Comparison of the neuroprotective effects of gossiping on cisplatin-induced neurotoxicity in vitro and in vivo

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Abstract

Aims: The aim of this study is to investigate the possible protective neuroprotective effects of gossypine (GOS) against cisplatin (CIS) (cis-diamminedichloroplatin [II]) toxicity.

Materials and methods:CIS (In-vitro; 50 and 100 μM, In-vivo; 10 mg/kg/day) toxicity was created In-vitro and In-vivo, and GOS administration (In-vitro; 50, 75 and 100 μM, In-vivo; 5, 10 and 20 mg/kg/day).

Key findings: We used different methods that supported each other. In primary neuron culture, cell proliferation was protected against CIS toxicity in a time-dependent manner with applying GOS. Oxidative/antioxidative markers were normalized in a dose-dependent manner with gossypin administration. In the mechanisms of neurotoxic/neuroprotective (iNOS/nNOS) and inhibition of inflammatory mediators (TNF-a and NF-kB), gossypin has been shown to inhibit NF-kB activation to suppress CIS-induced inflammation in primary neuron culture cells. It has been shown to have antiapoptotic effects in healthy tissues and cells by inhibiting CASP-3 and CASP-9 mRNA levels by regulating the ratio of BCL2/BAX, which are pro-apoptotic and anti-apoptotic indicators.

Significance: We conclude that It has been shown that gossypin may be an ideal neuroprotective agent with potent antioxidant capacity and does not reduce the antitumor activity of the chemotherapeutic drug.

1. Introduction

Cancer represents a significant medical challenge, impacting both individual well-being and healthcare economics globally. Treatment modalities for cancer vary based on the disease's type and progression, encompassing conventional methods like surgery, chemotherapy, and radiation therapy, as well as advanced therapies such as immunotherapy, targeted therapy, hormone therapy, gene therapy, and photodynamic therapy. Among these, chemotherapy remains a predominant and efficacious strategy, employing cytotoxic or alkylating agents to combat cancerous cells (Eastman, 2017; Shewach and Kuchta, 2009).

Cisplatin (CIS), a pioneering metal-based chemotherapeutic, is noteworthy for its widespread application, with reports suggesting that it is utilized in the treatment of half of the cancer patients worldwide (Galanski et al., 2005). The annual expenditure on platinum-based anticancer drugs, including CIS, is estimated to be around $2 billion (Ross et al., 2006). CIS is versatile, treating a broad spectrum of solid tumors, including those affecting the testicles, ovaries, bladder, lungs, cervix, head and neck, stomach, and more (Tchounwou et al., 2021; Yang et al., 2018). Its antineoplastic efficacy is attributed to its ability to bind with both genomic DNA (gDNA) and mitochondrial DNA (mtDNA), resulting in DNA lesions that hinder DNA, mRNA, and protein synthesis, disrupt DNA replication, and trigger signal transduction pathways leading to cell death via necrosis or apoptosis (Sigel et al., 2018).

However, CIS's full therapeutic potential is often not realized due to adverse effects and the development of drug resistance. Resistance mechanisms involve reduced drug uptake, drug neutralization by protein binding, enhanced DNA repair capabilities, and alterations in apoptosis-related proteins (Skowron et al., 2018). The toxic side effects of CIS are significant concerns, with nephrotoxicity, ototoxicity, hepatotoxicity, gastrointestinal issues, and neurotoxicity being the most severe (Astolfi et al., 2013; Manohar and Leung, 2018; Oun et al., 2018; Tchounwou et al., 2021). Notably, CIS-induced neurotoxicity has been substantiated through various animal studies, highlighting the need for cautious application and monitoring during treatment (Fumagalli et al., 2020; Perse, 2021).

It would be beneficial to understand the mechanism of these side effects of CIS as a dose-limiting factor that negatively affects patients' quality of life and to eliminate and reduce these side effects if possible.

Gossypin (3,5,8,3,4-pentahydroxy-7-O-glucosylflavone 8-glucoside) isolated from *Hibiscus vitifolius* is a bioflavnoid. It has antioxidant, anti-inflammatory (Cinar et al., 2022) and analgesic (Viswanathan et al., 1993) properties. In addition, it has been reported to protect pancreatic β-cells from glucotoxicity in diabetes (Venkatesan and Sorimuthu Pillai, 2012). It was also said to have neuroprotective activity in a model of cerebral ischemia in rats (Chandrashekhar et al., 2013b). In a neurotoxicity study with rat cortex primary cells, it was reported that gossypin is neuroprotective and does this by suppressing oxidative stress (Yoon et al., 2004). In addition, gossypin has not only antioxidant but also anticancer effects. Its antiproliferative and apoptotic effects have been demonstrated in gastric cancer (Wang et al., 2019), melanoma (Bhaskaran et al., 2013), and prostate cancer (PC-3 cells) (Cinar, 2021b). Since it shows both antioxidant and anticancer effects, it suggests that its combination with CIS may reduce its side effects with a neuroprotective impact while supporting the chemotherapeutic effect.

Although there are many studies on CIS toxicity in the current literature, we have seen that an effective treatment method still needs to be determined. When the literature is examined, as an ideal neuroprotective agent, an agent with no side effects, has a strong chemical protective capacity, and does not reduce the antitumor activity of the chemotherapeutic drug is sought. No research has been done on the CIS toxicity of gossypsin, and it was thought that gossypin may be an ideal neuroprotective agent due to its chemical structure and the mechanisms of CIS toxicity formation described above.

While the number of studies on gossypin in the literature is low, the antioxidant properties of gossypin are prominent in these studies. Our study is the first to investigate its effects against neurotoxicity due to CIS in vivo and in vitro.

2. Materials and Methods

Ten newborn (5-hour-old) animals were used for primary neuron culture formation. 50 Mus Musculus mice (25–30 g) were used in the in-vivo toxicity model. The animals were treated with 12 hours of light and dark in plastic cages with sawdust floors, and one-week adaptation care was provided with regular feed and water at average room temperature (24 ± 2 0C). All study and animal care procedures were

performed by the decisions of the local animal ethics committee of Kafkas University, numbered KAU/HADYEK/2019-066 and KAU/HADYEK/2019-070.

2.1 In-vitro and In-Vivo investigations

Primary neuron culture

The newborn Sprague dawley rat puppies were quickly decapitated, their cortex neurons were removed, and they were kept in the ready neurobasal medium (CAS number: SCM003 (Sigma-Aldrich In. (St. Louis, MO)). After adding 1:1 trypsin and keeping it in the incubator for 30 minutes, it was centrifuged 3 times, and the supernatant was discarded each time, and a new medium was added. The medium was prepared by adding a neurobasal medium of 1000:1 penicillin 50:1 B27 and 10:1 FBS (fetal bovine serum) in a separate tube. Cells were added to the conditioned medium. 150 microliters of medium were added to each chamber of the 96 healthy plate. For PCR analysis, 2000 ml of medium was added to 6 well plates using the same medium and method. Cells were kept in the incubator for ten days to adhere to the bottom of the wells, to coat the bottom, and to grow. With the same method, a 6-well plate was planted for biochemical analysis. CIS doses were kept similar to our previous study. (Binnetoglu et al., 2019). The cell cultures were examined at an inverted microscope (Leica) for approximately 24 hours. Then, IC_{50} was determined in this cell line.

2.2 Determination of Cell Proliferation

MTT (Cell Viability Test)

Doses of gossypin (10–100 μM) (Sigma Aldrich (CAS Number 652-78-8)) were administered 2 hours before toxicity. Cell proliferation was determined by applying the MTT method 24 hours after CIS (Sigma Aldrich (CAS Number 15663-27-1)). The cells incubated with the MTT kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (11465007001, Sigma-Aldrich Chemie Gmbh Munich, Germany) method by the manufacturer's protocols, as in my previous studies, were measured with a microplate reader spectrophotometer (Epoch Microplate Spectrophotometer, BioTek, USA) at 620 nm absorbance value in 3 replicates. The cell viability was then calculated using the following equation: Inhibitory rate (%)= (1 – [ODtreatment-ODcontrol])* 100%. By taking the average of these measurements, the IC_{50} value of primary neuron cells for gossypin was determined from the results. While creating the biochemical and PCR groups, three different doses were used considering the IC_{50} value.

2.3 Quantitative Determination of mRNA Expression by Real-Time PCR

For PCR analysis, 2000 ml of medium was added to 6 well plates using the same medium and method. Cells were kept in the incubator for ten days to adhere to the bottom of the wells, cover the bottom, and grow.

PCR groups (6th and 12th-hour groups are indicated below with the same). Three groups were formed based on IC50 values for group determination.

1. Control, 2. Control+ % 0.1 lik DMSO, 3. CIS 50 µM, 4. CIS 100 µM, 5. CIS 50 µM + Goss 50 µM, 6. CIS 50 µM + Goss 75 µM, 7. CIS 50 µM + Goss 100 µM, 8. CIS 100 µM + Goss 50 µM,

9. CIS 100 µM + Goss 75 µM, 10. CIS 100 µM + Goss 100 µM

CIS was administered two hours after Gossypin administration. After 6 and 12 hours, the cells were removed by scraping with a plate scraper, and centrifuged at 1000 Rpm for 2 minutes. Total RNA extraction and cDNA synthesis were performed according to the methods described in our previous studies (Cinar et al., 2021; Cinar et al., 2022), according to the manufacturer's instructions, and TNF-α (Mm00562055_m1), NF-KB (Mm01399572_m1), iNOS (Mm00561646_m1), nNOS (Mm00583793_m1), Bcl (Mm00821025_g1), Bax (Mm01480161_g1), Caspase-3 (Mm00563902_m1), Caspase-9 (Mm00581212_m1) and β-actin (Mm00667869_m1) mRNA expression as a housekeeping gene were analyzed. Compared with the control group, All data were expressed as fold change in expression compared with the cell groups using the $2^{-\Delta\Delta}$Ct method. The groups' differences in oxidative stress, inflammation, and apoptosis were evaluated.

2.4 Biochemical Parameters

For oxidative stress analysis, 2000 ml of medium was added to 6 healthy plates using the same medium and method. Cells were kept in the incubator for ten days to adhere to the bottom of the wells, cover the bottom, and grow. CIS was administered 2 hours after Gossypin administration. After 6 and 12 hours, the cells were scraped with a plate scraper, and the supernatant was removed by centrifugation at 1000 Rpm for 2 minutes. The cell lysate was subjected to the same steps as the brain tissues by the manufacturer's instructions, and SOD activity, GSH, and MDA level parameters were measured. Briefly, Mouse cortex, cerebellum tissues, and primary cell lysates were stored at -80˚C. 100 mg of tissue from each sample was homogenized with Tissue Lyser in a specific homogenate buffer on ice. It was then centrifuged according to the instructions in the kit. For biochemical studies, SOD activity, MDA levels, and GSH levels were measured from each supernatant by ELISA kit measurement methods. Superoxide dismutase (SOD) activity and glutathione (GSH) and malondialdehyde (MDA) levels were measured manually from the supernatants, as described in our previous studies (Cinar et al., 2022).

2.5 Hoechst 33258 staining assay

The nuclear morphology of cells treated with CIS and GOS was observed with a Hoechst 33258 staining assay. Primary neuron cells were seeded in 6-well plates and cultured for 10 days. CIS was administered 2 hours after Gossypin administration. The method was performed as in our previous study (Cinar et al., 2021). Changes in nuclear morphology were monitored with a LEICA inverted microscope (Leica, DMIL LED, Germany).

2.6 Animals

2.7 Ethical statements and animals

Ten newborn Balb/C Mice were used for the in-vitro part of the study. For the in-vivo phase of our research, a total of 50 male Mus Musculus mice (25–30 g) were obtained from the Atatürk University Experimental Animals Research and Application Center. We studied at Atatürk University Experimental Animals Research and Application Center. In our study, 50 male mice were randomly divided into five equal groups and taken into a separate study compartment. The animals were treated with 12 hours of light and dark in plastic cages with sawdust floors, and one-week adaptation care was provided with regular feed and water at average room temperature (24 ± 2 ^{0}C). All study and animal care procedures were performed by the decisions of the local animal ethics committee of Kafkas University, numbered KAU/HADYEK/2019-066 and KAU/HADYEK/2019-070.

2.8 Experimental Protocols of Neurotoxicity

After a week of acclimatization, the experimental animals were divided into five groups (comprising six rats each) as follows:

1.Healthy Control (n = 10) i.p saline + oral % 0.1 lik DMSO
2. CIS 10 mg/kg/ day (n = 10) (i.p) 3. CIS 10 mg/kg/ day + Gossypin 5 mg/kg/ day (n = 10) (i.p + oral)
4. CIS 10 mg/kg/ day + Gossypin 10 mg/kg/ day (n = 10) (i.p + oral)
5. CIS 10 mg/kg/day + Gossypin 20 mg/kg/ day (n = 10) (i.p + oral)

In our study,

CIS and gossypin were applied to 4 groups except the healthy group.

CIS (CIS-DICHLORODIAMINEPLATINUM(II), 99.99%,(TRACE METAL BASIS), Sigma Aldrich, MO, Germany) 10 mg/kg/day was administered intraperitoneally for 14 days.

Gossypin (Cat. no: 652-78-8, Sigma Aldrich, Germany) dissolved in 0.1% DMSO and administered with oral gavage of 5, 10, 20 mg/kg/day for 14 days (Chandrashekhar et al., 2013b).

On the 15th day, all mice were anesthetized with 30 mg/kg of thiopental sodium, the brain tissues were removed, some of them were treated with liquid nitrogen for biochemistry and molecular analysis, then kept at -80 0C for examination, and the other part was preserved in formaldehyde for histopathological examinations. Brain tissues were divided into two hemispheres. Ten brain tissues were allocated for histopathology and ten brain tissues for biochemistry and molecular analysis in a randomized manner.

2.9 Histopathological Examinations

Hematoxylin-Eosin Staining Procedure

At necropsy, tissue samples from the cerebral cortex and cerebellum were taken and fixed in 10% formaldehyde solution. The method was performed as in our previous study (Cinar et al., 2022).

Immunohistochemical Staining

For immunohistochemical analysis, the cerebral cortex and cerebellum region tissues were stained with apoptosis-initiating BAX and inflammation-initiating NFkB (Bax and NFkB, Santa Cruz Biotechnology). The method was performed as in our previous study (Salakou et al., 2007).

2.10 Statistical analysis

Values were expressed as mean ± S.D. Numerical data obtained from biochemical analyses and Real-Time PCR results were analyzed with the One Way ANOVA Post Hoc Duncan test. SPSS-20 for Windows was used for all statistical analyses. P values less than 0.05 were considered significant.

3. Results

3.1 Primary Neuron Cells Viability Results

In the primary neuron culture, As shown in Table 1, gossypin conservation the cell viability of primary neuron cells in a time- and dose-dependent manner. Gossypin, IC50 values in primary neuron culture were found to be 60.40ug/ml (Fig. 1). Especially the application of 100 µM dose of Gossypin, the viability level, which decreased to 50% with the application of 100 µM cisplatin, increased up to 80% (Table 1)

Table 1
Primary Neuron Culture Time-Dependent Cell Viability Results (Mean ± SE*).*

Groups	24 h	48 h	72 h
Control	100.41 ± 0.42^{f}	99.74 ± 0.25^{f}	100.07 ± 0.67^{f}
DMSO (%0.01)	98.92 ± 0.59^{f}	98.98 ± 0.88^{f}	99.67 ± 1.22^{f}
CIS 50	$81.75 \pm 2.05^{b,c}$	$72.05 \pm 2.07^{b,c}$	58.27 ± 1.86^{c}
CIS 100	74.05 ± 2.74^{a}	63.55 ± 2.81^{a}	43.20 ± 3.24^{a}
CIS 50 + GOS 50	$86.22 \pm 1.74^{c,d}$	$75.67 \pm 1.74^{c,d}$	$56.25 \pm 1.73^{b,c}$
CIS 50 + GOS 75	$89.76 \pm 2.45^{d,e}$	$76.29 \pm 1.69^{c,d}$	68.71 ± 1.79^{d}
CIS 50 + GOS 100	93.15 ± 2.24^{e}	83.05 ± 2.23^{e}	84.76 ± 1.89^{e}
CIS 100 + GOS 50	74.99 ± 2.20^{a}	65.05 ± 2.29^{a}	51.80 ± 0.98^{b}
CIS 100 + GOS 75	$78.86 \pm 1.76^{a,b}$	$68.22 \pm 1.70^{a,b}$	65.55 ± 1.44^{d}
CIS 100 + GOS 100	$87.36 \pm 1.95^{c,d,e}$	$79.79 \pm 1.11^{d,e}$	80.11 ± 1.46^{e}

$^{a–f}$ Different letters in the same column are statistically significant ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM.

3.2 Oxidative Stress Markers in Primary Neuron Cells And Brain Tissues

Compared to the control group, SOD activity (34.60 ± 1.18^{a}) and GSH levels (2.14 ± 0.07^{a}) were decreased, while MDA levels (5.11 ± 0.11^{e}) were increased with CIS application in the primary neuron cells. With the application of Gossypin, these parameters approached their normal levels. For the 12th hour, respectively, It was found to be 70.88 ± 3.75^{e}, 4.59 ± 0.25^{e}, 3.17 ± 0.22^{b} (Table 2).

Table 2
Antioxidant and oxidant parameters in primary neuron culture (Mean ± SE).

Groups	SOD Activity Levels (nmol/mg protein)		MDA Levels (nmol/mg protein)		GSH Levels (nmol/mg protein)	
	6 h	12 h	6 h	12 h	6 h	12 h
Control	99.61 ± 0.91^{e}	99.29 ± 0.57^{g}	1.58 ± 0.11^{a}	1.55 ± 0.17^{a}	5.88 ± 0.22^{e}	5.77 ± 0.07^{g}
DMSO (%0.01)	99.26 ± 0.71^{e}	98.67 ± 1.53^{g}	1.55 ± 0.06^{a}	1.47 ± 0.05^{a}	5.89 ± 0.13^{e}	5.86 ± 0.10^{g}
CIS 50	74.96 ± 0.92^{b,c}	63.96 ± 1.34^{c}	3.30 ± 0.17^{d,e}	3.78 ± 0.29^{d}	3.89 ± 0.13^{b,c}	2.95 ± 0.10^{b,c}
CIS 100	61.76 ± 1.88^{a}	34.60 ± 1.18^{a}	4.02 ± 0.16^{f}	5.11 ± 0.11^{e}	2.96 ± 0.17^{a}	2.14 ± 0.07^{a}
CIS 50+ GOS 50	79.95 ± 0.91^{c}	68.31 ± 1.63^{c,d}	3.05 ± 0.14^{d,e}	3.61 ± 0.20^{c,d}	3.82 ± 0.08^{b,c}	3.02 ± 0.05^{c}
CIS 50+ GOS 75	81.00 ± 1.48^{c}	71.81 ± 2.01^{d}	2.52 ± 0.14^{c}	3.22 ± 0.03^{b,c}	4.29 ± 0.19^{c,d}	3.81 ± 0.03^{d}
CIS 50+ GOS 100	92.14 ± 1.75^{d}	85.66 ± 2.48^{e}	1.66 ± 0.17^{a,b}	1.75 ± 0.05^{a}	5.86 ± 0.15^{e}	5.31 ± 0.18^{f}
CIS 100+ GOS 50	64.70 ± 1.85^{a}	44.42 ± 2.02^{b}	3.44 ± 0.15^{e}	4.77 ± 0.15^{e}	3.06 ± 0.08^{a}	2.55 ± 0.21^{a,b}
CIS 100+ GOS 75	72.29 ± 3.19^{b}	63.09 ± 3.94^{c}	2.97 ± 0.16^{d}	3.58 ± 0.15^{c,d}	3.69 ± 0.22^{b}	3.33 ± 0.19^{c}
CIS 100+ GOS 100	79.85 ± 3.31^{c}	70.88 ± 3.75^{e}	2.08 ± 0.15^{b}	3.17 ± 0.22^{b}	4.72 ± 0.32^{d}	4.59 ± 0.25^{e}

$^{a–f}$ Different letters in the same column are statistically significant ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM.

Similar findings were found in brain tissue analyses of mice. Decreased SOD activity and GSH levels and increased MDA levels with CIS application were dose-dependently regulated by GOS application. In addition, TNF-α level, measured by the elisa method in terms of inflammation, also supported our findings (Fig. 3).

3.3 Primary Neuron Cells and Brain Tissues mRNA expression Results

In comparison to the control group, we found significant increases ($p < 0.05$) in pro-apoptotic markers, CAS3 (5.85 ± 0.28^{f}), inflammatory markers TNF-α (6.99 ± 0.49^{a}), NF-kB (2.68 ± 0.09^{g}) and iNOS (7.85 ± 0.09^{h}) after cisplatin challenge. In addition, significant reductions ($p < 0.05$) in anti-apoptotic markers, CAS9 (1.79 ± 0.12^{g}) gene expression, Bcl/Bax ratio (0.28 ± 0.03^{a}), and nNOS (0.36 ± 0.02^{a}) after cisplatin injection were observed. Moreover, our results revealed that gossypin treatment significantly decreased (2.54 ± 0.22^{b}) CAS3 (2.54 ± 0.22^{b}) gene expressions, CAS9 ($1.31 \pm 0.02^{c,d}$), TNF-α (2.97 ± 0.19^{a}), NF-kB ($1.73 \pm 0.14^{d,e}$) and iNOS (4.32 ± 0.25^{e}). And increased Bcl/Bax ratio (0.80 ± 0.05d) and nNOS (0.75 ± 0.03^{c}) gene expression (Tables 3, 4, and 5).

Table 3
Primary Neuron Culture TNF-α, NF-kB and Bcl-2/Bax mRNA Expression Results (Mean ± SE).

Groups	TNF-α mRNA Expression		NF-kB mRNA Expression		Bcl-2/Bax mRNA Expression	
	6 h	12 h	6 h	12 h	6 h	12 h
Control	0.98 ± 0.01^{a}	0.97 ± 0.03^{a}	1.00 ± 0.02^{a}	1.01 ± 0.01^{a}	0.97 ± 0.02^{d}	0.98 ± 0.03^{e}
DMSO (%0.01)	1.03 ± 0.03^{a}	0.94 ± 0.13^{a}	1.02 ± 0.03^{a}	1.05 ± 0.07^{a,b}	0.99 ± 0.01^{d}	1.01 ± 0.06^{e}
CIS 50	3.64 ± 0.21^{g}	5.30 ± 0.20^{a}	1.96 ± 0.02^{d,e}	2.20 ± 0.10^{f}	0.74 ± 0.04^{a,b}	0.67 ± 0.05^{c,d}
CIS 100	5.13 ± 0.24^{h}	6.99 ± 0.49^{a}	2.38 ± 0.09^{f}	2.68 ± 0.09^{g}	0.65 ± 0.02^{a}	0.28 ± 0.03^{a}
CIS 50+ GOS 50	3.18 ± 0.09^{d,e}	3.96 ± 0.16^{a}	1.81 ± 0.05^{c,d}	1.85 ± 0.05^{e}	0.75 ± 0.05^{b}	0.51 ± 0.05^{b}
CIS 50+ GOS 75	2.85 ± 0.08^{c,d}	3.10 ± 0.09^{a}	1.62 ± 0.09^{c}	1.53 ± 0.09^{c,d}	0.96 ± 0.07^{d}	0.99 ± 0.02^{e}
CIS 50+ GOS 100	2.08 ± 0.10^{b}	2.48 ± 0.10^{a}	1.32 ± 0.08^{b}	1.30 ± 0.12^{b,c}	1.21 ± 0.05^{e}	1.69 ± 0.05^{f}
CIS 100+ GOS 50	3.55 ± 0.17^{e,f}	5.04 ± 0.12^{a}	2.10 ± 0.07^{e}	2.60 ± 0.14^{f,g}	0.72 ± 0.02^{a,b}	0.46 ± 0.03^{b}
CIS 100+ GOS 75	3.09 ± 0.10^{d}	4.08 ± 0.14^{a}	1.96 ± 0.03^{d,e}	2.01 ± 0.05^{e,f}	0.79 ± 0.03^{b,c}	0.59 ± 0.01^{b,c}
CIS 100+ GOS 100	2.50 ± 0.09^{c}	2.97 ± 0.19^{a}	1.81 ± 0.05^{c,d}	1.73 ± 0.14^{d,e}	0.90 ± 0.04^{c,d}	0.80 ± 0.05^{d}

$^{a–f}$ Different letters in the same column are statistically significant ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM.

Table 4
Primary Neuron Culture nNOS and iNOS mRNA Expression Results (Mean ± SE).

Groups	nNOS mRNA Expression		iNOS mRNA Expression	
	6 h	12 h	6 h	12 h
Control	1.00 ± 0.02^{d,e}	1.03 ± 0.02^{f}	1.00 ± 0.01^{a}	1.01 ± 0.02^{a}
DMSO (%0.01)	1.03 ± 0.03^{d,e}	0.99 ± 0.01^{f}	1.03 ± 0.04^{a}	1.03 ± 0.04^{a}
CIS 50	0.91 ± 0.02^{c,d}	0.81 ± 0.02^{c,d}	4.15 ± 0.12^{e}	4.33 ± 0.12^{e}
CIS 100	0.62 ± 0.05^{a}	0.36 ± 0.02^{a}	7.05 ± 0.12^{h}	7.85 ± 0.09^{h}
CIS 50+ GOS 50	0.97 ± 0.01^{d,e}	0.86 ± 0.01^{d}	3.40 ± 0.17^{d}	3.80 ± 0.02^{d}
CIS 50+ GOS 75	1.02 ± 0.03^{e}	0.94 ± 0.01^{e,f}	2.95 ± 0.03^{c}	2.77 ± 0.10^{c}
CIS 50+ GOS 100	1.19 ± 0.01^{f}	1.14 ± 0.02^{g}	2.29 ± 0.16^{b}	1.74 ± 0.03^{b}
CIS 100+ GOS 50	0.72 ± 0.01^{b}	0.50 ± 0.05^{b}	6.08 ± 0.07^{g}	7.00 ± 0.13^{g}
CIS 100+ GOS 75	0.85 ± 0.03^{c}	0.75 ± 0.03^{c}	5.05 ± 0.04^{f}	5.55 ± 0.24^{f}
CIS 100+ GOS 100	0.94 ± 0.01^{d,e}	0.87 ± 0.02^{d,e}	4.11 ± 0.15^{e}	4.32 ± 0.25^{e}

$^{a–f}$ Different letters in the same column are statistically significant ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM.

Table 5
Primary Neuron Culture CAS-3 and CAS-9 mRNA Expression Results (Mean ± SE).

Groups	CAS-3 mRNA Expression		CAS-9 mRNA Expression	
	6 h	12 h	6 h	12 h
Control	1.03 ± 0.02^{a}	1.02 ± 0.07^{a}	0.99 ± 0.02^{a}	0.97 ± 0.01^{a}
DMSO (%0.01)	1.08 ± 0.04^{a}	1.10 ± 0.06^{a}	1.00 ± 0.00^{a}	1.01 ± 0.06^{a,b}
CIS 50	3.75 ± 0.09^{e}	4.94 ± 0.19^{e}	1.42 ± 0.11^{d,e}	1.53 ± 0.07^{e,f}
CIS 100	4.34 ± 0.24^{f}	5.85 ± 0.28^{f}	1.66 ± 0.07^{f}	1.79 ± 0.12^{g}
CIS 50+ GOS 50	3.44 ± 0.11^{d,e}	4.34 ± 0.20^{d,e}	1.31 ± 0.01^{c,d}	1.43 ± 0.02^{d,e}
CIS 50+ GOS 75	3.17 ± 0.05^{c,d}	3.98 ± 0.05^{c,d}	1.26 ± 0.03^{b,c,d}	1.30 ± 0.02^{c,d}
CIS 50+ GOS 100	2.91 ± 0.04^{c}	2.09 ± 0.10^{b}	1.10 ± 0.00^{a,b}	1.17 ± 0.03^{b,c}
CIS 100+ GOS 50	3.75 ± 0.09^{e}	4.56 ± 0.30^{d,e}	1.49 ± 0.05^{e}	1.67 ± 0.04^{f,g}
CIS 100+ GOS 75	2.89 ± 0.06^{c}	3.44 ± 0.25^{c}	1.29 ± 0.04^{c,d}	1.51 ± 0.02^{e,f}
CIS 100+ GOS 100	2.45 ± 0.23^{b}	2.54 ± 0.22^{b}	1.18 ± 0.04^{b,c}	1.31 ± 0.02^{c,d}

$^{a–f}$ Different letters in the same column are statistically significant ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM.

TNF-α, NF-kB, iNOS, CASP-3, and CASP-9 values increased significantly with CIS application to mice, while nNOS and Bcl-2/BAX values were significantly decreased compared to control. These values were normalized depending on the dose (especially GOS 20) with the administration of Gossypin (Fig. 4).

3.4 Results of In-vivo Hematoxylin Eosin and Immunohistochemistry Findings

The most striking change was observed in Purkinje cells when the cerebellum tissues were examined in staining with hematoxylin-eosin. Purkinje cell bodies consisted of well-circumscribed nuclei and nucleoli

in the control group. In the CIS group, unlike the control group, Purkinje cell nuclei were pycnotic, and there was an increase in eosinophilia in the cytoplasm. It was observed that the nucleus and cell border were improved, and the cytoplasm became close to control in Purkinje cells, which was dose-dependent in the GOS-treated groups (Fig. 5).

The CIS group was compared with the control group, and the most prominent degeneration was seen in the capillaries. In the CIS group, it was observed that the capillary wall integrity was impaired, and the endothelial cells clustered into the lumen. The capillary degenerations were smoothed in the GOS-applied groups depending on the dose (Fig. 5). Purkinje and capillary base changes in hematoxylin-eosin stained sections were scored for Purkinje cell degeneration and capillary degeneration. (Table 6)

Table 6
Evaluation of degeneration from hematoxylin-eosin stained preparations.

	C	CIS	GOS 5	GOS 10	GOS 20
Picnotic Purkinje cell density	-	++++	++++	++	+
Eosinophilia in Purkinje cytoplasm	+	++++	++++	+++	++
Purkinje nuclear border nucleolus prominence	++++	-	+	++	+++
Capillary smoothness	++++	-	-	+++	++++
Endothelial cell aggregation	-	++++	++++	++	+
Grade 0: - (negative), Grade 1: + (mild), Grade 2: ++ (moderate), Grade 3: +++ (severe), Grade 4: ++++ (most serious)					

3.5 Immunohistochemical Findings

Immunopositive Purkinje cells in the ganglion cell layer of the cerebellum tissues were counted by marking with BAX primary antibody in the brain tissues (Fig. 6). A statistically significant difference was found between the groups ($P < 0.0001$). The number of positive cells was increased in the CIS group compared to the control group ($P < 0.0001$). It was observed that the number of immunopositive Purkinje cells decreased statistically depending on the increasing dose of gossiping ($p = 0.0004$, $p = 0.0055$, $p = 0.0053$, respectively) (Fig. 7a).

Positive cells were counted in the dentate gyrus of the hippocampus, especially in the subgranular zone (Fig. 7b). When the groups were compared for the number of positive cells, the difference was statistically significant ($P < 0.0001$). The number of positive cells was increased in the CIS group compared to the control group ($P < 0.0001$). Compared to the control group, it was observed that the number of positive cells increased due to the decreased dose of gossypine ($p = 0.0020$, $p = p < 0.0001$, $p = 0.0020$, respectively) (Fig. 7b).

When the H-score analysis of the BAX primary antibody marking of the images taken from the cerebral cortex was examined, it was observed that there was a statistically significant difference between the

groups ($p < 0.0001$). The H-score ratio increased in the control group compared to the CIS group, which was statistically significant ($p < 0.0001$). When the control group and GOS groups were compared, it was observed that the positivity decreased with increasing dose ($p < 0.0001$, $p < 0.0001$, $p = 0.0003$), respectively (Fig. 7c).

The H-score ratios obtained from labeling with NFKB primary antibody were statistically significant between the groups ($p < 0.0001$). When the control group was compared with the CIS group and GOS groups, it was observed that the H-score changed statistically (Graph 4) (CIS; $p < 0.0001$, GOS 5; $p < 0.0001$, GOS 10; $p < 0.0001$, GOS 20; $p < 0.0001$) (Fig. 7d)

4. Discussion

In the conducted research, gossypin was thoroughly investigated for its antioxidant, anti-inflammatory, and anticancer properties through a comprehensive array of in vitro and in vivo methodologies, followed by a detailed analysis of the findings.

The efficacy of CIS is dose-dependent, with higher doses yielding more pronounced therapeutic outcomes. However, dosage escalation is curtailed by the onset of severe toxicities, chiefly nephrotoxicity, ototoxicity, and neurotoxicity. Clinically, saline hydration and osmotic diuresis are preventative strategies that have shown partial effectiveness against nephrotoxicity (dos Santos et al., 2012). Nonetheless, no prophylactic measures have been established to counteract CIS's ototoxic or neurotoxic effects (Avan et al., 2015). The most prevalent approach to mitigate CIS-induced neurotoxicity involves either dose modification or cessation of treatment (Avan et al., 2015). Among platinum-based chemotherapeutic agents, CIS is recognized as the most neurotoxic.

The neurotoxicity caused by CIS is both cumulative and dose-dependent (Frisina et al., 2016). In our study, the available information was tested with the application of CIS 50 and CIS 100 in primary neuron culture, and a significant increase in cell death, ROS, inflammatory and apoptotic genes, and damage in the CIS 100 groups was observed in immunofluorescent staining. Therefore, neurotoxicity is a crucial dose-limiting factor for CIS chemotherapy, justifying the search for protective agents.

In our study, we first performed IC_{50} determination to examine the effect of gossypin on primary neuron culture cells, and our IC_{50} value was 60.40 µM for gossypin. Based on this dose and the data we obtained in our previous gossiping and in-vitro studies, we determined our doses (50, 75, and 100 µM). By applying CIS and available doses of gossypin to primary neuron culture cells, we examined their effects on cell viability (MTT) as time-dependent (24, 48, and 72 h). Our findings, in line with the literature, proved that CIS administration produces a time-dependent cytotoxic effect, which increases with CIS100. Gossypin, on the other hand, protected neuron cells in a dose-dependent manner. While CIS100's survival rate at 72 hours decreased to 43%, the CIS100 + GOS100 group's viability at 72 hours was 80%.

The pathophysiological processes associated with CIS-induced toxicity are not yet fully elucidated. One pivotal mechanism implicated in this toxicity is oxidative stress. Initial studies have observed a reduction

in the levels of endogenous antioxidants, such as SOD and GSH, coupled with an elevation in oxidative biomarkers like MDA in tissues treated with CIS. The oxidative damage prompted by CIS is known to cause structural alterations in neural tissue, potentially leading to demyelination and myelin degeneration. Concurrently, releasing NO and ROS during this process results in the depletion of tissue antioxidants (de Pinto et al., 2002). Several other mechanisms are believed to contribute to CIS toxicity, including hypoxia triggered by free radicals, inflammatory responses, lipid peroxidation, and apoptotic cell death. Specifically, in the spinal cord, CIS has been shown to cause a sustained increase in pro-inflammatory cytokines such as TNF-α and IL-1β within astroglial cells (Guindon et al., 2014). Under typical conditions, cellular homeostasis maintains ROS levels in check by a balance between ROS production and their neutralization by antioxidant defenses, including GSH, SOD, and CAT. However, when oxidative stress prevails, ROS overproduction can damage proteins, lipids, and DNA within cells, leading to lesions that may precipitate cellular transformation and carcinogenesis.

Furthermore, CIS exhibits a particular affinity for neuronal structures within the peripheral and central nervous systems. Neuronal cells are especially vulnerable to oxidative stress due to their limited antioxidant capabilities and high lipid concentration, predisposing them to oxidative damage (Krarup-Hansen et al., 1999).

Cancer cells experience heightened oxidative stress from ROS compared to normal cells. This increase is attributed to oncogene activation, which drives uncontrolled cell proliferation, enhanced metabolic demands associated with rapid growth, and dysfunctions within the mitochondria, the cell's energy-producing organelles. Chandrashekhar et al. It has been shown that gossypin has anti-inflammatory activity, which may also be related to I/R neuroprotection in the brain by inhibiting inflammation mediators, and it was stated that free radical scavenging activity is directly responsible for the antioxidant and neuroprotective effect of gossypin (Chandrashekhar et al., 2013a).

In our study, SOD activity, GSH, and MDA levels were measured from each supernatant with ELISA kit measurement methods for in-vitro biochemical studies at 6 and 12 hours and in-vitro on homogenates obtained from primary neuron culture cells and brain tissue. In our results, while SOD activity and GSH levels decreased significantly in the CIS groups compared to the control group, significant increases were observed in MDA levels. These harmful effects were observed more in the in-vitro CIS100 group. Gossypine dose-dependently normalized CIS-induced damage in in-vitro and iv-vivo groups. Our in vitro findings are consistent with the hippocampal tissue concentrations observed in in-vivo studies. It has been established that CIS treatment leads to increased lipid peroxidation and reduced glutathione levels (Ognjanovic et al., 2012). Consequently, interventions that inhibit lipid peroxidation and elevate intracellular antioxidant quantities could be advantageous for maintaining neuronal integrity, which is crucial for cognitive functions and memory retention.

Furthermore, the potential impact of CIS on the balance between neurotoxic and neuroprotective mechanisms, specifically iNOS/nNOS, was examined. The CIS-treated cohort exhibited elevated levels of the neurotoxic enzyme iNOS and diminished levels of the neuroprotective enzyme nNOS. NO serves a

dual role, acting as a neurotoxin and a neuroprotector (Boje, 2004). CIS-induced ROS have been implicated in the upregulation of neurotoxic iNOS and the downregulation of neuroprotective nNOS (Dawson and Dawson, 2004). The deleterious effects of iNOS may operate through pathways that are both dependent and independent of caspases (Dawson and Dawson, 2004), whereas the beneficial effects of nNOS are mediated by the activation of hypoxia-inducible factor-1, a transcriptional regulator of several genes associated with neuroprotection (Keswani et al., 2011).

TNF-α is a pivotal cytokine in the pathophysiology of brain injury. It is synthesized by various cells within the central nervous system, including astrocytes, neurons, and microglia. These cells are recognized as the predominant producers of TNF-α during neuroinflammatory processes (Gahring et al., 1996). Inflammation has been identified as a critical mechanism in cisplatin-induced systemic toxicity. Our research indicates that levels of NF-κB and TNF-α are markedly elevated in subjects administered with CIS. Notably, TNF-α is upregulated in the early stages following nerve injury, triggering the local inflammatory response (George et al., 2004). TNF-a, iNOS, and nNOS mRNA levels increased iNOS (NOS2) and decreased nNOS(NOS1) in primary neuron culture cells treated with CIS compared to control cells. However, changes in TNF-a, iNOS, and nNOS induced by gossypin and CIS were again normalized in a dose-dependent manner with gossypin administration. Furthermore, in our in-vivo study, the supernatant obtained from brain tissue and TNF-α levels were measured by the ELISA method, and the relevant parameter also supports our current findings. NF-κB activation induces proinflammatory mediators, including TNF-α and iNOS (Beinke and Ley, 2004).

CIS showed NF-kB phosphorylation compared to control; CIS significantly increased TNF-α, iNOS, and nNOSmRNA expression levels. These data indicated that gossypin inhibited NF-κB activation to suppress CIS-induced inflammation in primary neuron culture cells. Our in-vivo study supports the current situation in Fig. 4. The last possible mechanism of CIS studied is mitochondrial dysfunction and apoptosis. This process is pivotal in mediating the cytotoxic effects and cellular damage attributed to CIS (Peitsch et al., 1993).

The overproduction of ROS can trigger apoptosis through both the extrinsic and intrinsic signaling pathways. Bax, a pro-apoptotic protein within the Bcl-2 family, plays a vital role in the apoptotic regulatory mechanism and is acknowledged for its significance (Campbell and Tait, 2018). The integrity of mitochondrial function is crucial, as mitochondrial impairment is a predominant cause of redox disequilibrium and apoptosis, particularly in peripheral neurons. Oxidative stress can perturb normal biological operations, with mitochondrial dysfunction significantly contributing to neurotoxicity (Melli et al., 2008). Mitochondria are notably susceptible to CIS-induced oxidative stress, with the depletion of mitochondrial protein thiol groups leading to impaired calcium sequestration and a reduction in mitochondrial membrane potential (Saad et al., 2004). The mitochondrial route of apoptosis, modulated by Bcl-2 family proteins, is initiated by an upsurge in the pro-apoptotic protein Bax and a concurrent decline in the anti-apoptotic protein Bcl-2 (Senichkin et al., 2020). The Bcl-2/Bax ratio is a critical indicator in assessing cellular apoptosis and viability.

In previous studies, it also exerts a downstream effect on several molecular factors in CIS-mediated cytotoxicity, including activation of p53 and modulation of Bcl-2 family proteins, including the proapoptotic proteins BAX. CIS releases cytochrome c from BAX-displaced mitochondria (Giordano et al., 2014). In short, the molecular mechanism of CIS-induced apoptosis has been linked to activation of the p53 gene, Bax translocation, mitochondrial cytochrome c release, caspase-3 and caspase-9 activation (Kanat et al., 2017). In our in-vitro study, the BCL2/BAX ratio in the group treated with CIS was almost 3/4 lower (0.28 ± 0.03) compared to the control group. With the application of Gosyypin, improvement was achieved, especially at the 12th hour, in a dose-dependent manner. The improvement in the CIS50 groups was more significant than the CIS100 groups, and we thought it was related to the CIS dose. In our in-vivo study, CIS administration significantly reduced the BCL-2/Bax ratio and subsequent activation of CASP-3 cleavage. Previous studies have also shown that gossypin has antiapoptotic activity (Cinar, 2021a).

Our study corroborates the anti-apoptotic effects of gossypin in the cerebral neurons of mice. The compound achieved a notable reduction in cell death by enhancing the expression of the anti-apoptotic protein Bcl-2 while simultaneously decreasing the expression of the pro-apoptotic protein Bax. Furthermore, it inhibited CASP-3 and CASP-9 mRNA levels, critical apoptosis mediators. To visualize apoptotic cells, neurons were stained with Hoechst 33342, a dye that binds to DNA. Cells undergoing apoptosis were identified by their brightly stained fragmented nuclei with condensed chromatin—a hallmark of apoptosis. Treatment with CIS at 50 and 100 μM resulted in observable apoptotic nuclei. Contrastingly, cells treated with gossypin exhibited a substantial decrease in apoptotic nuclei, typically marked by dense chromatin, nuclear shrinkage, and irregular fragmentation. This observation is depicted in Fig. 2, illustrating that gossypin exerts a dose-dependent protective effect against neuronal apoptosis in primary cultures. Control cells, however, showed neither apoptotic nuclei nor diffuse staining. Additionally, the proportion of apoptotic cells within the culture well was quantified and is displayed in Fig. 2.

The beneficial effects of Gossypin against CIS-induced apoptosis were determined in our current study NF-kB and BAX IHC staining in brain tissue supported our findings. Combining our current data with in-vitro results provides evidence supporting the neuroprotective effect of gossypin through its antiapoptotic effect in healthy cells.

5. Conclusion

Our results also support the efficacy of gossypin, a possible neuroprotective anti-apoptotic agent, during cisplatin-induced neurotoxicity. Gossypin appears to regulate the apoptotic pathway by ameliorating nitro-oxidative stress and increased inflammatory markers caused by cisplatin challenge. Our results represent a step towards understanding the mechanism underlying the anti-apoptotic and antioxidant properties of gossypin.

Declarations

Author Contributions Irfan Cinar and Muhammed Yayla Investigation, Conceptualization, Methodology, and Writing- Original draft. Irfan Cinar, Muhammed Yayla, Pınar Bayram and Damla Binnetoğlu Data curation, Software and Validation. All authors read and approved the final version of the manuscript.

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Consent for Publication

All authors agreed to the publication of this paper.

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Figures

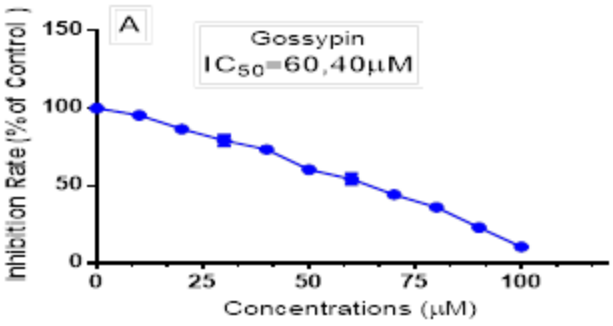

Figure 1

Fluorescent microscope findings and apoptotic cell rates after Hoechst 33342 staining in primary neuron culture cells.

a.Control, b. DMSO (%0.01), c. CIS 50: CIS 50 μM, d. CIS 100: CIS 100 μM, e. CIS 50+GOS 50: Gossypin 50 μM, f. CIS 50+GOS 75: Gossypin 75 μM, g. CIS 50+GOS 100: Gossypin 100 μM. h. CIS 100+GOS 50:

Gossypin 50 µM, ı. CIS 100+GOS 75: Gossypin 75 µM, i. CIS 100+GOS 100: Gossypin 100 µM. j. Apoptotic cell (%)

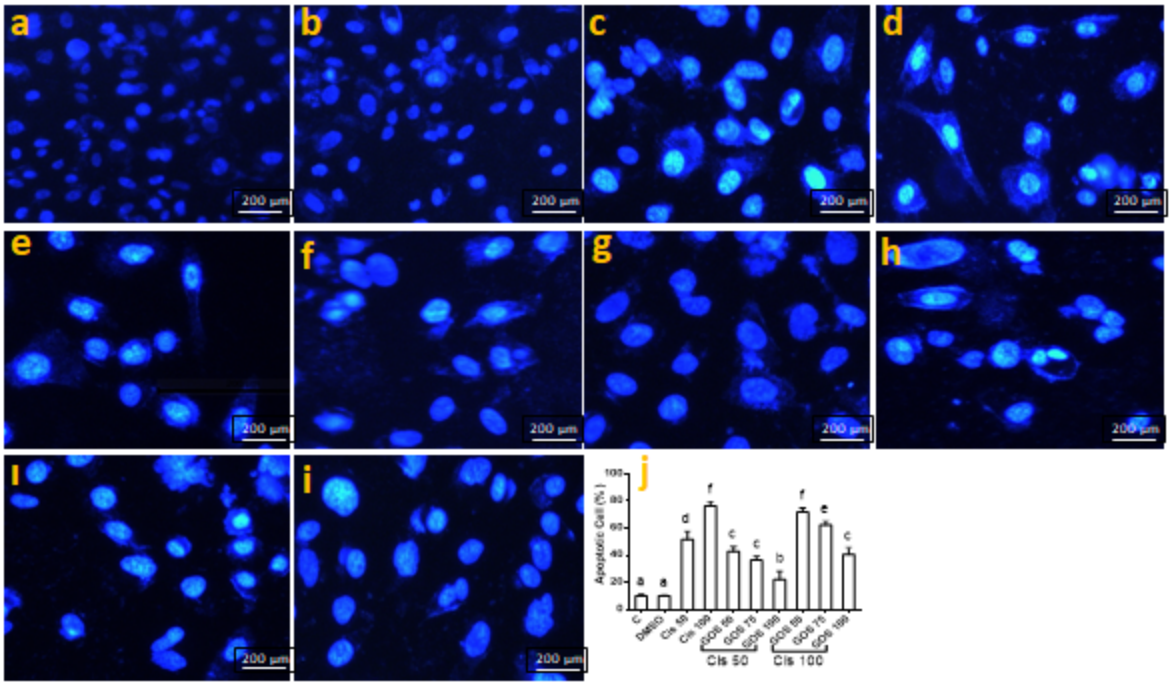

Figure 2

İn-vivo parameters. C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg

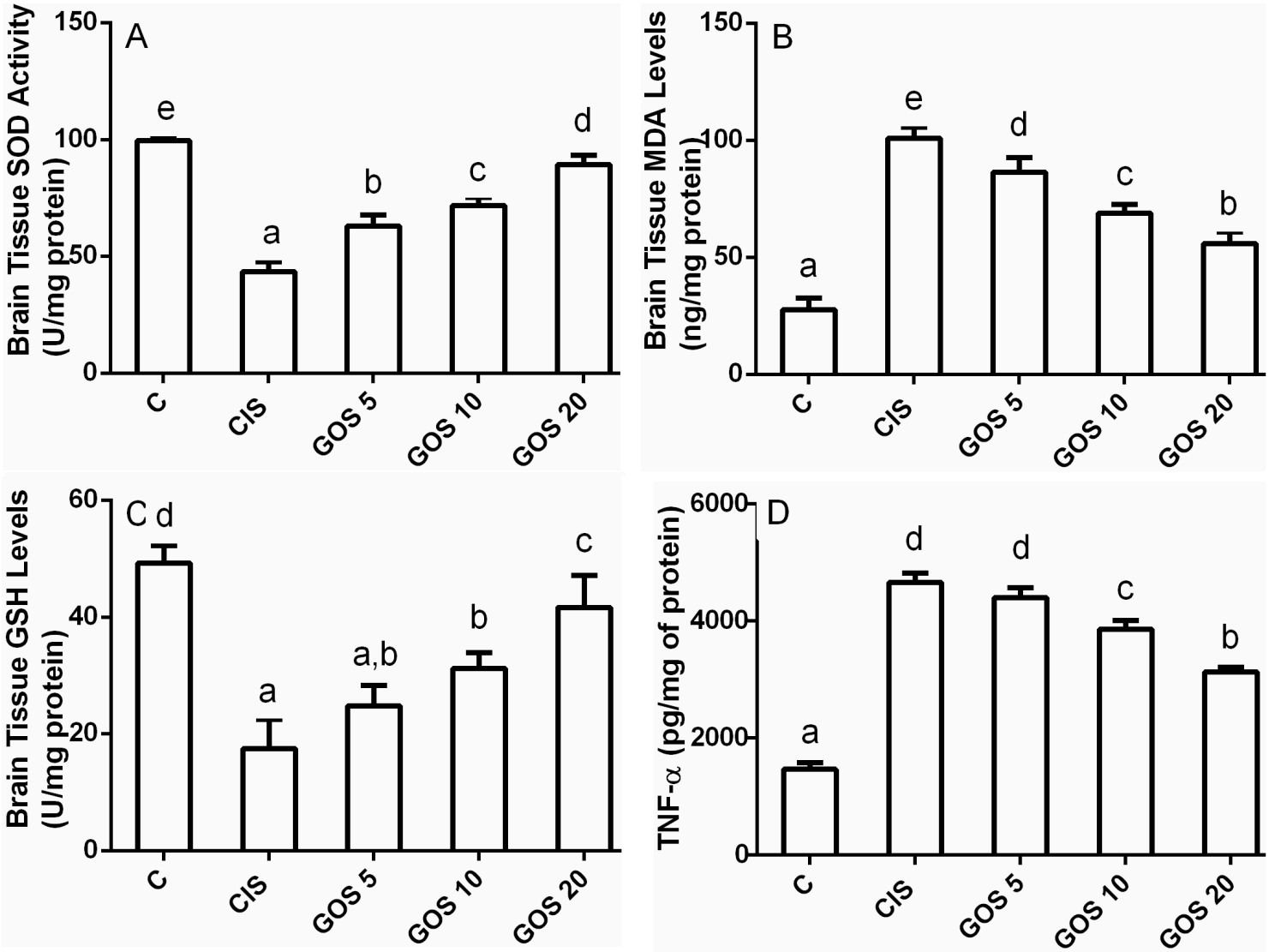

Figure 3

İn-vivo parameters. C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg

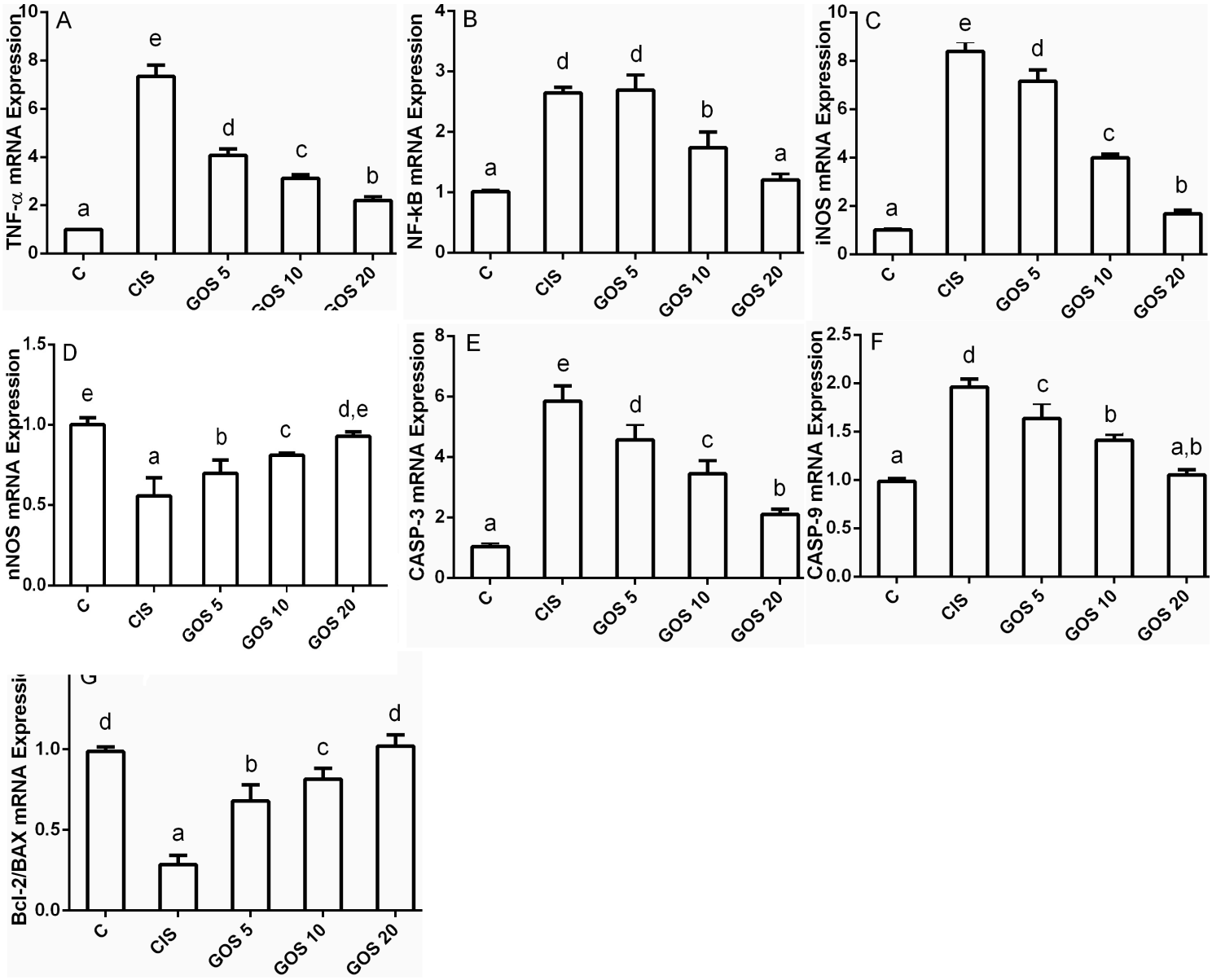

Figure 4

Brain Micrographs Stained With Hematoxylin Eosin.

C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg. OK: Picnotic cells, star; cells clustered into the lumen in capillaries. Bar: 200µm

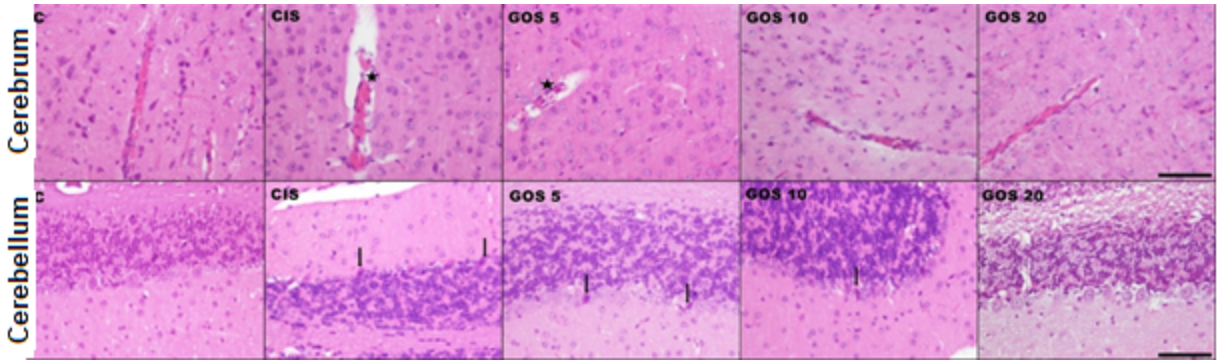

Figure 5

Labeling with BAX and NF-kB Primary Antibody.

Brown areas indicate immunopositivity. Arrow: positive cells (hippocampus and cerebellum). C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg. OK: Picnotic cells, star; cells clustered into the lumen in capillaries. Bar: 200µm.

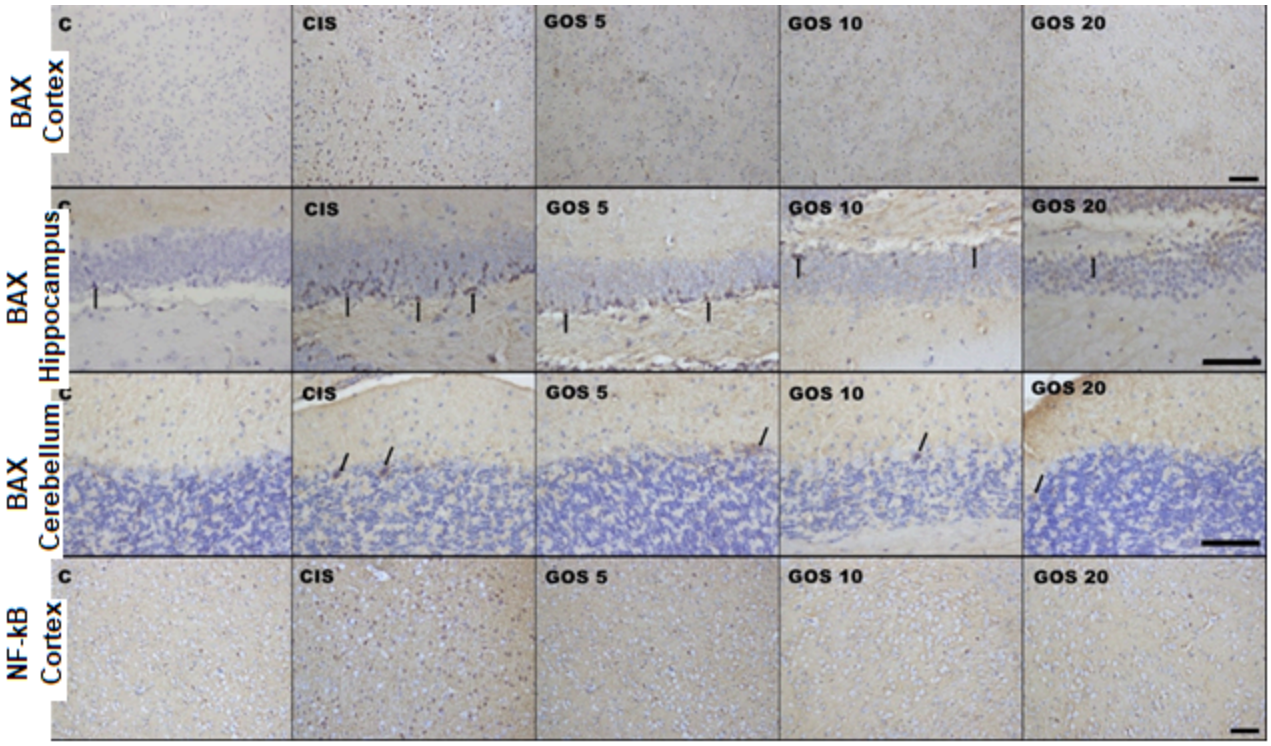

Figure 6

Graphical Expressions of Immunohistochemical Staining Results

Number of positive Purknje cells per area in labeling with BAX primary antibody. b. Number of hippocampus positive cells per area in labeling with BAX primary antibody. c. H-Score analysis results of the labeling with BAX primary antibody. D. H-Score analysis results of labeling with NFKB primary antibody. *: Statistically significant difference between control-CIS, GOS 5, GOS 10, GOS 20 groups. +: Statistical difference between CIS- GOS 5, GOS 10, GOS 20 groups.

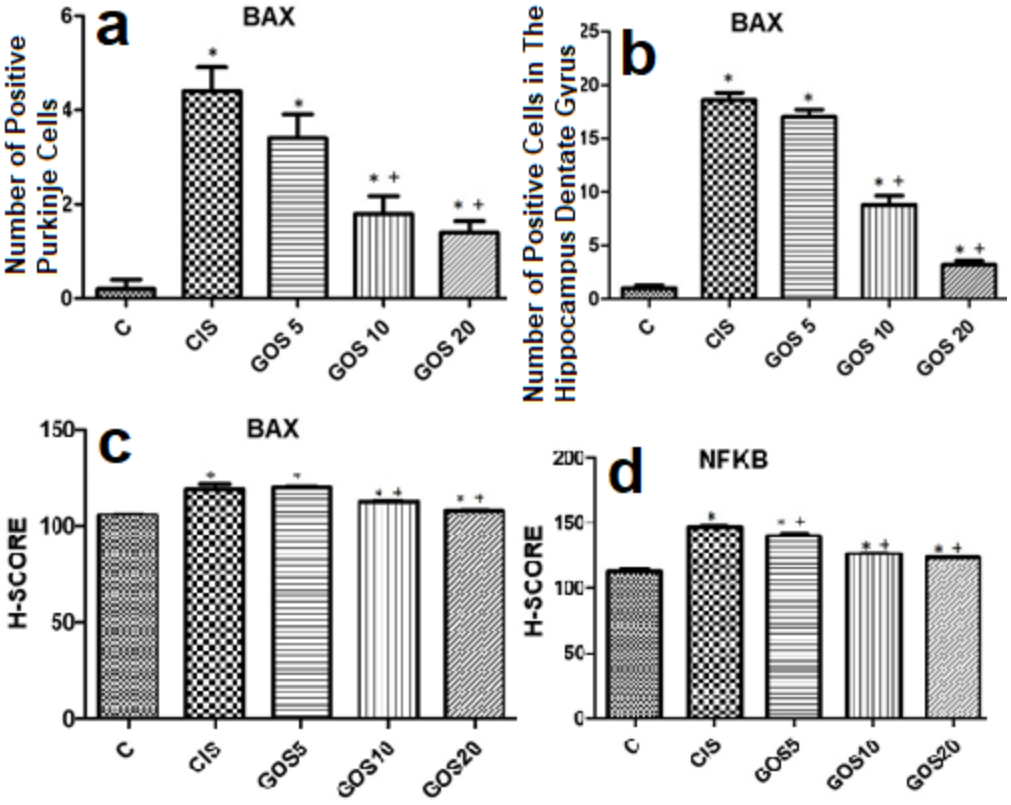

Figure 7

Graphical Expressions of Immunohistochemical Staining Results

Number of positive Purknje cells per area in labeling with BAX primary antibody. b. Number of hippocampus positive cells per area in labeling with BAX primary antibody. c. H-Score analysis results of the labeling with BAX primary antibody. D. H-Score analysis results of labeling with NFKB primary antibody. *: Statistically significant difference between control-CIS, GOS 5, GOS 10, GOS 20 groups. +: Statistical difference between CIS- GOS 5, GOS 10, GOS 20 groups.

Supplementary Files

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