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Neurocognitive and behavioural effects of sage-leaved rock-rose *Cistus salviifolius* L. extract in experimental non-alcoholic steatohepatitis*

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Summary

Non-alcoholic steatohepatitis (NASH) is the leading cause of chronic liver disease in the modern world. Besides metabolic and cardiovascular comorbidities, NASH is associated with increased prevalence of neurocognitive and psychiatric disorders. Sage-leaved rock-rose *Cistus salviifolius* L. (CS) is a plant with a wide spectrum of biological activities including possible psychotropic and procognitive effects. In view of the above, the present study was aimed at exploring the potential therapeutic effects of a CS aqueous extract in behavioural and memory dysfunction associated with experimental murine alimentary/toxic NASH. 90 male C57Bl/6 mice were randomized into the following groups: (1) Control: NASH + no treatment; (2) CS253: NASH + 253 mg·kg-1 b.w. CS ex-tract; (3) CS506: NASH + 506 mg·kg-1 b.w. CS extract. NASH was induced over 3 months, and the drugs were administered orally q.d. during the experimental period. As assessed by the Open field, Elevated plus maze, and Light/dark box test, both doses of the CS extract induced sedation with possible anxiogenic effect in mice. In addition, the CS extract alleviated spatial memory dysfunction but had no effect on object recognition memory. Possible mechanisms behind the extract's effects include the potentiation of calcium-dependent neuronal signaling as well as the modulation of central γ-aminobutyric acid, acetylcholine, or monoamine neurotransmission, which requires further elucidation.

Keywords: *Cistus salviifolius* L., sedative agents, memory deficits, cognitive dysfunction, anxiety, non-alcoholic steatohepatitis, behavioural testing, mice

Conflict of interests. The authors declare no conflict of interest.

* Illustration 2 to the article is on the colored inset of the Journal (p. II).

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Нейрокогнитивные и поведенческие эффекты экстракта ладанника шалфеелистного *Cistus salviifolius* L. при экспериментальном неалкогольном стеатогепатите*

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Резюме

* Иллюстрация 2 к статье – на цветной вклейке в журнал (стр. II).

Неалкогольный стеатогепатит (НАСГ) является ведущей причиной хронических заболеваний печени в современном мире. Помимо сопутствующих метаболических и сердечно-сосудистых заболеваний, НАСГ связан с увеличением распространенности нейрокогнитивных и психических расстройств. Ладанник шалфеелистный *Cistus salviifolius* L. (CS) — растение с широким спектром биологической активности, включая возможные психотропные и прокогнитивные эффекты.

Учитывая вышеизложенное, настоящее исследование было направлено на изучение потенциальных терапевтических эффектов водного экстракта CS при поведенческих дисфункциях и нарушениях памяти, связанных с экспериментальным алиментарным/токсическим НАСГ у мышей. 90 мышей-самцов C57Bl/6 были рандомизированы на следующие группы: (1) Контроль: НАСГ + отсутствие лечения; (2) CS253: НАСГ + 253 мг·кг-1 м.т. экстракт CS; (3) CS506: НАСГ + 506 мг·кг-1 м.т. Экстракт КС. НАСГ вызывали в течение 3 месяцев, и препараты вводили перорально один раз в день в течение экспериментального периода. По данным тестов «Открытое поле», «Приподнятый крестообразный лабиринт» и «Черно-белая камера» обе дозы экстракта CS моделировали седативный эффект с возможным анксиогенным эффектом у мышей. Кроме того, экстракт CS облегчал дисфункцию пространственной памяти, но не влиял на память распознавания объектов. Возможные механизмы действия экстракта включают усиление кальций-зависимой нейрональной передачи сигналов, а также модуляцию центральной нейротрансмиссии γ-аминомасляной кислоты, ацетилхолина или моноаминов, что требует дальнейшего выяснения.

Ключевые слова: *Cistus salviifolius* L., седативные средства, нарушения памяти, когнитивная дисфункция, тревога, неалкогольный стеатогепатит, поведенческое тестирование, мыши

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is an umbrella term covering a wide range of conditions characterized by excessive hepatic lipid accumulation, defined by the presence of steatosis in > 5% of hepatocytes, in the absence of significant ethanol consumption or other plausible causes of liver injury [1]. With a global prevalence of up to 30% and a constantly increasing incidence, NAFLD is considered the leading cause of chronic liver disease worldwide [2]. Due to the high heterogeneity of the pathogenesis of metabolic liver

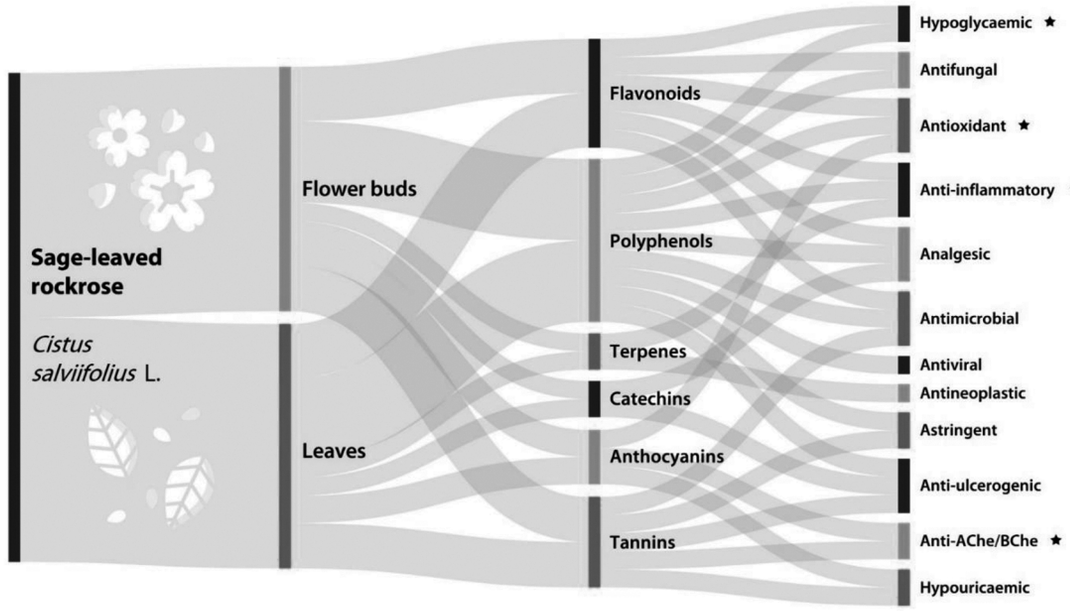

Figure 1.
Cistus salviifolius L. phytochemical composition and pharmacological activity. Stars indicate pharmacological properties potentially relevant to neuroprotection in non-alcoholic steatohepatitis. AChe, acetycholinesterase; BChe, butyrylcholinesterase.

disease, the term 'metabolic [dysfunction]-associated fatty liver disease' (MAFLD) has been proposed as a broader alternative to the conven-tional 'NAFLD' [3]. 20–25% of NAFLD cases are classified as non-alcoholic steatohepatitis (NASH), which has a significantly higher risk of progression to liver fibrosis, cirrhosis and end-stage liver disease, and hepatocellular carcinoma [4].

The prevalence of diabetes mellitus among the NAFLD and NASH patients is es-timated to be 22.51% and 43.63%, respectively, which is much higher than the preva-lence of diabetes in the general population (8.5%) [5, 6]. As diabetes and NAFLD share the main risk factors, pathogenetic features, and potential complications, many anti-diabetic medications have shown effectiveness against NAFLD and are being evaluat-ed in clinical trials [7]. Besides metabolic and cardiovascular comorbidities, NAFLD and NASH are associated with cognitive and neuropsychiatric disorders observed both in experimental animals [8] and human patients [9, 10]. According to clinical studies, NAFLD subjects tend to have poorer executive and frontal functions, visuospatial memory, and are predisposed to behavioral changes including apathy, mood imbalance, depression, and anxiety [9, 10, 11].

C. salviifolius (sage-leaved rock-rose, CS) is an evergreen perennial ligneous plant of the family Cistaceae. CS leaves and flowers, which are used in traditional medicine, contain several classes of chemical biologically active compounds, including flavonoids, polyphenols, tannins, catechins, terpenes, anthocyanines, catechines, and coumarins [12, 13]. Among other Cistus species, CS has been reported to posess antioxidant, hypoglycaemic [14], antiviral [15], antimicrobial, antifungal, anti-inflammatory [16], analgesic [17], antineoplastic, hypouricaemic, astringent, and acetylcholinesterase-blocking, activity [12] *in vitro* and *in vivo* (Figure 1).

Hypoglycaemic and antidiabetic properties of CS and its close relative *C. laurifolius* L. have been demonstrated in streptozotocin [18, 19] and streptozotocin/nicotinamide [14] rodent models of type II diabetes mellitus as well as primary glucose-induced hyperglycaemia [18]. Compounds isolated from cyclohexane and di-chloromethane CS extracts activate peroxisome proliferator-activated receptor-γ and stimulate glucose uptake by adipocytes in vitro [20]. Phenol-rich extracts of CS and Cistus monspeliensis L. inhibit α-amylase and α-glucosidase, key enzymes involved in the development of hyperglycaemia, and thus may have a therapeutic potential for T2DM [21]. Moreover, quercetin-3,7-dimethyl ether isolated from the leaves *C. laurifolius* has demonstrated antioxidant and hepatoprotective activity when administered orally to mice with acetaminophen-induced acute liver injury [22]. However, to the best of our knowledge, the effects of CS or its active consituents on the neuropsychiatric complications of NAFLD remain unknown.

In view of the above, this work was aimed at exploring the potential protective effects of a CS aqueous extract against the cognitive and memory dysfunction associ-ated with experimental NASH.

Materials and Methods

Animal experiments were carried out in compliance with the principles of the Ba-sel Declaration, the Order of the Ministry of Health of the Russian Federation No. 199n (April 1, 2016) "On the approval of the Rules of Good Laboratory Practice", and the recommendations of the Bioethics Committee of the St. Petersburg State Chemical and Pharmaceutical University of the Ministry of Health of the Russian Federation. 90 young adult male C57Bl/6 mice weighing 18–20 g were purchased from the Rappolovo laboratory animal supplier (Leningrad Oblast, Russia) in a single shipment, quaran-tined for 2 weeks, then housed in a standard animal facility with ad libitum access to normal chow (Laboratorkorm, Russia) and drinking water.

Prior to experimentation, the animals were randomized using the random number method into the following groups: (1) Control (n = 30): high-fat diet (HFD) + 42 g·L-1 fructose + 0.9% NaCl orally (p/o) q.d. + 0.32 mg·kg-1 b.w. CCl4 intraperitoneally (i/p) q.wk.; (2) CS253 (n = 30): HFD + 42 g·L-1 fructose + 253 mg·kg-1 b.w. CS extract (Gehrlicher Pharmazeutische Extrakte GmbH, Germany) p/o q.d. + 0.32 mg·kg-1 b.w. CCl4 i/p q.wk.; (3) CS506 (n = 30): HFD + 42 g·L-1 fructose + 506 mg·kg-1 b.w. CS extract p/o q.d. + 0.32 mg·kg-1 b.w. CCl4 i/p q.wk.

NASH was induced over 3 months using the model described by Tsuchida et al. [23], combining a "western"-like HFD and low-dose i/p CCl4 as an accelerant. CS doses were calculated as a single and a double one based on the doses recommended for use in humans [24]. Following the completion of all experiments, NASH was confirmed histologically using light microscopy with haematoxylin/eosin and Van Gieson staining following conventional protocols.

Animal locomotor activity and behaviour were assessed using the Open field (OF), Elevated plus maze (EPM), and Light/dark box (LDB) tests (Open Science, Russia). Animal movement was recorded on camera for 3 min and analyzed subsequently using the VideoMot2 3.0.1 (TSE Systems GmbH, Germany) (for OF and LDB) or RealTimer 1.30 (Open Science, Russia) (for EPM) software.

In the OF test, distance covered (cm), mean velocity ($cm \cdot s^{-1}$), time in centre (s), total freezing duration (s), and the number of freezes, line crossings, rears, grooming bouts, and hole pokes were registered [25]. In the EPM test, time spent in the open arms, closed arms, and centre (s), the number of entries into the open and closed arms, rears, grooming bouts, head dips, and peeking out frequency were registered [26]. In the LDB test, time spent (s) and distance covered (cm) in the white chamber (WC), mean velocity ($cm \cdot s^{-1}$), total freezing duration (s), freezing, grooming, rearing (min^{-1} in the WC) and peeking out frequency (min^{-1} in the dark chamber (DC)), the number of transitions, latency to enter the DC for the 1st time (s), and duration of the 1st DC visit (s) were registered [27].

Short-term spatial memory (STSM) and object recognition memory (ORM) were assessed using the Spontaneous alternation in the T-maze (SATM) and Novel object recognition (NOR) tests, respectively. In the SATM test, spontaneous alternation percentage was assessed live by an experienced researcher, in 3 trials per animal [28]. In the NOR test, total object exploration time (s) and novel object exploration time (s) were registered using the RealTimer software, and the discrimination index was calculated as described previously [29].

Following behavioural testing, the mice were euthanized by carbon dioxide inhalation, and the liver was excised for histological examination. Liver tissue samples were fixed in 10% buffered formalin, dehydrated, cleared in isopropanol, and embedded in paraffin according to standard protocols. 4 µm sections prepared from paraffin blocks were mounted on slides, stained with hematoxylin-eosin, cover-slipped, and examined using light microscopy.

For each sample, hepatitis activity, steatosis, hepatocellular ballooning (HCB), cholestasis, necrosis, central vein fibrosis (CVF), perisinusoidal fibrosis (PSF), and bridging fibrosis (BF) were scored as 0 (no), 1 (mild), 2 (moderate), or 3 (severe). Liver fibrosis was also staged according to the METAVIR-F scoring system as F0 (no fibrosis), F1 (portal fibrosis without septa), F2 (portal fibrosis with few septa), F3 (numerous septa without cirrhosis), or F4 (cirrhosis).

Statistical analysis was carried out in R 4.1.1 (R Foundation for Statistical Computing, Austria) with RStudio 1.4.1717 (RStudio PBC, USA) and Prism 9.0.0 (GraphPad Software, USA). The data were tested for normality using the Shapiro-Wilk W-test. For normally distributed data, the significance of differences between group means was tested using one-way ANOVA followed by the Dunn-Šidák *post hoc* test for Control vs. CS253, Control vs. CS506, and CS253 vs. CS506 pairwise comparisons. Otherwise, the Kruskal-Wallis test and Dunn's *post hoc* test were used. The significance of differences between zone preference in the EPM was tested using the pairwise chi-square test for contingency data. The significance threshold was set at $p < 0.05$. Data are presented as group mean ± standard error of mean except for the histological score totals, which are presented as median ± 95% confidence interval. Post hoc multiple comparisons of score frequency distributions were conducted using the RVAideMemoire 0.9–81–2 function package for R [30] according to the previously published protocol [31].

Results

Liver tissue morphology. In liver tissue samples obtained from Control mice, substantial evidence of non-alcoholic liver injury was found (Figure 2). All samples showed signs of moder-ate-to-severe cholestatic hepatitis, complicated by centrilobular and/or bridging necro-sis in 90% cases. Some of the samples exhibited mild medio- and macrovesicular stea-tosis involving up to 5% of the hepatocytes. Hepatocellular ballooning varied from mild to severe, and intracellular cholestasis was either moderate or severe in degree. The majority of the samples were graded as either F2 or F3 (moderate or severe fibrosis, respectively) according to the METAVIR scale [32]; no signs of cirrhosis were detected.

Liver tissue samples from the CS253 group showed signs of mild hepatocellular ballooning, moderate focal micro- and medio- vesicular steatosis involving up to 1% of the hepatocytes, and mild intracellular cholestasis. All samples were graded as F0 (no fibrosis) or F1 (mild fibrosis) according to the METAVIR scale [32]; no signs of cirrhosis were detected. No substantial differences between liver tissue morphology was found between the CS253 and CS506 groups except that hepatocyte ballooning was severe in degree in the majority of the samples.

Histological score totals were significantly reduced in the CS253 group compared to Control ($p<0.05$), and a strong trend towards score total improvement was observed in the CS506 group (Figure 2A), failing to

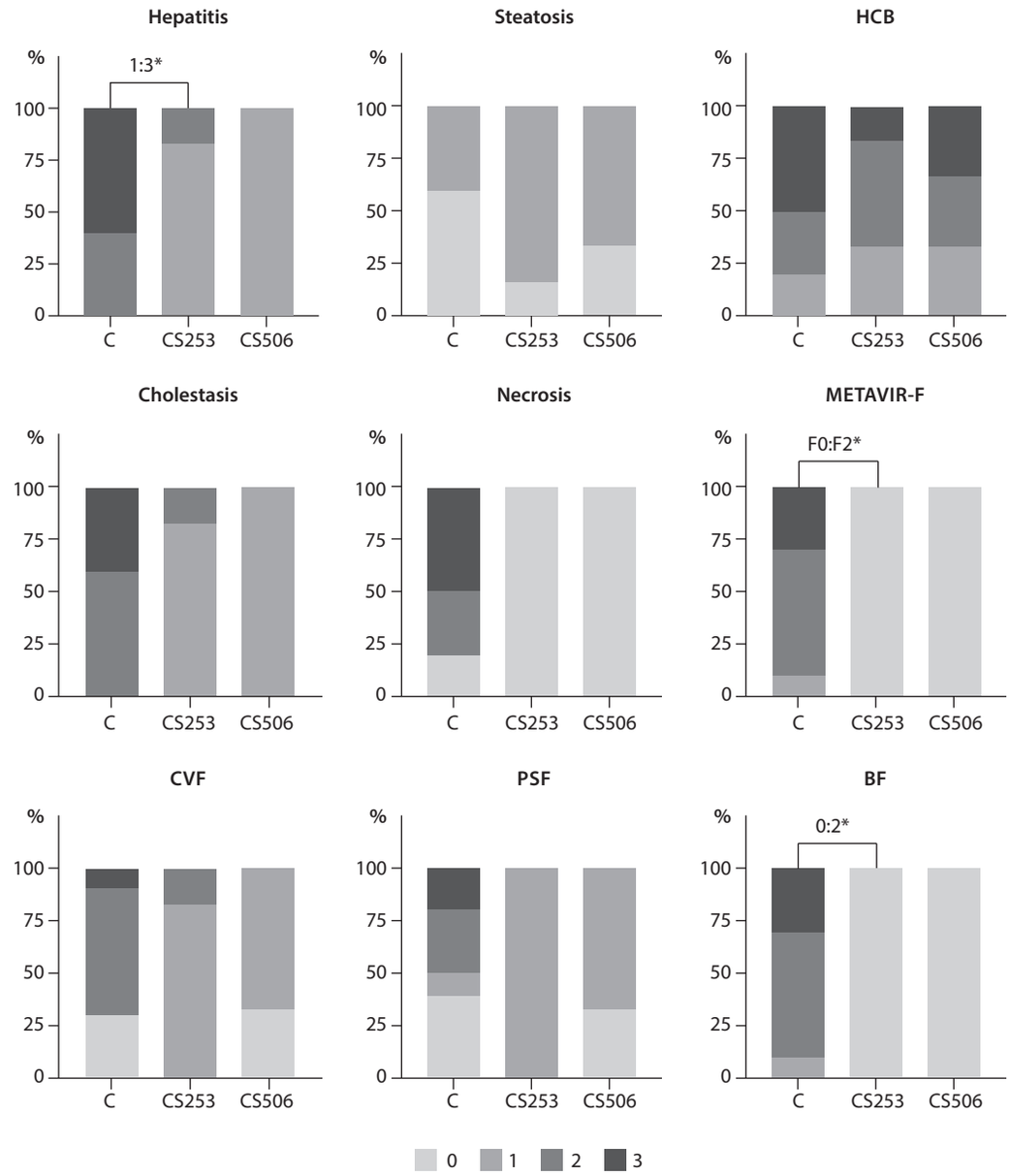

Figure 3.
Liver tissue histological score frequency distributions. C, Control; CS253, *C. salviifolius* extract (253 mg·kg^{-1} b.w.); CS506, *C. salviifolius* extract (506 mg·kg^{-1} b.w.); *, p<0.05.

reach significancy due to insufficient power. Significant improvements by the CS253 treatment were observed for hepatitis activity (p<0.05 for CS253 vs. Control), the METAVIR-F score (p<0.05 for CS253 vs. Control), and the presence of fibrotic septa (p<0.05 for CS253 vs. Control). No significant differ-ences were observed for the CS506 group due to the small sample size (Figure 3).

General locomotion and anxiety-like behaviour assessment. Both doses of CS extract caused significant reductions in animal locomotion, de-creasing the distance covered in the OF and the number of crossed lines (p<0.05 vs. Control for both groups) as well as increasing the total duration of freezing episodes (p<0.01 vs. Control). The number of freezes was reduced (p<0.01 vs. Control for CS253, p<0.05 vs. Control for CS506). No differences between the two doses were observed (Figures 4, 5).

Mice that had been receiving CS extract made significantly less entries into the closed arms of the EPM but spent more time in them (p<0.05 vs. Control for both groups), exhibiting a clear preference for the closed arms (p<0.05 vs. Control for CS253, p<0.01 vs. Control for CS506). Total number of arm entries was markedly re-duced in both groups (p<0.01 vs. Control for CS253, p<0.05 vs. Control for CS506). In the CS253 but not CS506 group an increase in the number of grooming bouts was also observed (p<0.05 vs. Control) (Figure 6).

In the light/dark box, the only significant change observed was a decrease of peeking out frequency in both the CS253 and CS506 groups (p<0.05 vs. Control) (Figure 7).

Memory assessment. In the SATM test, spontaneous alternation rate was significantly (p<0.05) increased in mice that had been receiving the CS extract at 253 but not 506 mg·kg^{-1} b.w. (Figure 8).

In the NOR test, no significant changes were observed, although a slight reduction of total exploration time was noted in the groups that had been receiving the CS ex-tract (Figure 9).

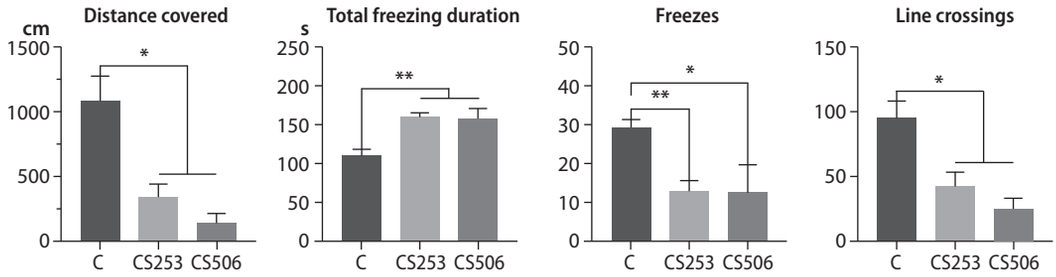

Figure 4.
Open field test results. C, Control; CS253, *C. salviifolius* extract (253 mg·kg^{-1} b.w.); CS506, *C. salviifolius* extract (506 mg·kg^{-1} b.w.); *, p<0.05; **, p<0.01.

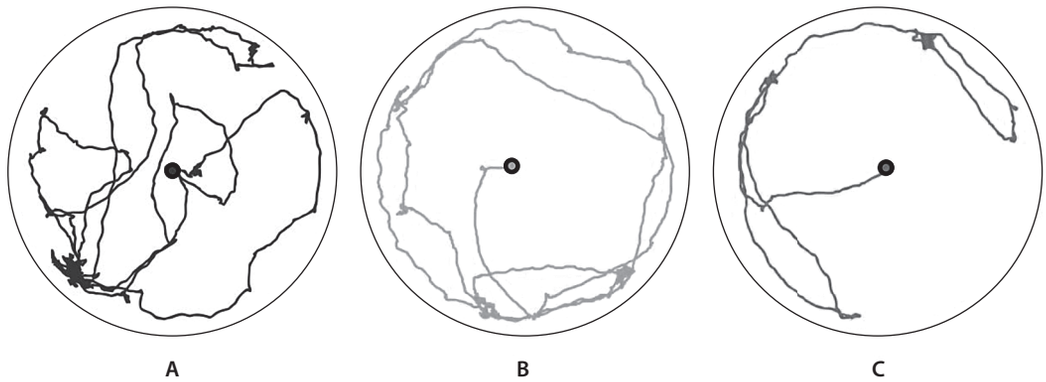

Figure 5.
Representative examples of animal movement tracks in the Open field test. (A) Control; (B) *C. salviifolius* extract (253 mg·kg^{-1} b.w.); (C) *C. salviifolius* extract (506 mg·kg^{-1} b.w.).

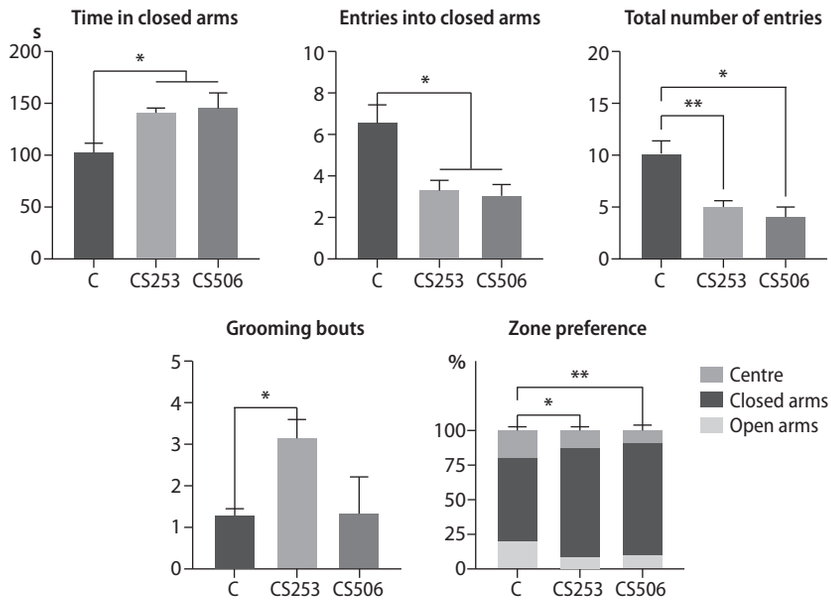

Figure 6.
Elevated plus maze test results. C, Control; CS253, *C. salviifolius* extract (253 mg·kg^{-1} b.w.); CS506, *C. salviifolius* extract (506 mg·kg^{-1} b.w.); *, p<0.05; **, p<0.01.

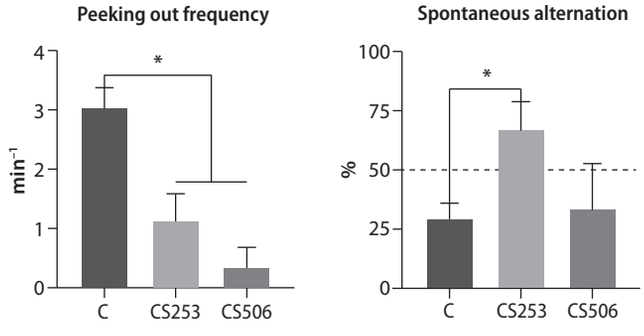

Figure 7.
Light/dark box test results. C, Control; CS253, *C. salviifolius* extract (253 mg·kg^{-1} b.w.); CS506, *C. salviifolius* extract (506 mg·kg^{-1} b.w.); *, p<0.05.

Figure 8.
Spontaneous alternation in the T-maze test results. C, Control; CS253, *C. salviifolius* extract (253 mg·kg^{-1} b.w.); CS506, *C. salviifolius* extract (506 mg·kg^{-1} b.w.); *, p<0.05.

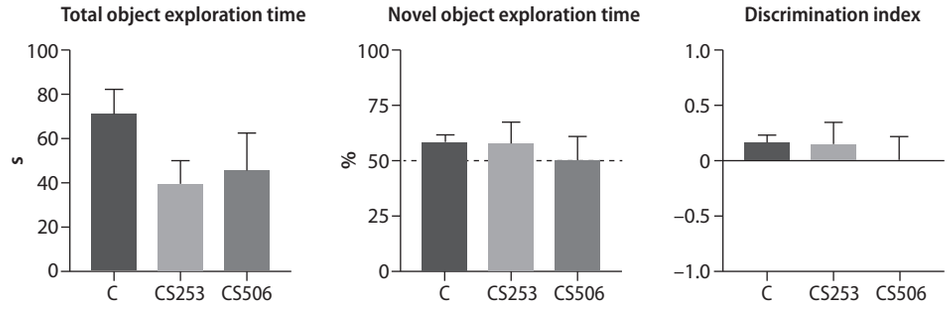

Figure 9.
Novel object recognition test results. C, Control; CS253, *C. salviifolius* extract (253 mg·kg^{-1} b.w.); CS506, *C. salviifolius* extract (506 mg·kg^{-1} b.w.); *, p<0.05.

Discussion

As reported previously, mice with experimental NASH develop an anxiety-like behavioural phenotype as well as a transient recognition and a progressive spatial memory impairments [8, 33]. In the OF test, both doses of the CS extract markedly re-duced animal locomotion and promoted freezing behaviour without affecting thigmo-taxis, which might indicate sedation. Similar behavioural changes in the OF have been reported for CNS depressant agents such as benzodiazepines (high-dose diazepam [34], midazolam [35], phenazepam [36], and lorazepam [37]), sedating antipsychotics (levomepromazine [38], chlorpromazine [39]), and sedating antidepressants (amitrip-tyline [40, 41]).

In the EPM, mice treated with the CS extract showed markedly decreased arm exploration as well as a clear preference for closed arms, which is generally interpreted as a sign of elevated anxiety [42]. Increased grooming frequency, which was observed in the CS253 group, may also indicate anxiety-like behaviour; however, the reliability of grooming frequency and duration as markers of anxiety in rodents is questioned [43, 44]. The decrease in exploratory activity but not the anxiety-like behavioural phenotype was further confirmed in the LDB test by a marked reduction in peeking out fre-quency. Therefore, it is most likely that the CS extract induced sedation and suppressed general locomotion and exploration in mice, while its effects on anxiety were quite contradictory and require further investigation. In a study in mice by de Andrés et al. [45], another Cistus species closely related to CS, *C. populifolius* L., was demostrated to cause significant reductions in spontaneous locomotion, exploratory behaviour, and motor coordination as well as a slight increase of sleeping time in the sodium pentobarbital-induced sleep test.

CS extract improved the spontaneous alternation rate in the SATM test, but did not affect object recognition in the NOR test, indicating a selective positive effect on short-time spatial memory. Both allocentric spatial and object recognition memory are regarded as hippocampus-dependent functions, however, the former may require more hippocampal tissue [46] and be more vulnerable to direct hippocampal damage [46, 47], prenatal ethanol exposure [48], and aging [49]. Moreover, in our previous work [7], we have shown that object recognition memory undergoes a transient decrease followed by complete recovery during the initial 3 months of experimental NASH induction, while spatial memory continues to decline. Therefore, the selectivity of CS's effect might be explained by the distinct nature of the two neural systems respectively involved in object and allocentric spatial recognition.

A selective effect towards working spatial but not recognition memory has been demonstrated for amifampridine (3,4-diaminopyridine) treatment in mice [50, 51] and long-time light treatment in rats [52]. Amifampridine blocks presynaptic voltage-gated potassium channels, allowing for prolonged calcium influx, enabling acetylcholine re-lease and thereby improving neuromuscular function in myasthenia. Amifampridine crosses the blood-brain barrier [53] and may improve neurocognitive dysfunction by promoting cholinergic neurotransmission, similarly to acetylcholinesterase inhibitors used to treat Alzheimer's disease, namely, galantamine, rivastigmine, and donepezil [50, 51, 54]. Since in vitro experiments have demonstrated acetylcholinester-ase-blocking activity for various CS constituents [12], CS extract might ameliorate spatial memory deficits via modulation of central cholinergic neurotransmission.

According to Huang et. al., light treatment supposedly improves spatial but not object recognition memory via its activation of CaMKIIα-positive neurons of the thalamic ventral lateral geniculate nucleus, intergeniculate leaflet, and nucleus reuniens [52]. In mice, CaMKIIα is the most abundantly expressed in the dentate gyrus of the hippocampus [55], and its deficiency causes spatial and contextual memory deficits as well as alterations in anxiety- and depression-like behaviour [56].

Myricetin, a flavonoid with sedative properties, has been shown to promote GABAA activity by inducing the phosphorylation and activation of CaMKII [57]. 3,5,6,7,8,31,41-Heptamethoxyflavone, a citrus flavonoid, ameliorated corticosterone-induced neurobehavioural dysfunction and restored neurogenesis and neuroplasticity in the hippocampus by restoring the decrease in the phosphorylation of CaMKII [58]. A similar mechanism of action was demonstrated for the flavonoid bai-calin having neuroprotective and antidepressant properties [59].

In contrast, vitexin, a flavonoid with prominent GABA-ergic sedative activity [60], has been found to suppress hyperactivated CaMKIIα signaling and the associated pro-apoptotic pathways, ameliorating brain damage and improving neurobehavioural outcomes in hypoxic-ischemic neuronal injury [61]. Since CS is rich in flavonoids, it is possible that its sedative and spatial memory-enhancing effects are mediated by mod-ulation of CaMKIIα signaling pathways in the hippocampus and/or associated structures.

Noteworthy, a similar decrease in locomotor activity with simultaneous im-provement in spatial memory has been reported for subchronic administration of the tricyclic antidepressant amitriptyline [41]. Amitriptyline has also been found to prevent or ameliorate spatial memory impairments in aging rats [62] as well as rats with anhedonia [63]. This observation might indicate the involvement of serotonergic, do-paminergic, and/or noradrenergic mechanisms in the behavioural and cognitive effects of CS, which requires further elucidation.

Conclusions

Experimental treatment with a CS aqueous extract induced behavioural changes in mice with experimental NASH. At 253 and 506 mg·kg^{-1} b.w., the extract reduced general locomotion and suppressed exploration, indicative of a sedative effect, and induced preference of closed space in the EPM test, which might indicate an additional anxiogenic effect. These behavioural effects might potentially be related to potentiat-ing GABAA activity by promoting CaMKII signaling, or modulation of central cholin-ergic and/or monoamine neurotransmission. In addition, both doses of the extract im-proved spatial but not recognition memory, possibly explained by the distinct nature of the mechanisms and neural pathways mediating the two types of memory. The effects of CS as well as its potential therapeutic value for NAFLD/NASH require further in-vestigation.

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To article

Neurocognitive and behavioural effects of sage-leaved rock-rose *Cistus salviifolius* L. extract in experimental non-alcoholic steatohepatitis (p. 54–63)

К статье

Нейрокогнитивные и поведенческие эффекты экстракта шиповника шалфейнолистного *Cistus salviifolius* L. при экспериментальном неалкогольном стеатогепатите (стр. 54–63)

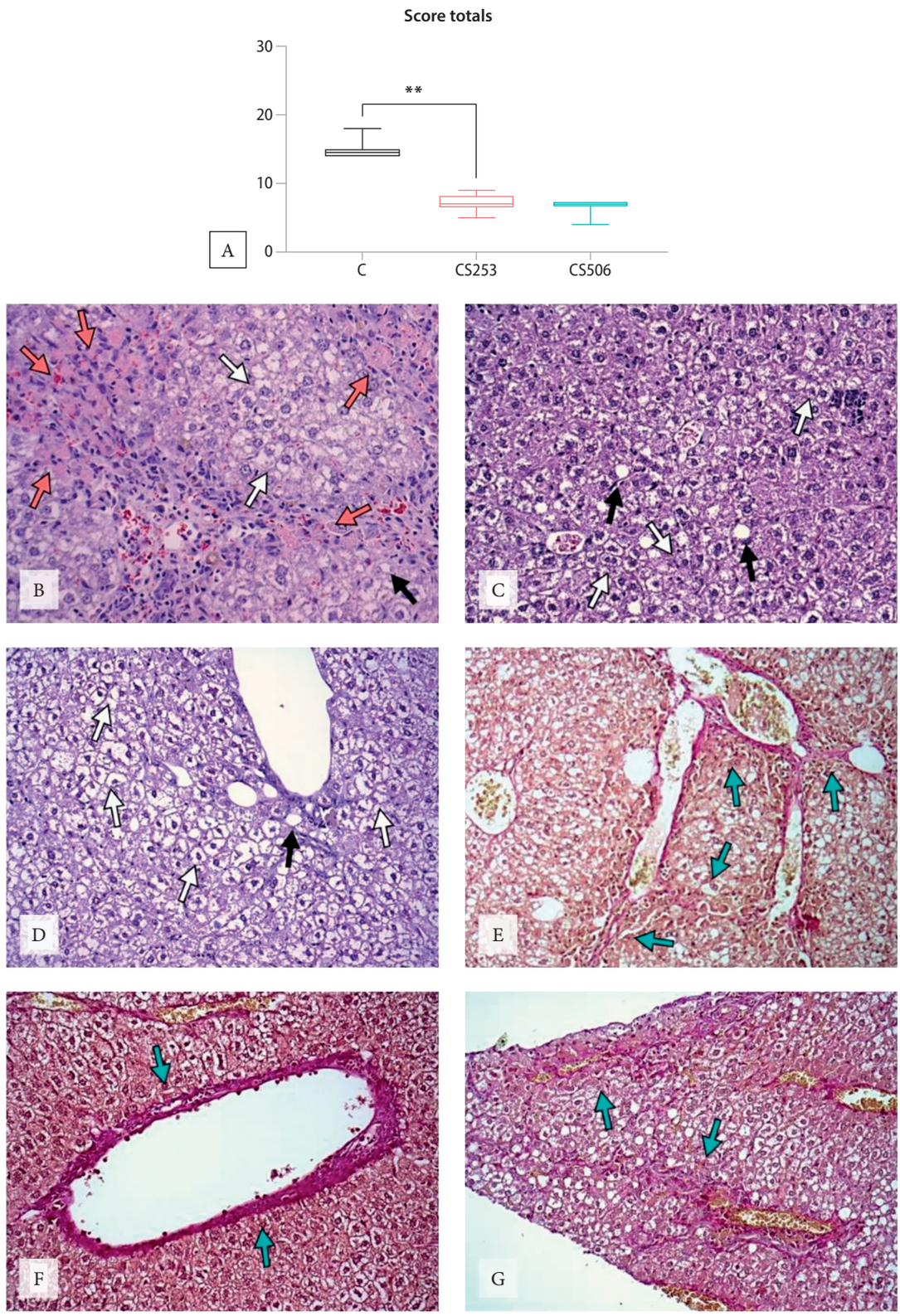

Figure 2.
Liver tissue morphology.
(A) Group score totals; (B, E) Control; (C, F) *C. salviifolius* extract (253 mg·kg^{-1} b.w.); (D, G) *C. salviifolius* extract (506 mg·kg^{-1} b.w.). Black arrows indicate steatosis; white arrows, hepatocyte ballooning; pink arrows, necrotic foci; teal arrows, fibrotic foci. Haematoxylin and eosin (B, C, D), van Gieson's picrofuchsin (E, F, G), ×200.