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Apoptosis overview of cerebellum Purkinje cell in mice (*Mus musculus* L.) after exposure to methanol extract of the seeds of bitter melon (*Momordica charantia*) and DMPA

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Abstract : Apoptosis of Purkinje cells of the cerebellum of mice can be used as an indicator of disruption to the cerebellum in the delivery of methanol extract of bitter-melon seeds (*Momordica charantia*) and DMPA. Experimental method used to determine the differences that occur in each of the control group and duration of administration methanol extract of bitter melon seeds and DMPA. The control group was divided to K0, K1, and K2 with duration time 0, 4, and 8 weeks respectively. The treatment group consisted of (P0) bitter melon seeds 0 week were given orally and intramuscular DMPA (@ 6 hours), (P1) bitter melon seeds and DMPA (@ 4 weeks), (P2) bitter melon seeds and DMPA (@ 8 weeks). Each group consisted of 5 mice so that the total of male mice is 30 individuals. Doses of methanol extract of bitter melon seeds is 5mg/10g body weight of mice were given orally)[1]. While DMPA dose of 0.175 mg/mouse were administered intramuscularly[2]. The results showed no significant difference ($p>0.05$) between control and treatment at 0, 4, and 8 weeks on cell apoptosis of purkinje of mice cerebellum. It was concluded that the administration of the methanol extract of bitter melon seeds and DMPA secure the histology of the cerebellum in mice.

Introduction

Administration of male contraception has proved no better efficacy with combined contraceptives. Provision of methanol extract of bitter melon seed (*Momordica charantia*) combined with DMPA has hope in reducing the quality and quantity of sperm without reducing libido in men as well as safe in aplication[2]. Activities bitter melon seeds and DMPA work in organ testis, pituitary and hypothalamus. There is a report that stated that the reproductive activity associated with the activity of the hypothalamus and also cerebellum[3]. In Purkinje cells in the cerebellum there which is great with lots of dendrites of neurons found in the cortex and cerebellum as well as play an important role in controlling movement motor[4,5,6].

Purkinje cell apoptosis was observed to be very important when the further development of research and DMPA combination with bitter melon seeds as a contraceptive. Apoptosis is programmed cell death and can be induced by the materials supplied redundantly *via* molecular mechanism[2,6,7,9–13].

Cell apoptosis involves various stages of molecular processes such as caspase cascade such as caspase 3, 6, 7, 8, 9, and 10[14,15]. Apoptosis consists of the intrinsic pathway and the extrinsic pathway. The intrinsic pathway involves the mitochondria such as the release of cytochrome pathway of the mitochondrial membrane. Extrinsic pathway involves the mitochondria as a molecular pathway in addition to activation of the caspase 3,

6, 7, 8, and 10[16,15,6]. In addition it also involves apoptosis signal molecules that pro- and anti apoptosis[9,11,12]. Pro-apoptotic molecule is bax19, bad, bid, cytochrome c and anti-apoptosis such as Bcl-2 and p53[14,15,6,20–22].

Material and Methods

a. Animal experiment

Male mice (*Mus musculus*) strain DDW, healthy, fertile and age 8-11 weeks with body weigh 25-30 g, healthy, fertile (ever given birth one) as many as 50 individuals. Mice of Animal Disease Investigation Center of Sumatra Utara, Medan and divided into treatment and control groups. Mice were fed and watered adlibitum, clean cages and arranged 12 hours light - 12 hours dark. Treatment of mice according to the code of conduct of experimental animals (Ethical clearance) of Ethics Committee of Animal Research - USU[23].

b. Sampling of Cerebellum

Mice were maintained and treated according to study design at 0 week (6 hours on the first day), week 4th and week 8th, and then sacrificed using ether to take their brains. Cerebellum that have been removed from the skull was then fixed in 10% formalin buffer solution over night[24].

c. Cerebellum tissue preparation and analysis

Brains were prepared using paraffin and staining Haematoxylin Eosin[2]. Brain sections were fixed after then washed in alcohol-rise began alcohol concentration of 30%, 40%, 50%, 60%, 70%, 80%, 90%, 96% up to 100% alcohol. Then the brains of mice inserted in xylol over night. Paraffin embedding or infiltration into the brain is processed in the incubator with a temperature of 58-65^{o}C and block creation. Specimen (paraffin block containing the brain) was then sliced with a rotary microtom with thick 5µm, pasted on the glass objects and stained with Haematoxylin Eosin[24].

b. Assessment of DNA fragmentation (Immuno HistoChemistry - TUNEL assay) of Purkinje cells in mice

Samples taken from the cerebellum in mice by autopsy, then fixed in Bouin, the end fixation included in paraffin and then cut with a microtome to a thickness of 5 µm. Fixative significantly improve the specificity and sensitivity of in situ 3'-end labeling apoptotic DNA fragmentation (TUNEL = terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling) while maintaining morphology in storage[8].

Assessment using the proposed method Leake et al.[25], such as the score indicated in Table 1 below. At least 100 cells were counted for each sample with a light microscope and a magnification of 100x.

Table 1. Scoring system used.

Score for proportion staining	Score for staining intensity	Scores result of the addition
0 = No nuclear staining	0 = No staining	0 = no treatment response
1 = <1% nuclei staining	1 = Weak staining	2-3 = small treatment response (20%)
2 = 1–10% nuclei staining	2 = Moderate staining	4-6 = middle treatment response (50%)
3 = 11–33% nuclei staining	3 = Strong staining	7-8 = good treatment response (75%)
4 = 34–66% nuclei staining		
5 = 67–100% nuclei staining		

Note: Adding the two scores together gives a maximum score of 8.[25].

c. Data Analysis

Data collected and analyzed by Anova level of 5% is in bootstrap with SPSS 23.

Results and Discussion

Based on research conducted on administration of seed extract of bitter melon and DMPA showed some parameters such as the number of apoptotic Purkinje cells of the cerebellum in mice (Table 1 and Figure 1), histological structure of Purkinje cells (Figure 2) and immunohistochemistry (Figure 3).

Table 1. Mean and standard deviation of apoptosis cell of cerebellum (%)

Treatment	Mean ± SD	P value
K0	3.33 ± 1.26	p>0.05
P0	4.50 ± 1.29	
K1	3.00 ± 0.82	
P1	3.50 ± 1.29	
K2	2.00 ± 0.82	
P2	2.75 ± 0.96	

Note: K0 = control of 0 weeks (± 6 hours), P0 = Treatment of the methanol extract of bitter melon and DMPA of 0 weeks (± 6 hours), K1 = Control of 4 weeks, P1 = Treatment of the methanol extract of bitter melon and DMPA of 4 weeks, K2 = Control of 8 weeks, P2 = treatment of the methanol extract of bitter melon and DMPA of 8 weeks.

Administration of seed methanol extract of bitter melon and DMPA to rat during 8 weeks can cause decreased fertility[2,8] and mice and has no significant effect ($p<0.05$) for death (apoptosis) Purkinje cells of the cerebellum in mice (Table 1). Although steroids are derived from the methanol extract of bitter melon seeds and DMPA may affect the activity of nerve cells signal transduction or cerebellum mice, but in this study does not provide negative effect on the function (Figure 1) and the structure (Figure 2 and 3). In Table 1, it is clear from the data view that the Purkinje cell apoptosis in all the control and treatment that is below 20%. This indicates that the seed extract of bitter melon and DMPA influence on developments of Purkinje cells become better. Possible dose of bitter melon seeds and DMPA are still within safe limits. Thus giving effect to support the development of Purkinje cells of the cerebellum. According of Peper et al.[26], progesterone acts to increase Purkinje cell dendrite length by binding with its receptor and presumably changing gene expression.

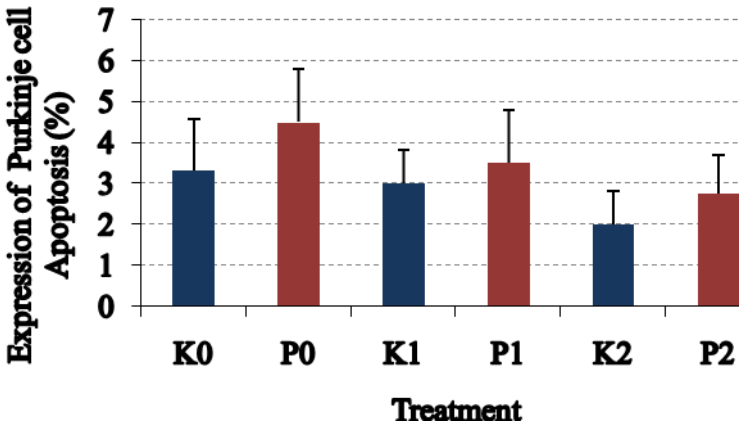

Figure 1. Error Bar of expression of cerebellum cell apoptosis of male mice (%) (mean±SD).

Pada kondisi fisiologis, antioksidan intraseluler akan mengkonversi radikal bebas menjadi senyawa yang tidak berbahaya bagi sel sehingga dapat melindungi sel dari kerusakan yang diinduksi oleh radikal bebas[29]. Hippocampus dan cerebellum merupakan bagian otak yang paling rentan terhadap kerusakan oleh stres oksidatif karena bagian tersebut memiliki aktivitas antioksidan yang rendah[30]. Kegagalan mekanisme proteksi antioksidan akibat produksi berlebihan dari radikal bebas dan penurunan aktivitas enzim *scavenger* menyebabkan peroksidasi lipid yang berujung pada kerusakan atau kematian sel[18].

Bitter melon seeds also contain flavonoids and polyphenols that are antioxidant[1,27] so as to prevent damage to the Purkinje cells of the mouse cerebellum[10,15,16]. In physiological conditions, will convert the intracellular antioxidant free radical into compounds which are harmless to the cell so it can protect of the cells from damage induced by radicals bebas[29]. Hippocampus and cerebellum part of the brain most vulnerable to damage by oxidative stress because that section has low activity of antioxidant[30]. Failure mechanisms of antioxidant protection due to excessive production of free radical and decrease of scavenger enzyme activity and lipid peroxidation causes that led to the damage or cell death (apoptosis)[18].

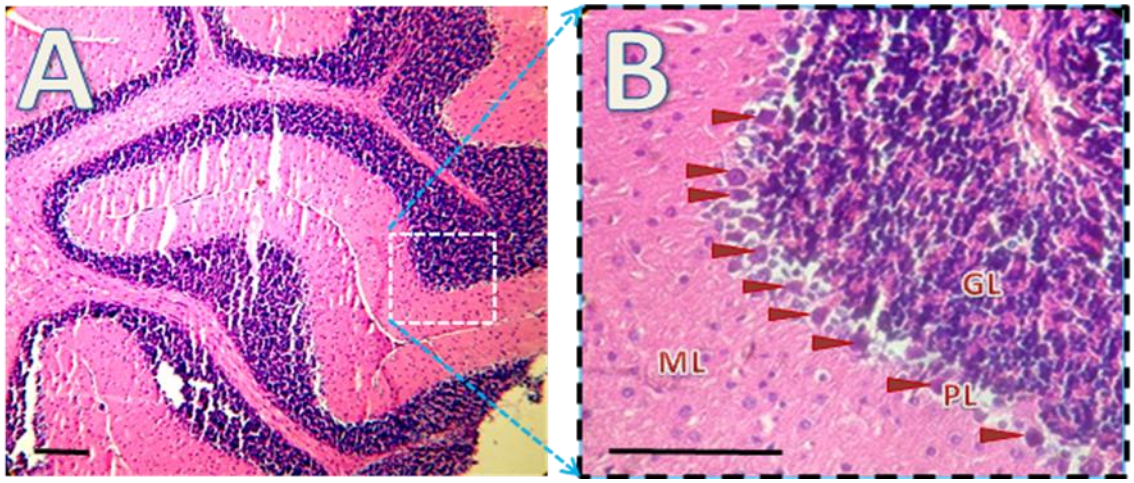

Figure 2. Histological Purkinje cells of the cerebellum (A) magnification of 100x and (B) magnification of 1000x (HE), yellow arrow = Purkunje cell of apoptosis, red arrow = Purkunje cell of normal, GL = Granule Layer, PL = Purkinje cell, ML = Molecular Cell , ─ = 100 µm

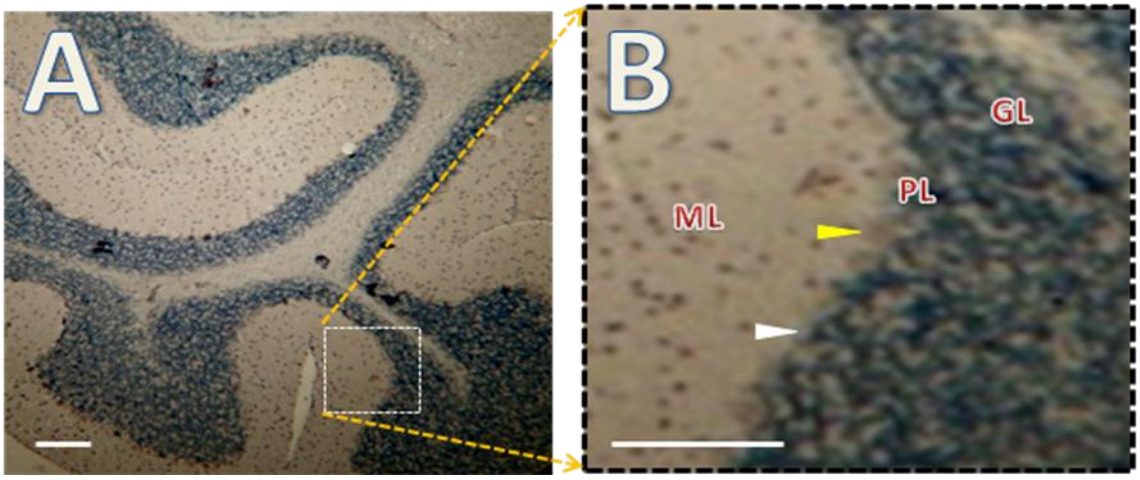

Figure 3. Histological apoptosis Purkinje cells of the cerebellum (A) magnification 100x and (B) 1000x magnification (TUNEL assay), yellow arrow = Purkunje cell of apoptosis, white arrow = Purkunje cell of normal, GL = Granule Layer, PL = Purkinje cell, ML = Molecular cell, ─ = 100 µm

Reference

1. Yama OE, Duru FI, Oremosu AA, Noronha CC, Okanlawon A. Suppressive effects of Momordica charantia on pituitary-testicular axis and sperm production in male Sprague-Dawley rats. 2011;3(12):353–359.
2. Ilyas S. Effect of methanolic *Momordica charantia* seed extract and depot medroxyprogesterone acetate (DMPA) to quantity and quality of rat sperm. *Int. J. PharmTech Res.* 2014;6(6):1817–1823.
3. Piasecka B, Kutalik Z, Roux J, Bergmann S, Robinson-Rechavi M. Comparative modular analysis of

gene expression in vertebrate organs. *BMC Genomics*. 2012;13(1):124.
4. Akosman MS, Gocmen-Mas N, Karabekir HS. Estimation of Purkinje cell quantification and volumetry in the cerebellum using a stereological technique. *Folia Morphol. (Warsz).* 2011;70(4):240–244.
5. Yuan J, Lipinski M, Degterev A. Diversity in the Mechanisms of Neuronal Cell Death Neurons may die as a normal physiological process. *Neuron.* 2003;40(2):401–413.
6. Peng J, Wu Z, Wu Y, et al. Inhibition of caspases protects cerebellar granule cells of the weaver mouse from apoptosis and improves behavioral phenotype. *J. Biol. Chem.* 2002;277(46):44285–44291.
7. Herwanto RY, Bashiruddin J, Ilyas S, Lubis MND. Correlation of Noise Intensity to Heat Shock Response with ultrastructure region of Rattus norvegicus ' s cochlea . 2015;7(1):80–84.
8. Ilyas S, Lestari SW, Moeloek N, Asmarinah, Siregar NC. Induction of rat germ cell apoptosis by testosterone undecanoate and depot medroxyprogesterone acetate and correlation of apoptotic cells with sperm concentration. *Acta Med. Indones.* 2013;45(1):32–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23585406.
9. Hadisahputra S, Ilyas S. Combinational effects of ethylacetate extract of. 2015;7(4):651–653.
10. Arianto A, Bangun H, Harahap U, Ilyas S. The comparison of swelling, mucoadhesive, and release of ranitidine from spherical matrices of alginate, chitosan, alginate-chitosan, and calcium alginate-chitosan. *Int. J. PharmTech Res.* 2014;6(7):2054–2063.
11. M, Harahap U, Nasution MP, Ilyas S. The activity of *Rhaphidophora pinnta* Lf. Schott leaf on MCF-7 cell line. *Adv. Biol. Chem.* 2013;03(04):397–402. Available at: http://www.scirp.org/journal/PaperDownload.aspx?DOI=10.4236/abc.2013.34042.
12. Masfria, Hap UH, Nasution MP, Ilyas S. Cytotoxic activity, proliferation inhibition and apoptosis induction of rhaphidophora pinnata (L.F.) schott chloroform fraction to MCF-7 cell line. *Int. J. PharmTech Res.* 2014;6(4):1327–1333.
13. Hasibuan R, Ilyas S, Hanum S. Effect of leaf extract haramonting (Rhodomyrtus tomentosa) to lower blood sugar levels in mice induced by alloxan. *Int. J. PharmTech Res.* 2015;8(6):284–291.
14. Singh AB, Kaushal V, Megyesi JK, Shah S V., Kaushal GP. Cloning and expression of rat caspase-6 and its localization in renal ischemia/reperfusion injury. *Kidney Int.* 2002;62(1):106–115.
15. Lossi L, Gambino G. Apoptosis of the cerebellar neurons. *Histol. Histopathol.* 2008;23(3):367–380.
16. Oliveira SA, Chuffa LGA, Fioruci-Fontanelli BA, et al. Apoptosis of Purkinje and Granular Cells of the Cerebellum Following Chronic Ethanol Intake. *Cerebellum*. 2014;13(6):728–738.
17. Ghoumari AM, Wehrle R, De Zeeuw CI, Sotelo C, Dusart I. Inhibition of protein kinase C prevents Purkinje cell death but does not affect axonal regeneration. *J Neurosci*. 2002;22(9):3531–3542. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? cmd =Retrieve&db=PubMed &dopt= Citation&list_uids=11978830.
18. Kumar A, LaVoie HA, DiPette DJ, Singh US. Ethanol neurotoxicity in the developing cerebellum: underlying mechanisms and implications. *Brain Sci.* 2013;3(2):941–63. Available at: http://www.mdpi.com/2076-3425/3/2/941/htm.
19. Dong J, Li A, Yamaguchi N, Sakaguchi S, Harris DA. Doppel induces degeneration of cerebellar Purkinje cells independently of Bax. *Am. J. Pathol.* 2007;171(2):599–607. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1934519&tool= pmcentrez&rendertype=abstract.
20. Bregano LC, Agostinho SD, Roncatti FTLB, et al. Expression of pro-and-anti-apoptotic antigens in the cerebellum of dogs naturally infected with canine distemper virus. *Medicina (B. Aires)*. 2010;3(2):80–85.
21. Sinha RA, Khare P, Rai A, et al. Anti-apoptotic role of omega-3-fatty acids in developing brain: perinatal hypothyroid rat cerebellum as apoptotic model. *Int. J. Dev. Neurosci.* 2009;27(4):377–383.
22. Kitagishi Y, Minami A, Nakanishi A, Ogura Y, Matsuda S. Neuron membrane trafficking and protein kinases involved in autism and ADHD. *Int. J. Mol. Sci.* 2015;16(2):3095–3115.
23. Wardani K, Y. Yazir, S. Ilyas. Effect Of Vitamin E Administration To The Level of Estrogen Hormone and Histopathology of Alveolar Bone of The Mice (*Mus musculus* L.) with Maximal Physical Exercise). 2012;17(2):2012.
24. BJ D, AO O. Histological Alteration of the Cerebellum of Adult Male Wistar Rat Treated with the Grapefruit Extract (Citrus paradisi). *Anat. Physiol.* 2012;02(03):2–4. Available at: http://www.omicsonline.org/2161-0940/2161-0940-2-107.digital/2161-0940-2-107.html.
25. Leake R, Barnes D, Pinder S, et al. Immunohistochemical detection of steroid receptors in breast cancer: a working protocol. UK Receptor Group, UK NEQAS, The Scottish Breast Cancer Pathology

Group, and The Receptor and Biomarker Study Group of the EORTC. *J. Clin. Pathol.* 2000;53(8):634–635.
26. Peper JS, van den Heuvel MP, Mandl RCW, Pol HEH, van Honk J. Sex steroids and connectivity in the human brain: A review of neuroimaging studies. *Psychoneuro-endocrinology*. 2011;36(8):1101–1113.
27. Rashmi T, Kamlesh W, Jayashri T, Milind U. Bitter melon : a bitter body with a sweet soul. *Int. J. Res. Ayurveda Pharm.* 2011;2(2):443–447.
28. Setiawan A, Sagi M, Asmara W. Quantitative Analysis of The Purkinje Cell in Mice Cerebellum After Induction of Ochratoxin A during Organogenesis Period. 2011;16(2):3.
29. Imosemi IO. The Role of Antioxidants in Cerebellar Development. A Review of Literature. *Int. J. Morphol*. 2013;31(1):203–210. Available at: file:://WOS:000321868 400034.
30. Henderson GI, Chen J, Schenker S. [Frontiers in Bioscience, 4, d541-550, June 15, 1999] Ethanol, Oxidative Stress, Reactive Aldehydes, And The Fetus George I. Henderson, JuanJuan Chen and Steven Schenker. 1999;1972(1):541–550.

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