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Article

Microstructure of the dentate gyrus and spontaneous alternation behaviour of male Wistar rats following *Rauvolfia vomitoria* and *Gongronema latifolium* extracts administration

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Abstract: *Rauvolfia vomitoria* (RV) and *Gongronema latifolium* (GL) are medicinal plants used for the local treatment of various health issues. Their activities on the brain motivated this investigation on the histology and immunohistochemistry of the dentate gyrus and spontaneous alternation behaviour (SAB) of adult Wistar rats following RV root bark and GL leaf extract administrations. Twenty young adult Wistar rats (130–160 g) were assigned into four groups: Group 1 served as the control (5 mL/kg of distilled water placebo), while the test groups 2–4 were, respectively, singly administered 200 mg/kg of RV, 200 mg/kg of GL, and their combination. The administrations were oral and lasted for seven days. A T-maze SAB test was carried out, and the animals were sacrificed immediately after ketamine hydrochloride intraperitoneal anaesthesia. Serial sections of the hippocampal region from perfused rat brains were stained with Cresyl fast violet and immunolabelled with neuronal nuclei (NeuN) for neurons and glial fibrillary acidic protein (GFAP) for astrocytes. Results indicated that SAB was significantly ($p < 0.05$) lower in the test groups. Histologically, Nissl was less distributed in the RV and GL-only groups but not in the combined group, while there was less NeuN positivity in the RV group, with the GL and RV + GL groups not affected. There was less positive GFAP expression in individual RV and GL groups, but not in the RV + GL combined group, all compared with the control. In conclusion, the combination of RV and GL did not improve SAB but modulated Nissl, NeuN, and GFAP expression in the dentate gyrus.

Keywords: *Rauvolfia vomitoria*; *Gongronema latifolium*; Nissl substance; hippocampal region

1. Introduction

Folkloric treatment against diseases utilises medicinal plants, and this is rampant in Africa and Asia. Such diseases as malaria, diabetes, epilepsy, and depression disorders, among others, are known to be managed by medicinal plants [1–3]. However, an individual medicinal plant reported to be effective in addressing a particular disease condition can become toxic to body tissues and other metabolic processes [4–6]. As such, the use of a combination of these medicinal plants can help overcome the adverse challenge [7,8].

Two important medicinal plants with diverse medicinal properties utilised in folkloric treatment and in the present study are *Rauvolfia vomitoria* and *Gongronema latifolium* [2,7,9,10]. *Rauvolfia vomitoria* (*R. vomitoria*), also known as the swizzler stick or serpent wood, is found in Africa [5]. It is a shrub of the family Apocynaceae, whose roots, root bark, stem bark, and leaves are commonly

used for herbal remedies [2]. Its phytochemical components include alkaloids, primarily reserpine, glycosides, polyphenols, and reducing sugars [11,12], and it is used in the management of fever, epilepsy, and hypertension, among other folkloric uses [12–14]. But even at these, there are reports of adverse effects of *R. vomitoria*, especially on the brain tissues [4,15,16] These adverse effects may erode the needed benefits derivable from the use of the plant material, especially in brain-related disease conditions. However, Ekong et al. [7,15,17] reported beneficial relationships with another medicinal plant, *Gongronema latifolium*, which this study seeks to explore.

Gongronema latifolium (*G. latifolium*) is also known as bush buck and is found in tropical Africa [18]. It is a climbing shrub of up to 5 m long belonging to the family Apocynaceae. It is an edible plant with a sharp, bitter, and sweet taste. The leaf, which is heart-shaped and highly nutritious, contains proteins, fatty acids, fibres, and elements such as sodium, potassium, calcium, copper, manganese, chromium, and selenium [18–20]. It is also made up of essential oils, alkaloids, saponins, tannins, amino acids, and vitamins [20]. *G. latifolium* is reported to have antimicrobial, cardioprotective, anti-inflammatory, antioxidant, antipyretic, hypotensive, and hypoglycaemic properties [21–24].

The effectiveness of *R. vomitoria* in combination with *G. latifolium* seems synergistic, as the cerebellum has been protected from the individual adverse effects of *R. vomitoria* [7,9,17], while still maintaining its useful properties. However, there is a report of non-effectiveness with the combination [25].

One critical target of most plant-based materials is the hippocampal formation, with the dentate gyrus being very important as it serves as a source of new cell formation to enhance memory [26–28]. The role of extraneous material on this region in memory activity cannot be overlooked, as some important medicinal plants with beneficial metabolic potential have been reported to cause adverse effects to the hippocampal formation [29]. These motivated this study on spontaneous alternation behaviour and the hippocampal formation histology in Wistar the rat following *R. vomitoria* and *G. latifolium* combination.

2. Materials and methods

2.1. Handling of the experimental animals

Twenty adult male Wistar rats weighing about 130–160 g were obtained from the Animal House Facility of the Faculty of Basic Medical Sciences, University of Uyo. The rats were acclimatised for two weeks at the animal house and were allowed normal rat chow (Vital Feed, Nigeria) and clean water ad libitum throughout the duration of the experiment. The rats were grouped into four groups of five rats each. Group 1 animals were the control, while groups 2–4 were the test groups. Each rat was handled within the shortest possible time and at the base of the tail, except during oral gavages and intraperitoneal injection.

2.2. Preparation of *R. vomitoria* and *G. latifolium* extracts

The *R. vomitoria* and *G. latifolium* plants were identified and authenticated in the Department of Botany and Ecological Studies of the University of Uyo, Nigeria. The roots of *R. vomitoria* and the leaves of *G. latifolium* were, respectively, obtained from local farms in Esit Eket and Ika in Akwa Ibom State, Nigeria. The barks of the roots of *R. vomitoria* were separated from the cambium for extraction, while the *G. latifolium* leaves were used for extraction. The plant parts were air-dried, crushed to a fine powder, and 75%–80% ethanol was used to macerate in a Soxhlet extractor. The extracts were concentrated by evaporation of the ethanol using a rotary evaporator, and the concentrates were dried in a Plus 11 Gallenkamp oven at 45–50 ℃. The dry extracts obtained were stored in a refrigerator at 4 ℃ until used.

2.3. Experimental protocol

Group 1 rats (control) were administered 5 mL/kg body weight of distilled water placebo, while groups 2–4 were administered oral gavages of 200 mg/kg body weight of root bark extract of *R.* vomitoria [17], 200 mg/kg of leaf extract of *G. latifolium* [17], and a combination of 200 mg/kg body weight of root bark extract of *R. vomitoria* and 200 mg/kg leaf extract of *G. latifolium* [7,17], respectively, for seven days (**Table 1**), which was followed by the T-maze spontaneous alternation test.

Table 1. Schedule of administration for experimental groups.

Groups (*n* = 5)	Treatments	Duration of treatment (days)
1 (Control)	Distilled water (5 mL/kg)	7
2	200 mg/kg of *R. vomitoria*	7
3	200 mg/kg of *G. latifolium*	7
4	200 mg/kg of *R. vomitoria* + 200 mg/kg of *G. latifolium*	7

2.4. Spontaneous alternation test

The spontaneous alternation test was carried out on day 8 of the experiment. This test was conducted using the T-maze, which was made up of a start arm and two cross-arms. Each rat was placed in the start arm of the maze and allowed to freely choose between the right and left arms of the maze for a minute. Once a choice was made, the rat was prevented from leaving the chosen arm with a shutter door for 10 s and then placed in a holding cage for 10 min. If no choice was made, nothing was recorded for the trial. Four subsequent trials were carried out. A spontaneous alternation occurred when a rat chose a different arm of the maze from a previously visited one. Ethyl alcohol (70%) was used to clean the maze in-between trials. The spontaneous alternation results were recorded, analyzed, and percentage alternations were calculated [30,31].

2.5. Termination of the experiment

Immediately after the neurobehavioural test, the animals were anaesthetized with ketamine hydrochloride (50 mg/kg) and were then sacrificed by perfusion fixation with 10% buffered formalin. The abdomens of the animals were dissected

through the diaphragm so as to access the heart, and 10% buffered formalin was transcardially perfused through the left ventricle. The skull was later excised, and the brains were removed and post-fixed in 10% buffered formalin for 48 h.

Whole brains were cryoprotected in a 30% sucrose solution overnight at 4 ℃, and sectioned at 40 μm on a freezing microtome at −30 ℃. Cut sections were picked up with a Carmel brush and floated on a well containing phosphate buffered saline (1 M, PBS at pH 7.35). The representative serial sections of the hippocampal formation were placed on glass slides and processed for Cresyl fast violet staining for Nissl substance and immunolabelled with anti-neuronal nuclei (NeuN) for neurons and glial fibrillary acidic protein (GFAP) for astrocytes.

For Cresyl violet staining, sections on slides were first incubated in a mixture of chloroform and methanol (1:1) for an hour and then stained in Cresyl fast violet solution for 30 min. They were then rinsed in distilled water and dehydrated in ascending grades of alcohol (50% to absolute), cleared in xylene, and mounted on Entellan.

Sections for immunohistochemistry were blocked with 3% hydrogen peroxide for 15 min, washed and incubated in 5% normal goat serum for an hour, and incubated in anti-NeuN and rabbit anti-GFAP in 1% normal goat serum and Triton-X overnight at room temperature (18 °C). Sections were washed with PBS and incubated with goat anti-mouse for NEUN and goat anti-rabbit for GFAP for 2 h. Thereafter, they were washed in PBS and incubated in an avidin-biotin complex (1:1) for an hour. The sections were washed in PBS and incubated in diaminobenzedine (chromogen) to develop the staining. Sections were then washed in PBS, mounted on slides, dehydrated through ascending grades of alcohol (50% to absolute), cleared in xylene, and mounted on Entellan.

2.6. Statistical analyses

GraphPad Prism 5 was used to analyse data obtained from the spontaneous alternation test. Data were analysed using a repeated one-way analysis of variance (ANOVA), followed by a Tukey multiple comparison post hoc test to compare individual group means. A P value ≤ 0.05 was considered statistically significant. Data are presented as the mean ± standard error of the mean.

3. Results

3.1. Spontaneous alternation behaviour

The animals in the test groups administered only *R. vomitoria* (200 mg/kg) and *G. latifolium* (200 mg/kg), and their combination had significantly ($P < 0.05$) lower spontaneous alternation behaviour compared with the control. No spontaneous alternation ($p > 0.05$) was observed in the group administered *R. vomitoria* and *G. latifolium* combination compared with their individual groups (**Figure 1**).

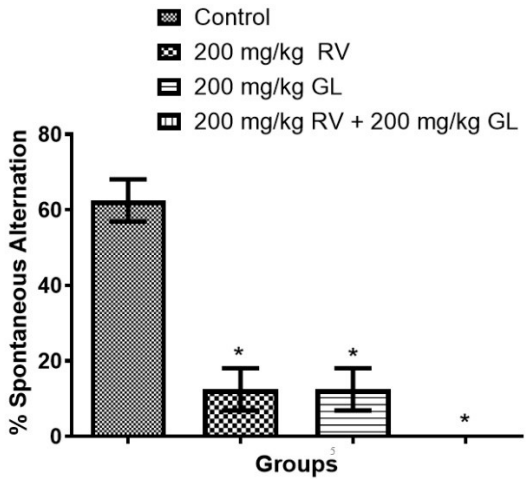

Figure 1. Spontaneous alternation behavioural test result.

Data are presented as the mean ± standard error of the mean, $n = 5$. ANOVA followed by a Tukey multiple comparison test * Significantly different from the control group at $P < 0.05$. RV = *R. vomitoria*, GL = *G. latifolium.*

3.2. Histology of the dentate gyrus

The hippocampal dentate gyrus of Wistar rats is made of three layers: from outside to inward, the molecular, granular, and polymorphic layers. In the control group, Nissl was well expressed and distributed in the small, sparse cells of the molecular layer. The granular layer consisted of a dense population of Nissl-expressed cells, while the polymorphic layer consisted of sparse Nissl-expressed cells (**Figure 2a**).

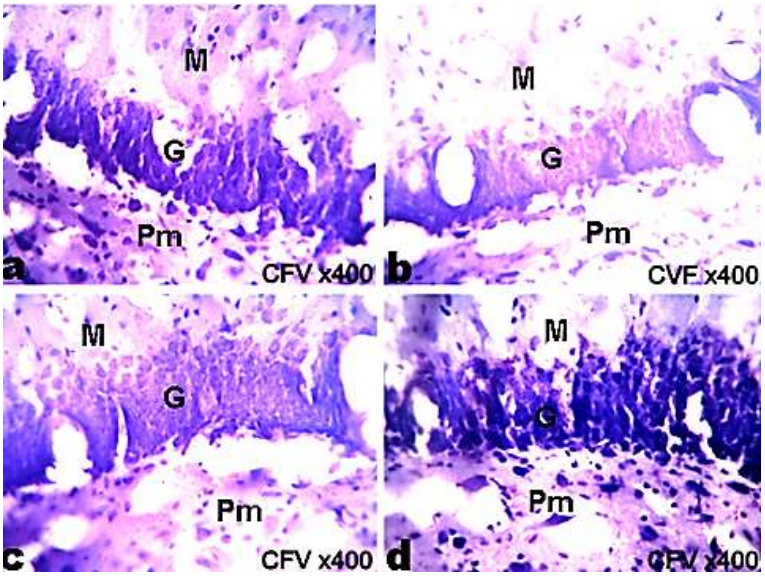

Figure 2. Representative sections of the dentate gyrus stained with Cresyl fast violet (CFV), indicating Nissl expression. Magnification: ×400. **(a)** The control group section showing well stained Nissl in the dentate gyrus layers; **(b)** the section of group 2 administered 200 mg/kg *R. vomitoria*; showing less-stained Nissl; **(c)** the section of group 3 administered 200 mg/kg *G. latifolium,* showing moderately stained Nissl; **(d)** the section of group 4 administered 200 mg/kg *R. vomitoria* and 200 mg/kg *G. latifolium* showing well stained Nissl.

Nissl—bluish.

In group 2, administered 200 mg/kg *R. vomitoria* only, Nissl was less stained compared with the control group (**Figure 2b**). In group 3, administered 200 mg/kg *G. latifolium*, Nissl was moderately stained compared with the control group (**Figure 2c**). In group 4, administered 200 mg/kg *R. vomitoria* and 200 mg/kg *G. latifolium*, Nissl was well stained compared with the control group (**Figure 2d**).

3.3. Immunohistochemistry

3.3.1. Neuronal Nuclei (NeuN)

The dentate gyrus of the control group showed normal deep expression of neuronal nuclei (NeuN) in the molecular, granular, and polymorphic layers (**Figure 3a**). In group 2, administered 200 mg/kg of *R. vomitoria*, NeuN was moderately expressed in the central granular cells, but the peripheral granular cells were well expressed compared with the control group (**Figure 3b**).

In group 3, administered 200 mg/kg of *G. latifolium*, NeuN expression appeared normal in the granular cells compared to the control group (**Figure 3c**). In group 4 administered 200 mg/kg *R. vomitoria* and 200 mg/kg *G. latifolium*, NeuN was moderately expressed in the granular cells and appeared like the control group (**Figure 3d**).

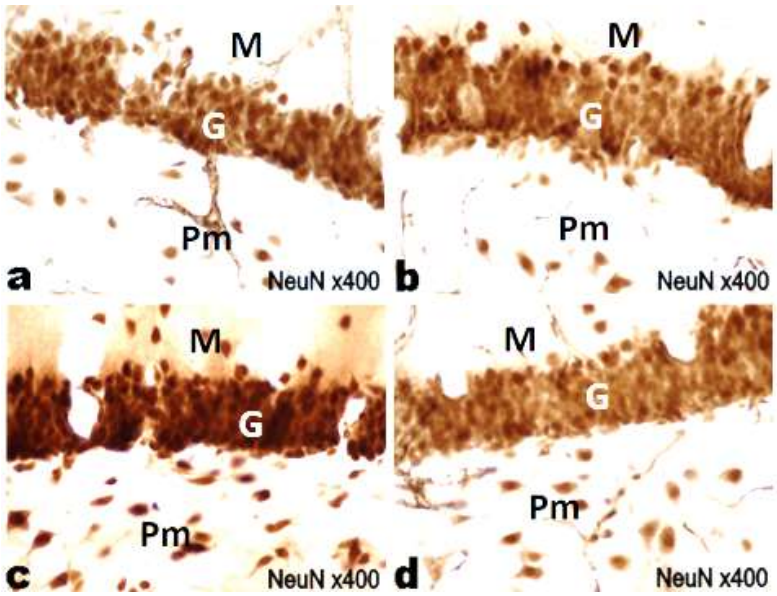

Figure 3. Representative sections of the hippocampal dentate gyrus immunolabelled with neuronal nuclei (NeuN), indicating neurons. Magnification: ×400. **(a)** The control group section showed the expression of neuronal nuclei (NeuN) in the soma of the granular cells throughout the molecular, granular, and polymorphic layers; **(b)** the section of group 2 administered 200 mg/kg *R. vomitoria*; NeuN expression was less in the soma of the pyramidal cells throughout the molecular, granular, and polymorphic layers; **(c)** the section of group 3 administered 200 mg/kg *G. latifolium, and* NeuN expression in the soma of the granular cells appeared like the control group; **(d)** the section of group 4 administered 200 mg/kg *R. vomitoria* and 200 mg/kg *G. latifolium in* combination, and NeuN expression in the soma of the granular cells appeared like the control group.
NeuN—brown.

3.3.2. Glial Fibrillary Acidic Protein (GFAP)

The section of the dentate gyrus of the control group animals showed the expression of GFAP within the astrocytes' soma and processes throughout the molecular, granular, and polymorphic layers (**Figure 4a**). In group 2, administered 200 mg/kg of *R. vomitoria*, GFAP expression was expressed in the processes but was less expressed in the soma of the astrocytes compared with the control group (**Figure 4b**).

In group 3, administered 200 mg/kg *G. latifolium*, GFAP expression was less in the astrocytes soma and processes compared with the control group. In group 4, administered 200 mg/kg of *R. vomitoria* and 200 mg/kg of *G. latifolium*, GFAP expression in the soma of the astrocyte cells appeared similar to the control group, while the processes were less expressed (**Figures 4c,d**).

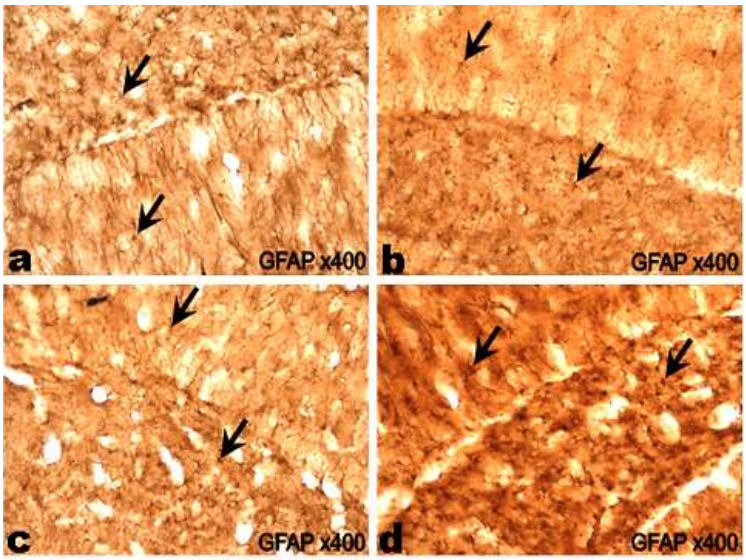

Figure 4. Representative sections of the hippocampal dentate gyrus immunolabelled with glial fibrillary acidic protein (GFAP) indicating astrocytes. Magnification: ×400. **(a)** The control group section showed the GFAP expression (arrows) within the astrocytes' soma and processes throughout the molecular, granular and polymorphic layers; **(b)** the section of group 2 administered 200 mg/kg *R. vomitoria* showing less expressed GFAP in the soma and processes of the astrocytes; **(c)** the section of group 3 administered 200 mg/kg *G. latifolium* showed less GFAP expression in the soma and processes of the astrocytes; **(d)** the section of group 4 administered 200 mg/kg *R. vomitoria* and 200 mg/kg *G. latifolium* combination, showing less expressed GFAP in the processes.
Result: GFAP—Astrocytes are brown.

4. Discussion

The present study investigated the microstructures of the dentate gyrus and spontaneous alternation behaviour of adult Wistar rats following *R. vomitoria* root bark and *G. latifolium* leaf extract administration. Results showed that spontaneous behaviour declined, along with Nissl, NeuN, and GFAP expressions.

To study the cognitive action of rats following *R. vomitoria* root bark and *G. latifolium* leaf extracts administration, the T-maze spontaneous alternation behaviour

was carried out. This test is based on the willingness of rodents to explore a new environment, which in this case was the new arm of the maze instead of the previous visited one [30,31]. The ability of the rats to perform the spontaneous alternation in groups 2 and 3 administered only *R. vomitoria* root bark and *G. latifolium* leaf extracts, respectively, was significantly lower than that of the control group, implying a poor spontaneous alternation [31], associated with the extracts, and invariably impaired working memories [32,33]. *R. vomitoria* root bark extract sedates, usually resulting in poor motor and cognitive activities [4,15,16], which may have played out in the present study. Although *G. latifolium* leaf extract is not known to cause adverse behaviour [17,22], it is possible that the rats in the *G. latifolium* group were anxious, as this could have influenced their poor spontaneous alternation [34].

Group 4 rats administered a combination of *R. vomitoria* root bark and *G. latifolium* leaf extracts did not move from the point of entry in any direction in the maze, suggesting a sedative action of the combined extracts or their anxious state. It is possible that there was a synergistic action of *R. vomitoria* root bark and *G. latifolium*, which could have further worsened the sedative state of the rats. However, the present result is at variance with previous actions of *R. vomitoria* and *G. latifolium* combination, which improved cognition [8,35].

Spontaneous alternation is indicative of spatial memory [32], which the hippocampal formation regulates [28]. A part of the hippocampal formation essential for memory consolidation is the dentate gyrus. Its structure is vital for memory function [27]. In the present study, groups 2 and 3 administered only *R. vomitoria* root bark and *G. latifolium* leaf extracts, respectively, showed reduced Nissl expression, which was even less expressed in the *R. vomitoria* group. This implies that *R. vomitoria* and *G. latifolium* extract administration may have resulted in chromatolysis. Chromatolysis results from ribosomal protein degradation, which often precedes cellular degeneration [36]. *R. vomitoria* is reported in structural alterations of brain cells, although *G. latifolium* does not show such a major adverse effect [8–10,15]. The effect of the combined extracts in group 4 did not seem to affect the granular cell layer as the Nissl appeared well expressed, suggesting that the combination may have protected the dentate gyrus Nissl as previously reported in other brain areas [7–9]. Cells adapt to the environment to protect themselves from injury [37], and chromatolysis may have been a result of such.

Immunohistochemically, neurons can be identified with a neuron-specific nuclear protein, NeuN, expressed mostly by the mature ones [38]. Reduced expression of NeuN indicates loss of cell viability or antigenicity [39,40]. In the present study, NeuN expression reduced moderately in the group administered only *R. vomitoria* extract. This result aligns with the actions of *R. vomitoria* reported in cellular structural changes [4,8] and in the Nissl result of the present study. A report showed that dentate granule cells undergo morphological changes in response to excessive excitation or trauma [29], which may have also been applicable in the present study.

The groups administered *G. latifolium* only showed positive NeuN that was well expressed in dentate gyrus, suggesting no adverse actions of the extract. *G. latifolium* administration is not known with adverse effects on most brain structures,

and this could have been applicable in the present study. The groups administered *G. latifolium* in combination with *R. vomitoria* showed moderately reduced NeuN expression, indicating the potential of *G. latifolium* in protection against the adverse effect of *R. vomitoria.*

Immunohistochemically, astrocytes can be demonstrated with GFAP, an intermediate filament protein they mostly express, and an increased or decreased expression may indicate a trauma to the brain [41–43]. In the present study, GFAP was less expressed in the astrocytes' soma within the molecular, pyramidal, and polymorphic layers of the dentate gyrus of the group administered only *R. vomitoria*. This indicates a downregulation of the GFAP protein, which may be associated with degeneration. This is at variance with reports on the cerebellum from previous studies [7,9]. The group administered only *G. latifolium* also showed reduced GFAP expression, which may be a protective mechanism, as neurotoxicity is often not reported in *G. latifolium.* The group administered a combination of *G. latifolium* and *R. vomitoria* also showed reduced GFAP expression.

The dentate gyrus is the integral region of the hippocampal formation that contributes to the formation of new episodic memories, spontaneous exploration of novel environments, and other functions [44,45]. It is observed that destruction of the dentate gyrus cells leads to poor maze activities and memory impairment [26,46].

From the present findings, *R.* vomitoria elicited its action through the down-regulation of dentate gyrus structural proteins, including Nissl, NeuN, and GFAP, which affected neuronal and astrocyte viability and resulted in memory impairment. A combination with *G. latifolium* partially protected against these proteins down-regulation, as it improved Nissl and NeuN, suggesting antagonistic action. However, GFAP expression and memory did not improve, suggesting a synergistic action.

5. Conclusion

The results of the present study showed that the combination of *R. vomitoria* and *G. latifolium* could not reverse the individual effects of *R. vomitoria* and *G. latifolium* on spontaneous alternation but was able to show modulation of the dentate gyrus Nissl distribution and immunohistochemical expressions of NeuN and GFAP. These findings suggest a beneficial synergistic role for the *R. vomitoria* and *G. latifolium* combination on the dentate gyrus, but care should be taken as their administration is not free of adverse effects. Due to limited data, further analysis involving specific neurochemicals and proteins could identify the pharmacology of the *R. vomitoria* and *G. latifolium* combination in the dentate gyrus and other brain areas.

Author contributions: Conceptualization, MBE; methodology, MBE; software, MBE; validation, MBE and IOGE; formal analysis, MBE; investigation, MBE, CKB and OMA; resources, MBE, IOGE, CKB and OMA; data curation, MBE, IOGE, CKB and OMA; writing—original draft preparation, MBE and IOGE; writing—review and editing, MBE; visualization, MBE and IOGE; supervision, MBE; project administration, MBE; funding acquisition, MBE and CKB. All authors have read and agreed to the published version of the manuscript.

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

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