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Impact of Alcoholic Extract of *Brassica olecera* on Endothelial Dysfunction of Lead Acetate Exposed Rabbits

[1]Dina S. Dheyab, [2]Majida A. J. Al-Qayim

[1,2] Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad

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Abstract: The present study aimed to investigate the modulatory effect of alcoholic extract of broccoli on lead acetate-induced endothelial dysfunction. The aim of the study alcoholic extract of *Brassica olecera* on endothelial dysfunction of lead acetate exposed rabbits. Three groups of female local rabbits (6 each group) aged 5 months with a verge weight 1500 Kg were handled as following:1st as a control group (C) and 2nd lead acetate (Pb-AC) ,and 3rd lead and broccoli (Pb-Ac-B) groups were orally gavage with 10 mg/kg/Bw of lead acetate for 4 weeks. Six adult rabbits of 3rd group were treated with 300 mg/kg alcoholic extract of broccoli orally after exposures to lead acetate for another 4weeks. Blood samples collected before and after treatment. Aorta was harvested after euthanasia, for endothelial nitric oxide synthase gene expression. Vascular endothelial growth factor and antioxidant markers assayed using Eliza. The results showed the ameliorative effects of alcoholic extract in reducing the significant elevation in e NOS expression ($p \leq 0.05$) and serum VEGF and MDA caused by lead acetate, accompanied with significant modulation of TAC. It was concluded the results confirmed the superiority of the *Brassica olecera* in treating the pb-acetate adverse effects on the endothelial, which contributed to its phytochemical and nutrient value.

Keywords: e NOS, VEGF, Broccoli, Endothelial dysfunction, Lead acetate

Corresponding author: (E-mail: dena.s269@covm.uobaghdad.edu.iq)

Introduction

Lead (Pb) is a contaminant in the environmenta with no recognizes biological purpose in organisms (1). Its negative effects can be seen in extremely low dosages, affecting many systems and organs and causing worries all around the world (2, 3). The Pb exposure from contaminated air, food, and water is a risk factor for cardiovascular disease and other linked disorders such as atherosclerosis, anemia, and hypertension (4, 5). The principal processes by which Pb causes injury include oxidative damage, apoptosis, inflammation, and cation dyshomeostasis (6, 7). Endothelial dysfunction caused by lead acetate is mediated by oxidative stress and inflammation (8). The enzyme nitric oxide synthase (NOS) has the remarkable ability to become "uncoupled" and produce superoxide anion (O_2) instead of nitric oxide (NO).Reduced NO bioavailability produced by NOS uncoupling is expected to have an essential role in cardiovascular disorders such as dilated cardiomyopathy, ischemia reperfusion damage, endothelial dysfunction, atherosclerosis, hypertension, and diabetes mellitus (9,10). Brassicaceae olecerea plants are among the earliest cultivated plants known to man (11).

Broccoli intake has inspired researchers to look at a variety of biological benefits, such as gastroprotective, antimicrobial, antioxidant, anticancer, hepatoprotective, cardioprotective, anti-obesity, anti-diabetic, anti-inflammatory, and immunomodulatory properties. It is a nutrient-dense plant rich in bioactive phytochemicals such as glucosinolates, phenolic compounds, and flavonoids, as well as antioxidants that are beneficial to your diet (12). Broccoli also includes a lot of indol-3-carbinol. These components are regarded to be fairly common in broccoli since they contain a wide variety of antioxidant characteristics and benefits. In broccoli sprouts, oleic and linoleic acids predominated, whereas blossoms included caproic, stearic, and oleic acids (13). Aim of the current study sought to provide a novel method to Pb adverse effects therapy that was both less toxic and more effective

Material and methods

Plant extract preparation

Brassica olecerae flowers were cleaned and gently washed under running water before being sliced into small pieces and dried at 45°C The dried flowers were pulverized into a fine powder. In a clean, well-sealed conical flask Fifty grams of powder were mixed with 250 ml of 70% ethanol and incubated in a water bath (37° C)for 24 hours, agitated for one hour, and then dried using a rotating evaporator. The collected substance was stored at -20 ° C (14)

Experimental design

Before commencing this experiment, the local committee of animal care and use at the University of Baghdad's College of Veterinary Medicine granted ethical permission.In this study, 18 female local rabbits weighing between 1250 and 1750 Kg were housed in cages (3 animals/cage) at room temperature with controlled illumination for 24 hours in the physiology, biochemistry, and pharmacology department at the University of Baghdad's College of Veterinary Medicine .The animals had full access to water and pellets during the study. For four weeks, the first, second, and third groups were gavaged with 10mg/kg/lead acetate daily. After 4 weeks of Lead (Pb-AC) were as 2nd group had no treatment, the 3rd group (pb-AC-B) treated with 300mg/kg/Bw of *Brassica olecerea*. Ethanolic extract (15). Blood samples were taken after the 4th week of Pb-Ac and 8th week of treatment . Aorta sample were collected at the end of the 8th week of the experiment from euthanized animals .

Molecular analysis of endothelia nitric oxide synthase gene expression in aorta tissue (eNOS)

Total RNA was extracted from aorta samples (50mg) using the Easy-spin TM (DNA free) total RNA extraction Kit from intron biotechnology in Korea. Total RNA samples were utilized in the cDNA synthesis stage with the Accu Power ® Goscript RT Mix Kit from Korea.The extracted RNA samples were quantified (ng/L) and validated by detecting the absorbance at 260 and 280 nm in a Thermo USA nano drop spectrophotometer. This depends on the normalization of the target genes' RT-qPCR (CT values) values in comparison to the housekeeping gene (GAPDH) used as a reference gene in the control and different treatment groups. The primers for the housekeeping gene (GAPDH) and the endothelial nitric oxide synthase gene (eNOS) are shown in Table 1. Real-Time qPCR (Gene expression assay) was performed using the GoTaq® 1-Step RT-qPCR System protocol show in (Table 2).

Table (1): Forword and reverse primers for endothelium nitric oxide synthase gene (eNOS) and the house keeping gene (GAPDH)

Organism	Target gene	Primer name	5'-3'	PCR Product	Accession number
Rabbit	**eNOS**	F	ATGAAGCACCTGGAGAATGAG	106 bp	AY964103.1
		R	CTTGATGGAAGACAGGAGTGAG		
Rabbit	**GAPDH**	F	TGGTGAAGGTCGGAGTGAAC	121 bp	NM_001082253.1
		R	ATGTAGTGGAGGTCAATGAATGG		

Table (2): Real-Time PCR conditions (According to the instruction of GoTaq® 1-Step RT-qPCR System)

Stage	Ta (°C)	Time	Cycles
Reverse transcription	42	15 min	1
RT inactivation/Hot-start activation	95	10 min	1X
Denaturation	95	10 sec.	40X
Annealing/data collection	60	30 sec.	
Extension	72	30 sec.	
Dissociation	72	2 min	1X

Determination of vascular endothelial growth factor (VEGF)

The VEGF was analyzed using Rabbit ELISA kit (ELIK biotechnology). Sulphuric acid solution stops the enzyme-substrate reaction, and the color change is monitored spectrophotometric ally at 450 nm..

Determination of serum malondialdehyde (MDA) concentration

Level serum MDA was measured by methods of enzymol according to (16). Serum-100 µL serum is diluted to 500 µL with distilled water. The samples are kept in boiling water bath for 15 min. The reaction mixture is cooled and centrifuged. The supernatant is taken and the optical density of the pink color formed is read at 535 nm..

Determination total antioxidants capacity(TAC)

The TAC were measured by Colorimetric assay kit for total antioxidant status. TAC in serum was determined using a colorimetric technique. By using a suitable oxidant, ABTS is oxidized to green ABTS•+, which may then be reduced to colorless ABTS in the presence of antioxidants.

Statistical analysis

The effect of various treatments and time on study parameters was determined using the Statistical Analysis System- SAS (2018) application. ANOVA was applied in two different ways. To evaluate significant changes between means, least significant difference (LSD) post hoc tests were run (P 0.05 is regarded as statistically significant (17).

Results and discussion

Table 3 and (Figure 1),recorded the comparison mean value of the eNOS gene of the aorta in the pb-AC group, Pb-Ac_B, is contrasted with control groups. The results revealed significant differences between the pb-AC and pb-AC-B groups ($p<0001$), as well as between the pb-AC and control group ($p=0.0002$), whereas the pb-AC-B and control groups did not exhibit any differences of significance ($P=0.0938$) The lead-treated coronary endothelial cells displayed a significant($P<0.01$) up regulation of e NOS expression when compared to the control and pb-AC-B as shown in (Figure 2).

Table (3): The comparison of the mean value of e NOS gene between lead acetate,broccoli and control groups

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Control vs. pb-AC-B	0.5586	-0.07910 to 1.196	0.0938
Control vs. Pb-AC	-1.414	-2.051 to -0.7759	0.0002
Pb-AC-B vs. pb-AC	-1.972	-2.610 to -1.334	<0.0001

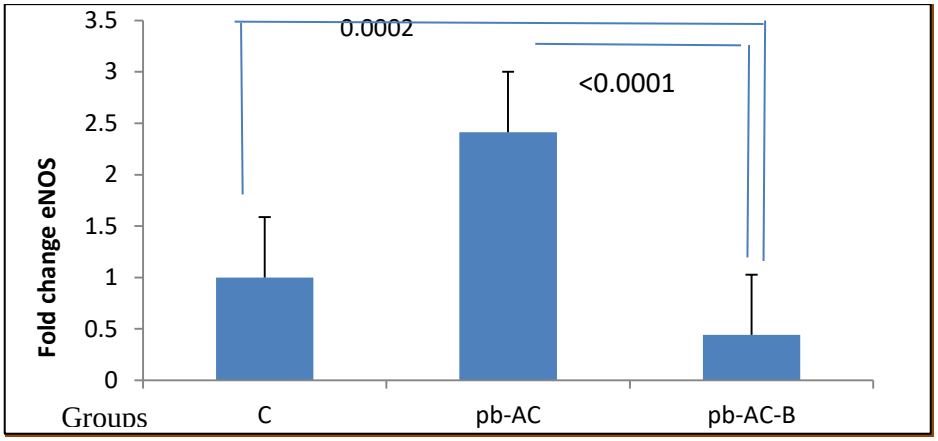

Figure (1): Effects of alcoholic extract of *Brassica oleracea var. Italica*, in e NOS gene expression against lead acetate

Table (4): Comparison between different groups in Ct value of e NOS and housekeeping gene expression

Gene/Sample	Ct	Gene/Sample	Ct	ΔCt	ΔΔCt	Fold change
eNOS-C	35.35	GAPDH-C	28.76	-6.59	-5.853333333	1
eNOS-C	35.33	GAPDH-C	29.96	-5.37	-5.853333333	1
eNOS-C	36.19	GAPDH-C	30.59	-5.6	-5.853333333	1
eNOS-pb-AC	27.32	GAPDH- pb-AC	20.21	-7.11	-1.256666667	2.38943027
eNOS-pb-AC	33.67	GAPDH- pb-AC	26.61	-7.06	-1.206666667	2.308037504
eNOS-pb-AC	26.04	GAPDH- pb-AC	18.84	-7.2	-1.346666667	2.543238333
eNOS-pb-AC-B	25.64	GAPDH-pb-AC-B	20.56	-5.08	0.773333333	0.585064127
eNOS-pb-AC-B	24.52	GAPDH-pb-AC-B	20.38	-4.14	1.713333333	0.30495466
eNOS-pb-AC-B	25.36	GAPDH-pb-AC-B	20.71	-4.65	1.203333333	0.434270743
P value	****				<0.0001	

Mean having the different letters in same column significantly differed **(p<0.01)**

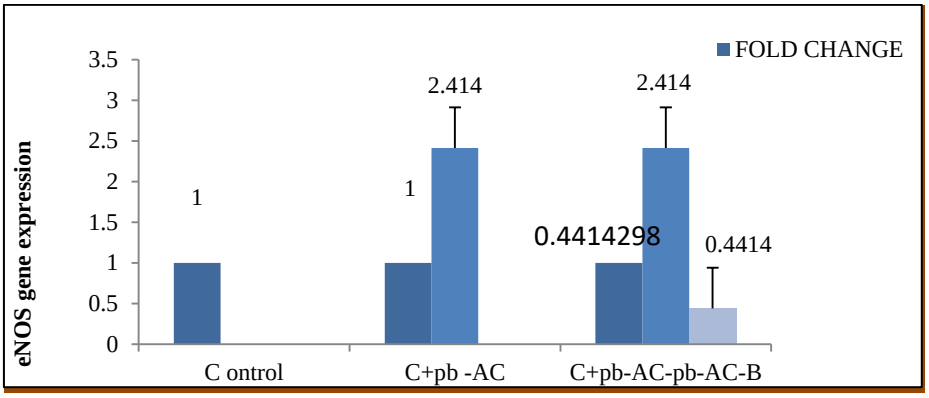

Figure (2): Mean Fold of gene expression of e NOS gene expression before and after treatment

There are many defined lead toxicity mechanisms, however it appears that lead accumulation-induced oxidative stress is the primary cause of the toxicity of Lead (18, 19). The current investigation sought to examine if a supplement containing broccoli extract may alleviate endothelial dysfunction induced by lead acetate. Surprisingly, our research discovered that despite the overexpression of eNOS, it did not help to the reduction of endothelial dysfunction caused by lead acetate. According to the comparison's results, lead acetate group eNOS average folding was significantly higher than treatment (pb-AC-B) and control group eNOS gene expression in Pb-AC, Pb-AC-B, and control groups. There are numerous reasons why gene expression may increase. Nonetheless, the most plausible mechanism of Pb-induced vascular damage is an increase in oxidative stress caused by a change in oxidant-antioxidant balance (20). This resulted in a significant increase in e NOS folding and upregulated eNOS expression in Pb-AC compared to pb-AC-B and control groups. According to Table 4, lead acetate considerably decreased ($P<0.01$) the Ct, ΔCt and ΔΔCt levels with increase in fold change of the NOS gene .Mean while these changes were significantly ($P<0.01$) reversed by changes in the CT, ΔCt , and ΔΔCt levels with depress fold change of the treated broccoli and control groups. Effects of Pb on eNOS are multifactorial. One of these factors may be the influence of cellular hypoxia caused by Pb-Ac on the activation of genes expression that related to hypoxia responses for angiogenesis (21). The effects of hypoxia on eNOS uncoupling appear to be especially relevant since uncoupled eNOS is a generator of damaging radicals such as superoxide and peroxynitrite, and the consequent oxidative stress is central to the pathogenesis of atherosclerosis. On the other hand Pb has Ca ions on its biding sites of different Ca-binding proteins, particularly calmodulin and can replace calcium in Ca2+-dependent signaling pathways (22), resulting in eNOS uncoupling since activity is also dependent on Ca2+ / calmodulin binding (23). Uncoupled eNOS generates superoxide anion (O2) rather than NO, becoming a source of damaging free radicals and aggravating oxidative stress. One of the key underlying reasons of endothelial dysfunction seen in the current experiment is likely to be eNOS uncoupling. Additionally, the current results of the comparison of the e NOS gene expression in the Pb-AC and control groups revealed that there were substantial differences between the expression .It appears that a gene superfamily with various activities in

cells has connections between apoptosis and inflammation. In the current study, we propose that endothelium pathology is mediated by oxidative stress caused by Pb and ROS generation lead to inflammation (24). Results in (Table 5**)** showed significant ($p \leq 0.05$) increased of serum VEGF by Pb-Ac when compared to control group, meanwhile, treatment with broccoli returned VEGF to the semi-normal level of control after 4 weeks of treatment.

Table (5): Effect of alcoholic extract of *Brassica oleracea var. Italica* in VEGF (Pg/ML) against lead acetate

Groups	VEGF(Pg/ML)	
	Before treatment	After 4Weeks of treatments
Control	237.92 ±0.66 ^{Ac}	236.04 ±1.63 $^{A c}$
Pb-Ac	461.92 ±42.25 $^{B a}$	576.42 ±47.56 $^{A a}$
Pb-Ac-B	577.91 ±48.69 $^{A b}$	235.02 ±27.10 $^{B c}$
LSD	87.301	

Means with a different small letter in the same column are significantly different (P≤0.05), Means with a different capital letter in the same row are significantly different (P≤0.05).

VEGF plays a crucial role in the maturation of newly created blood vessels, the development of atherosclerosis and other CVDs and cardiovascular diseases is influenced by the vascular endothelial growth factor (25). Pb-induced angiogenesis in myocardium via different molecular mechanisms, mainly mediated by hypoxia inducible factor (26,27). Figure (3) showed the significant increase in MDA and significant decrease of TAC in rabbits of Pb-Ac group. From other side and after treatment with the broccoli extract serum MDA significantly ($p \leq 0.05$) lowered and boosted TAC activity**.**

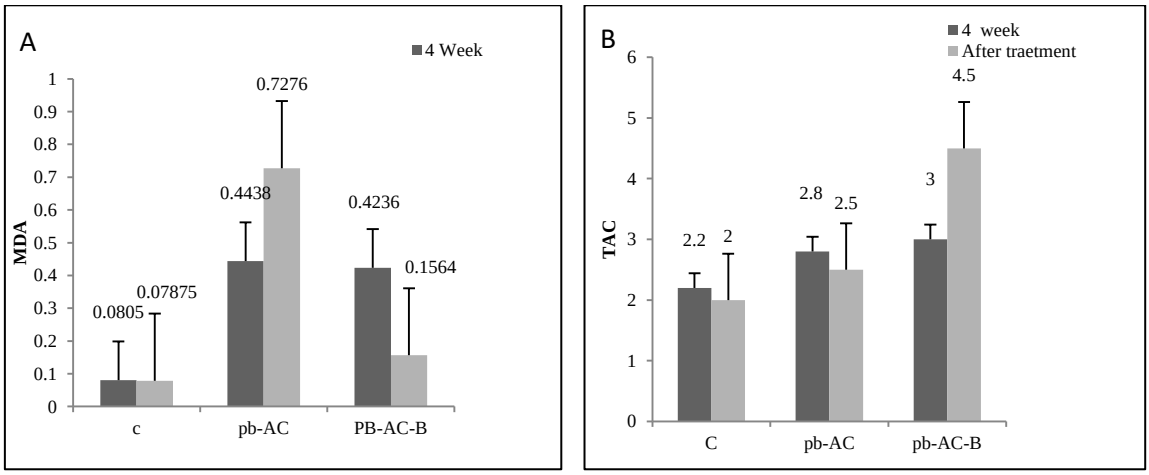

Figure (3): Effect of alcoholic extract of Brassica oleracea var. Italica in MDA(A) and Total antioxidant capacity (B) against lead acetate

An increase in MDA levels as a sign of lipid peroxidation and a decrease in TAC activity in serum are significant indicators of free radical generation and cause an imbalance in the oxidant/antioxidant system. In the present study Pb exposure increased the levels of MDA and decreased TAC . Pb- induced oxidative stress may entail increased ROS generation as a result of

damage to enzymes involved in mitochondrial respiration degradation (28**).** Broccoli is a vegetable that contains a variety of bioactive substances, such as vitamins, minerals, dietary fibers, plant proteins, and phytochemicals. Treated with broccoli that lead to the decrease in the value of gene expression this due to effectiveness exposure of 300mg/kg of extract alcoholic of broccoli highly effected on expression, in addition broccoli contains tannins, which have an antiplatelet impact due to their antioxidant characteristics and the resultant reduction in ROS generation in platelets. According to **(**29**)** the inhibitory action of tannins on oxidative stress also entails an increase in the concentration of antioxidant enzymes and avoidance of stress-induced protein modification. It was proposed that phenolic chemicals, known to act as antioxidants through various chemical processes, including directly as reducing agents or hydrogen donors, may be partially responsible for the extract's antioxidant effects and indirectly as metal chelates Phytochemicals that are present in the fruits and herbs can preserve upon free radical damage (30) various phytochemicals which can be utilized as a treatment option to reverse the effect of the toxicity caused by the ingestion of heavy metals in our body through various environmental or lifestyles ways(31).

Conclusion

The present results confirmed the superiority of the Brassica olecera in treating the Pb adverse effects on the vascular endothelial, which contributed to its phytochemical and nutrient value.) Broccoli may assist to minimize oxidative stress by increasing TAC and decreasing MDA in lead acetate-treated blood.

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