Mangiferin Produces Neuroprotective Effect By Attenuating H_2o_2 Induced Oxidative Stress In Primary Rat Hippocampal Neural Progenitor Cells.

Hiba Kanwal Majeed[1], Saba Majeed[2*], Qurratul Ain Leghari[3], Saima Mahmood Malhi[4], Ali Asgher Shuja[5], Syed Ali Jasim Rizvi[6], Uzma Zaman[7], Sadia Suri Kashif[8]

[1]Department of Pharmacology, Faculty of Pharmacy, University of, Karachi, Pakistan.
[2]Department of Pharmacology, Faculty of Pharmacy, Ziauddin University, Pakistan.
[3] Department of Pharmaceutical chemistry, Faculty of Pharmacy, Hamdard University, Karachi, Pakistan.
[4]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Dow university of Health Sciences, Ojha Campus Karachi, Pakistan.
[5]Department of Pharmacology, Faculty of Pharmacy, Hamdard University, Pakistan
[6]Department of Pharmacy Practice, Faculty of Pharmacy, Hamdard University, Karachi, Pakistan.
[7]Department of Biochemistry, Dow International Medical College, Ojha Campus, Karachi. Pakistan.
[8]Department of Pharmacology, Faculty of Pharmacy, Ziauddin University, Pakistan.

***Corresponding Author:** Saba Majeed
[*]Department of Pharmacology, Faculty of Pharmacy, Ziauddin University, Pakistan, Email: saba.majeed@zu.edu.pk

Abstracts

Background: To develop the novel therapeutic strategies for treatment of neurodegenerative disorder, the exploration of new neuroprotective compounds can play a great role to modulate the neurogenesis and treat the neuronal defecit. Formerly the Mangiferin (MNG), well known Xanthone glucoside and phenolic constituent of Mangifera indica has shown to inhibit H_2O_2 induced oxidative damage in rat Pheochromocytoma (PC12) and nucleus pulposus (NP) cultures, but its neuroprotective and antioxidant property on primary hippocampal neural progenitor cells (NPCs) has not evaluated. Targeting proneurogenic approach we designed to evaluate the in vitro antioxidant and neuroprotective potential of Mangiferin extracted from *Mangifera indica*.

Methodology: We examined the proliferative, antioxidant and neuroprotective actions of Mangiferin against H_2O_2 induced oxidative stress in primary isolated hippocampal neural progenitor cells (NPCs).

Results; The screening results displayed prominent neuroprotective effects by rescuing the hippocampal NSPCs against H_2O_2 induced oxidative damage. The proliferative effects of Mangiferin on hippocampal cells was also observed in dose dependent manner. We further demonstrated that the selected concentrations of Mangiferin did not produced any cytotoxic effect on hippocampal cell viability and significantly inhibited reactive oxygen species generation.

Key Words; Mangiferin (MNG), oxidative stress, hippocampal neural progenitor cells (NSPCs), Reactive oxygen species (ROS)

Abbreviations

CAT - Human Catalase Enzyme
DMEM - Dulbecco's Modified Eagle Medium
FBS – Fetal bovine serum
H2O2 – Hydrogen per oxide
MDA - Molandehyde
MNG - Mangiferin
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NP – Nucleus pulposus cells
NPCs – Neural progenitor cells
PBS – Phosphate buffer saline
PC12 – Pheochromocytoma cells
ROS – Reactive oxygen species
SOD – Super oxide dismutase

Introduction

The cure of neurodegenerative diseases can be made easy by restoring and protecting the neuronal cells , Current evidences suggest that the activation of neural progenitor cells (NPCs) can play crucial role to recover neurological deficit (Ebert & Svendsen, 2010).The hippocampus is the most extensively studied region of the brain possessing neural progenitor or

stem cells particularly subgranular zone (SGZ) of dentate gyrus (DG) capable of adding new neurons in the central nervous system, which is associated with learning and memory, mood regulation, depression, and stress response (Gage, 2000). Various neurodegenerative ailments such as Alzheimer and Parkinson disease are initiated in in response to reduced capability of neurons to self renew and lack of proliferation and integration of pre-existing neural progenitor towards injured region of brain (Nakatomi et al., 2002).

The proliferation and integration of newly progenitor cells are highly sensitive to oxidative stress, the ROS overproduction and inadequate redox balance can enhance the apoptosis of neuronal cells (Kohen and Gati, 2000) , additionally free reactive oxidative species (ROS) radicals accelerates mitochondrial dysfunction, oxidation of proteins, genomic instability and eventually perturbs the CNS homeostasis which leads to neurodegenerative disorders due to altered neurogenic capacity of hippocampus and other brain regions. Natural antioxidants play great role to neutralize free radicals and to combat the over production of ROS. Mangiferin is a Xanthone glucoside significantly present in Mangifera indica (Mango) and papaya fruit. Evidences revealed that it possess potent antioxidant, neuroprotective and cytoprotective functions against neurotoxicity in animal models of anxiety, learning and memory (Agarwala et. al., 2012, Marquez, et al., 2012) In vitro studies on Mangiferin has proven to shown its antioxidant action against H2O2 induced oxidative stress in rat pheocytochroma PC12 cells and Nucleus pulposus cells (Peng et al., 2019) despite these extensive reportings no study executed to evaluate its antioxidant effects on rat hippocampal NPCs. The objective of this research is to identify neuroprotective potential of mangiferin in rat hippocampal neural progenitor cells (NPCs).

.

Methodology

Animals:

The neonatal rats 2-3 days old were raised in the breeding colony at animal house facility of Ziauddin University were used. In the present study, experimental protocols for animal care were conducted in accordance with the procedures and ethical guidelines set by the Scientific Animal Ethics Committee on Animal Use of Ziauddin University.

Isolation and Culturing of Hippocampal NPCs:

For isolation of hippocampal neural stem/ progenitor cells, neonatal rat pups were sacrificed under sterile conditions. The hippocampi were carefully dissected out from brain of neonatal rat pups .Subsequently hippocampal sections were mechanically dissociated with scalpel and trypsinized with (0.05% of trypsin) for 7 minutes. After centrifugation for 4 min at 100rpm and washing with PBS, the resultant cell pellets were finally cultured in the DMEM medium supplemented with 10% FBS. The growth was monitored until cells achieved 90% confluency. The characterization of hippocampal neural progenitor cells for has done in our previous studies (Majeed, Aziz & Simjee, 2020).

Preparation of Test Doses:

Stock solution of mangiferin (1000 µg/mL) was prepared in sterile 100% DMSO and stored at -20°C. The working solutions of the testing compound were prepared fresh from the stock by diluting in Dulbecco Modified Eagle Medium (DMEM). Five different working concentrations of Mangiferin (from Mangifera indica, Sigma) were used i.e., (2 µg/mL, 5 µg/mL, 10 µg/mL, 50 µg/mL and 100 and 200µg/mL) in order to evaluate their neuroprotective effect. All doses were selected in accordance to literature search demonstrating neuroprotective effects of Mangiferin.

Cell viability evaluation:

The growth proliferation or cytotoxic effects of the mangiferin was tested on the basis of % cell viability using MTT assay. Briefly, the cultured hippocampal NSPCs 104 cells/200 µL were plated to each well of 96-wells plate and re-incubated for 24 h at 37ºC. Next day media was aspirated from each well and 200µl of aforementioned test doses of *Mangiferin* were added. After incubation of 24 h with test doses the supernatant was removed and MTT dye (5 mg/mL) was added to each well followed by 3 h incubation. After incubation, supernatant was removed and 100 µL of DMSO was added into each well. The plates were then kept for shaking on an orbital shaker for 10-20 min until formazan crystals were solubilize. The absorbance was then measured at 570 nm by using spectrophotometer (TECAN Trading AG, Switzerland). The assays were performed in triplicates

The viability of hippocampal NSPCs were expressed as percentage of control, represented by untreated group, which were set at 100%.

Mitochondrial REDOX Activity.

The effect of mangiferin against H_2O_2 induced oxidative stress was determined by means of REDOX activity using resazurin (Sigma, USA), a fluorescent salt indicator of viable cells as a result of determining oxidation−reduction mitochondrial activity. The hippocampal cells (1.4 × 103 cells /well) were pre-treated with selected doses of *Mangiferin* and incubated for 4h. After drug treatments, the cultures was exposed to H_2O_2 and kept for 24h incubation to induce oxidative stress. The only cells were kept as control group and not pretreated or treated with compounds or H_2O_2 respectively. To assess the neuroprotective efficacy of mangiferin against oxidative stress, the resazurin salt solution 10 µg/ml was added to each well, and incubation was done for 2 h. Finally, the fluorescence intensity was determined at dual wave lengths of 530 nm (an excitation wavelength) and 590 nm (an emission wavelength) using spectrophotometer. The

proliferative effect was expressed as % AB reduction with respect to control cells. Data was shown as the mean ± SEM of at least three individual experiments.

Determination of ROS production.

The assessment of ROS generation was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) sensitive dye for ROS. (Sigma, USA). After exposure with test doses of Mangiferin and 4h post treatment with H_2O_2. The supernatant was aspirated and 10µM H2DCFDA dye was added in to each well and plates were kept for 2h at 37 °C in the dark. The ROS levels were indicated by intracellular conversion of non fluorescent dye H2DCFDA to the fluorescent molecule 2′,7′- dichlorofluorescein (DCF) due to oxidation. Fluorescence intensity was calculated at an excitation wavelength of 495 nm and an emission wavelength of 520 nm using a microplate ELISA reader.

Statistical Analysis

Measurements of absorbance showing reduction of MTT and resazurin were proportional to the number of viable or proliferating cells. The absorbance of the control group (vehicle alone) was set as 100%. The rate of growing cells treated with various doses of tested drugs was expressed as a percentage of control groups. Statistical analysis was performed using One-Way ANOVA followed by boneferroni's multiple comparison test, with $p < 0.05$ being assumed statistically significant.

Results

Effects of Mangiferin on hippocampal neural progenitor cell Viability.

A 24 h incubation with various doses of Mangiferin (MNG), the remarkable growth supportive effect on hippocampal cells was observed at dose of 50 ug/ml (Fig1) and Mangiferin at dose of 2,5 and 10 ug/ml stabilized the growth and did not produced any cell death. No cytotoxicity was observed with treatment of all doses *of Mangiferin* on hippocampal cells which indicates its neuroprotective potential. Interestingly, the treatment with dose of 100 and 200 ug/ml (Fig 1) produce significant growth proliferative effect demonstrating its neurognic potential on hippocampal neurons.

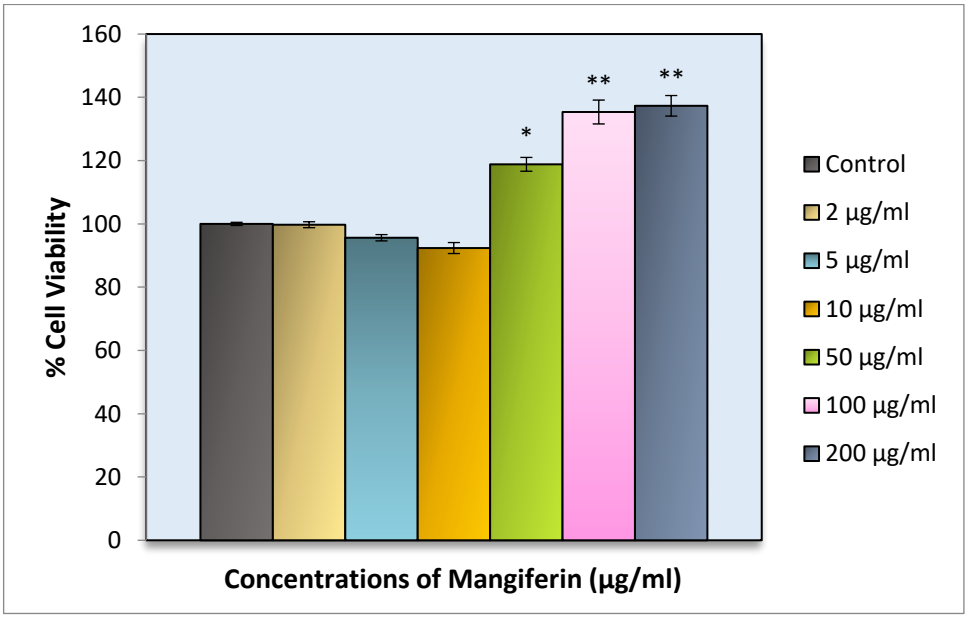

Figure 1: Hipoocampal cultures were treated with different doses of Mangiferin for 24 h. Percent cell viability was increased at the dose of 50, 100 and 200 ug/ml**.** Statistical analysis was performed by using ANOVA followed by post hoc bonferroni test for multiple comparisons among treatment groups. Values are expressed as mean ± SEM of triplicate experiments . ** $P < 0.001$ and * $P < 0.05$ vs untreated hippocampal control cells.

Mangiferin protects hippocampal NPCs against H_2O_2 induced Oxidative stress

The neuroprotective potential of Mangiferin against brain damage have been linked with its antioxidant capacity (Ren, W., & Guo, W. (2018), Siswanto, S.et, al, 2016) but its antioxidant effect on primary hippocampal cells not yet been explored. H_2O_2 promotes intracellular ROS accumulation in neonatal hippocampal NSPCs resulting in neuronal cell damage. we evaluated the effects of *mangiferin* on generation of intracellular ROS by H_2O_2 in hippocampal cells. As shown in Figure 2, *Mangiferin* (at 50, 100 and 200µg/ml) inhibit the neuronal damage under oxidative stress and protect hippocampal cultured cells in comparison to H_2O_2 group where viable cells were significantly reduced around 50-60% compared with the only cells (Fig 2). Moreover, cells exposed to *Mangiferin* (200 µg/ml) showed highest inhibition of oxidative stress among all test doses.

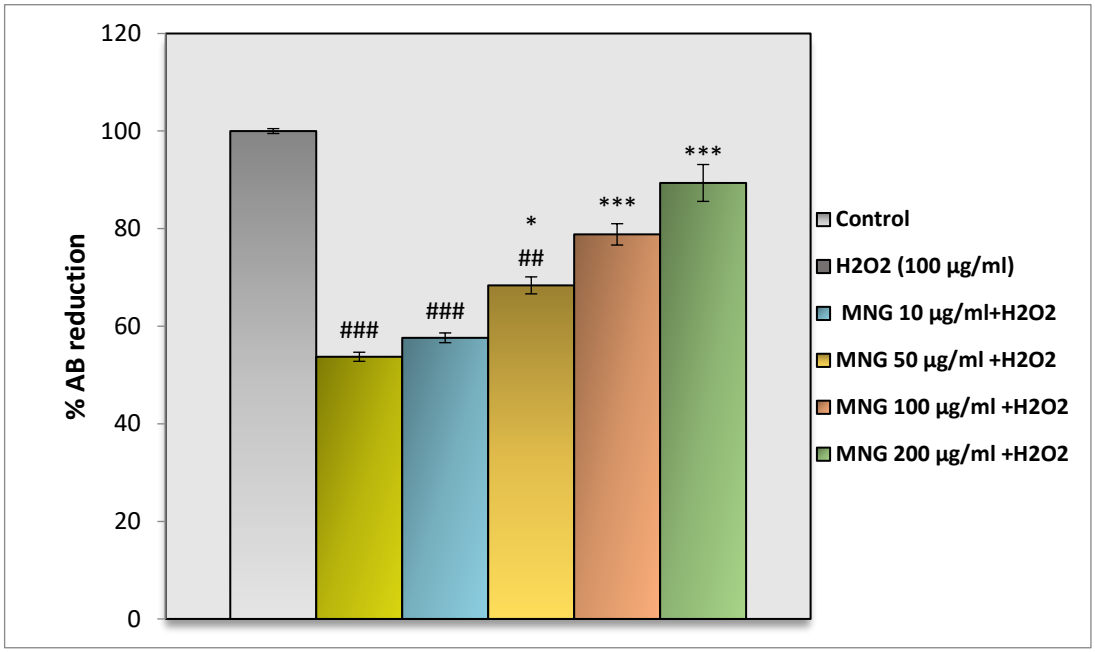

Figure 2: Hippocampal culture were pretreated with Mangiferin at doses of 10, 50, and 100 and 200µg/mL, after 4 h of incubation with test doses, the ROS was induced by exposure of cells with H_2O_2 (100ug/ml) and neuroprotective effects of mangiferin on hippocampal cells was investigated by comparing with control cells ($p < 0.05$) and H_2O_2 treated cells. Pretreatment with Mangiferin shown significant neuroprotection in dose dependent manner and exhibit marked proliferative effect by reducing % Ab at the doses of 100 and 200µg/mL ($p^* < 0.05$ or $p^{***} < 0.001$) compared with cells treated with H_2O_2 alone and control cells ($p^{\#\#} < 0.01$ or $p^{\#\#\#} < 0.001$ respectively (Figure 2).

Neuroprotective effects of Mangiferin associated with inhibition of ROS accumulation.

The intracellular damage of hippocampal neuronal cells upon ROS generation was demonstrated was demonstrated in H_2O_2 treated group in comparison to control cells ($^{\#\#\#}P < 0.001$) as shown in Figure 3. Exposure with mangiferin at concentrations of 50, 100 and 200 µg/mL significantly ($p^{**} < 0.05$ or $p^{***} < 0.001$) inhibited ROS generation at cellular level produced by H_2O_2 . However, the dose of 10 µg/mL did not shown any significant results in comparison to H2O2 treated group. Our findings confirmed that Mangiferin had inhibitory effect on H2O2-induced oxidative damage in hippocampal NPCs cultures.

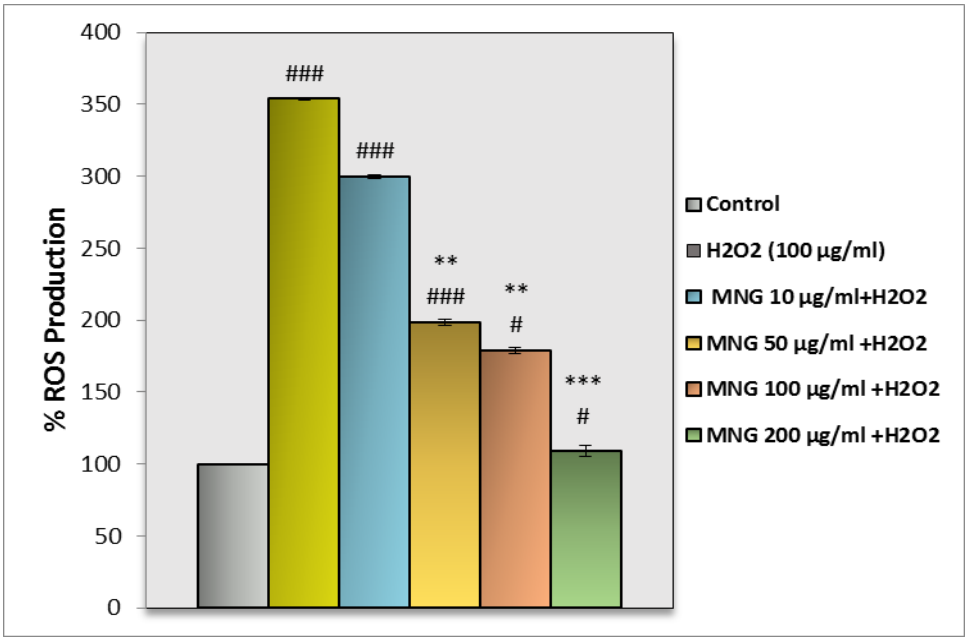

Figure 3: Percent ROS values with treatment of Mangiferin and H_2O_2 in hippocampal NSPCs for 24 h. Total ROS values were determined by flourimetric method using DCFDA dye where H_2O_2 served as positive control and untreated cells as control. Statistical significance was analysed by one way ANOVA with Bonferroni as post hoc analysis by using SPSS version 20, where ($^{**}p < 0.01$ $^{***}p < 0.001$) express comparison of treatment groups with H2O2 alone and $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ to control cells respectively.

Discussion

Mangiferin is a natural component isolated from *Mangifera indica*, Since Mangiferin has shown a neuroprotective effects in doxorubicin and H_2O_2 induced neuronal toxicity studies in PC 12 cultures (Peng et al., 2019) and MPTP induced Parkinson's models (Kavitha et al., 2013, Pardo et. al, 2010, Márquez al. ,2012, Guo et al., 2018) .We set out to determine its effects on neuronal modulation, as the hippocampal dentate gyrus (DG) generates neural progenitor-derived neurons throughout life and it involves the proliferation and subsequent differentiation of neuronal progenitors (REF). In order to determine the in vitro effects of Mangiferin on proliferation and viability of the hippocampal neurons under oxidative stress environment triggered by H_2O_2 treatment, we utilize MTT assay to assess the cell viability. Among all tested doses of MNG, the concentrations of 100 and 200 µg/mL has shown significantly increased the proliferation of cells. Whereas the doses of 2,5,10, and 50 µg/mL did not exhibit any significant growth reduction effect on hippocampal NPCs. These outcomes suggest that Mangiferin has ability to proliferate and support growth of the neural progenitor cells, which are in accordance of previous findings indicating neuronal protection capability of mangiferin in neurodegenerative diseases such as (Kavitha, et al. 2013).

High level of free radical aacumulation in the brain is linked with neuronal loss leading to impairments in memory, cognition, and neurogenesis. H_2O_2 induced oxidative stress is well – recognized model that initiates the production of ROS abundantly which, in turns results in a impaired mitochondrial activity associated to neuronal cell death or apoptosis (Andreu et al., 2005). Numerous studies suggested H_2O_2 elicits a oxido-nitrosative stress and mitochondrial dysfuctioning in CNS (Kasbe et al., 2015, Zhu et al., 2009), this investigation assessed mitochondril redox activity in hippocampal NPCs treated with H_2O_2 and Mangiferin. We found that, MNG exposure significantly protects the mitochondrial function in hippocampal NPCs via marked elevation in percent AB reduction in comparison to H_2O_2 group. It is obvious from our results that pretreatment with MNG significantly attenuated H_2O_2 mediated hippocampal cell death. Our findings also supports a previous investigations reported that MGF protects mitochondrial loss due to its potent antioxidant capacity in H_2O_2 mediated loss in NP cells and PC 12 cells (Ren, W., & Guo, W. 2018, Peng et al., 2019)

To further evaluate the antioxidant potential of Mangiferin we aimed to identify that the neuronal protection by MNG is associated with inhibition of ROS. Former Studies showe by Siswanto et al., 2016, revealed that MNG strongly inhibit oxidative stress via reduction in MDA , GSH and eNOS levels and upregulaion of SOD and CAT Jaishakar et al., 2014). Mangiferin has also shown to reduce ROS levels and e NOS signalling mechanism (Amazzal et, al. 2007, Viera et al., 2013) H2O2 produces ROS mediated oxidative damage by easily penetrating into the cell and form highly reactive hydroxyl radicals, consequently imbalancing cellular oxidants and antioxidant enzymes like SOD and CAT works as defensive scavenger components in response to increase levels of ROS, which cause cellular damage and disease progression (Kohen and Gati., 2000). Our investigation represents that MNG found to have inhibitory effects on ROS production in hippocampal NPCs. The treatment with doses of 50, 100 and 200µg/mL shown significant reduction in ROS levels in comparison to H_2O_2group. The neuroprotective efficacy exerted by MNG on H_2O_2 induced neuronal damage are further supported by findings of MTT assay and mitochondrial REDOX activity.

Conclusions:

The Xanthone polyphenolic compound mangiferin has found to be growth supportive for neonatal hippocampal cells and it may have an important role in neuroprotection and can be consider as potential antioxidant and neurogenic compound for the treatment of neurodegeneratives diseases.

Conflict of Interest: There is no conflict of interest between authors

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript

References

1. Agarwala S, NR B, Mudholkar K, Bhuwania R, Satish Rao BS. 2012. Mangiferin, a dietary xanthone protects against mercury-induced toxicity in HepG2 cells. Environ Toxicol, 27(2):117–27.
2. Amazzal L, Lapôtre A, Quignon F, Bagrel D.2007. Mangiferin protects against 1-methyl- 4-phenylpyridinium toxicity mediated by oxidative stress in N2A cells. Neurosci Lett., 418(2):159–64.
3. Andreu GL, Delgado R, Velho JA, Curti C, Vercesi AE. 2005. Mangiferin, a natural occurring glucosyl xanthone, increases susceptibility of rat liver mitochondria to calcium-induced permeability transition. Arch Biochem Biophys, 439(2):184–93.
4. Ebert, A.D. and Svendsen, C.N., 2010. Human stem cells and drug screening: opportunities and challenges. Nature Reviews Drug Discovery, 9(5), pp.367-372.
5. Gage, F.H., 2000. Mammalian neural stem cells. Science, 287(5457), pp.1433-1438.
6. Guo,J.D.,Zhao,X.,Li,Y.,Li,G.R.,Liu,X.L.,2018.DamagetodopaminergicneuronsbyoxidativestressinParkinson'sdiseas e.Int.J.Mol.Med.41,1817–1825.
7. Jaishankar,M.,Tseten,T.,Anbalagan,N.,Mathew,B.B.,Beeregowda,K.N.,2014.Toxicity,mechanismandhealtheffectsof someheavymetals.Interdiscip.Toxicol.7,60–72

8. Kasbe P, Jangra A, Lahkar M. 2015. Mangiferin ameliorates aluminium chloride induced cognitive dysfunction via alleviation of hippocampal oxido-nitrosative stress, proinflammatory cytokines and acetylcholinesterase level. J Trace Elem Med Biol.;31:107–12.
9. Kavitha M, Nataraj J, Essa MM, Memon MA, Manivasagam T. 2013. Mangiferin attenuates MPTP induced dopaminergic neurodegeneration and improves motor impairment, redox balance and Bcl-2/Bax expression in experimental Parkinson's disease mice. Chem Biol Interact, 206(2):239–47.
10. Kohen, R., Vellaichamy, E., Hrbac, J., Gati, I. and Tirosh, O., 2000. Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues. Free Radical Biology and Medicine, 28(6), pp.871-879.
11. Majeed, S., Aziz, A. and Simjee, S.U., 2020. Neuronal transcription program induced in hippocampal cells cocultured with bone marrow derived mesenchymal cells. *Heliyon*, *6*(10), p.e05083.
12. Márquez L, García-Bueno B, Madrigal JL, Leza JC. 2012. Mangiferin decreases inflammation and oxidative damage in rat brain after stress. Eur J Nutr, 51(6):729–39.
13. Nakatomi, H., Kuriu, T., Okabe, S., Yamamoto, S.I., Hatano, O., Kawahara, N., Tamura, A., Kirino, T. and Nakafuku, M., 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell, 110(4), pp.429-441..
14. Pardo Andreu GL, Maurmann N, Reolon GK, de Farias CB, Schwartsmann G, Delgado R, Roesler R. 2010. Mangiferin, a naturally occurring glucoxilxanthone improves long-term object recognition memory in rats. Eur J Pharmacol, 635 (1-3):124–8.
15. Peng, S., Hou, Y., Yao, J. and Fang, J., 2019. Neuroprotection of mangiferin against oxidative damage via arousing Nrf2 signaling pathway in PC12 cells. BioFactors, 45(3), pp.381-392.
16. Ren, W., & Guo, W. 2018. Protection of mangiferin on hydrogen peroxide-induced injury in nucleus pulposus cells through inhibition of oxidative stress and the endothelial nitric oxide synthase pathway. *International Journal of Clinical and Experimental Medicine*, *11*(5), 5343-5349.
17. Siswanto, S., Arozal, W., Juniantito, V., Grace, A., & Agustini, F. D. 2016. The effect of mangiferin against brain damage caused by oxidative stress and inflammation induced by doxorubicin. HAYATI Journal of Biosciences, 23(2), 51-55.
18. Vieira AB, Coelho LP, Insuela DB, Carvalho VF, dos Santos MH, Silva PM, Martins MA. 2013. Mangiferin prevents guinea pig tracheal contraction via activation of the nitric oxide-cyclic GMP pathway. PLoS One, 8(8):71759.
19. Zhu,Y.,Kalen,A.L.,Li,L.,Lehmler,H.J.,Robertson,L.W.,Goswami,P.C.,Spitz,D.R.,AykinBurns,N.,2009.Polychlorinatedinducedoxidativestressandcytotoxicitycanbemitigatedbyantioxidantsafterexposure.FreeRadic.Biol.Med47,1762–.71.