

Article

Molecular Insights of Neuroprotective Effect of *Cornulaca monacantha* Extract Against LPS-Induced Neuroinflammation Supported by Metabolic Profiling and Protein Interaction Analysis

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Abstract

Natural medicines with neuroprotective, antioxidative, and anti-inflammatory characteristics may act as promising neuroprotective agents against neurodegenerative disorders. This study aims to determine the essential components of the methanolic extract of *Cornulaca monacantha*, and to explore their neuroprotection against lipopolysaccharides (LPS)-induced neuroinflammation in Neuro-2a mouse neuroblastoma cells, and also to investigate the possible underlying molecular mechanism through tracing the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. LC-ESI-TOF-MS/MS was conducted for metabolomic profiling, together with the determination of bioactive compounds. The MTT assay was performed to select an appropriate cytoprotective dose for further analyses. Then, the cells were divided into three groups: control, LPS, and LPS + *C. monacantha* extract. Inflammatory cytokines, gene expression of Nrf2-related genes, and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α)-mediated mitochondrial adaptation were also detected. Protein–protein interaction (PPI) network analysis and gene ontology (GO) enrichment analysis based on biological process were also performed. *C. monacantha* crude extract showed meaningful contents of flavonoids and phenolic compounds, together with other 49 additional hits detected by LC-ESI-TOF-MS/MS. It also showed a significant antioxidant capacity by 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and ferric reducing antioxidant power (FRAP) assays. The extract also exhibited a significant decline in the level of inflammatory biomarkers, along with modulation of the Nrf2 signaling pathway. *C. monacantha* showed beneficial phytochemical composition, which may be responsible for the neuroprotective effect that might be mediated through modulation of Nrf2 expression and related genes, together with the anti-

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inflammatory capability. Other molecular pathways were found to be interconnected with the Nrf2 pathway, as revealed by PPI and GO, which may act as further molecular targets in neuroinflammation.

Keywords: *Cornulaca monacantha*; antioxidant; LC-ESI-TOF-MS/MS; neuroprotective; oxidative stress; Neuro 2a

1. Introduction

Neuroinflammation is now widely recognized as one of the major features in the development and progression of neurodegenerative diseases [1,2], such as Alzheimer's disease and Parkinson's disease, both of which typically result in neuronal loss [3,4]. The hallmark of neuroinflammation is glial activity, particularly activation of microglial cells. Microglia are the primary innate immune cells found in the brain. When stimulated by stimuli such as pathogens; inflammation, or brain injury, microglia are activated and release a significant number of neurotoxic substances such as tumor necrosis factor- α (TNF- α), which then work in concert to promote neurodegeneration [5,6].

The brain is especially responsive to alterations in the redox state; therefore, sustaining redox homeostasis in the brain is essential for averting cumulative oxidative damage [7]. On the other hand, oxidative stress arises from an imbalanced redox state, characterized by either insufficient antioxidant defenses or excessive production of reactive oxygen species (ROS) [3]. Oxidative stress is significantly related to the etiology of various neurodegenerative diseases [8].

Multiple signaling pathways play a significant role in modulating neuroinflammation, including, but not limited to, the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Activation of the Nrf2 pathway may slow the progression of neuroinflammation and eventually, neurodegeneration [3]. Antioxidant response elements (AREs) are associated with the basic leucine-zipper transcription factor Nrf2, which is essential for mitigating oxidative stress by upregulating Nrf2-related antioxidants and protecting neurons from ROS-induced damage [9,10]. Likewise, Nrf2 directly regulates the expression of the heme oxygenase-1 gene (Hmox1). From this point of view, a possible neuroprotective approach for neurodegenerative diseases could involve significant suppression of neuroinflammation or significant activation of Nrf2 [11].

Natural products including plant or herb extracts with antioxidative and neuroprotective characteristics may offer neuroprotection for neurodegenerative diseases [12]. *Cornulaca monacantha* is a member of the Amaranthaceae family, which is widely distributed in Egypt, and is generally known as had and djouri [13,14]. Decoction of the leaves is used in folk medicine to cure jaundice, and it is also regarded as a valuable pasture for camels, particularly for its galactagogue and purgative properties. It has prickly leaves that are used to cure scabies [14]. These beneficial usages are attributed to its possession of many pharmacological properties including, antioxidant, antimicrobial, antidiabetic, antiarthritic, hepatoprotective, and cytotoxic activities [13]. There are limited findings regarding the chemical composition and biological activities of *C. monacantha*. Previous studies have identified a number of secondary metabolites, including isoflavones, flavonoid derivatives such as luteolin and quercetin, gallotannin analogs, triterpenes, and several alkyl amides [13–16]. Among these constituents, certain isoflavones and cinnamoyl tyramine derivatives have demonstrated noteworthy anticancer activity [15]. In addition, selected isoflavone and feruloyltyramine derivatives were reported to exhibit significant antioxidant activity, supporting the potential bioactivity of *C. monacantha* phytochemicals [14].

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria. LPS is widely used to induce neuroinflammation due to its ability to activate innate immune signaling pathways by stimulating the toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF- κ B) inflammatory axis, with subsequent excessive production of pro-inflammatory cytokines [17]. This sustained neuroinflammation promotes microglial overactivation, leading to neuronal damage, synaptic dysfunction, and histopathological degeneration. Moreover, LPS induces oxidative stress and increases the pool of ROS, thereby overwhelming antioxidant defenses [18].

The purpose of the current study was to prepare a methanolic extract of *C. monacantha* and perform metabolomic profiling by LC-ESI-TOF-MS/MS, together with in vitro evaluation of antioxidant capacity. Moreover, we aimed to investigate whether *C. monacantha* extract has any neuroprotective properties against LPS-induced neuroinflammation in Neuro-2a neuroblastoma cells and determine the possible underlying protective mechanism through investigating the Nrf2 pathway and its related genes.

2. Results

2.1. Evaluation of Total Phenolic Content and Total Flavonoid Content

C. monacantha methanolic extract had a total phenolic content (TPC) of 29.91 ± 1.61 μ g GAE/mg extract and a total flavonoid content (TFC) of 6.30 ± 0.37 μ g RE/mg extract.

2.2. Evaluation of the Antioxidant Activity of *C. monacantha* Extract In Vitro

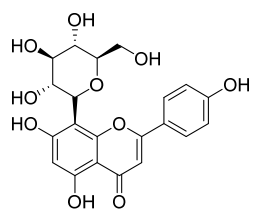
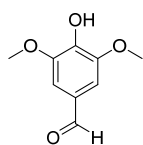
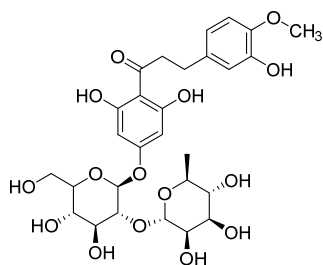
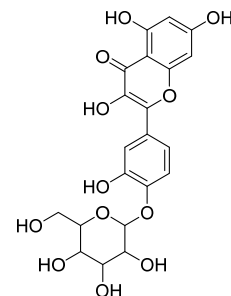
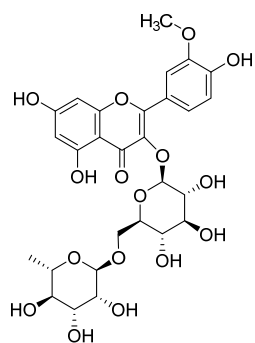
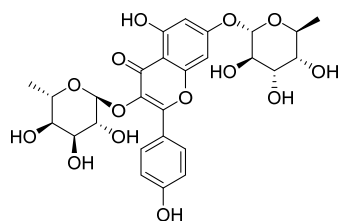
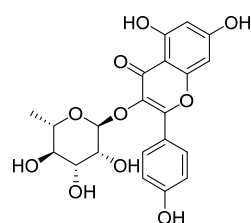
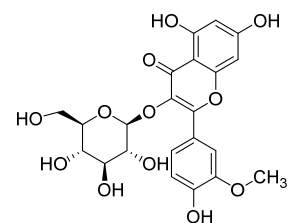
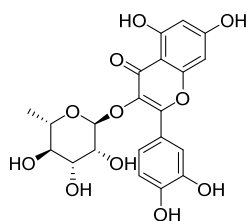
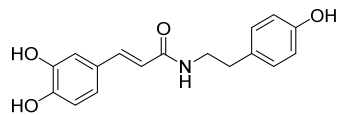
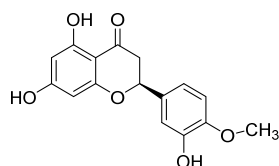
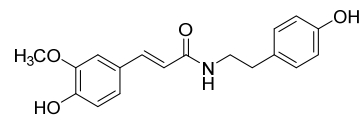
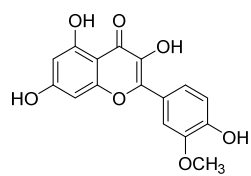
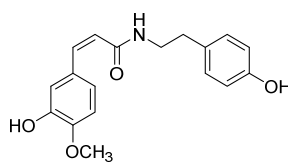
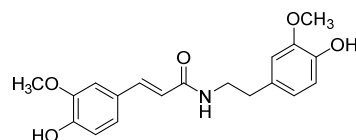
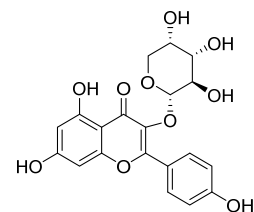
C. monacantha crude extract demonstrated significant antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and ferric reducing antioxidant power (FRAP) assays. *C. monacantha* crude extract ($IC_{50} = 346.4 \pm 8$ μ g/mL) showed significant DPPH radical scavenging activity compared to Trolox reference standard ($IC_{50} = 6.57 \pm 0.449$ μ g/mL). *C. monacantha* ferric reducing capacity was 122.86 ± 5.69 μ M TE/mg, indicating promising activity compared to the Trolox reference standard.

2.3. Analysis of *C. monacantha* Methanolic Crude Extract by LC-ESI-TOF-MS/MS.

Forty-nine hits in *C. monacantha* were identified (Figure 1 and Table 1). Two of them were isoflavones; formononetin and the recently isolated compound, cornulacin. In addition, six other flavonoid aglycones were observed, including quercetin, hesperetin, 3'-methoxy-4',5,7-trihydroxyflavonol, naringenin, luteolin, and apigenin. Procyanidin B2, an epicatechin dimer, was also recorded. Moreover, ten glycosylated flavonoids were recognized: acacetin-7-O-rutinoside, cyanidin-3-O-rutinoside (anthocyanin), kaempferol-7-O-neohesperidoside, vitexin, quercetin-4'-glucoside, isorhamnetin-3-O-rutinoside, kaempferol-3,7-O-bis- α -L-rhamnoside, kaempferol-3-O- α -L-rhamnoside, isorhamnetin-3-O-glucoside, quercitrin, and kaempferol-3-O- α -L-arabinoside.

It is noteworthy that kaempferol and its glycosides were reported in *C. monacantha* for the first time, as well as the anthocyanin cyanidin-3-O-rutinoside and the proanthocyanidin procyanidin B2. In addition, six alkyl amides (nitrogenous compounds) belonging to cinnamoyl tyramines were identified in the current study. These compounds were N-cis-feruloyl tyramine, N-trans-feruloyl tyramine, N-trans-caffeoyl tyramine, N-trans-feruloyl-3-methoxy tyramine, (2aS,3aS) lyciumamide D, and cannabisin F. All the detected alkyl amides were reported previously in the plant [14,15].

Two coumarins, esculin and its glycosylated form, were also recorded. Furthermore, six phenolic acids derivatives, including p-hydroxybenzoic acid, ferulic acid, 2-hydroxyphenyl acetic acid, protocatechuic acid, syringaldehyde, and sinapyl aldehyde, were detected in *C. monacantha* crude extract for the first time. However, vanillic acid, previously isolated from the plant, was not identified [14]. In the current investigation, stigmasterol

**25****26****27****28****29****30****31****32****33****34****35****36****37****38****39****40**

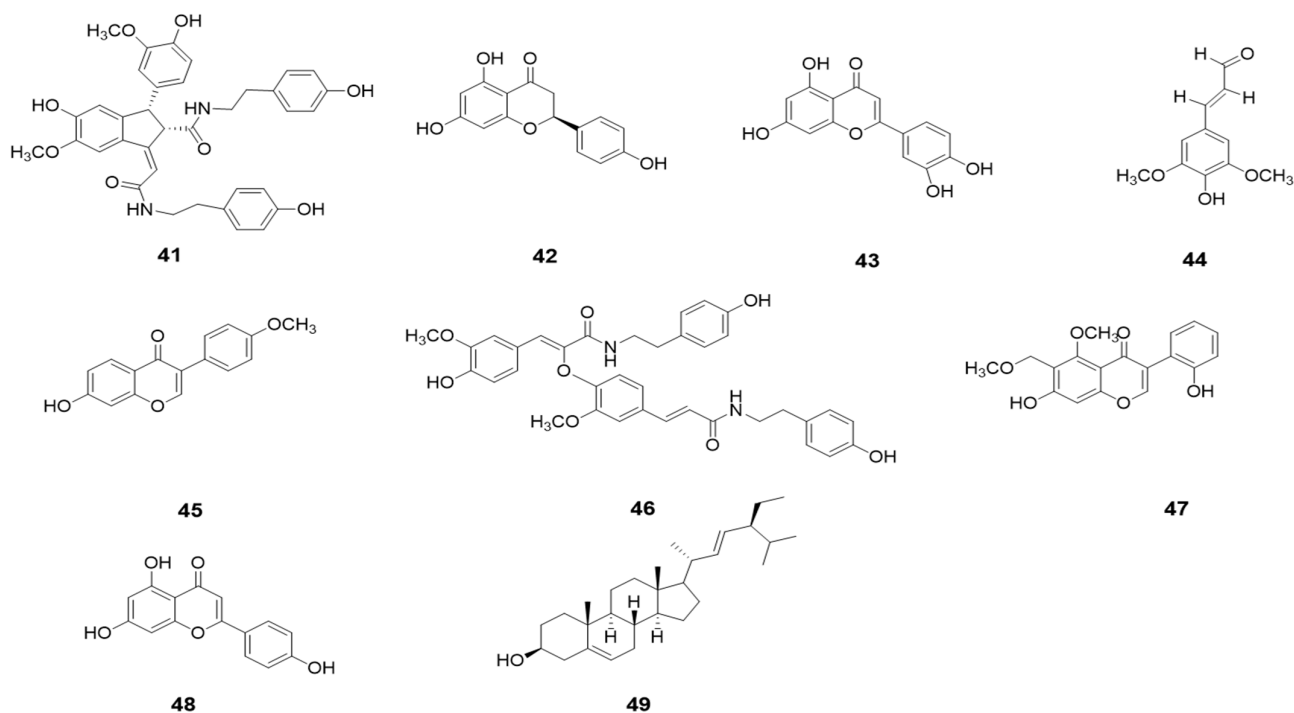


Figure 1. Chemical structures of the identified compounds by LC-ESI-TOF-MS/MS.

Table 1. Metabolites identified in *C. monacantha* crude extract using LC-ESI/TOF/MS/MS.

No.	Ret. Time (min)	Deduced Compound	Molecular Formula	Adduct	Calc. <i>m/z</i>	Observed <i>m/z</i>	Mass Error (ppm)	MS/MS Fragments	Ref.
1	1.152	Succinic acid	C ₄ H ₆ O ₄	[M – H] [–]	117.0193	117.0181	–10.25	117, 99, 73	[19]
2	1.2013	Citric acid	C ₆ H ₈ O ₇	[M – H] [–]	191.0192	191.0204	6.28	173, 111	[20]
3	1.2013	D-(+)-Malic acid	C ₄ H ₆ O ₅	[M – H] [–]	133.013	133.0132	1.5	115, 89, 71	[21]
4	1.2141	Citraconic acid	C ₅ H ₆ O ₄	[M – H] [–]	129.0184	129.019	4.65	129	[22]
5	1.2141	Gluconic acid	C ₆ H ₁₂ O ₇	[M – H] [–]	195.051	195.0526	8.2	195, 129, 87, 75	[23]
6	1.2919	N, N-Dimethylglycine	C ₄ H ₉ NO ₂	[M + H] ⁺	104.0711	104.072	8.64	104, 58	[24]
7	1.4573	Mannitol	C ₆ H ₁₄ O ₆	[M – H] [–]	181.0718	181.0721	1.65	181, 163, 89, 59	[25]
8	1.4693	<i>P</i> -Hydroxybenzoic acid	C ₇ H ₆ O ₃	[M – H] [–]	137.0247	137.0239	–5.83	137, 93	[26]
9	1.5175	D-(+)-Galacturonic acid	C ₆ H ₁₀ O ₇	[M – H] [–]	193.0325	193.0345	10.36	193	[24]
10	1.7972	L-5-Oxoproline	C ₅ H ₇ NO ₃	[M + H] ⁺	130.0497	130.0493	–3.07	130, 84, 71, 70, 56	[26]
11	1.834	Guanosine	C ₁₀ H ₁₃ N ₅ O ₅	[M + H] ⁺	284.0991	284.096	10.91	284, 152, 135, 110	[24]
12	1.883	Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	[M + H] ⁺	268.1028	268.1011	–6.34	268, 136, 119	[26]
13	2.17	Ferulic acid	C ₁₀ H ₁₀ O ₄	[M – H] [–]	193.0501	193.05	–0.51	193, 178, 134	[20]
14	2.4883	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	[M + H] ⁺	355.098	355.0981	0.28	192	[27]
15	4.1228	Esculin	C ₁₅ H ₁₆ O ₉	[M – H] [–]	339.0716	339.0725	2.65	339, 177	[28]
16	4.2497	2-Hydroxyphenyl acetic acid	C ₈ H ₈ O ₃	[M – H] [–]	151.04	151.0385	–9.93	151	[29]
17	5.0213	Protocatechuic acid	C ₇ H ₆ O ₄	[M – H] [–]	153.0193	153.0181	–7.94	153, 109	[28]

18	5.6675	Procyanidin B2	C ₃₀ H ₂₆ O ₁₂	[M + H] ⁺	579.1487	579.1486	−0.17	579	[30]
19	5.9511	Acacetin-7-O-rutino- side	C ₂₈ H ₃₂ O ₁₄	[M − H] [−]	591.16	591.161	1.69	591	[31]
20	6.4682	Kynurenic acid	C ₁₀ H ₇ NO ₃	[M − H] [−]	188.03479	188.0337	−5.31	188, 144	[32]
21	6.4763	Cyanidin-3-O-rutino- side	C ₂₇ H ₃₁ O ₁₅	[M] ⁺	595.1657	595.1629	−4.70	595, 287	[33,34]
22	6.5704	Kaempferol-7-neohe- speridoside	C ₂₇ H ₃₀ O ₁₅	[M − H] [−]	593.15	593.1537	6.23	593	[35]
23	6.7049	Quercetin	C ₁₅ H ₁₀ O ₇	[M + H] ⁺	303.0505	303.0496	−2.96	303	[36]
24	6.7609	Rosmarinic acid	C ₁₈ H ₁₆ O ₈	[M − H] [−]	359.0767	359.0761	−1.67	359	[28]
25	6.8464	Vitexin	C ₂₁ H ₂₀ O ₁₀	[M + H] ⁺	433.1134	433.1115	−4.38	415, 397, 379, 313, 283	[37]
26	6.902	Syringaldehyde	C ₉ H ₁₀ O ₄	[M − H] [−]	181.0501	181.0491	−5.52	181, 151	[36]
27	6.9912	Neohesperidin dihy- drochalcone	C ₂₈ H ₃₆ O ₁₅	[M − H] [−]	611.1976	611.1998	3.59	449	[38]
28	7.0864	Quercetin-4'-glucoside	C ₂₁ H ₂₀ O ₁₂	[M + H] ⁺	465.1033	465.1021	−2.58	465, 303	[36]
29	7.1125	Isorhamnetin-3-O-ruti- noside	C ₂₈ H ₃₂ O ₁₆	[M − H] [−]	623.1631	623.1594	−5.93	623, 315, 300, 271	[24]
30	7.161	Kaempferol-3,7-O-bis- α-L-rhamnoside	C ₂₇ H ₃₀ O ₁₄	[M − H] [−]	577.1557	577.1564	1.21	431, 285	[39]
31	7.5128	Kaempferol-3-O-α-L- rhamnoside	C ₂₁ H ₂₀ O ₁₀	[M − H] [−]	431.1	431.1025	5.79	431	[40]
32	7.7175	Isorhamnetin-3-O-glu- coside	C ₂₂ H ₂₂ O ₁₂	[M + H] ⁺	479.1193	479.1183	−2.08	479, 317, 285, 273, 153	[41]
33	7.87	Quercitrin	C ₂₁ H ₂₀ O ₁₁	[M − H] [−]	447.0927	447.0918	−2.01	447	[37]
34	8.3578	<i>N-trans</i> -caffeoyl tyra- mine	C ₁₇ H ₁₇ NO ₄	[M − H] [−]	298.107	298.1082	4.02	298, 178, 161, 136	[42]
35	9.4266	Hesperetin	C ₁₆ H ₁₄ O ₆	[M − H] [−]	301.0712	301.0712	0	301, 271	[43]
36	9.4676	<i>N-trans</i> -feruloyl tyra- mine	C ₁₈ H ₁₉ NO ₄	[M + H] ⁺	314.1387	314.1381	−1.90	314, 177, 163	[44]
37	9.5229	3'-Methoxy-4',5,7-tri- hydroxyflavonol	C ₁₆ H ₁₂ O ₇	[M − H] [−]	315.0505	315.0527	6.98	315, 300	[36]
38	9.5586	<i>N-cis</i> -feruloyl tyramine	C ₁₈ H ₁₉ NO ₄	[M − H] [−]	312.1236	312.1242	1.92	312, 297, 178	[45]
39	9.8094	<i>N-trans</i> -feruloyl-3'- methoxytyramine	C ₁₉ H ₂₁ NO ₅	[M − H] [−]	342.1351	342.1347	−1.16	342, 178, 148, 135	[46]
40	10.217	Kaempferol-3-O-α-L- arabinoside	C ₂₀ H ₁₈ O ₁₀	[M − H] [−]	417.1502	417.1536	8.15	417, 415	[47]
41	10.22	(2aS,3aS) lyciumamide D	C ₃₆ H ₃₆ N ₂ O ₈	[M + H] ⁺	625.2553	625.2552	−0.15	625	[48]
42	10.29	Naringenin	C ₁₅ H ₁₂ O ₅	[M − H] [−]	271.0633	271.0632	−0.36	271	[28,49]
43	10.852	Luteolin	C ₁₅ H ₁₀ O ₆	[M − H] [−]	285.0399	285.0412	4.56	285	[50,51]
44	11.175	Sinapyl aldehyde	C ₁₁ H ₁₂ O ₄	[M − H] [−]	207.0652	207.0664	5.79	207, 192, 177, 133	[52]
45	11.306	Formononetin	C ₁₆ H ₁₂ O ₄	[M + H] ⁺	269.08	269.0774	−9.66	269	[53]
46	11.4	Cannabisin F	C ₃₆ H ₃₆ N ₂ O ₈	[M + H] ⁺	625.2556	625.2538	−2.87	625	[48]
47	11.465	7-hydroxy-3-(2-hydrox- yphenyl)-5-methoxy-6- (methoxymethyl)-4H-	C ₁₈ H ₁₆ O ₆	[M + H] ⁺	329.1022	329.1023	0.3	329, 298, 297, 227	[15]

chromen-4-one (Cornu-lacin)									
48	11.765	Apigenin	C ₁₅ H ₁₀ O ₅	[M – H] [–]	269.045	269.0461	4.08	269, 225, 181	[51]
49	18.67	Stigmasterol	C ₂₉ H ₄₈ O	[M + H] ⁺	413.3633	413.3631	–0.48	413, 395	[54]

2.4. Biological Assays

2.4.1. Cell Viability and Selection of a Non-Cytotoxic Concentration

Figure 2 illustrates the effect of the extract on the cell viability, which was used to determine the safest concentration associated with the highest cellular viability. Cell viability decreased with increasing extract concentration in the following descending order: 31.25 > 62.5 > 125 > 250 > 500 > 1000 µg/mL. The concentration of 31.25 µg/mL exhibited significantly higher cell viability compared with the other tested concentrations.

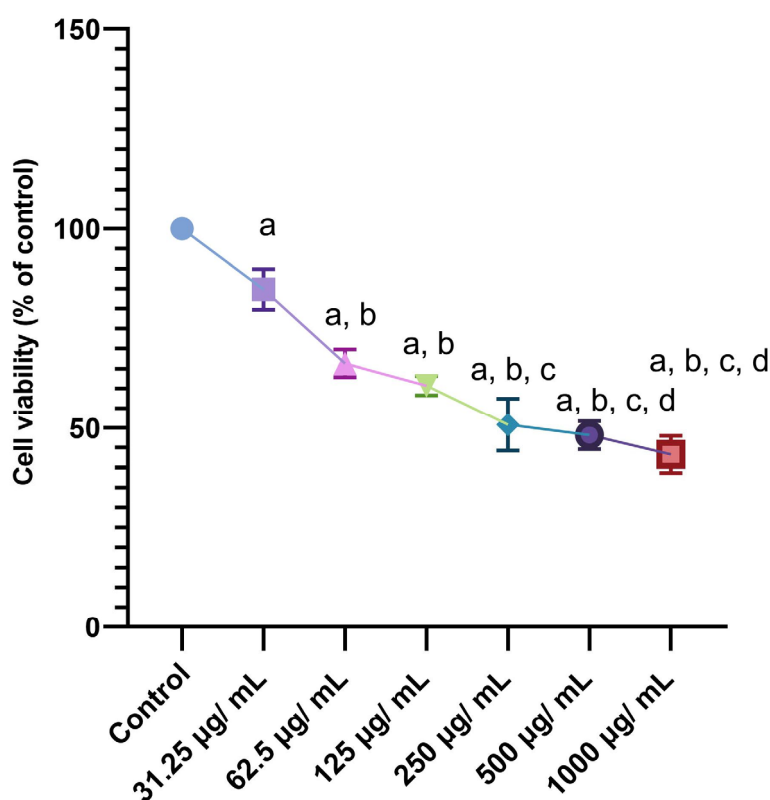


Figure 2. Effect of *C. monacantha* methanolic extract on Neuro-2a cell viability. The tested concentrations of the methanolic extract ranged from 31.25 to 1000 µg/mL. Data are expressed as percentage of the untreated control (set at 100%) and presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc multiple-comparison test ($p < 0.05$). a: significant vs. control; b: significant vs. 31.25 µg/mL; c: significant vs. 62.5 µg/mL; d: significant vs. 125 µg/mL.

2.4.2. *C. monacantha* Extract Decreases Inflammatory Cytokines in Neuro-2a Cells

Figure 3 shows that the LPS-treated group exhibited a significant increase in the levels of interleukin 1β (IL-1β) (Figure 3a) and monocyte chemoattractant protein-1 (MCP-1) (Figure 3b) compared to the control group. In contrast, pretreatment with *C. monacantha* extract significantly reduced IL-1β levels compared to the LPS group, whereas the reduction in MCP-1 levels was not statistically significant.

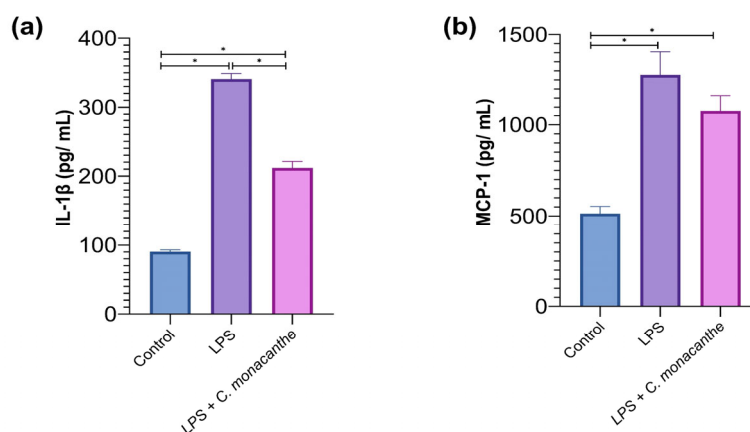


Figure 3. Effect of *C. monacantha* extract on LPS-induced neuroinflammation in Neuro-2a cells. (a) IL-1 β and (b) MCP-1. Data are expressed as mean $\pm$ SD. * $p < 0.05$. LPS: lipopolysaccharide; IL-1 β : interleukin-1 β ; MCP-1: monocyte chemoattractant protein-1.

2.4.3. *C. monacantha* Extract Modulates Nrf2 Signaling Pathway in Neuro-2a Cells

Figure 4 shows that LPS significantly downregulated the mRNA expression of Nrf2, Hmox1, and NAD(P)H quinone dehydrogenase 1 (NQO-1), while significantly upregulating NF- κ B compared to control cells. Pretreatment of the cells with *C. monacantha* extract significantly reversed the gene expression profiles for these genes compared to the LPS group.

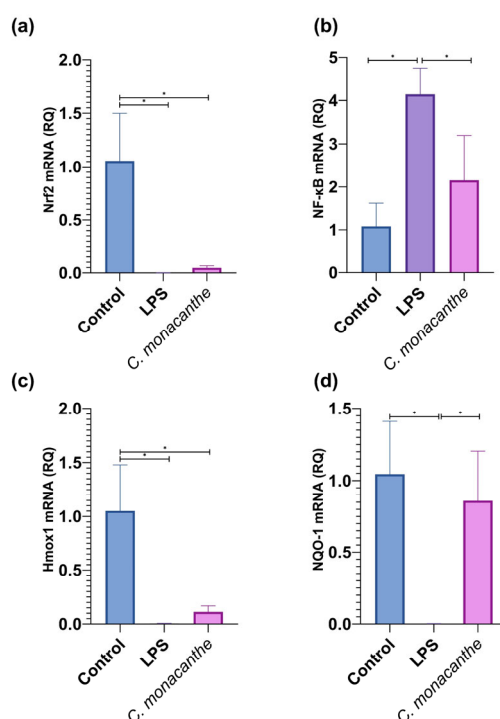


Figure 4. Effect of *C. monacantha* extract on the mRNA expression of the Nrf2 pathway and related genes in Neuro-2a cells. (a) Nrf2, (b) NF- κ B, (c) Hmox1, and (d) NQO-1. Data are expressed as mean $\pm$ SD. * $p < 0.05$. LPS: lipopolysaccharide; Nrf2: nuclear factor erythroid 2-related factor 2; NF- κ B: nuclear factor kappa B; Hmox1: heme oxygenase 1; NQO-1: NAD(P)H quinone dehydrogenase 1.

2.4.4. *C. monacantha* Extract Modulates Regulatory Control of Nrf2 Signaling and Mediates Mitochondrial Adaptation in Neuro-2a Cells

Figure 5 shows that LPS significantly increased the level of the negative regulator of Nrf2, Kelch-like ECH-associated protein 1 (Keap1), compared with the control cells. In contrast, the level of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) was significantly decreased relative to the control group. Pretreatment of the cells with *C. monacantha* extract resulted in a significant reduction in Keap1 levels, along with a non-significant increase in PGC-1 α levels compared with the LPS group.

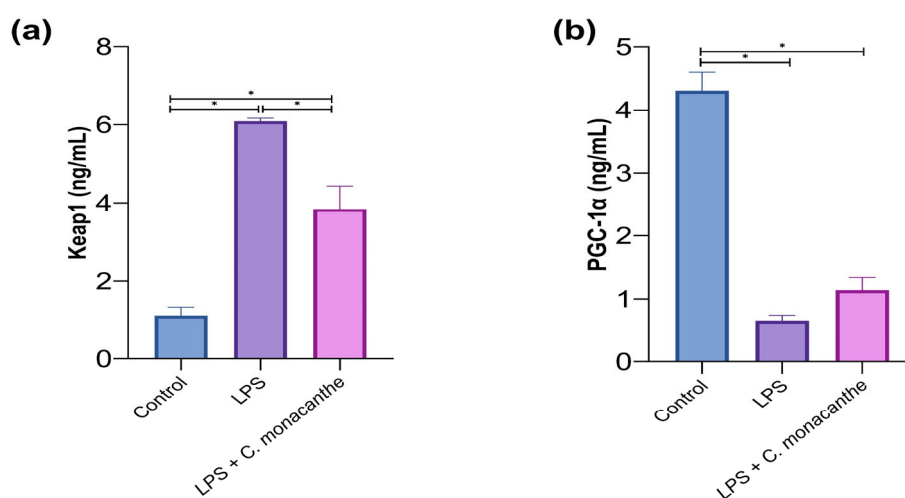


Figure 5. Effect of *C. monacantha* extract on the levels of (a) Keap1 and (b) PGC-1 α . Data are expressed as the mean $\pm$ SD, * $p < 0.05$. LPS: lipopolysaccharide; Keap1: Kelch-like ECH-associated protein 1; PGC-1 α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

2.5. Protein–Protein Interaction Analysis

A protein–protein interaction (PPI) network was built using the STRING online database to discover the functional associations among principal antioxidant proteins involved in our study. The network (Figure 6a) revealed a crosstalk between hypoxia- and stress response-related proteins, represented by HMOX1; NFE2L2 (NRF2); and KEAP1, and members of the glutathione peroxidase (GPX) family, which are mainly involved in GSH metabolism and detoxification. The network was clustered using K-means clustering, which classifies the nodes into functionally related groups based on their possible interactions and involvement in a shared biological pathway. The number of clusters was three, and each cluster was illustrated by a distinct color. Cluster 1 (represented by red nodes) contained eight proteins that are involved in ROS scavenging and GSH metabolism. The second cluster (represented by green nodes) contained three proteins that are responsible for terpenoid-quinone biosynthesis, protein carboxylation, and flavodoxin-like folding. Cluster 3 (shown in blue nodes) illustrated two proteins that are involved in the cellular response to thyroxine stimulus and glutamate–cysteine ligase complex, reflecting regulatory functions in antioxidant capacity and cellular metabolism. Overall, this clustering underscores the coordinated involvement of these proteins in the oxidative stress response and redox homeostasis.

To provide further clues regarding the association between these proteins and pathways, gene ontology (GO) enrichment analysis based on biological processes was performed (Figure 6b). The most significantly enriched process was the response to oxidative stress (GO:0006979) with false discovery rate (FDR) = 2.83×10^{-16} , followed by the detoxification process (GO:0098754, FDR = 4.08×10^{-9}); indicating a strong functional association

within the dataset. In summary, The PPI network suggests a strong functional linkage between the key antioxidant proteins, with Nrf2 and its interactors forming a central regulatory module. Moreover, GO enrichment analysis demonstrated a significant involvement of the studied proteins in oxidative stress response, detoxification, and glutathione metabolism. These findings highlight additional molecular pathways that could be targeted against neuroinflammation by alleviating oxidative stress and enhancing the cellular antioxidant capacity.

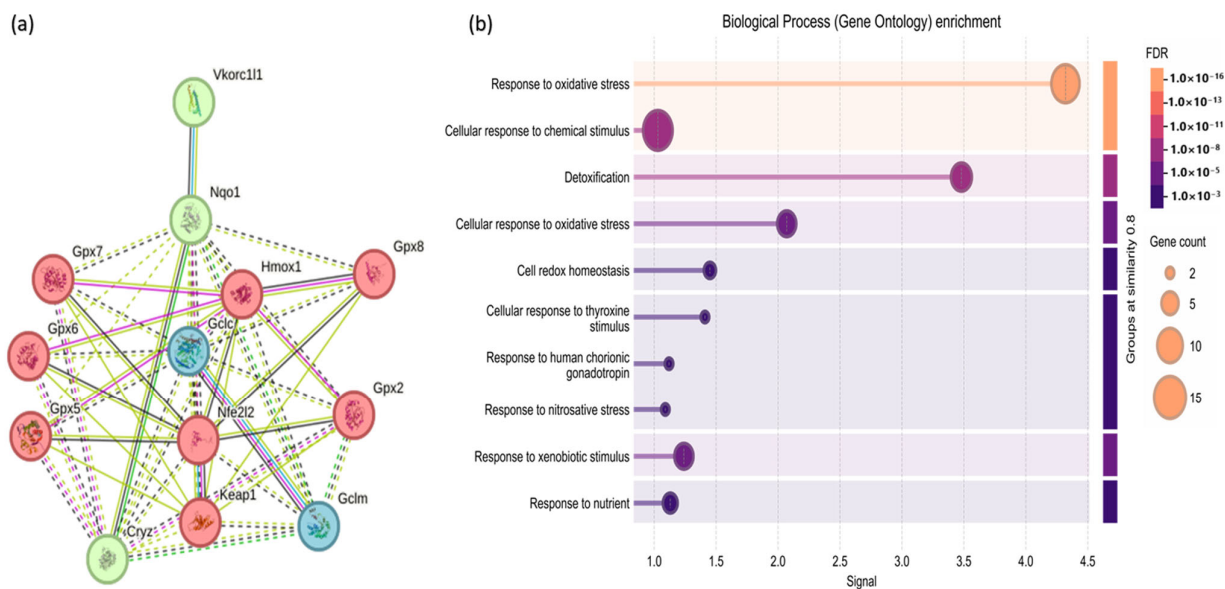


Figure 6. Protein–protein interaction (PPI) analysis. Nrf2, Hmox1, and NQO-1 were selected as central proteins using the STRING online database. (a) PPI network in which each protein is represented by a node. Total nodes = 13, clustered by color based on K-means clustering. Number of edges = 52; PPI enrichment p -value $< 1.0 \times 10^{-16}$; average local clustering coefficient = 0.87. Nodes represent proteins and edges represent interactions between them (b) Gene ontology (GO) enrichment analysis based on biological processes.

3. Discussion

The plant kingdom contains an enormous number of phenolic compounds, which fall into two categories: flavonoids and non-flavonoids. Phenolic compounds have recently attracted interest for their biological activities and medicinal benefits [55]. Thus, to evaluate the antioxidant properties of *C. monacantha* crude extract, the TPC was assessed. The Folin–Ciocalteu colorimetric technique was used to measure the TPC of the *C. monacantha* methanolic extract, which was found to be $29.91 \pm 1.61 \mu\text{g GAE/mg}$ [56]. Using rutin as a standard and the AlCl_3 reagent, spectrophotometric analysis revealed that the TFC of the *C. monacantha* extract was $6.30 \pm 0.37 \mu\text{g RE/mg}$ [57].

In this study, *C. monacantha* crude extract showed antioxidant potential as evaluated by two tests (DPPH, FRAP) using Trolox as a standard. Modern techniques, such as liquid chromatography with tandem mass spectrometry (LC-ESI-TOF-MS/MS), are used to identify phytochemicals that might be beneficial to human health. This is the first analysis of *C. monacantha* methanolic crude extract using LC-ESI-TOF-MS/MS (Agilent, Santa Clara, CA, USA). This metabolomic investigation revealed the existence of isoflavonoids, flavonoids, glycosylated flavonoids, glycosylated coumarins, simple phenolics, cinnamoyl tyramine alkyl amide derivatives, amino acid derivatives, carboxylic acids, and alkaloids. These metabolites were discovered by comparing fragmentation patterns, m/z values, and

chromatographic behavior with existing literature. Additionally, their mass accuracy, which is measured in parts per million (ppm) error, was considered [36,58,59].

As far as we are aware, our research is the first to investigate the effect of *C. monacantha* extract on LPS-induced neuroinflammation in Neuro-2a mouse neuroblastoma cells. Subjecting the cells to *C. monacantha* extract reduced the LPS-induced inflammation and oxidative stress responses. This effect was found to be mediated through its action on the Nrf2/NF- κ B axis. LPS is a well-known activator of the immune system and inducer of brain macrophages. The mechanism by which LPS functions is by activating microglial cells, which results in the production of proinflammatory cytokines and mitochondrial dysregulation [18,60].

Several lines of evidence have been provided to support that inflammation and oxidative stress have long been thought to be key factors in the development of neuroinflammation [17,61]. Previous investigations revealed that LPS activates microglia, triggering the release of proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, interferon-gamma (IFN- γ), and MCP-1 with the involvement and activation of NF- κ B and activator protein-1 (AP-1) [18,62]. Thus, blocking NF- κ B signaling has been demonstrated to reduce neuroinflammation [63]. Conversely, Nrf2 is a crucial regulator of two major cytoprotective mechanisms: anti-inflammatory and antioxidant responses [64]. Nrf2 inhibits NF- κ B activation, resulting in reduced production of proinflammatory cytokines [9]. Furthermore, the Nrf2 pathway suppresses NF- κ B activation by increasing Hmox1 expression, thereby reducing ROS levels and inhibiting NF- κ B activation [65].

Our results showed that *C. monacantha* extract significantly lowered the level of NF- κ B transcription factor and, consequently, the levels of the released proinflammatory cytokines, IL-1 β and MCP-1, which were elevated by LPS. These data imply that *C. monacantha* extract may possess a potential anti-inflammatory effect, which is beneficial in combating LPS-induced neuroinflammation. This favorable effect could be attributed to the detected constituents in the *C. monacantha* extract. Our results are in harmony with recent investigations showing that naringenin, an active compound in our extract, suppresses the NF- κ B signaling pathway in experimental stroke, supporting its neuroprotective action [66]. Moreover, luteolin has been found to reduce neuroinflammation by suppressing the TLR4/TRAF6/NF- κ B pathway following intracerebral hemorrhage [67].

Nrf2 is a transcription factor that acts as an antioxidant and has neuroprotective effects in CNS diseases [68]. Under physiological conditions, Nrf2 interacts with Keap1 in the cytoplasm. Under oxidative stress, Nrf2 dissociates from Keap1 and translocates to the nucleus, activating the Nrf2/antioxidative response element (ARE) system [69]. Activation of Nrf2 promotes the transcription of antioxidant enzymes such as Hmox1 and NQO-1, which could reduce the progression of neurodegenerative diseases [69–71]. It has been demonstrated that activation of the Nrf2 pathway is a promising approach to protect against neurodegenerative disorders [72]. In the current work, our results showed that *C. monacantha* extract activated the Nrf2 pathway in LPS-stimulated Neuro-2a cells. This protective effect could be attributed to several putative contributors in *C. monacantha* extract, such as hesperetin [73], quercetin [74], and naringenin [75], which have been reported to activate the Nrf2/ARE signaling pathway. These effects may also be attributed to the intrinsic antioxidant capacity of the extract, as demonstrated by the DPPH and FRAP assays. Furthermore, our findings are consistent with previous studies reporting the antioxidant properties of *C. monacantha* [76,77].

It is well-known that LPS disrupt redox homeostasis and mitochondrial function through dysregulation of the Keap1/Nrf2 signaling axis. In the current work, LPS increased the Keap1 protein level, demonstrating repression of Nrf2 signaling. These observations are in harmony with previous studies demonstrating that inflammatory stress can

promote the accumulation of Keap1, thereby enabling Nrf2 ubiquitination and proteasomal degradation. These events consequently weaken the cellular antioxidant defense mechanisms [78,79]. On the other hand, pretreatment with *C. monacantha* extract reduced the Keap1 protein level compared with the LPS group, suggesting the release of Nrf2 from Keap1. Suppression of Keap1 represents a critical upstream mechanism for restoring Nrf2 activity and promoting the transcription of protective genes, including Hmox1 and NQO-1. Our findings are in line with a previous report demonstrating the antioxidant activity of *C. monacantha* [80]. Other studies also revealed that polyphenol-rich plants can activate the Nrf2 signaling cascade by targeting Keap1, thereby improving antioxidant capacity during inflammatory stress conditions [81,82].

PGC-1 α is a key regulator of mitochondrial function, oxidative homeostasis, and neuronal survival. Downregulation of PGC-1 α has been implicated in the pathogenesis of neurodegenerative and neuroinflammatory disorders [83]. It is well-known that mitochondrial dysfunction contributes significantly to cognitive impairment in neurodegenerative diseases, whereas Nrf2 activation confers neuroprotective effects that improve these impairments [84]. LPS exhibited remarkable reduction for PGC-1 α , revealing impaired mitochondrial biogenesis and metabolic adaptation during neuroinflammation, along with suppression of Nrf2 signaling. Our results are consistent with others that reported the inhibitory effect of LPS on PGC-1 α [85,86]. On the other hand, pretreatment with *C. monacantha* extract increased PGC-1 α to a considerable level, indicating restoration of mitochondrial signaling. This effect may be mediated by the upregulation of Nrf2 and its related defense genes, Hmox1 and NQO-1, as well as through the resulting antioxidant effect.

The PPI network was constructed to strengthen our hypothesis regarding the association between the studied proteins and prediction of further molecular targets. The PPI revealed a highly interconnected network centered on Nrf2 (Nfe2l2) and Keap1, together with principal downstream antioxidant and detoxification enzymes, including Hmox1, NQO1, and several glutathione peroxidases. This network underscores the central regulatory role of Nrf2 in directing cellular antioxidant defense, glutathione metabolism, and redox homeostasis under stress conditions. Consistent with these interactions, GO enrichment analysis demonstrated strong overrepresentation of biological processes related to oxidative stress response, detoxification, and cellular redox balance, highlighting the critical role of Nrf2 as a cytoprotective signaling pathway. The enrichment of the pathways was also associated with nitrosative stress and xenobiotic response, further suggesting that this network extends beyond classical antioxidants to broader stress-adaptive mechanisms.

4. Materials and Methods

4.1. Plant Materials

In November 2019, the entire *C. monacantha* plant utilized in this investigation was collected from North Sinai, Egypt. Prof. Dr. Rim Hamdy, professor of plant taxonomy and flora, Department of Botany and Microbiology, Faculty of Science, Cairo University, confirmed the authenticity of the plant. The plant's voucher specimen is held in the Herbarium of Suez Canal University's Department of Pharmacognosy, Faculty of Pharmacy, Ismailia, Egypt (Reg. No. SAA-300). After two weeks of air drying in the shade at 25 °C, the plant was powdered.

4.2. Preparation of Plant Extract

The extract was prepared by cold maceration of *C. monacantha* (0.5 kg) in 90% aqueous MeOH at 25 °C until exhaustion (3 L, three times, 7 days for each run). Exhaustion was confirmed when the solvent became colorless and no additional spots were detected

by TLC analysis. The combined extracts were filtered through Whatman's No. 1 filter paper and vacuum-dried at 40 °C to obtain 20 g of crude *C. monacantha* extract.

4.3. Estimation of the Total Phenolic Content (TPC)

The TPC of *C. monacantha* extract was estimated using the Folin–Ciocalteu (FC) technique described in [56], with gallic acid (GA) as a standard. The results were expressed as µg of gallic acid equivalent per mg of extract based on the following equation:

$$\text{TPC } (\mu\text{g GAE/ mg dry extract}) = \frac{cV}{m}$$

where c is the concentration of gallic acid obtained from the calibration curve, V is the volume of the extract solution in mL, and m is the weight of the extract in g.

4.4. Estimation of the Total Flavonoid Content (TFC)

The TFC of *C. monacantha* was assessed using the AlCl_3 method [57]. Rutin was used as the standard compound, and the results were expressed as µg of rutin equivalent per mg of extract based on the following equation:

$$\text{TFC } (\mu\text{g Rutin/ mg dry extract}) = \frac{cV}{m}$$

where c is the concentration of rutin obtained from the calibration curve, V is the volume of the extract solution in mL and m is the weight of the extract in g.

4.5. Evaluation of the Antioxidant Activity of *C. monacantha* Extract

4.5.1. DPPH Free Radical Scavenging Activity

Using Trolox as a standard, the DPPH free radical assay of *C. monacantha* crude extract was performed according to the methodology described in [87,88]. The data were expressed as means ± SD using the following equation:

$$\% \text{inhibition (PI)} = \frac{\text{Average absorbance of blank} - \text{Average absorbance of test}}{\text{Average absorbance of blank}} \times 100$$

The IC_{50} value was determined using GraphPad Prism 6®.

4.5.2. Assay for FRAP

The FRAP assay of *C. monacantha* extract was performed using the method described in [89], with minor modifications conducted in microplates and using Trolox stock solution as a reference standard. The data were expressed as means ± SD. The ferric reducing capacity of the samples was expressed as µM TE/mg of sample.

4.6. LC-ESI-TOF-MS/MS Metabolomic Analysis of Crude *C. monacantha*

C. monacantha metabolomic profiling was performed by LC-ESI-TOF-MS/MS in both positive and negative ionization modes (+ve and -ve) following the previously reported method [59]. The details of the chromatographic separation process, mass analysis, and compound identification are provided in the Supplementary Materials.

4.7. Biological Assays

4.7.1. Cell Culture

In this study, the Neuro-2a (Cat. # ABC-TC0819) mouse neuroblastoma cell line (Accugen Biotech Fairfield, NJ, USA) was used. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). The culture media was supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. All cell culture reagents were obtained from Elabscience® (Houston, TX, USA).

4.7.2. MTT Cellular Proliferation Assay

MTT cell proliferation and cytotoxicity assay kit (Elabscience®, Houston, TX, USA) was used to select the cytoprotective dose of the extract that maintains cellular viability and could be used for the subsequent analyses. Based on a pilot preliminary experiment, the examined concentrations of the extract were 31.25, 62.5, 125, 250, 500, and 1000 µg/mL. Each concentration was applied in three wells, and the average reading was taken. Briefly, the cells were seeded in 96-well plate (5 × 10³ cells/well) and incubated with the respective extract concentrations for 24 h. Then, 50 µL of MTT solution, prepared according to the manufacturer's instructions, was added to each well and incubated for 4 h. The supernatant was carefully removed, and the formazan crystals were solubilized in 150 µL DMSO. The optical density (OD) was measured at 570 nm to determine cellular viability. The results were normalized to control cells and expressed as percentage viability.

4.7.3. Experimental Design and Grouping

Following the MTT assay, 31.25 µg/mL of *C. monacantha* crude extract was incubated with the cells for 24 h. Subsequently, neuroinflammation was induced by LPS at a concentration of 1 µg/mL, which was left with the cells for an additional 24 h [90]. Consequently, there were three groups of Neuro-2a cells: control, LPS only, and LPS + *C. monacantha*. LPS and *C. monacantha* extract were dissolved in DMSO/DMEM (0.1% v/v). The study protocol was approved by the Research Ethics Committee, Faculty of Pharmacy, Menoufia University (approval number: MPIR 24/02).

4.7.4. Determination of Inflammatory Cytokines

Commercial ELISA kits from Elabscience® (USA) were used to measure IL-1β (Cat. # E-EL-M0037) and MCP-1 (Cat. # E-EL-M3001), following the manufacturer's recommendations. Both kits are based on the sandwich ELISA principle, and the optical density (OD) was measured spectrophotometrically at 450 nm.

4.7.5. RT-PCR for Determining Gene Expression

qRT-PCR was used to evaluate the expression levels of Nrf2, NF-κB, Hmox1, and NQO-1. Briefly, total RNA was extracted from the cells using the RNeasy Mini Kit. The QuantiTect® Reverse Transcription Kit was used to synthesize cDNA from the isolated RNA. The amplification step was carried out using QuantiNova™ Probe PCR Kit. Reactions were performed using a StepOnePlus™ Real-Time PCR thermal cycler (Applied Biosystems, USA). All kits used were from Qiagen (Hilden, Germany). Primer-Blast (Primer designing tool) was used to design specific primers for the target genes (Table 2). β-actin served as the housekeeping gene, and the data were presented as the means of relative quantification (RQ).

Table 2. Sequence of the used primers.

Gene	Forward	Reverse
Nrf2	AACAGAACGGCCCTAAAGCA	CCTTGAGCTGGTGACAGAGG
NF-κB	ATGTAGTTGCCACGCACAGA	GGGGACAGCGACACCTTTTA
Hmox1	GTCAGGTGTCCAGAGAAGGC	TGTTTGAACCTGGTGGGGCT
NQO-1	CGAGGATGGGAAAAGGAGTAAGT	TGCCCTGAGGCTCCTAATCT
β-actin	TGGTGGGAATGGGTCAGAAG	TGTAGAAGGTGTGGTGCCAG

Nrf2: nuclear factor erythroid 2-related factor 2, NF-κB: nuclear factor kappa B, Hmox1: heme oxygenase 1, NQO-1: NAD(P)H quinone dehydrogenase 1.

4.7.6. Determination of Keap1 and PGC-1α

The concentrations of Keap1 and PGC-1α in the cells were determined using commercial ELISA kits purchased from ELK Biotechnology (Houston, TX, USA) (Cat. # ELK8105 and ELK6745, respectively) according to the supplier's instructions. Both kits are based on the sandwich ELISA principle, and the optical density (OD) was measured spectrophotometrically at 450 nm.

4.8. Protein–Protein Interaction

Nrf2, Hmox1, and NQO-1 were chosen as central proteins to build a network analysis between these proteins and other molecular target pathways using STRING online database. The interaction score was high confidence (0.7) with maximum of 10 interactors at the first shell. This was followed by K-means clustering to group the proteins based on their centroids. GO analysis based on biological process was also performed to explore the functional connection between the pathways and the FDR was calculated automatically using the Benjamini–Hochberg procedure.

4.9. Statistical Analysis

Results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to assess significant differences, followed by Tukey post hoc test with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) version 8.0.2. Differences were considered statistically significant at $p < 0.05$. To ensure precision, all experiments were carried out in triplicate.

5. Limitations of the Study

The use of the Neuro-2a cell line, while suitable for mechanistic investigations, does not fully reflect the complexity of neuroinflammatory processes involving primary neurons or microglia. The extract was evaluated within a limited concentration range, and assessment of a wider range, particularly under the induced inflammatory condition, is recommended. In addition, the study relied on a crude extract rather than isolation of specific functional bioactive compounds that were detected by LC-ESI-TOF-MS/MS. While antioxidant capacity was evaluated using DPPH and FRAP assays, intracellular redox markers, antioxidant enzyme activity, and protein-level confirmation of Nrf2 activation or nuclear translocation were not assessed. Finally, further investigations and validation studies are warranted.

6. Conclusions

C. monacantha extract was found to contain multiple bioactive compounds with reported antioxidant and anti-inflammatory properties, which may underlie the attenuated LPS-induced neuroinflammatory responses in Neuro-2a cells. These effects were associated with modulation of Nrf2-related gene expression, indicating a possible involvement

of antioxidant signaling pathways. Bioinformatic analyses further supported the relevance of Nrf2-associated networks in oxidative stress responses. Overall, the results suggest that *C. monacantha* extract may represent a promising source of antioxidant and anti-inflammatory compounds, but further protein-level investigations as well as functional and in vivo studies are highly recommended to confirm its neuroprotective potential.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052263/s1>.

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Conflicts of Interest: The authors declare no conflicts of interest.

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