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Investigating Umbelliferone's Neuroprotective Potential on the Glutamatergic System in PTZ-Induced Epileptic Rats

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Abstract:

This thorough investigation meticulously examines the neuroprotective effectiveness of umbelliferone, with a specific focus on the complex dynamics of the Glutamatergic System in brain segments influenced by pentylenetetrazol (PTZ)-induced epilepsy in rats. Epilepsy, a multifaceted neurological disorder characterized by disrupted brain electrical activity and cellular processes, serves as the focal point of this investigation. Employing meticulous experimentation, the study deliberately induced epilepsy in the animal model, presenting a unique opportunity to comprehensively assess the effects of umbelliferone on the intricacies of the Glutamatergic System.The findings not only illuminate the robust neuroprotective qualities inherent in umbelliferone but also provide a nuanced understanding of the specific alterations induced by PTZ. This research enhances our knowledge of potential therapeutic interventions within the complex framework of the Glutamatergic System amidst epileptic conditions. A significant contribution to advancing neuroprotective strategies, this study encourages further exploration of umbelliferone as a promising candidate for refining therapeutic approaches to epilepsy, providing a foundation for future research endeavors aimed at unraveling the intricate molecular mechanisms involved in managing epileptic conditions.

Key words: Glutamatergic System, Epileosy, Coumarin, UMB, PTZ, Diazepam, Male Albino Rats.

Introduction:

Epilepsy, a neurological disorder characterized by abnormal, synchronized neuronal activity in the brain, poses significant challenges to cognitive, psychological, and behavioural well-being, ultimately diminishing the quality of life for affected individuals **(Thurman et al., 2017; Beghi et al., 2016).** Globally, approximately 50 million people grapple with epilepsy, with prevalence rates ranging from 4 to 10 individuals per 1000 in the general population. Developing countries exhibit a higher burden, with prevalence rates ranging from 7 to 14 per 1000 people **(Camfield and Camfield, 2015).** Recognized for centuries, epilepsy manifests through diverse symptoms such as altered awareness, tingling sensations, convulsions, and sensory auras **(Kaiboriboon et al., 2013).**

The pathophysiology of epilepsy encompasses both seizure induction (ictogenesis) and the transformation of a normal brain into an epileptic-prone state (epileptogenesis) **(Fisher et al., 2017).** Currently, there is a lack of antiepileptogenic medications to halt the progression of epileptogenesis **(Fisher R.S et al., 2014),** underscoring the pressing need for innovative treatment approaches. With an urgency for novel strategies, consideration should be given to compounds inhibiting both ictogenesis and epileptogenesis **(Scheffer et al., 2017).** The ideal anticonvulsant should effectively mitigate seizures triggered by excessive neuronal firing. The

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gravity of the epilepsy burden emphasizes the imperative for groundbreaking interventions informed by the latest research findings.

Normal neural signaling requires a complex orchestra of pre-and postsynaptic events mediated by intracellular signaling and genetic expression. Standard signaling can go wrong with symptomatic conditions such as epilepsy since the resulting abnormal cellular and network activity produces a change in connectivity and synchrony that contributes to disease progression. In epilepsy, chronic dyssynchronous network activity induces additional neuronal firing and pathological changes in signalling arising from multiple pathways. The glutamate is one of the most frequently implicated toxic amino acid categories of neurotransmitter in the pathology of epilepsy.

Glutamate is the most common excitatory neurotransmitter in adult mammal's brains and is critical for various functions. Neuronal depolarization causes calcium-dependent presynaptic glutamate release into the synaptic cleft. Glutamate, like the inhibitory neurotransmitter γ-Aminobutyric acid (GABA), mediates its excitatory effects through various ionotropic and metabotropic receptor subclasses. AMPA receptors (AMPARs) are essential for fast excitatory neurotransmission, whereas NMDA receptors (NMDARs) mediate much of the slow postsynaptic excitatory potentials required for global information processing. Glutamate can also interact with the kainate receptor, another ionotropic glutamate receptor (KAR), plays a less prominent role in neuronal signalling, which may include both pre-and postsynaptic modulation of excitatory neurotransmission **(Lerma and Marques, 2013).** Glutamate's effect on these receptor subtypes stimulates a variety of pre-and postsynaptic events, all of which underpin normal and abnormal neuronal activity.

About coumarins: Umbelliferone (UMB), a natural coumarin derivative, has emerged as a compelling subject in the realm of therapeutic exploration, particularly in the context of epilepsy treatment. This bioactive compound possesses a unique chemical structure, characterized by a benzene ring fused to an alpha-pyrone ring. Its systematic nomenclature is 7-hydroxy-2H-chromen-2-one, with a chemical formula of C9H6O3, resulting in a molecular weight of 162.15 g/mol **(PubChem).** Noteworthy for its abundance in various plants, UMB exhibits a spectrum of pharmacological properties, encompassing anti-inflammatory, antioxidant, and potential anti-cancer effects **(Salehi et al., 2019).**

The spotlight on UMB has intensified in recent studies that delve into its neuroprotective potential, specifically examining its impact on the glutamatergic system - a pivotal element in the pathophysiology of epilepsy. The modulation of glutamate neurotransmission, a critical facet in epilepsy management, positions UMB as a promising candidate for therapeutic interventions (**Salehi et al., 2019).**

While the precise mechanism of UMB's action in epilepsy treatment remains under scrutiny, its capacity to influence glutamatergic neurotransmission presents intriguing prospects for therapeutic applications. The compound's natural origin and diverse biological activities render it a captivating subject for ongoing research, with potential implications for epilepsy treatment.

Recent research findings suggest that UMB may exert neuroprotective effects through its modulation of the glutamatergic system. Glutamate, a pivotal excitatory neurotransmitter, plays a crucial role in seizure generation, and disruptions in glutamatergic signalling contribute

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to the process of epileptogenesis **(Zhao et al., 2022).** These insights further underscore UMB's potential as a therapeutic agent in the intricate landscape of epilepsy treatment.

MATERIALS & METHODS

Procurement of Chemicals:

The present study utilised only Analar grade (AR) chemicals, and solvents were procured from the following scientific companies: Sigma (USA), Fisher (USA), Merck (India), Himedia (India), TCI (China), Molychem (India), and SRL (India).

Procurement of Animals:

The Anti-Convulsant activity of Umbelliferon was tested in three months old Wistar strain male Albino Rats (180 ± 20 grams), purchased from Sri Venkateswara Enterprises, Bangalore. The maintenance of Experimental Rats and all the protocols followed in this study were approved by the ethical committee of Sri Venkateswara University and ethical permission **Resolution No: 34/2012-2013/(i)/a/CPCSEA/IAEC/SVU/KY/ dt. 01. 07.2012.** Healthy rats were made into 6 experimental groups of 6 rats each within ranges of (180 ± 20) grams body weights as follows.

Preparation of Dosage for: Umbelliferon, Pentylenetetrazol and Diazepam.

All doses, prepared in 1% Tween 80 distilled water, were intraperitoneally administered to Experimental Rats in Groups III, IV, and V **(Zagaja et al., 2015).** Convulsions were induced by intraperitoneal injection of Pentylenetrazole (60 mg/kg.b.w) in saline **(Gupta et al., 1999).** Diazepam (4 mg/kg.b.w), a standard reference drug, was intraperitoneally administered to Group VI for comparison **(Shivakumar et al., 2009).**

Grouping of rats

Total Groups = 6 : Each Group with 6 Male Albino Rats (*Rattus norvegicus*)	
Group: I	Normal 1% tween 80 Saline Treated Control Rats
Group: II	Rats Treated with PTZ 60 mg/kg body weight
Group: III	Epileptic rats pretreated with PTZ + UMB 50 mg/kg body weight)
Group: IV	Epileptic rats pretreated with –PTZ + UMB 100 mg/kg body weight
Group: V	Epileptic rats pretreated with PTZ + UMB 150 mg/kg body weight
Group: VI	Epileptic rats pretreated with PTZ + DZ 4 mg/kg body weight

Biochemical Analysis:

All the below mentioned biochemical estimations were done in 4 selected regions Viz. Cerebral Cortex, Hippocampus, Cerebellum and Pons Medulla of both control and experimental groups of rats on 15th day of Epilepsy induction.

Isolation of Tissues:

For biochemical estimations, all six groups of Rats were sacrificed on 15th day by cervical dislocation. The brain was isolated immediately placed on a chilled glass plate. Four

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selected regions of the brain viz. Cerebral Cortex, Hippocampus, Cerebellum and Pons Medulla were separated by following standard anatomical marks **(Glowinski and Iverson, 1966),** frozen in liquid nitrogen (-180^0 C) and then stored at -40^0 C until further use. At the time of biochemical analysis, the selected tissues were thawed and used. The results obtained on various biochemical parameters were analysed statistically.

Biochemical Constituents Analysis:

A. Glutamine Content Estimation:

Glutamine content was determined following the acid hydrolysis method by **Colowick and Kaplan (1967).** Brain region homogenates were prepared in cold distilled water, treated with H_2SO_4, boiled, cooled, and centrifuged. The supernatant underwent NaOH addition, and the mixture was made up to 2.0 ml with distilled water. Ammonia formed was estimated by Nesslerization, and glutamine content was expressed as µ moles of ammonia/gm wet weight of tissue **(Colowick & Kaplan, 1967).**

B. Glutaminase (GA) Activity Assay:

Glutaminase activity was assayed as per **Meister et al. (1995).** Tissue homogenates were prepared in cold double-distilled water, used as an enzyme source, and incubated with L-glutamine. The reaction was stopped with TCA, and the developed color was measured at 490 nm. Enzyme activity was expressed in µ moles of ammonia released/mg protein/hr (**Meister et al., 1995).**

C. Glutamine Synthetase (GS) Activity Assessment:

Glutamine synthetase activity was measured using the method of **Wu et al. (1963).** Region-specific homogenates were prepared, and the supernatant served as an enzyme source. The reaction mixture, incubated at 37°C, was terminated, and the color intensity was measured at 535 nm. Glutamine synthetase activity was expressed as µ moles of γ-glutamyl hydroximate formed/mg protein/hr **(Wu et al., 1963).**

D. Glutamate Dehydrogenase (GDH) Activity Analysis:

Glutamate dehydrogenase (GDH) activity was assessed following **Lee and Lardy (1965).** Region homogenates were prepared, and the supernatant was used as an enzyme source. The reaction mixture was incubated, the reaction was stopped, and the color intensity was measured at 495 nm. Enzyme activity was expressed as µ moles of formazan formed/mg protein/hr **(Lee & Lardy, 1965).**

RESULTS:

This study evaluated four crucial components in glutamate metabolism: A. Glutamine Content (GC), B. Glutaminase Activity (GA), C. Glutamine Synthetase (GS), and D. Glutamate Dehydrogenase (GDH). These assessments aimed to understand Glutamate Activity in rats undergoing PTZ-induced seizures, with pre-treatment using UMB. The results are summarized below.

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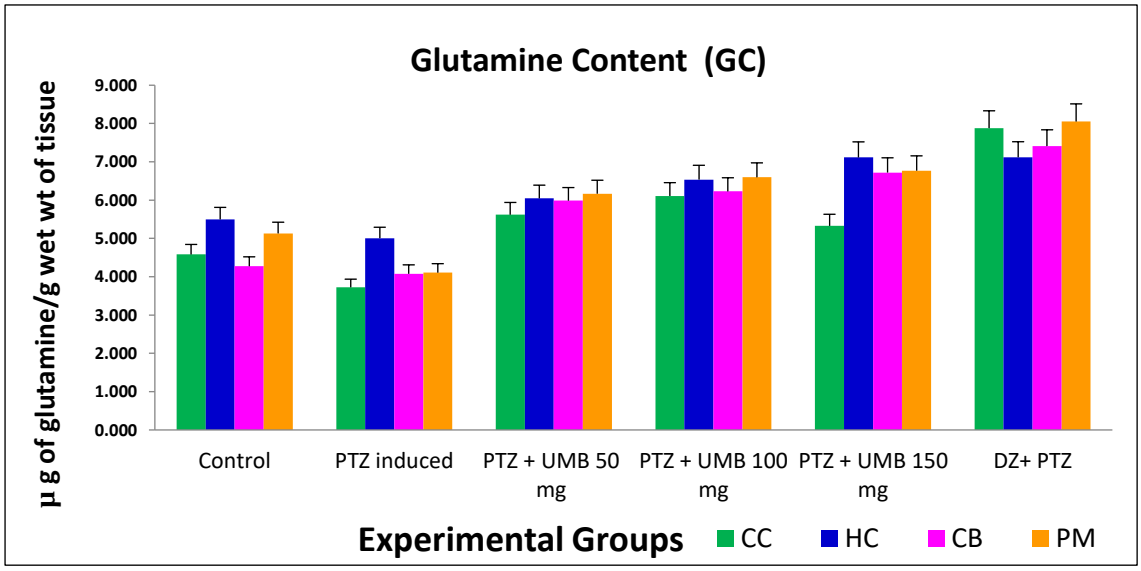
A. Glutamine Content (GC) : (Graph – 1)

From the results, it was observed that in control rats, the Glutamine content was found to be highest in Hippocampus. However, it was observed that there was a significant decline in Glutamine Content of all the above selected brain regions in the PTZ-induced experimental rats when compared to control rats. Interestingly it was noticed that the Glutamine Content was gradually increased in the experimental group of rats pre-treated with PTZ+UMB at 50 mg, 100 mg, 150 mg and DZ+PTZ doses in a dose-dependent manner from lower to higher, but for a significant decrease in the Cerebral Cortex of PTZ+UMB 150 mg group.

Graph - 1. Changes in the Glutamine Content (GC) in selected brain regions of Control and Experimental Rats (Values are expressed in µg of glutamine/g wet wt of tissue)



B. Glutaminase Activity (GA) : (Graph – 2)

In the control rats, the Glutaminase activity was found to be highest in the Pons medulla , followed by the Cerebral Cortex, Cerebellum. When compared to the Control rats, Glutaminase activity in all the experimental groups showed great changes in the selected brain regions of the rats. Findings in the current study revealed that Glutaminase Activity is increased in all the selected brain regions of the rats induced with PTZ compared to the control rats. Among all, a significant increase in Pons Medulla and Cerebral Cortex regions was observed and the remaining two regions viz Cerebellum and Hippocampus, recorded lower levels. Among all the experimental groups of rats pre-treated with PZT-UMB 50 mg was shown lowest values in descending order as follows Cerebral cortex, Pons medulla, Hippocampus and Cerebellum.

Results on epileptic rats pre-treated with UMB of 100 mg showed a significant improvement in Glutaminase Activity in all the selected brain regions in the descending order of Pons Medulla, Cerebral Cortex, Cerebellum and Hippocampus when compared with the above group, but lower than the levels recorded in PTZ-induced group. In experimental rats pre-treated with UMB 150 mg a significant increase in Glutaminase Activity in all the selected

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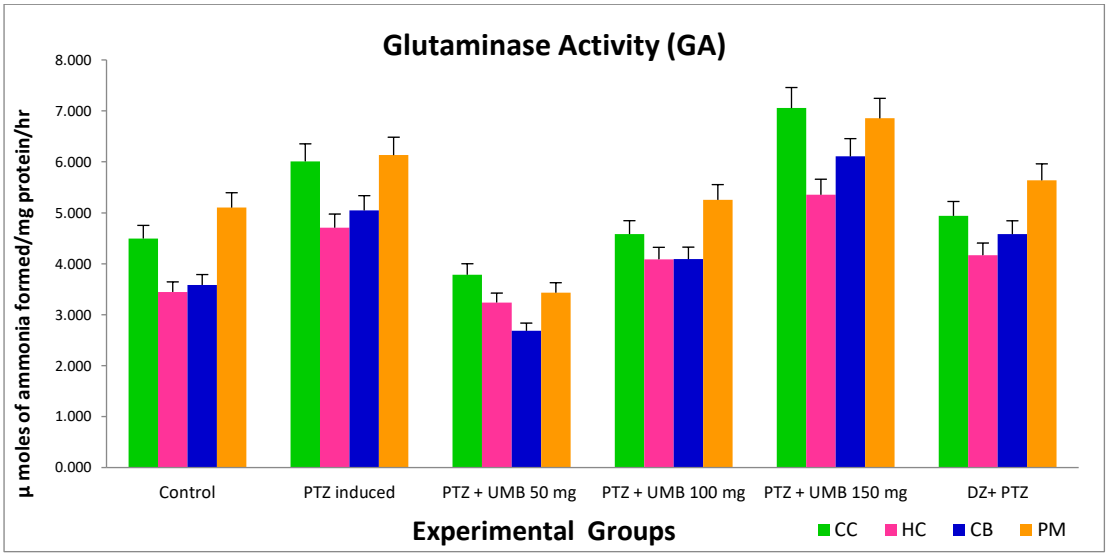
brain regions was noticed. Maximum increase was observed in Glutaminase Activity in the Cerebral Cortex followed by the Pons Medulla, Cerebellum and Hippocampus.

Interestingly, the Glutaminase Activity in the reference-drug treated rats (DZ-PTZ) showed the following values in the order from higher to lower i.e., Pons Medulla, Cerebral Cortex, Cerebellum and followed by the Hippocampus. The Glutaminase Activity values of the UMB 100 mg group were found to be similar to those of the reference drug group.

Graph - 2. Changes in the Glutaminase Activity (GA) levels in selected brain regions of Control and experimental Rats (Values are expressed in μ moles of ammonia formed/mg protein/hr)



C. Glutamine Synthetase (GS): (Graph - 3)

The changes in the Glutamine Synthetase (GS) activity levels in selected brain regions of Control and Experimental groups were shown in Table - 9. In the 1st Group (control group), the highest GS activity was observed in the Hippocampus, followed by the Cerebral Cortex, Cerebellum, and Pons Medulla. In the 2nd group (PTZ-induced), a significant decrease in GS activity was observed in all selected brain regions compared to the control group, with the maximum decrease observed in the Pons medulla and the remains as follows Cerebellum, Cerebral Cortex and Hippocampus.

Among the UMB pre-treated groups, the 3rd group (50 mg) showed a significant increase in GS activity levels in all selected brain regions compared to the PTZ-induced group. The highest GS activity was observed in the Hippocampus, followed by the Cerebral Cortex, Cerebellum and Pons Medulla. The GS activity of the 4th group (UMB 100 mg) showed a significant improvement in all selected brain regions compared to the previous group. The highest GS activity was observed in the Cerebral Cortex, followed by the Hippocampus, Cerebellum and Pons Medulla.

In the 5th group (UMB 150 mg), a much more increase in GS activity was observed in all selected brain regions in a significant manner compared to the control group. The highest GS activity was observed in the Cerebral Cortex, followed by the Hippocampus, Cerebellum

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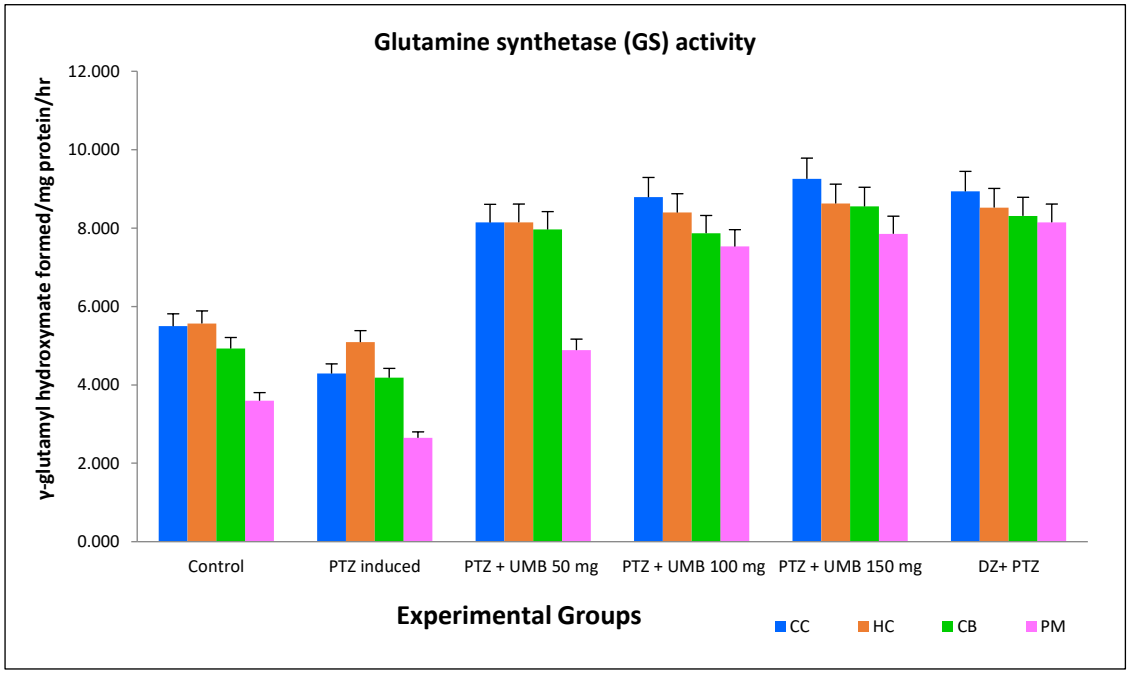
and Pons Medulla. Regarding the GS activity of the 6th group (DZ-PTZ), it was higher in all selected brain regions compared to the UMB pre-treated groups in the order of Cerebral Cortex, followed by the Hippocampus, Cerebellum and Pons Medulla.

Graph - 3. Changes in the Glutamine Synthetase (GS) activity levels in selected brain regions of Control and experimental Rats (Values are expressed in µ moles of γ-glutamyl hydroxymate formed/mg protein/hr)



D: Glutamate Dehydrogenase (GDH): (Graph – 4)

In this study, the effects of UMB on the activity of Glutamate Dehydrogenase (GDH) in various brain regions of rats induced by PTZ was investigated. The results indicated that in the 1st group (Control), the highest GDH activity was observed in Cerebellum (3.780 µ moles of formazan formed/mg protein/hr). Compared to the 1st group (Control), the 2nd group (PTZ-induced) showed a significant decrease in GDH activity in all selected brain regions. The maximum decrease was observed in the Pons medulla (2.365 µ moles of formazan formed/mg protein/hr) and the Cerebral Cortex (2.656 µ moles of formazan formed/mg protein/hr) regions.

In the 3rd group (PTZ + UMB 50 mg), a significant increase in GDH activity was observed in all selected brain regions. The highest GDH activity was observed in the Hippocampus. The GDH activity in 4th group of rats (PTZ + UMB 100 mg) showed a significant improvement in all selected brain regions compared to the control group, but lower than the 3rd group (UMB 50 mg). The highest GDH activity was observed in the Hippocampus. In the 5th group (PTZ + UMB 150 mg), a highest increase in GDH activity was observed in all selected brain regions, with the maximum increase observed in the Hippocampus, Finally, the GDH activity in the 6th group (DZ + PTZ) was higher in all regions. Overall, our findings suggest that UMB treatment can significantly increase GDH activity in PTZ-induced rats in a dose-dependent manner and thus, may have therapeutic potential for the treatment of neurological disorders.

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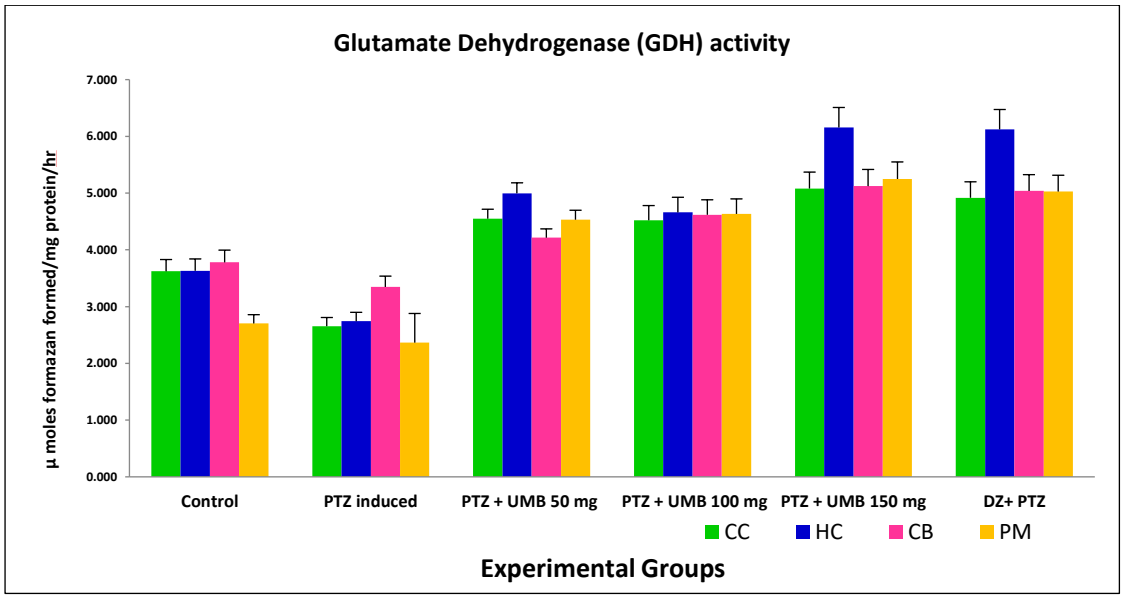
Graph - 4. Changes in the Glutamate Dehydrogenase (GDH) activity levels in selected brain regions of Control and Experimental Rats (Values are expressed in µ moles formazan formed/mg protein/hr)



DISCUSSION:

In the present study, the efficacy of Umbelliferon in PTZ-induced epilepsy with reference to 4 constituents of Glutamatergic system (Glutamine, Glutaminase, Glutamine Synthetase and Glutamic Acid Dehydrogenase, in four selected brain regions viz. the Cerebral Cortex, Hippocampus, Cerebellum and Pons medulla of male albino rats were evaluated and compared with the reference drug Diazepam.

A. Glutamine Content:

The graph illustrates a decrease in Glutamine content across selected brain regions in PTZ-induced rats compared to control rats. However, PTZ-induced epileptic rats, pre-treated with various doses of Umbelliferon, exhibited a significant dose-dependent increase in Glutamine content, approaching control levels by the 15th day. This positive effect resembled that of the reference drug, Diazepam.

Considering Glutamine's role in excitatory neurotransmission and potential association with seizures and neurological disorders, reducing Glutamine content in PTZ-treated brain regions may mitigate excitotoxicity. Evidence suggests Glutamine's involvement in certain brain tumours, and its depletion may inhibit tumour growth. Additionally, Glutamine plays a role in brain energy metabolism, and reducing its content may conserve energy, reduce oxidative stress, and protect against inflammation caused by PTZ.

Earlier reports highlighted varying effects of PTZ on different brain regions, impacting Glutamate, Glutamine, GABA, Aspartate, and Taurine levels. Natural compounds, including α-asaronol and α-asarone, demonstrated antiepileptic activity in PTZ-induced seizure rats, reducing seizure frequency and intensity. Recent studies indicated that natural compounds, such as omega-3 fatty acids, curcumin, and resveratrol, could increase Glutamine content in brain regions, offering protection against inflammation and oxidative stress, ultimately improving cognitive function **(Wollen, 2010).**

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B. Glutaminase Activity (GA):

Contrary to Glutamine Content, the Glutaminase Activity was increased in all the selected brain regions of the rats induced with PTZ when compared to the control rats. Among all, a significant increase in Cerebral Cortex and Pons Medulla regions was observed in PTZ-induced epileptic rats. As in the case of Glutamine, normal levels of Glutaminase were brought by pre-treatment PTZ-induced rats with Umbelliferon.

Glutaminase is an enzyme that plays a crucial role in regulating the level of Glutamate in the brain, which is an essential neurotransmitter. Epilepsy is a neurological disorder characterized by recurrent seizures and it has been suggested that increased glutaminase activity could be involved in developing epilepsy. A study conducted by **Suresh et al. (2008)** demonstrated that curcumin and resveratrol inhibit glutaminase activity. In addition, quercetin has been shown to reduce glutaminase activity by up to 80% **(Liu and Zhang, 2002).** These findings indicate that natural compounds can potentially reduce glutaminase activity and thereby improve glutamine metabolism in the body.

B. Glutaminase Activity (GA):

In contrast to Glutamine Content, Glutaminase Activity exhibited an increase in all selected brain regions of PTZ-induced rats compared to the control group. Significantly elevated levels were particularly observed in the Cerebral Cortex and Pons Medulla regions of PTZ-induced epileptic rats. However, akin to the observations with Glutamine, pre-treatment of PTZ-induced rats with Umbelliferon restored Glutaminase levels to normal. Glutaminase, a pivotal enzyme in regulating brain Glutamate levels, an essential neurotransmitter, has been implicated in epilepsy development. Studies, such as the one by **Suresh et al. (2008),** have demonstrated that compounds like curcumin and resveratrol can inhibit glutaminase activity. Additionally, quercetin has shown the potential to reduce glutaminase activity by up to 80% **(Liu and Zhang, 2002).** These findings suggest that natural compounds may have the ability to decrease glutaminase activity, thereby potentially improving glutamine metabolism in the body.

C. Glutamine Synthetase (GS):

In tandem with the observed changes in Glutamine content, Glutamine synthetase activity exhibited a decrease in all selected brain regions of PTZ-induced rats when compared to control rats. Notably, a significant decline in Pons Medulla and Cerebellum regions was evident in PTZ-induced epileptic rats. Conversely, a noteworthy increase in Glutamine Synthetase activity was discerned in PTZ-induced epileptic rats pre-treated with varying doses of UMB in a dose-dependent manner. Glutamine synthetase, an enzyme pivotal in catalysing the conversion of Glutamate to Glutamine, plays a crucial role in nitrogen and carbon metabolism, amino acid metabolism, and the synthesis of various nitrogen-containing metabolites **(Chahma et al., 2016).** Past research indicates that glutamine synthetase activity is subject to regulation at both transcriptional and post-transcriptional levels. Transcriptional modulation responds to nitrogen availability, along with other regulators such as carbon sources and energy, as well as different stressors. Post-transcriptional regulation involves mechanisms like mRNA stabilization, mRNA editing, and protein-protein interactions **(Kim et al., 2013).**

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D. Glutamate Dehydrogenase (GDH):

The activity of Glutamate Dehydrogenase exhibited a decrease in all selected brain regions of PTZ-induced rats compared to the control group. In contrast, similar to the other three constituents, a significant increase in Glutamate Dehydrogenase activity was observed in PTZ-induced epileptic rats pre-treated with UMB in a dose-dependent manner. These findings align with previous research attributing decreased GDH activity in epileptic rats to elevated Reactive Oxygen Species (ROS) and Nitric Oxide (NO) levels, leading to oxidative damage, disruptions in energy metabolism, and neuronal cell death. Additionally, reduced GDH activity has been linked to heightened Glutamate concentrations, contributing to excessive excitatory neurotransmission and neuronal hyperexcitability **(Vezzani et al., 2015).** Dysfunction in GDH has also been associated with disruptions in the glutamate-glutamine cycle, further contributing to epilepsy-associated excitatory neurotransmission (Notarangelo et al., 2009). These results collectively suggest a positive impact of UMB on all four constituents of the glutamatergic system in PTZ-induced epileptic rats. Nevertheless, comprehensive research is essential to ascertain the efficacy of UMB in greater detail.

CONCLUSIONS:

In conclusion, UMB demonstrates promising neuroprotective effects on the glutamatergic system in PTZ-induced epileptic rats. The observed dose-dependent modulation of key components, including glutamine content, glutaminase activity, glutamine synthetase activity, and GDH activity, highlights UMB's potential as a therapeutic candidate in epilepsy management. Further research is warranted to elucidate the underlying mechanisms and optimize UMB dosage for enhanced efficacy.

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