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Diclofenac sodium and dexamethasone co-therapy restores brain neuron-specific enolase (NSE), S-100 Beta and glial fibrillary acid protein (GFAP) proteins in experimental rat's model: A possible inhibition of P-glycoprotein

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ABSTRACT

Non-steroidal anti-inflammatory drugs decrease pain and fever while corticosteroids regulate inflammation and immune response, both are prescribed to reduce inflammation and control pain. The present study aimed to study the effects of their monotherapy and co-administration on the brain tissue structure of experimental rats. P-glycoprotein (PGP), a transporter membrane protein, plays an important role in various physiological and physio-pathological conditions, drug-drug and drug-food interactions, and multi-drug resistance. Male rats were divided into four groups and received normal saline, dexamethasone, diclofenac sodium and their dual therapy respectively, then after one-month rats were sacrificed and brain tissues proceeded for hematoxylin and eosin staining to study their histopathology and immunohistochemically staining of NSE, S100-B and GFAP biomarkers were performed. Additionally, in silico molecular docking studies were conducted to elucidate interactions between PGP and used compounds. Resultsshowed that dexamethasone or diclofenac sodium treatments showed abnormalities like edema, neuronal vacuoles, astrocytes hyperplasia and microglial cells with positive reaction to NSE, S100 and GFAP antibodies while the dual therapy displayed less edema and other signs of damage with negative and weak positive staining of NSE, S100 and GFAP antibodies respectively. The molecular docking showed that there were different affinities toward the involved PGP active site. These interaction results were great with Dexamethasone -9.6 kcal/mol forming hydrophobic interactions with the highest affinity when compared with Diclofenac sodium which gave -8.4 kcal/mol. In conclusion, the side effects of the two types of anti-inflammatory drugs may be minimized through their interactions. However, Molecular Dynamic Simulations studies are required to explain the exact dynamic behaviors and protein-ligand stability.

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Introduction

Pain control and complication reduction are the main challenges for pharmacologic protocols with diverse procedures. For instance, combined therapy with steroidal and non-steroidal types of anti-inflammatory drugs inhibit the same chain reactions that degrade released phospholipids by injured cell membranes, reduce important pro-inflammatory mediators and are very effective in controlling pain and inflammation. Both topical NSAIDs and steroids have been effectively used to control postoperative inflammation after phacoemulsification (1). Diclofenac sodium is a nonsteroidal anti-inflammatory drug that has a partition coefficient of 13.4 and an acidity of 4.0. The structural elements include a secondary amino group, a phenylacetic acid group and a phenyl ring containing chlorine atoms (2). Diclofenac and the combination of dexamethasone and diclofenac sodium showed superior results when compared to their monotherapy in minimizing central retinal thickness change and the incidence of pseudophakia cystoid macular oedema (3). Also, effective results were reported to reduce knee joint inflammation in osteoarthritis rats over 17 days by the co-administration of diclofenac sodium and dexamethasone (4). Both drugs showed effective results in reducing intraocular pressure eyes and in improving visual acuity (5). Moreover, in our previous research, we proved that the co-administration of diclofenac sodium and dexamethasone gave better results when compared to their usage alone on the histological structure of livers and kidneys of the experimental animals (6), also they inhibit the secretion of tumor necrosis factor (TNF-α) and interlukin-1 beta (IL-β) cytokines (7). The present study aimed to test the effect of diclofenac sodium, dexamethasone, and their combination on the brain tissues of rats by monitoring specific brain proteins. P-glycoprotein (PGP) is an efflux pump synthesized in blood-brain, blood-testicle barriers, as well as in most organs and tissues. PGP plays an important role in drug behavior in the body, primarily in the absorption, distribution, and elimination of drugs (8). Several studies have demonstrated that this protein is a factor in drug-drug and drug-food interactions, drug resistance, etiology of some diseases, drug sensitivity, and treatment failures (9). It has been reported that dexamethasone, a strong corticosteroid,

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induces PGP synthesis in the liver, brain, intestinal, and lung tissue, and this effect is dependent on glucocorticoid concentration. The modulation that dexamethasone causes in PGP synthesis in normal tissues, due to its wide use in chemotherapy and multi-drug treatment, may affect the effectiveness of treatment and drug side effects (10). PGP is one of the transmembrane proteins that restrict the passage of substances to tissues and organs, and it is the most abundant and researched in organs and tissues. This protein mediates drug-drug and drug-food interactions. The modification of the PGP function by various substances has led to PGP based studies to reduce unwanted drug effects and increase drug efficacy (8).

Materials and Methods

Experimental animals

Forty laboratory male rats (180-200 grams) were divided into four separated groups injected intramuscularly with the following every day: Group one with (4ml/kg) normal saline, Group two with dexamethasone (1.6mg/kg), Group three rats with Diclofenac Sodium (30mg/kg) and Group four with both dexamethasone (1.6mg/kg) and Diclofenac Sodium (30mg/kg). The experiment lasted for one month then the rats were weighed & sacrificed then the brains were extracted, weighed, and fixed in (10% formalin) for 48 hours then dehydrated with increasing alcohol concentrations in an automated tissue processing machine. The processed tissue was embedded in paraffin for tissue sectioning. And a microtome was used to cut the tissue into 5 µm sections for staining with haematoxylin–eosin and stained with specific immunohistochemical antibody stains for Brain Neuron-specific enolase (NSE), S-100 Beta and Glial fibrillin acid protein (GFAP) proteins. The process of tissue section preparation was investigated following the manufacturer's manual and performed in PAR private hospital/ Erbil city. Semi-quantitative immunohistochemical analysis of brain samples with protein expression and localization within tissues was done using Fiji (Image J) software.

Preparation of P-glycoprotein protein and compounds

P-Glycoprotein (PDB ID: 4XWK) was chosen for docking studies. The protein was prepared for docking after being downloaded from PDB (https://www.rcsb.org/)(8). After being converted to (PDBQT) file format which is required to supply AutoDock Vina (11) using Molecular Graphics Laboratory (MGL) Tools 1.5.6 (http://mgltools.scripps.edu) (11), the studied compounds Diclofenac sodium and Dexamethasone downloaded from (https://pubchem.ncbi.nlm.nih.gov/) (12) and converted to PDBQT file format.

The lowest energy (higher affinity) conformations for molecular docking were determined via default parameters. The protein-ligand docking analysis was conducted using AutoDock Vina (version 1.5.6), which can provide studied compound binding flexibility with the binding site residues. The images were finally generated using Discovery Studio Visualizer (13).

AutoDock Vina

AutoDock Vina was run on Windows 8.1 operating system with 12 GB of RAM. The configuration file coordinate required to supply AutoDock Vina involving; PGP PDB ID: 4XWK as (receptor), used compounds as (ligand) and the dimensions of the active site for PGP which was defined by establishing a line cube with 20 Å with a grid point spacing of 1.0 Å, and centre grid boxes (X, Y, Z) with (78.924, -4.383, -5.917) respectively.

Results

In the current study, the body weights of rats were chosen in the range of 180-200 grams as mentioned earlier however, on the day of surgery the body weights and brain weights were measured and decreased ranges were observed especially at the dexamethasone group followed by diclofenac treated rats though no significant differences were found between the control group and Dexamethasone/Diclofenac treated group as shown in Figure 1.

The normal brain delineation in tissues stained with haematoxylin & eosin is shown in Figure 2. With negative staining and weak positive reaction of NSE and S100 antibodies respectively in contrast to the positive staining of GFAP protein that indicates the normal brain tissue architecture however, many differences can be observed when compared to brain tissues treated separately with dexamethasone (Figure 3) or diclofenac sodium (Figure 5)

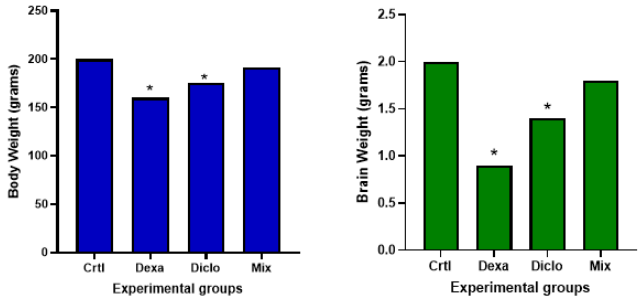

Figure 1. Body weight and brain weights of experimental rats. Values are expressed as mean±SD (*) indicating significance when $P<0.05$ when compared to the control group (Ctrl).

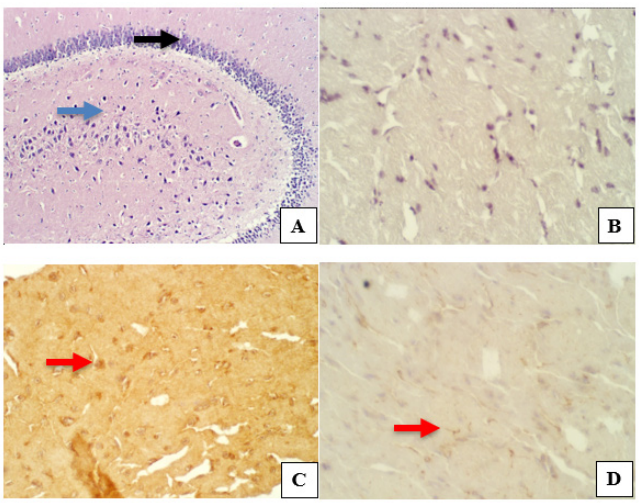

Figure 2. The brain of rats treated with Normal saline (Control group). A: (H&E stain 400x) Showed normal layers of the brain such as the granular layer (arrow), and pyramidal cells (blue arrow). B: (IHC-*NSE*-DAB stain 400x) Showed negative reaction with *NSE* antibody. C: (IHC-*S100*-DAB stain 400x) Showed weak positive reaction with *S100* antibody in few neurons (red arrow), note nonspecific staining with Dab stain at the background. D: (IHC-*GFAP*-DAB stain. 400x) Showed positive reaction with *GFAP* antibody which appears as golden-brown fine bristles in the cytoplasm of neurons (red arrow).

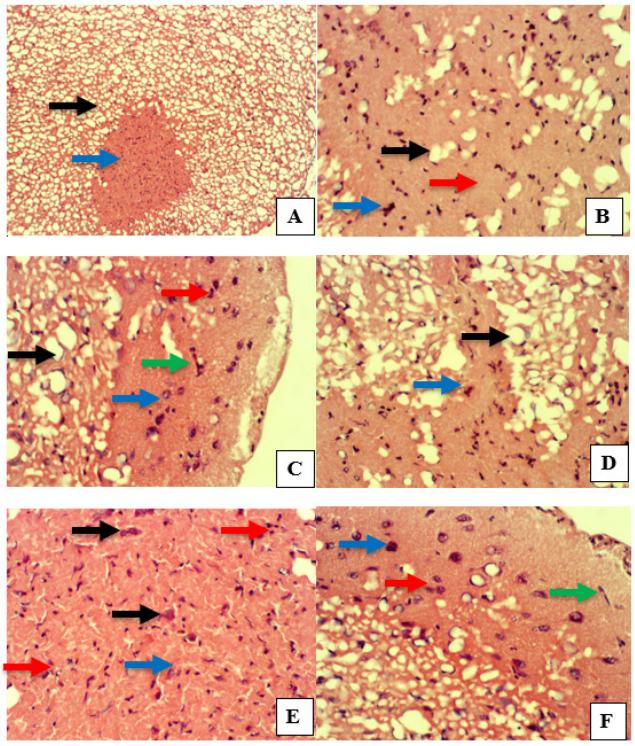

Figure 3. The brain of the rats treated with Dexamethasone. A: Showed large clear vacuoles in neuronal tissue (black arrow), surrounding normal tissue (blue arrow). B: Showed large clear vacuoles in neuronal tissue (black arrow), hyperplasia of astrocytes (blue arrow), with vasogenic edema (red arrow). C: Showed different size clear vacuoles in the granular layer (black arrow), neuronal necrosis (blue arrow), with an increase in Gitter cells (red arrow), and vasogenic edema (green arrow). D: Showed different size clear vacuoles in neuronal tissue (black arrow), and hyperplasia of microglial cells (blue arrow). E: Showed neuronal necrosis (black arrow), and vasogenic edema (blue arrow), with hyperplasia of astrocytes (red arrow) and microglia cells (green arrow). F: Showed different size clear vacuoles in the granular layer (black arrow), neuronal necrosis (blue arrow), with an increase in astrocytes (red arrow), vasogenic edema (green arrow) (H&E. 400x).

in which signs of edema, the appearance of neuronal vacuoles, hyperplasia of astrocytes and microglial cells with neuronal necrosis are well observed in variation. Also, positive reactions to NSE, S100 and GFAP antibodies were noted (Figures 4 and 6 respectively). On the other hand, as can be observed from Figure 7 the co-administration of dexamethasone and diclofenac sodium restored the brain cell and tissue structure better than the other treatments with less signs of vasogenic edema. These results are confirmed by the negative and weak positive staining of NSE and S100 antibodies and the positive staining of GFAP antibodies respectively (Figure 8).

A successful docking protocol was detected, Dexamethasone and Diclofenac sodium was successfully docked into the active site of PGP, ten runs were performed for each compound, and the average of docking scores (kcal/mol) were calculated (Table 1, Figure 9). The suitable and correct orientations were the most important requirements of these interactions that fitted to the binding site of the protein-forming protein-ligand complexes.

Discussion

The brain is an organ that is exposed to oxidative stress

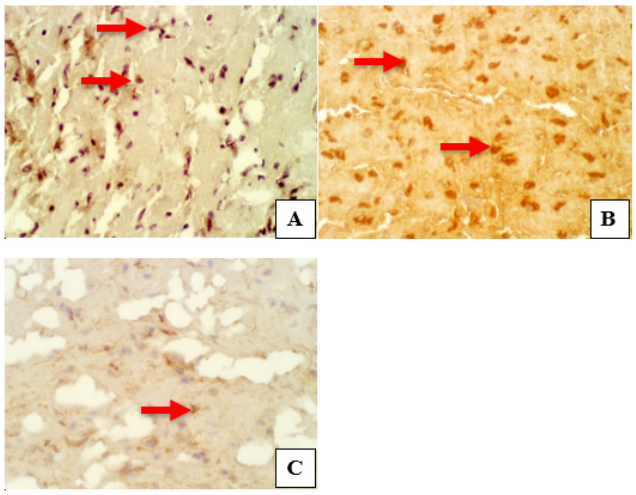

Figure 4. The brain of the rats treated with Dexamethasone. A: Showed positive reaction with *NSE* antibody which appears as a dark brown stain in the cytoplasm and nucleus of astrocytes and necrotic neurons (red arrow). B: Showed positive reaction with *S100* antibody which appears as golden-brown batches in the cytoplasm of neurons (red arrow), note nonspecific staining with Dab stain at the background. C: Showed positive reaction with *GFAP* antibody which appears as golden-brown fine bristles in the cytoplasm of neurons (red arrow). IHC-*GFAP*-DAB stain. 400x.

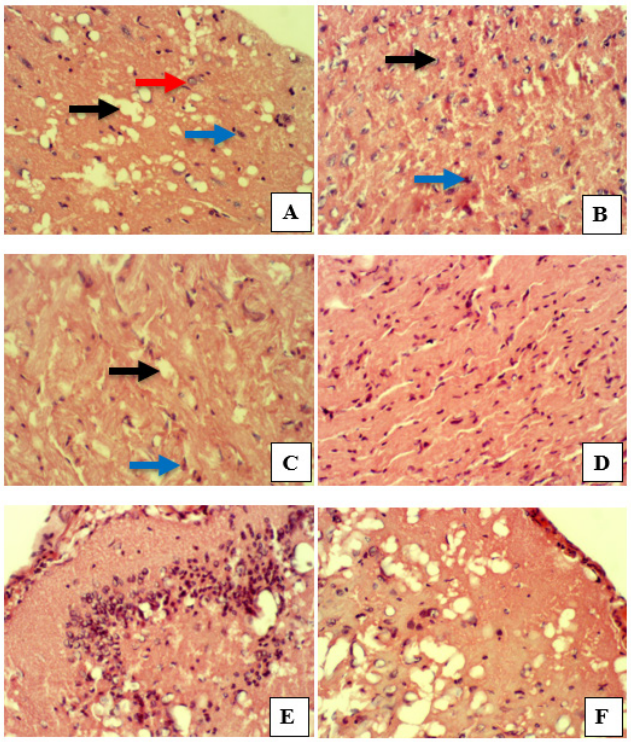

Figure 5. The brain of rats treated with Diclofenac sodium. A: Showed clear vacuoles in the neuropil (black arrow), increase in astrocytes number (blue arrow), with few necrotic neurons (red arrow). B: Showed necrotic and degenerative changes in neurons (black arrow), and hyperplasia of astrocytes (blue arrow). C: Showed different size vacuoles (black arrow), with vasogenic edema (blue arrow). D: Showed astrocytosis phenomena (black arrow), and hyperplasia of microglial cells (blue arrow). E: Showed clear vacuoles in the neuropil (black arrow), neuronal necrosis in the granular layer (blue arrow) with an increase in thickness of meninges (red arrow). F: Showed clear vacuoles in neuropil (black arrow), and neuronal necrosis (blue arrow) with an increase in thickness of meninges (red arrow). H&E. 400x.

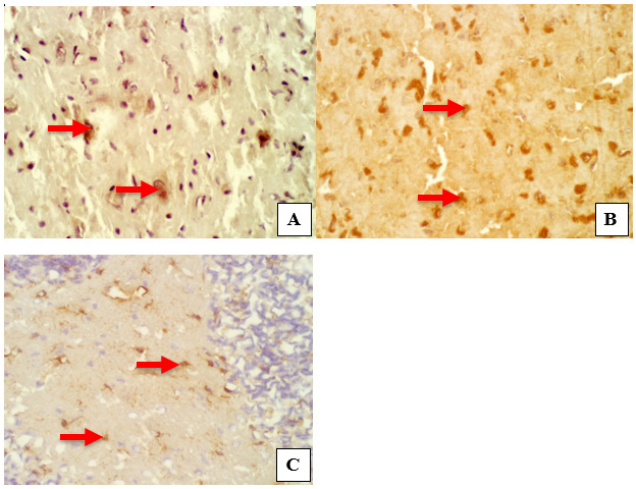

Figure 6. Brain of rats treated with Diclofenac sodium. A: Showed positive reaction with *NSE* antibody which appears as a dark brown stain in the cytoplasm and nucleus of astrocytes and necrotic neurons (red arrow). IHC-*NSE*-DAB stain. 400x. B: Showed positive reaction with *S100* antibody which appears as golden-brown batches in the cytoplasm of neurons (red arrow), note nonspecific staining with DAB stain at the background. IHC-*S100*-DAB stain. 400x. C: Showed positive reaction with *GFAP* antibody which appears as golden-brown fine bristles in the cytoplasm of neurons (red arrow). IHC-*GFAP*-DAB stain. 400x.

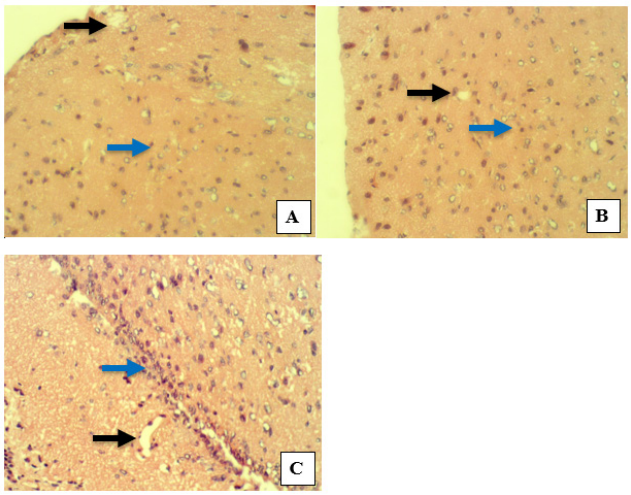

Figure 7. Brain of rats treated with both dexamethasone and Diclofenac sodium. A: Showed vasogenic edema (black arrow), few astrocytes (blue arrow). B: Showed vasogenic edema (black arrow), few microglial cells (blue arrow). C: Showed vasogenic edema (black arrow), with normal granular cells (blue arrow). H&E. 400x.

more than the other organs because of its active biochemical and physiological functions. It consumes around twenty percent of total oxygen intake due to its moderately small weight and lack of antioxidant defenses in its tissues(14). Brain injuries affect different growth patterns, response to therapy, invasion and prognosis. Around one hundred and eighty diverse brain proteins were investigated, (located either in the mitochondria or the cytoplasm of the cells) including the structural proteins and enzymes with various catalytic activities (15). One of the well-identified brain biomarkers is the immune-reactive neurons specific enolase (NSE) which is a biomarker for the determination of the number of metabolically active neurons in different brain regions (16). NSE is a glycolytic enzyme found in the synaptic plasma membrane and the cytosol of

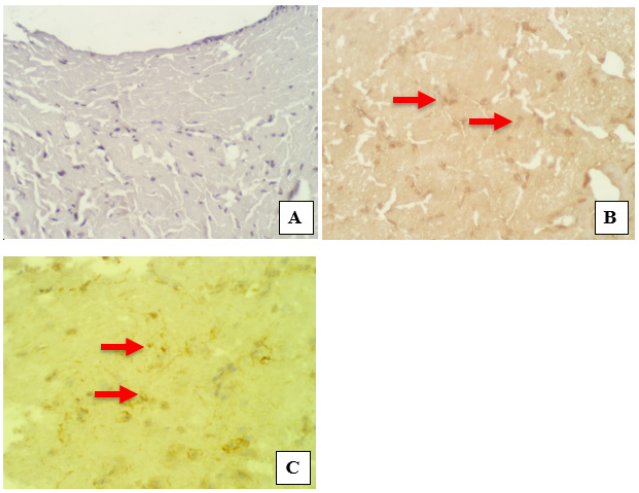

Figure 8. Brain of rats treated with both dexamethasone and Diclofenac sodium. A: Showed negative reaction with *NSE* antibody. IHC-*NSE*-DAB stain. 400x. B: Showed weak positive reaction with *S100* antibody in few neurons (red arrow), note nonspecific staining with Dab stain at the background. IHC-*S100*-DAB stain. 400x. C: Showed positive reaction with *GFAP* antibody which appears as golden-brown fine bristles in the cytoplasm of neurons (red arrow). IHC-*GFAP*-DAB stain. 400x.

Table 1. Average docking scores (kcal/mol) for dexamethasone and diclofenac sodium docked to PGP.

#	Compound	Docking Score* (kcal/mol)
1	Diclofenac sodium	-8.4
2	Dexamethasone	-9.6

* Average of ten runs performed for each compound.

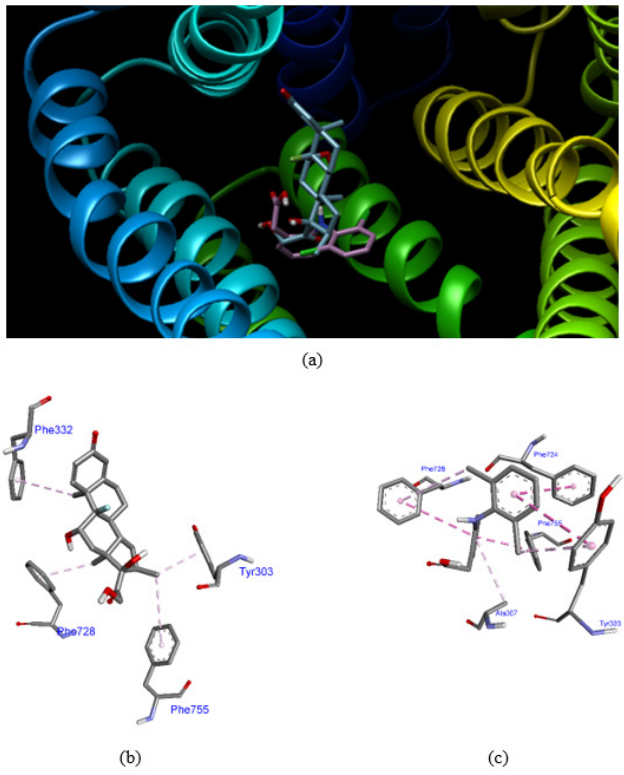

Figure 3. Dexamethasone and Diclofenac sodium docked to P-Glycoprotein; (a) Showing compounds in the active site of PGP (b) Showing interacted residues of protein with Dexamethasone (c) Showing interacted residues of protein with Diclofenac sodium.

the neurons (17). Studies reported that NSE levels were decreased in the serum of male rats during spinal cord injury, The reason was postulated to be due to inflammation status and activation of glial cells which are regulated by active NSE expression. While the levels of NSE were elevated because it can produce inflammatory chemokines, promote extracellular degradation and yield metabolic factors that stimulate glia and damage neurons (18). Our results proved that treatment with diclofenac sodium and dexamethasone restores the NSE levels in brain tissues near to normal control group after it has been destroyed to abnormal levels by using diclofenac or dexamethasone monotherapy. This indicates that the NSE marker reflects the situation of the neurons due to different treatments that cells are exposed to(19). On the other hand, S-100 protein occurs in some types of neurons and glial cells. It is found in two forms (soluble and membrane-bound) and has the capability to cooperate with natural and artificial membranes. S-100 is documented to be involved in numerous actions including diffusion of cations through membranes, memory processes, membranes physical state modulation, assembly-disassembly of microtubules and regulation of the phosphorylation of several proteins, phosphorylation of some proteins(20). In research, it has been postulated that for choosing any medication as neuroprotective medication the S-100 protein dynamics activity should be tested (21). Also, the increased S-100 levels is associated with traumatic brain injuries(22). Very similar to our results in which the staining with S-100 protein varied between the rat groups depending on the type of treatment used. Moreover, Glial fibrillary acidic protein (GFAP) a cytoplasmic filament protein originates in astrocytes (nerve cells that respond to all types of neuronal injury) and preserves these cells structural integrity when undergoing hyperplasia and hypertrophy during cell injuries. GFAP protein is a biomarker of neurotoxicity, when up-regulated it indicates anatomical damage in the brain tissue **(23)**. In the present study, the positive staining of GFAP was observed in different treated groups which designate more numbers of astrocytic processes in the brain tissues of treatment groups as compared with control, which express less levels of GFAP staining. These results are similar to Liu *et al.* (24) who suggests that after intoxication with diisopropylfluorophosphate glial cells activates which inturn lead to neuronal repair and neuropathological changes and when traumatic brain insults occurs GFAP and further brain-derived proteins with their collapse yields are released into body fluids such as blood and CSF (25). Also, another study showed an increase in GFAP levels in the male rats brain areas after exposure to 900 MHz electromagnetic field signal (26). In conclusion, the dual therapy with dexamethasone and diclofenac reduced the degenerative processes in the nervous tissue, which was displayed by the repair of normal morphological structures, opposite to rats that receive the drugs alone and separately. Immunohistochemically, S-100 and GFAP glial markers were reduced which indicates nerve tissue degenerative changes. However, the increased expression of the NSE marker indicates elevated metabolic action of the neurons. The higher affinity was observed when Dexamethasone docked into PGP binding site (Table 1, Figure 9), it formed hydrophobic interactions with TYR303, PHE332, PHE728, and PHE755 when compared with Diclofenac sodium forming hydrophobic interactions with TYR303, ALA307, PHE724, PHE728, and PHE755, revealed that Dexamethasone interacts more effectively than Diclofenac sodium with PGP.

The docking study provides a computational energetic measure that approves that both studied compounds can inhibit the PGP protein. Dexamethasone showed the lowest docking scores (-9.6) kcal/mol interacting with the PGP more tightly than Diclofenac sodium.

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Conflict of interest
The authors declare no conflict of interest.

Authors contribution
BH and AS designed the experiment, AS and ZA performed the work, HB performed the docking method, BH and ZA interpreted the data, and all authors revised the manuscript.

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