Role of *T. vulgaris* in protection of DNA damage induced by Lead acetate in Rats

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Abstract

Objective: The purpose of this study was to investigate *Thymus vulgaris* (*T.vulgaris*) ethanolic extract protective role for lymphocytes viability and prevent DNA damage by long-term exposure to Lead acetate (Pb) via ingestion.

Material and method: A number of 24 females adult rats were divided into equal groups; 1st Group, as control, 2nd Group administered with lead acetate 5 mg/kg B.W oral gavage lead acetate for 60 days and 3rd Group treated with lead acetate at dose 5mg/kg B.W and *T.vulgaris extract* at doses 75mg/kg B.W for 60 days.

Result: Results of leukocytes (WBCs) and lymphocyte count revealed a significant ($P \leq 0.05$) decrease in the 2nd group and an increase in the 3rd group in comparison to the 1st group. Also the result of Lymphocyte/Neutrophil ratio increased significantly ($P \leq 0.05$) in the 2nd and 3rd groups as compared to the 1st group. lymphocytes viability were evaluated using trypan blue, showed statistically ($P \leq 0.05$) less viable lymphocytes cells were in the 2nd group and more viable lymphocytes cells were found in the 3rd group when comparing to 1st group. Ingestion of *T.vulgaris* showed a protective role in preventing DNA damage according to comet assay.

Conclusion the ethanolic *T.vulgaris* polyphenols extracted molecules with their unique chemical molecular structure withstand lead toxicity, could be due to chelate lead activity.

Key words: Comet assay, *T.vulgaris*, lymphocytes viability, lead acetate.

Introduction

Accumulation of heavy metals in soil, water, and air from industrial waste are considered to be toxic to the environment as well as their effects on health. Lead is a major environmental pollutant that harms living things in a variety of ways. In Iraq, the percentage of lead exceeded the permissible limits in some areas (1, 2). Lead as a pollutant was studied extensively in Iraq. The Heavy Pollution Index (HPI) results show that the drinking water sources in the city of Baghdad have been significantly contaminated with Cd, Co, and Pb (3). It was found that led as one of the heavy metals that cause birth defect in Falluja children (4). Many pollutants of heavy metals exceeded the national and international standards criteria, among Iraqi provinces Baghdad showed the highest level of Pb (5). When compared to other worker, refinery worker are more likely to have higher serum levels of Pb, Cd, and Hg, which can enhance oxidative stress, lower TAC, and lower amounts of the vital trace elements Zn, Cu, and Mg.

Sex, age, exposure time, route of exposure, rate of absorption, frequency of intake, solubility, and retention percentage are all factors that can contribute to lead poisoning (6). Living organisms, body organs affected by lead toxicity by different ways associated with number of physiological, biochemical and morphological alteration (7, 8). Pb alters cell signaling, cell function, and enzyme activities to cause disruption in biological systems (9, 10, 11), In addition it produces alteration in gastrointestinal activities because of the significant alterations in the gut microbiota and metabolite in children (12). Despite its toxicity, lead is classified by the International Research Agency On Cancer as probably harmful to humans. (13, 14). Lead is toxic even in very low concentration (15).

Studies showed that lead toxicity cause leukocytosis and lymphocytosis when analysis of Total Leukocyte Count of exposure animals. Exposure of animals and human to heavy metal, such as lead caused oxidative stress increasing of ROS production. According to some reports, ROS can directly cause various types of DNA damage, such as oxidizing nucleoside bases or damaging the sugar backbone of a cell's DNA strand (16). Lymphocyte are type of white blood cell in the immune system of vertebrates. Despite the fact that the specific method by which Pb affects the immune system is still unknown, some damaging aspects have been identified.

According to El-Boshy study (17) Administering *T. vulgaris* ethanolic extracts to lead (Pb)-toxicated rats improves antioxidant parameters and reduces overall toxic effects. *Thymus* is a Latin name for a plant that some authors believe derives from the Greek word thyo (perfume), while others believe it to indicate strength and courage. (18). There are over 350 aromatic species in the *Thymus* genus, most of which are abundant in the west Mediterranean and are members of the Lamiaceae family. (19, 20) These species, which are perennials, are described as herbaceous subshrubs or shrubs with large clusters of pink, white, cream, or violate flowers, and branches that are divergent and prostrate. They range in height from 10 to 30 cm (20). *Thymus* plants are thought to be therapeutic because to their antimicrobial, antioxidant, anti-inflammatory, neuroprotective, ant-carcinogenic, and hypoglycemic properties. (21, 22, 23, 24). The phenolic content and/or specific phenolic composition of *Thymus* species are connected to their antioxidant action. (25). The present experiment tend to clarify the extent of damage of lead acetate to lymphocytes activity in blood circulation and the protective role of *Thymus vulgaris* against the deleterious effects of Pb on lymphocytes production, viability and DNA.

Material and methods

Experimental design:

A number of 24 females adult rats were equally grouped to ; 1st Group , as control, 2nd Group administered with lead acetate 5 mg/kg B.W by oral gavage (26) for 60 days; 3rd Group treated with lead acetate at dose 5mg/kg B.W (26) and *T.vulgaris* extract at doses 75mg/kg B.W (27) for 60 days by oral gavage.

***T. vulgaris* extract preparation:**

The ethanolic extract of *T.vulgaris* was prepared according to (28) by soaking the 30 gm from plant dried leaves powder in 300ml of ethanol alcohol 70% for 72 hour with intermittent shaking at room temperature, then filtration through filter paper Whatman -2, this step repeated three times the solvent was rotary evaporated, the net extract weight evaporated and the extract dried at 31C˚.

Lymphocytes in circulation:

Fresh blood sample, WBCs count (10^9/l), Lymphocyte (%) by auto heamato analyzer, neutrophil/lymphocyte ratio, lymphocyte viability (%) and concentration (cell/ml)

Lymphocytes viability:

Lymphocyte isolation done using the Ficoll standard method in fresh Lithium Heparin blood sample following the procedure described by (15), briefly,750µl Ficoll- Paque (lymphocyte isolation media) was powered to 1 ml blood sample. Then centrifuge for 30 min at 2500 rpm, after plasma discard, to obtain the cell disc, the white color layer containing the lymphocytes was suspended in RPMI 1640 and centrifuged at 1500 for 15 minutes. The supernatant then discarded. Cells were diluted in 1 ml of RPMI 1640 with 10% FBS but no antibiotics. Using an identical volume of cell suspension and Trypan blue stain 0.4%, the number of living and dead cells was counted using a hemocytometer under a light microscope at a magnification of 10 X to determine the level of cell viability. All the steps of the isolation and staining were achieved under aseptic condition under bacterial hood cabinet. The viability estimated as living cells ration from total cells and as a number of viable cells in 1 ml of the cells suspension by followings equations according to BioNetwork Lab technique (29):

1) Percentage of viable cells were calculated according to equation :

$$cell\ viability\ \% = \frac{number\ of\ vaible\ cells}{total\ nuber\ of\ cells} \times 100$$

2) Average number of cells per square :

$$cells/square = \frac{number\ of\ vaible\ cell}{number\ of\ square}$$

3) Dilution factor :

$$dilution\ factor\ = \frac{final\ dilution}{volume\ of\ cell}$$

4) Concentration of viable cells / ml :

Concentration of cells (cells/ml)= average of cell/square × dilution factor × 10^4

Where 10^4 equal to depth of hem cytometer chamber

DNA Damage Assessment by Comet Assay

The ability of DNA fragments to migrate when subjected to electric fields is used to measure DNA damage in isolated mononuclear white blood cells using the comet assay.A tail-like morphology was created by the migration of the DNA pieces under the electric field. Following Comet Assay Kit-assisted cell separation from heparinized whole blood, Trevigen (USA). In a microcentrifuge tube10µl of cells supernatant with 90 µl/ml molten LMA garose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipette 50 µl on to Comet Slide. Two slides for each sample were prepared. For 10 minutes, slides were stored in the refrigerator at 4 °C in the dark. Slides were immersed in lysing solution (ice) in 4 °C for 30 to 60 minutes .DNA unwinding by immersed slides in freshly prepared alkaline unwinding solution, pH>13.The samples were electrophoresed at 21°C for 30 minutes with mA for 20 to 60 minutes at room temperature in the dark .Slides were cautiously dried after electrophoresis, twice soaked in d H_2O for 5 minutes each, and then immersed in 70% ethanol for 5 minutes. Additionally, slides were dried for 10-15 minutes at ≤ 45°C .Drying arranges all of the cells into a flat plane to make observation easier. Slides were stained with SYBER, and cells were scored for each sample using the Comet scolar image analyzer software to determine the ratio of DNA damage in 100 cells for each slide, the head diameter and area, the percentage of DNA in the head, the tail length and area, and the percentage of DNA in the tail. This information was used to assess the protective role of *T. vulgaris* against lead acetate oxidative damage.

Statistical Analysis

Analysis of variance (ANOVA) and one-way analysis were used to examine the data from the current experiment using SAS (Statistical Analysis System) and Microsoft Office Excel (Microsoft Office Excel for windows; 2010). Multiple comparisons were made using least significant differences (LSD) to assess significant differences.

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Results

Results of (**Figure 1**) represent white blood cells (WBCs) and lymphocyte count showed significant ($P \leq 0.05$) drop in the 2nd with an increase in the 3rd groups after 60 days, as compared to the 1st group. After 60 days, the Lymphocyte/Neutrophil ratio increasd significantly ($P \leq 0.05$) in the 2nd and 3rd group, as compared to the 1st group.

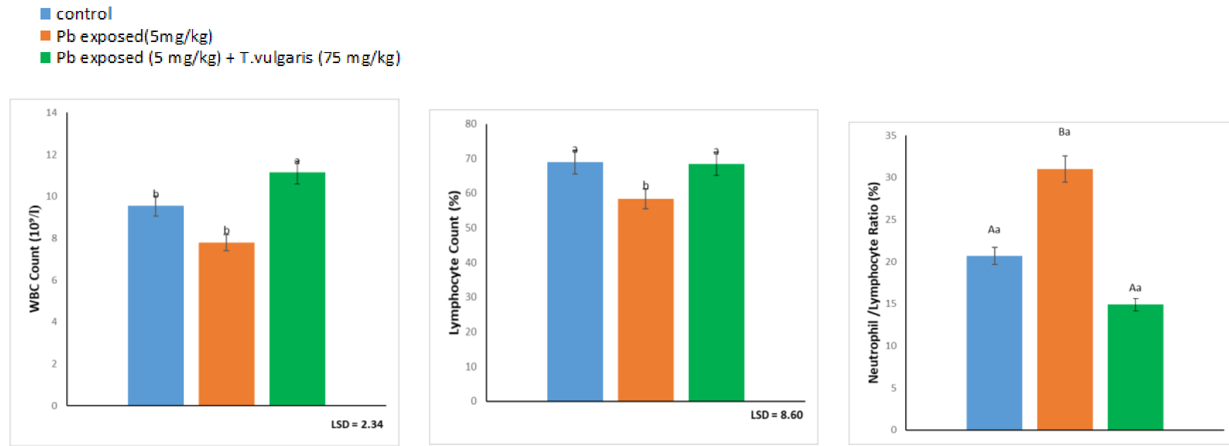

Figure (1): Protective role of *T.vulgaris* in Total WBCs, Lymphocyte count (%)and Neutrophil / Lymphocyte Ratio (%) against lead acetate at different experimental times. Means ± SE. n= 8 animals /group. Different letters mean significant differences ($P \leq 0.05$) between means, capital letters within group, small letters between groups, LSD = 2.34 .

And the result of **Figure (2)** showed statistically ($P \leq 0.05$) less viable lymphocytes cells were found in the 2nd group and more viable lymphocytes cells were found in the 3rd group compared to 1st group.

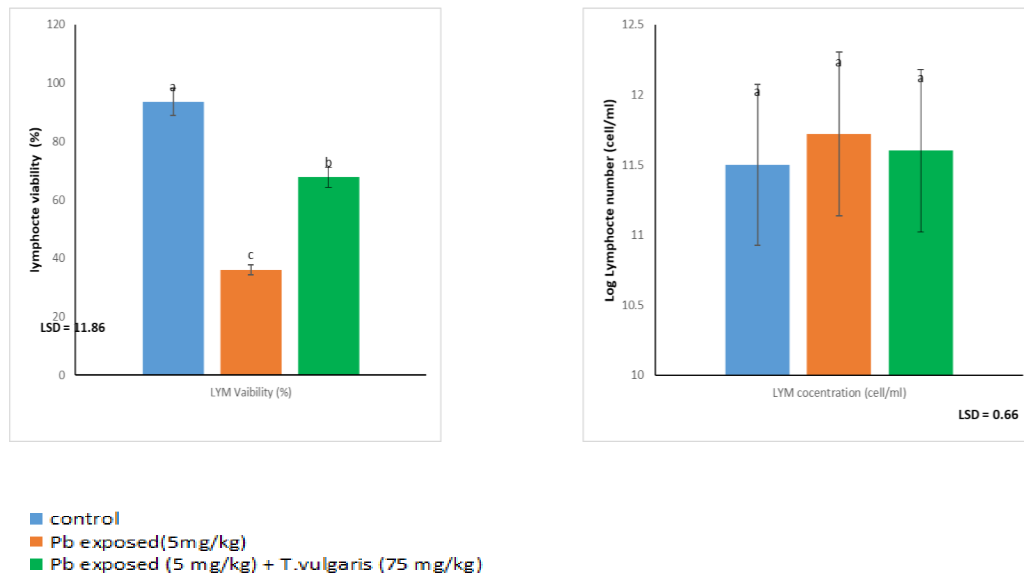

Figure (2): Protective role of *T.vulgaris* to Lymphocytes viability and Lymphocyte number (cell/ml of suspention) exposed to lead acetate at end of experiment .Means ± SE. n= 8 animals /group. Different letters mean significant differences ($P \leq 0.05$) between means, capital letters within group, small letters between groups, LSD = 0.66

DNA damage Assessment by comet assay

The cells image obtained using fluorescent microscope for agarose gel electrophoresis slid (Figure 3) showed that control group with no damage in the DNA and the cell looked like a complete circle but Pb exposed group showed cell with long tail and distended head, the Pb +*T.vulgris* treatment group showed a medium length tail in some cells and absent in others (Figure 3).

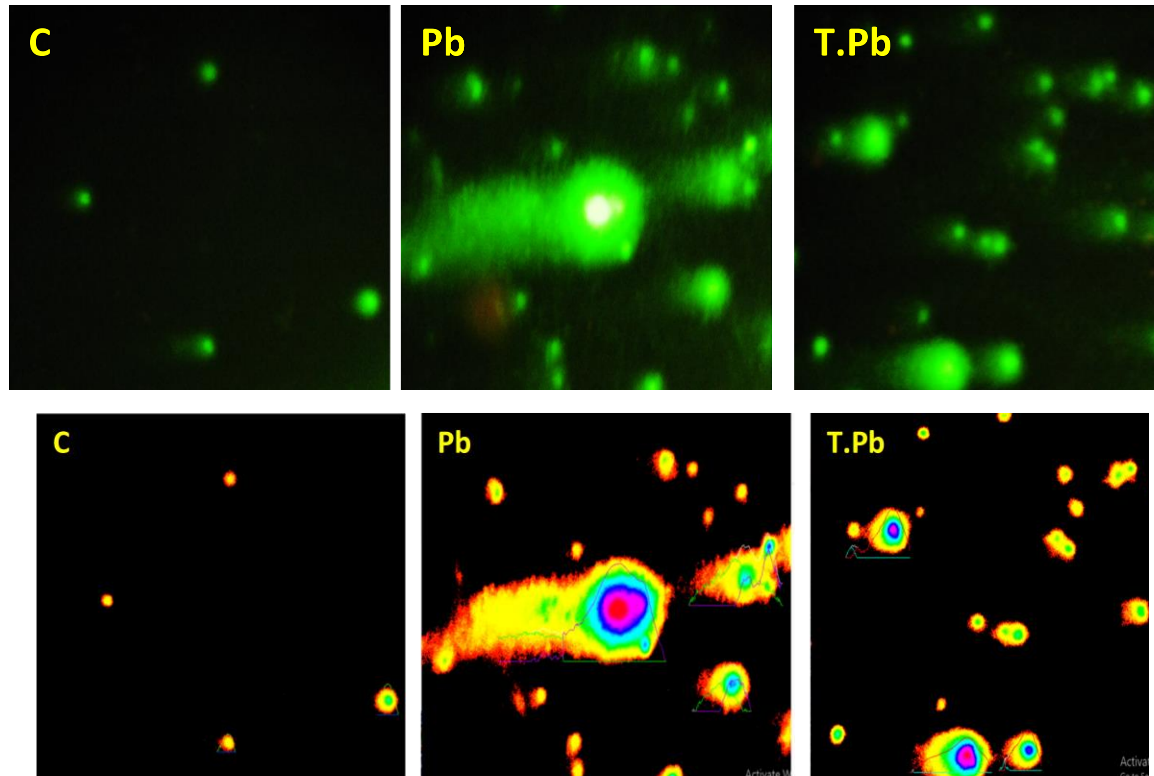

Figure 3 fluorescent microscope for agarose gel electrophoresis slid and in comet scholar image analyzer software (Comet Score software) for DNA damage in C= control group, Pb =lead acetate group, T.Pb =lead acetate and *Thymus vulgaris* group

Percentage of DNA in head (%) in control group was the significantly (P≤0.05) highest and no DNA in tail, on the contrary Pb exposed group had lowest DNA in head and highest in tail, while Pb + *T.vulgaris* treatment group showed less DNA in head and tail DNA than control and Pb groups respectively (**Figure 4**).

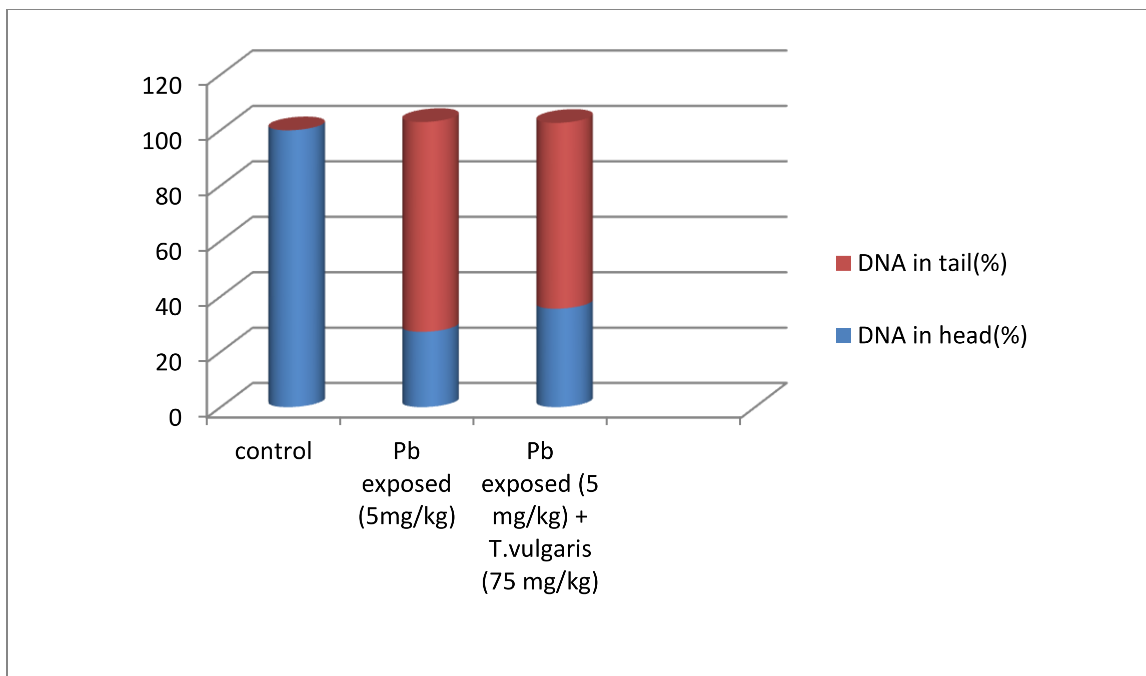

Figure (4): Protective role of *T. vulgaris* in ratio of DNA damage against lead acetate at the end of experiment. Means ± SE. n= 3 animals / group

Severity of DNA damage (%), as shown in **Table (1)** showed that cells of rats exposed to Pb had the highest DNA damage.

Table (1): Protective role of *T. vulgaris* in ratio of DNA damage against lead acetate at the end of experiment. Means ± SE. n= 3 animals / group.

Groups	cells with no damaged DNA (%)	cells with low damaged DNA (%)	cells with medium damaged DNA (%)	cells with high damaged DNA (%)
control	44.12±1.00A	44.87±0.90A	5.15±0.31C	5.86±0.71C
Pb exposed (5mg/kg)	35.52±0.79B	36.87±0.68B	13.10±1.61A	14.51±0.41A
Pb exposed (5 mg/kg) + T.vulgaris (75 mg/kg)	41.27±0.46A	39.98±1.94AB	8.94±1.01B	9.81±1.06B
LSD	4.78	5.01	4.10	3.9

Using Comet Score analyzer software to measure the a mount and percentage of DNA in head, and comet (tail),comet length (Px), head diameter (Px), tail length (Px), head and tail areas (Px), and tail moment, in different experimental groups revealed the protective role of *T. vulgaris* against Pb on DNA fragmentation (**Table 2**)

Table (2): Protective role of *T. vulgaris* in comet score assay of DNA damage against lead acetate at the end of experiment

Groups	Comet length (Px)	Head diameter (Px)	Head area (px)	DNA in head (%)	Tail length (Px)	Tail area (Px)	DNA in tail (%)	Tail moment
Control	35.50±1.15 C	35.18±1.13 B	833.84 ±55.29 B	99.97 ±0.01 A	0.31 ±0.08 C	1.06 ±0.04 C	0.02 ±0.01 C	0.000 ±9.163 C
Pb exposed (5mg/kg)	142.63±10.92 A	79.39 ±6.76 A	4265.18 ±789.43 A	27.233 ±2.12 C	68.94 ±7.49 A	4893.24 ±729.90 A	75.67 ±2.53 A	53.65 ±4.87 A
Pb exposed (5 mg/kg) + T.vulgaris (75 mg/kg)	98.50±6.84 B	37.88±3.46 B	1617.53 ±251.62 B	33.58 ±2.95 B	52.86 ±4.45 B	2968.92 ±357.60 B	67.20 ±2.82 B	35.69 ±3.67 B
LSD	22.27	13.248	1443.5	6.244	15.025	1406.3	6.477	10.459

Discussion

Immune system components and in particular lymphocytes for being the first defensive line in the body are in challenges with environmental pollutants such as lead (30, 31). For lymphocytes viability, Trypan blue exclusion dye, which gives dead cells' membranes a blue tint and cannot penetrate live cells with intact cell membranes, was used (32). However, the effects of lead on peripheral blood lymphocytes have not been well recognized and investigated and the mechanism of lead induced injury needs to be revealed. In the present experiment, we evaluated the level of damage in rats peripheral blood lymphocytes exposed chronically to lead acetate. The lymphopenia in rats suffered from Pb exposure, although total WBCs have not significantly changed indicate the inflammatory response mediated by Pb, which may have increased with increased Pb in blood. Lead causes the immune system's constitutive cells to become less effective, and this effect is caused by either the degree of gene expression or the production of proinflammatory proteins (33). Immunological parameters were impaired due to chronic lead (Pb) exposure, which was characterized by a slight decrease in lymphocyte count (34). Moreover, Pb-intoxicated rats exhibited an increase in neutrophils/ lymphocytes ratio in responses to lead inflammatory effects (35). Nonetheless, Pb decreases the formation of Neutrophils extracellular traps proteins, causing defects in the important part of the body's innate immunity (36).

The higher DNA fragmentation (genotoxicity) using agarose gel electrophoresis in Pb intoxicated rats triggered through different mechanisms. Primarily concerned with the biomedical implications of lead-induced generation of free radicals and the defect in antioxidant system (37). A positive proportional relationship recorded between the blood's lead content and the malaldehyded acid with the degree of DNA fragmentation among the lead-exposed workers DNA (38). Pb is one of the heavy metals that contributes to the pathophysiology of tissue deterioration and causes problems with protein dysregulation, oxidative inflammation, apoptosis induction, and DNA damage (39). Additionally, lead binds to DNA's main grooves and reacts with phosphate oxygen atoms to damage DNA (40). Because heavy toxic metals are not easy to be degraded so to avoid their toxicity, organism bind them to 'accessory proteins'. Pb can also binds and interacts with small proteins like nuclear proteins causing a loophole for DNA damage (41). Furthermore DNA methylation can be altered by Pb and is a reversible and regulatory modification (42).

According to research, a number of plant polyphenols may be helpful in reducing lead poisoning in both people and animals (43).

According to the findings of our study, prolonged lead acetate exposure in rats resulted to inflammatory responses, defect in lymphocytes number and viability, and DNA fragmentation while accompanying giving *T.vulgaris* with Pb prevent in somehow these changes. *T. vulgaris* could be a proper candidate to modulate the compromised immunity in response to Pb for its anti-oxidant, anti-inflammatory, and antidote action. *T. vulgaris*, the good medicinal plant for its phenolic acids, terpenoids, flavonoids, saponins, steroids, tannins, alkaloids, and polysaccharides use in pharmacotherapy (36, 44). *T. vulgaris* polyphenols regulate immunity of Pb exposed rats by interfering with immune cell regulation, proinflammatory cytokines' synthesis, and gene expression (45).

Metal poisoning is frequently treated with chelation therapy (46). Chelation is a chemical reaction that produces a complex ring-like structure when a central metal atom or ion interacts with a ligand. A plant phytochemical compound with a ring structure that has a high affinity to donate ions serves as the ligand's donor ion or molecule.

Since it contains substances like thymol and carvacrol, two potent antidotes,*Thymus vulgaris* is beneficial as a natural antidote. (47, 48). Plant polyphenols can chelate lead using their unique chemical molecular structure to protect against lead toxicity. Seabuckthorn (*Hippophae rhamnoides*) leaf-extract (SLE) supplementation in lead acetate-intoxicated Wistar rats. Worked as a toxicity-protective force against lead (49). For people who live in surroundings where lead toxicity is a risk, Daflon-Enriched foods could be a viable preventative measure. (50).

Among polyphenols, Tannins are utilized in medicine primarily because of their potent qualities, which include the potential to bind harmful metals and to prevent their absorption while enhancing their bodily elimination. (51). this is especially important in the present case of *T.vulgaris* against Pb, tannin have been evaluated in *T.vulgaris* alcoholic extract (52).

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دور الزعتر البري في حماية الدنا من التلف المحدث بخلات الرصاص في الجرذان

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الخلاصة

الهدف من الدراسة:الغرض من البحث كان تحديد الدور الوقائي للمستخلص الكحولي لنبات الزعتر البري ضد تأثير مادة خلات الرصاص على تلف الدنا بعد التعرض لمدة طويلة.

المواد وطرق العمل: تم استخدام 24 من اناث الجرذان حيث تم تقسيمها الى ثلاث مجاميع متساوية العدد، المجموعة الاولى مجموعة السيطرة (الطبيعية) اما المجموعة الثانية تم معاملتها بمادة خلات الرصاص بجرعة 5ملغم/كغم من وزن الجسم لمدة 60 يوم عن طريق التجريع والمجموعة الثالثة تم معاملتها بمادة خلات الرصاص بجرعة 5ملغم/كغم من وزن الجسم لمدة 60 يوم عن طريق التجريع ومادة مستخلص الزعتر بجرعة 75 ملغم/كغم من وزن الجسم لمدة 60 يوم عن طريق التجريع.

النتائج: اظهرت النتائج فرق معنوي بين المجاميع من حيث تعداد كريات الدم البيضاء وخلايا اللمفوسايت ونسبة خلايا اللمفوسايت الى الخلايا البيضاء العدلة وكذلك نسبة قابلية خلايا اللمفوسايت للحياة حيث كانت قليلة في المجموعة المعرضة للرصاص واكثر قابلية للحياة في المجموعة المعرضة لمادتين الرصاص والمستخلص الزعتر عند المقارنة مع المجموعة الاولى، كذلك اظهرت النتائج دور الزعتر في حماية الدنا من التلف باستخدام فحص المذنب حيث خلايا المجموعة الثالثة ذو تلف اقل مقارنة بالمجموعة الثانية.

الاستنتاج: ان المركبات الكيميائية الفينولية الموجودة ضمن مستخلص الزعتر البري الكحولي لعب دور في سحب مادة خلات الرصاص وبالتالي قامت بحماية الدنا من التلف.

كلمات مفتاحية : خلات الرصاص ، الزعتر البري، فحص المذنب، وفرة اللمفوسايت

* البحث مستل من رسالة ماجستير للباحث الاول