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Antioxidant and Neuroprotector effect of *Lepidium meyenii* (MACA) methanol leaf extract against 6-hydroxy dopamine (6-OHDA)-induced toxicity in PC12 cells

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Abstract

Reactive oxygen species (ROS) are normally produced during cell metabolism, there is strong evidence to suggest that ROS produced in excess impair the cell and may be etiologically related to various neurodegenerative diseases. This study was undertaken to examine the effects of *Lepidium meyenii* (MACA) methanol leaf extract on neurotoxicity in PC12 cell exposed to 6-hydroxydopamine (6-OHDA). Fresh samples of "maca" leaves were processed in order to obtain foliar extracts and to evaluate the neurobiological activity on PC12 cells, subjected to the cytotoxic effect of 6-hydroxydopamine (6-OHDA) through the determination of the capacity antioxidant, cell viability and cytotoxicity assays on PC12 cells. The results of the tests of antioxidant activity, showed maximum values of 2262.37 and 1305.36 expressed in Trolox equivalents (TEAC), for the methanolic and aqueous fractions respectively. Cell viability assays at a dose of 10 µg extract showed an increase of 31% and 60% at 6 and 12 hours of pretreatment, respectively. Cytotoxicity assays at the same dose and exposure time showed a 31.4% and 47.8% reduction in Lactate Dehydrogenase (LDH) activity and an increase in superoxide dismutase (SOD) activity. The results allow us to affirm that the methanolic foliar extract of "maca" presents *in vitro* neurobiological activity of antioxidant protection, increase in cell viability and reduction of cytotoxicity against oxidative stress generated by 6-OHDA. In conclusion, the present study shows a protective role for *Lepidium meyenii* leaf extract on 6-OHDA induced toxicity by an antioxidant effect.

Introduction

Parkinson's disease (PD) is an idiopathic degenerative process of the nigrostriatal dopaminergic pathways and the accumulation of α-synuclein (Lewy bodies) in surviving neurons (Doria et al., 2016). This disease is primarily affecting the aging population (Gao and Wu, 2016), and it is considered a heterogeneous neurological disorder including a variety of motor and non-motor symptoms (Hall et al., 2016).

Oxidative stress plays a fundamental role in the pathophysiology of PD, and excessive reactive oxygen species (ROS) can lead to DA neuron vulnerability and eventual death (Xie and Chen, 2016). On this basis, therapy with antioxidants has been proposed for the prevention and treatment of PD (Kim et al., 2015). Antioxidants are widely distributed in the plant kingdom and they become in potential source for treatment of different diseases associated to oxidative stress including PD (Sarrafchi et al., 2016).

The main antioxidant agents present in plants are the polyphenols that includes flavonoids, which may contribute for treatment of central nervous system diseases (Pandareesh et al., 2015).

Sulforaphane a compound derived from glucosinolates and present in cruciferous vegetables, is a potent indirect antioxidant and recent advances have shown its neuroprotective activity in various experimental models of neurodegeneration (Morroni et al., 2013).

Lepidium meyenii (maca) is also a cruciferous plant growing over 4000 m in the Peruvian central Andes. Maca belongs to the family of Brasicáceas (Cruciferae) and is the only species of the genus *Lepidium* domesticated in the Andes. Originally, the crop was restricted to the departments of Junín and Pasco, in the Peruvian Central Andes (Gonzales et al., 2014).

Maca is characterized because high content in glucosinolates (Valerio and Gonzales, 2006). Several studies have demonstrated that aqueous extract of black maca improved experimental memory impairment induced by ovariectomy or by alcohol, due in part, by its antioxidant and acetyl cholinesterase (Ache) inhibitory activities (Rubio et al., 2011; 2011a). The effect of maca extracts on cognition suggests that active compounds after oral consumption are available at the central nervous system (CNS). A recent clinical trial controlled with placebo showed that maca improves mood in healthy adult men and women after 12 weeks of treatment (Gonzales-Arimborgo et al., 2016).

The neuroprotective activity of maca has been also studied in other models *in vivo* but also *in vitro* (Pino-Figueroa et al., 2010).

Mosty of the biological effects on maca has been studied using the hypocotyls, the edible part of the plant as traditionally described (Gonzales et al., 2014). A pionner investigation using extracts maca leaves showed a prevention of development of sunburn cells, epidermal hyperplasia, leukocytic infiltration, and other alterations produced by ultraviolet B (UVB) radiation. These effects seem to be due to the presence of significant antioxidant activity and the inhibition of lipid peroxidation (Gonzales-Castañeda et al., 2011).

PC12 cells are a cell line derived from a pheochromocytoma of the rat adrenal medulla and is used as an *in vitro* model to study neurotherapy for PD among others (Walkinshaw and Waters, 1994). These cells secrete dopamine (Seo et al., 2016) and contain dopamine transporters and it is an adequate model to study neuronal toxicities induced by drugs (Magalingam et al., 2016).

6-hydroxydopamine (6-OHDA) induces apoptosis in rat pheochromocytoma PC12 cells (Guo et al., 2013). Administration of 6-hydroxy dopamine (6-OHDA) produces reactive oxygen species (ROS) and impairs mitochondrial function (Masoudi et al., 2015). In turn, oxidative stress induced by 6-OHDA activates MAPK family members such as ERK1/2 and promotes DA neuronal death (Kulich and Chu, 2001).

The presebt study evaluates the neuroprotective effects of methanolic extract of leaves from maca (*Lepidium meyenii*) in 6-OHDA-induced neurotoxicity in PC12 cells, and it relates these effects with the presence of antioxidant compounds.

Methods

Materials

Collection of the biological material of *Lepidium meyenii* Walpers "Maca" occurred in August 2015 in the district of Ramancancha (south latitude 11°9'23", north latitude 75°59'44") province of Junin, in the Junin Department. PC12 cells line, 6-hydroxydopamine, Dulbecco´s modified Eagle´s medium (DMEM), horse serum, fetal bovine serum, pen-strep and Sodium Nitroprusside (NPS) were obtained from Sigma Aldrich.

Preparation of the maca leaves extract

Fresh maca leaves were dried at 40° C for 48 hours with plant position changes in a Binder natural convection oven until maximum moisture of 15% was reached. Subsequently, the dry leaves were pulverized, sieved in a sieve of 600 µm pore size and stored in darkness with temperature control at 20° C until analysis.

To obtain the maca extracts, 10 g of the homogenized powdered sample were placed in a balloon for maceration of 250 ml. Then 80 ml of methanol was added and

the mixture was allowed to soak for 20 hours. Subsequently, reflux was made for 4 hours. The resulting solution was vacuum filtered with Whatman No 4 paper and brought to 100mL. From 10 grams of raw material, the extraction procedure yields 1.2 grams of methanol extract.

Antioxidant Capacity

The analysis of the antioxidant capacity was performed by the ABTS method reported by Arnao et al (2001) with some modifications. To prepare the stock solution of ABTS 38.2 mg of ABTS and 6.6 mg of potassium persulfate were weighed and 10 ml was stocked. This solution was diluted in 80% methanol until an absorbance of 1.1 ± 0.02 at a λ of 734 nm was obtained.

For the standard curve, TROLOX solutions at 100, 200, 300, 400 and 500 nM were used. To prepare the reaction tubes, 150 µL of the sample, standard solution or methanol (blank) to be measured was added and 2850 µL of the ABTS moiety was added. The reaction was allowed to proceed at room temperature for 30 minutes, at which time the absorbance at 734 nm was measured.

For antioxidant capacity assessment, five fractions from polar to non polar were used. For the lipophilic phase analyzes the same steps were followed, changing the solvent methanol to dichloromethane.

Cell culture

PC12 cells were cultured in a 5% CO_2 incubator at a temperature of 37°C in DMEM medium supplemented with 5% horse serum and 5% fetal bovine serum and Pen-Step antibiotics (100 U/ml). The initial growth was done with the flask T-75 with adherent until reaching a confluence of 80%, then, the cells were disaggregated and passed to microplates of 96 wells with adherent, at a density of $1x10^5$ cells/ml and incubated overnight to achieve cell adhesion to the wells.

PC12 cells were treated with 3 different concentrations of leaf maca methanolic extract: 2 µg, 5ug and 10µg for 6 and 12 hours. Subsequently the pre-treated cells were induced with 6-OHDA at 100uM or with 200uM Sodium Nitroprusside (NPS) for 12 and 24 hours, respectively.

The negative control group was PC12 cells alone with DMEM medium, and the positive control group was PC12 cells treated only with 6-OHDA or NPS. All assays were performed in triplicate for each dose or incubation time.

Determination of Cell Viability

The cytotoxic effect of 6-OHDA on PC12 cells pretreated with the methanol extract of Maca were determined using the MTT (3- (4,5 Dimethylthiazol-2) -2,5-

diphenyltetrazolium bromide) assay, wherein the yellow tetrazole is reduced to a purple insoluble compound known as formazan, in the mitochondria of viable cells (Liu et al, 2009).

After pretreatment and induction with 6 OHDA for 12 hours, 20 µl of MTT solution (0.25%) was added to each well and incubated for 4 hours at 37°C. The solubilization of the formazan was then performed with 150 µl of DMSO and the colored solution quantified using a plate reader at a wavelength of 570 nm. Cell viability data are expressed as a percentage with respect to the cells of the negative control group.

Cell Cytotoxicity

Cytotoxicity was quantified by measuring the activity of lactate dehydrogenase (LDH) released from damaged cells in the culture medium (Kong et al,2013). After treatment of PC12, the cells were treated with 0.5% Triton X-100, the whole medium was centrifuged and the supernatant was used for the determination of the LDH activity by spectrophotometry at 440nm according to the instruction of the kit (LDH Cytotoxicity assay kit II, Abcam). LDH was expressed as a percentage according to the following equation:

$$\% \text{ LDH} = (\text{LDH of DMEM} / \text{total LDH medium}) \times 100.$$

Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined by a sensitive kit (Superoxide dismutase activity colorimetric Assay kit, Abcam) using WST-1 to produce a water-soluble formazan compound due to reduction with a superoxide anion. One unit of SOD is the amount of the enzyme necessary to produce 50% dismutation of superoxide radical was determined from a standard curve and expressed in U/ml.

Release of Nitric Oxide

The release of nitric oxide (NO) produced by exposure to sodium nitroprusside (NPS) was spectrophotometrically estimated by nitrite measurement (Pandaresesh et al,2014). After 24 hours of NPS treatment at 200 uM, 100 µl of the culture medium was taken and incubated with 100 µl of 1% Griess reagent, incubated at room temperature for 10 minutes. The absorbance of the colorimetric reaction was measured at 540 nm using a plate reader. The nitrite content was calculated based on a calibration curve constructed with sodium nitrite, and was expressed in ug/ml.

Statistical analysis

Data are expressed as means and standard deviation. The results were analyzed using analysis of variance (ANOVA) with the statistical package STATA 12.0. A value of $p < 0.05$ was considered as statistically significant.

Results

Antioxidant Capacity

Trolox equivalents (TEAC) values for different fractions evaluated ranging from 74.33 to 2262.37. The antioxidant activity observed shows higher values for the methanolic extract than the separated fractions. Considering the different solvents applied, the chloroform extract has the lowest values of antioxidant activity (Table 1). Most of the antioxidant activity observed in the methanol extract is observed in the aqueous fraction.

Cell viability

Induction with 6-OHDA reduced PC12 cell viability from 100% in the negative control to 56.1%. This effect of 6-OHDA was prevented by pretreatment with the methanol maca leaves extract in a dose-response manner (Figure 1). This is observed after 6 or 12 hours of incubation. Under these conditions, cell viability was higher at 10 ug of methanolic extract in the 2 incubation times (72.6% and 85.1%), being greater at 12 hours ($p < 0.001$).

Cellular cytotoxicity

The LDH is a stable cytoplasmic enzyme in most cells that is quickly released when there is a damage to the plasma membrane, therefore an increase in membrane damage or cell death results in an increase in LDH activity in media cell culture.

As shown in Figure 2, when PC12 cells were incubated with 6-OHDA for 12 hours, the percentage of LDH released was increased from 12% (control) to 54.6% ($p<0.001$). Pretreatment with maca extract in PC12 treated with 6-OHDA produced a significant reduction in LDH activity released by the cells, the percentage reduction LDH directly proportional to the concentration of the extract (2 µg: 43.6%; 5 µg: 10 µg 34.3% and 29.3% for 6 hours; $p<0.001$) and time-dependent treatment (5 µg: 29%; 10 µg: 22.3% for 12 hours, $p<0.001$).

Superoxide dismutase activity

SOD levels were significantly decreased in PC12 cells treated with 6-OHDA (0.6 U/ml) compared to negative control ($p<0.001$). Pretreatment with methanolic maca extract reduced the inhibitory effect of 6-OHDA in a dose-dependent fashion

($p<0.001$). The effect was observed at 6 h and 12 h incubation with a better effect at 12 hours of incubation (Figure 3).

Nitric Oxide Release

In our experiments (Figure 4), exposure of PC12 to SNP produced an increase in nitrite, nitric oxide indicator, relative to the control group (3.4ug/ml and 3.7ug/ml at 6 and 12 hours respectively). Pretreatment with maca extract significantly decreased nitrite concentration at 2, 5 and 10ug (2.54/ml, 2.38ug/ml, 2.15ug/ml, $p<0.001$ respectively) for 6 hours of incubation. At 12 hours of incubation, nitrite levels were further reduced at 2, 5 and 10ug (2.05 ug/ml, 1.65ug/ml and 1.3ug/ml, $p<0.001$ respectively).

Discussion

Oxidative stress contributes, at least in part, to the pathogenic cascade that leads to degeneration and neuronal death in various neurodegenerative diseases (Kim et al., 2015; Kumar and Khanum, 2012; Magalingam et al., 2016). The induction of endogenous antioxidant proteins by plants seems to be a reasonable strategy to decrease the progression of this type of diseases. Parkinson's disease is a chronic, debilitating neurodegenerative movement disorder characterized by progressive degeneration of dopaminergic neurons in the substantia nigra pars compact region in human midbrain. To date, oxidative stress is the well accepted concept in the etiology and progression of Parkinson's disease (Magalingam et al., 2014; Magalingan et al.,2015; Morroni et al., 2013). Hence, the therapeutic agent is targeted against suppressing and alleviating the oxidative stress-induced cellular damage. Within the past decades, an explosion of research discoveries has reported on the protective mechanisms of flavonoids, which are plant-based polyphenols, in the treatment of neurodegenerative disease using both *in vitro* and *in vivo* models (Pandareesh et al., 2015; Sarrafchi et al., 2016; Valerio and Gonzales, 2005).

In our antioxidant evaluation of the methanol extract of maca, the results are related to the previous phytochemical studies (Gonzales-Castañeda et al., 2011; Pino-Figueroa et al., 2010; Gonzales et al., 2014), where the presence of molecules involved in the antioxidant activity (phenols, flavonoids and terpenes) was observed in greater quantity than in the aqueous fraction. Other study mentions that extracts of Maca have a large number of polyphenols, which would be involved in the cytoprotective capacity against UV radiation in the skin of animals treated with Maca (Gonzales-Castañeda et al., 2011).

Among the methods used to determine the ability of an antioxidant to capture free radicals, the $ABTS^{\bullet+}$ radical is one of the most applied, considering a highly sensitive, practical, fast and very stable method (Arnao et al., 2001). Treatment with herbal medicine may be active by increasing antioxidants present in plants, inhibiting intracellular ROS generation or activating antioxidant defense in the target cell (Kim et al., 2010). Maca extract showed scavenging activity in the ABTS assay. Better antioxidant activity was observed in the whole methanol extract than in the fractions and among fractions best antioxidant activity was observed in the aqueous frcation. Previous study has demonstrated a better biological property observed after maca administrations in the hydroalcoholic extract that in its fractions (Yucra et al., 2008).

We evaluated the neuroprotective capacity of the methanolic extract of maca in a cell model of Parkinson's, inducing neurotoxic damage in PC12 cells, through an analogue of dopamine (DA), 6 OHDA. In cell viability assays, pretreatment with maca extract increased cell viability, especially at 10 ug for 12 hours of pretreatment (85.1%, $p<0.001$). Increased oxidative stress has been implicated as one of the causes of DNA, protein and lipid damage and is considered an important mechanism in the process of neuronal death induced by OHDA. Increased cell viability and decreased cell cytotoxicity in PC12 cells pretreated with the methanol extract of maca leaves confirms the antioxidant capacity of maca. A mechanism of cellular defense to compensate for the state of oxidative stress is given by endogenous antioxidant enzymes, of which superoxide dismutase (SOD) is one of the most important and abundant in PC12 (Guo et al., 2013; Liu et al., 2009; Magalingam et al., 2014).

SOD is in large quantities in the brain and catalyzes the dismuttion of superoxide anion to oxygen molecules and hydrogen peroxide, which are less toxic molecules (Kong et al., 2013; Magalingam et al., 2016). In our trial we saw an increase in the activity of SOD in the cells pretreated with maca extract in all doses analyzed, especially at 5 and 10 ug ($p <0.001$), suggesting that the neuroprotective effect could be linked to the inhibition of oxidative stress by oxygen free radicals (Magalingan et al., 2015; Masoudi et al., 2014).

The neuronal damage induced by sodium nitroprusside (NPS) is mainly due to the production of free radicals such as nitric oxide, peroxynitrate and other reactive nitrogen species. Excessive levels of nitric oxide lead to oxidative stress to neurons and their subsequent damage or cell death (Pandaresesh and Anad, 2014). In our trial, we saw that NPS increased nitrite levels in PC12 cells, and after pre-treatment with maca extract at 5ug and 10ug these levels decreased significantly. Nitric oxide and peroxynitrate are potent inducers of cell damage by forming nitrosamines in DNA and inhibiting DNA repair mechanisms (Pandaresesh and Anad, 2014).

It is known that mitochondrial ROS can activate signaling pathway mediating neuronal death in PD (Onyango et al., 2017). Our findings suggest that maca a nutritional

plant may reverse PD probably by a mechanism involving mitochondrial function and upregulate autophagy-related proteins as it has been previously demonstrated by slowing down age-related cognitive decline (Guo et al., 2016).

Conclusions

Methanolic extract of *Lepidium meyenii* leaves presents an *in vitro* neurobiological effect of antioxidant protection, increasing the cellular viability and reduction of the cytotoxicity against oxidative stress generated by the 6-OHDA.

In addition, it is proposed that the mechanisms that explain this action are the enhancement of the oxidation-reduction balance enzyme mechanism, through the increase of the activity of the enzyme superoxide dismutase; and direct uptake of free radicals, such as nitric oxide.

Conflict of interest

The authors declare that there is no conflict of interests of any kind in this research.

Acknowlegments

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Table 1. Antioxidant activity of different fractions of ethanolic extract of *Lepidium meyenii* determined by ABTS (µmol of trolox EQ/100g)

FRACTION	A	B	C	D	E
PHASE	METHANOLIC	CHLOROFORMIC	DICHLOROMETHANE	CHLOROFORM-ETHANOLIC	AQUEOUS
µmol of trolox EQ/100g	2262.37	74.33	246.42	149.96	1305.36

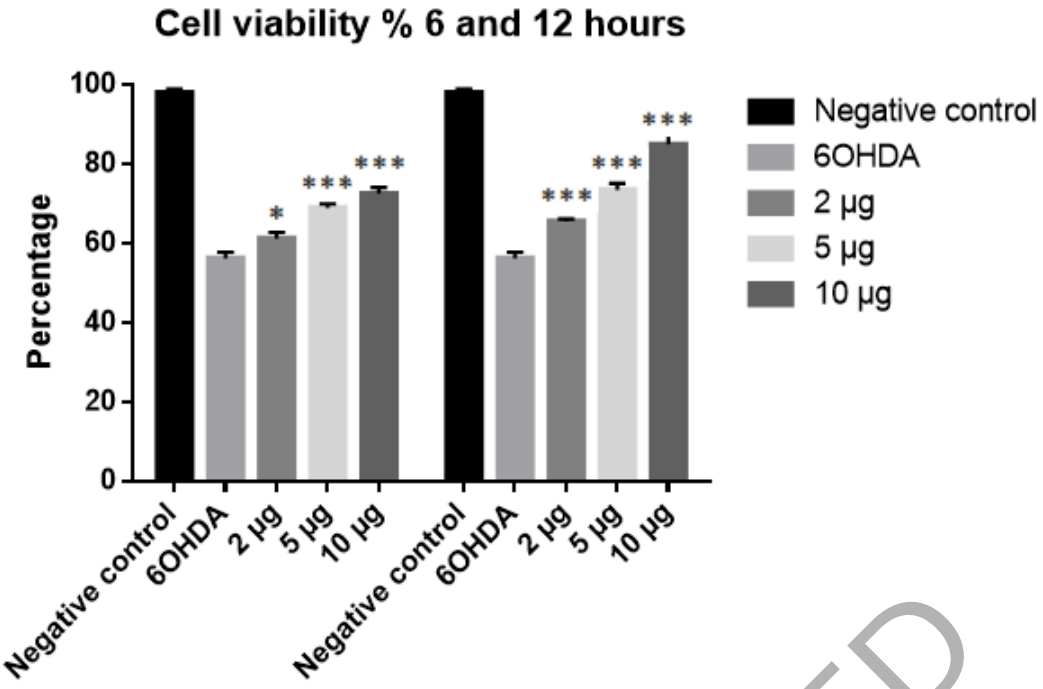

Figure 1. Cell viability after pretreatment with methanolic maca extract. The pretreatent with methanolic maca extract showed a significant increase in cell viability compared to the 6-OHDA. The cell viability was highest at 10 µg in 12 hours. Values are the percentege of viable cells. Data are mean and SD (n=3), **p<0.01, ***p<0.001, relative to cells with only 6-OHDA.

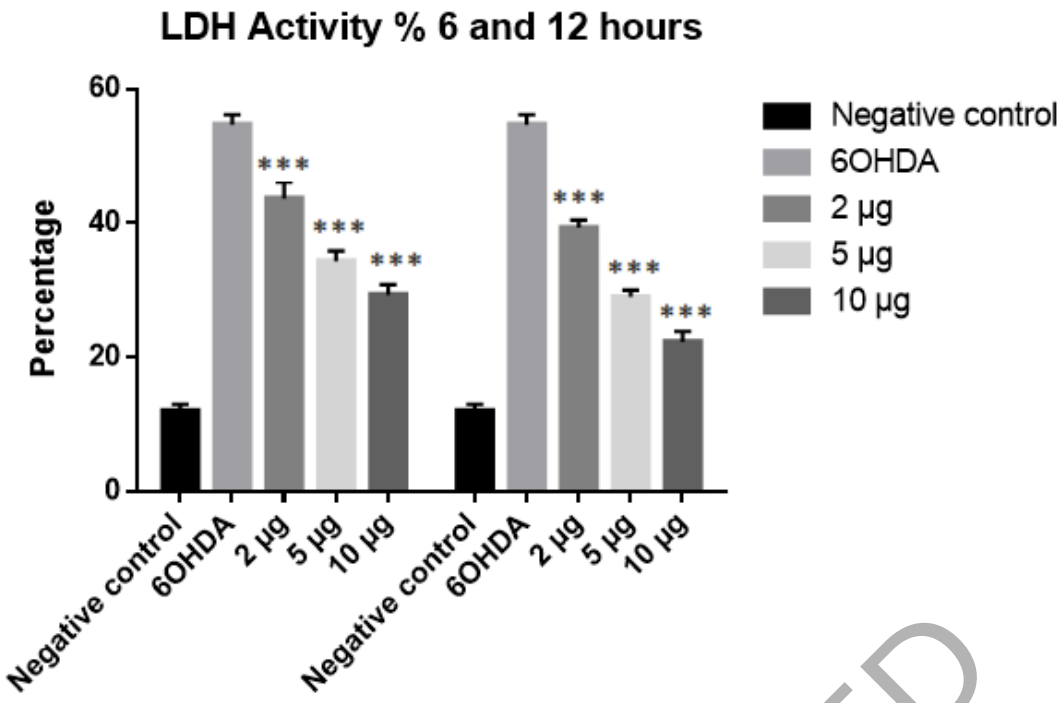

Figure 2. LDH activity after pretreatment with methanolic maca extract. The pretreatent with methanolic maca extract showed a significant decrease in LDH activity compared to the 6-OHDA. The LDH activity was lowest at 10 µg in 12 hours. Values are the percentege of activity. Data are mean and SD (n=3), **p<0.01, ***p<0.001, relative to cells with only 6-OHDA.

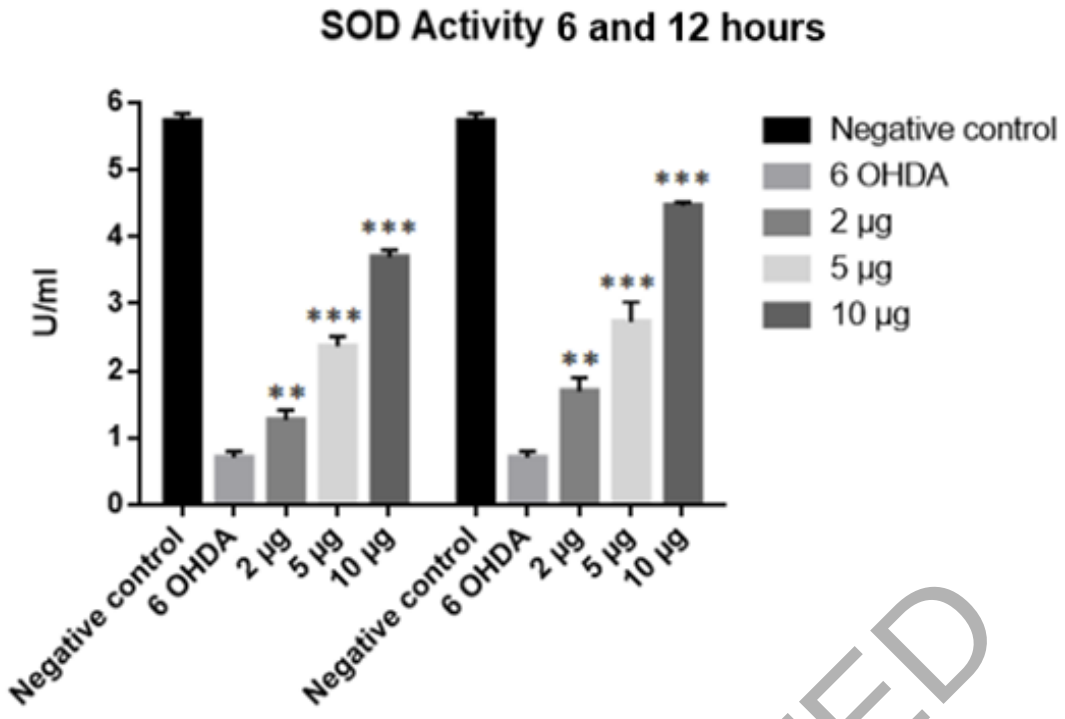

Figure 3. SOD activity after pretreatment with methanolic maca extract. The pretreatent with methanolic maca extract showed a significant decrease in SOD activity compared to the 6-OHDA. The SOD activity was lowest at 10 µg in 12 hours. Values are the unidades per milliliter. Data are mean and SD (n=3), **p<0.01, ***p<0.001, relative to cells with only 6-OHDA.

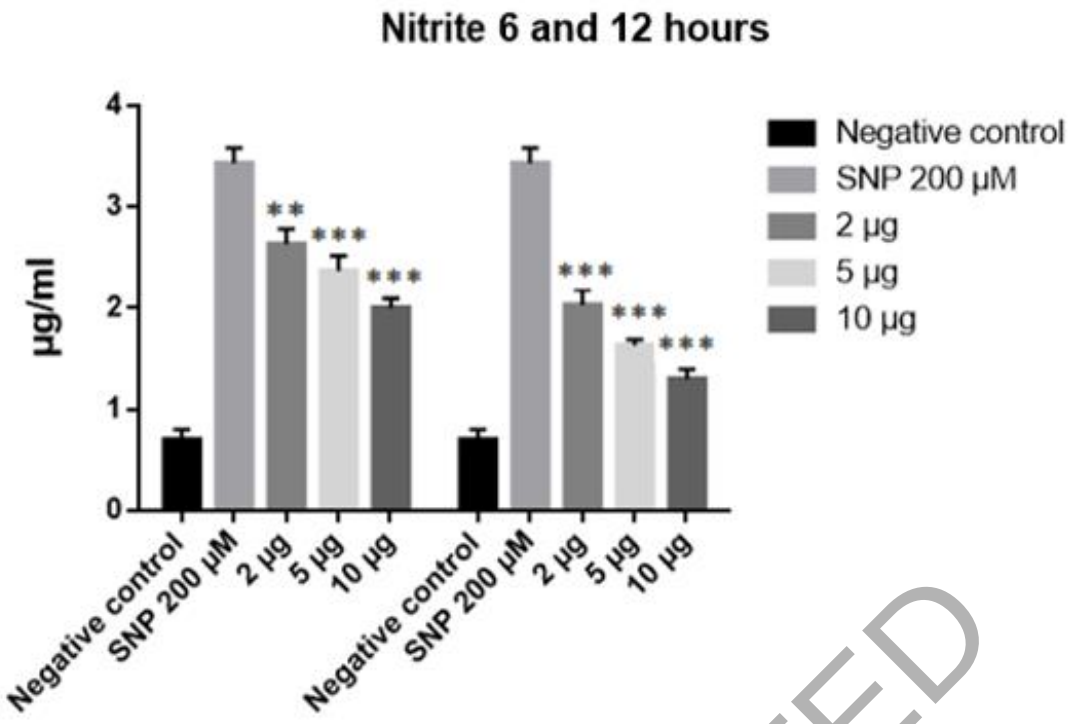

Figure 4. Nitrite load after pretreatment with methanolic maca extract. The pretreatent with methanolic maca extract showed a significant decrease in nitrite load compared to the 6-OHDA. The nitrite level was lowest at 10 µg in 12 hours. Values are the micrograms per milliliter. Data are mean and SD (n=3), **p<0.01, ***p<0.001, relative to cells with only 6-OHDA.