นิพนธ์ต้นฉบับ . Original Article

การศึกษาผลของสารสกัดจากลูกไหน ต่อความบกพร่องของความจำที่ถูกเหนี่ยวนำด้วยยาเมโธเทรกเซท ในหนูแรทตัวเต็มวัย

รติมา คูณหอม[1,2], ปุญชรัสมิ์ ปานนิล[1,2], อนุสรา อารณะโรจน์[1,2], นัตยา ศรีตะวัน[1,2], จริยา อำคา เวลบาท[1,2], ระวิวรรณ์ เจริญทรัพย์[3,4], อภิวัฒน์ ศิริโชติ[1,2*]

[1]สาขาวิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย

[2]กลุ่มวิจัยการสร้างเซลล์ประสาท มหาวิทยาลัยขอนแก่น จ.ขอนแก่น ประเทศไทย

[3]ศูนย์นวัตกรรมสมุนไพรครบวงจร มหาวิทยาลัยแม่ฟ้าหลวง จ.เชียงราย ประเทศไทย

[4]สำนักวิชาการแพทย์บูรณาการ มหาวิทยาลัยแม่ฟ้าหลวง จ.เชียงราย ประเทศไทย

The Impact of *Prunus domestica* L. Crude Extract on Methotrexate-induced Cognitive Deficits in Adult Rats

Ratima Koonhom[1,2], Puncharatsm Pannin[1,2], Anusara Aranarochana[1,2], Nataya Sritawan[1,2], Jariya Umka Welbat[1,2], Rawiwan Charoensup[3,4], Apiwat Sirichoat[1,2*]

[1]Department of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

[2]Neurogenesis Research Group, Khon Kaen University, Khon Kaen, Thailand

[3]Medicinal Plant Innovation Center of Mae Fah Luang University, Mae Fah Luang University, Chiang Rai, Thailand

[4]School of Integrative Medicine, Mae Fah Luang University, Chiang Rai, Thailand

Received: 2 December 2024/ Review: 2 December 2024/ Revised: 20 January 2025/ Accepted: 22 January 2025

บทคัดย่อ

หลักการและวัตถุประสงค์: เมโธเทรกเซท (methotrexate, MTX) เป็นยาเคมีบำบัดที่นิยมใช้ในการรักษาโรคมะเร็งชนิดต่าง ๆ การศึกษาที่ผ่านมาพบว่าการรักษาด้วยยาเคมีบำบัด ส่งผลทำลายเซลล์ประสาทต้นกำเนิด (neural progenitor cells) ในสมองส่วน hippocampus ทำให้เกิดภาวะความทรงจำบกพร่อง นอกจากนี้ MTX มีผลทำให้เกิดการลดลงของการทำงานของระบบสารต้านอนุมูลอิสระในร่างกาย *Prunus domestica* L., (PD) หรือลูกไหน อยู่ในตระกูลพรุน มีการศึกษาพบว่า PD มีสารยับยั้งเซลล์มะเร็ง ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบ และฤทธิ์ป้องกันต่อเซลล์ประสาท

วิธีการศึกษา: หนูแรทเพศผู้สายพันธุ์ Sprague-Dawley ถูกสุ่มออกเป็น 8 กลุ่ม ได้แก่ กลุ่มควบคุมได้รับน้ำยา, MTX (75 มก./กก.), PD (75,100 และ 150 มก./กก.) และ MTX (75 มก./กก.) + PD (75,100 และ 150 มก./กก.) จากนั้นหนูได้ถูกทดสอบความจำโดยวิธี novel object location (NOL) และ novel object recognition (NOR)

ผลการศึกษา: ผลการทดสอบพฤติกรรมโดยวิธี NOL และ NOR พบว่า สารสกัดจากลูกไหนและยา MTX ไม่มีผลกระทบต่อการเปลี่ยนแปลงน้ำหนักตัว และกิจกรรมการเคลื่อนที่ ภายหลังจากการได้รับยาพบว่า ค่าดัชนีการจำแนกของสัตว์ทดลองทุกกลุ่มมีค่าต่างจากศูนย์อย่างมีนัยสำคัญทางสถิติ ยกเว้นกลุ่มที่ได้รับยา MTX ก่อให้เกิดความบกพร่องทางด้านความจำ สารสกัดจากลูกไหนสามารถลดความบกพร่องที่เกิดจาก MTX ได้

สรุป: ยา MTX ส่งผลต่อการเรียนรู้และความจำ ซึ่งภาวะบกพร่องดังกล่าวส่งผลดีขึ้นเมื่อได้รับสารสกัดจากลูกไหน

คำสำคัญ: ลูกไหน, เมโธเทรกเซท, ความจำ

*Corresponding author: Apiwat Sirichoat, E-mail: apiwsi@kku.ac.th

Abstract

Background and Objective: Methotrexate (MTX) is a chemotherapy drug used to treat various cancers. MTX is toxic to neural progenitor cells in the hippocampus and affects hippocampus dependent behavior. *Prunus domestica* L. (PD), a species of plum belonging to the Rosaceae family and Prunus genus, is rich in phenolic compounds. These phenolic-rich fruits contain a mixture of polyphenolic compounds that can exert several biological effects, including antibacterial, antioxidant, and anti-inflammatory properties.

Methods: Male Spraque-Dawley rats were divided into eight groups: vehicle, MTX (75 mg/kg), PD (75, 100 and 150 mg/kg), and MTX + PD (75 mg/kg plus 75, 100 and 150 mg/kg). After drug administration, spatial and recognition memories were investigated using the novel object location (NOL) and the novel object recognition (NOR) tests, respectively.

Result: The NOL and NOR tests analysis indicated that neither PD extract nor MTX adversely affected body weight or locomotor activity. Following drug administration, the discrimination index (DI) of all experimental groups differed significantly from zero, except for the MTX group. This suggests that MTX induced impairments in spatial and recognition memory, and PD extract was shown to mitigate the memory deficits caused by MTX.

Conclusion: These results showed that MTX treatment caused learning and memory deficits. These impairments were alleviated by co-treatment with PD.

Keywords: *Prunus domestica* L., Methotrexate, memory

Introduction

Adult neurogenesis is a process of neural stem/progenitor cells (NSCs) that occurs in two regions of the brain: the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle[1]. The production rate of neurogenesis is regulated and affected by various factors such as physical activities, stress, aging and treatment with certain drugs[2].

Methotrexate (MTX), a chemotherapeutic drug, is widely used in the treatment of various cancers[3] . MTX acts as a folic acid antagonist, inhibiting the activity of dihydrofolate reductase (DHFR), an enzyme responsible for converting folic acid into tetrahydrofolate (THF). THF is a crucial coenzyme in the synthesis of purine nucleotides[4]. As the levels of THF decrease, the synthesis of purine and thymidylate, which are essential for DNA and RNA synthesis is disrupted[5]. Additionally, MTX can induce neurotoxic effects that affect learning and memory within the brain system.

Prunus domestica L., (PD) is a valuable fruit belonging to the *Rosaceae* family. *P. domestica* possesses bioactive properties, including antioxidant, anticancer, anti-inflammatory and neuroprotection effects[6–8]. However, there are a few reports on the effect of PD extract on cognition and its impact remains largely unclear.

Therefore, the aim of this study is to determine the protective effect of PD extract against memory impairments induced by MTX in adult rats MTX. The novel object location (NOL) and the novel object recognition (NOR) tests were used to investigate spatial and recognition memory, respectively.

Methods

Plant material and extraction

Fresh PD fruits were harvested in late February 2021 from Mae Chan District, Chiang Rai province, Thailand, located at latitude/longitude coordinates 20° 11’ 57” N/99° 53’ 5” E. The crude extract was prepared at the Medical Plants Innovation Center, Mae Fah Luang University, Thailand. Each fruit was carefully selected, washed with distilled water, and then soaked in 95% ethanol for 7 days. The extract was then filtered using filter paper, and the supernatant was concentrated with rotary evaporator. The final extract was stored at -20° C until use.

Animals

Sixty-four adult male Sprague-Dawley rats (age: 4-6 weeks, body weight: 180-200 g) were obtained from the Nomura Siam International Co., Ltd. Pathumwan, Bangkok. The experimental animal model used in this study complied with ethical guidelines and was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Record No. IACUC-KKU-80/64).

Rats were housed in 4 animals per plastic cage at a controlled temperature (23 ± 2 °C) with a 12-hour light-dark cycle. Each rat had free access to standard food and water *ad libitum*.

Drugs administration and experimental design

The rats were acclimated for 7 days before the procedures began and divided into 8 groups (n=8/group). All rats were treated for 15 days.

The vehicle group, rats received 0.9% normal saline solution via intraperitoneal (i.p.) injection and distilled water by oral gavage.

The MTX group, rats were administered MTX at 75 mg/kg body weight (BW) via intravenous (i.v.) injection on day 8 and 15.

The PD extract groups (75, 100 and 150 mg/kg/day): Rat received *P. domestica* 75, 100, 150 mg/kg/day dissolved in distilled water by oral gavage.

The MTX + PD extract (75, 100 and 150 mg/kg/day): Rat received MTX (75mg/kg, i.v. injection) and were co-administered PD extract at doses of 75, 100 and 150 mg/kg/day via oral gavage, respectively.

Rats that received MTX were also administered with leucovorin (LCV) 6 mg/kg BW 18 hours post-MTX and at 3 mg/kg BW at 26, 42, and 50 hours via i.p. injection. LCV enters cells via the reduced folate carrier (RFC), similar to MTX, and subsequently

competes with MTX during its transformation into polyglutamate. As a result, LCV can mitigate the toxic effects of MTX.

At the end of the treatment, the rats were evaluated using the novel object location (NOL) and novel object recognition (NOR) tests over 4 days.

Behavioral tests

Behavioral tests were employed to assess cognitive function, spatial memory, and recognition memory using the NOL and NOR tests, both conducted before and after drug administration. The experimental setup consisted of an open arena with dimensions of 50 x 50 x 50 cm and plastic bottles filled with water. Data recording was conducted using video tracking software (EthoVision®, XT version 12, Noldus, Wageningen, Netherlands) for both tests.

The protocol for both the NOL and NOR tests consisted of three phases: habituation, familiarization, and choice trials. Prior to testing, rats were habituated by being allowed to freely explore an empty arena for 30 minutes. On a subsequent day, they underwent a second habituation session, exploring the arena without any objects for 3 minutes. During the familiarization phase of both tests, two identical objects were placed in different locations within the arena, and each rat was allowed 3 minutes to explore them. Afterward, the rats were returned to their cages. In the NOL test, following a 15-minute interval, one object was left in its original position (familiar location; FL), while the other was moved to a new location (novel location; NL). Each rat was then placed back in the arena for 3 minutes to explore the objects.

In the choice phase of the NOR test, one familiar object (FO) and one novel object (NO) was placed at the same location as during the familiarization trial. The rats were then allowed to explore objects in the arena for 3 minutes.

The exploration time for each object or location was meticulously recorded and calculated for each trial of both behavioral tests. The difference in exploration time between the novel and familiar objects or locations were used to compute the discrimination index (DI). The DI, regarded as a measure of memory sensitivity, is crucial in assessing object recognition in rats, as it can be evaluated based on the difference in exploration time between novel and familiar objects. To eliminate potential olfactory cues, the arena and objects were thoroughly cleaned with 20% ethanol after each trial.

Statistical analysis

Total exploration time in both NOL and NOR tests was analyzed using one way ANOVA followed by Bonferroni's post hoc test. The discrimination index was analyzed using a one-sample t-test. Two-way repeated measures ANOVA was used to evaluate differences in body weight between a group.

All statistical analyses were performed using GraphPad Prism (Version 9.0; Graphpad Software Inc., San Diego, CA, USA). All data were presented as mean ± SEM, and the comparison was made using the student's *t*-test and ANOVA, A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Effects of MTX and PD on weight gain

Throughout the experiment, the daily body weight of the ratss was recorded. No significant differences in body weight were observed among the groups ($p>0.05$). These results suggest that neither MTX nor PD had any adverse effects on body weight (Figure 1).

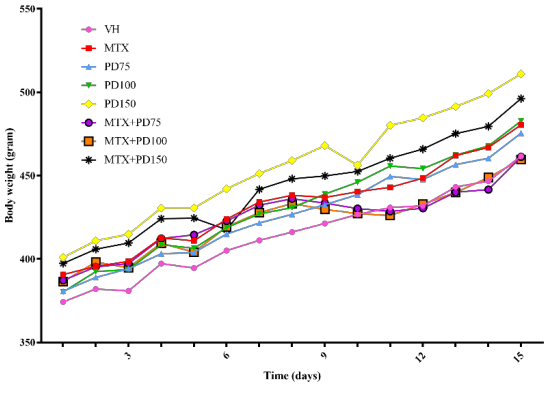

Fig. 1 Body weight of rats throughout the drug administration.

Effects of MTX and PD crude extract on locomotor activities

Total exploration time refers to the cumulative time that rats spent exploring objects during both the familiarization and choice trials. The findings showed no significant differences in total exploration time in either the NOL or NOR tests, both before and after drug administration ($p > 0.05$ Figure 2 and 3). These results indicate that the drug administration did not have a detrimental effect on locomotor activity.

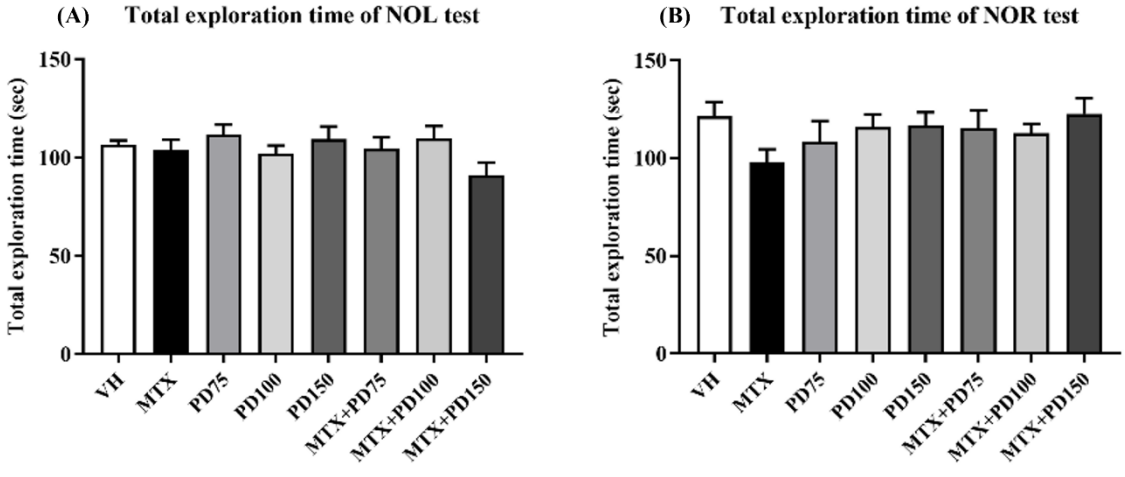

Fig. 2 Total exploration time of all rats in the NOL (A) and NOR (B) tests before drug administration.

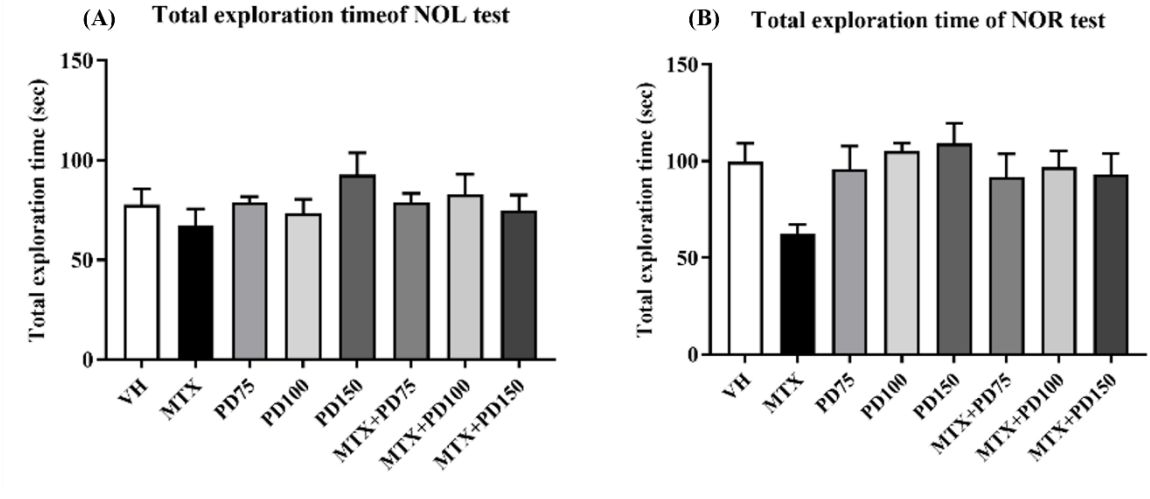

Fig. 3 Total exploration time of all rats in the NOL (A) and NOR (B) tests after drug administration.

Effects of MTX and PD crude extract on discrimination Index (DI)

The DI measures the time spent by animals exploring novel versus familiar objects during the choice trial in both the NOL and NOR tests. Before drug administration, there were significant differences in DI between groups in either the NOL or NOR tests ($p<0.05$, according to one-way ANOVA, as shown in Figure 3A and 3B), indicating that all groups were able to discriminate between novel and familiar objects.

However, after drug administration, the DI of the vehicle group, all PD groups, and the co-treatment group remained significantly higher than zero ($p<0.05$, Figure 4A and 4B). In contrast, the DI of the MTX group did not differ from zero ($p>0.05$, Figure 5A and 5B), suggesting that MTX led to cognitive impairment. Notably, in the PD-treated groups, the time spent investigating novel objects increased, indicating that PD has the potential to prevent and alleviate MTX-induced cognitive impairment.

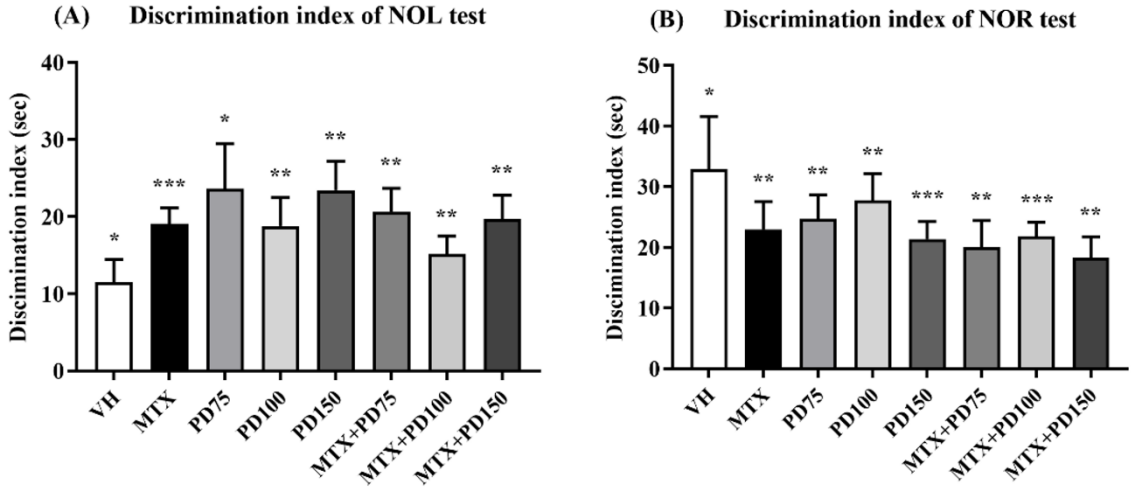

Fig. 4 Before drug administration, the DI of the rats in the NOL (A) and NOR (B) tests. (*p<0.05, ** p<0.01, and *** p<0.001 significant differences compared to zero)

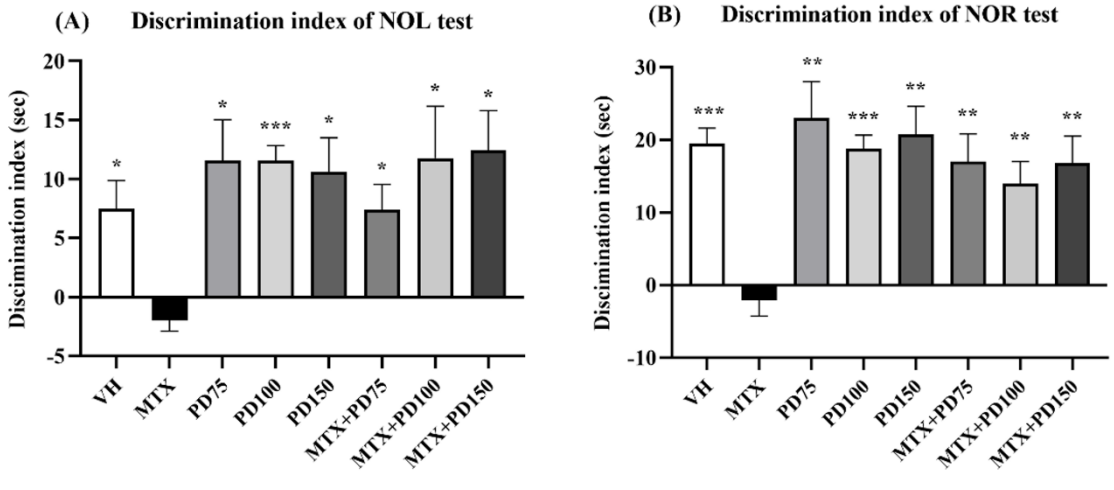

Fig. 5 The DIs of the rats in the NOL (A) and NOR (B) tests after drug administration (*p<0.05, ** p<0.01, and *** p<0.001 significant differences compared to zero)

Discussion

The present study, PD crude extract demonstrated positive effects in the MTX-induced rat model, effectively preventing cognitive impairment. This consistent with previous research, which has shown that PD play a beneficial role in supporting learning and memory[8,9].

There was no significant difference in body weight among all groups. In this study, the body weight of rats in the MTX group and the co-treatment group did not differ significantly from those in other groups. This contrasts with previous studies, which reported a decrease in body weight in MTX-treated rats. Additionally, prior research has shown that treatment with PD did not result in significant changes in the rats' body weight between the beginning and end of the experiment[8].

MTX is a folate antagonist widely used in the treatment of various cancers. Numerous studies have shown that high dose of MTX can induce cognitive deficits[10] following a single intravenous injection of MTX (37.5-300 mg/kg. MTX promotes the formation of reactive oxidative species (ROS), leading to

oxidative stress. Additionally, MTX disrupts DNA and RNA synthesis by inhibiting the Dihydrofolate reductase (DHFR) reaction, which ultimately leads to cell apoptosis, contributing to negative effects on memory and cognition. In this study, behavioral tests were used to evaluate changes in memory related mechanisms, allowing for the assessment of behavior linked to specific brain regions. Animals with memory impairment typically showed a lack of interest and an inability to discriminate between objects or locations[11,12]. The NOL and NOR tests were selected because they do not involve external stimuli, rewards, or emotional stress, allowing to naturally explore based on their innate preference for novelty[13,14].

In this study, behavioral testing revealed that total exploration time did not differ significantly among groups in either test, indicating that the drug treatment did not negatively impact the rats' mobility or locomotion ability. The rats co-treated with PD extract demonstrated the ability to discriminate between objects and locations in the NOL and NOR tests. This finding aligns with earlier reports suggesting that PD extract treatment can alleviate MTX-induced memory deficits[15,16]. Furthermore, the DI in the NOL and NOR tests was significantly different from zero in the MTX-treated group, indicating that the rats experienced spatial and recognition memory impairments following MTX administration. These results reinforce previous findings that chemotherapy can lead to spatial memory deficits in rat models.[17,18] These studies are limited as they only assess the acute or extremely short-term effects of chemotherapy on cognition (e.g. within 1 month of treatment. MTX has been shown to cause abnormality of cell division and increase cell apoptosis by inducing toxicity in neural stem cells within the subgranular zone (SGZ) of the dentate gynes (DG) in hippocampus, leading to memory system impairment[19]. In contrast, rats treated with both MTX and PD crude extract were able to discriminate between the objects in both NOL and NOR tests, as indicated by positive discrimination index values significantly higher than zero. This is consistent with previous research, which has demonstrated that the antioxidant effects of phenolic phytochemicals in plums inhibit enzymes responsible for superoxide production, contribute to their positive effects on learning and memory[8,20]. These findings support the conclusion that PD can prevent and ameliorate memory deficit caused by MTX.

Conclusion

This study demonstrates that MTX can adversely impact spatial and recognition memory in adult rats. However, the administration of PD extract, either alone or in combination with MTX, can help mitigate memory deterioration. These findings suggest that PD extract holds potential as a therapeutic agent to alleviate MTX-induced memory impairment in rats.

Acknowledgement

The authors would like to thank the Invitation Research from Faculty of Medicine (IN66060), Khon Kaen University for financial support.

References

1. Bond AM, Ming GL, Song H. adult mammalian neural stem cells and neurogenesis: five decades later. Cell Stem Cell 2015;17(4):385-95. doi:10.1016/j.stem.2015.09.003
2. Kitabatake Y, Sailor KA, Ming GL, Song H. Adult neurogenesis and hippocampal memory function: new cells, more plasticity, new memories? Neurosurg Clin N Am 2007;18(1):105-13. doi:10.1016/j.nec.2006.10.008
3. Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP. Mechanisms of action of methotrexate. Immunopharmacology 2000;47(2-3):247-57. doi:10.1016/s0162-3109(00)00189-2
4. Baik JY, Han HJ, Lee KH. DNA double-strand breaks affect chromosomal rearrangements during methotrexate-mediated gene amplification in Chinese hamster ovary cells. Pharmaceutics 2021;13(3):376. doi:10.3390/pharmaceutics13030376

5. Xie L, Zhao T, Cai J, Su Y, Wang Z, Dong W. Methotrexate induces DNA damage and inhibits homologous recombination repair in choriocarcinoma cells. Onco Targets Ther 2016;9:7115-22. doi:10.2147/OTT.S116387
6. Kayano S, Kikuzaki H, Yamada NF, Aoki A, Kasamatsu K, Yamasaki Y, et al. Antioxidant properties of prunes (Prunus domestica L.) and their constituents. Biofactors 2004;21(1-4):309-13. doi:10.1002/biof.552210160
7. Miljić U, Puškaš V, Velićanski A, Mašković P, Cvetković D, Vujić J. Chemical composition and *in vitro* antimicrobial and cytotoxic activities of plum (*Prunus domestica* L.) wine: composition and functional properties of plum wine. J Inst Brew 2016;122(2):342–9. doi:10.1002/jib.329
8. Shahidi S, Setareye S, Mahmoodi M. Effect of Prunus domestica L. (mirabelle) on learning and memory in mice. Anc Sci Life 2013;32(3):139-43. doi:10.4103/0257-7941.122996
9. Shukitt-Hale B, Lau FC, Joseph JA. Berry fruit supplementation and the aging brain. J Agric Food Chem 2008;56(3):636-41. doi:10.1021/jf072505f
10. Seigers R, Schagen SB, Beerling W, Boogerd W, van Tellingen O, van Dam FS, et al. Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat. Behav Brain Res 2008;186(2):168-75. doi:10.1016/j.bbr.2007.08.004
11. Faraji J, Lehmann H, Metz GA, Sutherland RJ. Rats with hippocampal lesion show impaired learning and memory in the ziggurat task: a new task to evaluate spatial behavior. Behavioural Brain Research 2008;189(1):17–31. doi: 10.1016/j.bbr.2007.12.002
12. Wenk GL. Assessment of spatial memory using the radial arm maze and Morris water maze. Curr Protoc Neurosci 2004;Chapter 8:Unit 8 5A. doi:10.1002/0471142301.ns0805as26
13. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process 2011;13(2):93-110. doi:10.1007/s10339-011-0430-z
14. Vogel-Ciernia A, Wood MA. Examining object location and object recognition memory in mice. Curr Protoc Neurosci 2014;69(1):1-17. doi:10.1002/0471142301.ns0831s69
15. Sirichoat A, Anosri T, Kaewngam S, Aranarochana A, Pannangrong W, Wigmore P, et al. Neuroprotective properties of chrysin on decreases of cell proliferation, immature neurons and neuronal cell survival in the hippocampal dentate gyrus associated with cognition induced by methotrexate. Neurotoxicology 2022;92:15-24. doi:10.1016/j.neuro.2022.06.010
16. Sirichoat A, Krutsri S, Suwannakot K, Aranarochana A, Chaisawang P, Pannangrong W, et al. Melatonin protects against methotrexate-induced memory deficit and hippocampal neurogenesis impairment in a rat model. Biochem Pharmacol 2019;163:225-33. doi:10.1016/j.bcp.2019.02.010
17. Fardell JE, Vardy J, Logge W, Johnston I. Single high dose treatment with methotrexate causes long-lasting cognitive dysfunction in laboratory rodents. Pharmacol Biochem Behav 2010;97(2):333-9. doi:10.1016/j.pbb.2010.08.019
18. Seigers R, Schagen SB, Coppens CM, van der Most PJ, van Dam FS, Koolhaas JM, et al. Methotrexate decreases hippocampal cell proliferation and induces memory deficits in rats. Behav Brain Res 2009;201(2):279-84. doi:10.1016/j.bbr.2009.02.025
19. Yang M, Kim JS, Kim J, Kim SH, Kim JC, Kim J, et al. Neurotoxicity of methotrexate to hippocampal cells in vivo and in vitro. Biochem Pharmacol 2011;82(1):72-80. doi:10.1016/j.bcp.2011.03.020
20. Shukitt-Hale B, Kalt W, Carey AN, Vinqvist-Tymchuk M, McDonald J, Joseph JA. Plum juice, but not dried plum powder, is effective in mitigating cognitive deficits in aged rats. Nutrition 2009;25(5):567-73. doi:10.1016/j.nut.2008.10.018

SMJ