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Research Article

Inhibition of NO Production in LPS-Stimulated Primary Rat Glial Cells by Gnidilatimonoein and Extract of *Daphne mucronata*

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Background: In the CNS, glial cells are involved in neuroinflammation and neuropathic pain. The glial cells are activated by a variety of pathological conditions and release pro-inflammatory mediators, including nitric oxide (NO). Overexpression of iNOS (inducible nitric oxide synthase) and extra NO is detrimental to neurophysiology and neuronal viability.

Objectives: This study aimed to examine the effect of Gnidilatimonein isolated from *D. mucronata* and its leaves extract (as natural phytochemicals) on NO production in the LPS-induced primary glial cells.

Materials and Methods: A preparative HPLC method was used to isolate gnidilatimonoein from leaves ethanolic extract. Various doses of Gnidilatimonoein, the ethanolic extract were applied to primary glial cells inflamed by lipopolysaccharide. A Colorimetric test, an MTT assay, and a RT-PCR analysis were then performed to analyze and compare NO production, cell viability, and iNOS expression.

Results: Gnidilatimonoein treatment of pretreated primary glial cells significantly inhibited iNOS expression and decreased NO synthesis. Plant extracts also reduced NO production in inflamed microglial and glial at 0.1-3 $mg.mL^{-1}$. At these concentrations, none of these compounds exerted a cytotoxic effect, suggesting that their anti-inflammatory effects were not due to the death of cells.

Conclusion: This study indicates that *D. mucronata* and its active compound, Gnidilatimonoein, could have restrained effects on the expression of iNOS on the induced glial cells; however, further investigation is warranted.

Keywords: Anti-neuroinflammation, *Daphne mucronata*, Glial, Gnidilatimonoein, Nitric Oxide

1. Background

Glial cells comprise 70% of brain and spinal cord cells. The glial cell population includes microglial (5% to 10% of the total glial population) and macroglial, such as astrocytes and oligodendrocytes. These primary immune cells contribute to several essential functions, including developing, repairing, and protecting the nervous system, modulation of neuronal activity, and regulation of the nervous environment (1). Controlling the activities of these cells is essential for the proper functioning of the central nervous system (CNS). The most common types of brain damage include stroke, MS, AD (Alzheimer's Disease), Parkinson's (Parkinson's Disease), ALS (Amyotrophic Lateral Sclerosis), viral neurotrophic infections, neoplasia, aging, etc. occur as a result of inflammation (2-7). During such inflammatory conditions, glial cells are stimulated by factors such as pathogens, protein mass, or damaged neurons. This may result in the production and expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, reactive oxygen species (ROX), and nitric oxide (9). These inflammatory mediators may kill neurons as they activate microglial and astrocytes. The process of

activating microglial and astrocytes is characterized by various mechanisms, such as intense phagocytic NADPH oxidase (PHOX) activity in microglial, inducible nitric oxide synthase (iNOS) expression in glial, and phagocytosis of neurons. These processes have been shown to be effective in neurodegeneration (10). The production of nitric oxide (NO) by iNOS and the overexpression of iNOS play an essential role in the pathogenesis of both acute and chronic inflammation (11, 12). Consequently, NO inhibitors and negative regulators of glial activation are considered important therapeutic advances in the treatment of NO-induced neuroinflammation. Various stimuli, such as lipopolysaccharides (LPS), can induce glial cell proliferation in vitro. Upon stimulation of these cells, LPS activates the inflammatory responses and produces inflammatory molecules (14-16).

Several species of Thymelaeceae (*Daphne mezereum*) have been used over the years to treat rheumatism, skin diseases, joint pain, neuropathic pains, and certain cancers (17). However, it has not yet been determined how *Daphne mucronata* exerts its anti-inflammatory action.

Daphne mucronata is a shrub of the Thymelaeceae family found in many regions throughout Asia. In some studies, the leaves of *D. mucronata* have been reported to exhibit anti-irritant, anti-tumor, and anti-proliferative properties. Currently, multiple components of the Thymelaeceae family have been isolated, and their main structural components are coumarins and diterpene esters. Gnidilatimonoein, a diterpene (20), Daphnecin, a coumarinolignan (21), and other components such as 7,8-dimethoxycoumarin, Cinnamic acid, triterpenoids (Lupeol, β-amyrin, Betulin), aquillochin, umbelliferone, glucosides, Stigmasterol 3-O-β-D-Glucopyranosid, Hexa methoxy flavone, and other flavonoids have been isolated and elucidated from *D. mucronata* (17, 18).

Gnidilatimonoein was first isolated in 2003 from Daphne mucronate onsist leaf extract and identified as 12-0cinnamoyl-3-hydroxy-5-hydroxy-6,7-epoxy-resiniferonol-9,13,14-ortho-2-decadienoate (a diterpenoid daphnan). According to the studies, it had a significant effect on cell metabolism, and it exhibited high anti-tumor activity against some cancer cell lines (22-24).

2. Objectives

This study aimed to determine whether ethanolic *Daphne mucronata* leaf extract and its purified active component, Gnidilatimonoein, are effective at reducing inflammation in primary rat mixed glial cells. As glial cells interact (25), we conducted experiments on the mixed glial cells, representing a plenary model of the CNS immune system, similar to what would occur in vivo. We also examined the effects of *D. mucronata* extract on induced microglial cells. Cells were cultured in the presence of LPS and these compounds. Following this, we investigated cytotoxicity, oxidative and inflammatory factors release, NO, and the expression of iNOS. It has been shown in previous studies that treatment of cell cultures with LPS after 48 hours results in three times more NO production than treatment after 24 hours, resulting in greater stimulation of cells. Therefore, the Griess test was performed 48 hours after treatment in this examination.

3. Materials and Methods

3.1. Chemicals and Plant Species

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and Griess reagent were obtained from GibcoBRL (Grand Island, NY, USA). The trypan blue dye, lipopolysaccharide (LPS) (E5:055), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all obtained from Sigma Chemical Co. (St. Louis, MO). We purchased penicillin, streptomycin, trypsin, EDTA, dimethyl sulfoxide (DMSO), phosphate-buffered solution (PBS), chloroform, and ethanol from Merk (Darmstadt, Germany).

Total RNA extraction kit from MBST (Tehran-Iran). cDNA synthesis Kit from vivantis (USA). Ampliqon master mix and Primers were purchased from Cinnagen (Tehran-Iran).

A random sampling of *Daphne mucronata* was conducted in the wild park of Yasouj in the Iranian province of Kohgiluyeh and Boyer-Ahmad. The species was determined by the botanist of yasouj university of Kohgiluyeh and Boyer-Ahmad Province, Iran.

3.2. Extracts of the Daphne mucronata Leaves

Drying and powdering of plant samples were performed in a darkroom. For more extended storage, some samples are stored at -20 °C. We extracted the leaves of *Daphne mucronata* using the Pourmorad method (25) with a few modifications and without heating the leaves.

3.3. Isolation of Gnidilatimonoein by HPLC

The reversed-phase HPLC analysis of the extract was performed using a KNAUER (Germany) HPLC equipped with an ODS (C18) 4.6 mm × 25 mm (Beckman, USA) analytical Zorbax column. According to the flow program of **Table 1**, an isocratic method involving a mixture of deionized water and acetonitrile as a mobile phase was employed. Detectors were set to measure at 326 nm for UV-Visible measurements. The injection volume was 20 µL. Standardization was performed using pure Gnidilatimonoein (2 $mg.mL^{-1}$) (20).

Table 1. Flow gradient program of mobile phase on the staid phase in HPL

Acetonitrile%	Time (min)
5	0
15	5
45	25
50	30

Having obtained the analytical chromatogram, the HPLC was regulated at the preparative system under the same analytical conditions previously described. For the first time, 100 µL of the concentrated extract was used to purify Gnidilatimonoein. Considering the retention time of Gnidilatimonoein, a fraction collector system was applied for collecting purified components. The active component of *D. mucronata* leaf extract was isolated by infusing 500 µL of concentrated leaf extract into the system. As a final step, the active component was infused into the analytical system, and the resulting chromatogram was compared with the standard chromatogram to verify its accuracy.

The fractionated soluble was frozen using liquid nitrogen and put in a freeze dryer under -95 ºC and 0.1 hPa vacuum pressure. After one overnight, the solubility completely dried, resulting in a pure milky powder.

3.4. Primary Mixed Glial Cell Culture and Treatment

With the approval of the Iran Ethics Committee of the National Institute of Genetic Engineering and Biotechnology (permit number: IR. NIGEB.EC.1395. 4.1.C), primary glial cell cultures were prepared from brain cortex tissues of newborn Wistar rats (1 to 3 days old). As soon as the brains were removed from the skull, they were carefully removed from the meninges and blood vessels and then mechanically separated into DMEM. The cells are then transferred to flasks containing DMEM complemented with 100 $UI.mL^{-1}$ penicillin G, 100 $mg.mL^{-1}$ streptomycin, and 10% heat-inactivated FBS. A 5% CO_2 incubator (Binder, USA) was used to maintain mixed glial cultures at 37 ºC, and the medium was renewed twice a week. To obtain primary microglial, after about 12 days' flasks were confluent, they were shaken to remove floating microglial, and seeded onto 96 well tissue culture plates (26, 27).

20×10^3 and 5×10^4 mixed glial cells were seeded into each well of 96 and 24 well plates, respectively, and microglial into 96 well plates. The cells were incubated with 10% fetal bovine serum (FBS) for 24h. After that, Primary cells were pretreated with various doses of Gnidilatimonoein and *Daphne mucronata* extract in a fresh medium containing 1% FBS for 1 h before LPS (10 $mg.mL^{-1}$ for mixed glial cells and 1 $mg.mL^{-1}$ for microglial cells) was added and then were incubated for 48 h. All assays were examined in three replicates.

3.5. NO Measurement

A colorimetric test based on the Griess reagent was used to assess NO production in cells 48h after treatment. Standard curves were constructed using a standard NO solution, and p values less than 0.01 were considered significant.

3.6. Cell Viability Assay

After various treatments, MTT (10 µL, 0.5 $mg.mL^{-1}$) was added to 100 µL of the medium of each treatment after 48 h and incubated at 37 ºC for 4 h. Then, the MTT solution was removed. Cell formazan crystals were dissolved in 100 µL l DMSO, and spectra were analyzed for absorbance at 580 nm on a Multiskan RC microplate reader (Labsystems, Finland).

3.7. RNA Extraction and RT-PCR

The total RNA was isolated from treated cells 48 hours after LPS stimulation using an MBST kit, as instructed by the manufacturer (Investigating group Molecular Biological System Transfer). To obtain cDNA from RNA samples, viva 2–step RT-PCR kits (vivantis technologies sdn bhd) were used following the manufacturer's instructions. RT-PCR was conducted using the cDNA, master mix (Ampliqon), and specific primers (Cinnagen) (**Table 2**).

Table 2. Primers of RT-PCR

Gene	Primer Pair F, forward; R, reverse	Size (bp)
iNOS	F: 5`-GAC ATC GAC CAG AAG CTG TC-3` R: 5'-GGG CTC TGT TGA GGT CTA AAG-3`	253
GAPDH*	F: 5`-CCC CCA ATG TAT CCG TTG TG-3` R: 5'-TAG CCC AGG ATG CCC TTT AGT-3`	118

* Glyceraldehyde 3-phosphate dehydrogenase

3.8. Statistical Analysis

Statistical comparisons between treatments were conducted with SPSS software (IBM SPSS Statistics 16) using One-way ANOVA and Duncan and Dunnett tests. Data are presented as mean ± SD.

4. Results

4.1. Detection and Isolation of Gnidilatimonoein by HPLC

Gnidilatimonoein (**Fig. 1A**) was infused into HPLC under the mentioned conditions. A chromatogram was obtained and retention time was determined (**Fig. 1B**). To obtain an analytical chromatogram, 20 µL of *Daphne mucronata* were infused into HPLC (**Fig. 1C**). Using HPLC, 500 µL of the concentrated extract was purified based on the retention time of the component.

4.2. Morphology of Glial Cells in Different Culture Days

The first branching of neural cells appears three days after primary culture and binding of small fragments of cortex tissue to the flask floor. A week later, the types of glial cells were recognizable. Also, microglial are well propagated around two weeks after the primary mixed culture. Their shape is round, and they stick lightly to the surface of the astrocytes (**Fig. 2A, 2B, 2C**).

4.3. The Effect of Daphne mucronata Leaf Extract on the Morphology of Mixed Glial Cells

A contrast phase microscope was used to compare the morphology of glial cells in control and treated samples with plant extracts (**Fig. 2D, 2E, 2F, 2G**). Upon increasing the concentration of plant extract, the cells accumulate their solids and become spherical, rather than broad and elongated, and they are observed as vesicles.

4.4. The Effect of Daphne mucronata Leaf Extract and Gnidilatimonoein on Mixed Cell Survival

The survival of mixed glial cells after 48 hours was evaluated by MTT assay using different concentrations of crude leaf extract of *Daphne mucronata* and Gnidilatimonoein. Based on statistical analysis, mixed glial cells treated with *Daphne mucronata* leaf extract between 0.1 and 10 $mg.mL^{-1}$ had a similar survival rate to the control group. Still, there was no significant difference at the 1% probability level. At higher concentrations of 20 and 40 $mg.mL^{-1}$ compared to other concentrations, there is a significant decrease in survival at the 1% probability level in the control group (**Fig. 3A**).

According to Statistical Analysis, mixed glial cells treated with Gnidilatimonoein compound at 0.25, 0.6, 10, and 100 $mg.mL^{-1}$ survived similarly to the control cells.

They did not differ significantly at a probability of less than 1% (**Fig. 3B**).

4.5. The Effect of Daphne mucronata Leaf Extract on Microglial Cell Survival

MTT assay was used to investigate the effect of different concentrations of crude extract of *Daphne mucronata* leaf on the survival of microglial cells after 48 hours (**Fig. 3C**). Based on statistical analysis, microglial cells treated with 0.1 to 1.5 $mg.mL^{-1}$ plant extract survived similarly to control cells. At 1% probability, there is no significant difference, but at higher concentrations (3 and 5 $mg.mL^{-1}$), there is a significant decrease in cell survival at less than 1%.

4.6. Effect of D. mucronata Leaf Extract on the Inflamed Microglial Cells

One hour before stimulation with 1 $mg.mL^{-1}$ LPS, microglial cells were pretreated with 0.1, 0.25, 0.5, 1.5, 3, and 5 $mg.mL^{-1}$ *D. mucronata* extract.

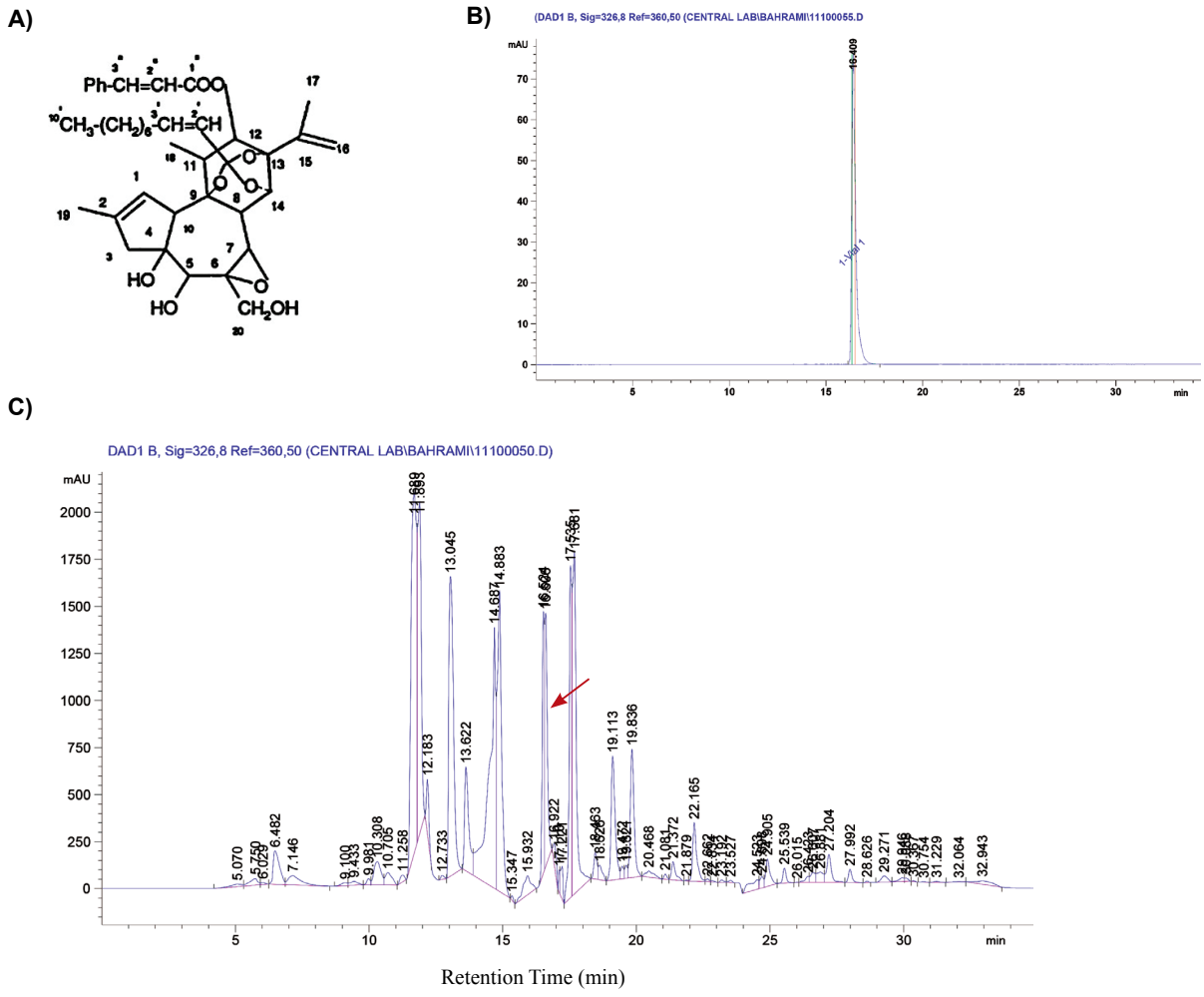

Figure 1. Gnidilatimonoein and its Chromatogram. A) chemical structure of Gnidilatimonoein (20, 23). **B**) Chromatogram of Gnidilatimonoein obtained from HPLC. (Flow gradient of Deionized water and acetonitrile, flow rate: 1 mL.min^{-1}, the UV-Visible detector:326 nm). **C**) Chromatogram of *Daphne mucronata* leaf extract. The flash shows the peak of Gnidilatimonoein with a retention time of 16.9 min.

NO production in the media cells was measured 48 hours after treatment by Griess reagent. A significant reduction in NO levels was observed in the media of microglial treated with the extract from 1.5 to 5 mg.mL^{-1} compared to the group treated with LPS without the extract (**Fig. 3D**).

4.7. Evaluation of Toxicity of D. mucronata Leaf Extract Concentrations in the Microglial Cells

The effect of *D. mucronata* leaf extract concentrations (0.1, 0.25, 0.5, 1.5, 3, and 5 mg.mL^{-1}) on cell viability was investigated to distinguish between those that tend to inhibit LPS-stimulated NO production and those that do not. Cell viability was not affected by extract concentrations up to 1.5 mg.mL^{-1} compared with the control group; however, 3 and 5 mg.mL^{-1} of the plant extract were cytotoxic (**Fig. 3E**).

4.8. The Effect of Daphne mucronata Leaf Extract on The Morphology of LPS-inflamed Microglial

Inflammation results in the rounding of the resting microglial, which is attached to the bed (**Fig. 4A**). **Figure 4B** shows inflamed microglial caused by LPS. After 48 hours, inflamed microglial were treated with 1.5 mg.mL^{-1} *Daphne mucronata* leaf extract. In **Figure 4C**, the arrow shows that the cells are returning to ramifications.

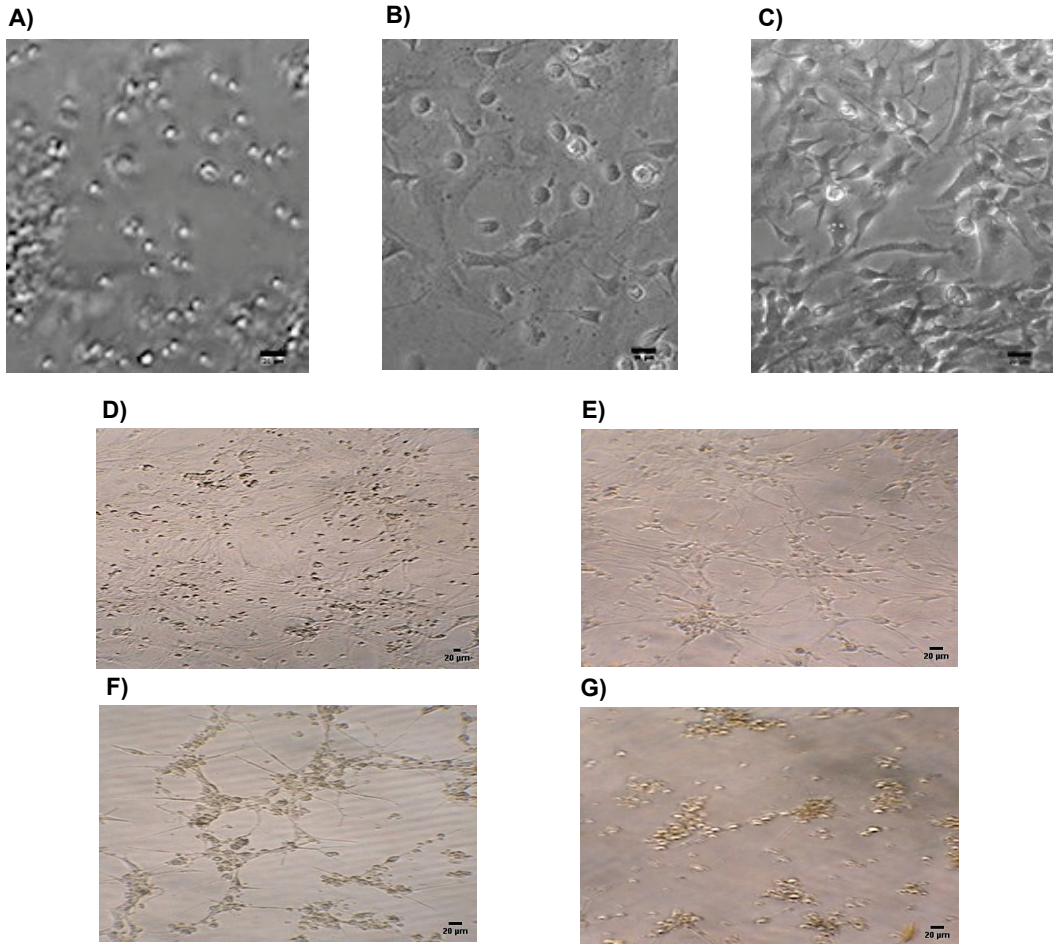

Figure 2. Morphology of Glial Cells in Different Culture Days. A) Mixed Glial cells in first, **B**) seventh, and **C**) eleventh days of culture (20x), respectively. Morphology of mixed glial cells treated with different concentrations of *Daphne mucronata* extract. **D**) Control, with x10 magnification. **E**) Treatment with a concentration of 5 mg.mL^{-1}, with x20 magnification. **F**) treatment with 10 mg.mL^{-1}, with x20 magnification and **G**) treatment with 20 mg.mL^{-1}, with x20 magnification.

4.9. Effect of D. mucronata Leaf Extract on the NO Production in Inflamed Mixed Glial Cells

Before stimulation with LPS, glial cells were pretreated with 0.1, 0.5, 1, 1.5, 3,5, 10, 20 mg.mL^{-1}, and after 1 h were stimulated with 10 g.mL^{-1} LPS. According to the experiment's results in mixed glial cells, 48 h after the application of all doses of the extract, significant decreases in NO were observed (**Fig. 5A**).

4.10. Evaluation of Toxicity of D. mucronata Leaf Extract Concentrations in the Mixed Glial Cells

Cell viability was investigated to delineate the cytotoxic activity of *D. mucronata* leaf extract concentrations (0.1, 0.5, 1, 1.5, 3,5, 10, 20 mg.mL^{-1}) in the inflamed mixed glial cells. A higher concentration of *D.mucronata* leaf extract (5,10, and 20 mg.mL^{-1}) had a cytotoxic effect on mixed glial cells than concentrations less than 3 mg.mL^{-1}.

However, other doses, from 0.1 to 3 mg.mL^{-1}, inhibited NO production and did not cause any cell toxicity (**Fig. 5B**).

4.11. Effect of Gnidilatimonoein on the NO Production in Inflamed Mixed Glial Cells

The cells were pretreated with Gnidilatimonoein, and the Griess test was performed like the plant extract. A significant reduction in NO production was observed with Gnidilatimonoein at all applied doses (0, 0.6, 1, 5, 10, and 50 mg.mL^{-1}) (**Fig. 5C**).

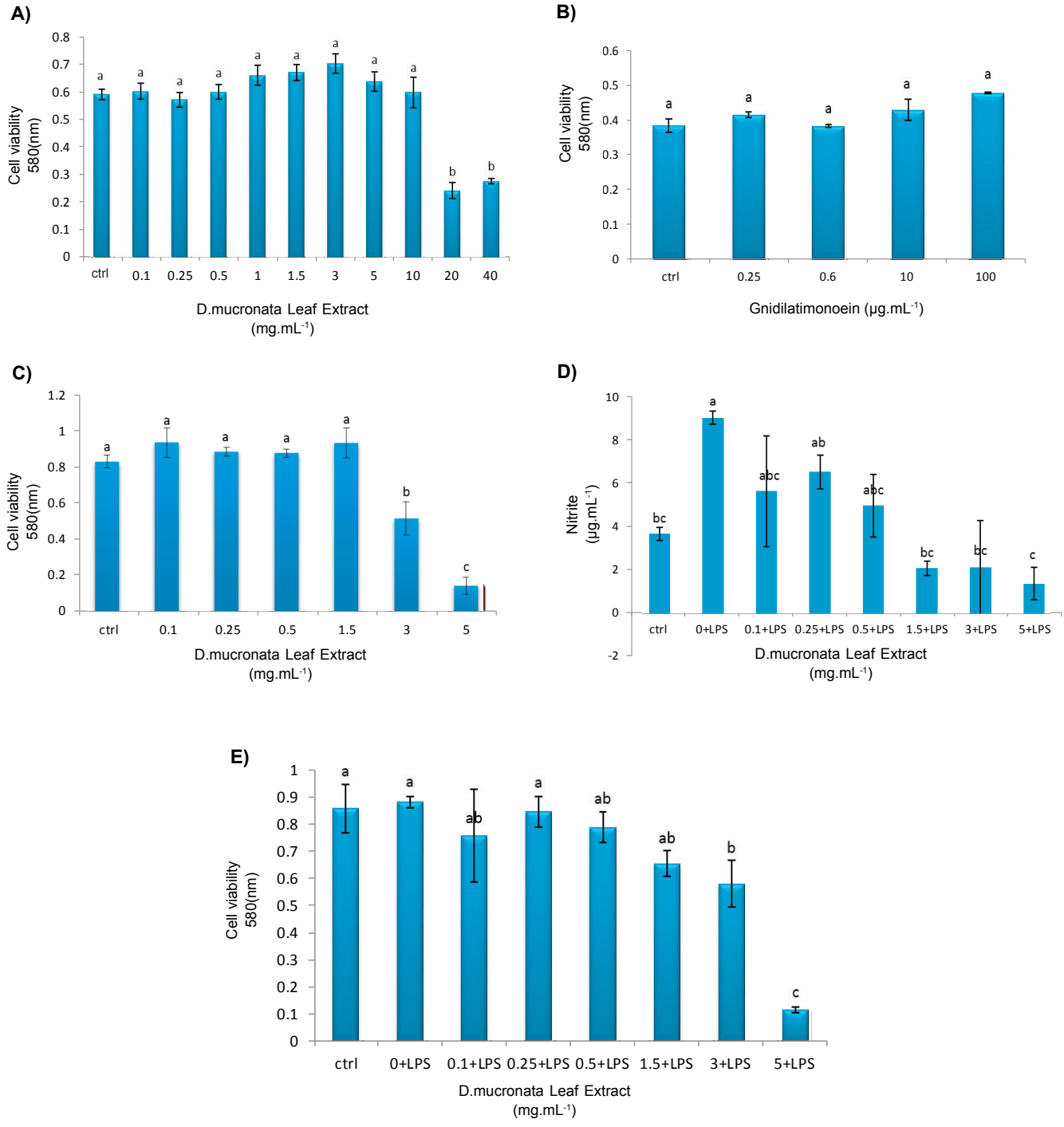

Figure 3. Cell viability measurement of plant extract and Gnidilatimonoein on the mixed glial cells. **A**) Effect of different concentrations of *Daphne mucronata* leaf extract (mg.mL^{-1}) on the survival of mixed glial cells. **B**) Different concentrations of Gnidilatimonoein (mg.mL^{-1}) affect the survival of mixed glial cells. **Cell viability measurement of plant extract on the microglial cell. C**) Effect of *Daphne mucronata* leaf extract on microglial cell survival. (Each column in the A, B, and C graphs, represents the average data ± standard error. Columns with at least one letter in common with the Duncan test are not significantly different at the 1% probability level). **NO, and cell viability measurement of plant extract on LPS-stimulated microglial cells. D**) Effects of *D. mucronata* leaf extract on NO production and cell viability in LPS-stimulated microglial. The cells were pretreated with various amounts of the extract before stimulation with LPS (1 mg.mL^{-1}), and after 48 h, NO production was measured, and **E**) cell viability was evaluated. In the D and E graphs, Columns of at least one common letter by the Duncan test Indicate the level of significance P<0.05.

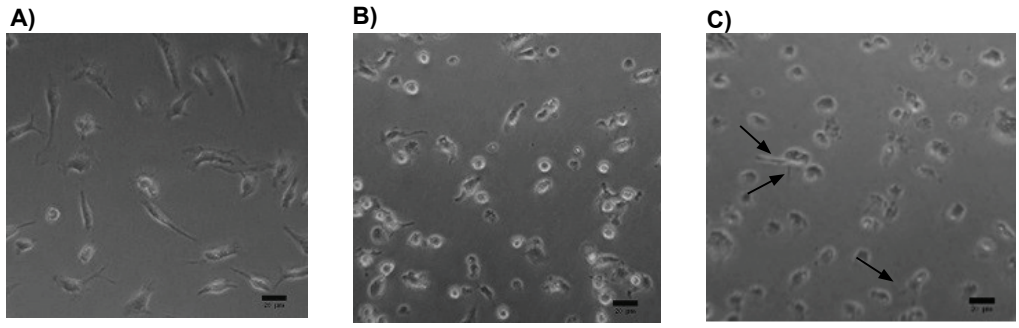

Figure 4. Morphology of LPS-inflamed Microglial treated with plant extract. A) Microglial in the control group. **B**) LPS-inflamed microglial. **C**) Inflamed microglial treated with 1.5 mg.mL^{-1} *Daphne mucronata* leaf extract. Photos were at 20x magnification.

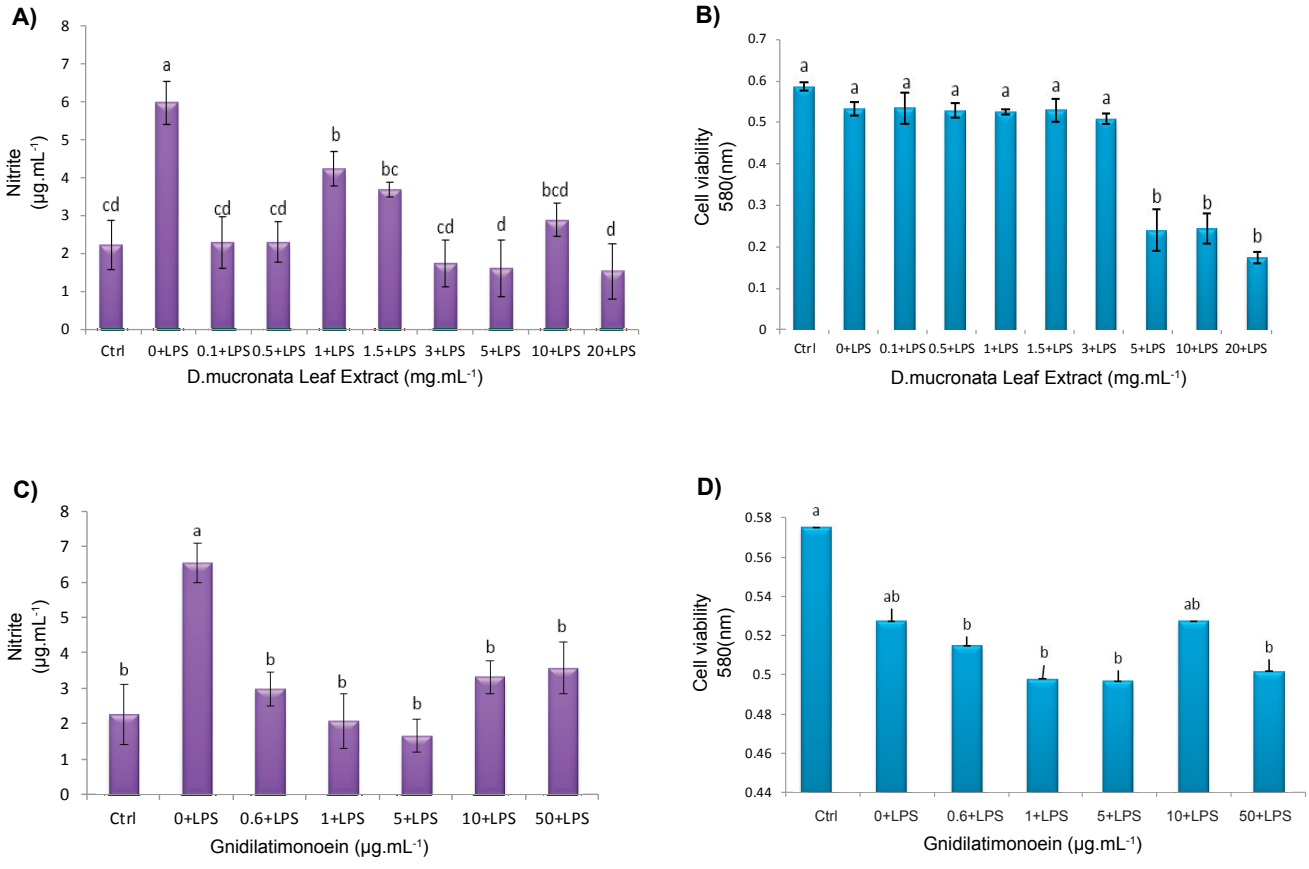

Figure 5. NO measurement and cell viability assay of plant extract and Gnidilatimonoein on the LPS-stimulated mixed glial cells. A) Primary mixed glial cells were incubated in the absence or presence of LPS (10 mg.mL^{-1}). The cells were pretreated with various amounts of *D. mucronata* extract before LPS was added. After 48h, the cultures were examined with a nitrite assay and **B**) a cell viability assay. **C**) Primary mixed glial cells were incubated in the absence or presence of LPS (10 mg.mL^{-1}). The cells were pretreated with various amounts of Gnidilatimonoein before LPS was added. After 48h the cultures were examined with a nitrite assay and **D**) a cell viability assay. Columns of at least 1 common letter by the Duncan test Indicate the level of significance P<0.05.

4.12. Evaluation of Toxicity of Gnidilatimonoein Concentrations in the Mixed Glial Cells

Cell viability was examined in inflamed mixed glial cells to determine the cytotoxic activity of *D. mucronata* leaf extract concentrations (0.6, 1, 5, 10, and 50 mg.mL^{-1}). Analytical results showed there was no cytotoxicity in all applied doses (**Fig. 5D**).

4.13. Gnidilatimonoein Inhibits Expression of iNOS Gene in the Inflamed Mixed Glial Cells Treated with LPS

An RT-PCR was conducted to determine whether the decrease in LPS-induced NO measured in supernatants corresponded to a reduction in iNOS transcription at the transcriptional level. After pretreatment with 0.6 and 5 g.mL^{-1} Gnidilatimonoein for 1 hour, primary glial cultures were stimulated with LPS (10 g.mL^{-1}) for 48 hours. As shown in **Figure. 6**, LPS increased the mRNA expression of iNOS, and Gnidilatimonoein inhibited its expression (**Fig. 6**).

5. Discussion

Glial cells play an important role in inflammation and nervous system toxicity. A macrophage's functions include phagocytosis (29, 30), the secretion of proinflammatory cytokines, such as cytokines, chemokines, and nitric oxide (31, 32), and antigen presentation (33). Under pathological conditions, induced nitric oxide synthase (iNOS) (the key enzyme responsible for NO production) is overexpressed in activated glial cells (32). A low concentration of NO is neuroprotective, but a high concentration causes neuronal death due to its destructive effects on neurophysiology. In this study, it has been shown that natural phytochemicals can target neurodegeneration by attenuating the expression of iNOS (35, 36).

Daphne extracts and active compounds isolated from them have been shown to have anti-inflammatory properties. A few species of Daphne plants are used to treat certain cancers and inflammatory diseases. The aerial parts and roots of *Daphne oleoide* are used in Turkish traditional medicine to treat rheumatic pain, low back pain, and fever (37). In traditional Chinese medicine, some species of Daphne have been used in the formulations prescribed for inflammatory diseases; for example, *Daphne Jenkwa* is used to treat edema (38), *Daphne acutiloba* is used to treat wounds and bruises, *Daphne tangutica* is used to treat rheumatoid arthritis, and *Daphne mezereum* is used to treat chronic rheumatism, skin diseases, gout, and lymphatic inflammation. One study found *Daphne pontica* L roots to have anti-inflammatory activity in an ethyl acetate extract (41). Various skin disorders, such as spots and pace, are treated with ethanolic extract of aerial parts of *Daphne mucronata* Royl native to Iran and Turkey. The leaves of *Daphne mucronata* are used in northern Pakistan to treat inflammatory conditions, acute arthritis, and similar conditions for patients with the flu. They are also used topically for treating muscle inflammation.

Already, it was reported several active components of

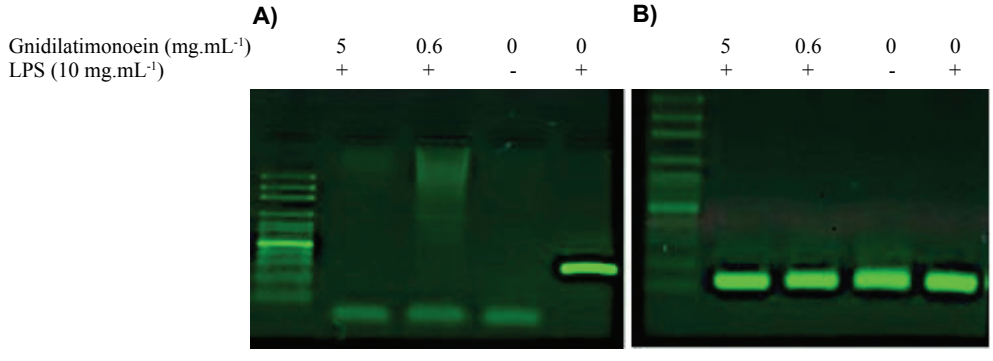

Figure 6. Electrophoresis of RT-PCR productions. A) iNOS gene products, 253 bp. **B**) GAPDH gene products, 118 bp. Primary glial cultured were pretreated with 0.6 and 5 mg.mL^{-1} Gnidilatimonoein for one hour and stimulated with LPS (10 mg.mL^{-1}) for a 48h incubation period. Gnidilatimonoein inhibits iNOS expression the same as the control group without LPS stimulation.

Daphne oleoides spp. Oleoides have inhibitory activity against inflammatory cytokines (37). The ability of aptosimon (as a lignan) and several other components isolated from *Daphne Jenkwa* to inhibit iNOS and COX-2 expression has been demonstrated in LPS-induced RAW 264.7 (42). *Daphne jejudoensis* reduced LPS-Induced Inflammation by Inhibiting TNF-α, IL-1β, IL-6, iNOS, and COX-2 Expression in Periodontal Ligament Cells (43). Radical/antioxidant scavengers and coumarin derivatives also present in *D. mucronata* extracts exhibit anti-inflammatory activity through various mechanisms (44, 45). Some types of ent-kauranoid diterpenoids have also been reported to reduce nitric oxide (NO) generation (46, 47). Gnidilatimonoein and *Daphne mucronata* extract has not been shown to reduce inflammation, especially an inflammation of primary glial cells.

6. Conclusion

Using primary rat mixed glial cells and microglial, we found that *D. mucronata* leaf extract significantly reduced NO production stimulated by LPS. Additional-ly, it has been demonstrated that Gnidilatimonoein is significant-ly effective in inhibiting the expression of the iNOS gene in the primary rat mixed glial cells and decreasing NO production at all applied doses. In the absence of LPS stimulation, Gnidilatimonoein protects cell morphology like the control group, suggesting attenuation of proinflammatory responses. Gnidilatimonoein and the plant extract do not exhibit cytotoxicity at any of the doses mentioned, thus indicating that their anti-inflammatory effects are not caused by cell death. It is possible to evaluate upstream inflammatory pathways. The biological activity of *D. mucronata* extract containing such active components, considered an effective therapeutical agent, could be further explored.

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