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Neuroprotective activity of methanolic extract of leaves from *Oenothera rosea* in pc-12 cell line

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Abstract
In this study, we evaluated the neurotoxicity and neuroprotective properties of the methanolic extract obtained from leaves of *Oenothera rosea* in PC-12 cell line. The PC-12 cell line was used to evaluate neurotoxicity and neuroprotection (MTT cell viability assay) and to evaluate radical scavenging activity (DCFDA assay) using different concentrations of the extract. DPPH was also used to evaluate antioxidant activity. Low neurotoxicity was observed, and all concentrations maintained a cell viability percentage above 80%. The concentration of 25 µg/mL was the most active in the neuroprotection assay, with a cell viability of 82.41±5.97, even higher than the control used for neuroprotection Trolox 50µM (76.19±2.98%). The antioxidant properties were evaluated with DCFDA and DPPH, an inhibition of free radicals was reduced in more than an 80%. Methanolic extract of leaves from *O. rosea* present neuroprotective activity and low cytotoxicity *in vitro*.

Keywords: *O. rosea*, neuroprotection, plant extract, neurodegenerative diseases.

Introduction
Neurodegenerative diseases enlarge a wide range of conditions that are disabling and incurable due to progressive neuronal loss. The etiology of these conditions is unclear, but they are associated with protein aggregation, mitochondrial dysfunction, blood-brain barrier damage, oxidative stress, and inflammation. These diseases have an important increase each year and include disorders such as Alzheimer´s disease, Parkinson´s disease, amyotrophic lateral sclerosis, and Huntington´s disease. In the US, between 2016 and 2017 up to 6 million individuals were affected, and 272,644 died due to these disorders. The economic U.S burden in 2020 was around $695 billion, including direct costs for medical attention, indirect costs from caregiving hours, and lost productivity [1-4].
Oenothera rosea L'Hér. Ex Aiton (*O. rosea*) is a plant used in Mexican traditional medicine to treat wounds, inflammation, renal disease, hepatic pain, diarrhea, and skin infections. This plant is widely distributed around the globe with a major presence from the south of the U.S. to South American countries such as Peru and Argentina [5, 6]. Several pharmacological activities like anti-inflammatory, antibacterial, antioxidant, anticancer, and anti-adipogenic have been reported in polar extracts of this plant (ethanol-water, methanol, and butanol). The main compounds in these extracts are phenolic compounds and flavonoids [7-11]. Given the pharmacological activity of extracts of *O. rosea* and the need for neuroprotectors to treat neurodegenerative disease, the main of this work was to evaluate the neuroprotective activity of the methanolic extract of this plant in an *in-vitro* excitotoxicity model.

Materials and Methods
Methanolic extract
Leaves from *O. rosea* (voucher HJAAA_04_2022_0002) were harvested in spring in Durango, Dgo, Mexico. They were dried and macerated in methanol for 48 hours at 25 °C, the resulting mixture contained the extract dissolved in the solvent, the latter was evaporated using a rotary evaporator, and finally a dried greenish-brown extract was obtained. For biological analysis, the final extracts were dissolved in DMSO to a concentration of 10,000 µg/mL.

Cell line
The assays were performed using the PC-12 cell line (ATCC®, CRL-1721™).

The cells were maintained frozen (-80°C) until the experiments in which cells were cultured in RPMI- 1640 medium (Caisson) containing 10% of Fetal Bovine Serum (Caisson) and penicillin - Streptomycin 1% at 37 °C in an atmosphere containing 5% CO_2.

Phytochemical screening.

Phenolic compounds: A few drops of 12.5% iron chloride in water were added to 1mL of extract dissolved in solvent (hexane, chloroform, or methanol depending on the case). The appearance of green coloration was taken as positive in the presence of phenols [12].

Sterols (Salkowski Test): To 2mL of the sample, 2mL of chloroform and 2mL of concentrated sulfuric acid were added, shaken, and allowed to settle. The formation of a greenish-yellow layer was taken as the presence of sterols [13].

Coumarins test: 1-2 mg of sample was dissolved in 10% NaOH, if a yellow coloration appears that disappears when acidifying, it is positive [14].

Terpenes: To 1mL of extract, 2mL of chloroform, and 1.5mL of sulfuric acid were added. The appearance of a reddish-brown interface indicates the presence of terpenes [14].

Alkaloids (Dragendorff test): To 1mL of extract, 1mL of Draggendorff's reagent (Sigma-Aldrich) was added. The appearance of a white precipitate was taken as a positive result [13].

Saponins: To 1mL of extract, 9mL of distilled water was added, after which it was shaken vigorously for 1 minute. Foam formation indicates the presence of saponins [14].

Total Phenolic Content

The Folin-Ciocalteu assay was performed. The following reagents were utilized: 80% ethanol, 1 N Folin Ciocalteu reagent (prepared by diluting 2 N Folin-Ciocalteu reagent with an equal volume of water), 5% sodium carbonate, standard stock solution (prepared by dissolving 50 mg of gallic acid in methanol and adjusting the volume to 50 ml in a standard flask), and working standard solution (obtained by diluting 5 ml of the stock solution to 100 ml with distilled water, resulting in a concentration of 50 µg/ml of gallic acid). The experimental protocol involved pipetting 0.2, 0.4, 0.6, 0.8-, and 1-mL aliquots of the working standard solution into labeled test tubes, S1, S2, S3, S4, and S5, respectively. Subsequently, 50 µL of phenolic extract from the sample was taken in a series of test tubes, with all analyses conducted in triplicate. The contents of all test tubes were then brought to 1 ml with distilled water. Next, 0.5 mL of 1 N Folin Ciocalteu reagent was added to each test tube, including the blank. After thorough agitation, all test tubes were allowed to rest for 5 minutes at room temperature. Following this, 2.5 ml of 5% sodium carbonate was added to all test tubes, including the blank, followed by another round of agitation. Subsequently, the test tubes were incubated in the dark at room temperature for 40 minutes. The absorbance of the developed blue color was measured at 725 nm using a spectrophotometer. The results were used to calculate the total phenolic content in the sample, expressed as mg of gallic acid equivalents per gram of the sample.

Antioxidant activity

DPPH. Methanol solutions were prepared using the extracts in concentrations from 200 to 3.125 µg/mL, then 0.1 mL were added to each well in a 96-well plate by triplicate. Then, 0.1 mL of DPPH (100 µM) was added to each well. The plaque was incubated at room temperature in darkness, finally, the absorbance was measured at 517 using a U-V spectrometer. Trolox 50 µM was used as a positive control of antioxidant activity and DPPH solution as a negative control.

DCFDA. 10,000 cells per well were transferred into a 96-well plate and incubated until monolayer formation (24 hours). Then, 0.1 mL of the extract (25 µg/mL) was added to each well in triplicate and incubated at 37 °C with an atmosphere of 5% CO_2 for 4 hours. Thereafter, a solution of H2O2 0.5mM and a solution of DCFDA 0.005 µg/mL was added and the plaque was incubated at the same conditions. The absorbance was measured at 485/538 nm.

Neurotoxic activity. 10,000 cells per well were transferred into a 96-well plate and incubated until monolayer formation (24 hours). Then 0.1 mL of the extract dissolved in RPMI-1640 medium was transferred into each well by triplicate by serial dilution from 100 to 3.125 µg/mL. Glutamate 40mM was used as positive control for neurotoxicity and cells with RPMI-1640 medium as negative control. Then the plaque was incubated at 37 °C, 5% CO_2 for 24 hours. The medium was removed, and 0.1mL of the MTT M5655 (Sigma-Aldrich) solution with medium (0.5 mg/mL) was added and incubated at the same conditions for 3 hours. After this time, the MTT solution was removed and 0.1mL of the 10% isopropyl alcohol/hydrochloric acid solution was added, then it was incubated for 30 minutes at room temperature in the dark. Finally, the absorbance was read at a wavelength of 570nm.

Neuroprotective activity. 10,000 cells per well were transferred into a 96-well plate, then serial dilution from 100 to 3.125µg/mL of the extract was added as pretreatment. Trolox at 50 µM was the positive neuroprotection control. They were incubated at 37 °C, 5% CO2 for 4 hours, then 0.1mL of 30mM glutamate was added, as well as 0.5% DMSO to rule out the possible activity of the solvent, thereafter they were incubated for another 20 hours. Cells with 30 mM glutamate were used as a negative control of neuroprotection. Finally, the MTT test was carried out with the same methodology and conditions mentioned above.

Statistical analysis. The differences among the concentrations of the extract and controls were tested with ANOVA using a Dunnet as a post hoc test. GraphPad Prism 5 software was utilized for this analysis.

Results

Neurotoxic activity. The concentrations studied showed cell viability higher than 80% and decreased as increase the concentration. The highest concentration, 100 µg/mL, presented a viability of 82.49±5.44%, while the minor concentration 3.125 µg/mL of 99.20±3.49%. The difference among the concentrations was statistically significant (p<0.01). Dunnet's post hoc test indicates that the cell viability percentage of the positive control of death (Glutamate 40 mM), and the concentrations of methanolic extract (50 and 100 µg/mL) present a significant difference concerning the negative control (p<0.01), (Figure 1).

Neuroprotective activity. All concentrations increased the percentage of cell viability in relation to the death control glutamate (30mM) (cellular viability = 57.23±4.31%). Only the extract concentration of 25 µg/mL presented a higher neuroprotection than Trolox (50 µM) with a viability of 82.41±5.97 and 76.19±2.98% respectively. The concentrations of 100, 50, 12.5, 6.25, and 3.125 µg/mL presented values of 10.71±4.97%, 70.55±6.69%, 74.61±11.14%, 72.97±1.99% and 70.02±9.79% respectively.

Among the concentrations evaluated, no significant differences were observed (ANOVA, P=0.3677). However,

Dunnett's post hoc test was carried out in which the concentrations were compared, as well as the positive control of neuroprotection of Trolox 50 µM with the death control of glutamate 30mM, it was obtained that the extracts of 25, 12.5, and 6.25 µg/mL presented a significant difference (p<0.0001).
Phytochemical screening and Total Phenolic Content.
The presence of multiple metabolites was observed in the methanolic extract of *O. rosea;* these metabolites were diverse, including polar (phenols, coumarins, alkaloids) and non-polar molecules (sterols, terpenes, saponins).
A high concentration of Total Phenolic Compound was observed (278.44±18.4 mEq GA).

Antioxidant activity.
DCFDA. The group without pretreatment, H2O2, presented a higher relative fluorescence 38.92±0.75, than the rest of the groups. The values of Trolox and the control group were 8.69±1.55 and 6.04±0.39 respectively. The extract present 20.19±0.96. All groups present significant differences among them (p<0.01). The extract showed a significant difference with the H2O2 group and with the Trolox group (Dunnet´s test; p<0.01) (Figure 3a).
DPPH. An increase in scavenging activity was observed as an increase concentration of extract. The highest percentage of radical scavenging was observed with 200 µg/mL (91.69±4.55) and was similar to Trolox (Figure 3b). The IC50 of the extract was 24.35.

Discussion
In this study, the neurotoxic and neuroprotective activity of methanolic extracts of leaves from *O rosea* are reported for the first time. With respect to neurotoxic activity, results indicate that methanolic extracts have no neurotoxic activity since none of the concentrations used decreased cell viability considerably. At the highest concentration (100 µg/mL), the viability percentage remained above 80%. This is similar to the reported previously in methanolic extract of whole aerial parts that showed cell viability higher than 80%, however, they use another type of cell, the mouse fibroblast (NIH/3T3) [15]. A compound isolated from this type of extract, quercetin 3-O-β-dalopyranoside-3",6" diacetate, which has pharmacological activities such as antioxidant and anti-inflammatory, has been subjected to cytotoxicity tests in mouse fibroblasts (3T3 cell line -NIH) showing low toxicity with an IC50 <30µM [10, 16, 17].
In the case of neuroprotective activity, all concentrations evaluated increased the percentage of cell viability compared with the death control (Glutamate 30Mm), this suggests that methanolic extract has neuroprotective activity. However, this activity is in a non-linear concentration relation. The higher activity was to low concentrations (25, 12.5, and 6.25 µg/mL) with a peak at 25 µg/mL. In this last concentration, the neuroprotective activity was better than positive control of neuroprotection Trolox (50 µM). In higher concentrations the neuroprotection was decreasing, this suggests that the extract contain compounds that are neurotoxic when reach some concentrations that antagonize the neuroprotective effect.
Concerning the antioxidant activity, in a previous study Marquez-Flores *et al*. reported the antioxidant activity of a methanolic extract obtained from the same plant. Their results showed that at a concentration of 500 µg/mL, 89.51% of free radical scavenging activity is observed. Results of the present study, the free radical scavenging activity was higher at a lower concentration (200 µg/mL). The better antioxidant activity observed in the present study could be due to the environmental conditions of the plant before its recollection, low water availability, U-V radiation, and other stress factors play an important role in the secondary metabolites production [18].
In the phytochemical tests, phenolic compounds and terpenes were observed, phenolic compounds were present in a higher concentration compared with previous studies [18], so their neuroprotective activity can be attributed to the presence of this type of compounds since some of them have antioxidant capacity such as ursolic acid, gallic acid and the quercetin derivative quercetin 3 -O-β-D-alopyranoside- 3´´, 6´´-diacetate which have been previously reported [19]. Gallic acid has been shown to have different effects on the central nervous system, it has been tested in different models of Alzheimer's disease, Parkinson's disease, ischemia, depression, and anxiety in which it has been effective through certain mechanisms, in terms of its effect on excitotoxicity, it acts as an antioxidant and decreases intracellular Ca^{2+} levels [20]. Ursolic acid has similarly shown effects on different models of neurological diseases such as Alzheimer's disease, cerebral ischemia, cognitive deficit, anxiety, and depression [21]. In the PC12 cell line, it has shown a good antioxidant effect, decreased the formation of reactive oxygen species, and increased the expression of antioxidant enzymes [22].

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Conflicts of Interest
The authors declare no conflicts of interest.

Conclusion
The methanolic extract of *Oenothera rosea* demonstrates promising neuroprotective and antioxidant properties, potentially offering a therapeutic avenue for neurodegenerative diseases characterized by neuronal loss and oxidative stress. Our findings indicate that lower concentrations of the extract exhibit significant neuroprotective effects against glutamate-induced toxicity, with the 25 µg/mL concentration showing superior activity compared to the positive control, Trolox. Furthermore, the phytochemical composition reveals a high concentration of phenolic compounds, which may contribute to these protective effects. Given the increasing prevalence of neurodegenerative conditions and the economic burden they impose, further investigation into the therapeutic potential of *O. rosea* is warranted.

Figures

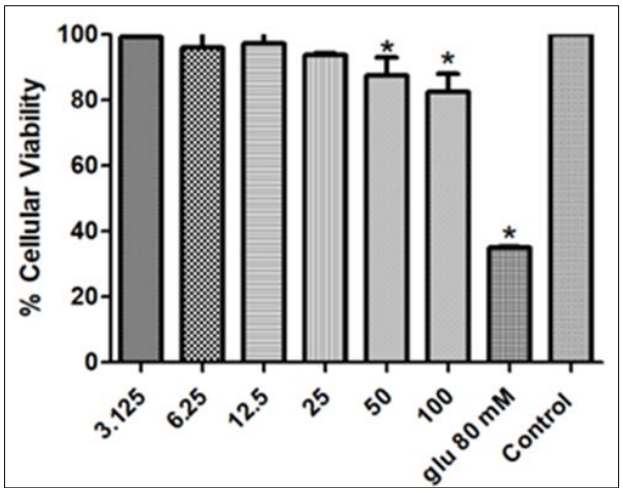

Fig 1: Neurotoxic evaluation of methanolic extract of *O. rosea* (Concentration of extract ex-pressed in µg/mL) (OM: methanolic extract of *O. rosea*), (P=0.0059)

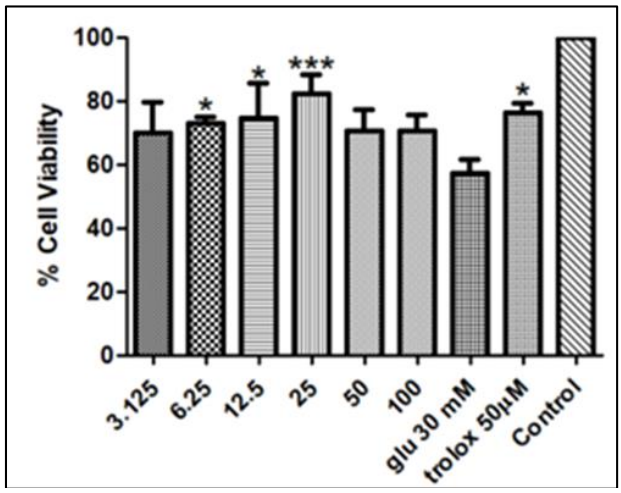

Fig 2: Neuroprotective evaluation of methanolic extract obtained from leaves of *O. rosea* (Concentration of extract expressed in µg/mL) (p<0.0001) (OM: methanolic extract of *O. rosea*)

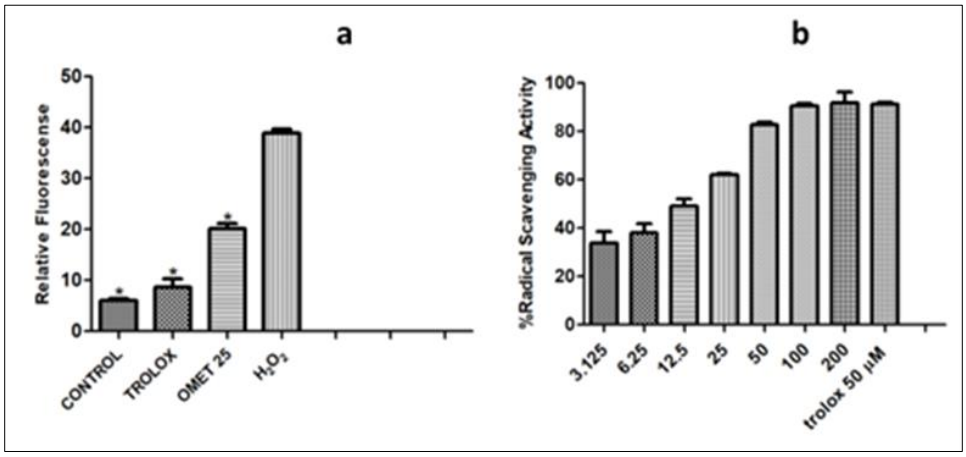

Fig 3: Antioxidant evaluation of methanolic extract obtained from leaves of *O. rosea*. a) DCFDA assay, b) DPPH assay (Concentration of extract expressed in µg/mL) (H2O2: 0.5 µM) (OM: methanolic extract of *O. rosea*)

References

1. Feigin VL, Vos T, Alahdab F, Amit AML, Bärnighausen TW, Beghi E, *et al*. Burden of Neurological Disorders across the US from 1990-2017: A Global Burden of Disease Study. JAMA Neurol. 2021;78(2):165-176.
2. Yang W, Hamilton JL, Kopil C, Beck JC, Tanner CM, Albin RL, *et al*. Current and projected future economic burden of Parkinson's disease in the U.S. npj Park Dis [Internet]. 2020 Dec 1 [cited 2021 May 4];6(1):1-9. Available from: https://doi.org/10.1038/s41531-020-0117-1
3. Logroscino G, Piccininni M, Marin B, Nichols E, Abd-Allah F, Abdelalim A, *et al*. Global, regional, and national burden of motor neuron diseases 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2018;17(12):1083-1097.
4. Hernández PJ, Machorro ZVJ, Porras VD, Ávila VE, Chavarría A. A Potential Alternative against Neurodegenerative Diseases: Phytodrugs. Oxid Med Cell Longev; c2016, 2016.
5. GBIF Backbone Taxonomy. *Oenothera rosea* Aiton in GBIF Secretariat [Internet]. 2021 [cited 2022 Feb 1]. Available from: https://www.gbif.org/species/3188875
6. García ER, Calix PE, Zarzosa OA, Pérez GME. Ethnomedicinal plants used for the treatment of dermatological affections on the Purépecha Plateau, Michoacán, Mexico. Acta Bot Mex. 2018;2018(125):95-132.
7. Pinedo AY, Acevedo AJ, Calderon HO, Cerrate CV, Quispe HR, Jayo TA, *et al*. Preventive effect of *Oenothera rosea* on N-methyl-N-nitrosourea-(NMU) induced gastric cancer in rats. Clin Exp Gastroenterol [Internet]. 2017 Dec 11 [cited 2022 Jan 25];10:327-332. Available from: /pmc/articles/PMC5729836/
8. Chirinos R, Pedreschi R, Rogez H, Larondelle Y, Campos D. Phenolic compound contents and antioxidant activity in plants with nutritional and/or medicinal properties from the Peruvian Andean region. Ind Crops Prod [Internet]. 2013;47:145-152. Available from: http://dx.doi.org/10.1016/j.indcrop.2013.02.025
9. Flores GR. Antibacterial Activity of *Oenothera rosea* (L 'Hér) Leaf Extracts. Br J Med Med Res [Internet]. 2012 Jun 5 [cited 2022 Jan 25];2(3):396-404. Available from: https://journaljammr.com/index.php/JAMMR/article/view/12142
10. Rashid R, Hameed R, Choudhary M, Mukhtar F, Khan A. Anti-adipogenic and Cytotoxic Effects of a New Compound from Hartmannia rosea G. Don. Curr Pharm Anal. 2015;11(4):300-307.

11. Ruiz VR, Ruiz MRM, Ruiz HM, Cortazar GM, González BEA, Aparicio JAR, *et al*. Effect of phenolic compounds from *Oenothera rosea* on the kaolin-carrageenan induced arthritis model in mice. J Ethnopharmacol [Internet]. 2020;253(February):112711. Available from: https://doi.org/10.1016/j.jep.2020.112711
12. Emmanuel E, Emmanuel. Phytochemical and anti-nutritional studies on some commonly consumed fruits in Lokoja, Kogi State of Nigeria. Gen Med Open. 2018;2(3):1-5.
13. Marka R, Talari S, Penchala S, Rudroju S, Swamy Nanna R. Preliminary phytochemical analysis of leaf, stem, root and seed extracts of *Arachis Hypogaea* L. Int J Pharm Sci Rev Res. 2013;20(1):134-9.
14. Rajesh KD, Vasantha S, Rajesh NV, Panneerselvam A. Qualitative and quantitative phytochemical analysis in four pteridophytes. Int J Pharm Sci Rev Res. 2014;27(2):408-412.
15. Flores MYK, Pérez EAR, Quijano VJS, Urrutia MZM, Hernández RMC, Morales FLG, *et al*. LC-MS metabolomic evidence metabolites from *Oenothera rosea* L´ Hér ex Ait with antiproliferative properties on DU145 human prostate cancer cell line. Biomed Pharmacother. 2023;165(April).
16. Flores MYK, Rosales MH, Aldrete CME, Camargo MME. Anti-inflammatory activity of aqueous and methanolic extracts of *Oenothera rosea* Ĺ Hér ex Ait in the rat. Rev Mex Ciencias Farm [Internet]. 2009 [cited 2022 Jan 25];40(3):11-6. Available from: http://www.redalyc.org/articulo.oa?id=57912963003
17. Rashid R, Kalsoom Khan A, Ul Haq I, Mir S, Mehmood S, Lu Y, *et al*. In vitro biological screening of hartmannia rosea extracts. Biomed Res Int; c2017, 2017.
18. Márquez-Flores YK, Meléndez-Camargo ME, García-Mateos NJ, Huerta-Anaya MC, Pablo-Pérez SS, Silva-Torres R. Phytochemical composition and pharmacological evaluation of different extracts of *Oenothera rosea* L´Hér ex Ait (Onagraceae) aerial part. South African J Bot. 2018;116:245-50.
19. Munir R, Semmar N, Farman M, Ahmad NS. An updated review on pharmacological activities and phytochemical constituents of evening primrose (genus Oenothera). Asian Pac J Trop Biomed. 2017 Nov 1;7(11):1046-54.
20. Shabani S, Rabiei Z, Amini-Khoei H. Exploring the multifaceted neuroprotective actions of gallic acid: a review. Int J Food Prop [Internet]. 2020;23(1):736-52. Available from: https://doi.org/10.1080/10942912.2020.1753769
21. Habtemariam S. Antioxidant and Anti-inflammatory Mechanisms of Neuroprotection by Ursolic Acid: Addressing brain injury, cerebral ischemia, cognition deficit, anxiety, and depression. Oxid Med Cell Longev. 2019;2019.
22. Tsai SJ, Yin MC. Antioxidative and anti-inflammatory protection of oleanolic acid and ursolic acid in PC12 cells. J Food Sci. 2008;73(7):174-178.