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DAPAGLIFLOZIN/ CARVEOL COMBINATION AMELIORATES PENTYLENETETRAZOLE INDUCED EPILEPSY IN RATS: ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTI-APOPTOTIC EFFECTS: TARGETING TLR4/MIR-181B/NFKB

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Background: *Epilepsy is a significant public health problem, which encompasses socio-cultural, psychological, and economic dimensions. Dapagliflozin is an SGLT2 inhibitor that has antiseizure activity. Carveol is a naturally occurring monocyclic monoterpenoid compound with antioxidant properties. This study examines the impacts of Dapagliflozin and Carveol, either monotherapy or in combination, on PTZ-induced epilepsy in rats.* ***Methods:*** *45 Male Wistar rats were randomly categorized in 5 equal groups: Group I: Control Group, Group II: PTZ Group, Group III: Dapagliflozin-Treated Group, Group IV: Carveol-Treated Group and Group V: Dapagliflozin + Carveol Treated Group. Hippocampal tissues were obtained for assay of tissue biomarkers including, SOD, MDA, NFκB, IL-6, TNF-alpha, Beclin-1, GABA, glutamate, gene expression of TLR4, miR-181b, NKCC1 and KCC2, immunohistochemical analysis of caspase 3 and histopathological analysis.* ***Results:*** *Dapagliflozin and Carveol treated group (Group V) ameliorated PTZ induced epilepsy evidenced by increased GABA, decreased glutamate, improvement of histopathology and oxidative stress via upregulation of SOD and downregulation of MDA, improved inflammation via significant reduction of NFκB, IL-6, TNF-α. Moreover, modulation of apoptosis and autophagy process via significant reduction of caspase3, TLR4 gene expression and up regulation of miR-181b gene expression and beclin-1, a significant increase in GABA and gene expression of KCC2 accompanied with a significant decrease in glutamate and gene expression of NKCC1, compared to other groups.* ***Conclusion:*** *The combination of Dapagliflozin and Carveol exhibited significantly better antioxidant, anti-inflammatory and anti-apoptotic effects than monotherapy by either of each of both drugs against PTZ induced epilepsy*

Keywords: *Dapagliflozin, Carveol, Pentylenetetrazol, Epilepsy, Experimental Model*

INTRODUCTION

Epilepsy stands as the predominant chronic neurological ailment, exerting its impact on 70 million individuals across diverse age groups on a global scale [1]. Epilepsy and convulsive seizures persist as significant public health challenges, which encompass socio-cultural, psychological, and economic dimensions [2].

While transient abnormal cortical nerve stimulation constitutes a prominent pathway to seizures, many additional mechanisms contribute to their genesis, such as excitatory

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neurotransmitter induced neuronal injury, mitochondrial dysfunction, inflammation and oxidative damage [3]. Side effects of existing antiepileptic drugs (AEDs) make their efficient use difficult, coupled with chronic toxicities targeting vital organs [4].

Elevated levels of oxidative stress and inflammation may contribute to the exacerbation of epileptic manifestations[5, 6.] The pathophysiology of seizures has shown higher levels of inflammatory cytokines[7], lipid peroxidation (LPO), and impairment of the blood-brain barrier (BBB)[8, 9]. Furthermore, increased cytokine levels correlate directly with impairment of BBB[10]. These inflammatory cytokines include TNF-α and IL-6[11.]

Pentylenetetrazol (PTZ) is commonly used for causing epilepsy. It acts by inhibition of the GABA receptor, which is a crucial neurotransmitter responsible for inhibitory functions in the brain [12.] PTZ demonstrates convulsions similar to that occurring in humans, rendering it suitable for generating rodent models of epilepsy [13.] Numerous studies have indicated diminished antioxidant activity following administrating PTZ [14]. Consequently, concentration of reactive oxygen species (ROS) increases in brain [15].

Autophagy activity is an intracellular degradation process allowing cells to recycle damaged intracellular components such as bulk proteins and aging organelles. This process has increased in many diseases such as Alzheimer's disease, models of traumatic brain damage and excitotoxicity, demonstrated by disruption of Beclin1, which is a critical regulator of the autophagic process [16].

Nuclear factor kappa B (NF-κB) is a transcription factor that has an important role in the innate defense mechanisms of cells by regulating the expression of numerous genes associated with inflammation and it is highly activated at site of inflammation[17.]

KCC2 and NKCC1 are neuron-specific cotransporters, which play an important role in Cl^- homeostasis and electrical signaling transduction in neurons. Down-regulation of KCC2 and up-regulation of NKCC1 expression are implicated in the pathogenesis of many neurological disorders [18.]

TLR4 is a key activator of innate immunity, which is a transmembrane protein that is encoded by TLR4 gene regulating apoptosis and inflammation process[19], while microRNAs (miRNAs) are types of tiny RNA molecules that do not code for proteins and have a significant role in controlling gene expression regulating apoptosis process. Also, caspase 3 is an important apoptotic marker modulating brain apoptosis[20, 21.]

Carveol is a naturally occurring monocyclic monoterpenoid compound with antioxidant properties that is abundant in caraway seeds, mandarin, black tea, dill and essential oils of orange peel [22]. It has the ability to protect the brain from damage caused by lack of blood flow, as seen in ischemic brain injury models[23)] Moreover, Carveol has exhibited hepatoprotective effects through its antioxidant and anti-inflammatory effects [24]. It stimulates antioxidant pathways and ameliorates inflammation through various mechanisms.

Dapagliflozin is a pharmaceutical agent, selectively and competitively inhibits SGLT2 in a reversible mechanism. SGLT2 is a cotransporter that facilitates the coupled reabsorption of sodium and glucose from the proximal convoluted tubules of kidney. Consequently, Dapagliflozin increases the elimination of glucose in the urine by blocking its reabsorption in the proximal convoluted tubules. Improved glycemic control reduces seizure activity making it a good choice for patients suffering from both diabetes mellitus and seizures. Dapagliflozin was found to decrease the frequency and severity of seizures represented as myoclonic jerks induced by PTZ in rats[25]. Furthermore, recent findings suggest that Dapagliflozin may reduce the risk of readmission arises from mortality causes like heart failure and renal disease in diabetic patients [26]. Therefore, this study aims to examine the role of Dapagliflozin and Carveol, each alone and in combination, on PTZ induced epilepsy, with determination of the levels of tissue biomarkers of oxidative stress as SOD, MDA and inflammatory parameters as NFκB, IL-6 and TNF-alpha. Apoptosis and autophagy markers such as caspase 3, TLR4, miR-181b and Beclin-1 were assessed, in addition to GABA, glutamate, NKCC1 and KCC2 as indicators of tissue excitability.

MATERIALS AND METHODS

Drugs and Chemicals

PTZ, Dapagliflozin, Carveol (PubChem ID: 24851543), mixture of isomers at a purity of 97%, and 3,3-diaminobenzidine tetrahydrochloride hydrate (PubChem ID: 57654109) were purchased from Sigma-Aldrich (USA). Normal saline was obtained from national pharmaceutical solution laboratories, while other chemicals employed in this experiment met analytical quality standards.

Induction of seizure using PTZ:

A dosage of 60 mg/kg of PTZ was administered intraperitoneally (IP)[27] after it had been dissolved in normal saline to cause the seizure model in rats. Within 30 minutes after administering PTZ, the seizure activity was immediately assessed.

Study Design, Treatment Protocol and Samples Collection

The study included 45 Male Wistar Rats, ranging in weight from 150 to 200 grams, and with an age range from 6 to 8 weeks. The rats were accommodated in wire mesh cages, provided with standard rat feed ad libitum and had unrestricted access to water. A period of two weeks was allotted for acclimatization. Subsequently, each group of the five groups of rats was made up of nine chosen at random. **Group I (Control Group):** A 5% DMSO saline injection was given for a duration of fifteen days. **Group II (PTZ Group):** A solution of 0.9% saline containing 60 mg/kg of PTZ was administered to cause the seizure model in rats until stage 5 convulsions [28]. The evaluation of seizure episodes was conducted promptly, within half an hour after PTZ administration. Seizure manifestations were categorized according to Modified Racine scale into five distinct stages: stage 0, characterized by a lack of activity; stage 1, marked by twitching of the ears and facial muscles; stage 2, involving convulsive waves propagating throughout the body; stage 3, characterized by myoclonic jerks and rearing behavior; stage 4, signified by the animal turning over onto its side; and stage 5, typified by the animal turning onto its back, accompanied by generalized clonic-tonic seizures. Seizure latency is the measurement of how long it takes an animal to experience their first convulsive wave after receiving a PTZ injection. Each animal's total behavioral seizure activity duration was measured[29]. **Group III (Dapagliflozin-Treated Group):** Dapagliflozin was delivered IP at a dose of 75 mg/kg, dissolved in a solution of 0.9% saline and injected 30 minutes before PTZ administration[25]**. Group IV (Carveol-Treated Group):** The rats were given Carveol (20 mg/kg) 30 minutes before PTZ injection, in an IP injectable solution of 0.9% saline with 5% DMSO [23]**. Group V (Dapagliflozin + Carveol):** The rats received both Dapagliflozin and Carveol in the same dosing regimen 30 minutes prior to PTZ administration.

The treatment with Dapagliflozin and Carveol started concomitantly with administration of PTZ and continued daily for 15 days. Each day's infusions began with freshly prepared drug solutions.

At the end of the experiment (after 15 days), rats were anaesthetized by thiopental sodium at a dose of 20 mg/kg administered intraperitoneally and sacrificed by cervical dislocation.

This experiment was carried out according to the guidelines of our institutional " Research Ethics Committee " (REC), with an approval code (36264PR185/4/23), and adhered to the protocols established by the National Institutes of Health for the treatment and care of animals used in research (NIH Publications number: 85-23, amended in 1996).

Tissue Sampling

The skull was opened and the brain was excised. A part of hippocampal tissues was processed and subjected to histopathological and immunohistochemical examination. The other part was subsequently homogenized in a chilled phosphate buffer solution with a pH of 7.4. The homogenate then underwent centrifugation at 3,000 rpm/10 min at a temperature of 37°C. After the centrifugation, the resulting supernatants were carefully transferred into clean plastic storage tubes and preserved at a temperature of -80°C. These supernatant samples were subsequently utilized for the quantification of various tissue biomarkers, including SOD, MDA, NFκB, IL-6, TNF-alpha, Beclin-1, GABA and glutamate. Total protein content was also determined in

both lung tissue homogenates method by reaction of protein molecules with the Folin-Ciocalteu reagent. That led to the formation of blue colour due to reduction of the reagent by aromatic acids and copper-treated proteins. The absorbance was measured at 750nm [30].

Histopathological and Immunohistochemical Assessment

Histopathological Assessment

For the preservation of brain tissues, they were immersed in a 10% neutral buffered formalin solution. Subsequent to the fixation process, the tissues underwent rapid routine tissue processing procedures. The brain samples were embedded in paraffin and allowed to solidify overnight, after which slices were cut using a rotating microtome at a thickness ranging from 5 to 6 micrometers. The tissue sections were then subjected to staining protocols, commencing with immersion in hematoxylin for 1 minute, followed by a 1-minute water wash. Next, a solution of 1% hydrochloric acid and ethanol was used to differentiate the pieces for a duration of 30 seconds. Afterward, their rinsing with water lasted for twenty minutes before being immersed in an eosin solution for a period of 5 to 10 minutes, with all staining steps performed at room temperature. The stained sections were dehydrated using a graded ethanol series. Finally, a light microscope was used to inspect and view the prepared slides.

Immunohistochemical Assessment for Caspase 3

Immunohistochemical staining was conducted on tissue sections with a thickness of 6 micrometers. The sections underwent a dewaxing process followed by rehydration through a descending series of alcohol solutions. Subsequently, for 10 minutes, they were exposed to a 10% H_2O_2 solution in methanol in order to decrease the activity of their own peroxidase. After that step, the sections were subjected to microwave irradiation in a 0.01 M sodium citrate buffer (pH 6.0) for 10 minutes, allowed to cool to room temperature and then washed three times in PBS, with each wash lasting 5 minutes. Following the washing steps, antigen retrieval was facilitated by autoclaving the sections for 11 minutes in citrate buffer. The sections were subsequently exposed to primary antibodies at a temperature of 4°C overnight. Afterward, a goat polyclonal secondary antibody was added to the tissues and left to incubate for 30 minutes at room temperature with 3,3′-diaminobenzidine. Finally, the tissue sections underwent light counterstaining with Hematoxylin (Sigma), dehydration in alcohol, clearing in xylene (Sigma) and mounting for visualization. The prepared slides were examined under a light microscope at 400X magnification[31].

Assessment of Glutamate and GABA Neurotransmitters

ELISA method was used to assess the level of GABA and glutamate in the hippocampus according to the manufacturer guidelines (MyBioSource, USA Cat No. # MBS756400, MBS269152, respectively)

Assessment of Oxidative Stress

SOD and MDA were measured calorimetrically in hippocampal tissue using colorimetric kits (Bio-Diagnostic, Giza, Egypt Cat No. # ab65354, ab118970 respectively) following the kit's instructions.

Assessment of Inflammatory and Autophagy Biomarkers

ELISA kits were used to detect inflammation markers, NFκB, IL-6 and TNF alpha in hippocampal tissue (MyBioSource, USA Cat No. #MBS704916, Life Span BioSciences, Inc. Cat No. # LS-F24088 and LS-F12799, respectively). Beclin-1, as a marker of autophagy in hippocampal tissue, was measured using ELISA kit provided by Abcam, USA (Cat No. # MOFI00319).

Quantitative Real Time PCR for TLR4, miR-181b, NKCC1 and KCC2

The miRNeasy Mini Kit (Qiagen, USA, #217661) was employed to extract total RNA from samples of frozen brain tissue. In order to measure the concentration and purity of the RNA, a NanoDrop spectrophotometer (Analytik Jena, Germany) was used and stored at −80 °C. Using the FastGene 55-Scriptase cDNA synthetic kit (Nippon Genetics Europe, LS-61), an aliquot of 5µg of the extracted RNA was reverse-transcribed to cDNA. We maintained cDNA at −20 °C until used as a

template for detecting TLR4, miR-181b, NKCC1, and KCC2 relative gene expression. Expression data were normalized to GAPDH as a housekeeping gene using the comparative cycle threshold $2^{-\Delta\Delta CT}$ method [32]. Gene-specific primers are given in **Table 1**.

Statistical Analysis

The statistical analysis of the acquired data was performed using the Statistical Program for Social Sciences (SPSS), version 14.0 for Windows. After assessing the normality of distribution, parametric data was subjected to the One-Way Analysis of Variance (ANOVA) test (F value) to identify differences among more than two arithmetic means. Subsequently, the post-hoc Scheffe test was employed to determine the specific differences between each pair of means. The results were expressed as mean ± standard deviation (SD). Statistical significance was established at a threshold of $P < 0.05$.

RESULTS AND DISCUSSION

Results

Histopathological and Immunohistochemical Assessment

The normal control group's hippocampal CA1, CA3 and DG regions had well-organized neurons with rounded or oval nuclei and normal structures. In the epilepsy group II, the cellular structure was disrupted in the different hippocampal regions. Some of the neurons in this group were loosely arranged, enlarged and thick while others appeared to degenerate with unclear boundary, condensed or shrunken pyknotic nucleus and cytoplasmic vacuolation **(Fig. 1)**. These changes persisted to a lesser extent in both groups III (Dapagliflozin-Treated Group) and IV (Carveol-Treated Group). On the contrary, in group V (Dapagliflozin + Carveol), the regular cellular arrangement was mostly restored and structural disruption, such as pyknosis, red neurons and cytoplasmic vacuoles, was obviously decreased. The cells in the hippocampus regions CA1, CA3 and DG of this group were properly arranged with large, central nuclei and distinct clear membranes and nucleoli **(Fig. 2).**

Caspase 3 immunohistochemistry staining results **(Fig. 3)** showed that groups differed significantly with respect to the proportion of CA1 region of the right hippocampus immune stained with caspase 3. The area percentage of active caspase3-immunostaining was markedly elevated in Group II (PTZ Group) compared to all other 4 groups. On the other hand, the area percentage of active caspase3-immunostaining in CA1 in Group V (Dapagliflozin + Carveol) was significantly lessened than that detected in both Group III (Dapagliflozin-Treated Group) & Group IV (Carveol-Treated Group).

Table 1: Gene-Specific Primers**.**

Gene	Accession Number	Sense Primer	Anti-Sense Primer
TLR4	NM_003266.4	5′-CTGCAGGTGCTGGATTTATCC-3′	5′-GGTGGCTTAGGCTCTGATATGC-3′
miR-181b	NR_129611.1	5′-AACATTCATTGCTGTCGGTGG-3′	5′-TTTGGTCCGCAGTTTGCATTC-3′
NKCC1	NM_001046.3	5′-AGACTTCAACTCAGCCACTGT-3′	5′-CAAGGTCAAACCTCCATCATCA-3′
KCC2	NM_020708.5	5′-AGGTGGAAGTCGTGGAGATG-3’	5’-CGAGTGTTGGCTGGATTCTT-3′
GAPDH	NM_002046.7	5'-ATGACATCAAGAAGGTGGTG-3´	5´- CATACCAGGAAAATGAGCTTG-3´

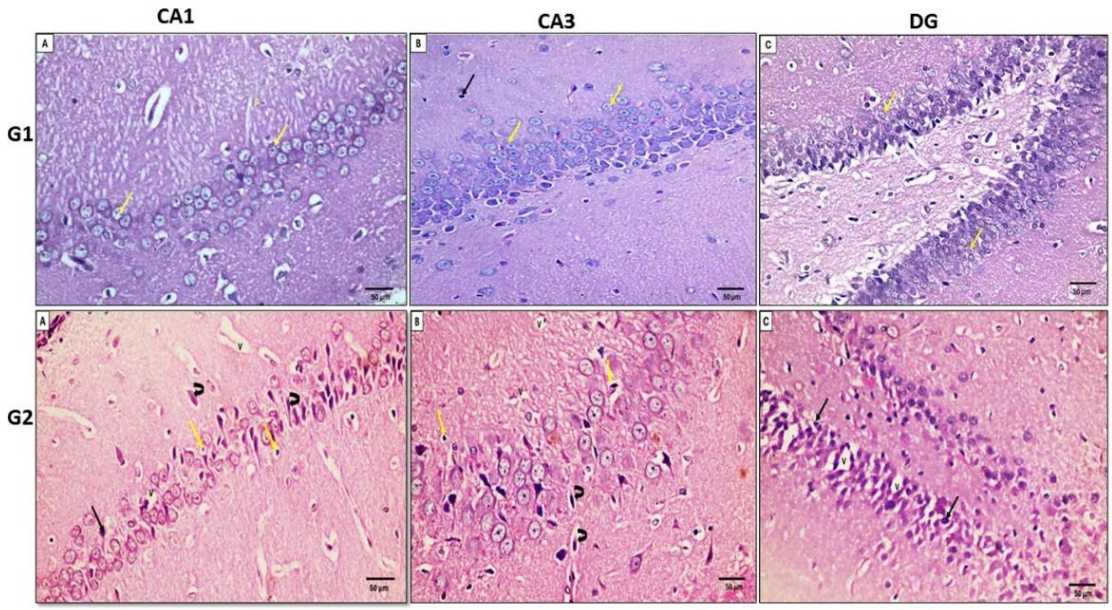

Fig. 1: Photomicrographs of H & E staining in the CA1 (A), CA3 (B) and DG (C) regions of the hippocampus of G I & II. The cells in Group I are densely packed, granular (yellow arrows) with rounded vesicular nuclei and prominent nucleoli. In Group II, the cells are disordered with pyknotic nuclei (yellow arrows), hyperdense nuclei (black arrows), multiple red neurons (curved arrows) and neuropil vacuolations (V). (H & E × 400, scale bar = 50 μm).

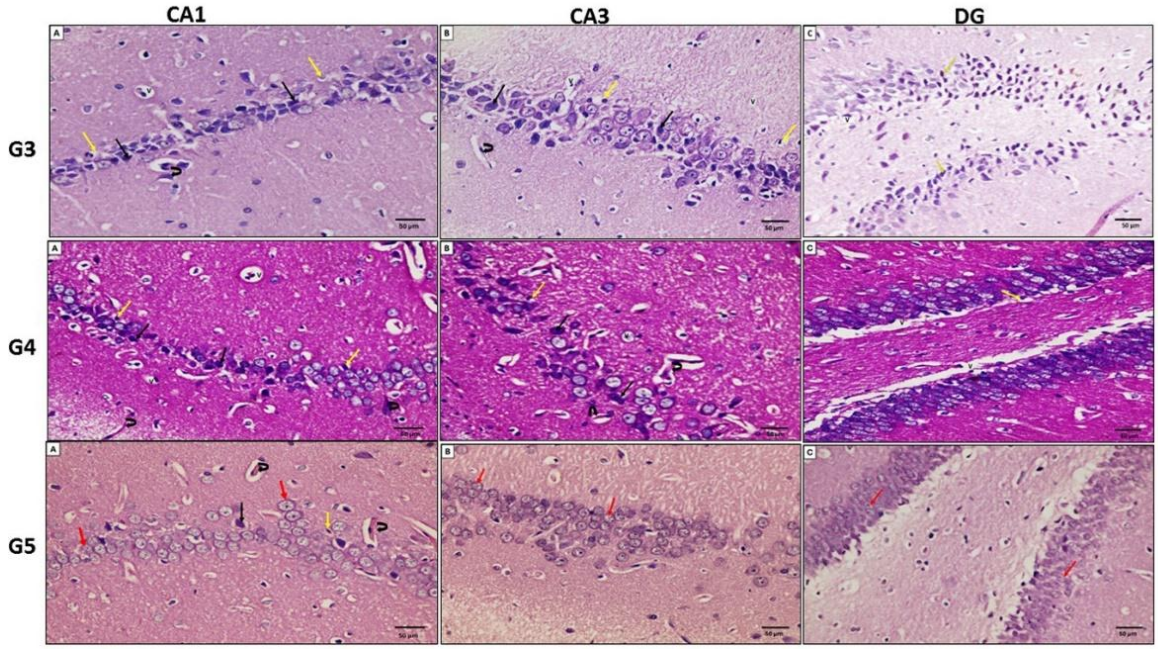

Fig. 2: Photomicrographs of H and E staining in the CA1 (A), CA3 (B) and DG (C) regions of the hippocampus of G III, IV and V. Group III (Dapagliflozin-Treated Group) and Group IV (Carveol-Treated Group) show less disrupted structure (Pyknotic (yellow arrow), hyperdense (black arrow) nuclei, red neurons (curved arrows) and neuropil vacuolations (V). Neurons of Group V (Dapagliflozin + Carveol) are well arranged with granular cells (red arrows) of prominent nuclei. Few cells with pyknotic (yellow arrow) hyperdense (black arrows) nuclei and red neurons (curved arrows) persist. (H & E × 400, scale bar = 50 μm).

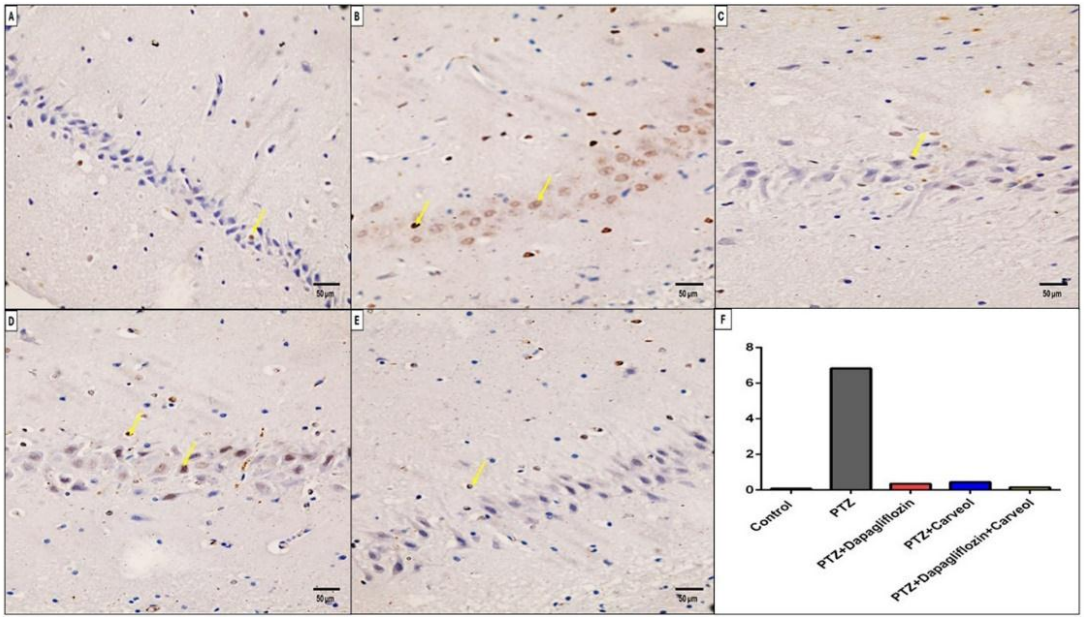

Fig. 3: Photomicrographs depicting caspase-3 immunohistochemical staining within the CA1 region of the hippocampus across distinct experimental cohorts: (A) Group I, (B) Group II (PTZ-induced seizures), (C) Group III (Treated with Dapagliflozin), (D) Group IV (Treated with Carveol), and (E) Group V (Treated with Dapagliflozin + Carveol). Notably, yellow arrows delineate the presence of active caspase-3 positive cells. The magnification scale is set at x400 with a scale bar of 50 µm. Additionally, (F) presents the quantitative assessment of the mean area percentage of caspase-3 across the various experimental groups. All results are presented as mean ± standard deviation (SD), with each experimental group consisting of n = 9 rats. A significance level of $p < 0.05$ was adopted for statistical analysis.

Effect of Dapagliflozin and Carveol on Hippocampal Neurotransmitters

The PTZ-induced epilepsy group (Group II) demonstrated a significant increase in glutamic acid levels, compared to the Control Group (Group I) and other groups (Groups III, IV, and V). Furthermore, a notable reduction in GABA concentrations is evident in PTZ-induced epilepsy (Group II) compared to Control Group (Group I) and other groups (Groups III, IV, and V). Additionally, Group III (Dapagliflozin-Treated Group) and Group IV (Carveol-Treated Group) exhibit a non-significant decrease in glutamic acid and a non-significant increase in GABA concentrations, compared to PTZ-induced epilepsy group (Group II). Conversely, the combination therapy of Dapagliflozin and Carveol (Group V) is associated with a significant decrease in glutamic acid levels and a significant increase in GABA concentration compared to Group II and the other treated groups (Groups III and IV). **(Table 2).**

Effect of Dapagliflozin and Carveol on Hippocampal Oxidative Stress

The PTZ-induced epilepsy group (Group II) showed a decreased antioxidant activity and increased oxidative stress evidenced by a significant decrease in SOD and a significant increase in MDA concentrations, compared to the Control Group (Group I) and the other treated groups (Groups III, IV, and V). Additionally, Dapagliflozin-Treated Group (Group III) and Carveol-Treated Group (Group IV) displayed a non-significant difference in SOD and MDA concentrations, as compared to PTZ-induced epilepsy group (Group II). While Dapagliflozin and Carveol-Treated Group (Group V) showed a significant increase in SOD and a significant decrease in MDA concentrations as compared to Group II and the other treated groups (Groups III and IV) **(Table 3)**.

Table 2: Levels of Glutamic Acid and GABA in Hippocampal Tissue among the Studied Groups.

	Groups					F-value	P-value
	I	II	III	IV	V		
Glutamic Acid (ng/mg Protein)	0.10± 0.011 b,c, d	0.23± 0.13 a,c, d,e	0.16± 0.01 a, b, e	0.15± 0.011 a, b, e	0.12± 0.007 b, c,d	182.275	<0.001*
GABA (pg/ mg Protein)	93.44± 1.24 b,c, d	32.72± 0.67 a,c, d,e	66.33± 1.0 a, b, e	65.56± 1.01 a, b, e	82.78± 1.09 a,b, c,d	4589.14	<0.001*

Data is presented as mean ± SD. Significance between groups was denoted as *p < 0.05: a denotes significance from Group I, b from Group II, c from Group III, d from Group IV, and e from Group V. The degrees of freedom were (4) between groups and (40) within groups, with a total of 44 observations. GABA: Gamma-aminobutyric acid. a: Group 1 compared to other groups, b: Group II compared to other groups, c: Group II compared to other groups, d: Group IV compared to other groups, e: Group V compared to other groups.

Table 3: Levels of SOD and MDA Levels in Hippocampal Tissue among the Studied Groups.

	Groups						
Parameter	I	II	III	IV	V	F-value	P-value
SOD(U/mg Protein)	7.59± 0.13 b,c, d	3.24± 0.15 a,c, d,e	6.20± 0.48 a, b, e	6.22± 0.51 a, b, e	7.17± 0.16 b, c,d	662.186	<0.001*
MDA (mmol/ g Protein)	7.9± 0.27 b,c, d	16.84± 0.63 a,c, d,e	12.42± 0.41 a, b, e	12.0± 0.27 a, b, e	8.22± 0.43 b, c,d	233.416	<0.001*

Data is presented as mean ± SD. Significance between groups was denoted as *p < 0.05: a denotes significance from Group I, b from Group II, c from Group III, d from Group IV, and e from Group V. The degrees of freedom were (4) between groups and (40) within groups, with a total of 44 observations. SOD: superoxide dismutase, MDA: Malondialdehyde. a: Group 1 compared to other groups, b: Group II compared to other groups, c: Group II compared to other groups, d: Group IV compared to other groups, e: Group V compared to other groups.

Effect of Dapagliflozin and Carveol on Inflammatory and Autophagy Biomarkers

The PTZ-induced epilepsy group (Group II) showed an increased inflammatory activity evidenced by a significant increase in NFκB, IL-6 and TNF- α concentrations, compared to the Control Group (Group I) and the other treated groups (Groups III, IV, and V). Also, autophagy biomarker Beclin-1 levels are considerably lower in the PTZ-induced epilepsy (Group II) compared to the Control Group (Group I) and the other treated (Groups III, IV, and V) groups. Additionally, Dapagliflozin-Treated Group (Group III) and Carveol-Treated Group (Group IV) exhibited a nonsignificant difference in these studied parameters, as compared to PTZ-induced epilepsy group (Group II). While Dapagliflozin and Carveol-Treated Group (Group V) exhibited a significant decrease in NFκB, IL-6 and TNF- α and a significant increase in Beclin-1, as compared to Group II and the other treated groups (Groups III and IV) **(Table.4)**

Effect of Dapagliflozin and Carveol on Hippocampal Relative TLR4 Gene Expression

The PTZ-induced epilepsy group (Group II) showed an upregulation of TLR4 expression levels, compared to the Control Group (Group I) and the other treated groups (Groups III, IV, and V). Additionally, Dapagliflozin-Treated Group (Group III) and Carveol-Treated Group (Group IV), exhibited a nonsignificant difference, as compared to PTZ-induced epilepsy group (Group II). While Dapagliflozin and Carveol-Treated Group (Group V) resulted in a pronounced reduction in the relative expression of the TLR4 gene, as compared to Group II and the other treated groups (Groups III and IV). **(Table 5, Fig. 4A).**

Table 4: Levels of NFκB, IL-6, TNF- α and Beclin-1 levels in Hippocampal Tissue.

	Groups					F-value	P-value
	I (n=9)	II (n=9)	III (n=9)	IV (n=9)	V (n=9)		
NFκB (pg/mg Protein)	2.91± 0.34 [b,c, d]	16.92± 0.54 [a,c, d,e]	9.22± 0.59 [a, b, e]	9.04± 0.39 [a, b, e]	3.07± 0.31 [b, c,d]	1493.776	<0.001*
IL-6 (pg/mg Protein)	23.44± 0.88 [b,c, d]	46.67± 1.0 [a,c, d,e]	35.78± 0.83 [a, b, e]	35.89 ± 0.93 [a, b, e]	24.67± 1.22 [b, c,d]	845.32	<0.001*
TNF-α (pg/mg Protein)	84.67± 1.58 [b,c, d]	474 ± 9.82 [a,c, d,e]	311.33± 6.16 [a, b, e]	314.44 ± 7.6 [a, b, e]	87.89± 2.93 [b, c,d]	6173.25	<0.001*
Beclin-1(ng/mg Protein)	8.29± 0.41 [b,c, d]	2.32 ± 0.1 [a,c, d,e]	6.61± 0.13 [a, b, e]	6.59 ± 0.13 [a, b, e]	8.08± 0.18 [b, c,d]	1082.77	<0.001*

Data is presented as mean ± SD. Significance between groups was indicated as *p < 0.05: a denotes significance from Group I, b from Group II, c from Group III, d from Group IV, and e from Group V. The degrees of freedom were (4) between groups and (40) within groups, resulting in a total of 44 observations. NFκB: nuclear factor kappa B, IL-6: Interleukin-6, TNF-α: tumor necrosis factor α. a: Group 1 compared to other groups, b: Group II compared to other groups, c: Group II compared to other groups, d: Group IV compared to other groups, e: Group V compared to other groups.

Table 5: Effect of Different Treatments on Hippocampal Relative Gene Expression of TLR4, NKCC1, miR181b and KCC2 in all Studied Group.

	Groups					F-value	p-value
	I (n=9)	II (n=9)	III (n=9)	IV (n=9)	V (n=9)		
TLR4	1.06± 0.87 [b,c, d]	2.11± 0.12 [a,c, d,e]	1.6± 0.07 [a, b, e]	1.62± 0.67 [a, b, e]	1.166± 0.05 [b, c,d]	237.008	<0.001*
NKCC1	1.0± 0.05 [b,c, d]	1.63± 0.07 [a,c, d,e]	1.24± 0.053 [a, b, e]	1.2 ± 0.07 [a, b, e]	1.13± 0.087 [b, c,d]	111.268	<0.001*
miR181b	1.02± 0.083 [b,c, d]	0.45 ±0.037 [a,c, d,e]	0.83± 0.067 [a, b, e]	0.8 ± 0.087 [a, b, e]	0.96± 0.088 [b, c,d]	77.652	<0.001*
KCC2	1.02± 0.067 [b,c, d]	0.44 ± 0.022 [a,c, d,e]	0.74± 0.039 [a, b, e]	0.78 ± 0.031 [a, b, e]	0.93± 0.087 [b, c,d]	149.464	<0.001*

Data is presented as mean ± SD. Significance between groups was indicated as *p < 0.05.: a denotes significance from Group I, b from Group II, c from Group III, d from Group IV, and e from Group V. The degrees of freedom were (4) between groups and (40) within groups, resulting in a total of 44 observations. TLR4: toll-like receptor 4, NKCC1: sodium-potassium-chloride cotransporter 1, miR181b: microRNA-181b, KCC2: potassium-chloride cotransporter 2. a: Group 1 compared to other groups, b: Group II compared to other groups, c: Group II compared to other groups, d: Group IV compared to other groups, e: Group V compared to other groups.

Effect of Dapagliflozin and Carveol on Hippocampal Relative NKCC1 Gene Expression

The PTZ-induced epilepsy group (Group II) showed a significant upregulation of NKCC1 expression when compared to the Control Group (Group I) and the other treated groups (Groups III, IV, and V). Additionally, Dapagliflozin-Treated Group (Group III) and Carveol-Treated Group (Group IV), exhibited a non-significant difference, as compared to PTZ-induced epilepsy group (Group II). While Dapagliflozin and Carveol-Treated Group (Group V) resulted in a significant reduction in the relative expression of the NKCC1 gene, as compared to Group II and the other treated groups (Groups III and IV). **(Table 5, Fig. 4B).**

Effect of Dapagliflozin and Carveol on Hippocampal Relative miR181-b Gene Expression

The PTZ-induced epilepsy group (Group II) showed a significant downregulation of miR181-b expression when compared to the Control Group (Group I) and the other treated groups (Groups III, IV, and V). Additionally,

Dapagliflozin-Treated Group (Group III) and Carveol-Treated Group (Group IV), exhibited a non-significant difference, as compared to PTZ-induced epilepsy group (Group II). While Dapagliflozin and Carveol-Treated Group (Group V) resulted in a significant upregulation in the relative expression of the miR181-b gene, as compared to group II and the other treated groups (Groups III and IV). **(Table 5, Fig. 4C).**

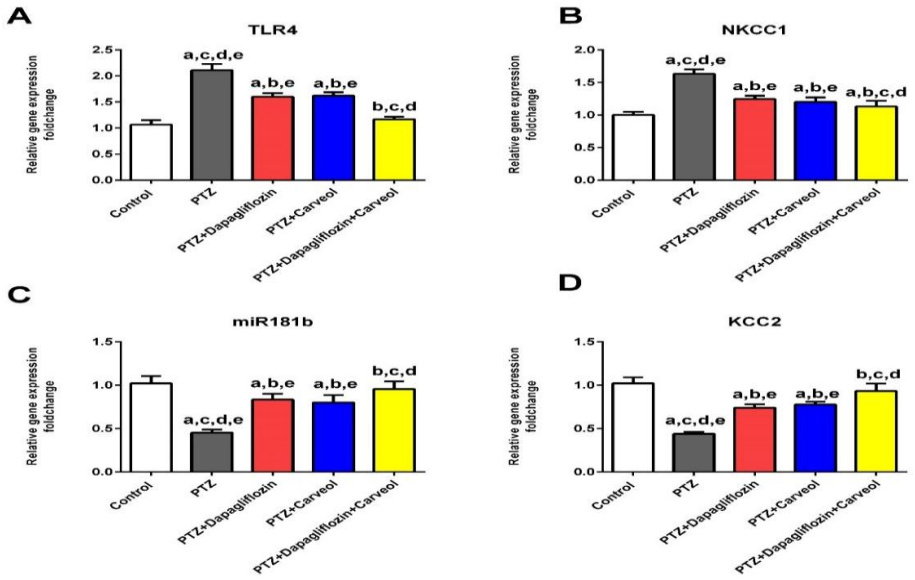

Fig. 4: Effect of Different Treatments on Hippocampal Gene Expression A): Relative Gene Expression of TLR4 in all Studied Group. B): Relative Gene Expression of NKCC1 in all Studied Groups. C): Relative Gene Expression of miR181-b in all Studied Groups. D): Relative Gene Expression of KCC2 in all Studied Groups. a: Group 1 compared to other groups, b: Group II compared to other groups, c: Group II compared to other groups, d: Group IV compared to other groups, e: Group V compared to other groups.

Table 6: Person's Correlation between miR181b Gene Expression as regards all the Studied Parameters.

Correlations		
	CIMT (mm)	
	r	**P-value**
SOD (U/mg protein)	0.913	<0.001*
MDA (mmol/ g protein)	-0.91	<0.001*
NFκB (pg/mg protein)	-0.919	<0.001*
IL-6 (pg/mg protein)	-0.902	<0.001*
TNF-α (pg/mg protein)	-0.881	<0.001*
Beclin-1(ng/mg protein)	0.934	<0.001*
TLR4 gene expression	-0.915	<0.001*
NKCC1 gene expression	-0.891	<0.001*
KCC2 gene expression	0.895	<0.001*

Table (6) shows a correlation analysis between miR181b & other studied parameters. There was a significantly positively correlated with SOD, KCC2 gene expression but significantly negatively correlated with MDA, NFκB, IL-6, TNF-α, Beclin-1, TLR4, and NKCC1 gene expression.

Effect of Dapagliflozin and Carveol on Hippocampal Relative KCC2 Gene Expression

The PTZ-induced epilepsy group (Group II) showed a significant downregulation of KCC2 expression when compared to the Control Group (Group I) and the other treated groups (Groups III, IV, and V). Additionally, Dapagliflozin-Treated Group (Group III) and Carveol-Treated Group (Group IV), exhibited a non-significant difference, as compared to PTZ-induced epilepsy group (Group II). While Dapagliflozin and Carveol-Treated Group (Group V) resulted in a significant upregulation in the relative expression of the KCC2 gene, as compared to Group II and the other treated groups (Groups III and IV). **(Table 5, Fig. 4D).**

Discussion

Epilepsy is a very common neurologic disorder that requires long-term therapy with antiepileptic drugs to provide long periods without seizures. It is distinguished by many symptoms, including changes in behavior and consciousness[33]. Low levels of certain antioxidants cause oxidative stress and may damage or kill neuronal cells[34].

In the present study, PTZ induced epilepsy was manifested by structural changes in different hippocampal regions in the form of nuclear pyknosis, hyperdenstity and red neurons, which were similar to the work of Flores-Soto M et al. [35]. Also, by oxidative stress evidenced by downregulation of SOD and upregulation of MDA, significant increase of NFκB, IL-6, TNF-α indicating activation of inflammatory process, a significant increase of caspase3, gene expression of TLR4 and reduction of gene expression of miR-181b with down regulation of beclin-1 indicating increased apoptosis and autophagy activity. Also, there was a significant decrease in GABA and a significant increase in glutamate, besides a significant decrease in the expression of KCC2 and a significant increase in NKCC1 gene expression indicating increased hippocampal excitability, compared to normal Control Group.

PTZ is a well-established GABA receptor antagonist which serves as a well-established model for inducing epilepsy. The principal inhibitory neurotransmitter in the brain, GABA, may be blocked by inhibiting its receptor and the balance between inhibitory and excitatory impulses is disrupted by PTZ, thereby precipitating epileptic seizures. Several studies have reported a decrease in antioxidant activity seen in the brains of rats following the treatment of PTZ[36].

Carveol is a natural antioxidant compound with diverse therapeutic properties, including antispasmodic and astringent effects, commonly employed to address issues such as indigestion and dyspepsia. Earlier investigations have highlighted Carveol's potential in mitigating hepatic cell necrosis through the reduction of oxidative stress, alongside its notable anti-hyperlipidemic and anti-inflammatory properties via regulating the NF-κB pathway [37].

Dapagliflozin is a SGLT2 inhibitor and its mechanism of action involves the reduction of blood glucose levels through the augmentation of glucose elimination via urine excretion[38]. Prior investigations have elucidated that the concurrent administration of Dapagliflozin showed advantageous neuroprotective outcomes in murine models afflicted with diabetes[39].

Dapagliflozin and Carveol-Treated Group exhibited a general amelioration of PTZ induced epilepsy evidenced by amelioration of histopathology and oxidative stress via upregulation of SOD and downregulation of MDA, improved inflammation via significant reduction of NFκB, IL-6, TNF-α. Also, modulation of apoptosis and autophagy process via a significant reduction of caspase3 and gene expression of TLR4 with up regulation of miR-181b gene expression and beclin-1. Moreover, decreased hippocampal excitability is evidenced by a significant increase in GABA and a significant decrease in glutamate, besides a significant increase in the gene expression of KCC2 and a significant decrease in NKCC1 gene expression, compared to other groups.

Supporting our findings, Arab HH et al. [40] Alvi AM et al. [3] documented that Dapagliflozin and Carveol have neuroprotective effects evidenced in many neurological disorders, like epilepsy, Alzheimer's disease, brain ischemia and traumatic brain damage due to their anti-apoptotic and antioxidant properties.

In rats, where PTZ- seizures were induced, our results showed a significant increase in glutamic acid and a significant decrease in

GABA levels. While the combination group treated with Dapagliflozin and Carveol showed a significant reduction in glutamic acid versus a significant elevation in GABA concentration, Dapagliflozin exhibited a reduction in seizure activity. This anticonvulsant effect is attributed to its ability to decrease glucose availability and reduce sodium transportation across neuronal membranes. These mechanisms collectively contribute to stabilizing neuronal excitability and preventing undesired depolarization [25]. Additionally, Dapagliflozin and Carveol treatment attenuated seizures through increasing the expression of KCC2 and decreasing NKCC1 gene expression indicating decreased hippocampal excitability, which was similar to the work of Wu et al. [18].

Interestingly, the combination treatment with Dapagliflozin and Carveol showed a notable anti-apoptotic and anti-inflammatory effect evidenced by reduction in caspase3, TLR-4 gene expression, TNF-α and IL-6 levels, as well as an inhibition of the NF-κB pathway in comparison to PTZ-induced epilepsy group. Similar to our study, Arab et al. have demonstrated that Dapagliflozin suppressed neuro-inflammation via decreasing the activation of NF-κB pathway and TNF-α levels[41]. These results corroborate those of the study carried out by Elmahdy et al.[42]. Additional studies by Abdollahi et al.[19] asserted that Dapagliflozin reduces apoptosis and inflammation via lowering TLR-4 expression and blocking the NF-κB pathway. Furthermore, Muhammad et al. demonstrated the anti-inflammatory effect of Carveol mediated by the regulation of the NF-κB pathway[37].

Additionally, the combination treatment group receiving both Dapagliflozin and Carveol demonstrated a remarkable antioxidant effect evidenced by a significant increase in SOD and a significant decrease in MDA concentrations when compared to the other studied groups. These results agree with the results demonstrated by the study of Samman et al.[43].

Moreover, Dapagliflozin and Carveol-Treated Group exhibited a significant reduction in TLR4 and upregulation of miR-181b gene expressions in the hippocampus of rats, in contrast with the results in the PTZ induced epilepsy group, suggesting an anti-apoptotic effect conferred by Dapagliflozin and Carveol treatment. Similar outcomes have been reported by Abdollahi et al.[19], Wei et al.[44]and Hu et al.[21].

Conclusions

The combination of Dapagliflozin and Carveol exhibited significantly better antioxidant, anti-inflammatory and antiapoptotic effects than monotherapy by either of each of both drugs against PTZ induced epilepsy. So, this combination might represent a promising therapeutic modality against PTZ induced epilepsy**.**

Abbreviations

AEDs: Antiepileptic drugs
DPG: Dapagliflozin
GABA: Gamma aminobutyric acid
IL-6: Interleukin-6
LPO: Lipid peroxidation
PTZ: Pentylenetetrazol
SGLT2: Sodium-Glucose Cotransporter 2
TNF-α: Tumor Necrotic Factor-Alpha
NFkB: Nuclear Factor Kappa Beta
KCC2: Potassium-Chloride Cotransporter 2
NKCC1: Sodium-Potassium-Chloride Cotransporter 1
TLR4: Toll-Like Receptor 4
miR-181b: Microrna-181b
SOD: Superoxide Dismutase
MDA: Malondialdehyde

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استخدام داباجليفلوزين وكارفيول يخفف من الصرع المستحث بالبنتيلينتيترازول في الفئران: تأثيرات مضادة للأكسدة وللالتهابات وموت الخلايا المبرمج: استهداف بروتينات تول ريسيبتور ٤ و ميكرو ار ان أي ١٨١ بي و نيوكلر فاكتور كابا بي

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الصرع هو مشكلة صحية عامة كبيرة، تشمل الأبعاد الاجتماعية، والثقافية، والنفسية، والاقتصادية. داباجليفلوزين له نشاط مضاد للنوبات. كارفيول هو مركب أحادي الحلقة موجود بشكل طبيعي وله خصائص مضادة للأكسدة. دراسة تأثيرات داباجليفلوزين وكارفيول، سواء كعلاج وحيد أو مجتمعًا، على الصرع المستحث ببنتيلينتترازول في الفئران.

تم إجراء البحث على (٤٥) فأر تجارب، تم تقسيمهم الى خمس مجموعات: المجموعة الأولى: المجموعة الضابطة ، المجموعة الثانية: المجموعة المعالجة بالبنتيلينتيترازول ، المجموعة الثالثة: المجموعة المعالجة بالداباغليفلوزين، المجموعة الرابعة: المجموعة المعالجة بالكارفيول، المجموعة الخامسة: المجموعة المعالجة بالداباغليفلوزين و الكارفيول. . بعد انتهاء التجربة، تم الحصول على أنسجة الحصين من جميع المجموعات لفحص المؤشرات الحيوية للأنسجة بما في ذلك، سوبر أكسيد ديسميوتاز، المالونديالدهيد، نيوكلر فاكتور كابا بي ، الانترلوكين ٦، عامل نخر الورم ألفا ، بيكلين ١، حمض جاما–أمينوبيوتيريك ، الغلوتامات ، التعبير الجيني لتول ريسيبتور ٤، ميكرو ار ان أي ١٨١ بي ، ان كيه سي سي ١ و كيه سي سي ١ ، والكاسبيز ٣ وقد اظهرت النتائج ان المجموعة الخامس و هي المجموعة المعالجة بالداباغليفلوزين و الكارفيول قد تحسنت من الصرع المستحث ببنتيلينتترازول من خلال زيادة حمض جاما–أمينوبيوتيريك ، وانخفاض الغلوتامات، وتحسين والإجهاد التأكسدي من خلال تنظيم سوبر أكسيد ديسميوتاز وخفض تنظيم المالونديالدهيد ، وتحسين الالتهاب عن طريق تقليل كبير في نيوكلر فاكتور كابا بي ، الانترلوكين ٦، عامل نخر الورم ألفا.و أيضًا من خلال تعديل عملية موت الخلايا المبرمج تقليل كبير في التعبير الجيني للكاسبيز ٣ و تول ريسيبتور ٤ وتنظيم التعبير الجيني لميكرو ار ان أي ١٨١ بي ، ان كيه سي سي ١ و كيه سي سي ١ مقارنة بالمجموعات الأخرى. و لقد اثبتت النتائج ان الجمع بين داباجليفلوزين وكارفيول له تأثيرات مضادة للأكسدة ومضادة للالتهابات ومضادة لموت الخلايا المبرمج أفضل بكثير من العلاج الأحادي بأي من كلا العقارين ضد الصرع المستحث ببنتيلينتترازول.