Pharmacol Toxicol Nat Med 2025, 5(3&4): 19-28
DOI: 10.52406/ptnm.v5i3-4.116

Methanol Leaf Extract of *Andrographis paniculata* Reverses Behavioral and Neuro-biochemical Changes Induced by Chronic Restraint Stress in Mice

Joseph A. Badejo[1], Olasubomi F. Sotunde[1], Mary O. Ologe[3], Obaro S. Michael[1], Babatunde A. Alabi[2], Emmanuel B. Ileri-Oluwa[1], Ezekiel O. Iwalewa[1*]

[1]Neuropharmacology, Cardiovascular Pharmacology and Ethnopharmacology Units, Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria. [2]Department of Pharmacology & Therapeutics, Faculty of Basic Clinical Sciences, Bowen University, Iwo, Nigeria. [3]Department of Pharmacology and Therapeutics, College of Health Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria.

***Corresponding Author:** eoiwalewa@yahoo.com* ***Phone:** +234 8033540641*

ARTICLE HISTORY
Received: 9th May, 2025
Accepted: 26th June, 2025

KEYWORDS
Andrographis paniculata, Chronic restraint stress, Hyperglycemia, Oxidative stress, Pro-inflammatory cytokines, Corticosterone

Pharmacology and Toxicology of Natural Medicines ISSN: 2756-6838

Published by ***Phytomedicine Research Group,*** *Department of Pharmacology & Toxicology, Faculty of Pharmacy, University of Benin, Benin City 300001, Nigeria*

ABSTRACT

Background and Purpose: Chronic stress and behavioral activities have recently been linked with hyperglycemia. The neurobehavioral effect and neuro-biochemical changes following the administration of *Andrographis paniculata* leaf extract were evaluated in mice using the chronic stress paradigm.

Methods: Male mice were assigned to 6 groups (n=6). Groups 1and 2 received distilled water and groups 3, 4, 5 were administered 80, 160 and 320 mg/kg of methanol extract of *Andrographis paniculata* (MEAP) respectively while group 6 which was the positive control was administered pioglitazone 15 mg/kg. All treatments were by the oral route. The animals were subjected to 4 cycles of restraint stress (2 h per day) in which a cycle lasted 10 days. Animals were stressed for 7 days and allowed to rest for 3 days. On the 39th day in the 4th cycle, animals were tested for neurobehavioral phenotypes: depression, anxiety and memory impairment. Blood was collected for corticosterone and insulin quantification, while the adrenal gland was weighed. Brains were collected for estimation of malondialdehyde (MDA), superoxide dismutase (SOD), catalase, (CAT), nitric oxide (NO), reduced glutathione (GSH), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6) and acetylcholinesterase (AchE).

Results: MEAP reversed hyperglycemia caused by chronic restraint stress (CRS), reduced anxiety, and memory impairment. It caused an increase in the level of SOD with a corresponding reduction in the levels of NO. Adrenal weight were not significantly changed. The levels of MDA were not significantly reduced while the levels of GSH and CAT were not significantly elevated. MEAP also reduced CRS-induced elevation in corticosterone, AChE, TNF-α, and IL-6 levels and reversed CRS-induced decrease in serum insulin levels.

Conclusion: The results suggest that the extract of *Andrographis paniculata* reversed the complications of hyperglycemia induced by exposure to chronic stress through the modulation of antioxidant system and reduction of pro-inflammatory cytokines.

INTRODUCTION

Stress is a state of being overwhelmed by demands exceeding the personal and social means of coping in the prevailing situation (Doreddula *et al.,* 2014). Living organisms exposed to stress undergo some physiological, morphological and biomedical modifications to survive. These modifications are necessary to maintain the normal homeostatic environment of the living organism. At the same time, insufficient or excessive activation of the stress system can disturb the innate physiological and behavioral functions of the organism. One of the modifications that occur during stressful situations in living organisms is the release of glucose which helps in combating stressful situations by enabling the flight or fight response (MacCarty, 2016). When the body of a living organism is stressed, the body through the anterior pituitary and adrenal cortex releases cortisol which acts on the liver to produce glucose via gluconeogenesis and glycogenolysis. When the body is stressed, it undergoes various strains to cope with the demands. This brings about various physiological and biological changes that can cause illnesses. If stress is continuous, it causes adverse effects on the body's central nervous system, cardiovascular system and the neuro-endocrine system. This continuous stress, which is also referred to as chronic stress may result in serious diseases such as high blood pressure, immune-suppression, anxiety, obesity, depression, heart disease, and metabolic disorders (Pakhetra *et al*., 2011). These effects are due to the release of hormones aimed at restoring homeostasis during stress. The hypothalamic-pituitary axis (HPA), sympatho-adrenal system and pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) function synergistically to trigger hyperglycemia (Marik and Bellomo, 2013). Also, studies carried out on animals have suggested that stressful experiences occurring throughout life are important to the pathogenesis of mental diseases such as schizophrenia, anxiety, depression and mood disorders (Heim *et al.,* 1999, Cattaneo and Riva 2016., Lee, 2022)

Chronic restraint stress, a well-known model, is a preferred means of stressing animals largely because it is straightforward and painless and without lasting debilitation (Mao et al., 2022). Immobilization, a procedural variation of restraint, is commonly described as restricting range of locomotion but does not attempt to limit specific limb movement as do most other restraint techniques. While both restraint and immobilization constitute physical operations, they are also important as models for psychiatric stress (Molina et al., 2023). The psychological and physiological changes associated with restraint appear to result from the distress and aversive nature of having to remain immobile, rather than any concurrent activation of pain mechanisms or irreversible discomfort (Grandin and Deesing, 2002). Thakur *et al.* (2019) reported that oxidative stress and hyperglycemia in patients with diabetes give rise to complications such as depression, anxiety and memory impairments. Depression, anxiety and memory impairments occur because of imbalances in monoaminergic, GABAergic and cholinergic transmissions respectively, coupled with decrease within the antioxidant defense system.

Andrographis paniculata is a plant that belongs to the family Acanthaceae. It is also known as king of bitters and grows annually. It is cultivated extensively in South Asia, some parts of Europe and in China (Josselin and Jeeva, 2014). It has been reported to be beneficial in the treatment of diseases affecting the respiratory system, and infections affecting the skin and lower urinary tract infections (Jarukamjorn and Nemoto, 2008). It is also used in the treatment of acute infections, fever, pneumonia, dysentery, tonsillitis, gastroenteritis (Deng *et al.,* 1982; Bensky *et al.*, 1993), and in the treatment of snakebites (Duke and Ayensu, 1985; Mills and Bone, 2000). In the Malaysian folklore, it is used in the treatment of diabetes and hypertension (Zhang and Tan, 1996), while in Ayurvedic medicine, it is used as a bitter tonic for treating diabetes, debility, hepatitis and as an anthelminthic (Bensky *et al.,* 1993). The plant has also been reported to have antibacterial (Hossain *et al* 2021), antioxidant (Hamid *et al* 2023), anti-inflammatory (Zhu *et al.,* 2021) and antihyperglycemic activities (Ogunlana *et al.,* 2021, Sanower *et al.,* 2021).

The beneficial effects of *Andrographis paniculata* in neurological disorders caused by stress have not been fully explored, hence this study was carried out to investigate its effects on stress induced by chronic restraint on hyperglycemic complications, neuro-biochemical and neurobehavioral changes in mice.

MATERIALS AND METHODS

Laboratory Animals

Thirty-six male Swiss mice weighing between 22-27 g were used in the study. They were purchased from the Central Animal House, University of Ibadan, and housed in plastic see- through cages at room temperature. These animals always had free access to food and water and were acclimatized for 2 weeks before the beginning of the study. They were handled in accordance with the National Institutes of Health (NIH) guidelines for Care and Use of Laboratory Animals (NIH 2011) and the institutional ethics committee of the University of Ibadan (Reference: UI-ACUREC/180053).

Drugs and Chemicals

Pioglitazone (International Drug & Chemical Co., India); dimethyl sulfoxide (DMSO), 5,5' dithiobis-2-nitrobenzoic acid (DNTB), and thiobarbituric acid, were obtained from Sigma-Aldrich, USA; sodium carbonate, potassium

carbonate and sodium chloride were manufactured by BDH (Poole, England).

Plant preparation and treatment

Cold maceration of *A. paniculata* leaves was carried out using methanol as the solvent. Ten percent (10%) concentration of extracts were prepared by adding 200 mL of the solvent to a conical flask containing 20 g of *A. paniculata* dry powder. The conical flask was wrapped with cotton and aluminum foil and was placed on an incubator shaker for a day at 37°C with gentle shaking. The mixture was filtered using Whatman No. 1 filter paper. The filtrate was evaporated using a rotary evaporator at 50°C. The concentrated methanol extract of *A. paniculata* (MEAP) was then transferred to a petri dish and dried in an oven at 40°C and packed in air-tight containers (Malahubban, *et al*., 2013). The extract was dissolved in distilled water prior to administration.

Chronic Restraint Stress Procedure and Experimental Design

This is a modified form of restraint whereby each animal was restrained in a 50 mL plastic centrifuge tube with holes made on the tip through which the animal could breathe. Each animal was put in the restrain tube for 4 h every day for 40 days. The chronic restraint stress (CRS) paradigm used for this study was adapted from Zheng *et al.,* 2018 with slight modification. The mice were randomly allotted to groups (n=6). Group 1 administered 10 mL/kg of distilled water; groups 2,3,4, were administered doses of 80, 160, and 320 mg/kg of MEAP respectively; and group 5 administered 15 mg/kg of pioglitazone. All administrations were by the oral route. The doses of MEAP used (80, 160, 320 mg/kg) and pioglitazone were obtained from a previous study (Adegbite *et al.,* 2019).

Animals in groups 2-5 were exposed to CRS for 4 h for 7 days and then allowed to rest for 3 days per cycle, in a total of 4 cycles that spanned 40 days (Figure 1). The fasting blood sugar was taken on the 10th day of each cycle while the neurobehavioral experiments were carried out on the 38th day of the study after exposure to CRS. The weight of adrenal glands and biochemical assays were carried out on the 40th day of the experiment after all animals were sacrificed.

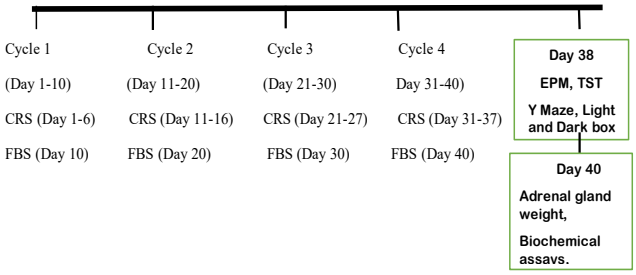

Figure 1: The study design according to the modified model of Zheng *et al*. (2018).

Behavioral Experiments

Test for memory impairment using Y maze

The maze is made up of three equally spaced arms at 120^{o} one to another, each was 41 cm long and 15 cm high. The floor of each arm was 5 cm wide. The mouse was put in arm A and allowed to move in all three arms for 5 min and the percentage alternation was measured. The apparatus was cleaned with 70% ethanol after each animal's removal to prevent odor bias. The neurobehavioral effects of MEAP on scopolamine (SC)-induced memory impairment in mice was assessed by utilizing the Y-maze (Kim *et al*., 2023). Mice in the various groups were pretreated with SC (3 mg/kg) once daily for 7 days. Thirty minutes after the last treatment, the animals were tested for the memory function tests.

Test on anxiety using elevated plus maze

The apparatus was validated for mice by Lister (1987). It is made up of two open arms 16×5×12 cm and two closed arms 16 × 5 × 12 cm (Umukoro *et al*., 2014) emanating from a central platform 5×5 cm. Each mouse was put at the center with the head facing an open arm and allowed to move in the maze for 5 min (Umukoro *et al.,* 2015). The time spent in both open arms was recorded. The apparatus was cleaned with 70% ethanol after each animal's removal to prevent odor bias.

Test on depression using forced swimming test

In this test (Umukoro *et al.,* 2015), animals were dropped one at a time and made to swim in a jar of 10 cm diameter and 25 cm height, containing 19 cm height of water. The animals forced swam for 6 min while duration for immobility and swimming were observed and recorded in the last 4 min. The mouse was taken to be immobile when it stopped swimming and started floating motionlessly.

Test on depression using tail suspension test

Each mouse was individually suspended on an iron bar 55 cm above the floor with an adhesive tape, 1 cm from the tail tip. Duration of immobility was observed in the last 4 min of the total of 6 min of suspension. The mouse was taken to be immobile when it hung passively without struggling (Steru *et al*., 1985).

Determination of Blood Glucose, Serum Insulin and Corticosterone Concentrations

Blood glucose concentration was determined on the last day of each cycle using tail tip blood applied on the strip of a glucometer (Accu-Chek Actives, Roche Diagnostics India Pvt., Ltd). The tip of the tail of each mouse was gently pricked, and blood was collected by gently squeezing the tail. The blood was allowed to clot at room temperature for 30 min, and centrifuged at 1000 *g* for 20 min at 4°C. The supernatant for testing (approximately 20 µL of serum for a single test) was taken. The serum samples for corticosterone determination were treated for an additional 1 h in a 75°C

water bath (to denature the corticosteroid binding globin). The concentration of corticosterone in serum was carried out with the aid of an ELISA kit (Biossay Laboratory Technology, Shanghai 200090, China) and the test was carried out according to manufacturer's instruction. The concentration of insulin in the serum was carried out with the aid of an ELISA kit according to manufacturer's instruction. (EIA-4164; DRG Instruments GmBH, Maburg, Germany).

Assessment of Body and Adrenal Gland Weight

The initial weight of each mouse was taken on the last day of each cycle. Thereafter, the animal was sacrificed by cervical dislocation and adrenal gland was removed and weighed. The relative organ weight was calculated as organ weight/100 g of body weight (Umukoro *et al.,* 2016).

Preparation of Brain Tissues for Neuro-biochemical Assays

The animals were sacrificed by cervical dislocation and the brains were homogenized in ice-cold saline and sodium phosphate buffer (0.1 M, pH 7.4) was added before keeping the mixture in a deep freezer for 30 min. The mixture was centrifuged at 10,000 *g* for 15 min at 4°C and the resultant supernatant was frozen at −20°C for the assay of oxidative parameters (Umukoro *et al.,* 2018).

Determination of Reduced Glutathione (GSH) Concentration

The concentration of glutathione in the whole brain was measured using the method described by Jollow *et al*. (1974), based on the formation of a relatively stable (yellow) color when sulfhydryl compounds are treated with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). Supernatant (0.4 mL) of brain homogenates were combined with 0.4 mL of 20% trichloroacetic acid (TCA) in a gentle swirling motion and then centrifuged at 5400 *g* for 20 min. The solution was then made up to 3 mL with phosphate buffer and 0.25 mL of the supernatant was applied to 2 mL of 0.6 mM DTNB (0.2 M, pH 8.0). A spectrophotometer (Laboratory 752 automatic BH, China) was used to measure absorbance at 412 nm against a blank reagent [2 mL of 0.6 mM DTNB + 1 mL phosphate buffer (0.2 M, pH 8.0)]. Reduced GSH concentrations in brain tissues were measured in nanomoles per milligram protein (nmol/mg protein).

Determination of Catalase (CAT) Activity

Sinha (1972) method for measuring catalase activity was used. It relies on the disappearance of hydrogen peroxide (H_2O_2) in the presence catalase. Brain supernatant (1 mL) was combined with 19 mL of distilled water to make a 1:20 dilution. Then 1 mL of this was mixed with 5 mL of phosphate buffer (pH 7.0) and 4 mL of H_2O_2 to make a solution. A gentle swirling motion was used to blend the reaction mixture at room temperature. Then 1 mL of the reaction mixture was taken out and mixed with 2 mL dichromate/acetic acid reagent. At 570 nm, the absorbance was determined with a spectrophotometer (Visible Spectrophotometer Ultraviolet Spectrophotometer Laboratory 752 automatic BH, China) and the change in absorbance was measured every 60 s. Catalase activity was measured in units of H_2O_2 decomposed per minute per mg protein (Unit/mg protein).

Determination of Lipid Peroxidation

Malondialdehyde (MDA) levels in the whole brain were calculated using the method described by Ohkawa *et al*. (1979). The basis for this assay is that lipid peroxidation produces unstable lipid peroxides, which decompose into a complex series of compounds, including reactive carbonyl compounds. Decomposition of the polyunsaturated fatty acid peroxides produces MDA. When heated in an acidic pH, MDA forms a 1:2 adduct with thiobarbituric acid (TBA), resulting in a pink colour product with a maximum absorbance of 532 nm. In this case, an aliquot of 0.4 mL of the sample was combined with 1.6 mL of Tris-KCl buffer, to which 0.5 mL of 30% trichloroacetic acid was added. After that, 0.5 mL of 0.75% TBA was added, and the mixture was put in a water bath set at 80°C for 45 min. The mixture was cooled in ice and centrifuged for 15 min at 1200 *g*. The absorbance of the clear supernatant was measured at 532 nm against a reference blank of distilled water. The molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used to measure the MDA concentration, which was expressed in nanomoles MDA per mg protein (nmol MDA/mg protein).

Estimation of Brain Nitrite Level

Nitrite levels, a measure of nitric oxide (NO), were measured in the mice whole brain homogenates according to the method described by Green *et al*. (2000). The output of nitrite was calculated using the Griess reaction. For 10 min at room temperature, 10 mL of supernatant was incubated with 100 mL of Griess reagent (sulfanilamide in 1% phosphoric/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/1% phosphoric acid/distilled water, 1:1:1:1) (Sigma-Aldrich, St. Louis, MO, USA). A UV spectrophotometer was used to take the absorbance at 550 nm. Several concentrations of $NaNO_2$ were used to create the typical curve (ranging from 0.75 to 100 µM) and was expressed as µmol/mg tissue.

Estimation of Superoxide Dismutase (SOD) Concentration

The Misra and Fridovich (1972) method was used for the estimation of SOD activity in whole brains of mice. The inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer set at 480 nm is the basis for this process. A 1: 10 dilution was made by adding 1 mL of brain supernatant to 9 mL of distilled water. To

equilibrate in the spectrophotometer, an aliquot of 0.2 mL of the diluted sample was applied to 2.5 mL of 0.05 M carbonate buffer (pH 10.2), and the reaction was initiated by adding 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture, which was easily combined by inversion. The blank in the reference cuvette was made up of 2.5 mL of carbonate buffer, 0.3 mL of substrate (adrenaline), and 0.2 mL of distilled water. A UV-spectrophotometer (752N INESIA, China) was used to take the absorbance at 480 nm for 30 s and 150 s. The amount of SOD required to inhibit the oxidation of adrenaline by 50% was regarded as one unit of SOD, which was then represented as Unit/mg protein.

Estimation of Brain Acetylcholinesterase Activity

The activity of acetylcholinesterase (AChE) enzyme, a marker for cholinergic neurotransmission, was measured for the cognitive effect of naringin using the method defined by Ellman *et al*. (1961). Brain supernatant (0.4 mL) from each mouse was mixed with 2.6 mL of phosphate buffer (0.1 M, pH 7.4) and 0.1 mL of 5,5′-dithio-bisphosphate (2-nitrobenzoic acid). The reaction mixture was incubated for 5 min at room temperature and 0.1 mL of acetylthiocholine iodide solution was added. The absorbance was measured with a spectrophotometer at a wavelength of 412 nm, and the change in absorbance was recorded for 10 min at 120-min intervals. The increase in color created by thiocholine when it reacts with 5,5′-dithio-bisphosphate (2-nitrobenzoic acid) was used to determine the rate of acetylcholinesterase activity. The rate of change in absorbance per minute was calculated and expressed as µmol/min/mg tissue.

Determination of Brain Levels of TNF-α and IL-6

The concentrations of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were determined from brain supernatant using an ELISA MAXTM Deluxe kit (BioLegend Catalog # 430904-BL) and the tests were carried out according to manufacturer's instruction.

Statistical Analysis

The data obtained are expressed as mean ± standard error of mean (SEM). The data were analyzed using one-way ANOVA followed by Tukey's post hoc test (GraphPad Prism version 9). P-values less than 0.05 were considered statistically significant.

RESULTS

Effect of MEAP CRS-induced Hyperglycemia

In Figure 2, all doses of MEAP significantly (P<0.05) reduced CRS-induced hyperglycemia in rats. The level of glucose reduction by the dose of 160 mg/kg was comparable with that of pioglitazone (15 mg/kg).

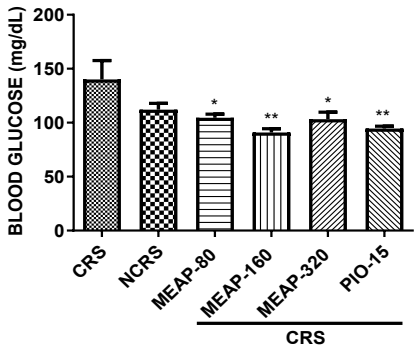

Figure 2: Effect of methanol leaf extract of *A. paniculata* (MEAP) on chronic restraint stress (CRS) mice induced hyperglycemia. *P<0.05, **P<0.01 versus CRS group, n=6. PIO=pioglitazone; NRCS=non chronic restrain stress. Values after abbreviations represent oral doses in mg/kg.

Effects of MEAP on CRS-induced Behavioral Disorder

Figures 3A-3C show the effect of MEAP in CRS-induced neurobehavioral disorders: anxiety (EPM), depression (TST) and memory (Y maze). Animals exposed to CRS spent considerably less time in the open arms when compared to closed arms (P<0.05) in the EPM test (Figure 3A). For the TST test (Figure 3B), the CRS-only mice had a higher duration of immobility when compared to the unstressed group (P<0.05). For the Y maze, there was a difference in % alternation between CRS-only and NCRS groups (P<0.05). Treatment with MEAP significantly increased time spent in open arms in the EPM test at the dose of 160 mg/kg, significantly reduced the duration of immobility at all doses, significantly increased % alternation in Y Maze test at 160 mg/kg.

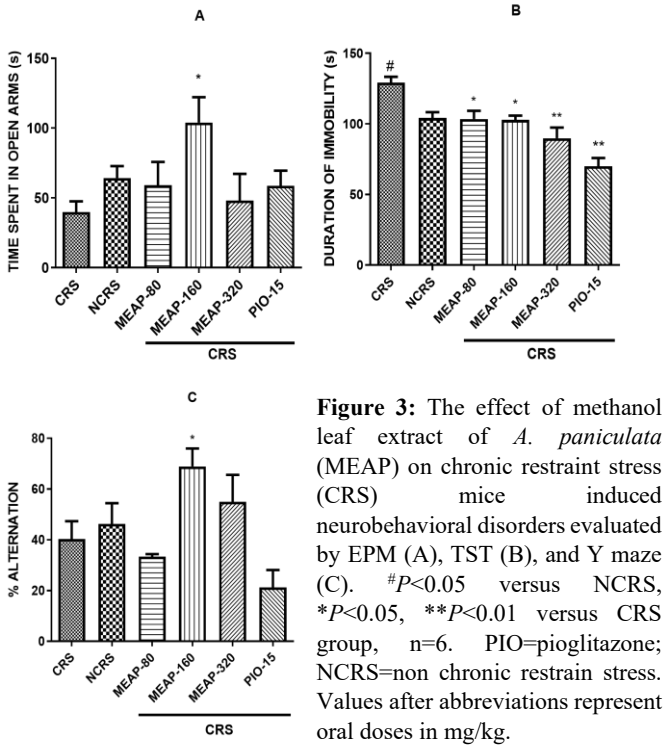

Figure 3: The effect of methanol leaf extract of *A. paniculata* (MEAP) on chronic restraint stress (CRS) mice induced neurobehavioral disorders evaluated by EPM (A), TST (B), and Y maze (C). #P<0.05 versus NCRS, *P<0.05, **P<0.01 versus CRS group, n=6. PIO=pioglitazone; NCRS=non chronic restrain stress. Values after abbreviations represent oral doses in mg/kg.

Antioxidant Effects of MEAP in CRS-Exposed Mouse Brain

Figures 4A – 4E show the effect of MEAP on CRS-induced oxidative stress in the brain of mice. The decrease in brain level of MDA was not significant in the CRS group when compared with the control (NCRS) and doses of MEAP did not significantly reverse the CRS-induced decrease in MDA levels (Figure 4A). Similarly, the reduction in GSH levels due to CRS was not significant and MEAP did not significantly reverse the decrease (Figure 4B). In Figure 4C, although CRS-induced decrease in SOD levels was not significant, the dose of 320 mg/kg/day significantly increased the levels. CAT levels were not significantly decreased by CRS in comparison with the NCRS group and were not significantly reversed by treatment with MEAP (Figure 4D). Nitrite levels which was a direct indication of NO levels was decreased by CRS although not significantly but reversed by the dose of 320 mg/kg of MEAP (Figure 4E).

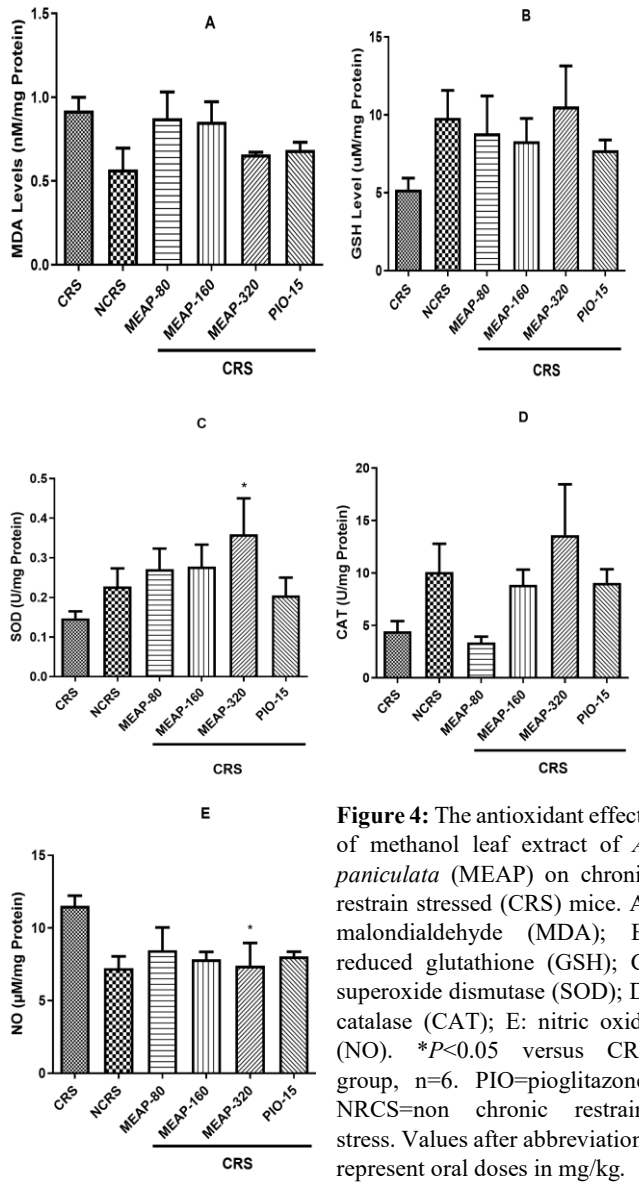

Figure 4: The antioxidant effects of methanol leaf extract of *A. paniculata* (MEAP) on chronic restrain stressed (CRS) mice. A: malondialdehyde (MDA); B: reduced glutathione (GSH); C: superoxide dismutase (SOD); D: catalase (CAT); E: nitric oxide (NO). **P*<0.05 versus CRS group, n=6. PIO=pioglitazone; NRCS=non chronic restraint stress. Values after abbreviations represent oral doses in mg/kg.

Effect of MEAP on CRS Mice Adrenal Gland Weight, Corticosterone and Insulin Concentrations

Figures 5A – 5C show the effect of MEAP on CRS induced changes in the adrenal cortex. The weight of the adrenal gland was not significantly changed in all the groups when compared with the control (NCRS) group (Figure 5A). CRS-induced increase in corticosterone levels was significantly reduced by the dose of 80 mg/kg/day MEAP. Other doses of MEAP did not significantly reduce the levels of corticosterone (Figure 5B). Serum insulin levels were reduced by CRS and reversed by 160 and 320 mg/kg of MEAP (Figure 5C).

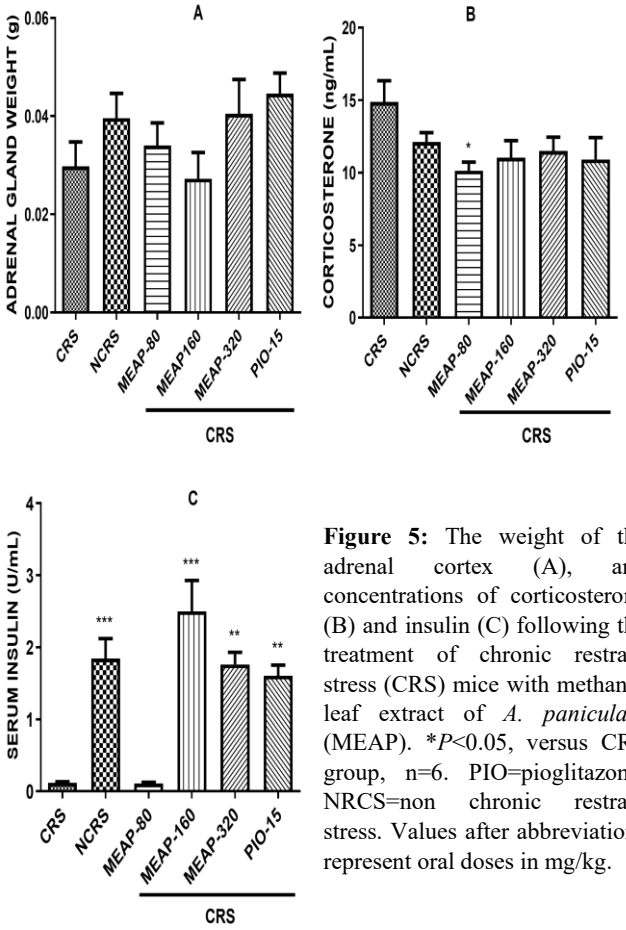

Figure 5: The weight of the adrenal cortex (A), and concentrations of corticosterone (B) and insulin (C) following the treatment of chronic restrain stress (CRS) mice with methanol leaf extract of *A. paniculata* (MEAP). **P*<0.05, versus CRS group, n=6. PIO=pioglitazone; NRCS=non chronic restrain stress. Values after abbreviations represent oral doses in mg/kg.

Effect of MEAP on Brain Concentration of Pro-inflammatory Cytokines

Mice exposed to CRS had significantly increased concentration of AchE in their brain compared to the non-CRS group but only the dose of 80 mg/kg MEAP significantly reduced it (Figure 6A). CRS also induced an increase in brain concentration of TNF-α compared to the NCRS group but the doses of 160 and 320 mg/kg MEAP significantly attenuated the increase (Figure 6B). The brain concentration of IL-6 increased significantly in the CRS group but was reduced significantly by the dose 80 mg/kg MEAP (Figure 6C).

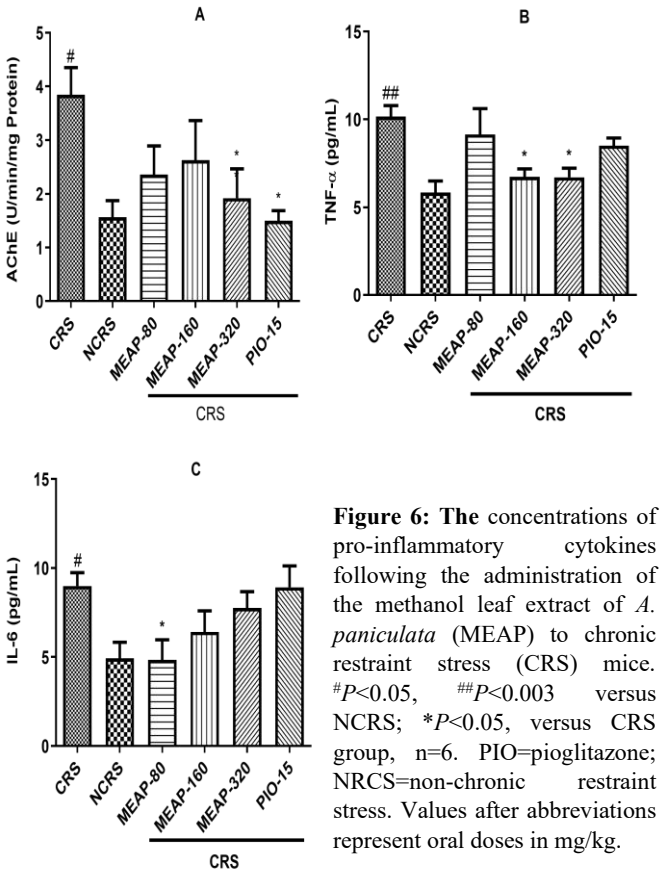

Figure 6: The concentrations of pro-inflammatory cytokines following the administration of the methanol leaf extract of *A. paniculata* (MEAP) to chronic restraint stress (CRS) mice. $^{\#}P<0.05$, $^{\#\#}P<0.003$ versus NCRS; $^{*}P<0.05$, versus CRS group, n=6. PIO=pioglitazone; NRCS=non-chronic restraint stress. Values after abbreviations represent oral doses in mg/kg.

DISCUSSION

This study was carried out to investigate the effects of methanol extract of *Andrographis paniculata* (MEAP) on stress induced by chronic restraint on hyperglycemic complications, neuro-biochemical and neurobehavioral changes in mice. These were done to explore the activity of MEAP and the putative mechanism of action in mice exposed to CRS for 4 cycles. The CRS paradigm mimics the stress humans experience on a day-to-day basis. This method has been widely used in studies to examine the various interplays and pathways with stress and its physiological and neurochemical responses.

The response to a stressful situation is interceded by the HPA axis and the sympathetic and adrenal mechanisms (Marik and Bellomo, 2013). When the body is stressed, a signal is sent to the brain, which activates the paraventricular nucleus (PVN) that in turn activates corticotropin releasing hormone leading to the discharge of adrenocorticotropic hormone (ACTH). The CRS model is an adopted version of restraint stress in which the animals are stressed for longer periods of time for them to have chronic stress. It also indicates an increase in the frequency in restraint of experimental animals. Buynitsky and Mostofsky (2009) reported that the CRS model is a preferred means of stressing animals because it is straightforward, painless and without lasting effects. Restraint is a method of locomotion restriction, and it is a major animal model of psychiatric stress. This method is also inexpensive and does not cause bodily harm to animals. It is preferred because it ensures that long-term effects of stress are observed due to the stressor, which was applied, rather than to the physical repercussions of an irreversible or chronic injury.

Stress has also been shown in humans to play an important role in hyperglycemia (Thakur *et al*., 2019). Some authors also stated that oxidative stress and hyperglycemia are responsible for depression, anxiety and memory impairment in diabetes. It has been stated that imbalances in monoaminergic transmission, GABAergic, cholinergic, and antioxidant defense systems result in stress induced-hyperglycemic complications (González *et al*., 2023). In depression, the monoaminergic system and antioxidant defense system are compromised during hyperglycemia and oxidative stress (Hogan *et al*., 2023). This also applies in anxiety, where the GABAergic and antioxidant defense systems are also compromised.

Various studies have implicated an increase in oxidative stress as a cause for the release of pro-inflammatory cytokines in neurobehavioral deficits caused by chronic stress in mice (Umukoro *et al*., 2018). The role of oxidative stress and inflammatory cytokines in chronic stress induced neuronal degeneration especially the cholinergic pathways has been reported (Umukoro *et al*., 2018), and monoaminergic and GABAergic pathways (Thakur *et al*., 2019). This prompted the investigation of acetylcholinesterase, GABAergic and monoaminergic activities in this study. Previous studies in which mice exposed to stress had deficits in spatial memory, and displayed anxiety- and depression-like behaviors, and elevated AChE activity (Okoh *et al*., 2020; Umukoro *et al*., 2018). In the present study, MEAP significantly attenuated CRS-induced anxiety-like, depressive-like behaviors and memory impairment although not in a dose-related manner.

Andrographis paniculata is a plant that is used in the treatment of tonsillitis, gastroenteritis (Deng *et al.,* 1982; Bensky *et al.,* 1993), which are inflammatory disorders (Duke and Ayensu, 1985; Mills and Bone, 2000). In the Southeast Asian folklore, the plant is essentially used in the treatment of diabetes and hypertension (Zhang and Tan, 1996), while in the Ayurvedic medicine, it is used as a bitter tonic for treating diabetes (Bensky *et al.,* 1993). The plant has also been reported to have antibacterial (Hossain *et al* 2021), antioxidant (Hamid *et al* 2023), anti- inflammatory (Zhu *et al.,* 2021) and antihyperglycemic activities (Ogunlana *et al.,* 2021, Sanower *et al*. 2021). Coupled with these ethnomedicinal values of the plant, our study justifies its uses in reducing hyperglycemia induced by chronic

stress as distinct from hyperglycemia caused by other factors. Animals exposed to stress exhibit certain parameters such as adrenal gland hypertrophy, increase in serum corticosterone and increase in blood glucose (Ulrich Lai *et al*., 2006). The adrenaline and noradrenaline released during stressful conditions act on the liver to release glucose. Constant stress on the body leads to constant release of adrenocorticotropin hormone which can lead to adrenal gland hypertrophy (Bertagna, 2017). So, increased glucose concentrations and increased cortisol concentrations are considered as indicators of chronic stress (Umukoro *et al*., 2018). Although there was no significant change in the weight of adrenal gland in the present study, serum corticosterone concentrations were reduced, and serum insulin concentration was increased with resultant decrease in blood glucose by doses of MEAP.

Stressful conditions have been associated with generation of reactive oxidative species (Umukoro *et al*., 2018; Patki *et al*., 2013). Several studies have also reported that stress causes the depletion of antioxidant enzymes (Patki *et al*., 2013). Although in the present study, reduction in the levels of antioxidant enzymes was not significant in the CRS-only group, MEAP significantly increased the levels of SOD while decreasing NO levels indicating a pattern of enhanced antioxidant mechanisms. In addition to the array of neuro-biochemicals, oral administration of MEAP prevented an increase in CRS-induced increase in the brain levels of acetylcholinesterase and pro-inflammatory cytokines TNF-α and IL-6.

CONCLUSION

This study provides evidence that MEAP reverses chronic stress-induced hyperglycemia, memory impairment, anxiety and depression via mechanisms related to reduction in AchE activity, reduction in the levels of pro-inflammatory cytokines and upregulation of the activity of antioxidant enzymes.

ACKNOWLEDGMENTS

The authors acknowledge the contributions of Dr. A.M. Ajayi and other members of the Neuropharmacology Unit of the Department of Pharmacology and Therapeutics, University of Ibadan.

CONFLICT OF INTEREST

Authors declare no conflict of interest

AUTHORS' CONTRIBUTION

JAB, OFS EOI, OMO and OSM designed the concept and took part in the execution, writing and proofreading of the manuscript. BAA and EBI contributed to the execution and writing of the manuscript.

FUNDING

The study was not funded from any external source.

AUTHORS' DECLARATION

The authors declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

REFERENCES

Adegbite MA, Fakunle GO, Amwe JV, Adedapo ADA, Ologe MO, Sotunde OS, Alabi AB, Iwalewa EO, Adesina SK (2019) Antidiabetic and antihyperlipidemic activity of *Peristrophe bicalyculata* leaves extract in Streptozotocin, and alloxan induced diabetic rats. *Archives of Basic and Applied Medicine* 7: 95-100.

Alla T, Handral M, Nandakumar K, Venkatrao N, Shantakumar SM (2007). Anxiolytic and anticonvulsant activity of alcoholic extract of heartwood of *Aquilaria agallocha* Roxb (Thymelaeceae) in mice. *Pharmacology Online* 1: 564-572.

Bensky D, Gamble A, Kaptchuk T (1993). Chinese Herbal Medicine Materia Medica, Revised Edn, Eastland Press, Seattle, Pp 70-71,277-278,331-332.

Bertagna X (2017). Effects of chronic ACTH excess on human adrenal cortex. *Frontiers in Endocrinology* (Lausanne) 8: 43. doi: 10.3389/fendo.2017.00043.

Buynitsky T, Mostofsky DI (2009). Restraint stress in biobehavioral research: Recent developments. *Neuroscience & Biobehavioral Reviews* 33(7): 1089-1098. doi: 10.1016/j.neubiorev.2009.05.004.

Cattaneo A, Riva MA (2016). Stress-induced mechanisms in mental illness: a role for glucocorticoid signalling. The Journal of Steroid Biochemistry and Molecular Biology 160:169-174.

Deng WL, Nie RJ, Liu JY (1982). Comparison of pharmacological effects of four andrographolides.

Doreddula SK, Bonam SR, Gaddam DP, Desu BSR, Ramarao N, Pandy V (2014). Phytochemical analysis, antioxidant, antistress, and nootropic activities of aqueous and methanolic seed extracts of ladies finger (*Abelmoschus esculentus* L.) in mice. *The Scientific World Journal* 2014: 1-14. DOI:10.1155/2014/519848

Duke, JA, Ayensu ES (1985). Medicinal plants of China (Vol. 2). Reference Publications Inc. Algonac. Michigan.

Ellman GL, Courtney KD (1961). Andres Jr V, Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7(2): 88-95.

González P, Lozano P, Ros G, Solano F (2023). Hyperglycemia and oxidative stress: An integral, updated and critical overview of their metabolic interconnections. *International Journal of Molecular Sciences* 24(11): 9352. doi: 10.3390/ijms24119352.

Grandin T, Deesing M (2002). *Distress in animals: is it fear, pain or physical stress?* Available from https://www.researchgate.net/publication/268341386_Distress_in_Animals_Is_it_Fear_Pain_or_Physical_Stress#fullTextFileContent. Accessed 25 June, 2025.

Green MF, Kern RS, Braff DL, Mintz J (2000). Neurocognitive deficits and functional outcome in schizophrenia: Are we measuring the "right stuff"? *Schizophrenia Bulletin*, *26*(1): 119-136.

Hamid MA, Adam RA, Ramli F, Wahab R, (2023). Antioxidant activity of andrographolide from Andrographis paniculata leaf and its extraction optimization by using accelerated solvent extraction: Antioxidant activity of andrographolide from *Andrographis paniculata* leaf. *Journal of Tropical Life Science* 13(1): 157-170.

Heim C, Nemeroff CB (1999). The impact of early adverse experiences on brain systems involved in the pathophysiology of anxiety and affective disorders. *Biological Psychiatry* 46(11): 1509-1522.

Hogan IA, Kuo YC, Abubakar AN, Lawal B, Agboola AR, Lukman HY, Onikanni SA, Olawale F, Fadaka AO, Ibrahim YO, Babalola SB, Batiha GE, Albogami SM, Alorabi M, De Waard M, Huang HS (2023). Attenuation of hyperglycemia-associated dyslipidemic, oxidative, cognitive, and inflammatory crises via modulation of neuronal ChEs/NF-κB/COX-2/NOx, and hepatorenal functional deficits by the *Tridax procumbens* extract. *Biomedicine & Pharmacotherapy* 158: 114114. doi: 10.1016/j.biopha.2022.114114.

Hossain S, Urbi Z, Karuniawati H, Mohiuddin RB, Moh Qrimida A, Allzrag AMM, Ming LC, Pagano E, Capasso R (2021). *Andrographis paniculata* (Burm. f.) Wall. ex Nees: An updated review of phytochemistry, antimicrobial pharmacology, and clinical safety and efficacy. *Life (Basel)* 11(4): 348. doi: 10.3390/life11040348.

Hossain S, Urbi Z, Karuniawati, H, Mohiuddin RB, Moh Qrimida A, Allzrag AMM, Ming LC, Pagano E, Capasso R (2021). *Andrographis paniculata* (Burm. F.) wall. Ex Nees: An updated review of phytochemistry, antimicrobial pharmacology, and clinical safety and efficacy. *Life* 11(4): 348-360.

Jarukamjorn K, Nemoto N (2008). Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. *Journal of Health Science 54*(4), 370-381.

Jollow DJ, Mitchell JR, Zampaglione N, Gillette JR (1974). Bromobenzene-induced liver necrosis: Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. *Pharmacology* 11(3): 151-169.

Joselin J, Jeeva S (2014). *Andrographis paniculata*: A Review of its traditional uses, phytochemistry and pharmacology. *Medicinal and Aromatic Plants* 3: 169. doi: 10.4172/2167-0412.1000169

Kim SB, Ryu HY, Nam W, Lee MS, Jang MR, Kwak YG, Kang G, Song KS, Lee JW (2023). The neuroprotective effects of *Dendropanax morbifera* water extract on scopolamine-induced memory impairment in mice. *International. Journal of Molecular Sciences* 24(22): 16444; https://doi.org/10.3390/ijms242216444

Lee RS (2022). The physiology of stress and the human body's response to stress. In: Youssef NA (ed), *Epigenetics of Stress and Stress Disorders, Academic Press,* Pp 31: 1-18.

Lister RG (1987). The use of a plus-maze to measure anxiety in the mouse *Psychopharmacology* 92(2): 180-185.

Malahubban M, Alimon AR, Sazili AQ, Fakurazi S, Zakry FA (2013). Phytochemical analysis of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts for their antibacterial and antioxidant potential. *Tropical Biomedicine* 30(3): 467-480.

Marik PE, Bellomo R (2013). Stress hyperglycemia: An essential survival response! *Critical Care* 17(2): 305.

Mills S, Bone K (2000). Principles and Practice of Phytotherapy: Modern Herbal Medicine. Churchill Livingstone, Edinburgh, Pp 439-447.

Misra HP, Fridovich I (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry* 247(10): 3170-3175.

Molina P, Andero R, Armario A (2023). Restraint or immobilization: A comparison of methodologies for restricting free movement in rodents and their potential impact on physiology and behavior. *Neuroscience and Biobehavioral Reviews* 151: 105224. doi: 10.1016/j.neubiorev.2023.105224.

National Institute of Health (2011). Guide for care and use of laboratory animals. Available from https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf. Accessed 25 June, 2025.

Ogunlana OO, Adetuyi, BO, Esalomi EF, Rotimi MI, Popoola JO, Ogunlana OE, Adetuyi OA (2021). Antidiabetic and antioxidant activities of the twigs of *Andrograhis paniculata* on streptozotocin-induced diabetic male rats. *BioChem* 1(3): 238-249.

Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95(2): 351-358.

Okoh L, Ajayi AM, Ben-Azu B, Akinluyi ET, Emokpae O, Umukoro S (2020). d-Ribose-l-cysteine exhibits adaptogenic-like activity through inhibition of oxido-inflammatory responses and increased neuronal caspase-3 activity in mice exposed to unpredictable chronic mild stress. *Molecular Biology Reports* 47(10): 7709-7722.

Pakhetra R, Garg MK, Suryanarayana KM (2011). Management of hyperglycemia in critical illness: Review of targets and strategies. *Medical Journal Armed Forces of India* 67(1): 53-57. doi: 10.1016/S0377-1237(11)80015-8.

Patki G, Solanki N, Atrooz F, Allam F, Salim S (2013). Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. *Brain Research* 1539: 73-86.

Sinha AK (1972). Colorimetric assay of catalase. *Analytical Biochemistry* 47(2): 389-394.

Steru L, Chermat R, Thierry B, Simon P (1985). The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacology* 85(3): 367-370.

Thakur AK, Tyagi S, Shekhar N (2019). Comorbid brain disorders associated with diabetes: Therapeutic potentials of prebiotics, probiotics and herbal drugs. *Translational Medicine Communications* 4: 12.

Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman JP (2006). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *American Journal of Physiology-Endocrinology and Metabolism* 291(5): E965-E973.Umukoro S, Eduviere AT, Ogunbiyi R, Adeoluwa OA (2014). Pharmacological evaluation of the analgesic and anxiolytic activities of Jobelyn® in mice. *Journal of Basic and Clinical Physiology and Pharmacology* 25(4): 439-444.

Umukoro S, Kalejaye HA, Ben-Azu B, Ajayi AM (2018). Naringenin attenuates behavioral derangements induced by social defeat stress in mice via inhibition of acetylcholinesterase activity, oxidative stress and release of pro-inflammatory cytokines. *Biomedicine & Pharmacotherapy* 105: 714-723.

Umukoro S, Omorogbe O, Aluko OM, Eduviere TA, Owoeye O, Oluwole OG (2015). Jobelyn®, a sorghum-based nutritional supplement attenuates unpredictable chronic mild stress-induced memory deficits in mice. *Journal of Behavioral and Brain Science* 5(13): 586.

Ye M, Yongkang X, Xia Y (2022). Validity of chronic restraint stress for modeling anhedonic-like behavior in rodents: A systematic review and meta-analysis. *The Journal of International Medical Research* 23: 50(2):03000605221075816. doi: 10.1177/03000605221075816

Zhang CY, Tan BKH (1996). Hypotensive activity of aqueous extract of *Andrographis paniculata* in rats. *Clinical and Experimental Pharmacology and Physiology* 23(8): 675-678.

Zheng X, Bi W, Yang G, Zhao J, Wang J, Li X, Zhou X (2018). Hyperglycemia induced by chronic restraint stress in mice is associated with nucleus tractus solitarius injury and not just the direct effect of glucocorticoids. *Frontiers in Neuroscience* 12: 983.

Zhu N, Hou J, Yang N (2021). Network pharmacology integrated with experimental validation revealed the anti-inflammatory effects of *Andrographis paniculata. Scientific Reports* 11(1): 9752