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Research Article

Histological study of the Toxic Effect of Methotrexate on the Cornu Ammonis pyramidal cells of the Adult Male Albino Rat Hippocampus and the possible Protective Role of Moringa Leaves Extract.

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Abstract:
Purpose: We aimed to detect the ameliorating effects of Moringa leaves extract (MLE) against Methotrexate (MTX) induced hippocampus damage. **Methods:** 50 male Albino rats weighing 150-170 g were divided into equal five groups; Group I: served as a control group, Group II: received MLE orally (300 mg/kg b w) twice a week for four weeks, Group III: injected intraperitoneal with MTX (0.5mg/kg b w) twice a week for four weeks, Group IV: rats received MTX and MLE at the same timeline for four weeks with the same dose and route of administration, Group V: received MTX firstly followed by MLE for four weeks with the same dose and route of administration. **Results**: the histological examination showed that in the control group, and group II there was a normal hippocampal features. Conversely, Group III revealed pronounced alterations, including a contracted CA size and a C-shaped DG with a lost angle, accompanied by pyramidal cell degeneration. In the group IV, the DG angle partially restored. In group V, showed persistent challenges in mitigating MTX induced alterations, with the contracted CA size and C-shaped DG configuration persisting. The immunohistochemical study of P53 immunoreactivity showed that the contrasting findings in P53 expression between the control and MLE-treated groups suggest that MLE may modulate the expression of this tumor suppressor gene in the hippocampus. **Conclusions**: MLE has a therapeutic effect against MTX induced hippocampus damage as evidenced by the stimulation of the pro-apoptotic effect via increasing the P53 expression in the brain tissues.

Keywords: Methotrexate, Moringa, Hippocampus, pyramidal cells, Rats

Introduction

In order to bridge the divide between clinical and population-based cerebral palsy registries and to advance research in the field, the Cerebral Palsy Research Registry was established. We regret to inform you that there is a scarcity of precise data regarding its prevalence in Alexandria, Egypt. One notable opportunity to bridge this knowledge divide and enhance the well-being of individuals with CP is the establishment of an internet-based registry for CP in Alexandria.[1] Laminar organization is uniform throughout the hippocampus's domains. Three distinct layers comprise the CA regions: the polymorphic layer, the pyramidal layer, and the molecular layer[2].

Chemotherapy is a therapeutic approach employed to address various forms of cancer through the inhibition of cancer cell proliferation and eradication. Nevertheless, it results in neurotoxicity. Methotrexate (MTX) is an antifolate, chemotherapeutic, anti-metabolite, and antineoplastic agent[3].

MTX exhibits cytotoxic properties against neoplastic cells through inhibition of cellular metabolism, in conjunction with its immune-suppressive activity. Methotrexate finds widespread application in the treatment of various autoimmune and inflammatory conditions, including cancer (e.g., acute lymphoblastic leukemia), rheumatoid arthritis, and psoriasis[4].

MTX infiltrates cells with the purpose of impeding their proliferation by means of inhibiting the folic acid reductase enzyme. Folate is a critical component in the biosynthesis of DNA and RNA; therefore, methotrexate will impede their synthesis[5].
Conversely, heightened treatment protocols resulted in a concomitant escalation in neurotoxicity. Specifically distressing complications of therapy for survivors are deficits in neurological and cognitive function, which may persist for years following treatment and impair occupational performance or learning. [6]

Neurotoxicity is presumed to occur via direct neuronal injury or disruption of folate homeostasis in the central nervous system. Neurotoxicity has been established through the administration of high doses or protracted low-dose oral MTX, according to numerous studies. The neurotoxic effects include symptoms resembling stroke, encephalopathy, seizures, and aphasia. Involvement of reactive oxygen species (ROS) overproduction in MTX neurotoxicity has been suggested. [7]

TX exerts detrimental effects on the mitochondrial apparatus, leading to the production of an inordinate amount of reactive oxygen species (ROS); this has been linked to its neurotoxic properties.[8] Peroxidation of cellular membranes and damage to cellular macromolecules, which ultimately results in cell mortality, can be initiated by reactive oxygen species (ROS). Moringa Oleifera is a widely distributed edible tree found in subtropical and tropical regions of Asia and Africa. Almost every component of the plant has been employed for its traditional medicinal properties[7].

As a source of natural antioxidants and anti-inflammatory compounds, moringa is of critical importance. As a topical treatment, extract of moringa leaves is applied to skin inflammation brought on by fungal, bacterial, or insect attacks[9].

The tumor suppressor gene P53 is a transient protein. With the greatest frequency of mutations in oncogenesis. It controls the rate at which a number of genes that govern genomic stability, the cell cycle, and apoptosis are translated.[10]

Consequently, the objective of this research endeavor was to ascertain whether Moringa leaf extract (MLE) could mitigate the detrimental effects of methotrexate (MTX) on the hippocampus.

Materials and methods:

Experimental Animals

Fifty male Albino rats, each weighing between 150 and 170 g, were obtained from the animal center of the Faculty of Pharmacy at Deraya University for the purpose of this research. The animals were confined in plastic enclosures with the following environmental parameters: a 12-hour light/dark cycle, a temperature of 22±2ºC, a humidity of ±10%, and unrestricted access to food and water. All procedures involving animals were carried out in accordance with the guidelines outlined in the guide for the care and use of laboratory animals, which received approval number 341/06/2022 from the Institutional Animal Ethics Committee.

Animal groups

There were five distinct types of rats: Group I comprised ten rats and was administered water and sustenance at will for a duration of four weeks as a control group. Group II also consisted of ten rats and was administered Moringa leaf extract (MLE) orally at a dosage of 300 mg/kg b w twice weekly for the same duration of four weeks[11], Group III: Consisted of 10 rats injected intraperitoneal with Methotrexate (MTX) (0.5mg/kg b w) twice a week for four weeks[12], Group IV: Ten rats were administered MTX and MLE concurrently for four weeks at the same time using the same dose and route of administration as described previously. Group V: Ten rats were administered MTX first, followed by MLE for four weeks using the same dose and route of administration as described previously.

Collection of blood and tissue samples

At the end of the experiment, the rats were weighed. Then, animals were sacrificed by

cervical dislocation, and the brains were removed and processed for histopathological and immunohistochemical studies.

Histopathological investigation:
Following fixation in buffered formalin, the brain tissues were encased in paraffin. Five micrometer-thick sections of paraffin were subjected to hematoxylin and eosin (H&E) staining. Following dehydration, the specimens were enumerated, covered, shifted, and scrutinized using an Olympus light microscope (Japan).[13, 14]

Immunohistochemical stainig:
The p-53 protein was immunohistochemically stained utilizing a dilution of 1:100 of the labeling avidin biotin complex (ABC). Following a PBS rinse, the transparencies were subjected to DAB staining in order to ascertain the response color. The tissue sections underwent dehydration, counterstaining with hematoxylin, mounting on a cover slip, and subsequent analysis in five randomly selected fields on each slide using an Olympus light microscope.[15]. The presence of dark brown staining on the epithelial cells of the injured region signifies the expression of p-53. Negative control samples were stained in the absence of primary antibodies (p-53 proteins), with the negative being indicated by a blue color. The immunohistochemical outcomes of p-53 were assessed semi-quantitatively in accordance with the immunostain's IOD values: +1 for feeble, +2 for moderate, +3 for strong, and +4 for intense.

Statistical analysis
The expression of p-53 is indicated by the dark brown staining observed on the epithelial cells of the injured region. Negative control samples, which were stained without primary antibodies (p-53 proteins), were designated with a blue color to denote the absence of the specific antibody. The semi-quantitative evaluation of the immunohistochemical outcomes of p-53 was conducted using the IOD values of the immunostains: +1 denoted weak, +2 moderate, +3 strong, and +4 intensive intensity, respectively.

Results

The effect of moringa leaves extract on the hitological structure of hippocampus exposed to methotrexate

In the control group, the microscopic observations of coronal sections from the hippocampus revealed normal CA3 region a characteristic feature consistent with the anatomical distinctions within the hippocampus. The layers, Stratum Radiatum (R) and Stratum Oriens (O), demonstrated their expected structural integrity (Fig 1).

In group II, CA3 region: highlighted the maintenance of normal cellular morphology, the pyramidal cells (P) in this region exhibited a notable enlargement (Fig 2).
In group III (MTX), CA3 region highlighted a field with degenerated, deeply stained pyramidal cells (P) across the entire region (Fig 3).

In group IV (MTX + MLE Simultaneously), CA3 region highlighted a mixed cellular landscape. Most pyramidal cells (Arrow heads) retained normal morphology, while some exhibited signs of degeneration (Stars) (Fig 4).

In group V, CA3 region highlighted a complex cellular landscape. Degenerated, deeply stained pyramidal cells (P) were marked by Stars, while others displayed a normal morphology, marked by Arrow heads. Both Stratum Radiatum (R) and Stratum Oriens (O) layers demonstrated a coexistence of normal and degenerated cellular structures (Fig 5).

The effect of moringa leaves extract on the P53 immunoreactivity of hippocampus exposed to methotrexate
Group I (Control) revealed faint P53 expression within the pyramidal cells (P) of the hippocampus as shown in figure 6.
Group 2 (Moringa treated group) showed a robust positive reaction for P53 within the pyramidal cells (P). This outcome indicates a heightened expression of P53 in response to MLE treatment, suggesting a notable influence of Moringa Leaves Extract on the immunereactivity of P53 within the examined hippocampal pyramidal cells as shown in figure 7.
Group 3 (MTX treated) showed an inconspicuous reaction for P53 within the pyramidal cells (P) indicating a few immunoreactivity pattern within the examined hippocampal pyramidal cells. The faint expression of a substantial P53 reaction in this

context implies potential regulatory effects of Methotrexate on P53 expression in the hippocampus as shown in figure 8.

Group 4 (MTX and Morina co-treated) revealed a discernible yet moderate expression of P53 within the pyramidal cells. The presence of this intermediate level of P53 expression underscores the potential interplay between the two treatments and hints at a regulatory influence of Moringa Leaves Extract on P53 immunoreactivity within the examined hippocampal pyramidal cells as showin in Figure 9.

In group 5 (MTX then Moringa), the staining for P53 immunoreactivity unveils a mild of positive reaction within the pyramidal cells (P). This observation denotes a little immunoreactivity for P53 in response to the sequential treatment, indicating a distinct regulatory influence of Moringa Leaves Extract subsequent to Methotrexate administration as shown in figure 10.

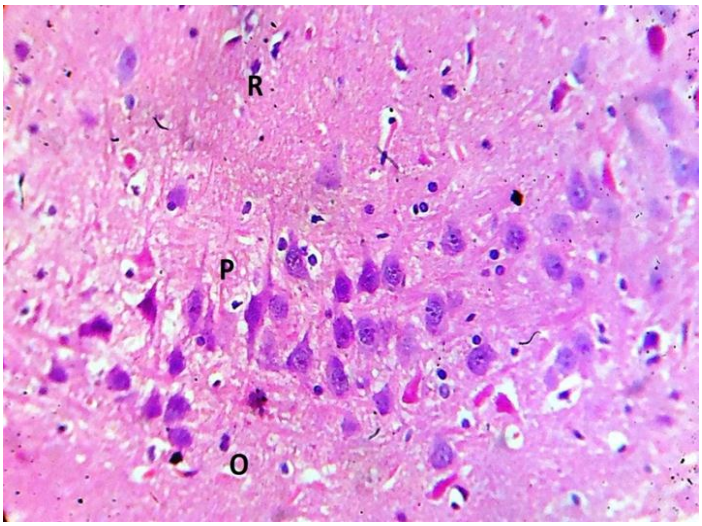

Figure 1 (Group1): A photomicrograph of control group showing coronal section of Cornu Ammonis CA3 of adult male albino rat Hippocampus. Normal structure of pyramidal cells (p) is observed. Stratum Radiatum (R), Stratum Oriens (O). (H&E X400)

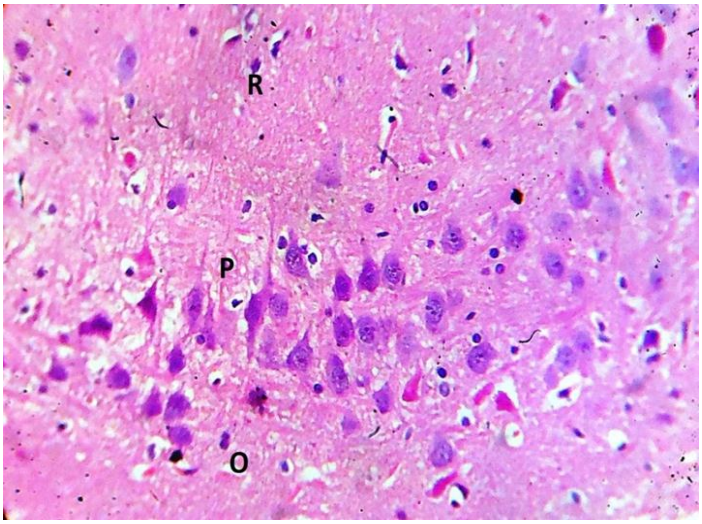

Figure 2 (Group 2): A photomicrograph of Moringa Leaves Extract (MLE) treated group showing coronal section of hippocampus of adult male albino rat. and CA3 showed normal structure of pyramidal cells (P) with vesicular nucleus. Stratum Radiatum (R), Stratum Oriens (O) (H&E X400)

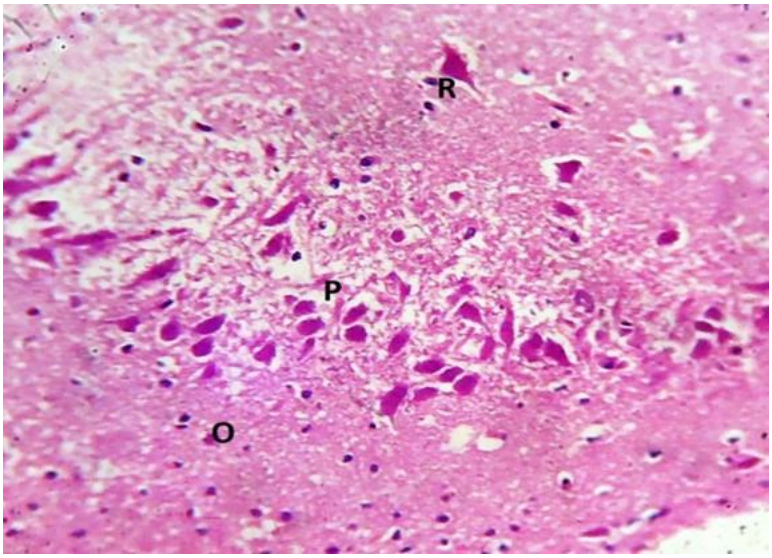

Figure 3 (group 3): A photomicrograph of Methotrexate treated group (MTX) showing coronal section of Cornu Ammonis CA3 of adult male albino rat Hippocampus. Degeneratedt deeply stained pyramidal cells (P) are noticed in the whole field. Stratum Radiatum (R), Stratum Oriens (O) (H&E X400)

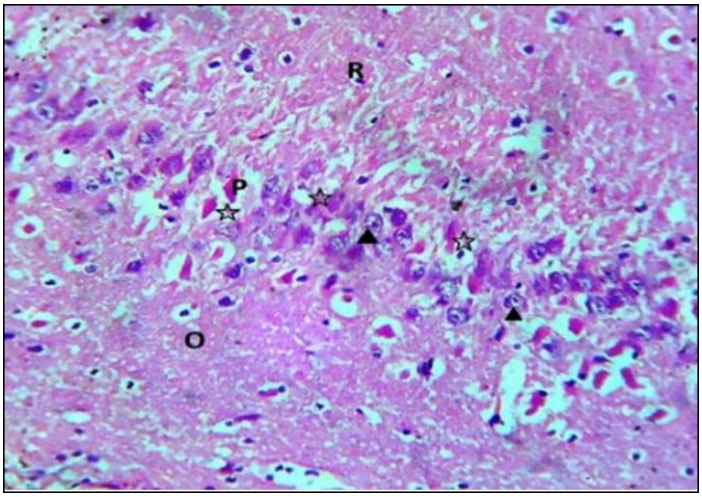

Figure 4 (Group4): A photomicrograph of Methotrexate-Moringa simultaneous treated group showing coronal section of hippocampus and showed *CA3* of adult male albino rat. Also, most of pyramidal cells (P) are still normal (Arrow heads) with some degenerated cells (Stars) (H&E x400)

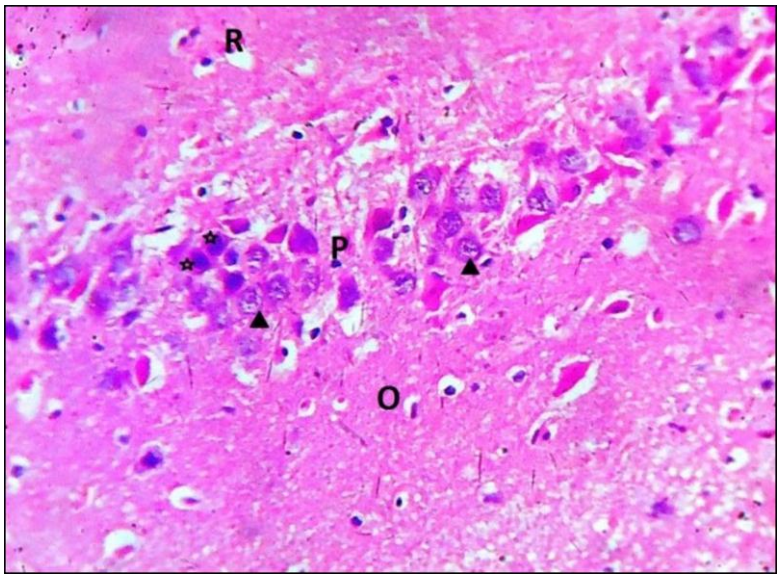

Figure 5 (Group 5): A photomicrograph of Methotrexate treated group followed by Moringa Leaves Extract showing coronal section of Cornu Ammonis CA3 of adult male albino rat Hippocampus. Degenerated deeply stained pyramidal cells (P) marked by Stars. Other normal cells are marked by arrow heads (H&E X400)

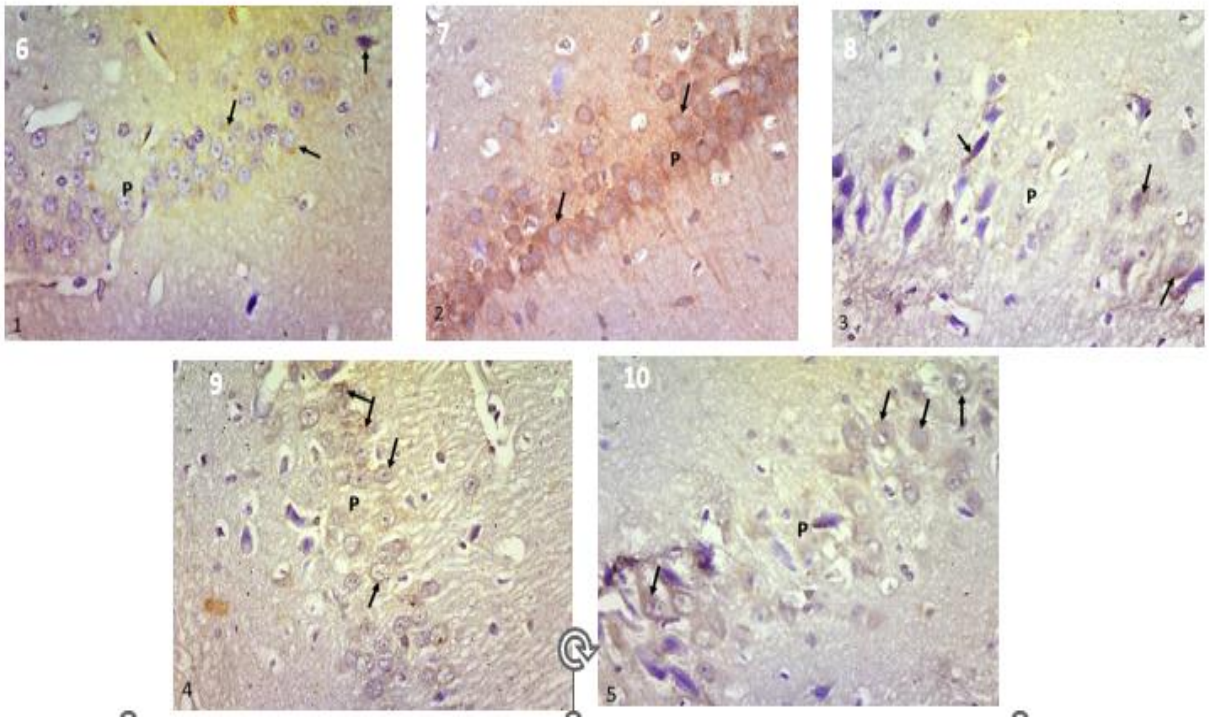

Fig 6: A photomicrograph showing coronal section of adult male albino rat hippocampus of control group. Some p53 immune positive pyramidal neurons with faint expression (arrows) is observed. Fig 7: Extensive p53 immune-positivity in most of pyramidal neurons (arrows) is noticed in MLE group. Figure 8: Few p53 immune positive pyramidal neurons with faint expression (arrows) are seen in MTX group. Figure 9: Positive p53 immune expression in many pyramidal neurons (arrows) is detected in MTX-MLE simultaneous treated group.
Figure 10: Few pyramidal neurons with mild positive p53 immune expression in (arrows) is remarked in MTX treated group followed by MLE (X400).

Discussion

The histological examination of the group that received MLE offers significant contributions to our understanding of the possible impacts of MLE on the cellular structure of the hippocampus. It is worth mentioning that the intact pyramidal cells in both the CA1 and CA3 regions provide evidence for the protective effect of MLE. This result is consistent with Hussein's research.[16], which identified ameliorating effects of MLE against hippocampus injury induced by amethopterin (AMP), thereby demonstrating that the hippocampus in the MLE group maintained its normal architecture.

Similarly, Mohamed et al.,[17] observed The histological structure of the cerebral cortices in the control group, which was ostensibly treated with Moringa Oleifera (MO), was observed to be normal. The results of our research, which imply that MLE may have neuroprotective properties on the hippocampus, are consistent with the conclusions drawn in Mohamed's study.[17]

Group III (MTX) histological observations demonstrate that MTX has a detrimental effect on the architecture of the hippocampus. The observed reduction in the extent of the CA, modification in the configuration of the dentate gyrus (DG), and degeneration of pyramidal cells collectively indicate that MTX has induced structural changes. Consistent with these results, prior research has documented comparable adverse impacts of MTX on neuronal structures.

Hussein's study [16], which AMP was employed to induce hippocampal injury in rats, and an extensive array of histopathological alterations

were observed. These included vacuolated neurocytes, degenerated pyramidal cells, and damaged neurons. Our findings in Group III corroborate these results, emphasizing the MTX-induced degeneration of pyramidal cells. Moreover, Ahmed et al.,[3] noted disarrangement of hippocampal layers, degeneration, and decreased thickness in the pyramidal cell layer in a MTX-treated group. This aligns with our findings, suggesting that MTX induces structural alterations, particularly in the pyramidal cell layer of the hippocampus.

Similarly, Mohamed et al.,[17] reported Destructive alterations were observed in the molecular layer and purkinje cells of MTX-treated rats' cerebellar sections. Although our research was specifically concerned with the hippocampus, the correlation between MTX's broad impact on the central nervous system and the destructive changes observed in neuronal structures serves to support this notion.

Additionally, Soliman et al.,[9] observed significant hydropic degeneration, focal coagulative necrotic areas, and multiple pyknotic nuclei in the MTX group. These findings parallel our observations, further supporting the notion that MTX induces histological changes indicative of cellular damage.

Our findings in Group III are consistent with and add to the current corpus of evidence that emphasizes the adverse impacts of MTX on structures in the hippocampus and cerebellum. The observed alterations in cellular architecture, degeneration, and necrotic changes in multiple studies emphasize the critical nature of comprehending and addressing the potential neurotoxicity that may result from the administration of MTX.

Our histological findings in Group IV (MTX + MLE Simultaneously) indicate that MLE may have the capacity to alleviate specific aspects of the hippocampal alterations induced by MTX. A mixed cellular morphology and partial restoration of the dentate gyrus (DG) angle are observed despite the persistence of CA size contraction. This suggests that administering MLE and MTX concurrently may provide some protection against alterations induced by MTX, specifically in the DG region.
In Group V, on the other hand (MTX followed by MLE), careful histological examinations reveal ongoing difficulties in resolving MTX-induced changes in the hippocampus. Based on the diminished dimensions of the CA regions, the mixed cellular morphology, and the preserved C-shaped DG configuration, it can be inferred that the use of MLE subsequent to MTX exposure might have restricted effectiveness in completely halting or reversing the hippocampal changes induced by MTX. By juxtaposing our findings with Hussein's research [16], where rats in the 4th group received AMP and MLE simultaneously, moderate tissue changes, including mild diffuse vacuolar degeneration and a few apoptotic astrocytes, were observed.

In the 5^{th} group, where rats were exposed to AMP for 4 weeks followed by MLE treatment for another 4 weeks, a good degree of improvement with more or less normal neuronal structure was noted, albeit with mild neuronal atrophy[16]. These findings align with our observations, suggesting that simultaneous administration of MLE may have a mitigating effect, while sequential treatment may have limitations in fully reversing MTX-induced alterations.

Also, Mohamed et al.,[17] An examination was conducted on the cerebral cortices of subjects receiving prophylactic co-treatment (MO/CoCl2) and therapeutic co-treatment (CoCl2/MO). The potential preventive effects of MLE when administered prior to and simultaneously with a damaging agent are highlighted by these results, lending credence to the notion that timing may be critical to the effectiveness of MLE.

In addition, Soliman et al.,[9] It was documented that the MTX plus Moringa Oleifera Leaf Extract (MOLE) group demonstrated a typical histological structure, which is consistent with the results we observed in Group IV. Simultaneous administration of MLE or MOLE with MTX may have protective effects on histological structures, according to both studies, which highlights the potential of MLE as a protective agent against MTX-induced changes.
P53 immunoreactivity detection of apoptotic cells within the hippocampus functions as a method to identify and quantify cells undergoing programmed cell death for the purposes of our research. The potent technique

of immunohistochemistry enables the localization and visualization of particular proteins within tissues. The expression of P53, which is indicative of the activation of apoptotic pathways, is the primary focus in this instance.

In Group I (Control), our immunohistochemical analysis of P53 immunoreactivity in the The pyramidal cells of the hippocampus of adult male albino rats exhibited a modest indication of expression. The aforementioned observation revealed that the control group had a baseline condition marked by almost no P53 immunoreactivity, indicating that the apoptotic pathway is minimally activated under typical conditions. On the contrary, a conspicuous positive reaction for P53 was observed in the pyramidal cells of Group II (treated with MLE), indicating an increased expression of P53 as a result of the MLE treatment. The observed result suggests that MLE significantly affected the immunoreactivity of P53 in the pyramidal cells of the hippocampus under investigation.

The observed differences in P53 immunoreactivity between our study and Hussein's [16] may arise due to discrepancies in experimental protocols, concentrations, or our investigation's particular emphasis on the hippocampus. The intricate nature of the interactions between MLE and P53 expression is underscored by inconsistencies in the results; therefore, a comprehensive analysis of the experimental conditions is required to ensure precise interpretation [16].

The heightened P53 immunoreactivity in our MLE-treated group suggests a potential modulatory effect of MLE on apoptotic pathways. The contrasting findings with Hussein's study [16] encourage a more thorough investigation into the particular circumstances that could impact the expression of P53 in reaction to MLE. The aforementioned distinctions underscore the importance of employing standardized methodologies and meticulously evaluating experimental parameters when contrasting findings across investigations. In general, the aforementioned findings serve to enhance our comprehension of the complex correlation between P53 immunoreactivity and MLE. This emphasizes the significance of situating results within particular experimental frameworks.

We concentrated our immunohistochemical analysis on P53 immunoreactivity in the hippocampus of adult male albino rats in Group 3 (MTX-treated rats). The photomicrograph that resulted depicted a coronal section of the subject, and the examination unveiled a subtle P53 reaction occurring within the pyramidal cells. This observation implies that the examined hippocampal pyramidal cells exhibited a decreased or negligible expression of P53 in response to MTX treatment, indicating the presence of a distinct immunereactivity pattern.

In contrast to our findings, Hussein's study[16] reported a significant positive response for the P53 gene in the group treated with AMP. The observed inconsistency indicates a distinction in the control of P53 expression between treatments with AMP and MTX, thereby emphasizing the drug-specific impacts on apoptotic pathways Ahmed et al.,[3] observed an intense positive immunoreactive expression of caspase in the hippocampus of the MTX-treated group, indicating heightened activation of apoptotic pathways.

The observed differences in P53 immunoreactivity between our study and Hussein's [16], Ahmed et al. [3], and El-ghazouly et al.,[18] Diverse regulatory mechanisms and responses to MTX-induced cellular stress have been suggested by research. Contrary to findings from other research that suggest MTX induces robust positive responses for P53 and upregulation of caspase, our study suggests that P53 expression is minimal or nonexistent. These disparities emphasize the necessity of taking into account the effects of MTX that are specific to each context and underscore the need for additional research to clarify the exact mechanisms that govern the observed differences in P53 immunoreactivity.

Our immunohistochemical analysis of Group 4, which received co-treatment with MTX and Moringa, indicated a noticeable albeit moderate level of P53 expression in the pyramidal cells of the hippocampus. This observation indicates that the concurrent administration of MTX and MLE resulted in a moderate upregulation of P53. The identification of this moderate degree of P53 expression suggests that MLE may exert a regulatory effect on the immunoreactivity of

P53. This finding enhances our complex comprehension of the reciprocal mechanisms by which MTX and MLE impact cellular processes in the hippocampus. In a similar vein, a marginal positive response for P53 was detected within the pyramidal cells of Group 5 (MTX followed by Moringa), indicating a small amount of immunoreactivity in reaction to the consecutive treatment. The observation of mild P53 expression provides significant contributions to our understanding of the potential mitigating effects of MLE subsequent to MTX-induced changes, highlighting the intricate dynamics of their interaction within the hippocampus's cellular processes.

Conclusion

In conclusion, these variations underscore the complexity of the interactions between MTX, MLE, and cellular processes within the hippocampus. Differences in dosages, timing, and the specific pathways targeted by each treatment may contribute to the observed variations in P53 immunoreactivity.

Declarations

Ethics approval:

All care and procedures adapted for the present study was according to the NIH guide, and animal used the approval of the Institutional Animal Ethics Committee of Faculty of Medicine, Minia University.

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Declarations of interest: none

Consent for publication:

Not applicable

Availability of data and materials:

All data generated or analyzed during this study are included in this published article

Competing Interests:

The authors declare that they have no competing interests.

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