

Research Paper

Ameliorating Effect of Morin Hydrate on Chronic Restraint Stress-induced Biochemical Disruption, Neuronal, and Behavioral Dysfunctions in BALB/c Mice

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ABSTRACT

Introduction: Morin hydrate (MH) is a bioflavonoid component of many fruits and vegetables. Our previous research demonstrated that MH provides neuroprotection in mouse models of acute restraint stress and sleep deprivation by attenuating hippocampal neuronal damage and enhancing memory. Based on these findings, our study investigated the role of MH in chronic stress-induced neuronal and biochemical perturbations in BALB/c mice.

Methods: Male BALB/c mice were divided into 6 groups (n=6). Groups 1 and 2 received vehicle (10 mL/kg normal saline), groups 3-5 received MH (5, 10, 20 mg/kg IP), while group 6 received ginseng (25 mg/kg) daily and 30 minutes afterward were restrained in a plastic cylindrical restrainer for 14 days.

Results: Immobility time in the forced swim test increased in the MH-treated group, indicating an antidepressant-like effect. Also, a reduction in frequency and duration of open arms exploration was observed in the elevated plus-maze (EPM) test in stressed mice, and administration of MH (5, 10, 20 mg/kg, IP) reversed these effects. An increase in blood levels of glucose, triglycerides, total cholesterol, and brain malondialdehyde and nitrite levels was observed in the stressed groups, which was reversed by MH. Furthermore, MH reversed the stress-induced reduction in HDL cholesterol and glutathione (GSH) levels and attenuated stress-induced alterations in the prefrontal cortex and hippocampus.

Conclusion: Our findings suggest that MH attenuated chronic restraint stress-behavioral and biochemical perturbations, probably due to its capability to decrease oxidative stress and brain neuronal damage

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Highlights

- Chronic stress perturbs physiological and psychological homeostasis;
- Morin hydrate normalized chronic stress-induced biochemical disruptions;
- Morin hydrate attenuated structural changes in prefrontal cortex and hippocampus.

Plain Language Summary

Stress is a state of being overwhelmed by demands exceeding the personal and social means of coping. Exposure to excessive stress has resulted in disruption of neurochemical and physiological processes, which sometimes manifest as behavioural abnormalities. Therefore to cope with the stressful life style, there is need to develop a therapeutic agent of plant origin. Morin hydrate is a flavonoid with known antioxidant and neuroprotective properties; however, its effect in a stressful condition has not been studied. The study thus evaluated ameliorating effect of Morin hydrate on chronic restraint stress-induced biochemical disruption, neuronal and behavioral dysfunctions in BALB/c mice. To achieve this, mice were exposed to chronic restraint stress protocol for fourteen days. Behavioural changes were examined using various techniques. The vital parameters like antioxidant, glucose and nitrite levels were also taken. Our findings show that Morin hydrate prevented behavioral abnormalities and damage to the brain cells. It also inhibited stress-induced biochemical disturbance.

1. Introduction

Stress critically perturbs the physiological and or psychological homeostasis in biological systems. Chronic stress results in the inability of the adaptive stress response to withstand the frequency or intensity of the stressor leading to a state of allostasis (Saraswathi, Sreemantula, & Prakash, 2010). This status triggers a series of complex and integral physiological processes which promotes a coordinated response to the stressful stimuli with outcomes observed in different organs and systems, particularly cardiovascular and central nervous systems (Pardon, M. Ma, & Morilak, 2003; Munhoz, Garcia-Bueno, B., Madrigal, Lepsch, Scavone, & Leza, 2008; Musazzi et al., 2010).

The cardiovascular system's response to stress is directly linked to the sympathetic system hyperactivation, which comprises increased cardiac output and vascular resistance, hyperlipidemia, and stimulation of platelet aggregation, which could engender various cardiovascular diseases (Esch, Stefano, Fricchione, & Benson, 2002). Moreover, several clinical, experimental, and epidemiological studies indicate that hyperlipidemia is a risk factor for dementia, Alzheimer disease, and several neurodegenerative diseases via induction

of oxidative stress, cortico-cerebral mitochondrial dysfunction, and eventual neuronal apoptosis (Kivipelto et al., 2002; Solomon, Kåreholt, Ngandu, Winblad, Nissinen, Tuomilehto, 2007; de Oliveira et al., 2011; Kosari, Badoer, E., Nguyen, Killcross, & Jenkins, 2012; Zhao et al., 2017).

Three major brain regions that are particularly vulnerable to stress and targets of stress hormones are the hippocampus, Prefrontal Cortex (PFC), and amygdala (McEwen, Nasca, & Gray, 2016). These regions undergo structural remodeling, altering behavioral and physiological responses (Arnsten, Raskind, Taylor, & Connor, 2015). Studies indicate that chronic stress activates hippocampal glucocorticoid receptors, promotes dendritic atrophy, and decreases neurogenesis and cell survival (Munhoz et al., 2008; Rothman and Mattson, 2010). In addition, chronic stress exposure dynamically regulates dendritic complexities by increasing or decreasing the complexity of dendrites depending on the brain area (Lucassen et al., 2010). Researchers have reported architectural changes induced by chronic stress in the PFC and hippocampus compared with other brain regions (McEwen, 2004). Also, they have reported abnormal alterations in plasticity in the hippocampal CA3, CA1, and dentate gyrus regions, where a reduction in total volume was observed (McEwen et al., 2016). Findings of the hippocampus provide an insight into the involve-

ment of the PFC in stress and stress-associated behaviors (McEwen and Morrison, 2013). As is the case for hippocampal cells, chronic stress also causes reversible shrinkage of neuronal dendrites in the medial prefrontal cortex (Gray, Rubin, Hunter, & McEwen, 2014).

Poor stress management can result in several neuropathologies, as well as contribute to impairment of behavior performance, memory deficits, depressive-like behavior, and anxiety. Thus, stress coping strategies must be devised and personalized to every individual. Successful coping strategies include using natural plant-based compounds called adaptogens, which augment resistance to stress while normalizing the body's overall physiological functions (Panossian, Wikman, Kaur, & Asea, 2010; Pawar and Hugar, 2012). Recent researchers have focused on flavonoids because strong evidence supports the beneficial roles of these plant molecules on the brain (Spencer, 2007; Kumar, & Pandey, 2013; Rendeiro, Rhodes, & Spencer, 2015; Wang et al., 2016; Ramezani et al., 2016). One of the most important characteristics of flavonoids is their antioxidant activity and their ability to suppress the formation of reactive oxygen species, which justifies their neuroprotective effects (Gottlieb et al., 2006; Kim et al., 2010; Jung et al., 2010; Lee et al., 2012; Wang et al., 2016; Ramezani et al., 2016). Among these flavonoids is the flavonoid Morin Hydrate (MH).

Morin hydrate (3, 5, 7, 2', 4'-pentahydroxyflavone) is a polyphenolic compound found in many fruits and vegetables such as guava, orange, sweet chestnut, almond, and onions (Wijeratne et al., 2006; Bhakuni et al., 2017). Several studies have demonstrated the neuroprotective (Gottlieb et al., 2006; Campos-Esparza et al., 2009; Zhang et al., 2010), anti-inflammatory (Gálvez et al., 2001; Manna, Aggarwal, Sethi, Aggarwal, & Ramesh, 2007), and cardioprotective (Al-Numai, Chandramohan, & Alsaif, 2012; Wang, Yang, Qin, Shan, F., & Ren, 2013) activities of MH both in vitro and in vivo by its potent antioxidant and free radical scavenging capacities (Wijeratne et al., 2006; Subash, & Subramanian, 2009; Jonnalagadda, Pittala, Lahkar, & Pradeep, 2013; Singh, Jakhar, & Kang, 2015). We reported that MH reduced distortion of CA1 pyramidal neurons in mice exposed to REM sleep deprivation via its antioxidant activities (Olonode et al., 2019) and also protected against some behavioral and biochemical perturbations induced by acute stress (Olonode et al., 2018). However, a paucity of evidence indicates its protective effect on chronic stress. Therefore, the present study investigated the effect of MH on structural changes in the PFC and hippocampal CA3 neurons and behavioral disorders induced by chronic restraint stress in mice.

2. Materials and Methods

Animals

We used adult male BALB/c mice (22-25 g) in the study. The animals were housed six per cage and maintained at ambient temperature and 12/12 h light-dark cycle in a controlled environment and fed standard laboratory food and water ad libitum. The mice were allowed to acclimate to laboratory conditions for 7 days before the commencement of the experiment. The experimental protocol was approved by the University of Ibadan Animal Ethics Committee (UI-ACUREC/App/2015/067) and carried out following the National Institute of Health Guide for the Care and Use of Laboratory Animals. All experiments were conducted between 9 AM and 2 PM, and efforts were made to minimize animal suffering and reduce the number of animals used in the experiments.

Drug preparation and administration

Morin hydrate, MH, the test compound (Sigma-Aldrich, USA), and ginseng, which serves as a standard adaptogen (Korea Pharma Ltd, South Korea), were used in the study. Doses were selected based on pilot studies and available literature (Olonode et al., 2018; 2019). Both agents were reconstituted with normal saline with the particles uniformly distributed and administered intraperitoneally 30 minutes before daily stress exposure for 14 days. Both were freshly prepared before daily administration. A total of 36 mice were evenly grouped as follows: 1) non-stressed+vehicle, 2) stressed+ vehicle, 3) stressed+5 mg/kg MH, 4) stressed+10 mg/kg MH, 5) stressed+20 mg/kg MH, and 6) stressed+25 mg/kg ginseng.

Stress protocol

Chronic immobilization stress was conducted by the method described (Rai, Bhatia, Palit, Pal, Singh, & Singh, 2003). Animals were pretreated for 30 minutes and then immobilized for two hours per day for 14 days using a plastic hemicylindrical tube of 25 mL capacity with holes for ventilation which restrained all physical movements without inflicting pain. All mice were maintained at room temperature and deprived of food and water throughout immobility. The control group's animals (non-stressed) received the vehicle and were also deprived of food and water for two hours per day during the same period of stress. On the 14th day, 30 minutes after the last stress exposure, all animals were subjected to the elevated plus maze (EPM) and forced swimming test (FST) and, after that, sacrificed for the biochemical and histological studies.

Behavioral tests

Elevated plus-maze test

The test was carried out by the protocol previously described (Walf and Frye, 2007). The EPM apparatus comprises two opposite open arms (30×5×0.25 cm) and two closed arms (30×5×15 cm) emerging from a common central platform (5×5 cm) and elevated to a height of 50 cm above the floor level. Mice were placed individually in the center of the apparatus facing an open arm. The time spent in each arm and the number of entries into each arm were observed and recorded by a blind observer for 5 minutes. After each trial, the maze was cleaned with 70% ethanol to prevent olfactory cues. A mouse is considered to make an entry when all four paws have crossed the line between the arm and the central area. Anxiolytic action was defined by increasing time in and or some entries into open arms and consequently decreasing in time in and or entry into the closed arms.

Forced swimming test (FST)

A FST was carried out according to the method described by Porsolt et al. (Porsolt, Bertin, & Jalfre, 1977) with slight modifications. Briefly, mice were individually forced to swim in an open Plexiglas cylinder (height 25 cm, diameter 18 cm) filled with water to a 15-cm depth at 25°C±1°C for 6 minutes. The total duration of immobility in seconds was recorded by a blind observer during the last 4 minutes of a single 6-min test session. Each mouse was judged to be immobile when it stopped struggling and remained motionless in the water, except for the movements required to maintain its head above the water.

Biochemical estimations

Following behavioral tests, blood was withdrawn from the retro-orbital plexus of the animals into plain tubes. Whole blood was tested for glucose levels using a blood glucose monitoring meter (AccuCheck performer meter), while serum was used to determine cholesterol and triglycerides. After that, mice were sacrificed by cervical dislocation, and the whole brain was removed and homogenized (1:10 w/v) in NaHP04 (0.1 M, pH=7.4). Tissue homogenates were centrifuged at 10000 rpm at 4 °C for 15 minutes, and the supernatants obtained were used to quantify glutathione (GSH) levels, malondialdehyde (MDA), and nitric oxide. The protein content was quantified using bovine serum albumin as standard (Gornall, Bardawill, & David, 1949).

Histological studies

Animals were perfused with 0.9% dextrose saline followed by 4% paraformaldehyde (PFA). Brain tissues were excised and fixed in 4% w/v paraformaldehyde. Transverse sections (5–6 µm thick) were obtained from the PFC and CA3 region of the hippocampus using a microtome (Leica, Germany), and the sections were fixed on glass slides. Hematoxylin and eosin (H&E) staining was carried out to demonstrate the general histological profiles of the brain regions according to the method described (Eltony & Elgayar, 2014). Images were acquired using an Optronics digital camera connected to a computer interface (MagnaFire) and an Olympus BX-51 binocular research microscope. The general structure of the pyramidal cells, periglomerular, and granule cells was characterized using inter-reader variability. Viable neuronal cells were defined as round-shaped, cytoplasmic membrane-intact cells without any nuclear condensation or distorted aspect. The number of viable neurons was determined using ImageJ software and counted as a ratio of viable neuronal cell counts to the square area of the circular view in a section.

Statistical analysis

The results are presented as the Mean±SEM. Statistical significance was determined by 1-way ANOVA followed by a post hoc Newman-Keul's test. GraphPad Prism software v. 4.03 was used for statistical analysis. Statistical significance was set at P<0.05.

3. Results

Morin hydrate and reduced immobility time induced by chronic restraint stress

Chronic restraint stress enhanced immobility time in the FST compared with the vehicle unstressed group. This effect reflects a state of despair which is a symptom of depressive-like behavior. One-way ANOVA revealed a significant effect of MH and ginseng. However, post hoc comparison indicated that chronic treatment with MH (5, 10, and 20 mg/kg) significantly (P<0.05) reduced the stress-induced immobility compared with the stressed+ vehicle group, indicating an antidepressant effect (Figure 1).

Effect of morin hydrate on chronic restraint stress-induced anxiety-like behavior

Figures 2 and 3 illustrate the effect of chronic restraint stress on the frequency and time spent in the open and closed arms of the EPM and the modulatory role of

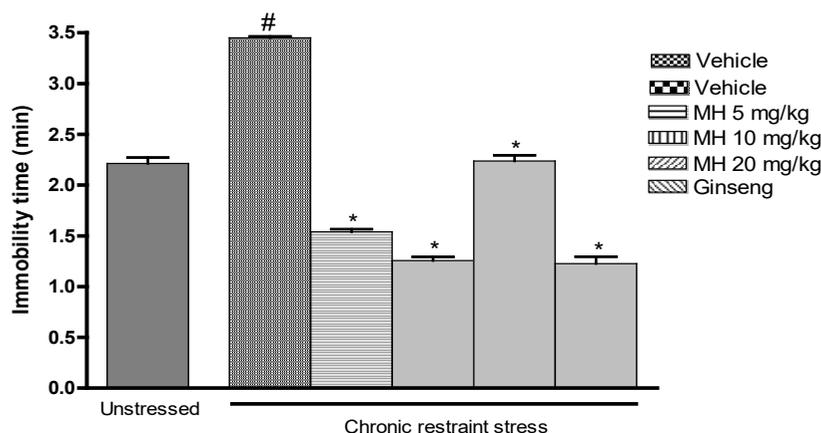


Figure 1. Effect of morin hydrate on immobility time in the FST after chronic restraint stress

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Each result is expressed as Mean±SEM (n=6). Statistical analysis was performed by 1-way ANOVA followed by the Student-Newman-Keuls post hoc test.

Indicates significant difference from the non-stressed+vehicle group (P<0.05), * indicates significant difference from stressed+vehicle group (P<0.05).

MH. One-way ANOVA revealed a significant difference among all treatment groups. Post hoc analysis indicated that chronic restraint stress significantly reduced the time spent in the open arms (P<0.01) and the frequency of open arms entry (P<0.001), as a result, significantly (P<0.001) increased time spent and frequency of closed arms entry in the EPM as shown in Figures 2 and 3, respectively. Treatment with MH (5, 10, and 20 mg/kg, IP) and ginseng (25 mg/kg) significantly prolonged the time spent in the open arm (P<0.01) and increased the

frequency of open arms entry (P<0.05) compared to stressed+vehicle (VEH) group.

Attenuating effect of morin hydrate on chronic restraint stress-induced hyperglycemia in mice

Figure 4 shows the effect of chronic restraint stress on blood glucose levels in mice. Chronic restraint stress increases glucose levels. One-way ANOVA revealed a significant difference among treatment groups. The post hoc test indicated a significant increase in serum

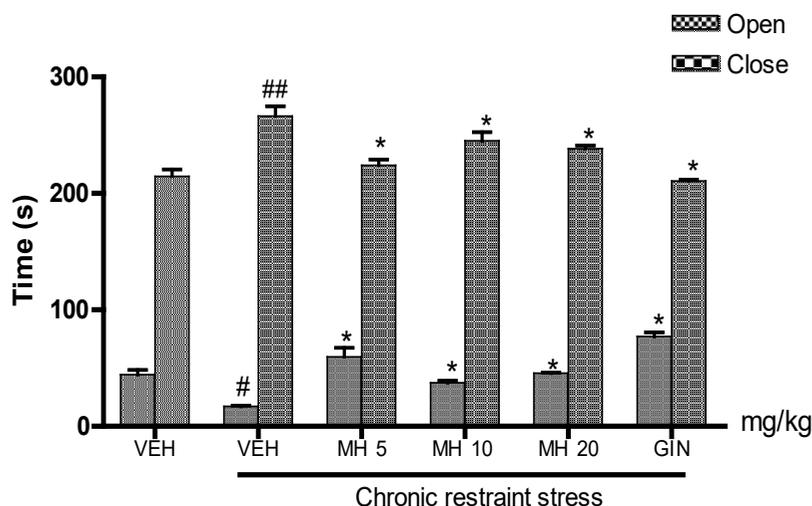


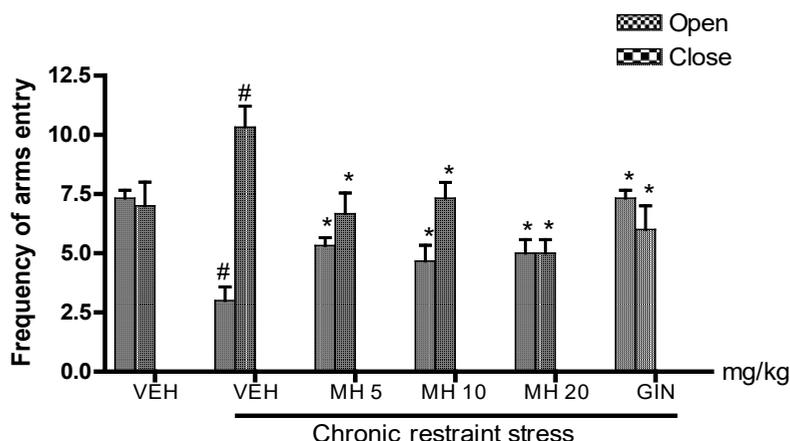
Figure 2. Effect of morin hydrate on time spent in both arms of the Elevated Plus Maze (EPM) after chronic restraint stress

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The values are expressed as Mean±SEM (n=6).

#, ## Indicates significant difference from the non-stressed+vehicle group (P<0.01 and P<0.001, respectively).

* Indicates significant difference from the stressed+vehicle group (P<0.001).



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Figure 3. Effect of Morin Hydrate on the frequency of entry into both arms of the EPM after chronic restraint stress

All values are expressed as Mean±SEM (n=6).

Indicates significant difference from the non-stressed+vehicle group (P<0.01); * indicates significant difference from the stressed+vehicle group (P<0.05).

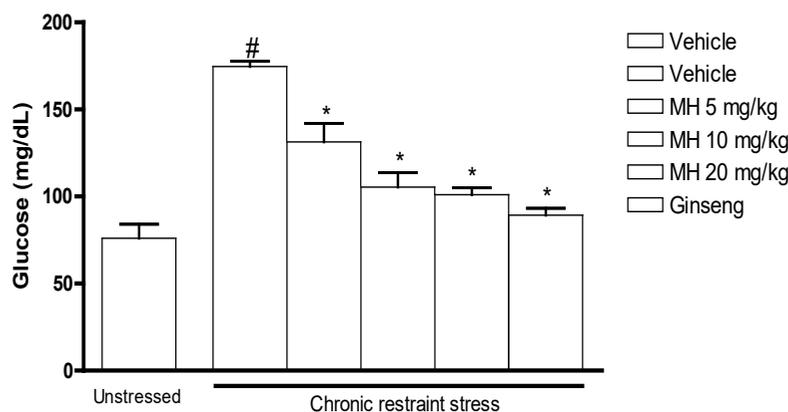
glucose level in the stressed+vehicle group compared to the non-stressed+vehicle group (P<0.05). Morin hydrate (5, 10 and 20 mg/kg) significantly (P<0.05) attenuated the effect observed.

The effect of stress on serum triglycerides is variable. In this study, chronic restraint stress-induced the mobilization of lipids as observed by the significant (P<0.05) increase in blood triglyceride and total cholesterol compared with the control. Pretreatment with MH (5, 10, and 20 mg/kg) significantly (P<0.05) reduced triglyceride and cholesterol levels. Consequently, chronic restraint stress significantly (P<0.05) decreased the level of HDL cholesterol which was potentiated by pretreatment with MH. Serum arteriosclerot-

ic index was significantly (P<0.05) reduced in the treatment group compared with the stressed+vehicle group (Table 1). This effect is attributed mainly to the suppression of the total cholesterol concentration and increased High-Density Lipoprotein (HDL) cholesterol concentrations.

Morin hydrate protects mice brains against lipid peroxidation induced by chronic restraint stress

Figure 5 shows the effect of MH on chronic restraint stress-induced lipid peroxidation. In this study, we measured brain MDA, a late product of lipid peroxidation. One-way ANOVA revealed a significant difference among the treatment groups. Post hoc comparison indi-



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Figure 4. Effect of morin hydrate on blood glucose in mice exposed to chronic restraint stress

All values are expressed as Mean±SEM (n=6).

indicates significant difference from the non-stressed+vehicle group (P<0.05), * indicates significant difference from the stressed+vehicle group (P<0.05).

3.4. Morin Hydrate suppresses chronic restraint stress-induced hyperlipidemia in mice

Table 1. Effect of Morin Hydrate on chronic restraint stress-induced changes in blood lipids (n=6)

| Treatment (mg/kg) | Mean±SEM | | | |
|-------------------|-----------------------|---------------------------|-------------------------|-----------------------|
| | Triglycerides (mg/dL) | Total Cholesterol (mg/dL) | HDL Cholesterol (mg/dL) | Atherosclerotic Index |
| VEH(uns) | 72.33±2.4 | 55.33±1.8 | 36.00±2.3 | 0.55±0.07 |
| VEH (str) | 107.0±2.9# | 76.33±3.5# | 25.00±0.6# | 2.06±0.2# |
| MH5 | 93.67±2.9* | 66.33±1.8* | 34.00±1.2* | 0.96±0.1* |
| MH10 | 89.67±3.2* | 64.67±1.5* | 34.33±0.9* | 0.89±0.1* |
| MH20 | 82.33±3.8* | 58.00±1.5* | 35.67±1.2* | 0.63±0.05* |
| GIN | 72.67±2.3* | 56.00±2.1* | 36.00 ±1.0* | 0.56±0.09* |

#Indicates significant difference from the non-stressed+vehicle group (P<0.05); *Indicates significant difference from the stressed+vehicle group (P<0.05).

ANOVA: analysis of variance; ATP: adenosine triphosphate; BDNF: brain derived neurotropic factor; Ca²⁺: calcium ion; CA3: cornus ammonis; CNS: central nervous system; DTNB: 5'-dithio-bis-[2-nitrobenzoic acid]); EDTA: ethylenediaminetetraacetic acid; EPM: elevated plus maze; FST: forced swim test; GSH: reduced glutathione; H&E: hematoxylin and eosin; HDL: high-density lipoprotein; HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A; HPA: hypothalamic pituitary adrenal; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; MDA: malondialdehyde; MH: morin hydrate; NaHP04: sodium phosphate buffer; PFC: prefrontal cortex; TCA: trichloroacetic acid; TBA: 2-thiobarbituric acid; Tris-KCl: tris- potassium chloride; VLDL: very low-density lipoprotein.

cated a significant (P<0.001) increase in the production of MDA in the stressed+vehicle group compared to the non-stressed+vehicle group, indicating oxidative damage to membranes. Pretreatment with MH (5, 10, 20 mg/kg) significantly (P<0.001) suppressed MDA production.

Morin hydrate ameliorate oxidative/nitrosative damage in mice brain

GSH is the main endogenous antioxidant in mammalian cells and plays a role in the maintenance of the redox

balance and prevention of cell damage via detoxification of reactive oxygen species. In this study, chronic restraint stress significantly (P<0.001) decreased GSH levels in the brain compared with the non-stressed vehicle group. Post hoc comparison revealed that pretreatment with MH (5, 10, 20 mg/kg) significantly (P<0.001) abolished the stress-induced reduction in GSH levels (Figure 6). Furthermore, chronic restraint stress caused a significant increase in nitrosative brain damage, as indicated by the elevation in brain nitrite level in the stressed+vehicle

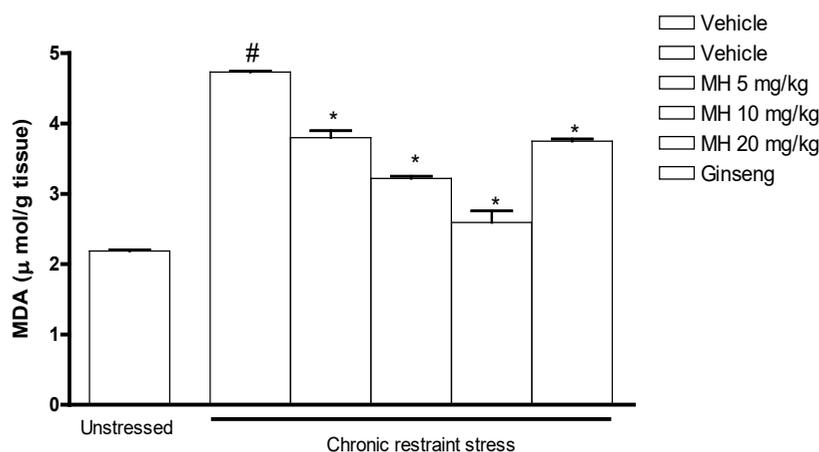
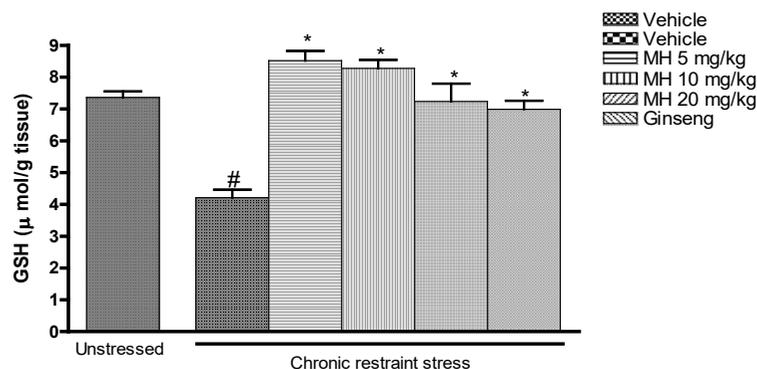


Figure 5. Effect of morin hydrate on MDA levels in mice brain after exposure to chronic restraint stress

Each result is expressed as Mean±SEM (n=6).

#Indicates significant difference from non-stressed+vehicle group (P<0.001), *Indicates significant difference from stressed+vehicle group (P<0.001).



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Figure 6. Effect of Morin Hydrate on Glutathione (GSH) levels in mice brain after exposure to chronic restraint stress

The values are expressed as Mean±SEM (n=6).

Indicates significant difference from non-stressed+vehicle group (P<0.001), *Indicates significant difference from stressed+vehicle group (P<0.001).

group compared to the non-stressed+vehicle group, as shown in Figure 7. Morin hydrate (5, 10, 20 mg/kg) significantly (P<0.001) abolished this effect.

Morin hydrate exhibits a protective effect on chronic restraint stress-induced neuronal damage

Histological studies of the PFC and hippocampus revealed marked alteration in neuron morphology in each stress group. The number of intact neurons in the PFC and hippocampal CA3 pyramidal layer was significantly (P<0.001) decreased in the stressed+vehicle group compared to the unstressed+vehicle group, and some irregularities in the CA3 pyramidal layer were indicated by the various dark chromatin nuclei which suggesting neuronal degeneration (Figures 8 and 9). Regardless of damaged neurons, MH (5, 10, and 20 mg/

kg, IP.) significantly increased the number of intact neurons containing large, round, and regular nuclei. Furthermore, quantitative assessment of neurons revealed that chronic restraint stress decreased significantly (P<0.001) the density of viable neurons in both regions as observed in the stressed+vehicle group compared to the unstressed+vehicle group. However, 1-way ANOVA followed by Newman-Keul post hoc comparison revealed that MH (5, 10, and 20 mg/kg, IP) significantly (P<0.001) reduced this effect. Magnification: (x400).

4. Discussion

The chronic restraint stress model has been suggested to trigger biochemical alterations which can be detrimental to CNS function. The model which involves the restriction of mobility represents a combination of physi-

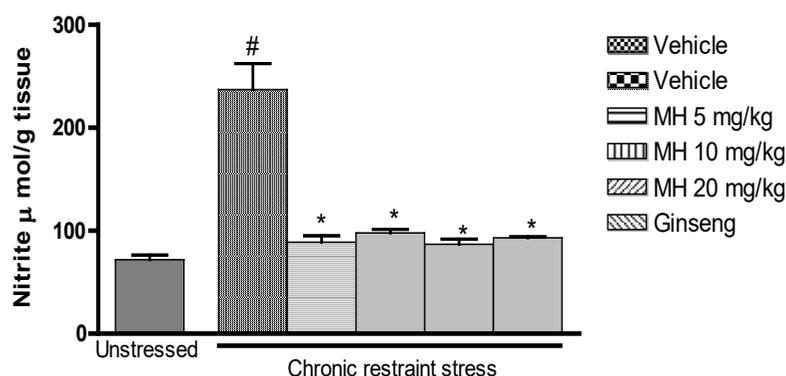


Figure 7. Effect of Morin Hydrate on nitrite levels in mice brain after exposure to chronic restraint stress

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All values are expressed as Mean±SEM (n=6).

#Indicates significant difference from non-stressed+vehicle group (P<0.001), *Indicates significant difference from stressed+vehicle group (P<0.001).

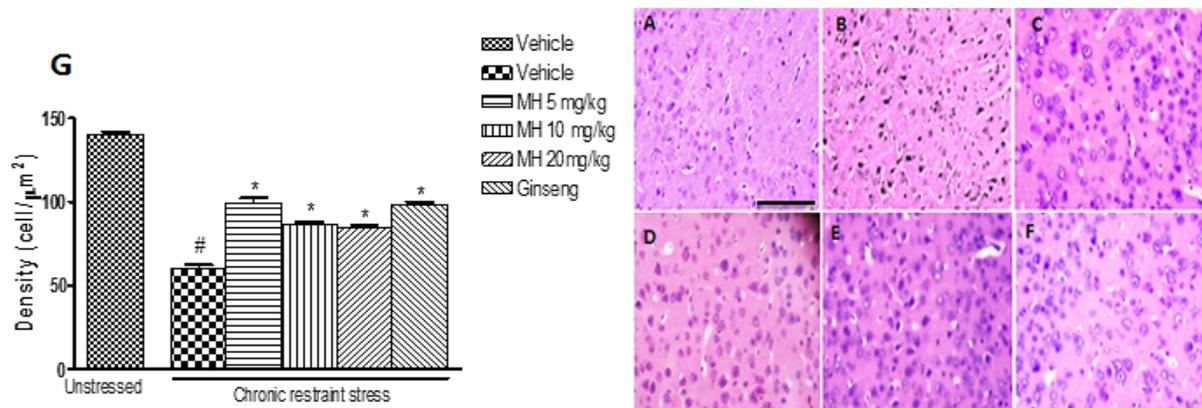


Figure 8. Photomicrograph of the PFC of mice exposed to chronic restraint stress

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A) Non-stressed+vehicle, B) Stressed+Vehicle, C) Stressed+5 mg/kg MH, D) Stressed+10 mg/kg MH, E) Stressed+20 mg/kg MH, F) Stressed+25 mg/kg Ginseng, G) Quantitative Assessment of the Data From Figure 8, Parts A-F

Counts are based on the number of neuronal nuclei in three rectangular boxes per slide using pre-calibrated ToupView software.

#Indicates significant difference from non-stressed+vehicle group ($P < 0.001$), *Indicates significant difference from stressed+vehicle group ($P < 0.001$).

cal and emotional stress resulting in aggression, depression, and anxiety (Kulkarni & Juvekar, 2008). The FST assesses depressive-like behavior in rodents. The test is based on a feeling of despair or helplessness and measures the immobility time of animals exposed to some inescapable and confined space (Doreddula, Bonam, S. Gaddam, Desu, Ramarao, & Pandey, 2014). An increase in immobility time indicates behavioral despair, which is thought to be an index of depression (Cryan & Mombereau, 2004; Moretti et al., 2013). In the present study, a decrease in immobility time was observed in the FST,

indicating that MH attenuates stress-induced depressive-like symptoms in mice.

The EPM paradigm is employed to assess innate anxiety-like behavior in rodents and the typical markers of anxiety behavior are the frequency and duration of exploration in open arms. When rodents are placed in the maze, they tend to avoid the open arms and prefer to stay in the enclosed arms, which is an index of anxiety or fear. One hallmark of anxiolytic drugs is their ability to reduce anxiety-like responses of rodents in the EPM. Anxiolytic-treated animals tend to explore open

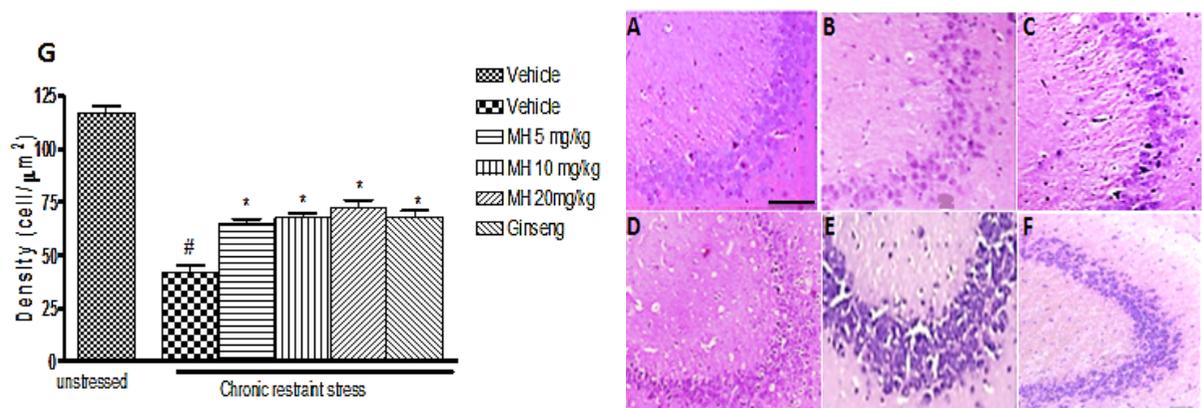


Figure 9. Photomicrograph of the CA3 hippocampal cells of mice exposed to chronic restraint stress

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A) Non-Stressed+Vehicle, B) Stressed+Vehicle, C) Stressed+5 mg/kg MH, D) Stressed+10 mg/kg MH, E) Stressed+20 mg/kg MH, F) Stressed+25 mg/kg Ginseng, G) Quantitative Assessment of the Data From Figure 9, Parts A-F

Effects of ginsenoside (Rg1) on neuronal injury in the frontal cortex and hippocampus in chronic DEX-exposed mice (H&E staining, x400).

Counts are based on the number of neuronal nuclei in three rectangular boxes per slide using pre-calibrated ToupView software.

#Indicates significant difference from non-stressed+vehicle group ($P < 0.001$), *indicates significant difference from stressed+vehicle group ($P < 0.001$).

arms more than their untreated counterparts. Several plants and flavonoids have demonstrated anxiolytic effects, as shown by their ability to increase exploration of the open arms and, therefore, are highly used as anxiolytics in traditional medicine (Almeida, Rafael, Couto, & Ishigami, 2009). The increase in duration, as well as frequency of open arm exploration of animals treated with MH, shows that it may possess anxiogenic-attenuating and anxiolytic-like properties.

Several lines of study on chronic stress have reported induction of type 1 diabetes mellitus in humans and different experimental animal models (Mirshekar, Arabmoazen, Parivar, & Sarkaki, 2015), in which corticosterone was found to play a crucial role in glucose metabolism via induction of glycogenolysis in the liver that results in glucose mobilization which ultimately contributes to the hyperglycemia observed. Also, according to Kyrou and Tsigos (2009), diabetes and depression share a common link in which chronic stress causes activation of the hypothalamus-pituitary-adrenal axis and sympathetic nervous system resulting in excessive production of cortisol. This condition increases obesity, insulin resistance, and disruption of neurogenesis in the hippocampus (Herbert et al., 2006). In this study, the observed significant rise in glucose level was significantly reversed by morin hydrate treatment, and this confirms the report of Singh, Bagachi, Semwal, Kaur, & Bharadwaj (2013) that *Morus alba*, the source of MH, had an anti-diabetic effect by increasing insulin production.

Elevated circulatory lipids and lipoproteins have characterized some cardiovascular and metabolic disorders (Artham, Lavie, Milani, & Ventura, 2008), which may lead to CNS changes and trigger co-morbid diseases. For instance, depression and diabetes mellitus are co-morbid diseases that affect millions of people worldwide, and people diagnosed with diabetes mellitus are more predisposed to depression. Reports indicate that chronic stress triggers metabolic diseases by elevating triglyceride and cholesterol levels via mechanisms associated with enhancement of HPA axis activity and increase of catecholamine (Lakshmi & Sudhakar, 2009; Nayanatara et al., 2012). Catecholamines activate lipolysis via stimulation of adipose tissues β_3 adrenoceptors and suppression of insulin secretion. Similarly, glucocorticoids and free fatty acids increase HMG-CoA reductase activity in hepatocytes to promote cholesterol synthesis. Additionally, catecholamines suppress hepatic lipase activity and raise blood low-density lipoprotein, intermediate-density lipoprotein, and very-low-density lipoprotein concentrations (Rosenfeld, 2012; Parker, 2013). Kulkarni and Juvekar (2008) reported elevated triglycer-

ide and cholesterol levels in rodents exposed to chronic restraint stress. Furthermore, Tan et al. (2006) reported a low HDL level in animals exposed to chronic restraint stress. In this study, MH significantly reverses chronic restraint stress-induced increase in triglyceride and cholesterol levels, attenuates the stressed-induced decrease in HDL, and consequently lowers atherosclerotic index, thus indicating MH's protective effect against stress-induced diseases.

Studies revealed that chronic restraint stress, like other stress models, produces an oxidant/antioxidant imbalance, resulting in excessive free radicals production, instigating lipid peroxidation, particularly in cell membranes (Sahin & Gumuslu, 2007; Kumar, Garg, & Prakash, 2010; Freitas et al., 2014). Several studies indicate that chronic restraint stress induces lipid peroxidation and nitrite production and attenuates the endogenous antioxidant system activity (Ahmad et al., 2012). Similarly, induction of oxidative stress (increased brain MDA and nitrite levels and decreased GSH activity) was observed following the chronic restraint stress paradigm. MH ameliorated these effects, indicating its antioxidant activity.

Neurons are the basic functional and structural components of the nervous system. Several factors, such as stress, alter the structural makeup of neurons, in particular their dendritic arborization, synaptic junctions, neurochemical components, and functions (Hemamalini, 2013). Chronic stress exposure dynamically alters the complexity of dendrites, specifically in the neurons of the PFC, amygdala, and hippocampus. Dendritic atrophy in these regions is observed during prolonged stress exposure which may affect their various functions (Krugers, Hoogenraad, & Groc, 2010). This effect could be due to reduced brain-derived neurotrophic factor expression, apoptosis of the neurons, glucocorticoid toxicity, reduced functionality of the GABAergic network, glutamate-induced excitotoxicity, or increased intracellular levels of Ca^{2+} (Hemamalini, 2013). Elevated Ca^{2+} levels cause the breakdown of microtubules and activation of calcium-activated neutral proteinase: the enzyme which controls cytoskeletal proteins disintegration (Hemamalini, 2013). This condition results in retraction and collapse of dendrite branches because the structural integrity of neuronal processes requires stable microtubules (Hemamalini, 2013).

Several anti-stress agents have been shown to rescue neurons from death and reduce dendritic atrophy in stress conditions via mechanisms such as induction of neurogenesis, formation of new dendrites (neurostimulation effect), or stimulation of corticotrophin-releasing factor, which may at the same time strengthen hippocam-

pal synaptic efficacy (Hemamalini, 2013). Recently, the researchers have focused on the neuroprotective activities of flavonoids since some flavonoids protect neuronal cells against stress-induced neuronal injury via their free radicals scavenging capacity and ability to mitigate excessive glutamate-induced oxidative damage (Lee et al., 2000; Ramezani et al., 2016). According to previous reports, this study confirms structural damage in the CA3 and PFC neurons caused by chronic restraint stress. Accordingly, MH significantly attenuated the structural damage in these brain regions; thus, it had neuroprotective effects, possibly due to its antioxidant activity. Moreover, evidence demonstrates that flavonoids can cross the blood-brain barrier (Youdim, Qaiser, Begley, Rice-Evans, & Abbott, 2004), and the ability of MH to cross the blood-brain barrier in various neurological disorders has been well-established (Gottlieb et al., 2006; Campos-Esparza et al., 2009; Zhang et al., 2010).

5. Conclusion

The outcome of our study indicates that MH protects against some detrimental changes induced by chronic restraint stress, such as oxidative stress, hyperglycemia, hyperlipidemia, and neuronal damage. Thus, MH may be suggested as a putative anti-stress agent because it has remarkably low cytotoxicity in cell cultures and animal models, especially since it is a common constituent of several fruits, vegetables, and herbs.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the University of Ibadan Animal (UI-ACUREC/App/2015/067).

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Authors' contributions

Conceptualization: Elizabeth Akinluyi; Supervision: Adegbuyi Aderibigbe; Methodology: Elizabeth Akinluyi, Adaeze Adebisin, Gladys Adeoluwa; Writing-original draft: Elizabeth Akinluyi; Writing-reviewing & editing: All authors; Data collection and data analysis: Elizabeth Akinluyi and Olusegun adeoluwa.

Conflict of interest

The authors declared no conflict of interest.

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