-Ciocalteu reagent was obtained from Merck Millipore, Darmstadt, Germany. A standard for the adrenaline was obtained from Terapia-Ranbaxy, Cluj-Napoca, Romania. For cholesterol determinations, the reagent was purchased from Bio Maxima, Lublin, Poland. All other chemicals and solvents used in the study were of analytical grade and were obtained from Chemical-Company, Iasi, Romania.

Animals and ethics

The experiment was conducted on adult female Wistar rats (8 weeks old), weighing 150 ± 20 g. The rats were procured and housed in the zoobase of the University of Veterinary Medicine in Cluj-Napoca, Romania, under standard conditions (12/12 light-dark cycle, humidity 30-70%, temperature 20-26°C), and provided with standard food and water *ad libitum*. All experimental procedures

described in this study adhered to the Directive 2010/63/EUand Romanian National Legislation (Law no. 43/2014). The experiments were approved by the Scientific Council and Ethical Committee of Babes-Bolyai University (Ethical Statement no. 2012/03.02.2022).

Experimental design

The animals were randomly divided into three experimental groups, each consisting of 6 animals: (I) negative Control group (C), (II) Restraint Stress group (RS), exposed to 3 hours of restraint per day for 5 consecutive days, and (III) Restraint Stress + *L. album* extract group (RS + LA). The experiment lasted for five days. The extract was administered before immobilization via intragastric gavage, in a dose of 100 mg of dry substance/kg of body weight daily, which was equivalent with approximately 1800 µg/kg 6-shanzhiside methyl ester and 2200 µg/kg of 8-acetyl shanzhiside methyl ester according to literature which described the ratio of the different iridoids in *Lamium*, following OECD guidelines for volume and dosage selection [43, 55-57]. Restraint stress was induced by immobilizing the rats in 20 cm × 7 cm plastic tubes for 3 hours per day for 5 days, with several 3 mm holes at the far end of the tubes to allow for breathing while restricting movement. Control and experimental animals were euthanized under isoflurane anesthesia one day after treatment and stress immobilization (on the 6^{th} day). The experiment was designed to assess the regenerative effect of iridoids, specifically 6-shanzhiside methyl ester and 8-acetylshanzhiside methyl ester, derived from the *L. album.*

Brain and blood preparations for biochemical assays

Immediately after euthanasia, the hippocampal formation was extracted and homogenized with a lysis buffer (PBS 100 mM, Triton X 0.5%, and protease inhibitor cocktail from Sigma-Aldrich, USA). The homogenate was then centrifuged at 10000 x g, +4°C, for 15 minutes, and the resulting supernatant was utilized for biochemical assays. Blood was collected in a plain vacutainer for biochemical analyses and allowed to clot for 30 minutes at room temperature. The serum was separated by centrifugation at 1200 x g for 10 minutes and subsequently stored at -80°C. The serum was utilized for determining catalase activity (CAT), thiobarbituric acid reactive substances (TBARS), cholesterol, corticosterone (CS), and adrenaline (AA) concentrations. Corticosterone and adrenaline levels in the blood were measured using ELISA methods (Bio Vision, CA), along with CAT activity (Abbexa, UK). ELISA plates were analyzed with a Biotek Synergy microplate reader (Biotek Instruments Inc., Winooski, VT, USA), following the manufacturer's instructions.

Molecular docking

The molecular docking was performed using the HDOCK online software. The main steps of the analysis involved preparing the structures, docking, scoring, clustering, ranking, and refinement of the results. Preparation: The structures of GLP1-R involved in the complex were prepared to ensure that the protein is in the correct format and contains all necessary information. Docking: HDOCK employs a hybrid docking algorithm that combines global docking with local refinement. Initially, a large number of initial docked poses are generated using a global docking approach. This involves sampling a wide range of possible orientations and conformations for the proteins in the complex. Scoring: Each generated pose is then scored based on various criteria, including shape complementarity, electrostatics, and desolvation energy between iridoid glycosides and GLP1-R. Clustering: The generated poses are clustered based on their structural similarity. Clustering identifies distinct groups of poses that represent similar binding modes. Ranking: The clustered poses are ranked based on their scores, with the top-ranking poses considered the most likely representations of the protein complex. Refinement: Finally, the top-ranking poses undergo local refinement to improve their accuracy and resolution further. This refinement step typically involves optimizing the side-chain conformations and fine-tuning the intermolecular interactions within the GLP1-R-iridoid glycosides complex.

Statistical analysis

All data are expressed as mean in Q1-Q3 quartile range and the error bars show the non-outlier range. The Gaussian distribution was assessed using the Shapiro-Wilk normality test. One-way analysis of variance (ANOVA) followed by post hoc Dunnett's range test procedures was conducted. Statistical significance was set at $p < 0.05$ (95% confidence interval). Statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, Boston, MA 02110, USA).

Abbreviations: AA, adrenaline; Akt, protein kinase B; Ang1, angiopoietin 1; CA, Cornus ammonis; CAT, catalase; CS, corticosterone; DG, dentate gyrus; GLP-1, glucagon-like peptide 1; GLP-1R, glucagon-like peptide 1 receptor; HHPA axis, hippocampal-hypothalamic-pituitary-adrenal axis; H&E, hematoxylin-eosin staining; HPLC, high-performance liquid chromatography; IκB-α, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha; *L. album*, LA; NF-κβ, nuclear factor κβ; PBS, phosphate buffer saline; RNS, reactive nitrogen species; ROS, reactive oxygen species; RS, restraint stress; MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances; Tie2,

tyrosine kinase receptor for angiopoietin 1; TLC, thin layer chromatography; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

Ethics Approval: All experimental procedures outlined in this study were conducted following the European Communities Council Directive 2010/63/EU and Romanian National Legislation (Law no. 43/2014). The experiments received approval from the Scientific Council and Ethical Committee of Babeș-Bolyai University (Ethical Statement no. 2012/03.02.2016).

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