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ALGERIAN POMEGRANATE PEEL DECREASES LEAD CONCENTRATION IN BRAIN AND IMPROVES NEUROLOGICAL DISORDERS

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Abstract

The purpose of this study was to determine the neuroprotective potential of pomegranate peel methanolic extract (500 mg kg^{-1}) on lead acetate (1000 ppm) induced neurotoxicity. After 12 weeks, the mice were subjected to behavioral tests. Brain injuries were determined with hematoxylin and eosin staining and lead accumulation was measured by graphite furnace atomic absorption with Zeeman correction. Lead exposure induced neurobehavioral alterations, reduced body growth, lead deposits in brain and histological change in the lead-treated group. Furthermore, co-administration of pomegranate extract with lead decreased locomotion, anxiety and depression in lead-exposed mice as indicated by the number of cells crossed by mice, the residence time in the dark compartment and the immobility time in forced swimming. Also, pomegranate extract improved weight loss and histological change of cortex cerebral and hippocampus by reducing the lead concentration in these sites. Pomegranate methanolic extract has neuroprotective effects against lead-induced neurological disorders probably by its various phytochemical components.

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Introduction

A widely known environmental toxicant, lead (Pb), adversely affects the majority organ systems and extremely impaired fetal brain development (MOUSA et al. 2015). SALEH et al. 2018) found that lead produces hazardous toxic effects including cerebellar damage especially in fetus and female pregnant rats. According to GURER et al. (2000) lead induces oxidative stress, reduces the antioxidative defense system, interfere with certain essential metals which are required for antioxidant enzyme activities, and/or modifying the integrity of the membrane and the fatty acid composition by increasing the cell sensibility to reactive oxygen species.

The beneficial role of antioxidant nutrients within the prevention of lead toxicity has investigated by several researchers however the mechanisms of antioxidant nutrients to restore effective via rebalancing the impaired prooxidant/antioxidant ratio are not completely clear (HSU and GUO 2002). Polyphenols are the most abundant antioxidants found fruits, vegetables, beverages, plants and some herbs. These natural extracts are able to protect neuronal cells through different biological processes like prevented apoptotic neural death, ROS scavenging, regulate of the kinase signal cascade and modulation of Ubiquitin-Proteasome pathway (CAMPOS-ESPARZA and TORRES-RAMOS 2010). Pomegranate is a high potential antioxidant and has several benefits health; antiatherogenic, antihypertensive, and anti-inflammatory properties which leads it's used in the prevention and treatment of diverse diseases. This fruit is rich with several classes of chemical components such as: flavonoids, anthocyanins, punicic acid, ellagitannins, alkaloids, some of carbohydrate, simple organic acids, and other components (ZARFESHANY et al. 2014).

In this regard, the current study is designed experimentally in female mice in order to investigate the beneficial role of pomegranate extract in the protection from brain damage that produced by administration of 1000 ppm lead for 12 weeks.

Materials and Methods

Extract preparation

Punica granatum were collected in the western region of Algeria. They were air-dried for one month and ground into fine powder by using a blender. The powder was kept in airtight container until use. 200 g of the dried powder was soaked in 2 L of methanol and stirred for 24 h using the method of DIALLO et al. (2004) after using 2 L of petroleum ether to remove

the chlorophyll. The operation was repeated on three successive days for the two solvents. The extract obtained after elimination of solvent with the aid of rotary evaporator was stored until at -20°C until used.

Polyphenols

The determination of the total polyphenols in the methanolic extract of *Punica granatum* peel was determined according to the Folin-Ciocalteu method (RAAFAT et al. 2014, MAVI et al. 2004). The concentration of the total polyphenols was deduced from a calibration range established with gallic acid (0–1 mg mL^{-1}). The results are expressed in mg of gallic acid equivalent/g of extract (mg EAG g^{-1} of dry weight).

The total flavonoid content in the methanolic extract from *Punica granatum* peel was determined using a method based on the formation of a flavonoid-aluminum complex having the maximum absorbance at 430 nm (HMID et al. 2017, LAMAISON and CARNAT 1990). Concentrations of flavonoids were deduced from the calibration range established with quercetin (0–40 mg mL^{-1}) and the flavonoid content was expressed in mg of quercetin equivalent per g of extract (mg EQ g^{-1} of dry weight).

The total tannin content was determined by the methods of POLSHETTIWAR et al. (2007). Tannin concentrations are deduced from the calibration range established with tannic acid (0–40 μg mL^{-1}) and the tannin content was expressed in mg of equivalent tannic acid per g of extract (mg ET g^{-1} of dry weight).

The determination of tannins condensed by the vanillin test was carried out according to the method of JULKUNEN-TITTO et al. (1985). The concentration of condensed tannins (proanthocyanidins) is deduced from the calibration range established with catechin (0–0.4 mg mL^{-1}) and is expressed in milligrams of catechin equivalent per gram of extract (mg ECat g^{-1} of dry weight).

Experimental animals

Twenty one healthy mice weighing from 20±2.52 g were taken from Pasteur Institute of Algiers. The animals were maintained room temperature 24 ± 5°C and standard 12 h day/night cycle. The animals were fed on a standard diet and fresh drinking water. The experimental protocol is in accordance to the Guide for the Care and Use of Laboratory Animals (8-th edition, 2011) and approved by the scientific committee of the university. Mice are divided in 3 groups, each group contains 7 mice. Group 1: control mice (*C*), animals receiving drinking water for 90 days. Group 2 (Pb): a dose of 1000 ppm of lead acetate was administered to mice orally for

12 weeks (DJEBLI et al. 2005). Group 3 (Pb-E): mice were treated with 500 mg kg^{-1} of methanol extract of *Punica granatum* peel for 4 h/day followed by acetate of lead at a dose of 1000 ppm orally for 20 h/day for 90 days. Weight gain and water intake were measured daily and weekly for 12 weeks.

Behavioral tests

The locomotor activity of mice was assessed by the number of crossing squares noted as scores per time of 5 min for 20 min investigated. The apparatus (a cage) contains a platform divided into 16 equal squares (SÁENZ et al. 2006).

The anxiety was established by the white/dark box test. A test by which the anxious animal presents conflict between the tendency to explore and the initial tendency to avoid the unfamiliar (CRAWLEY and GOODWIN 1980).

The depressive state was measured by Porsolt test since some aspects of human depression matches with the behavioral immobility of rats during forced swimming (PORSOLT et al. 1977). The mice were observed for 5 min and the immobility time was recorded.

Lead brain concentration

After sacrifice, the brain of each mice was digested in 5 : 1 nitric acid: perchloric acid, as described by GUPTA and GILL (2000). The concentration of lead in the brain was determined by graphite furnace atomic absorption with Zeeman correction (Agilent 240 ZAA/GTA 120) based on the atomization (HOLCOMBE 2010). The operating parameters were: wavelength: 283.3 nm, the slit: 0.5 nm, lamp current: 10.0, standard solution: 1000 mg L^{-1}.

Histological study

After fixation of the brain in 10% formalin, brain has been subsequently dehydrated by a series of alcohol dilution. Dehydrated cerebral tissue was included in paraffin. Sections of 5 μm thicknesses were stained with hematoxylin and eosin (H&E).

Statistical study

All data were expressed as the mean ± SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by student *t*-test. *p* values less than 0.05 were considered as statistically significant.

Results

The methanolic extract of pomegranate peel showed the following concentrations: 183.67 ± 1.91 mg GAE g^{-1} of dry weight for total polyphenol, 53.59 ± 0.53 mg EQ g^{-1} of dw for total flavonoid, 143.81 ± 10.53 mg EAT g^{-1} of dw for total tannin and 113.18 ± 21.38 mg EC g^{-1} dw for condensed tannin (Table 1).

Table 1

Total content of polyphenols, flavonoids and total tannins and condensed tannins in methanolic extract of pomegranate peel

Total phenol	Total flavonoid	Total tannin	Condensed tannin
183.67 ± 1.91 mg GAE g^{-1} of dw	53.59 ± 0.53 mg EQ g^{-1} of dw	143.81± 10.53 mg EAT g^{-1} of dw	113.18 ± 21.38 mg EC g^{-1} of dw

Table 2 shows the body weight of the mice and water intake during 12 weeks of experimentation. Mice intoxicated with lead acetate showed a decrease in body weight compared to control mice ($P > 0.05$). The mice receiving the methanolic extract from the pomegranate peel have body weights comparable to those of the controls ($P > 0.05$).

Table 2

Effect of Lead acetate on water intake and body weight in mice after 12 weeks of experiment

Group	Body weight [g]			Water intake [ml]		
	C	Pb	Pb-E	*C*	Pb	Pb-E
Week 1	20.78 ± 1.31	18.21±1.36	21.78±0.91	212.77± 48.65	126.61±42.56*	186.57±31.7
Week 4	30.32 ± 2.74	27.78±0.52	30.42±1.36	–	–	–
Week 8	34.54 ± 2.87	32.6±1.58	35.33±3.07	–	–	–
Week 12	36.18 ± 3.09	32.17±0.42	36.78±2.46	–	–	–

C – control without any treatment (© Lavoisier: Phytothérapie, GADOUCHE et al. (2018). Pb – lead-exposed mice (500 mg kg^{-1}) and (Pb-E) intoxicated treated. Pb vs. control, Pb-E vs. control. * $P < 0.05$.

The locomotor activity of the mice was evaluated by the score which is the number of cells visited by the mice in 20 minutes divided into four phases. Exposure to lead caused significant locomotor hyperactivity in the lead group compared to the control group in the 4 phases. However, methanol extract from pomegranate peel significantly decreased locomotor activity in the third phase, whereas it increased significantly in the last two phases $P < 0.05$ (Figure 1*a*).

The mice that received the lead acetate for 90 days spend significantly more time in the black compartment as compared with the control and the treated group. The light compartment is more aversive and anxious for

intoxicated mice group by lead ($P < 0.05$). However, the treated poisoning group (pomegranate peel) behaves similarly to that of the control group, but not significantly ($P > 0.05$) except in the last phase the methanol extract significantly decreases the residence time in compartment caused by lead (Figure 1*b*).

The state of depression of the mice was evaluated by the duration of immobility time after swimming forced. Exposure to lead caused significant increase of the immobility time in the lead intoxicated group compared to the control group and the treated group ($P < 0.05$). Mice treated with methanol extract of pomegranate peel experienced intermediate immobility between controls and mice that received lead acetate $P > 0.05$ (Figure 1*c*).

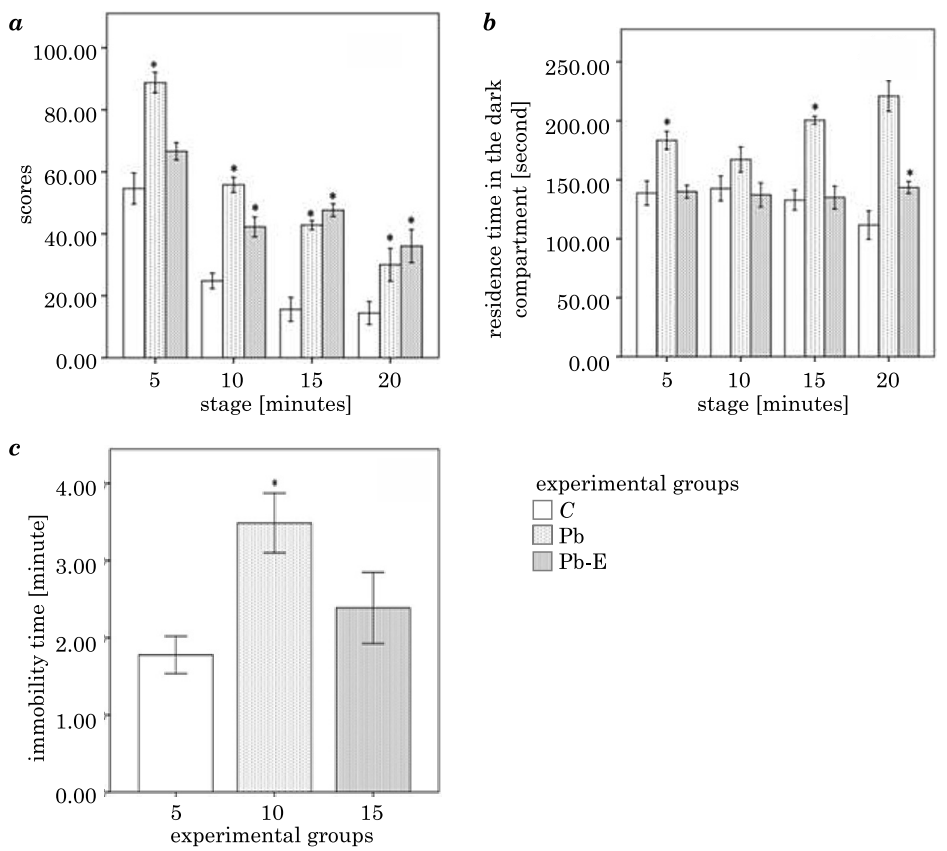

Fig. 1. Tests: *a* – locomotor activity test; *b* – black and white box test; *c* – swimming test. *C* – control group (© Lavoisier: Phytothérapie, GADOUCHE et al. 2018); Pb – mice intoxicated by lead acetate (1000 ppm); Pb-E – mice intoxicated treated with 500 mg kg^{-1} of methanol extract of *Punica granatum* peel. Values represent the means of 5 experiments ± SEM; * $P < 0.05$

The determination of the lead by atomic spectrophotometry is given in Figure 2. The results show a high lead level 7.87 ± 7.9 µg L in the brain, confirming its passage through the blood brain membrane and that the brain constitutes a site for the fixation of heavy metals. Treatment with methanol extract significantly reduced lead levels in the brain (1.07 ± 0.3 µg L^{-1}).

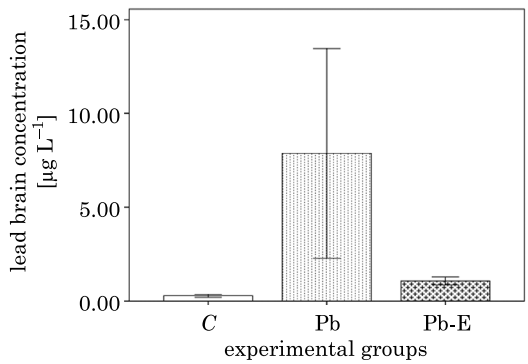

Fig. 2. Lead concentrations [µg L^{-1}] in brain of mice after 12 weeks

The cerebral cortex of the control mice showed normal cell structure. Within the histological sections of the brain of mice which has received lead acetate, we have observed microscopical lesions in many areas of the cerebral cortex and hippocampus, including neuronal degeneration, vacuolization, blood vessel congestion and inflammatory infiltrate with decrease in cell density in both cortex cerebral and hippocampus compared to the control group. Mice treated with the methanolic extract *Punica granatum* peel (500 mg kg^{-1}) showed less neurodegeneration and vacuolization than control and intoxicated mice.

Discussion

Due to its wide environmental distribution, lead alters several organ systems, including the nervous, renal, reproductive and hematological systems (AYKIN-BURNS and ERCAL 2006). Lead induced a significant decrease in body weight and water consumption in mice receiving lead acetate compared to the control group, which clearly shows that lead develop disorder of feed therefore this heavy metal is anorectic. These results are consistent with the work established by SEDDIKI et al. (2010) who observed reduced feed intake in rats with lead diluted acetate at 250 and 500 mg L^{-1} for 90 days.

MISSOUM et al. (2010) found that rats exposed to 1000 ppm of lead acetate had a slow body weight increase over control rats for 8 weeks and that water intake decreased in rats treated with lead during the experiment.

Lead increased the locomotor activity; this results agree with those obtained by SEDDIK et al. (2010), HASSAN and JASSMIN (2010) and KHAROUBI et al. (2011). Lead exposure may lead cognitive and motor impairment, with behavioral alterations as long-term (GARZA et al. 2006). These findings suggested that lead might interfere with catecholaminergic and particularly dopaminergic neurotransmission (DJEBLI et al. 2005).

In the light/dark choice assay, the duration of time spent in both compartments informs us about the degree of anxiety thus an increase in white compartment activity should reflect an anxiolytic effect, whereas a rise in dark compartment activity should reflect anxious behavior (MAXIMINO et al. 2010). In this context; lead intoxicated group spent more time in the dark compartment while mice intoxicated with lead and treated with the methanolic extract from the pomegranate peel have a longer residence time in the light compartment which implies an anxiolytic effect.

Our finding agrees with those obtained by KAHLOULA et al. (2013), who reported that lead increases significantly immobility time in the forced swimming test (FST). MANTOVANI et al. (1999) showed that in the forced swimming test, lead may exert their action repressing directly or indirectly of the *N*-methyl-D-aspartate receptor receptor complex. Within this test, antidepressant activity of drugs is related to reduction in immobility time (WOLAK et al. 2013). Pomegranate peel extract maybe improved the immobility time by modulation the NMDA receptor complex.

The quantification of lead by atomic spectrophotometer shows a high lead level in the brain which reveals that it crosses the blood-brain barrier and that the brain constitutes a fixation site of this metal. Lead damages the prefrontal cerebral cortex, the hippocampus and the cerebellum, which causes several neurological disorders following its ability to replace calcium ions which facilitate its passage through the blood-brain barrier (SANDERS et al. 2009).

STRUŻYŃSKA et al. (1997) reported that the functional state of the blood-brain barrier was altered in prolonged exposure of lead at the low doses. Indeed, these damages are typical for "leaky" microvessels confirmed by both light microscopy and by electron microscopic studies. Methanolic extract from pomegranate peel has decreased lead in the brain and can in part explain its protective effect. The chelating capacity of this metal is due to the flavonoids, tannins and phenolic compounds that potentiate the clearance of the lead of the body. The tannins quantified in the pomegra-

nate bark are polyphenols with excellent binding capacity with metals. PEKDEMIR et al. (2000) have shown that tannic acid under in vitro conditions is a very effective chelator for lead and cadmium.

Lead toxicity affects the normal histological structure of the brain and causes disturbances in the normal functions of the brain. According to BARKUR and BAIRY (2015) histological data indicate that lead exposure caused significant damage to neurons of hippocampus, amygdala and cerebellum regions.

Intake of pomegranate extracts protect brain by inhibition of cholinesterase, the stimulation of antioxidant capacity and decrease of oxidant stress markers; evidenced by the decrease of malondialdehyde and Protein carbonylation levels (AMRI et al. 2017). AHMED et al. (2014) showed that pre-administration of pomegranate extract to rats, can offer a neuroprotective activity against brain injury and DNA damage via decrease of inflammatory and oxidative stress markers, and ATP-replenishing effects. According, to these same authors, many researchers consider natural extracts as novel therapies for neurodegenerative disorders.

Conclusion

The results of the present report demonstrated that lead causes neurological, biochemical and histological alterations of the brain which are restored by daily supplementation of methanolic extract of *Punica granatum* peel. This extract showed anxiolytic and antidepressant activities as well as improved locomotor activity attributed to the high content of diversified bioactive molecules found in this extract polyphenols, flavonoids, and Tannins. These beneficial effects show that the grenade could have a neuroprotector effect.

Conflict of interest

The authors declare no conflict of interest.

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