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Rauwolfia vomitoria and Gongronema latifolium extracts influences cerebellar cortex

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Abstract

Oxidative stress and free radical production is an etiology to some neurodegenerative diseases which may be preventable by prior neuronal protection using herbs. *Rauwolfia vomitoria* and *Gongronema latifolium* are medicinal herbs with antioxidant, anti-diabetic and analgesic properties among others. While *R. vomitoria* acts as a brain stimulant, as well as a depressant, neurotoxic effects have also been reported, which *G. latifolium* has shown the potential to mitigate. This study therefore investigated the effects of the combination of *R. vomitoria* and *G. latifolium* on young rats' cerebellar cortex. Twenty young male Wistar rats (100-150 g) were divided equally into 4 groups (n=5). Oral doses of the vehicle (Tween 20™), ethanolic extracts of 200 mg/kg of *R. vomitoria* (RV), 200 mg/kg of *G. latifolium* (GL), and the combination of both (RV + GL) were given to the animals for 14 days. On day 15, the animals were sacrificed after ketamine sedation and perfusion-fixed with 10% buffered-formalin. The cerebella were excised and processed for histomorphology by silver impregnation technique and immunolabelled with anti- neuron specific enolase (NSE) and glial fibrillary acidic protein (GFAP). The histology results showed atrophied Purkinje and other neurons with increased NSE and GFAP expressions in the RV group, which were not as such in the GL and RV+GL groups, an indication of cerebellar cortical injury. In conclusion, RV was injurious to the cerebellar cortical neurons and also stimulated NSE and GFAP, but these RV-induced traumas were slightly mitigated with GL combination. This preliminary report of RV+GL combination may be considered an alternative to RV single treatment for better disease management and brain protection.

Introduction

Oxidative stress and free radical production is one of environment-induced etiology of some neurodegenerative diseases [1-3]. However, this does not rule out the hereditary aspect of some of these neurodegenerative diseases [4]. There are reports that degenerative diseases such as Alzheimer's, Parkinson and other forms of dementia related diseases may be preventable if there is prior neuronal protection using herbs and other food types [5,6].

In Africa, the abundance of herbs and herbal products has given a promising future towards the prevention of such disease as related to neuronal degeneration. The use of such plant as *Rauwolfia vomitoria* and *Gongronema latifolium* has been on the increase due to their known medicinal values. *R. vomitoria* and *G. latifolium* show promises as antioxidants, anti-diabetics and analgesics among others [7-11].

R. vomitoria (RV), a shrub of the family *Apocynaceae* is commonly called serpent wood or swizzler stick and is used locally in the treatment against snake bites, fever and some nervous disorders [12]. The root bark is extensively used, and this is reported to contain alkaloids such as; ajmaline, ajmalicine, reserpine, serpentine, serpentinine, yohimbine, among others [13-15]. It is reported that RV is effective as an analgesic, anticonvulsant, and antipsychotic among others [10,16,17]. Ekong *et al.* [18] on the other hand reported no adverse effect on behavioural and biochemical parameters; however, adverse effects of this plant have been reported. It causes depression and Parkinsonia syndrome [19], impede motor activity behaviour, and stimulates neurodegenerative features in the cerebellum and cerebral cortical cyto-architectures [20-23]. In foetal tissues, it is reported to be hepatotoxic, cardiotoxic, as well as stimulation of hypertrophy and hyperplasia of osteoblasts and osteoclasts in the femur [24-26].

Though this plant has very useful functions, its adverse effects tend to overshadow such. The potentials of this herb in combination with other viable herbs have been postulated as having better beneficial effect on the nervous system [21-23]. The benefits of this combination may be explored for health management including the prevention against neurodegenerative diseases. This study therefore explored the combination of RV with GL on the neurons of the cerebellum.

G. latifolium (GL), a climbing perennial plant of the family *Asclepiadaceae*, is commonly called amaranth globe or bush buck, and has both medicinal and nutritional values [27]. Its leaf is extensively used, and is reported to contain alkaloids, saponins, tannins, flavonoids and glycosides [28]. GL is reported to have hypoglycemic, anti-bacterial, antioxidant, anti-inflammatory, anti-ulcer, analgesic, anti-diabetic, anti-pyretic and cardio-protective properties [8,28-34]. These useful properties endeared the choice for its combination with RV.

It is reported that RV acts as a brain stimulant, as well as a depressant, with neurotoxic effects also reported [35,36]. GL has shown the potential to modulate some of these effects of RV [21,22]. This study therefore investigated the protective effects of the combination of RV and GL on young rats' cerebellum.

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Materials and methods

Twenty young male Wistar rats of body weight 100-150 g were obtained from the animal facility of the Faculty of Basic Medical Sciences of the institution. The animals were randomly assigned into 4 groups (1, 2, 3 and 4) of five (5) animals each, and were allowed to acclimatize for one week before the start of the experiment. The animals were allowed 12 hours dark and light cycles, and were handled according to the guidelines for animal care by the National Institute of Health of the United States of America.

RV and GL plants were obtained from local farms in Esit Eket and Ikono, respectively, all in Akwa Ibom State, Nigeria. The roots and leaves of RV and GL respectively, were washed off dirt and the cambium teased out exposing the phloem which was subsequently excised. The phloem of the RV and the leaves of GL were air dried for one week, and were grounded into fine powder.

Both plants parts were extracted by Soxhlet method using 70-80 % alcohol. Upon complete extraction, the alcohol was completely evaporated using a steam bath, and the extracts were stored at 4 °C. The actual dose of each extracts was re-constituted with Tween 20™.

Experimental design

Group 1 rats were the control and were given the vehicle, Tween 20™ (0.5 ml). Groups 2-4 were the test groups and were administered either 200 mg/kg ethanolic extract of RV, 200 mg/kg ethanolic extract of GL or the combination of the ethanolic extracts of both RV and GL (RV + GL) respectively. All administration was orally and lasted for 14 days.

On day 15, the animals were sacrificed after ketamine hydrochlode anaesthesia. Phosphate base saline (PBS) was transcardially perfuse to eliminate blood, and thereafter perfusion-fixed with 10% neutral buffered-formalin. The whole brains were excised and preserved for 48 hours, and the cerebellum was excised and processed for histomorphology study using silver impregnation technique, and also immunolabelled with neuron specific enolase and glial fibrillary acidic protein, antibodies.

Statistical analysis

One way analysis of variance (ANOVA) was used to compare the means for the cellular density, thereafter the post-hoc test using Dunnett's multiple comparison test was carried out to find the level of significance at $p < 0.05$. All the results were expressed as mean + standard error of mean.

Results

Silver impregnation

The section of the cerebellar cortex of the control group consists of three layers having dark-brown stained neurons. From the outside inwards it includes: molecular, Purkinje and granular layers. The molecular layers contained sparse small size neurons unequally distributed within the layer. The Purkinje layer had a single layer of large Purkinje cells, while the granular layer contained a dense population of small-size granule cells with intervening glomeruli (Figure 1a).

The cerebellar cortex of the RV group showed atrophic Purkinje cells, with no other apparent histopathology compared with the control (Figure 1b). The cerebellar cortex of the GL group showed no obvious histopathology compared with the control group (Figure 1c). The cerebellar cortex of the RV+GL group also showed no obvious histopathology compared with the control group (Figure 1d).

There was no difference in the cerebellar cortical density in all the test groups compared with the control group (Figure 2). There was significantly ($p < 0.05$) less average cerebellar cortical cell size in all the test groups compared with the control group (Figure 3). However, there was no difference in cell population and size among the test groups.

Neuron Specific Enolase (NSE)

NSE was expressed in the control group cerebellar cortex and was more pronounced in the Purkinje cell bodies (Figure 4a). There was increased expression of NSE in the Purkinje cell bodies of the RV

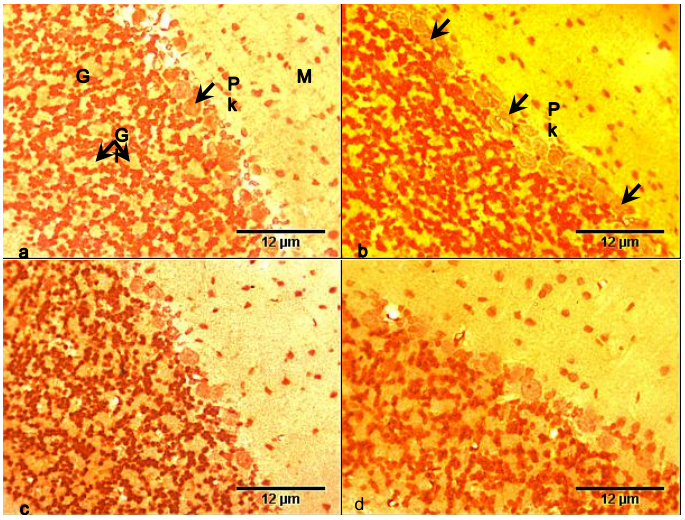

Figure 1. Photomicrographs of cerebellar cortex of the control and test groups (Bielschowsky silver impregnation, ×400):

a. The cerebellar cortex of the control group consists of three layers: molecular (M) with sparse small size neurons, Purkinje with large Purkinje (Pk) neurons, and granular (G) layer with dense small size neurons and intervening glomeruli (Gl).

b. The cerebellar cortex of the RV group showed atrophic Purkinje neurons (arrows).

c. The cerebellar cortex of the GL group did not show obvious histopathology.

d. The cerebellar cortex of the RV+GL group did not show obvious histopathology.

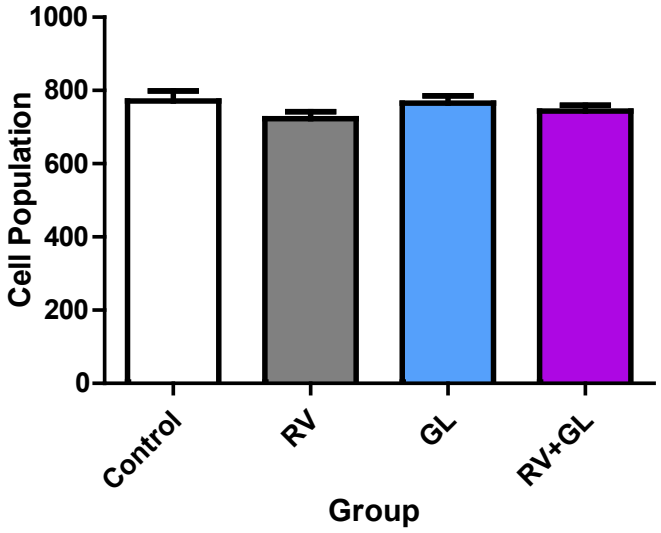

Figure 2. Average cell population of the cerebellar cortex in the experimental groups

Data are presented with mean ± standard error of mean

(n=5, F = 1.064, p = 0.3920, RV = *R. vomitoria*, GL = *G. latifolium*)

There is no significant difference between the test groups compared with the control group and among the test groups

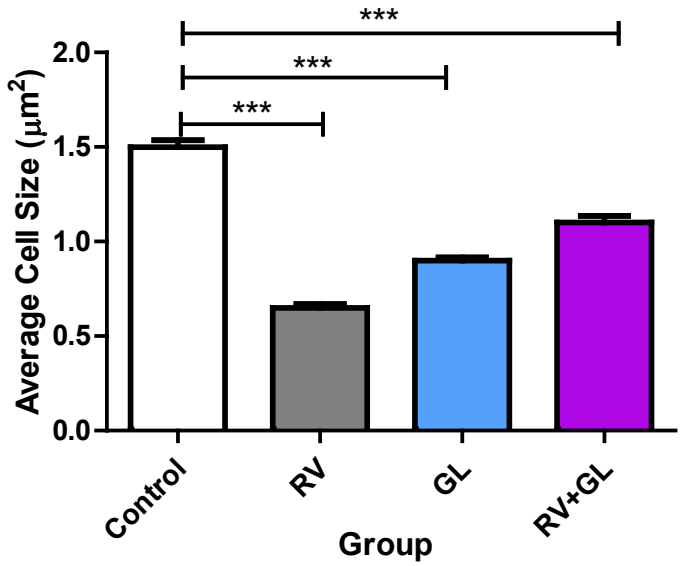

Figure 3. Average cell sizes of the cerebellar cortex in the experimental groups
Data are presented with mean ± standard error of mean
(n=5, F = 166.4, p = 0.0001, RV = *R. vomitoria*, GL = *G. latifolium*)
***RV group is significantly ($p < 0.001$) lower than the control group
There is no significant difference among the test groups

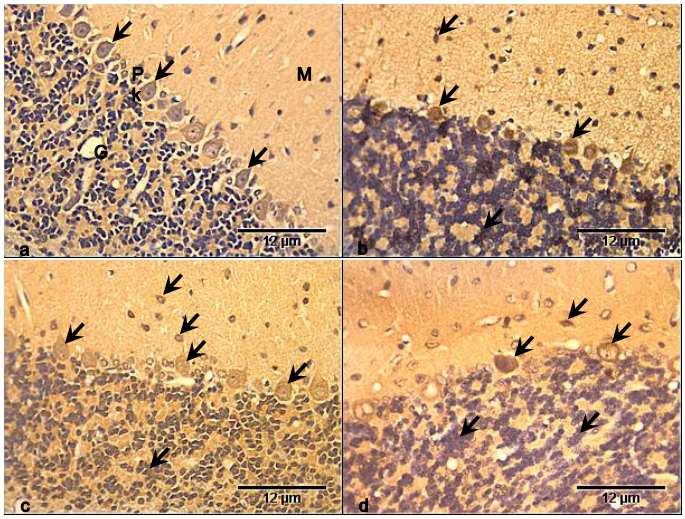

Figure 4. Photomicrographs of cerebellar cortex of the control and test groups (NSE ×400):

a. NSE is expressed in the control group cerebellar cortex and is more pronounced in the Purkinje cell bodies (arrows).

b. There is increased expression of NSE in the Purkinje cell bodies, granule cells and some cells in the molecular layers (arrows) compared with the control group.

c. NSE is expressed mostly in the Purkinje cells, with slight expression in the granule cells and cells in the molecular layer (arrows), though there is little difference in expression compared with the control group.

d. NSE is slightly more expressed especially in the Purkinje cell bodies. The granule cells and cells of the molecular layer (arrows) also expressed NSE compared with the control group.

group. The granule cells and some cells in the molecular layers also expressed NSE compared with the control group (Figure 4b). In the GL group, NSE was expressed mostly in the Purkinje cells, as well as the granule cells and cells in the molecular layer. There appear to be no difference in expression between this group and the control group (Figure 4c). There was slightly more expression of NSE especially in the Purkinje cell bodies of RV+GL group, while the granule cells and cells of the molecular layer also had NSE expression compared with the control group (Figure 4d).

There was significantly ($p < 0.05$) higher population of NSE expressed neurons in the cerebellar cortices in all the test groups compared with the control group. However, no difference was observed in NSE expressed neuronal population among the test groups (Figure 5).

Glial fibrillary acidic protein (GFAP)

GFAP was expressed in the cerebellar cortex of the control group. Astrocytic processes were mostly expressed in the molecular layer, with the cell bodies and processes being expressed in the granular layer (Figure 6a). There was increased GFAP expression in the RV group. Although the processes were mostly expressed in the molecular layer, the granular had more cell bodies expressing GFAP compared with control group (Figure 6b).

GFAP was expressed in the GL group mostly in the granular layer, while the molecular layer had expression in the astrocytic processes. However there appear to be no difference with the control group (Figure 6c). There was GFAP expression in the RV+GL group mostly in the granular layer, while the molecular layer had expression of the astrocytic processes. However there appear to be no difference with the control group (Figure 6d).

There was significantly ($p < 0.05$) lower population of GFAP expressed astrocytes in the cerebellar cortex of the RV + GL group compared with the control group. However, no difference was observed in GFAP expressed astocytes population in the other test groups compared with the control group, and among the test groups (Figure 7).

Discussion

Silver impregnation is a technique that demonstrates neuronal cell bodies and processes, with little or no interference with glial cells

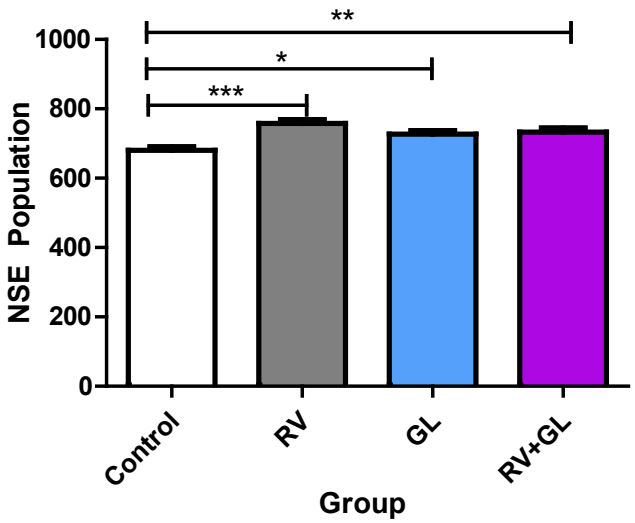

Figure 5. NSE labeled cell population of the cerebellar cortex in the experimental groups
Data are presented with mean ± standard error of mean
(n=5, F = 9.511, p = 0.0008, RV = *R. vomitoria*, GL = *G. latifolium*)
*RV group is significantly ($p < 0.05$) lower than the control group
**RV group is significantly ($p < 0.01$) lower than the control group
***RV group is significantly ($p < 0.001$) lower than the control group
There is no significant difference among the test groups

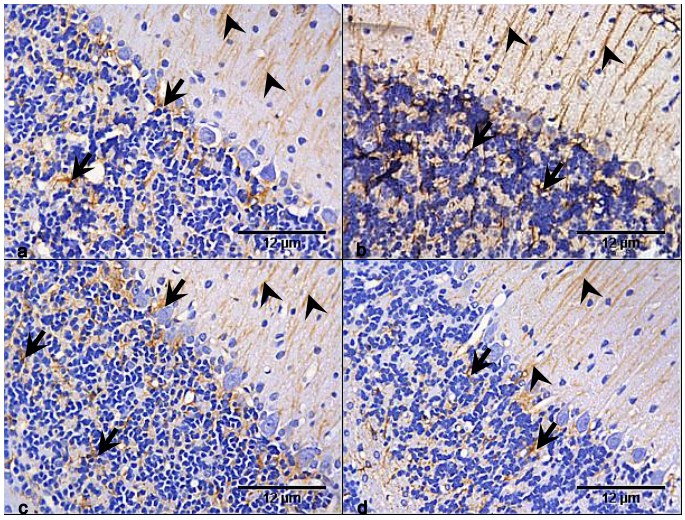

Figure 6. Photomicrographs of cerebellar cortex of the control and test groups (GFAP ×400):

a. There is expression of GFAP in the cerebellar cortex of the control group with astrocytic processes (arrow heads) mostly expressed in the molecular layer, while the cell bodies (arrows) were and processes were expressed in the granular and medullary layers.

b. There is increased GFAP expression in the RV group, though the processes (arrow heads) were mostly expressed in the molecular layer, the granular had more cell bodies (arrows) expressing the GFAP compared with the control group.

c. GFAP is expressed in the GL group mostly in the granular layer (arrows). In the molecular, the expression is only in the astrocytic processes (arrow heads). However there appear to be no difference with the control group.

d. GFAP is expressed in the RV+GL group mostly in the granular layer (arrows). The molecular layer had expression of the astrocytic processes (arrow heads), with no difference with the control group

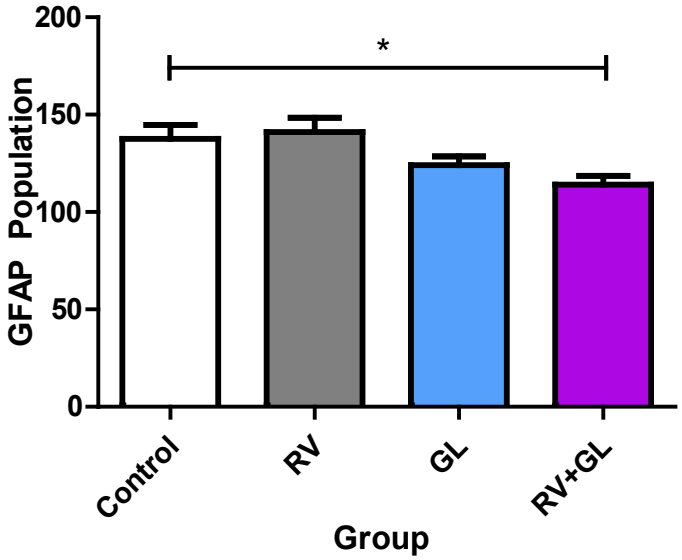

Figure 7. GFAP labeled astrocytic population of the cerebellar cortex in the experimental groups

Data are presented with mean ± standard error of mean

(n=5, F = 4.208, p = 0.0225, RV = *R. vomitoria*, GL = *G. latifolium*)

*RV group is significantly ($p < 0.05$) lower than the control group

There is no other significant difference with the control and among the test groups

[37,38]. It is reported to provide morphology insights into neuronal structure to detect damage or degeneration [39,40]. In the present study, the silver impregnation results showed atrophic Purkinje cells in the cerebellar cortex of the RV group, while there were slight atrophy also in the GL and RV+GL groups.

Cellular atrophy is a signal for degeneration and is known to occur when there is disruption of trophic signals to cells among other causes [41]. Some constituents of RV's have been reported to disrupt monoamines signals in the brain [35,36,42-44], thus, disrupting their roles in arousal, emotion and cognition [45]. The disruption of these roles may form a basis for neuronal degeneration, which atrophy may be one. The present result corroborates previous reports on the toxicity of RV. RV has been reported to cause cellular damage to the cerebellum and other brain regions [20,22], although its mechanism of action is not known.

The GL group also presented slight atrophy, an indication that GL extract at the given dose may lead to cerebellar tissue trauma. But cell atrophy may be an adaptive mechanism to cope with trauma, and may not be pathological. GL has been reported with beneficial role in different body tissues [46,47], and these beneficial effect are usually physiological without associated tissue morphology study. However, Ekong *et al.* [22,23] reported that GL altered neurons of the cerebellum, which the present study is in line with. Slight cellular atrophy was also observed in the cerebellar cortex of the RV+GL group, also indicating trauma to this brain tissues. Ekong *et al.* [22] also reported similar changes with the combination, indicating that although GL may have an antagonistic effect on RV, the dosage under study may not be sufficient to prevent RV toxic effect. The present RV+GL result is also in line with the works of Ekong *et al.* [21] and Ekong *et al.* [23].

Anti-NSE which labels the cell cytoplasm and dendrites of neurons was used to study the state of the neurons. The results showed increased ($p < 0.05$) NSE expression in the RV, GL and RV+GL groups. NSE is a cytosolic protein that functions as brain-specific glycolytic enzyme, and plays an important role in intracellular energy metabolism [48]. It is expressed by mature neurons and cells of neuronal origin, and thus regarded as a marker of the neuronal state [49,50], but becomes markedly expressed after brain injury [51]. It is reported that RV cause neuronal injury [20,22], and this may be a reason for the marked expression of the enolase, which may be deleterious to the normal function of the cerebellar cortical cells. On the other hand, increased NSE expression in the GL and RV+GL groups, indicate injury to the neurons which the combination was unable to ameliorate successfully.

Some glial cells under certain condition also express NSE [52,53]. As it was important to rule out NSE expression by these glial cells, anti-GFAP was also studied as well. The results showed slight increased GFAP expression in the RV group, with no difference in the GL group, but lower expression ($p < 0.05$) in the RV+GL group. Increased GFAP expression is an indication of the up-regulation of this protein, which usually occurs when brain tissues undergo injury or at diseased state [54]. Slight increased expression of GFAP, which is an intermediate filament protein of astrocytes and ependymal cells [55-57], is indicative of the traumatic effect of RV. Marked GFAP expression is also indicative of reactive astrogliosis [58], which may be detrimental on the long run as it usually underlie neural dysfunction and pathology in certain neurological disease states [59]. GFAP expression however, appeared unaffected in the GL and the RV+GL groups, which supports previous parameter results of the present study.

RV has been reported to induce a wide range of physiological, biochemical and structural alteration in the brain [10,20,22], and its sole use by locals in psychiatry management pose a threat to the normal function of the cerebellar cortex. While GL is reported with little or

no adverse effect on neural tissues, its combination has been reported to ameliorate RV-induced injury [21], which the present corroborates.

Decreased GFAP expression is invariably associated with detrimental conditions in the central nervous system [60], and could result from cytoskeletal destabilization or degradation and loss of GFAP antigenicity [61]. How-ever, it is reported that apparent decreased GFAP content reflects a decrease in GFAP expression, but may not be a decrease in the number of astrocytes [62].

RV root bark extract used in the present study is reported to contain active constituents such as reserpine, yohimbine, and ajmaline among others [13-15], and individually these constituents have powerful effects on the nervous system, which the cerebellum is part. As a combination in RV, their effects may be synergistic or antagonistic, and individually their mechanisms are needed.

The cerebellum function in the maintenance of equilibrium and muscle contraction coordination needed for carrying out movements and in the execution of the encoded instructions [63]. Alteration of the Purkinje cells which was the most affected neurons of the cerebellar cortex in this study prevents inhibitory projections to the deep cerebellar nuclei, and may lead to severe detrimental consequences. However, the combination of RV+GL may help to prevent such adverse functional and/or structural effects.

Conclusion

RV was observed to be toxic to the cerebellar cortical neurons as it caused cortical morphological change and stimulated marked expression of NSE and GFAP. GL on the other hand modulated the toxic effect of RV, thus protecting the cerebellar cortex from RV-induced toxicity. This preliminary report of RV+GL combination may be considered an alternative to RV single treatment for better disease management and brain protection.

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