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Pachira macrocarpa Schltdl. & Cham., HPLC Profile, and Neuroprotective Potential via Regulation of JNK, miRNA132, and miRNA-125b

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Cite This: *ACS Omega* 2023, 8, 27238−27246

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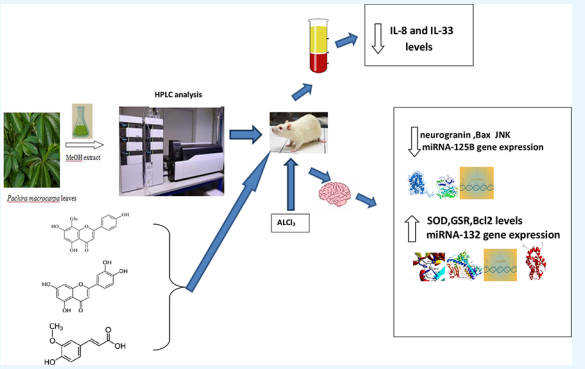
ABSTRACT: In this study, we investigated the polyphenolic profile of *Pachira macrocarpa* Schltdl. & Cham. by HPLC analysis and we also isolated three compounds from the ethyl acetate leaf extract, which were identified by different spectral data as vitexin 1, luteolin 2, and ferulic acid 3. Moreover, we investigated the three isolated compounds and the plant extract for their therapeutic potential against $AlCl_3$ exposure-induced neurotoxicity in rats. This investigation aims to determine whether vitexin, luteolin, and ferulic acid in *Pachira macrocarpa* Schltdl. & Cham. extract (*P. macrocarpa*) have the ability to treat $AlCl_3$-induced brain toxicity in rats. Six groups of rats were created: group 1 (normal group), group 2 treated with $AlCl_3$, and groups 3, 4, 5, and 6 treated with $AlCl_3$ with vitexin, luteolin, ferulic acid, and *P. macrocarpa* extract, respectively, for 28 days. Neurotoxicity was assessed by measuring plasma IL-8 and IL-33 as well as brain superoxide dismutase (SOD), glutathione reductase (GSR), B-cell lymphoma-2 (BcL-2), B-cell lymphoma-2 associated-x (Bax), and neurogranin using the ELISA technique and c-Jun N-terminal kinase (JNK), miRNA-125b, and miRNA-132 levels using western blot and PCR. HPLC analysis identified major phenolics and flavonoids. Among the phenolics identified, chlorogenic acid was prevalent (2159.14 μg/g), and regarding flavonoids, rutin was prevalent (204.69 μg/g). A significant elevation of IL-8 and IL-33 as well as brain Bax, neurogranin, and JNK levels and of miRNA-125b gene expression levels was observed following $AlCl_3$ exposure. However, significant depletion of SOD, GSR, BcL-2, total protein, and miRNA-132 gene expression was observed in $AlCl_3$-treated rats. Administration of the *P. macrocarpa* extract and its isolated compounds significantly increased SOD, GSR, BcL-2, total protein, and miRNA132 gene expression and decreased IL-8 and IL-33 as well as brain Bax, neurogranin, and JNK levels and brain miRNA-125b gene expression compared to $AlCl_3$-treated rats. *P. macrocarpa* extract and its isolated compounds ameliorated $AlCl_3$-induced oxidative stress and neurotoxicity in rats.



1. INTRODUCTION

Aluminum (Al) is one of the main heavy metals involved in the onset and development of neurodegenerative diseases[1] such as Alzheimer's disease (AD), which is a complicated, diverse, and advancing neurological disease disorder that affects several parts of the brain such as the hippocampus, olfactory bulb, cortical locations, spinal cord, and hippocampus and is classified by formation of beta-myloid (Aβ) neuropathology, as well as neurogenesis and astroglial amplification, resulting in neurodegeneration and inducing apoptosis.[2,3] Despite several theories being put out, the true genetic, epigenetic, and/or environmental cause of late-onset or sporadic AD (sAD) has not yet been identified.[4]

$AlCl_3$ is found in a wide range of products, including toothpaste, meals, pharmaceuticals, and bottled drinking water; its usage is exceedingly complicated.[5] The ingestion of certain metal toxicants, such as $AlCl_3$, which are absorbed into the body by occupational exposure, food pollutants, drinking water contaminants, and meals made in $AlCl_3$ cookware, was directly linked to the onset of AD.[6] $AlCl_3$ may alter the blood−brain barrier (BBB) and subsequently accumulate in the brain.[7]

Various plants have been reported to be therapeutically effective in cases of memory loss, AD, and disorders associated with aging. It is noted that during the past 20 years, various plant-based medicines have shown promise in reducing dementia and AD symptoms.[8]

Pachira macrocarpa is a tropical evergreen tree that grows in South America and Africa and belongs to the Malvaceae family, which contains about 244 genera with 4225 known species;

Received: April 14, 2023
Accepted: July 10, 2023
Published: July 21, 2023

well-known plants of this family include okra, cotton, and cacao.[9] *P. macrocarpa* trees grow up to 15 m in height. Their leaves are shiny green lanceolate or obovate, five on each branch, and their flowers are hermaphrodite with double-perianth long pinkish stamens. Their fruits are capsule-like, large with up to 30 cm length, brown, woody, and egg-shaped. Their seeds are brown, irregularly angulate, and 3−6 cm long at maturity.[10] *P. macrocarpa* is famed as the fortune tree as it is assumed to attract wealth and improve your financial prosperity; also, it purifies air by absorbing toxic components, which is believed to reduce stress and anxiety.[11]

Before modern medicine, humans were directed to use herbs and weeds for treating diseases and improving health. *P. macrocarpa* leaves have been used for healing wounds because of their antiseptic, antifungal, and antibacterial properties; they also prevent bruises, cramps, and rheumatism. Essential oil of the leaves is used as natural insect repellent and possesses strong anti-inflammatory activity. Boiled leaves are used to treat fever. Fruits are used to treat respiratory diseases, while roasted seeds are ground into flour to make bread or as a drink like hot chocolate. The bark of the tree is used as a blood tonic for anemia, low blood pressure, and fatigue.[12]

Most research done concerning our plant was focused on its volatile oil content. Scanty reports have dealt with its bioactive compounds; therefore, in this study, we wanted to reveal the plant's phytoconstituents and quantitative and qualitative HPLC analyses of the phenolic and flavonoid contents of the leaf methanol extract were carried out. In addition, we have isolated three bioactive compounds from the ethyl acetate leaf extract. The present study was extended to evaluate the neuroprotective and antioxidant activities of *P. macrocarpa* extract and its bioactive compounds vitexin, luteolin, and ferulic acid against $AlCl_3$-induced brain toxicity.

2. RESULTS

2.1. Spectral Data of the Isolated Compounds. Compound (1) was obtained as white powder. EI/MS showed a molecular ion peak at 432.4 *m/z*, ^{1}H-NMR (DMSO-d_6) δ ppm: 3.15 (1H, m, H-5″), 3.32 (1H, m, H-3″), 3.58 (1H, m, H-4″), 3.98 (1H, dd, J = 12, 5.8 Hz, H-6″a), 4.11 (1H, dd, J = 12, 5.8 Hz, H-6″b), 4.13 (1H, t, J = 9 Hz, H-2″), 6.96 (2H, d, J = 8.6 Hz, H-3′,5′), 7.13 (2H, d, J = 8.6 Hz, H-2′,6′). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 60.9 (C-6″), 70.2 (C-4″), 70.6 (C-2″), 73.1 (C-1″), 78.2 (C-3″), 81.1 (C-5″), 97 (C-8), 102 (C-3), 103.6 (C-10), 115.1 (C-3′, 5′), 121.5 (C-1′), 128.2 (C-2′, 6′), 156 (C-9), 160.3 (C-5), 162 (C-4′), 164 (C-7), 181.8 (C-4). Compound (1) was identified as vitexin.[13]

Compound (2) was obtained as yellow powder. EI/MS showed a molecular ion peak at 286.2 *m/z*, ^{1}H-NMR (DMSO-d_6) δ ppm: 7.35 (1H, d, J = 2 Hz, H-2′), 7.41 (1H, d, J = 8.5 Hz,H-6′), 6.89 (1H, d, J = 8 Hz, H-5′), 6.20 (1H, s, H-3), 6.44 (1H, d, J = 2 Hz, H-8), 6.25 (1H, d, J = 1.5 Hz,H-6). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 163.9 (C-2), 102.8 (C-3), 180 (C-4), 160.5 (C-5), 98.2 (C-6), 164 (C-7), 94.5 (C-8), 158.4 (C-9), 103.0 (C-10), 121 (C-1′), 113.5 (C-2′), 144.5 (C-3′), 148 (C-4′), 116 (C-5′), 122 (C-6′). Compound (2) was identified as luteolin.[14]

Compound (3) was obtained as buff powder. EI/MS showed a molecular ion peak at 194.18 *m/z*, ^{1}H-NMR (DMSO-d_6) δ ppm δ: 12.47 (1H, s, COOH), 7.51 (1H, dd, J = 8 and 2 Hz, H-5), 7.49 (1H, d, J = 2.5 Hz, H-3), 7.64 (1H, d, J = 15 Hz, H-1′), 6.85 (1H, d, J = 9HZ, H-6), 6.27 (1H, d, J = 15 Hz, H-2′), 3.84 (3H, s, H-4′). ^{13}C NMR (100 MHz, DMSO-*d6*): δ 179 (C-3′), 148.2 (C-1), 147.3 (C-6), 145.5 (C-1′), 136.26 (C-4), 122.49 (C-3), 116.1 (C-2′), 115.5 (C-2), 103.54 (C-5) 55.8 (C-4′). Compound (3) was identified as ferulic acid.[15]

2.2. RP-HPLC Analysis. The analysis identified the presence of 10 flavonoids and phenolics of the methanol leaf extract of *P. macrocarpa*. Major phenolics were chlorogenic acid (2159.14 μg/g), ferulic acid (915.71 μg/g), and gallic acid (881.07 μg/g), while major flavonoids detected were rutin (204.69 μg/g) and naringenin (136.11 μg/g) (Table 1). Chromatograms show that vanillin, kaempferol, daidzein, quercetin, syringic acid, ellagic acid, and hesperetin were absent.

Table 1. RP-HPLC Analysis of the Methanol Leaf Extract of *Pachira macrocarpa*[a]

identified component	area	conc. (μg/g)
gallic acid	329.05	881.07
chlorogenic acid	459.97	2159.14
catechin	105.48	62.81
methyl gallate	14.57	31.83
caffeic acid	157.48	447.85
pyrocatechol	32.03	41.59
rutin	46.18	204.69
coumaric acid	65.49	199.14
vanillin	ND	ND
ferulic acid	256.76	915.71
naringenin	10.66	136.11
daidzein	ND	ND
quercetin	ND	ND
kaempferol	ND	ND
hesperetin	ND	ND
syringic acid	ND	ND
ellagic acid	ND	ND

[a]ND: not detected.

2.3. Biological Study. Table 2 shows the activity of the *P. macrocarpa* extract and its bioactive compounds on levels of plasma interleukin-8 (IL-8) and interleukin-33 (IL-33) in control and treated rats. Our results revealed a notable increase ($p < 0.05$) in IL-8 and IL-33 in rats treated with $AlCl_3$ to 317.96 and 227.45%, respectively, in comparison with group I. The administration of luteolin clarified a decrease ($p < 0.05$) in plasma IL-8 and IL-33 in rats treated with $AlCl_3$ by 61.83 and 50.26%, respectively, in comparison with group II. Also,

Table 2. Effect of *Pachira macrocarpa* Extract and Its Bioactive Compounds on Levels of Plasma Interleukine-8 (IL-8) and Interleukine-33 (IL-33) in Control and Treated Rats[a]

groups	IL-8 (ng/mL)	IL-33 (ng/dL)
I	10.02 ± 1.13a	26.30 ± 2.62a
II	31.68 ± 2.95b	59.82 ± 4.35b
III	12.09 ± 1.25a	29.75 ± 2.76c
IV	12.50 ± 1.18a	26.85 ± 1.34a
V	16.21 ± 1.52c	32.47 ± 2.03d
VI	22.34 ± 2.32d	39.52 ± 3.89e

[a]Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at $P \leq 0.05$.

https://doi.org/10.1021/acsomega.3c02562
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administration of $AlCl_3$ with vitexin reveals a significant decrease ($p < 0.05$) in plasma IL-8 and IL-33 by 60.54 and 55.11%, respectively, in comparison with the $AlCl_3$-treated group ($P < 0.05$). On the other hand, treatment of $AlCl_3$-administrated rats with ferulic acid showed a significant decrease in plasma IL-8 and IL-33 by 48.83 and 45.72%, respectively, in comparison with group II ($P < 0.05$). However, administration of $AlCl_3$-treated rats with *P. macrocarpa* extract reveals a significant decrease ($p < 0.05$) in plasma IL-8 and IL-33 by 29.48 and 33.93%, respectively, in comparison with group II ($P < 0.05$).

Table 3 shows the effect of the *P. macrocarpa* extract and its bioactive compounds on levels of brain superoxide dismutase (SOD), glutathione reductase (GSR), and B-cell lymphoma 2 (Bcl-2) in control and treated rats. The present results reveal a significant decrease ($p < 0.05$) in SOD, GSR, and Bcl-2 in rats treated with $AlCl_3$ by 60.21, 71.88, and 53.82%, respectively, in comparison with group I. The administration of luteolin clarified the increase ($p < 0.05$) in brain SOD, GSR, and Bcl-2in rats treated with $AlCl_3$ to 197.46, 307.22, and 205.28%, respectively, in comparison with group II. Also, administration of $AlCl_3$ and vitexin (50 mg/kg) reveals a significant increase ($p < 0.05$) in brain SOD, GSR, and Bcl-2 to 192.61, 323.7, and 210.5%, respectively, in comparison with group II ($P < 0.05$). On the other hand, administration of $AlCl_3$ and ferulic acid showed a notable increase in brain SOD, GSR, and Bcl-2 to 180.58, 331.3, and 202.7%, respectively, in comparison with group II ($P < 0.05$). Furthermore, administration of $AlCl_3$ and *P. macrocarpa* extract reveals a notable increase ($p < 0.05$) in brain SOD, GSR, and Bcl-2 to 132.3, 224.6, and 130.7%, respectively, in comparison with $AlCl_3$-treated group ($P < 0.05$).

Table 3. Effect of *Pachira macrocarpa* Extract and Its Bioactive Compounds on Levels of Brain Superoxide Dismutase (SOD), Glutathione Reductase (GSR), and B-Cell Lymphoma 2 (Bcl-2) in Control and Treated Rats[a]

groups	SOD (U/mg protein)	GSR (μmol/min/mg)	Bcl-2 (ng/mg protein)
I	47.63 ± 3.17a	29.52 ± 2.93a	23.81 ± 2.42a
II	18.95 ± 1.45b	8.30 ± 1.51b	10.98 ± 1.21b
III	37.42 ± 3.28c	25.50 ± 1.82c	22.54 ± 2.84a
IV	36.50 ± 2.79c	26.87 ± 1.13c	23.11 ± 1.528a
V	34.22 ± 1.95c	27.50 ± 1.54ac	22.26 ± 1.59a
VI	25.07 ± 2.32d	18.64 ± 1.77d	14.35 ± 1.41c

[a]Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at $P \leq 0.05$.

Table 4 shows brain neurogranin, Bcl-2-associated X-protein (Bax), and protein in control and treated rats. Oral administration of $AlCl_3$ led to an increase in brain neurogranin and Bax significantly to 255.74 and 329.40%, respectively, as well as a significant decrease in brain protein by 63.45% ($P < 0.05$) in comparison with group I, which indicates acute brain toxicity. Treatment of rats with luteolin significantly decreased the level of brain neurogranin and Bax by 58.90 and 65.22%, respectively, as well as a significant increase in brain protein to 200.05% in comparison with group II ($P < 0.05$). Also, administration of $AlCl_3$ and vitexin significantly decreased the level of brain neurogranin and Bax by 56.92 and 54.67%, respectively, as well as a significant increase in brain protein to 225.33% as compared with group II ($P < 0.05$). The administration of ferulic acid clarified the decrease ($p < 0.05$) in brain neurogranin and Bax by 52.32 and 48.67%, respectively, as well as a significant increase in brain protein to 251.52%, as compared with group II ($P < 0.05$). Administration of *P. macrocarpa* extract showed a notable decrease in the level of brain neurogranin and Bax by 26.4 and 31.75%, respectively, as well as a notable increase in brain protein to 181.70%, as compared with group II ($P < 0.05$).

Table 4. Effect of *Pachira macrocarpa* Extract and Its Bioactive Compounds on Levels of on Brain Neurogranin, Bcl-2-Associated X-Protein (Bax), and Protein in Control and Treated Rats[a]

groups	neurogranin (ng/mg protein)	Bax (ng/mg protein)	protein (mg/g tissue)
I	497.51 ± 24.60a	6.53 ± 0.37a	52.70 ± 3.36a
II	1272.36 ± 67.12b	21.51 ± 1.71b	19.26 ± 2.74b
III	522.93 ± 24.78c	7.48 ± 0.72c	38.53 ± 3.42c
IV	548.07 ± 33.22d	9.75 ± 0.50d	43.40 ± 2.61d
V	606.56 ± 32.80e	11.04 ± 1.17e	48.52 ± 5.38e
VI	936.47 ± 53.41f	14.68 ± 2.26f	34.88 ± 3.69f

[a]Data shown are mean ± standard deviation of number of observations within each treatment. Data followed by the same letter are not significantly different at $P \leq 0.05$.

Figures 1 and 2 declare a significant ($P < 0.05$) increase in brain c-Jun NH2-terminal protein kinase (JNK) to 322.66% in group II compared to group I. The levels of brain JNK were significantly decreased by 57.68%, when treated with luteolin. When rats were administered vitexin and $AlCl_3$, there was a significant decrease in brain JNK by 62.59% as compared with group II. $AlCl_3$ and ferulic acid (50 mg/kg) were administered, which caused a significant decrease in brain JNK by 54.63% compared to group II. Also, administration of $AlCl_3$ and (50 mg/kg) significantly decreased the level of brain JNK by 38.50% compared to group II ($P < 0.05$).

Figure 3 shows a significant decrease in levels of brain miRNA-132 expression by 58.44% as well as a significant increase in brain miRNA-125b gene expression level to 572.64% in group II as compared with group I. Administration of luteolin shows a significant increase in levels of brain miRNA-132 expression to 228.20% as well as a significant decrease in the brain miRNA-125b gene expression level by 65.40% as compared with group II. Also, administration of vitexin with $AlCl_3$ shows a significant increase in levels of brain miRNA-132 expression to 200.0% as well as a significant decrease in brain miRNA-125b gene expression level by 73.49% as compared with group II. In addition, when $AlCl_3$ was administrated with ferulic acid, brain miRNA-132 expression levels showed a significant increase to 184.61% as well as significant decrease in brain miRNA-125b gene expression level by 44.48% as compared with group II. However, administration of *P. macrocarpa* extract showed a significant increase in the level of brain miRNA-132 expression to 171.79% as well as a significant decrease in brain miRNA-125b gene expression level by 19.93% compared to group II ($P < 0.05$).

3. DISCUSSION

Environmental pollution negatively impacts human beings, greatly affects our health, and is considered as one of the most serious global challenges. Our plants' life generates a positive

https://doi.org/10.1021/acsomega.3c02562
ACS Omega 2023, 8, 27238−27246

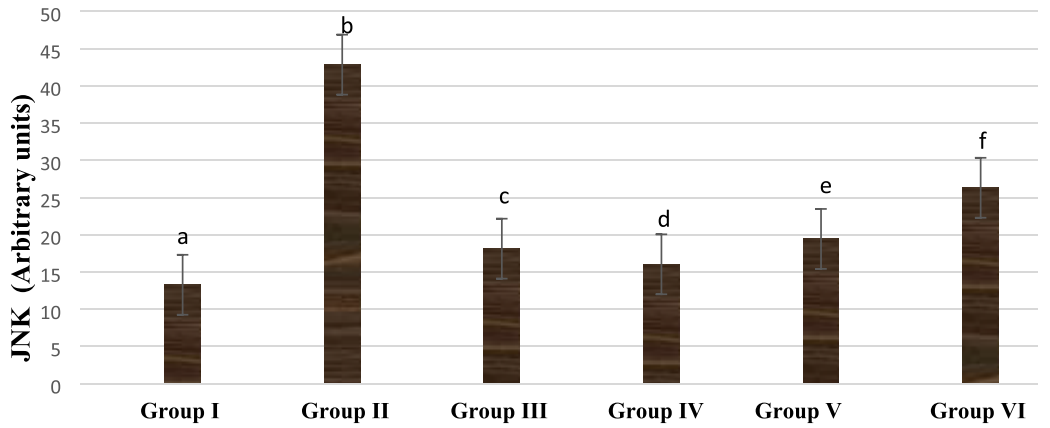

Figure 1. Effect of *Pachira macrocarpa* extract and its bioactive compounds on levels of brain c-Jun NH2-terminal protein kinase (JNK) in control and treated rats. Data followed by the different letters are significantly different at $P \leq 0.05$.

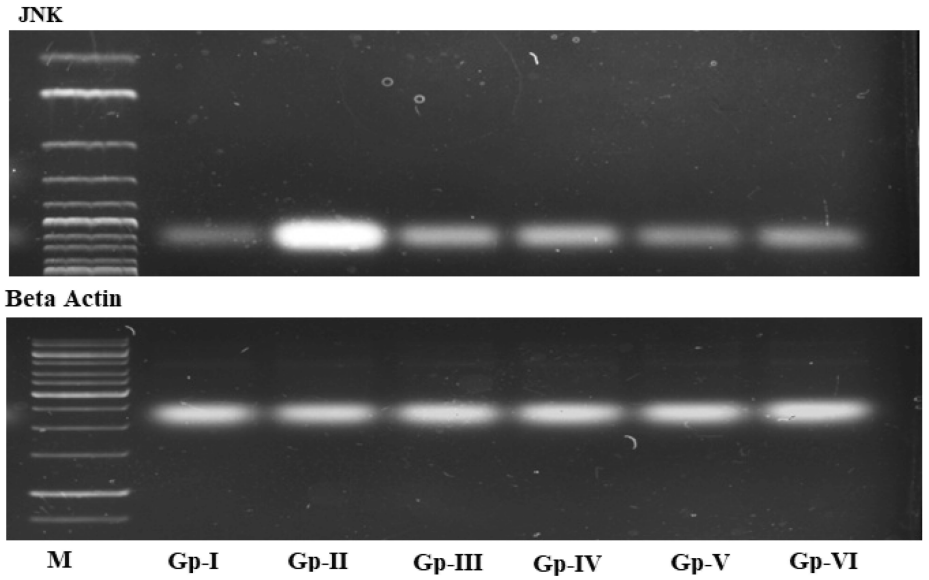

Figure 2. An agarose gel electrophoresis pattern of brain JNK and beta-actin in different studied groups. M: DNA marker with 100 bp.

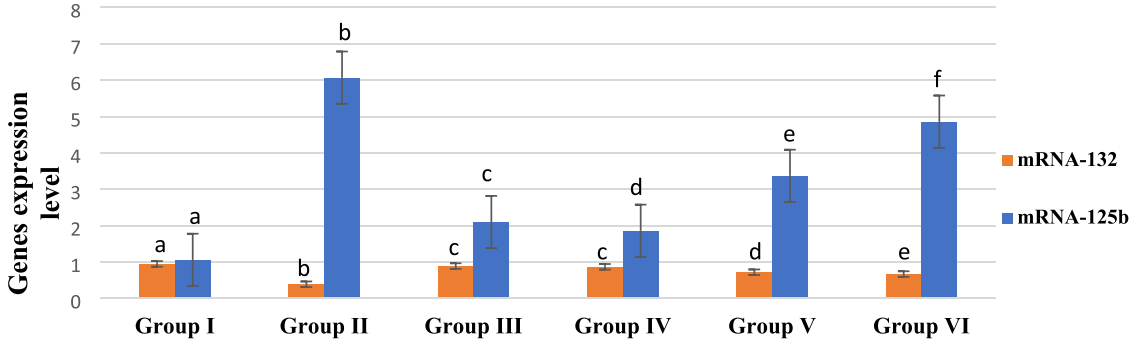

Figure 3. Effect of *Pachira macrocarpa* extract and its bioactive compounds on levels of brain miRNA-132 and -125b gene expression in control and treated rats. Data followed by different letters within each parameter are significantly different at $P \leq 0.05$.

influence on everyone and everything. *P. macrocarpa* is a common house plant; thus, keeping this plant indoors makes it a magical indoor plant, as it has the power to spread positivity and comfort everywhere. HPLC allows qualitative and quantitative analyses of the phenolic and flavonoid compounds contained in the plant extract. The analytes are then identified and quantified by comparison against standards. The HPLC profile indicates that the leaf extract contains 10 polyphenolic compounds; polyphenols have played an important role as antioxidants in the last decade.[16] Polyphenol induces inhibition of both apoptosis in neurons and a reduction of neuro-inflammatory reactions in microglia. Phenolic compounds have gained attention based on their role in improving health and treating a variety of human illnesses. Their chemical structures,

https://doi.org/10.1021/acsomega.3c02562
ACS Omega 2023, 8, 27238−27246

which are composed of an aromatic ring having mobile hydrogen, the 3′,4′-ortho-dihydroxy group in the B ring, and the 5-OH group in the A ring with a 4-carbonyl group are responsible for the antioxidant capacities.[17]

The activities of the three isolated compounds from *P. macrocarpa* were considered. First is vitexin, which is a flavone glycoside; it has plenty of pharmacological effects including anti-inflammatory, antioxidant, anticancer, and neuroprotective. Vitexin acts as an antioxidant against ROS, lipid peroxidation, and other oxidative damages with alterations in the nervous system's oxidative and defense biomarkers due to its ability to quench ROS and boost antioxidant enzymes.[18]

Second is ferulic acid, which is a free radical scavenger and has been approved as a food additive; it forms stable phenoxyl radicals, and it is more bioavailable than other dietary flavonoids.[19]

Finally is luteolin, which is a naturally occurring flavone that possesses various biological activities such as antimicrobial, antiallergic, antidiabetic, and antioxidant.[20] Luteolin reduces age-related inflammation in the brain and memory deficits as it inhibits the production of neurotoxic inflammatory mediators.[20]

In the present study, treatment of rats with $AlCl_3$ led to an elevation in the plasma levels of plasma IL-8 and IL-33. Numerous inflammatory substances, including cytokines, chemokines, and ROS,[21,22] were released by astrocytes and microglial cells in an active state. One of the important events occurring during AD is the activation of microglia and astrocytes, which can be caused by chronic Al buildup. It has also been reported that certain toxicants could up-regulate pro-inflammatory cytokines such as IL-8 and IL-33 in macrophages[7] as well as bronchial[23] and alveolar[24] epithelial cells. Several reports showed that the absence of IL-8 production in the presence of an NF-B inhibitor, dexamethasone, demonstrated that IL-8 expression is entirely dependent on NF-B activation.[25]

In the present study, administration of oral individual doses (50 mg/kg) of luteolin, vitexin, ferulic acid, and *P. macrocarpa* extract to $AlCl_3$-treated rats inhibited the secretion of IL-8 and IL-33 and reflected their anti-inflammatory activity. Our results were confirmed with the results of Cheetal et al.,[26] Costa et al.,[27] and Zheng et al.,[28] who reported that luteolin, vitexin, and ferulic acid have anticancer, antioxidant, anti-inflammatory, anti-nociceptive, antihypertensive, antispasmodic, antiviral, and antidepressant properties in vivo and in vitro. Several pharmacological actions including antibacterial, antifungal, nematocidal, antiproliferative, and anti-inflammatory (IL-8 and IL-33) activities have been linked to the presence of luteolin, vitexin, and ferulic acid in *P. macrocarpa* extract.

Our findings demonstrate that rats treated with $AlCl_3$ had lower levels of brain SOD, GSR, and Bcl-2. Superoxide anions and other reactive oxygen species are produced as a result of $AlCl_3$'s inhibition of NADH action, which weakens the antioxidant defense system.[29] These radicals harm lipids, proteins, DNA, and other biological components. An indicator of the level of oxidative stress in the brain is ROS, which is the byproduct of the peroxidation of polyunsaturated fatty acids. The enzyme antioxidants SOD, catalase, and GPx are able to eliminate ROS in the brain. $AlCl_3$ exposure increased ROS levels and reduced GPx, SOD, and other functions.[30,31] Additionally, $AlCl_3$ administration decreased anti-apoptotic indices such as BcL2, which is in line with several studies.[32,33] Co administration of luteolin, vitexin, and ferulic acid prevented $AlCl_3$ effects and enhanced the activities of SOD, GSR,[33−36] and Bcl-2 along with mitochondrial cytochrome-c release prevention.[37] The current study's findings are corroborated by reports that showed that decreased Bcl2 was involved in $AlCl_3$-induced apoptosis in rabbits.[38,39] Furthermore, the Bcl2 level inhibited the apoptotic niche in the cortex of rat brains treated with $AlCl_3$ due to the presence of luteolin, vitexin, ferulic acid, and other phenolic classes, which promotes the improvement of brain SOD, GSR, and BcL2 content levels.[40,41]

One of the processes causing the death of neurons in $AlCl_3$-induced neurodegeneration is apoptosis. During the administration of $AlCl_3$, Aβ oligomers significantly contribute to the death of neuronal cells via activating apoptotic proteins. A member of the apoptotic regulator protein family, Bcl-2 is crucial in controlling cell death in both healthy and pathological circumstances. In the current work, the induction of $AlCl_3$ led to a reduction in brain Bcl2 and total protein levels and an increase in neurogranin, Bax, and JNK protein levels in the cerebral cortex. Documented reports indicating the involvement of neurogranin, Bax, and JNK in $AlCl_3$-induced apoptosis[42−44] provide support for the findings of the current investigation. Furthermore, it was shown that luteolin, vitexin, and ferulic acid lower the neurogranin, Bax, and JNK levels inhibiting the apoptotic niche in the cortex of rat brains treated with $AlCl_3$. Our results were in accordance with the studies of Ahmad et al.,[42] Lyu et al.,[43] and Nakayama et al.,[44] who proved the improvement of neurotoxicity by treatment with luteolin, vitexin, and ferulic acid in $AlCl_3$-induced brain toxicity in rats. This showed that *P. macrocarpa* extract has a neuroprotective impact by preventing neuronal apoptosis, and it is more evidence that dietary flavonoids can prevent intrinsic neuronal death.

It is possible that synthesis and deposition are not the only negative impacts of changed microRNAs in AD neurons. miRNA-132, for instance, has a significant regulatory impact on the central nervous system. In the present study, levels of brain miRNA-132 gene expression were depleted in $AlCl_3$-treated rats. On the other hand, administration of $AlCl_3$ showed an elevation of miRNA-125b gene expression. Studies using a double knockout mouse model for miRNA-132 have shown that these mice had severe cognitive abnormalities in recognition, object identification, and spatial memory.[45] Furthermore, miR-132 was shown to be decreased in the frontal cortex of AD participants who had mild cognitive loss,[46] demonstrating that miRNA-132 plays a crucial regulatory function in cognitive ability. miRNA-125b is noticeably raised in animal models of AD causing hyperphosphorylation of the Tau protein. miRNA-125b injection in C57BL/6 wild-type mice may result in elevated Tau protein phosphorylation and reduced learning and memory function.[47] Similar to this, miRNA-125b overexpression in primary hippocampal neurons can speed up death, disrupt synaptic architecture, and cause Tau to become hyperphosphorylated.[47,48] Additionally, miRNA-125b can be suppressed in primary neurons to lower Tau phosphorylation and kinase expression/activity.

In this study, we demonstrated for the first time that luteolin, vitexin, and ferulic acid can improve the expression miRNA-125b and miRNA-132 gene impairment caused by $AlCl_3$ in mice. The neuroprotective effects of luteolin, vitexin, and ferulic acid are likely the result of the regulation of a certain miRNA expression through decreased release of cytokines and ROS. Our results were consistent with the

https://doi.org/10.1021/acsomega.3c02562
ACS Omega 2023, 8, 27238−27246

Table 5. Classification of Treatment Groups

group	animals	treatments
(I)	normal rats	treated with 3 mL distilled water + normal diet for 4 weeks
(II)	positive control ($AlCl_3$)	$AlCl_3$ (34 mg/kg s.c.)[55]
(III)	$AlCl_3$ + luteolin (50 mg/kg)	treatment of rats with $AlCl_3$ (34 mg/kg s.c.) + luteolin (50 mg/kg) orally for 4 weeks[56]
(IV)	$AlCl_3$ + vitexin (50 mg/kg)	treatment of rats with $AlCl_3$ (34 mg/kg s.c.) + vitexin (50 mg/kg orally) for 4 weeks[57]
(V)	$AlCl_3$ + ferulic acid (50 mg/kg)	treatment of rats with $AlCl_3$ (34 mg/kg s.c.) + ferulic acid (50 mg/kg orally) for 4 weeks[58]
(VI)	$AlCl_3$ + *P. macrocarpa* leaves extract (50 mg/kg)	treatment of rats with $AlCl_3$ (34 mg/kg s.c.) + *P. macrocarpa* extract (50 mg/kg orally) for 4 weeks[11]

studies of Lin et al.,[49] Wang et al.,[50] and Yao et al.,[51] who reported an improvement in the miRNA expression level in experimental rats treated with luteolin, vitexin, and ferulic acid in different animal models.

In the present work, oral treatment of the $AlCl_3$-treated rats with *P. macrocarpa* extract reverted this neurotrophic factor to normal levels as it showed a significant improvement of miRNA-125b and miRNA-132 gene comparable to group II. Our results were confirmed with certain researchers who reported the improved effect of several plant extracts.[52−54]

In this study, the levels of IL-8, IL-33, neurogranin, Bax, JNK, and miRNA-125b expression were significantly increased and the levels of SOD, GSR, BcL2, total protein, and miRNA-132 were decreased upon $AlCl_3$ exposure in the cortex, denoting that $AlCl_3$ induces neuronal apoptosis. Treatment with vitexin, luteolin, and ferulic acid in *P. macrocarpa* extract attenuates $AlCl_3$-induced neurotoxicity. This indicates that luteolin, vitexin, and ferulic acid have a neuroprotective effect by preventing neuronal apoptosis, which is proven by the fact that dietary flavonoids can inhibit neuronal apoptosis.

4. CONCLUSIONS

P. macrocarpa phytoconstituents have been identified through HPLC analysis; also, we have isolated three bioactive compounds and they were identified by different spectral data as vitexin 1, luteolin 2, and ferulic acid 3. The current study highlights the powerful neuroprotective potential of vitexin, luteolin, and ferulic acid in *P. macrocarpa*. According to our research, *P. macrocarpa*-isolated compounds and its polyphenolic constituents contribute to the maintenance of brain neurotransmitter levels and also control inflammation, localized microglial activation, and apoptotic neuronal cell death in the cortex. Since cognitive cytokine-associated illnesses may benefit from the use of luteolin, vitexin, and ferulic acid in *P. macrocarpa*, it makes sense to infer this from both the prior and current investigations. As far as we know, this is the first report of a kind to investigate the phenolic profile of *P. macrocarpa* and its neuroprotective potential. Future studies may focus on using *P. macrocarpa* in human clinical trials to examine its neuroprotective effects.

5. MATERIALS AND METHODS

5.1. Plant. Leaves of *P. macrocarpa* were collected since March 2022 from El Zohria Garden, Giza, Egypt. The plant was authenticated by Dr. Ehsan Sayed, Executive Manager of the garden. A voucher sample (P.m.-62022A) was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, October 6 University.

5.2. Chemicals and Equipment. HPLC standards were supplied by the Central Lab in National Research Centre, silica gel 60 from Fluka, and Sephadex LH-20 from Sigma. TLC-precoated plates (20 × 20 cm) were obtained from E. Merck (Darmstadt, Germany). All solvents were of highly pure analytical grade.

^{1}H-NMR (400, MH_Z) and ^{13}C-NMR (100, MH_Z) spectra were measured on a Bruker High-Performance Digital FT-NMR Avance III 400 MHz spectrometer. The NMR spectra were recorded in DMSO-*d6*, and chemical shifts were expressed in δ (ppm) relative to TMS as an internal standard. Mass spectrometer EI/MS was obtained from Thermo Scientific, ISO Single Quadrupole MS (USA).

5.3. Extraction. Powder of the leaves of *P. macrocarpa* (0.5 kg) was subjected to exhaustive extraction with methanol (5 L). Using a rotary evaporator, the MeOH extract was evaporated to form a residue (85 g). Water (200 mL) was added to the residue and partitioned successively with dichloromethane (2 L) and ethyl acetate (3 L). Under reduced pressure, the solvents were evaporated generating fractions of 7 and 15 g. The EtOAc fraction was then applied to a silica gel column and eluted with CH_2Cl_2-MeOH yielding two main subfractions, which were subjected to several runs using CH_2Cl_2-MeOH and C_6H_{14}-$C_4H_8O_2$ of different concentrations; similar fractions were pooled together by TLC. Further purification was done for the isolation of three compounds: 1 (64 mg), 2 (70 mg), and 2 (80 mg).

5.4. RP-HPLC Analysis. The methanol leaf extract phenolic composition of *P. macrocarpa* was investigated via RP-HPLC. An Agilent 1260 series was used for the analysis. An Eclipse C18 column (4.6 mm × 250 mm i.d., 5 m) was used for separation. The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 mL/min. The mobile phase was sequentially programmed in a linear gradient as follows: 0 min (82% A); 0−5 min (80% A); 5−8 min (60% A); 8−12 min (60% A); 12−15 min (85% A); and 15−16 min (82% A). The multi-wavelength detection device was examined at 280 nm. For each of the sample solutions, the injection volume was 10 μL. The column temperature was maintained at 35 °C.

5.5. Rats. Sixty adult albino rats weighing 150 ± 20 g were obtained from the National Cancer Institute, individually housed in cages in an air-conditioned environment with an 8:00 to 20:00 light cycle, a temperature of 22 ± 2 °C, and a relative humidity of 60%. Each animal was fed a standard food ad libitum during the acclimation phase.

5.6. Experimental Setup. The aim of this study was to determine whether *P. macrocarpa* extract, vitexin, luteolin, and ferulic acid could reduce the symptoms in $AlCl_3$-induced brain toxicity in rats.

As shown in Table 5, the animals were separated into six groups as follows.

The fasted rats were decapitated on day 29, 1 day after completion of the treatments. Blood was collected using sodium fluoride as an anticoagulant. The plasma was obtained after centrifuging and was used to estimate the levels of IL-8 and IL-33 by Quantikine R&D Systems, Inc. (Minneapolis,

https://doi.org/10.1021/acsomega.3c02562
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Minnesota 55413, USA) and Abcam ELISA Technology (Cambridge, CB2 0AX, UK).

Brain was isolated from each experimental animal for estimation of SOD, GSR, BcL-2, Bax, and neurogranin. In brief, 100 mg of brain tissue was homogenized in 1 mL of phosphate-buffered saline (pH 7.4). Under nitrogen stream (2.1), 0.2 mL of the mixture was extracted with 1 mL of chloroform and methanol. The homogenate extracted was divided into two parts. Using an ELISA kit from Cayman Chemical Company (Ann Arbor, Michigan), the initial fraction was used for calculating the levels of SOD and GSR. In the remainder, rat ELISA kits from Novus and MyBioSource, Inc., in the USA as well as ABclonal in the People's Republic of China were used to measure brain Bcl-2, Bax, and neurogranin.

5.7. Western Blotting of Brain Hippocampus JNK. Immunoblots of brain hippocampus JNK were done according to the method of Mascio et al.[59] Briefly, brain samples were homogenized in 50 mM Tris/10 mM EDTA/150 mM NaCl/0.1% mercaptoethanol with protease inhibitors such as soybean trypsin inhibitor, leupeptin, and PMSF and then centrifuged after 20 s of sonication. The total protein of the supernatant was estimated by the method of Henry.[60] Total protein (20 mcg) was isolated and transferred to a nitrocellulose membrane, and then Odyssey buffer was added for 1 h at 25 °C and placed in a primary antibody overnight at 5 °C. Incubation with secondary antibodies that were infrared-labeled (IRDye 800 or IRDye 700DX, LI-COR Biotechnology, Lincoln, Nebraska) allowed for the detection of the bound primary antibody, which was then read and quantitated using a LI-COR Odyssey Imaging System (LI-COR Biosciences, Lincoln, Nebraska).

5.8. Quantitative RT-PCR. Total RNA was taken out from brain tissues using Sepasol-RNA 1 Super (NakaraiTesque), as directed by the manufacturer. qRT-PCR was performed on isolated miRNA. The levels of brain miRNA-125b and miRNA-132 were quantified using a two-step RT-PCR. The PCR reaction mixture consisted of 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.4 M of specific primers (Table 6). The tests

Table 6. PCR Primers Used in qRT-PCR

gene	primer sequence
miRNA-125b	F:5′-TCCCTGAGACCCTAACTTGTGA-3′R: 5′-GCGAGCACAGAATTAATACGAC-3′
miRNA-132	F: 5′-TGGATCCCCCCCAGTCCCCGTCCCTCAG-3′R: 5′-TGAATTCGGATACCTTGGCCGGGAGGAC-3′
GAPDH (internal control for qRT-PCR)	F: 5′-GGGAAGGTGAAGGTCGGAGT-3′R: 5′-TTGAGGTCAATGAAGGGGTCA-3′

were carried out in 50 L of the single-plex reaction mixture. The reaction conditions comprised 40 cycles of 95 °C for 15 s and 60 °C for 1 min, as well as a 2 min pre-incubation at 50 °C and 10 min at 95 °C. The measurements were taken automatically. The qRT-PCR data are presented as a percentage of the control. GAPDH miRNA was employed as the internal control.

5.9. Statistical Analysis. The values are presented as mean ± SD. Except for body weight, all data were done by SPSS/20 software using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. When p was <0.05, differences were considered to be statistically significant.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful for Dr. Ehsan Sayed, Executive Manager of the El Zohria garden, for authentication of the plant identity.

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https://doi.org/10.1021/acsomega.3c02562
ACS Omega 2023, 8, 27238−27246