Ibn Al-Haitham Journal for Pure and Applied Sciences
Journal homepage: jih.uobaghdad.edu.iq

Anti-tumor Activity of Plantago *lanceolata* Aqueous Extract *In Vitro* and Genotoxicity by Micronucleus Assay *In Vivo*

Eman Ali Hussein Al-Mosawie
Department of Biology, College of Education for Pure Sciences (Ibn- Al-Haitham) University of Baghdad, Baghdad,Iraq.
eman.ali1202a@ihcoedu.uobaghdad.edu.iq

Worood Kamil Shalash Al-Maliky
Department of Biology, College of Education for Pure Sciences (Ibn- Al-Haitham) University of Baghdad, Baghdad,Iraq.
worood_almaliky@yahoo.com

Article history: Received 25 July 2022, Accepted 31 August 2022, Published in January 2023.

Doi.org/10.30526/36.1.2953

Abstract

The study is designed to evaluate the effect of the aqueous extract of the *P. lanceolata* plant, as well as to know the effect of the drug CCl_4 on the formation of micronucleus in *vivo* 48 female albino mice. In the study mice were separated into eight groups treated intraperitoneally for seven day first group Negative control, second positive control(CCl4 0.02%), third group aqueous extract (250 mg/kg), fourth group aqueous extract (500 mg/kg), fifth group (CCl4 0.02%) plus aqueous extract (250 mg/kg), sixth group (CCl4 0.02%) plus aqueous extract (500 mg/kg), seventh group aqueous extract (250 mg/kg) plus (CCl4 0.02%), and eighth group aqueous extract (500 mg/kg) plus (CCl4 0.02%). The genetic-cellular aspect involved measuring the coefficient of micronucleus formation in bone marrow cells in mice treated with CCl_4 and plant aqueous extract. The results showed that the treatment of mice with the drug led to a rise in the coefficient of micronucleus formation compared to the negative control group. In addition, it showed the plant's ability to reduce the drug CCl_4 effect in the totals of overlaps between the plant extract and the drug at the concentrations used for the plant 250 and 500 µg/ml and reduce the formation of micronucleus.

The cellular toxicity of the plant's aqueous extract on the liver cancer cell line was assessed in HepG2 (liver cancer cell line) and the WRL68 (hepatic human cell line) using concentrations (25, 50, 100, 200, and 400 µg /ml) from the plant's aqueous extract on the HepG2 liver cancer cell line. The results showed a decrease in cell viability depending on aqueous extract concentration. The vitality of cancer cells decreased with the increase in concentration; the viability of the aqueous extract of the plant on cancer cells reached the minimum at concentration 400 µg/ml 45.34±4.44, while it reached the maximum when concentration 25 µg/ml 84.53±2.41.

Keywords: *Plantago lanceolata,* CCL_4,Micronucleous , HepG2 cell line.

This work is licensed under a Creative Commons Attribution 4.0 International License

1.Introduction

Plantago lanceolata is a perennial plant that is extensively distributed. Plantago *lanceolata* is used to treat several malignant conditions and to enhance the immune, respiratory, digestive, urinary, and reproductive systems. [1,2]. Numerous active chemicals in the plant, such as flavonoids, which stop the oxidation of low-density lipoprotein and eliminate free radicals, give the plant its medicinal potential [3]. Flavonoids that are anti-oxidant and anti-mutagenic are present in plants [4].

Use test for micronucleus: It is one procedure used to investigate mammals' in *vivo* genetic toxicity as one of the most effective tests to analyze the induction of chromosomal abnormalities, one of the most significant causes of mutations, and assess the hazards of substances [5]. Numerous chemical, physical, and biological agents can kill cells, but a class of agents known as genetic toxins cans also damage genetic material without killing cells. These agents are not necessarily toxic to cells. Environmental pollutants expose modern humans to a variety of genotoxic substances. To determine this exposure, genotoxicity tests are used, the most significant and well-known of which are micronuclei tests performed on organisms both inside and outside their bodies [6].

2.Materials and Methods

Plantago lanceolata gathering and Identification Plant sample collection

The dried leaves of *Plantago lanceolata* were obtained from the local market in Baghdad / Herb Al-Razi in August of 2021 and the plant classifies by the College of Pharmacy, Al-Mustansiriya University, and Baghdad.

Preparation of plant aqueous extract

After drying, the plant leaves were chopped, and 150 grams of them were extracted for four hours in 250 ml of distilled water using the Soxhlet equipment. A water bath provided heating at 45°C. The leaf extract solution was put in plates and incubated in the oven at 37°C to prepare plant extract powder. The powder was added to create the necessary concentration for the laboratory mice [7].

Dose of plant aqueous extract

Two doses of plant extract were given based on LC50 (half lethal concentration) to the plant, to albino female mice, the first dose being 250 mg/kg and the second increased to 500 mg\kg.LC50 of *Plantago lanceolata* 2,940 mg/kg[8].

Dose of drug carbon tetrachloride (CCL_4)

The drug tetrachloride carbon CCL4 was used by Thomas Baker / India at 0.02 ml and olive oil was added at 99.98 [9].

Laboratory animals

This study utilized female white mice (*Mus musuls*) from the Biotechnology Research Center Al-Nahrain University. They ranged from 8-12 weeks and weighed 23-27 grams. They were placed in dedicated cages, each set in a cage. Each cage contained six female white rats while providing similar conditions for all groups of water, food (standard pellets), temperature, and lighting.

Scheme of Experiment

Forty eight mice were divided into 8 groups. The first group was a negative that received treatment with distal water, and the second group was received one dosage of the medication CCl_4 intraperitoneally (i. p.) daily for seven days. For a week, Third and fourth groups received 250 mg/kg and 500 mg/kg of plant extract, respectively. On the first day, CCl_4 was administered to groups fifth and sixth, and over the next six days, post treatment consisted of 250 mg/kg and 500 mg/kg of plant extract. Groups seven and eight received CCl_4 (i. p.) for the last day after receiving plant extract treatments of 250 mg/kg and 500 mg/kg i. p. for six days. 0.1 ml of the dosage was administered i. p. to the groups.

Micronucleus Formation Assay *in vivo*

To valuation of micronucleus formation, the method of [10] depended on the following steps: The mice were sacrificed and then dissected to obtain the femur. Then cutting, both ends of the bone were gripped from the middle with forceps in a vertical position over the edge of a test tube. Then the cellular content was collected with heat-inactivated human AB plasma 2 ml using a disposable insulin syringe.

1. After gentle mixing, the test tube was centrifuged (1000 rpm) for 10 minutes, and the supernatant was discarded. The cellular deposit was gently mixed; a thin smear was made on a clean slide, and air-dried at room temperature.

2. The smear was fixed with absolute methanol for 5 minutes, air-dried at room temperature, stained with Giemsa stain for 15 minutes, and rinsed with distilled water. The slides were examined under an oil immersion lens (100X), and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus formation. The micronucleus index was obtained using the subsequent equation:

$$\text{Micronucle us Index (micronucl eus/cell)} = \left(\frac{\text{Number of Micronucle i}}{\text{Total Count of PCE}} \right) \text{x } 100$$

Cytotoxicity MTT assay in *vitro*

This study used the Liver cancer cell line HepG2 obtained from the University of Malaya / College of Medicine / Department of Pharmacology / Center for Investigation of New Therapies in Malaysia.

A-The Kit Contents

• The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Solution: Vials 10 x 1ml.

• The Solubilization Solution: 50 ml × 2 bottle.

B. Assay procedure

The procedure consists of the following steps [11].

• Cells were planted at a density (1×10^4 to 1 x 10^6 cell/ml) 100 microliters of HepG2 cells were added to 96 pits in the microliter plate with repetition of each concentration as well as control.

• Covered the plate and incubated at 37°C for 4-2 hours with 5% C02 after which the culture medium (RPMI-1640) Roswell Park Memorial Institute was discarded.

• One hundred µL of *Plantago lanceolata* aqueous extract (25, 50,100,200, and 400 µg/ml) were added to each treated pit well in the plate for 48 hours at 37°C. Three duplicates for all concentrations were prepared. After the treatment with compounds, ten microliters of MTT solution and incubate for 4 hours at 37 °C. one hundred microliters of Solubilization solution per hole for 5 minutes. The results were read using the ELIZA reader (ASYS/Austria) to check the absorption at a wavelength of 575 nanometers. Percentage Growth inhibition (GI) for each concentration of the extract was calculated by the subsequent equation method:

Viability (%)=(Optical density of sample / Optical density of control) x100 Statistical analysis

One mode examination of variance ANOVA (Duncan) was made to test statistical significance was defined as ($p \leq 0.05$). Data were expressed as mean± standard error, and statistical significances were carried out using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla).

3.Results and Discussion

1-Micronucleus assay *in vivo*

The results showed that the rate of micronucleus in the negative control group was 0.011±0.001 MN/Cell, while the positive control group treated with CCl_4 increased significantly ($P \leq 0.05$) 0.062±0.008 MN/cell. While in groups treated with *P. lanceolata* aqueous extract 250 and 500 mg/kg the rate of micronuclei was 0.016±0.001 and 0.015±0.005, respectively where the difference was non-significant compared to a negative control**.** The two groups treated on the first day with CCl_4 and the remaining six days with plant aqueous extract 250 and 500 mg/kg were 0.025±0.004 and 0.020±0.001 respectively. The two groups were treated in the first six days with 250 and 500 mg/kg plant extract, and the seventh day with CCl4 was 0.030±0.001 and 0.027±0.004, respectively. This indicated that the plant has a protective effect as the proportion of micronuclei formation was significantly reduced ($P \leq 0.05$) when compared to the positive control group, as shown in **Table(1-1)** and **Figure(1-1).**

Table (1-1): The effect of *P. lanceolata* aqueous extract the formation of micronucleus in albino mice treatment with CCl_4

Group	Concentration	Micronucleus Count (Mean ±SD)
Negative control		0.011± 0.015^{D}
Positive control	0.02 %	0.062±0.008^{A}
P. lanceolata extract	250 mg/kg	0.016±0.001 ^{CD}
P. lanceolata extract	500 mg/kg	0.015±0.005^{CD}
P. lanceolata +CCl_4	250mg/kg+0.02	0.025±0.004 ^{BC}
P. lanceolata + CCl_4	500mg/kg+0.02	0.020±0.001 ^{C}
CCl_4+ *P. lanceolata*	250mg/kg+0.02	0.027±0.004^{BC}
CCl_4+ *P. lanceolata*	500mg/kg+0.02	0.030±0.001^{B}

Different letters: marked variation ($P \leq 0.05$) among means

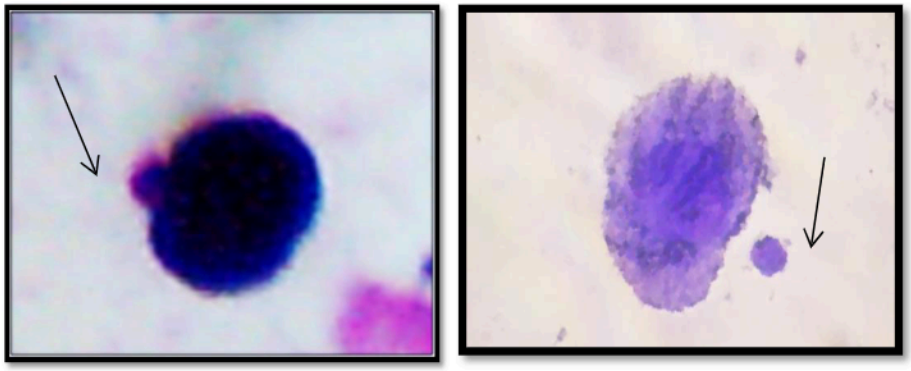

Figure (1-1): Two bone marrow cells of mice treated with CCl_4 show the formation of micronucleus using Giemsa stain magnification force (X100).

Micronucleus testing is a reliable application to many cells, such as red and white blood cells and epithelial cells. It is essential for assessing DNA damage and defects during cell division [12]. A study proved that the Sagittarius plant is rich in flavonoids, with an estimated ratio of

66.9 µg/ml[13]. The plant has an apparent effect on the decrease in the rate of micronucleus formation in the fifth, sixth, seventh, and eighth mice groups. Interactions between aqueous extract and ccl4 drug compared with the positive control group as shown in **Table 1-1**. Significant differences and different letters indicate significant differences between groups of mice.

2-Cytotoxicity MTT assay

Cytotoxicity screening was used to assess the toxic effects of the aqueous extract *P. lanceolata* in the cell line of liver cancer HepG2. Cell viability and cancer cell inhibition ratio were estimated by the MTT assay using different concentrations of the plant's aqueous extract ranging from 25-400 µg/ml. As for the effect of the plant's aqueous extract on the HepG2 liver cancer cell line, the results showed a decrease in cell viability depending on concentration. The vitality of cancer cells has declined as concentration has increased. The viability of the aqueous extract on cancer cells reached a minimum concentration of 400 µg/ml 45.34±4.44, while it reached the maximum concentration of 25 µg/ml 84.53±2.41 as shown in **Table (2-1)**. The inhibition efficacy of the plant's aqueous extract on the natural human cell line WRL68 withlC50 was 129.7 µg/ml. The inhibition efficacy of the liver cancer cell line HepG2 was compared with lC50 of 105.0 µg/ml (**Figure 2-1**).

Table (2-1) : The cytotoxicity effect of *P. lanceolata* aqueous extract in the HepG2 and WRL68 cell lines

Concentration mg/ml	Viable cell count of HepG2 Mean ±SD	Viable cell count WRL68 Mean ±SD
400	45.34±4.44 ^{E}	64.30±1.79 ^{D}
200	53.08±1.91 ^{D}	72.49±1.54 ^{C}
100	65.98±6.04 ^{C}	82.48±0.55 ^{B}
50	75.54±3.13 ^{B}	93.52±0.53 ^{A}
25	84.53±2.41 ^{A}	95.02±0.72 ^{A}

Different letters: marked variation (P ≤ 0.05) among means

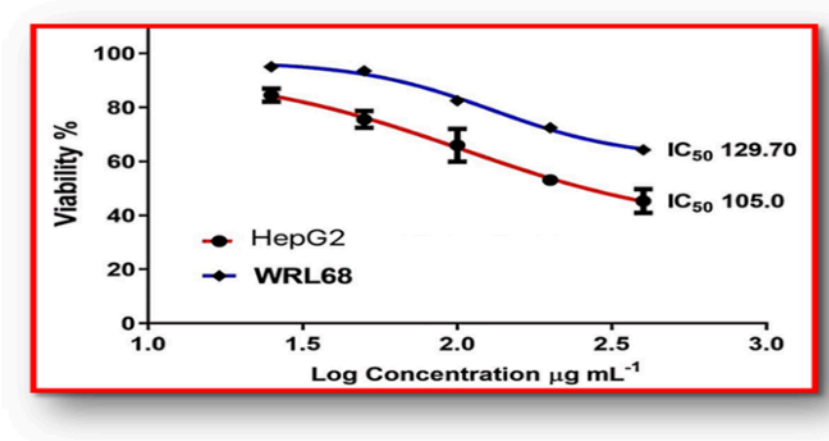

Figure 2-1: The effect *P .lanceolata* aqueous extract in the HepG2 and WRL68 cell lines

HepG2 is a common liver cancer cell line used in many studies to examine the cytotoxicity of substances in the liver [14]. *P. lanceolata* has a toxic effect on the HepG2 cell line in higher doses than in low doses. Through the results, the high dose was more toxic to cancer cells. This is

consistent with a study [15] that showed that the inhibition of cancer cells in plant extracts depends on the dose. A study has proven that the plant *P. lanceolata* is anticancer by possessing antioxidant properties and has a toxic effect on genes in all concentrations in HepG2 cells[16].

Plant toxicity to cancer cells is attributed to its high content of many anticancer compounds, such as glycosides and flavonoid compounds, the most important of which are rutin, myrictin, quarcetin, and kaempferol [17].

4.Conclusion

Plantago lanceolata modulates the mutagenic effect of the CCl4 drug through the reduced formation of a micronucleus. It has toxic activity on the cell line HepG2(liver cancer cell line).

References

1.Tutel, B.; Kandemir ,I.; Kufi ,S.; Kence, A. Classification of Turkish *Plantago L.* species using numerical taxonomy, Turk J Bot**. 2005**, *29*, 51-61.

2.Kurt, B .;Bilge, N.; Sozmen, M.,; Adyin, U.; Ö nyay, T.; Ozaydin ,I. Effects of *Plantago lanceolata* L. extract on full-thickness excisional wound healing in a mouse model, Biotech Histochem. **2018**,*93(4)*,249-257.

3.Ji, X.; Hou ,C.and Guo, X. Physicochemical properties, structures, bioactivities and future prospective for polysaccharides from *Plantago L.* (Plantaginaceae): A review. *Int J Biol Macromolecules*.**2019**,*135*, 637-646.

4.Harborne, J.B.and Williams, C.A. Advances in flavonoid research since 1992, Phytochemistry., **2000**,*55(6)*, 481-504,

5.BearaI,N.; Lesjak M.M.;OrcicD.Z.;SiminN.D.;SiminD.C.;BozinB.N.; Mimica-Dukic, N. M. Comparative analysis of phenolic profile, antioxidant anti inflammatory and cytotoxic activity of two closely-related Plantain species: *Plantago altissima L*. and *Plantago L.L*, Food Sci Technol., **2012**, *47*, 64-70.

6.De Stefano, A.; Caporali, S.; Di, Daniele N.; Rovella, V.; Cardillo ,C. Anti- Inflammatory and Proliferative Properties of Luteolin-7-O- Glucoside, *Int. J. Mol. Sci*.**2021**, *22,(3)*, 1321.

7.Sabhi, M.; Mansouri, S.H.; Ramezanian M. Gholam -Hoseinian A9Screening of plant from southeast of Iran antimicrobial activity. *Int J. crud drug Res*.**1987**,*25,(2)*,72-76,

8.Al-Obaidi, Z. F., and Okhti, Z. A. Histological effect of Plantago lanceolata on different Mice Organs. *Research Journal of Pharm. Technol*.**2022**,*15(7)*, 3241-3244.

9.Fua,W.;Chena,J.;Caia,Y;Leia,Y.;Chenb,L.;Peic,L.; Zhoua, D;Lianga, X.; Ruana, J. Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from Parathelypteris nipponica Franch. and Sav. *Ching. J. Ethno.pharm*.**2010**,*130*,521-528.

10.Schmid,A. W. The cell micronucleus test for cytogenes analysis in: Hollaender, A. Ed. Chemical Mutagens Principles and Methods for their Detection. Volume Four. Plenum Press, New York and London.**1979**, 31-53.

11.Rashid, H. ; Umamaheswari, G. Evaluation of the cytotoxic effect of ginger extract against prostate cancer model using in vitro study. *World journal of pharmacy and pharmaceutical science*, **2017**,*12*,1044- 1053.

12.Zehra, S. A. F. I. Micronucleus scoring: an available approach in the evaluation of genomic damage in exfoliative cervicovaginal cells. Anal. Cytol. Pathol.**2022**,*5,1*, 64-67.

13.Sanna, F.; Piluzza, G.; Campesi, G.; Molinu, M. G.; Re, G. A.; and Sulas, L. Antioxidant Contents in a Mediterranean Population of *Plantago lanceolata L.* Exploited for Quarry Reclamation Interventions. Plants, Basel, Switzerland.**2022**,*11*,*6*,791.

14. Arzumanian, V. A., Kiseleva, O. I., Poverennaya, E. V. The curious case of the HepG2 cell line: 40 years of expertise. *Inter. J. of Mol. Sci.* **2021**, *22(23)*,13135.

15.Dastan, T.; Daştan, S.; Cetinkaya, D. Atessahin, T. Karan.Evaluation of in vitro anticancer effect of *Plantago major L*. and *P. lanceolata L.* leaf extracts from Sivas," Cumhuriyet Universitesi Saglık Bilimleri Enstitusu Dergisi, **2016**, *1, 1*, 7–14.

16.Merve, S. ; Sengul ,Y. The Genoprotective Effects of *Plantago lanceolata* Extract and Luteolin-7-B-D-Glycoside on Lymphocyte and HEPG-2 Cells. (IJMPNP), **2022,** *8,2*, 1-8.

17.Alsaraf, K. M.; Mohammad, M. H.; Al-Shammari, A. M.; Abbas, I. S. Selective cytotoxic effect of *Plantago lanceolata* L. against breast cancer cells. *J. Egypt. Natl. Canc. Inst*.**2019**,*31,1,*1-7.