Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

# *Astragalus grahamianus* extract: a novel source of bioactive compounds with antioxidant and neuroprotective activities

## Extrato de *Astragalus grahamianus*: uma nova fonte de compostos bioativos com atividades antioxidantes e neuroprotetoras

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**Abstract**

The *Astragalus grahamianus* (AG) Royle ex. Benth is traditionally used for the treatment of various human disorders. The current research work is aimed to explore the neuroprotective anti-Parkinson effects of various fractions of *Astragalus grahamianus* (*A. grahamianus*). Fine powder of *Astragalus grahamianus* was extracted with 70% methanol and then fractionated with various solvents on the basis of polarity. Standard protocols were used to investigate the bioactive constituents present in the various plant fractions. *In-vitro* antioxidant potential of various fractions was checked using diverse free radicals. *In-vivo* rats model was used to determined the neuroprotective effects of methanol fraction of *A. grahamianus.* The results revealed that various fractions of *A. grahamianus* contain flavonoids, cardiac glycosides, steroids, gums, terpenes, proteins, and carbohydrates except chloroform fraction lake the presence of steroids, cardiac glycosides, gums and saponins, aqueous fraction of steroids, terpenoids, gums and saponins, n-Hexane fraction steroids, carbohydrates, alkaloids, gums and flavonoids. The highest amount of total phenolic contents was found in AGME (32.67 ± 2.3 mg GAE / g). The AGME also showed enhanced free radicals cations potential against DPPH, ABTS and $H_2O_2$, respectively. The correlation between AOA (antioxidant activity) and TPC (total phenolic contents) revealed to be substantial. Relative R2 values for ABTS, $H_2O_2$, and DPPH activity are 0.9974, 0.9845, and 0.9678, respectively. The *in-vivo* neuroprotective activities showed significant results. Our findings highlight significant antioxidant, and neuroprotective possessions of AGME attributed to powerful bioactive compounds.

**Keywords:** *Astragalus grahamianus*, total phenolic contents, memory dysfunction, pharmacological evaluation.

**Resumo**

O *Astragalus grahamianus* (AG) Royle ex. Benth pertence à família Fabaceae e o gênero Astragalus é tradicionalmente utilizado para o tratamento de diversas doenças humanas. O presente trabalho de pesquisa tem como objetivo explorar os aspectos benéficos das plantas que estão sendo investigados tanto *in vitro*, ou seja, antioxidantes, quanto *in vivo* (seu impacto no comprometimento da memória induzido pela neuroinflamação em ratos). Para tanto, extrato metanólico de *A. grahamianus* (AGME) e frações com diversos solventes foram sintetizados com base na polaridade. Os resultados revelaram que várias frações de *A. grahamianus* contêm flavonoides, glicosídeos cardíacos, esteroides, gomas, terpenos, proteínas e carboidratos, exceto a fração de clorofórmio, e a presença de esteroides, glicosídeos cardíacos, gomas e saponinas, fração aquosa de esteroides, terpenoides, gomas e saponinas, esteroides da fração n-hexano, carboidratos, alcaloides, gomas e flavonoides. A maior quantidade de conteúdo fenólico total foi encontrada no AGME (32,67 ± 2,3 mg GAE/g). O AGME também mostrou maior potencial de cátions de radicais livres contra DPPH, ABTS e $H_2O_2$. A correlação entre AOA (atividade antioxidante) e TPC (conteúdo fenólico total) revelou-se substancial. Os valores relativos de R2 para atividade ABTS, $H_2O_2$ e DPPH são 0,9974, 0,9845 e 0,9678, respectivamente. As atividades neuroprotetoras *in vivo* mostraram resultados significativos. Nossas descobertas destacam propriedades antioxidantes e neuroprotetoras significativas do AGME atribuídas a poderosos compostos bioativos.

**Palavras-chave:** *Astragalus grahamianus*, conteúdo fenólico total, disfunção de memória, avaliação farmacológica.

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Received: December 8, 2023 – Accepted: May 4, 2024

## 1. Introduction

Oxidative stress plays an important role in the degeneration of dopaminergic neurons in Parkinson's disease (PD). Interruption in the physiologic preservation of the redox possible in neurons interferes with more than a few biological progressions, eventually most important to cell death (Podkowińska and Formanowicz, 2020).

Parkinson's disease (PD) is a clinical disorder that may be diagnosed. It has a range of etiologies and clinical symptoms. The incidence of Parkinson's disease, a neurological disorder, is rapidly increasing globally, barring an infectious cause (Bucur and Papagno, 2023). Parkinson's disease (PD) is growing more and more common in the elderly. Its symptoms include memory loss, mobility issues, and sleep disturbances. Parkinson's disease (PD) is a complex neurological disorder characterized by the substantia nigra pars compacta (SNpc) losing dopaminergic neurons, which results in a dopamine shortage and unintentional movements. Neurodegenerative illnesses, including Parkinson's disease (PD), are also mostly caused by mitochondrial malfunction (Moradi Vastegani et al., 2023).

Medicinal plant plays a crucial role in the alleviation of Parkinson diseases. It was reported that ayurvedic doctors in India recommended numbers of medicinal herbs to treat neurodegenerative conditions like Alzheimer's, Parkinson's, memory damage, nerve degeneration, and other neuronal disorders (Mohi-Ud-Din et al., 2020). The *Astragalus* L. genus is divided into 2500-3000 taxa and more than 250 sections of vascular plants (İlçim and Behçet, 2016; Lysiuk and Darmohray, 2016) belongs to the *Astragaleae* tribe of *Papilionoideae* in the family *Fabaceae* (Chaudhary et al., 2008). *Astragalus* L. distributed widely in the temperate, arid and semiarid hilly regions of South America and northern hemisphere (100 and 400-450 species, respectively) (Azani et al., 2017), Southwestern part of Asia (1-1.5 k species), Himalayan region in China (0.5 k species), Europe (133 species), and the main diversity center of this genus is Iran (with 750 species) (Amiri et al., 2020; Souzani et al., 2009). The genus *Astragalus* L. contains *A. grahamianus,* which is widely distributed in Pakistan (Both in Khyber Pakhtunkhwa and Kashmir) and in Indian Punjab (Chaudhary et al., 2008). The leaves of *A. grahamianus* have abundant valuable compounds, such as *p*-hydroxybenzoic acid, taxiphyllin, sorgoleone prunasin, oxazolidinone, *p*-phloroglucinol, dihydrosorgoleone, chlorogenic acid, *p*-coumaric acid, hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde and aliphatic acids that are why it is used as a beneficial vegetable in daily life (Anand et al., 2010).

Leafy vegetables, fruits, and medicinal plants have played a critical part in the human health care system from the Stone Age to the present in modern era (Salehi et al., 2021). A variety of human diseases are treated with plant-based medications or formulations because medicinal plants contain many essential therapeutic components. Plant-derived drugs are most beneficial and valuable as compare to allelopathic medicines because it contains therapeutic agents which are easily accessible, relatively low prices and have no or little side effects (Ionkova et al., 2022). Phytochemicals are compounds produced by plants have beneficial effects in human health. These chemicals act as antioxidants, safeguarding cells from the harmful effects of Reactive Oxygen Species (ROS) (Husain and Kumar, 2012). ROS is a neutral such as hydrogen peroxide and superoxide, affect the mitochondrial electron transport chain and β-oxidation, can attack cell macromolecules, resulting in cell or tissue damage, that cause many chronic diseases such as asthma, rheumatism, cataracts, carcinoma, dementia, mongolism and Parkinson's are caused (Phaniendra et al., 2015).

The current research work was aimed to explore the bioactive phytochemical constituents, *in-vitro* and *in-vivo* neuroprotective anti-Parkinson effects of various fractions of *Astragalus grahamianus* (*A. grahamianus*).

## 2. Materials and Methods

### *2.1. Equipment's and chemicals*

Potassium Chloride (KCl), PBS (Phosphate Buffer), sodium chloride (NaCl), ammonium persulphate $(NH_4)_2S_2O_8$, Acrylamide, liquid nitrogen, dry ice, and Trizma Base respectively

### *2.2. Plant material*

The plants of *A. grahamianus* were collected from the mountainous area of District Karak, KP, Pakistan, and were carefully protected by freezing immediately in liquid nitrogen, and in dry ice for the purpose to protect it from contamination, and environmental stresses. Afterward, the plant material was grounded to powdered form in liquid nitrogen by using mortar and pestle (0.1 mm mesh size). The plant powder was kept in -80 °C until further use.

### *2.3. Synthesis of A. grahamianus plant methanolic extract*

The *A. grahamianus* methanol extract (AGME) was synthesized from the plant powder for the purpose of analyzing and extract the bioactive compounds present in it. For the aforesaid purpose we comprehensively mixed 800 g of plant powder in 2000 mL methanol (70%). After soaking, the methanolic mixture was kept in an incubator shaker for 72 hrs maintaining room temperature, to mix it thoroughly. After 72 hrs of mixing, the solution was filtered through Whatman No. 3. The filtrate was further concentrated using the rotary evaporator, and then at room temperature allowed to air dry entirely via methanolic evaporation. Finally, we obtained about 28.84 g sticky AGME, that was stored at 4 °C for further use.

### *2.4. Synthesis of various fraction's from AGME*

The different fractions i.e., aqueous, n-Hexane, and chloroform was synthesized following the protocol provided by the manufacturer. The separating funnel was used to divide 20 g of the previously prepared AGME into 400 mL fractions in each of the solvents i.e., aqueous (water), n-Hexane, and chloroform. The gummy residue from the filtrates of the aqueous, n-Hexane and chloroform fractions, respectively, was collected and stored at -20 °C for further investigation.

*2.5. Analysis of phytochemical screening*

The different phytochemical i.e., alkaloids, proteins, terpenes, saponins, flavonoids, cardiac glycosides, steroids and carbohydrates presence or absence in AGME and its different fractions was analyzed by following the standard procedures of Harborne (1973) and Sofowora and Hardman (1973).

*2.6. Determination of the total phenolic contents*

The folin-Ciocalteu reagent was used for the analyses of the total phenolic contents' presence in AGME and its different fractions in accordance with the approach utilized by McDonald et al. (2001). Final absorbance was calculated at wavelength (725 nm) by using a spectrophotometer and findings were displayed as gallic acid equivalents.

*2.7. Flavonoid contents determination*

According to the standard method, the accumulation of flavonoids in each sample was measured. A 10% (w/v) $AlCl_3$ solution in methanol, 0.2 milliliters of a 1 M potassium acetate solution and 5.6 mL of $dH_2O$ were added to one mL of extract solution (25-200 µg/mL). Before measuring the absorbance of the mixture at 415 nm, the mixture was kept at room temperature for 30 min. The results were given in milligram per gramme (mg QE/g) of quercetin equivalents for the dry extract.

*2.8. In-vitro determination of free radicals scavenging potential of AGME and its fractions*

The free radical scavenging or antioxidant potential of AGME and its fractions was accomplished following the procedure developed (Wojdyło et al., 2007). The 1 g of each sample was added to 10 mL methanol (80%), and the suspensions were stirred slightly. The tubes were vortexed 2 times for 15 min each time and were incubated at room temperature (25 °C) for 24 hrs. The mixtures were then centrifuged at 1500 rpm for 10 min, and the supernatants were kept at 4 °C prior to use within 24 hrs.

*2.8.1. DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging activity*

The DPPH (2,2-diphenyl-1- picrylhydrazyl) analysis of different concentrations (125, 250, 500, 1000, 1500 and 2000 µg/mL) of AGME and its fractions was accomplished by using the method conducted by Gyamfi et al. (1999). The DPPH free radical scavenging potential of all samples was comparatively analyzed with the standard solution of ascorbic acid. In detail, the freshly synthesized ethanolic 0.1 mM DPPH solution was used for the assay, after preparation, 1 mL of this mixture was mixed with 2 mL AGME and its fractions at different concentrations (125, 250, 500, 1000, 1500 and 2000 µg/mL). The resulted solution was vigorously agitated and stored in the dark for 30 minutes under control conditions at room temperature (25-28 °C). The degree of discoloration in the samples indicates the compounds' ability to scavenge free radicals. The reading was taken at wavelength of 517 nm on spectrophotometer. The comparative DPPH free radical scavenging efficiency of the samples as compare to control (ascorbic acid) was calculated using the Equation 1 below.

$$\%\ \text{DPPH free radicals scavenging effect} = (A1 - A2 / A1) \times 100 \quad (1)$$

where:
A1 = the absorbance of DPPH (Control), and
A2 = samples absorbance.

*2.8.2. ABTS free radical cation scavenging potential of AGME and its fractions*

The comparative free radical scavenging potential of AGME and its fractions at different concentrations (125, 250, 500, 1000, 1500, and 2000 µg/mL) were analyzed using an improved ABTS assay as conducted (Re et al., 1999). The 7 mM ABTS aqueous solution and 2.4 mM potassium persulfate were reacted in the dark for 12-16 hours at room temperature for the purpose to synthesize the ABTS•+. Before the experiment, this solution was diluted with ethanol (1:89 v/v) and allowed to equilibrize at 30 °C, yielding an absorbance of 0.70 ± 0.02 at 734 nm wavelength. The sample extracts' stock solution was diluted to generate between 20 and 80% inhibition of the blank absorbance when 10 µL aliquots were added to the experiment. The sample extracts' stock solution was diluted in a way that when 10 µL of aliquots added into the experimental solution, then we could obtain the inhibition absorbance value of the blank between 20 to 80%. After mixing of Trolox (final concentration 0-15 µM) in ethanol, or 10 µL of each sample with 1 mL of diluted ABTS, at 30 °C exactly 30 min after the primary mixing the absorbance was measured at 30 °C.

The essay was done in triplicates of each dilution, and the % inhibition values of all the samples were calculated as compare to ethanol (blank) absorbance at 734 nm wavelength, and then were plotted as a function of Trolox concentration. The reference chemical was ascorbic acid. The ABTS free radicals' scavenging activity was calculated using the Formula 2 below:

$$\textit{Scavenging effect}\ (\%) = \left[ \left( \begin{matrix} \textit{Control absorbance (ABTS)} - \\ \textit{samples absorbance} \end{matrix} \right) / \left( \textit{Control absorbance} \right) \right] x\ 100 \quad (2)$$

*2.8.3. Analyses of the $H_2O_2$ free radical scavenging capability of AGME and its fractions*

The $H_2O_2$ free radical scavenging activity of AGME and its fractions at different concentrations (125, 250, 500, 1000, 1500 and 2000 µg/mL) as compared to control (Ascorbic acid) was determined (Wettasinghe and Shahidi, 2000). About 0.6 mL $H_2O_2$ (4 mM) (prepared in PBS) was mixed with 4 mL extract containing different concentrations (125, 250, 500, 1000, 1500 and 2000 µg/mL) solutions and incubated for 10 minutes. The 230 nm wavelength at spectrophotometer was used to measure the absorbance values of the solution. The antioxidant potential of the samples was determined by % inhibition strength of $H_2O_2$. Decreased % accumulation of $H_2O_2$ shows strong free radical scavenging activity. The % $H_2O_2$ free

radical scavenging activity was calculated using the Equation 3 below;

$$\text{Scavenging effect}(\%) = \left[\left(\begin{array}{c}\text{Control absorbance}\left(H_2O_2\right) - \\ \text{samples absorbance}\end{array}\right) / \text{(Control absorbance)}\right] \times 100 \quad (3)$$

Each test was performed three times and the results were presented as means ± SD.

*2.9. In-vivo neuroprotective effects of methanol fraction*

The 40 adult male Swiss albino rats (150-160 g) were purchased from National Institute of Health (NIH) Islamabad, Pakistan. All the rats were properly labelled and randomly divided into the following 5 groups.

1. Normal control;
2. Rats were given a daily dose of 1.5 mg of Rotenone;
3. Rotenone + Levodopa-carbidopa (1.5 + 200 mg/kg/day, respectively);
4. Rotenone + AGME (1.5 + 200 mg/kg b.w, respectively);
5. Rotenone + AGME (1.5 + 400 mg/kg b.w, respectively).

In individual cages the rats were incubated under standard environmental conditions keeping the proper temperature, light and food, the individual cages contained the rats.

*2.9.1. The dose dependent injection of Rotenone to rates*

The rats in the groups were fasted for 16 hrs. Then, the rats weighing 150-160 g received a single IP dosage of the drug Rotenone, which contained 1.0 mL per kilogram of saline solution for the purpose of inducing psychological conditions and Parkinson effects.

*2.9.2. Test of Morris Water Maze (MWM)*

Morris Water Maze (MWM) test was conducted to the check the ability of the rats to learn spatial memory and learning. After 3 days of training, rats were adjusted to the environment, before the assessment. A 1-min escape time was calculated for each rat to locate the underwater platform for 05-days. Rats were incubated there for ten seconds in case, if they couldn't find the platform in the allotted time. The escape delay time (Sec) was tracked and reported every day. The platform was concealed during the rest period, which was given to the rats two days prior to the final probing test and the average amount of time spent in each rat's allocated zone was calculated.

*2.9.3. Test of Y-maze*

The aforementioned behavioral test was properly described in Atesyakar et al. (2020) and Moreyra and Lozada (2021). A 1200-degree angle connects the three 50×10×20cm$^3$ (LXWXH) Y-maze arms. The rats were allowed ten min each time to get adjusted to the new environment. Rats were then placed in the center of the labyrinth and given eight minutes to explore on their own as a follow-up experiment. The ratio of arms that alternated between each rat and its successive triplets might be calculated using the following Formula 4:

$$\%\ alternation = Successive\ triple\ sets / Total\ number\ of\ arm\ entries - 2\ x\ 100 \quad (4)$$

Positive correlations were found between the % of alternations and the rat's capacity to spatially store information.

*2.9.4. Exploration of biochemical inside the plasma*

When the drug treatment was completed then the rates were anesthetized and blood was taken for blood biochemistry, including the examination of HDL, VLDL, VDL and total cholesterol as well as TGL.

*2.9.5. Antioxidant enzymes analyses in brain homogenates*

*2.9.5.1. CAT (catalase) assay*

The CAT activity was quantified using an earlier protocol of Chance and Maehly (1954), with some minor modifications. The reaction mixture of 3 mL contained 400 µl of $H_2O_2$ (5.9 mM), 50 mM of phosphate buffer and brain supernatant (100 µl). At one-minute intervals, the reaction mixture's change in absorbance was measured at 240 nm. One unit of activity is described as a change in absorbance of less than 0.01 units per minute.

*2.9.5.2. POD (Peroxidase) assay*

The protocol of Chance and Maehly (1954) was used to examine the peroxidase activity with a few minor changes. For the peroxidase assay mixture, the 50 mM of phosphate buffer, 40 mM of $H_2O_2$, 20 mM of guaiacol and brain homogenate supernatant (1000 mL) are used. At regular intervals, the reaction mixture's change in absorbance at 470 nm was recorded.

*2.9.5.3. SOD (superoxide dismutase) assay*

According to Kakkar et al. (1984) this experiment was carried out with minor changes. The SOD activity was determined using a reaction mixture made up of 186 µM phenazine methosulphate, 0.052 mM sodium pyrophosphate buffer and brain homogenate supernatant (300 µl). The first to initiate an enzymatic reaction 780 µM of NADH were added to the reaction mixture and glacial acetic acid (1000 µl) were added for stop the reaction. After solution preparation the absorbance was calculated at 560 nm at spectrophotometer.

*2.9.5.4. GSH (reduced glutathione) assay*

To determine the levels of reduced glutathione, the proteins were separated via the addition of an equal amount of 4% sulfosalicylic acid solution to 1000 µl of brain tissue while the protocol was adapted from Salau et al. (2020). After one hour incubation at 4 °C, the reaction mixture was centrifuged for 20 min at 12000 rpm. The reaction mixture contained 200 µl of 100 mM DTNB, 100 µl of centrifuged aliquot and 2700 µl of pH 7.4 phosphate buffer (0.1 M). After that the absorbance were taken at 412 nm. 0.1 g tissue has been used to indicate the findings of reduced glutathione.

*2.9.5.5. TBARS assay*

A minor modification to an earlier approach was made to conduct the lipid peroxidation (TBARS) assay by following protocol of De Leon and Borges (2020). The test was conducted using a 1000 µl reaction mixture that contained 0.1 M of phosphate buffer, 100 mM of ascorbic acid, brain homogenate supernatant (200 µl) and 100 mM of ferric chloride. A wise bath incubated shaken and at 37 °C was used to incubate the reaction mixture for 1hr. After that, trichloroacetic acid (10%) solution was added to stop the reaction. The tubes were then quickly transferred to a cold bath and after being filled with 1000 µl of 0.67% thiobarbituric acid are heated to 95 °C for 20 min; the mixture was centrifuged at 12000 rpm for 10 minutes. The absorbance was taken at 535 nm by spectrophotometer.

*2.10. Statistical analysis*

Through SPSS 20.0., the results of all X-rays were scanned, assembled and statistically analyzed. Rats' average standard error of the mean (A.U.s) treated with Rotenone vs rats treated with normal saline is used to represent the density of proteins; *P<0.05, **## P<0.01 and ***### P<0.001. The results showed in the Figures are the means, ± standard deviation (SD) of 6 replications (n=06). The data were statistically analyzed by applying one-way ANOVA and Duncan tests (P < 0.05). All graphs were designed using GraphPad_Prism, version, 8.0.2.263, and TB Tools software.

## 3. Results

*3.1. Phytochemical investigations in AGME and its different fractions*

Phytochemical evaluation of AGME and its various fractions was conducted in accordance with a predetermined methodology in order to determine the presence or absence of certain bioactive chemicals in AGME and its different fractions i.e., flavonoids, proteins, alkaloids, steroids, cardiac glycosides, saponins and terpenes. The AGME have the highest no of bioactive compounds (09), the chloroform and aqueous fractions contains 5 phytochemicals, while the hexane fraction contains the lowest no of bioactive compounds (04). The presence and absence of the aforementioned phytochemicals in AGME and its fractions are shown in (Figure 1).

*3.2. Total phenolic and flavonoid contents*

By using Folin-Ciocalteu phenol reagent and gallic acid (standard), the total phenolic contents in AGME and its fractions were quantified. Phenolic compounds are present in different levels in medicinal plants, while the methanol extract in this study has the large quantity of phenolic contents (32.67 ± 2.3 mg GAE/g), the hexane fraction has the lowest amount (9.65 ± 2.67 mg GAE/g) and the chloroform and aqueous fractions have moderate levels (19.46 ± 2.58 mg GAE/g) and (14.65 ± 2.45 mg GAE/g), respectively). The enhanced flavonoid contents were detected in AGME as compared to chloroform and aqueous fractions, while lowest contents were detected in hexane fraction, as shown in Figure 2.

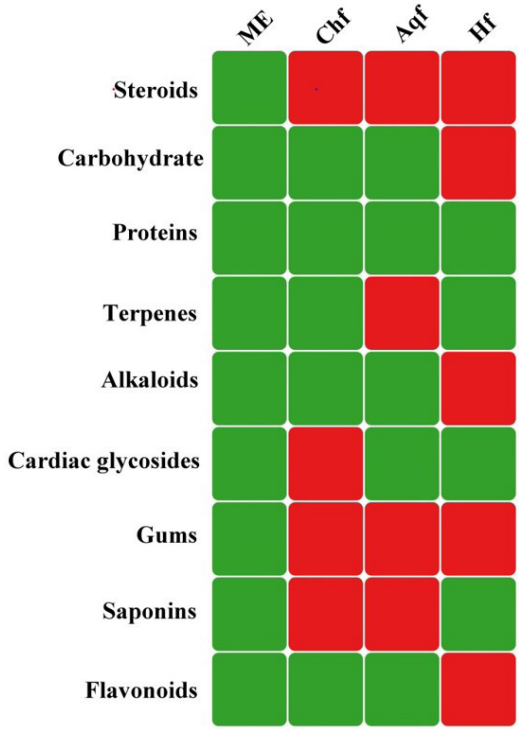


**Figure 1.** Analyses of the phytochemicals in AGME and its different fractions: Green color shows presence and red color shows absence of the phytochemicals.

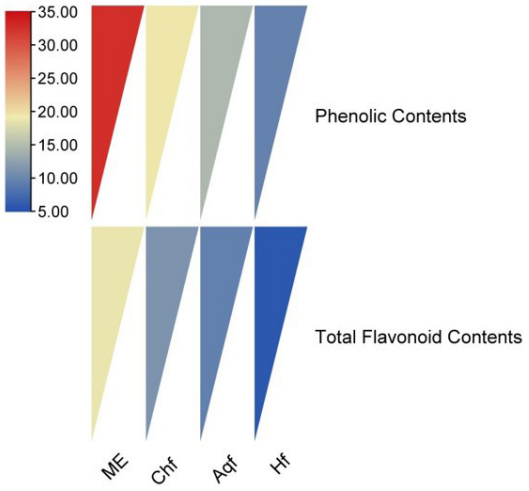


**Figure 2.** Quantification of Phenolic and Total flavonoid contents in AGME and its different fractions. The color spectrum from dark red to light yellow indicates the contents accumulation from higher to moderated extent, on the other hand, from light yellow to dark blue indicates low accumulation of flavonoids and phenolic contents.

### *3.3. In-vitro antioxidant potential investigation*

Reactive oxygen species (ROS) or free radicals release inside our bodies due to various reasons and can cause different types of serious ailments. Plants naturally produce antioxidants, that can mitigate the dangerous effects of the free radicals (Altaf et al., 2022). Organism must be protected from the detrimental effects of free radicals and their ROS (Sen et al., 2010). Following are some of the most common experiments used for the analyses of free radical scavenging potential of plants.

#### *3.3.1. The AGME and its fractions DPPH free radical scavenging potential analyses*

The ability of plant extracts to produce antioxidants was evaluated by using the DPPH free radical scavenging assay. The AGME and its fraction free radical scavenging was assessed at different concentrations i.e., 62.5, 125, 250, 500, 1000, 1500, and 2000 µg $mL^{-1}$, as compared to ascorbic acid (Control). The reading was taken at 517 nm at spectrophotometer. The reduced DPPH free radical's contents were observed in the methanolic extract (81%), as compared to different fraction i.e., chloroform fraction (69%), aqueous fraction (57%), and hexane fraction (51%), while the control (Ascorbic acid) showed the highest scavenging potential. The order of vindicating DPPH free radical scavenging potential of the experimental samples was; control > Me > Cf > Aqf > Hf. Our results express that AGME has the capability to neutralize the DPPH free radicals by hydrogen donating activity in a dose dependent manner (Figure 3). Concentration was shown to be a factor in the antioxidant properties of AGME and its various fractions.

#### *3.3.2. The AGME and its fractions ABTS free radical scavenging potential analyses*

Antioxidants (hydrophilic or lipophilic) free radical scavenging potential was analyzed by accomplishing ABTS free radical scavenging assay, which is simple and appropriate assay this analysis. The ability of ascorbic acid, AGME and its fractions to mitigate ABTS free radicals were determined (Johnston et al., 2006). The plant materials dramatically decreased the synthesis of the blue chromophore (ABTS•+), which is brought on by the interaction of potassium persulfate and ABTS. While, from the research findings of the present research project it was founded that at the most concentrated dose 2000 µg/mL the chloroform, aqueous, and hexane fractions, (68%), (59%), (55%), respectively, all have low free radical cation activity as compared to AGME that shows 80.3% scavenging activity in terms of inhibition potential, which is higher 12.3%, 21.3%, and 25.3%, from chloroform, aqueous, and hexane fractions inhibitive potential, respectively, while the standard i.e., ascorbic acid shows the enhanced inhibitive activity (93%), (Figure 4). The order of vindicating ABTS free radical scavenging potential of the experimental samples were; control > Me > Cf > Aqf > Hf.

#### *3.3.3. The AGME and its fractions $H_2O_2$ free radical scavenging potential analyses*

The ability of AGME and its fractions to scavenge $H_2O_2$ at different concentrations i.e., 2000, 1500, 1000, 500, and 250 µg/ml, was investigated. The highest value, however, was recorded at 2000 µg/ml, where methanol extract (81.2%), chloroform fraction (70%), aqueous fraction (69.5%), hexane fraction (56%) and standard ascorbic acid (83%) all shown scavenging activity (Figure 5). The order of mitigating $H_2O_2$ free radical scavenging potential of the experimental samples was; control > Me > Cf > Aqf > Hf. The Me shows significantly enhanced scavenging potential in dose dependent manner as compared to fractions. Further, studies are required to find out the mechanistic pathways.

### *3.4. Correlation of total phenolics with anti-oxidant properties*

The strong correlation between AGME antioxidant scavenging potential against (DPPH, ABTS, $H_2O_2$) and total phenolic contents were observed, while insignificant correlation between AGME total phenolic contents and its

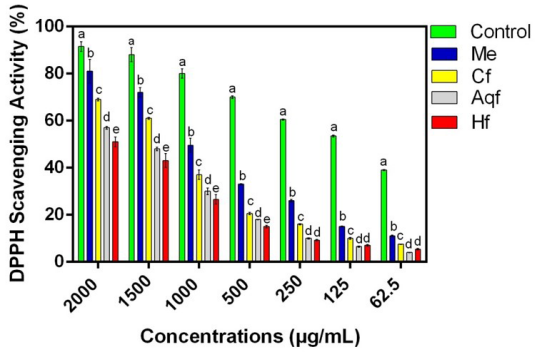


**Figure 3.** The scavenging potential of AGME and its fractions against DPPH free radicals. ME, Cf, Aqf, Hf and Asa stand for methanolic extract, chloroform, hexane, and ascorbic acid, respectively. The bars show the standard deviation of the means (n = 6). The different lower-case letters show statistical significance variations among treatments at $P < 0.05$.

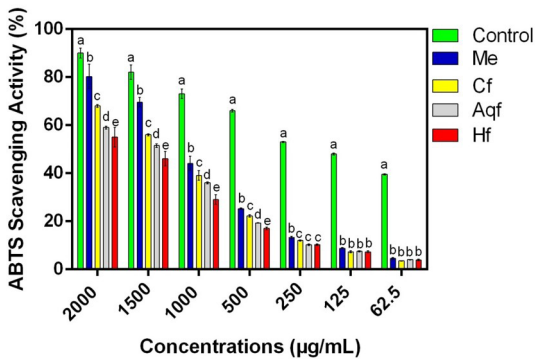


**Figure 4.** ABTS scavenging activity. ME, Cf, Aqf, Hf and Asa stand for methanol extract, chloroform, hexane, and ascorbic acid, respectively. The bars show the standard deviation of the means (n = 6). The different lower-case letters show statistical significance variations among treatments at $P < 0.05$.

anti-diabetic potential were recorded (Pearson correlation, two tailed), Table 1.

### *3.5. In vivo potential of AGME*

#### *3.5.1. Toxicity study*

The results of a study on acute toxicity indicate that the methanol extract (AGME) is not harmful. During the study, neither any deaths nor any drug-induced physical side effects occurred in the rats given the doses (200 and 400 mg/kg) of AGME. The highest effective dose (AGME, 400 mg/kg) for the treatment was found by acute toxicity testing.

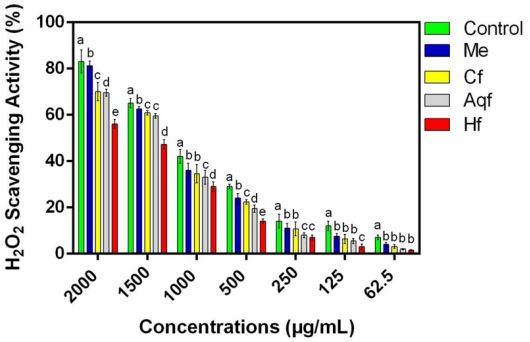


**Figure 5.** $H_2O_2$ scavenging activity of AGME and its fractions. ME, Cf, Aqf, Hf and Asa that are stands for methanolic extract, chloroform, hexane, and ascorbic acid, respectively. The bars show the standard deviation of the means (n = 6). The different lower-case letters show statistical significance variations among treatments at P < 0.05.

**Table 1.** Illustrates the correlation between the measured total phenolics in AGME and its fractions, and their respective levels of antioxidant and anti-diabetic activity.

| Antioxidant Activities | R2 values of Phenolic contents |
|---|---|
| DPPH activity | 0.9678* |
| ABTS activity | 0.9974* |
| $H_2O_2$ activity | 0.9845* |

*The 'P' (two-tailed) value is indicated, with asterisks denoting significance and 'ns' denoting non-significance.

### *3.6. Analyses neuroprotective activity of A. grahamianus*

#### *3.6.1. Analyses of AGME's effect on body weight*

When the experimental observation was completed and the data was collected, the rats' weight reduction was remarkable, but with the passage of time (21 days), it was not observable in the rats treated with Levadopa-carbidopa. The rats who continued taking the extracts reported an increase in body weight (b.w) when compared to controls. According to our findings (Table 2), the Rotenone causes an increase in lipid peroxidation, that cause diabetic control rats' body weight loss considerably ($P < 0.01$), in comparison to untreated rats. Rats receiving various AGME fractions constantly noticed a gain in body weight that was significant ($P < 0.01$), whereas untreated rats did not ($P > 0.01$).

#### *3.6.2. AGME impact on serum lipid profile*

Lipid profile is essential for many elements that make up the bodies of living things through a variety of catabolic and metabolic processes. When Rotenone is transformed in the liver, an intermediate reactive free radical is produced. This intermediate reactive free radical contributes significantly to oxidative stress and causes a neuro-degeneration. Both oxidative stress and brain damage are decreased by antioxidants. The detection and evaluation of oxidative stress can be done using a variety of antioxidant enzymes and biochemical indicators. Rotenone administration resulted in significant reductions ($P < 0.5$) in lipid profile of the rats in the current study had significantly lower lipid profiles after receiving Rotenone ($P < 0.5$). The data presented in the Table 3 shows definitely that the AGME extract successfully reduced blood serum levels of triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol and VLDL cholesterol with a dose-dependent effect. The findings demonstrate that diabetic rats still had higher blood lipid and cholesterol levels than control and extract-tested animals.

#### *3.6.3. AGME's impact on kidney profile*

The diabetic, healthy and treated groups all had significantly different serum concentrations of urea and creatinine. At dose (AGME, 200, 400 mg/kg b.w.), was significantly ($P < 0.05$) reversed the increased blood levels of renal profile in treated groups (Table 4).

**Table 2.** The analyses of AGME's impact on rats' body weight: Note: The normal SD (n=6) of each value in the table serves as its representation. At a 0.05 level of probability, the values a-f show statistical significance.

| Control | On day 0 | On day 7 | On day 14 | On day 21 |
|---|---|---|---|---|
| | **228.3 ± 6.5$^a$** | **229.8 ± 0.5$^b$** | **230 ± 0.3$^b$** | **230 ± 0.35$^b$** |
| Rotenone | 216.6 ± 4.7$^a$ | 211.6 ± 3.53$^a$ | 211 ± 3.5$^a$ | 206 ± 7.07$^a$ |
| Rotenone+200 mg/kg L. carbidopa | 230.5 ± 4.8$^a$ | 231.03 ± 0.7$^b$ | 233 ± 2.12$^b$ | 236 ± 4.24$^b$ |
| Rotenone+ 200 mg/kg AGME1 | 219.8 ± 10.2$^a$ | 201 ± 1.4$^a$ | 216 ± 4.9$^a$ | 223 ± 15.53$^b$ |
| Rotenone+400 mg/kg AGME2 | 225.9 ± 7.0$^a$ | 222.7 ± 2.3$^b$ | 226 ± 9.2$^b$ | 227 ± 29.1$^b$ |

Values are represented in Mean ± SEM ($n = 6$).

**Table 3.** Impact of AGME extract on lipid profile: Note: The mean and, ± SD (n=3) of each value in the table serves as its representation. At a 0.05 level of probability, the values a-f is significant.

| Treatment | Cholesterol (mg/dl) | VLDL (mg/dl) | TG (mg/dl) | LDL (mg/dl) | HDL (mg/dl) |
|---|---|---|---|---|---|
| Rotenone | 80.3 ± 4.8a | 14.4 ± 2.8a | 88.3 ± 5.9a | 22.3 ± 0.1a | 36.4 ± 2.9c |
| Rotenone+200 mg/kg | 90.5 ± 3.5c | 30.5 ± 2.4c | 165.4 ± 4.4e | 33.6 ± 3.2b | 21.3 ± 5.7a |
| L. carbidopa | | | | | |
| Rotenone+ 200 mg/kg AGME1 | 76.5 ± 4.4a | 20.1 ± 1.5b | 107.4 ± 5.5b | 24.9 ± 2.4a | 27.8 ± 7.0 b |
| Rotenone+400 mg/kg AGME2 | 79.9 ± 5.3a | 19.9 ± 2.1b | 88.8 ± 3.5b | 24.7 ± 2.1a | 37.8 ± 5.2c |
| Rotenone | 86.9 ± 3.1b | 20.4 ± 1.0b | 114.1 ± 4.6a | 17.8 ± 1.0a | 53.8 ± 3.1d |

Values are represented in Mean ± SEM (*n = 6*).

*3.6.4. The effect of AGME on antioxidant status*

The present research outcomes demonstrated that in diabetic rats, the AGME may successfully lower the oxidative damage brought on by Rotenone. MDA levels in normal control rats that weren't diabetic were considerably low in comparison to diabetic control. The concentration of the applied extract at the higher dose of 400 mg/kg b.w. dramatically (P<0.05) returned to a level that is essentially normal, in contrast to the modest reduction in MDA caused by the common medication Levadopa-carbidopa. In diseased rats, the formulation of the antioxidant-rich extract increased glutathione levels. Compared to the normal group, the diseased group has lower levels of superoxide dismutase (SOD) and catalase in the liver homogenate SOD and catalase levels were significantly (P< 0.05) increased after extract administration. Table 5 shows the effects of AGME on the relative levels of MDA, glutathione, catalase and SOD.

*3.7. Analyses of behavioural effects of AGME*

*3.7.1. Test for Morris Water Maze (MWM)*

The present study outcomes of the MWM test for the escape latency and swimming distances during spatial learning (Table 6). Among the experimental groups before and after treatments, there were no significant changes observed in escape delays, according to the test ($p > 0.05$). The distances swum to reach the platform and the mean escape delay for the control group were statistically shorter when compared to day-01, they were better on day-06 ($p < 0.05$), according to a comparison of the experimental groups' days. The mean escape delay time over the duration of the test days did not differ significantly between the Rotenone -treated group of rats and the control group ($p > 0.05$). The Rotenone-treated rats' mean escape latencies and swimming distances to the platform were considerably shorter on day 06 compared to day 01 (p< 0.01 and 0.05, respectively).

*3.7.2. Test of Y-maze*

The Y-maze test findings are shown in Table 7. In the Rotenone -treated group compared to the control group, the % of spontaneous variation was considerably lower ($p < 0.01$). Compared to the group that received Rotenone, the rats administered Levadopa-carbidopa (5 mg/kg) and AGME (400 mg/kg) had considerably more spontaneous alternation. The number of entries for each arm was similar across the 5 experimental groups ($p > 0.05$). These findings indicate that AGME injection with maintenance of locomotors activity may reverse Rotenone-induced Parkinson disease.

**Table 4.** AGME's Effect on creatinine and urea levels in serum Note: Comparing of AGME with the control group for diabetics and the normal group (Dunnett's test, p< 0.001 for both comparisons).

| Treatments | Urea (mg/dl) | Creatinine (mg/dl) |
|---|---|---|
| Rotenone | 33.6 ± 1.6 | 0.58 ± .1 |
| Rotenone+200 mg/kg | 50.2 ± 4.2 | 1.50 ± .2 |
| L. carbidopa | | |
| Rotenone+ 200 mg/kg AGME1 | 39.7 ± 2.4a | 0.98 ± .2a |
| Rotenone+400 mg/kg AGME2 | 37.9 ± .9a | 0.76 ± .2a |
| | 33.7 ± 2.4a | 0.54 ± .0a |

Values are represented in Mean ± SEM (*n = 6*).

## 4. Discussion

Phytochemicals are active substances found in all parts of a plant. They play a crucial role in shielding the plant from various diseases (Saxena et al., 2013). The results of the present investigation revealed that AGME and its various fractions contains different phytochemicals i.e., alkaloids, proteins, terpenes, cardiac glycosides, carbohydrates, steroids, terpenes, and flavonoids (Figure 1). Our findings agree with those of other studies, including in Hossain and Nagooru (2011) and Suresh and Nagarajan (2009). The cytotoxicity of AGME and its components were investigated using the brine shrimp lethality assay. The AGME and fractions i.e., ME, Cf, Aqf, Hf and Asa stand for methanol extract, chloroform, hexane, and ascorbic acid, respectively demonstrated brine shrimp lethality up to 80.5±1.3%, 60.4±1.0%, 60.7±2.3% and 50.5±2.3%, respectively, at a concentration of 1000 µg/mL (Figure 2). This could be attributed to the presence of chemicals renowned for their anticancer properties. Our research findings are entirely supported by studies on *Coscinium blumeanum, Fibraurea tinctoria, Arcangelisia flava Hapllophyllum*

**Table 5.** Effects of extract on antioxidant enzymes.

| Groups | SOD (U Min/mg protein) | CAT (U Min/mg protein) | GSH nMol/mL) | MDA (μMol/mg protein) |
|---|---|---|---|---|
| Control | 14.2 ± 0.6++ | 16.7 ± 0.5++ | 9.9 ±0.3++ | 19.6 ±1.0++ |
| 5 mg/kg Rotenone | 8.4 ± 0.3** | 9.7 ± 0.6** | 5.6±0.5** | 38.6 ±0.4** |
| 5 mg/kg Rotenone +200 mg/kg levodopa carbidopa | 13.7 ± 0.7++ | 15.9 ± 0.7++ | 9.7±0.7++ | 20.7± 0.7++ |
| 5 mg/kg Rotenone +200 mg/kg AGME1 | 12.8 ± 0.6++ | 12.3± 0.8++ | 7.5± 0.7++ | 25.5± 0.8++ |
| 5 mg/kg Rotenone +400 mg/kg AGME2 | 13.9± 0.3++ | 15.2± 0.5++ | 9.3±0.6++ | 19.3±0.6++ |

Values are represented in Mean ± SEM ($n = 6$). ** Indicates a statistically significant difference at the 0.01 level ($P < 0.01$). ++ Indicates a correlation coefficient above 0.6.

**Table 6.** Effects of extract on Morris's water maze test.

| Groups | Escape latency (s) | Number of Crossing over target quadrant |
|---|---|---|
| Control | 3.3 ± 0.5++ | 10.5 ± 0.2++ |
| 5 mg/kg Rotenone | 11.3 ± 0.2** | 3.0 ± 0.5** |
| 5 mg/kg Rotenone +200 mg/kg L.carbidopa | 4.7 ± 0.6++ | 8.3 ± 0.3++ |
| 5 mg/kg Rotenone +200 mg/kg AGME1 | 6.5 ± 0.3++ | 6.3 ± 0.4++ |
| 5 mg/kg Rotenone +400 mg/kg AGME2 | 4.1 ± 0.4++ | 9.2 ± 0.2++ |

Values are represented in Mean ± SEM ($n = 6$). ** Indicates a statistically significant difference at the 0.01 level ($P < 0.01$). ++ Indicates a correlation coefficient above 0.6.

*tuberculatum* (Al-Muniri and Hossain, 2017). Total phenolic contents are another important ingredient present in medicinal plants. The findings of the current study show that the methanolic extract, which is followed by the chloroform, aqueous, and the hexane fraction in terms of total phenolic content, has the highest concentration. According to Casquete et al., (2015) studies, phenolic compounds from medicinal plants obtainable extraction and isolated in similar ways. DPPH, $H_2O_2$ and ABTS free radicals scavenging assays were used to investigate the oxidants scavenging proficiency of different fractions of plant extracts (Mathew and Abraham, 2006). During the assay of DPPH free radicals, causing a change in color into dark red to light yellow. The most advanced activity for antioxidants, both lipophilic and hydrophilic, is the ABTS assay (Nenadis et al., 2004). The biological $H_2O_2$ regulation of free radicals is essential because hydrogen peroxide breaks down into single $O_2$ molecules and radicals of hydroxyl when exposed to ions of transitional metals (Ifeanyi, 2018). In DPPH, ABTS and $H_2O_2$, the greatest levels of methanol extracts' capacity to scavenge free radicals were discovered to be 81%, 80.3% and 81.2%. The findings of Khan et al. (2017) provide complete support for our findings. The varying free radical scavenging abilities of various extracts and fractions may be caused by differences in molecular weight, the quantity of aromatic rings and the nature active components within fractions (Akharaiyi, 2011; Anyasor et al., 2010). The fact that AGME had more antioxidant activity when compared to its fractions may be an indication of its high phenolic content. Antioxidant activity and total phenolic contents were shown to be significantly correlated. According to the correlations, a sample's phenolic and flavonoid content will determine how antioxidant-like an effect it is. The findings of earlier investigations (Awika et al., 2005; Dykes and Rooney, 2006), which shown a close link between the phenolic contents and antioxidant activities, correspond with our findings. Other cereals like finger millet and wheat have also shown significant correlations among phenolic content, flavonoids and antioxidant activity (Adom et al., 2003; Siwela et al., 2007).

**Table 7.** Effects of extract on Y- maze test.

| Groups | Normalize time (%) |
|---|---|
| Control | 80.6++ |
| 5 mg/kg Rotenone | 55.3** |
| 5 mg/kg Rotenone +200 mg/kg L.carbidopa | 78.7++ |
| 5 mg/kg Rotenone +200 mg/kg AGME1 | 70.5++ |
| 5 mg/kg Rotenone +400 mg/kg AGME2 | 79.1++ |

Values are represented in Mean ± SEM ($n = 6$). ** Indicates a statistically significant difference at the 0.01 level ($P < 0.01$). ++ Indicates a correlation coefficient above 0.6.

The goal of this study was investigating the therapeutic potential of AGME against neuro-degenerations caused. Numerous researches have discussed neuro-protection using natural products and medicinal plant remedies (Kumar and Khanum, 2012). According to research on a diet rich in

flavonoids, pollutants' negative effects on the brain can be reduced. Kumar and Khanum (2012) and Salehi et al. (2020) all found that *In-vitro* ethanol-encouraged apoptosis was inhibited by flavonoids found in supplements. To protect the growing brain, AGME was investigated in our present research outcomes. Our research suggests AGME reduces oxidative stress. These findings indicate that AGME may reduce neurotoxicity. It's also interesting to note that Rotenone significantly changed the activity of a number of antioxidant enzymes, including CAT, POD, GSH, SOD and LPO, which helped to reduce neuro-inflammation. According to earlier research (Zhao et al., 2018), chemicals are responsible for promoting inflammation in the CNS. Furthermore, the brain's hippocampus and cerebellum have significant oxidative resistance because of low vitamin-E levels. According to research by Wang et al. (2018), vitamin-E may inhibit the encouraged hippocampus stimulation.

## 5. Conclusions

In the present study, it was reported for the first time that A. *grahamianus* attenuated immature intoxication in rat brains caused by rotenone and showed potent ant-Parkinson effects. These effects are might be due the presence of bioactive metabolites.

## Acknowledgements

We would like to express our gratitude to HEC for funding which enabled the successful completion of this project. The HEC of Pakistan provided research funds for the completion of this work under HEC-NRPU project No. 17575.

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