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Cytogenetic *in Vivo* Effects of The Aqueous Extract of *Raphanus Sativus* L. Leaves in Mitosis of the Meristematic Cells of \Onion Roots

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Abstract

The current study included the preparation of the aqueous extract *of Raphanus sativus* (Brassicaceae), commonly known as radish which is widely available throughout the world and has been used in folk medicine as a natural drug against many toxicants. The study was designed to evaluate the *in vivo* cytogenetic activity of the crude aqueous extract of *R. sativus* on mitosis on *Allium cepa* root tips as a plant test system. Root tips of *A. cepa* were tested for four hours with four concentrations of the crude aqueous extract (0.00, 5, 10, 20, 40 mg/ml). The study included a number of cytogenetic analyses such as mitotic index, phase index, and chromosomal aberration. The data showed that this extract led to reducing the mitotic index (MI) to less than 50%. Specifically, when treated with 10 and 40 mg/ml the mitotic index reached 23.72 % and 41.89 %, respectively. This reduction is considered to have toxic and sublethal effects. The extract caused an arrest of the cells at metaphase (c-metaphase) with a high percentage of 80.76% at 5 mg/ml, along with chromosomal aberrations including sticky metaphase, polar deviation, and bridges in anaphase.

Keywords: *Raphanus sativus*, mitosis, *Allium cepa*, aberration, c-metaphase.

التأثير الوراثي الخلوي للمستخلص المائي لأوراق نبات الفجل *Raphanus sativus* على الانقسام الخلوي لخلايا جذور نبات البصل داخل الجسم الحي

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الخلاصة

تضمنت الدراسة الحالية تقييم التأثير الوراثي الخلوي للمستخلص المائي لأوراق نبات الفجل *Raphanus sativus* L. على الانقسام الخلوي باستخدام اختبار القمة النامية لجذور البصل *Allium cepa*. عوملت خلايا القمة النامية لجذور البصل لمدة اربع ساعات بأربعة تراكيز من المستخلص المائي, اضافة الى السيطرة (0.00, 5, 10, 20, 40 ملغم/مل). تضمنت هذه الدراسة بعض المعايير الوراثية الخلوية, مثل: دليل الانقسام الخلوي, دليل الطور ,ودليل الزيغ الكروموسومي (الحالات الشاذة), ادت المعاملة بهذا المستخلص الى خفض دليل الانقسام الخلوي كنسبة مئوية من السيطرة الى دون 50% وخاصة عند المعاملة بالتراكيز (10و40 ملغم/مل) حيث وصل دليل الانقسام الى 23.72% و 41.89% على التوالي , مما يدل على تأثيره السمي او شبه المميت, كذلك اظهر المستخلص قابلية تطفيرية اذ ظهرت حالات

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مختلفة من الزيغ الكروموسومي متمثلة بالاستوائي المتوقف بلغت اعلى نسبة لها 80.76% عند التركيز 5 ملغم/مل وبعض حالات الزيغ الكروموسومي مثل الاستوائي المتميع ,وانحراف الاقطاب ,و الجسور في الطور الانفصالي.

Introduction

Several plants incorporate many compounds with therapeutic and pharmaceutical properties that can protect or treat many diseases. One of these plants is radish which is progressively acquiring much attention. *R. sativus* is the scientific name of radish which belongs to the most common vegetable in the Brassicaceae family, mainly cultivated in Asian countries [1]. The parts of radish plant that are commonly consumed are the leaves, flowers, pods, roots and seeds which are used for medicinal purposes [2]. According to the USDA National Nutrient Database, radish is rich in various nutrients which include potassium, calcium, sodium, and vitamin C. It contains vitamins including B-vitamins (thiamin, niacin, riboflavin, folate, and vitamin B6), vitamin A and vitamin K. It also provides minerals such as iron, magnesium, phosphorus, and zinc [3]. The main constituents of radish are isothiocyanate, flavonoids such as kaempherol glycosides, peroxidases and antioxidants [4, 5]. It has been recently utilized in medicine as a natural drug against many toxicants. Radish has been used in many civilizations as a laxative, stimulant, digestive aid, and appetizer in the treatment of stomach disorders [6]. Radish extracts showed antimicrobial [7], antimutagenic [8], and anticarcinogenic effects [9], along with reduction of alkaline phosphatase and total bilirubin levels [10]. Thus, the aim of this study is to investigate the effectiveness of the crude extract of radish leaves on mitosis of the meristematic cells of onion roots *In vivo*.

Materials and Methods

Plant collection

This work was carried out in the Laboratory of Biology department, Collage of Science, University of Baghdad, Baghdad, Iraq during the period 1/10/2018 - 1/5/2019. The leaves of *R. sativus* were obtained from the local market in Baghdad. In November, the studied model was collected randomly from the plant, taking into account that the plant parts used in the extraction process were free from visible damage with a non-aligned size of the model. The parts of the plant used in this study included leaves, which were separated from the soil and washed well with tap water to remove dust. The leaves were then cut into small pieces, placed in the oven for drying at 50-55°C for 72 hrs, and then placed in the mill until they formed a powder**.** The powdered leaves of radish were transferred to a glass sealed cans and placed in the refrigerator before the extraction process.

Preparation of Different Concentrations of Plant Extracts

Stock solutions were prepared by mixing 10 g of the weighed plant leaves powder with 100ml distilled water, plugged with sterile cotton and kept in Shaking Incubator at 200 rpm for 24 h. The solution was filtered through muslin cloth; this process was repeated three times after which a clear aqueous extract of the plant was taken. Then, a concentration of 10 mg/ ml was prepared by mixing a known volume from the stock solution with distilled water. Following the equation C1V1 = C2V2, a series of different extracts solutions were prepared with concentrations of 5, 10, 20, and 40mg/ml [11,12].

Effects of aqueous extract on cell division of onion root tips *in vivo*

Onion bulbs were purchased from the local market. Old roots and dry scales were removed and allowed to germinate by placing in vials containing tap water. After 48 hrs, the roots reached 2-3 cm in length. The root meristem was exposed to various concentrations of radish aqueous extract at its peak mitotic time. Four concentrations (0.0, 5, 10, 20, and 40 mg/ml) were evaluated for four hours for their cytotoxic and genotoxic potential by *Allium* assay. Mitotic squash preparations of the root tips were achieved following an improved technique of Sharma and Sharma [13]. Each experiment was repeated three times and at least five micro glass slides were prepared for each parameter.

Treatment of roots and preparation of slides

Root tips were dissected from treated and non-treated onion root tips and subjected to karyotypic studies.

Excised roots from the germinated bulbs were grown at 25-30 ºC in dark for 48 hrs and dissected leaving 1-2 mm of the root tips. The root tips were stabilized in carnoy fluid to study cell division. Three volumes of absolute ethylene alcohol were combined with one volume of glacial acetic acid [14, 15].

Root tips were treated with 2-3 drops of 1 N HCl inside vials at 55-60 ºC, then placed in an oven for 10 min.

Root portions were washed with distilled water then transferred to another vial containing acetocarmin stain 2%, then placed in an oven for10 min.

Excess stain was removed carefully, then one drop of fresh stain was added on a dot-sized piece of the root tip before placing slide covers carefully, then root tips were squashed [13].

A compound light microscope was used to examine the meristematic region of the root tip at 40X and 100X magnification. Chromosomes at the various stages of mitosis were photographed using Iphone camera.

Mitotic index

The percentage of cells undergoing mitosis in each treatment was determined on the basis of a minimum of 1000 cells. This index, along with proportions of abnormal dividing cells , was calculated according to the following equations:

%Mitotic Index (MI) = Total number of dividing cells /Total number of cells examined x 100

% phase Index = Total number of phase /Total number of dividing cells x 100

% aberration Index = Total number of aberrant cells /Total number of dividing cells in the same phase x 100 [16, 17].

The percentage of control for mitotic index and phase index were calculated as in the following equations:

Percentage of control for Mitotic index = (Mitotic index in each treatments / Mitotic index in control)* 100

Percentage of control for phase index = (treatment phase index / control phase index) * 100 [18].

Experimental design and statistical analysis

The Statistical Analysis System (SAS) [19] program was used to study the effects of different factors on the studied parameters. The least significant difference (LSD) was used to compare the means in this study. Experiments were designed according to the complete random design and three replicates per treatment, at a probability level of $P \leq 0.05$.

Results and Discussion

Effects of the aqueous extract of radish leaves on cell division of onion root tips

Mitotic index

The mitotic index value was significantly reduced at all aqueous extract concentrations, recording 3.27, 1.87, 1.07, 1.67, and 1.37% for control and 5, 10, 20, 40 mg/ml of the treatment extracts, respectively (Figure-1 and Table-1). Mitotic index was decreased to less than 50% using 10 and 40 mg/ml of the extract, recoding 23.72 and 41.89 %, respectively, as shown in Figure-1 and Table- 2. The substances and extracts that cause a reduction in the mitotic index of up to 50% or less have a toxic or semi-lethal effect; they can interfere with the stages of division and thus prevent the nucleus from entering the mitotic phase [20, 21], then they stop the mitotic division during the interphase, prolong the G2 and S phases, or inhibit the processes of protein and DNA synthesis [22].

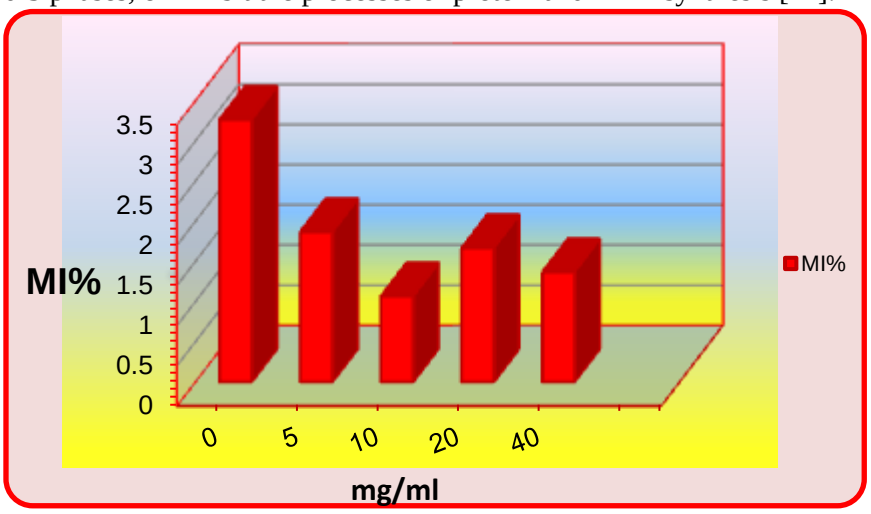


Figure 1-Mitotic index (%) in onion root tips treated with aqueous extract of radish leaves in different concentrations.

Prophase

The results in table 1 show the prophase index for the control as well as 5, 10, 20, and 40 mg/ml of the extract, which recorded values of 48.08, 31.53, 21.29, 2.67 and 7.35%, respectively. The percentage of the control was decreased to 65.57, 44.28, 5.55, and 15.28% using 5, 10, 20, and 40 mg/ml, respectively (Table-2).

Metaphase

Metaphase index was decreased significantly recording 27.78% using 5 mg/ml, compared with control (47.14%) (Table 1). The percentage of control was decreased using 5 mg/ml (58.93 %), while it was increased using 10, 20, and 40 mg/ml recording 105.07, 125.81 and 107.91 %, respectively, as shown in Table-2. Chromosomal aberrations such as Capture- metaphase (C-metaphase) and stickiness were also noticed. C-metaphase was very frequently seen even in lower concentrations of the aqueous extract of radish, recording 80.76 and 55.34% using 5 and 10 mg/ml, respectively (Figure- 2-A and B). This may indicate that the radish extract contains compounds that have anti-microtubules and polymerization of tubulin which is forming the spindle filaments, causing the chromosomes to fail to complete their alignment in metaphase. Alternatively, the effect may be on the mechanisms of nuclear division and thus have an effect on the formation and distribution of spindle threads, which stops cellular division [21,23]. Stickiness was more frequently observed in metaphase as well as anaphase chromosomes at higher concentrations of the extract, recording 16.66, 18.05, and 25.67% at 10, 20, and 40 mg/ml, respectively (Figu re-2-C). Chromosome stickiness is a clear evidence of genotoxicity, usually of an irreversible type leading to cell death [24].

Anaphase and telophase

The results in table 1 show that anaphase index was decreased significantly recording 3.65 and 7.87 % at 5 and 10 mg/ml, respectively, compared with that in the control which was 13.10% (Table-1). Also, the percentage of control was increased at 20 and 40 mg/ml of the extract, reaching 117.78 and 126.56%, respectively. The percentage of control was decreased at 5 and 10 mg/ml, recording 27.86 and 60.07%, respectively, as shown in Table-2. Bridges were more frequently observed in the aqueous extract at higher concentrations, recording 4.16, 5.55, and 3.33% at 10, 20, and 40 mg/ml, respectively as shown in the Table-1 and Figure -2-D. Bridges are the result of the translocation of the dicentric chromosomes, causing unequal exchange and structural chromosome mutations. The results showed that higher concentrations were able to significantly inhibit cell division [25].

According to the results, telophase in the control group was 28.47%, which was decreased significantly upon treatment with 5 mg/ml which recorded 7.48%, as shown in Table-1. The percentage of control was increased at 5, 10, 20, and 40 mg/ml, recording 26.27, 74.78, 78.92, and 88.83%, respectively (Table-2). Certainly, the polar deviation is one of the aberrant changes that were observed in cells treated with the aqueous extract; this may indicate that the studied extract contains compounds which may have anti-microtubules, long with the polymerization of tubulin. On the other hand, these compounds interfere with the S phase (DNA replication), since all the phases of mitosis are affected by the treatment Figure-(2-E) [22]. The *A. cepa* test is considered as an important test *in vivo* where the roots grow in a direct contact with the substance of interest, enabling prediction of possible damage to human DNA . In this study, this test enabled the assessment of different genetic endpoints that occurred as a result of exposure to aqueous extract. The changes in MI of *A. cepa* cells are indicators of the cytotoxic and genotoxic potential activities of the plant extract. In lower concentrations of the aqueous extract, non-clastogenic aberrations were observed. In higher concentrations of extract, a significant increase in the frequency and diversity of aberrations were observed, such as stickness in metaphase (Figure- 2-C). The results showed that higher concentrations were able to significantly inhibit cell division, as shown in Figure-1, indicating the toxicity of the tested concentration in *Allium* root tip cells [26]. The plant extract interfered with the replication of cellular DNA and prevented the proliferation of the cells. Devir and Thoppil [27] found that plant extracts showed significant toxicity in cell lines in higher concentrations (100µg/ml and 200µg/ml). Cytotoxicity *in vivo* was determined by the reduction of mitotic index (MI) and induction of chromosome aberration (CA) by the extracts in meristematic cells of *Allium*.

Conclusions

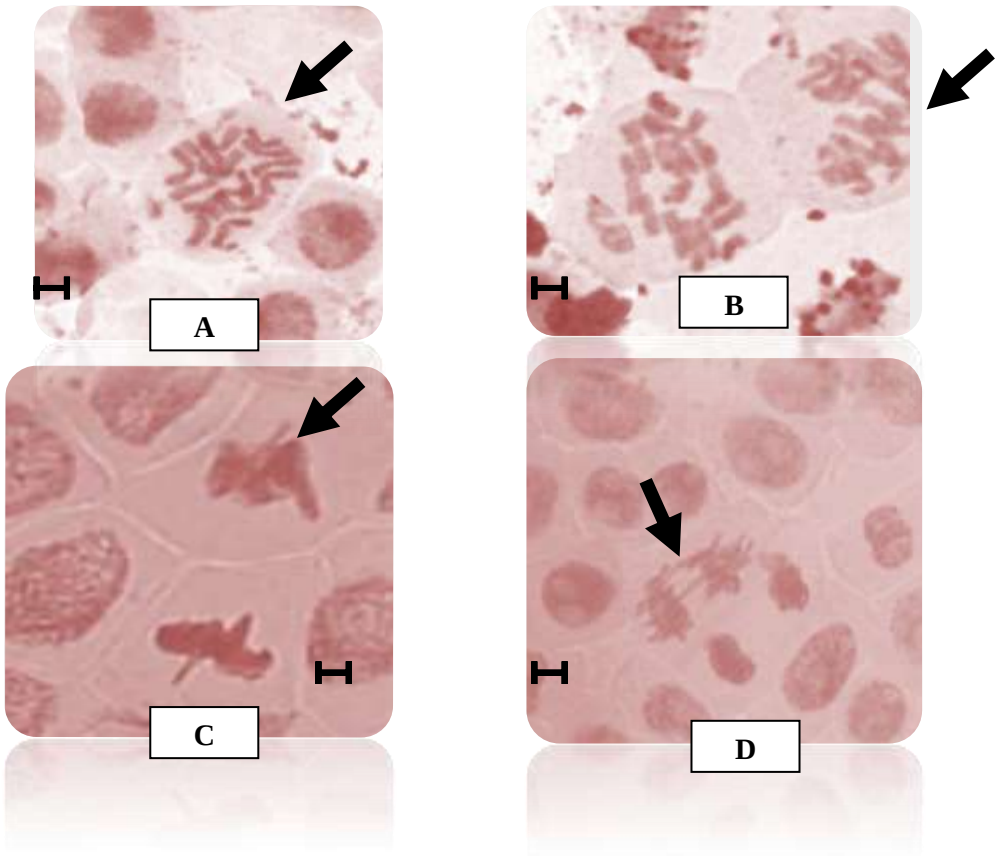
Bioactivity of the crude extracts may be ascribed to the active compounds present in the extract. Further phytochemical investigations may pave a new avenue to cancer drug research. Also, the cytotoxicity of the extract appraises the necessity of further studies in the mammalian systems.

Table 1-Mitotic index and its phases, and percentage of abnormality attributed to treatment of onion roots with different concentrations of aqueous crude extract of *Raphanus sativus* L. for four hours.

Concentration mg/ml	MI%	Phase index % ± S.D				Chromosomal aberration %			
		Prophase	Metaphase	Anaphase	Telophase	Metaphase		Polar deviation	Anaphase bridges
						c-metaphase	sticky		
Control	3.27 ± 0.59	48.08 ± 12.10	47.14 ± 5.42	13.10 ± 3.82	28.47 ± 0.34	0.00	0.00	0.00	0.00
5	1.87+ -0.96	31.53 ± 6.53	27.78 ± 9.72	3.65 ± 1.98	7.48 ± 4.86	80.76	0.00	0.00	
10	1.07± 0.37	21.29 ± 2.45	49.53 ± 8.79	7.87 ± 3.96	21.29 ± 2.45	55.34	16.66	4.16	4.16
20	1.67 ± 0.26	2.67 ± 2.67	59.31 ± 0.25	15.43 ± 4.30	22.47 ± 7.81	0.00	18.05	7.76	5.55
40	1.37 ± 0.07	7.35 ± 4.45	50.87 ± 1.94	16.58 ± 3.79	25.29 ± 7.39	0.00	25.67	10.9	3.33
LSD P ≤ 0.05	1.72	20.99	21.74	11.54	17.00				

Table 2-Percentage of the control of the mitotic division and the phases in the onion root tips treated with different concentrations of aqueous crude extract of *Raphanus sativus* L. for four hours.

Concentration mg\ml	MI%	Prophase%	Metaphase%	Anaphase%	Telophase%
5	57.18	65.57	58.93	27.86	26.27
10	23.72	44.28	105.07	60.07	74.78
20	51.07	5.55	125.81	117.78	78.92
40	41.89	15.28	107.91	126.56	88.83



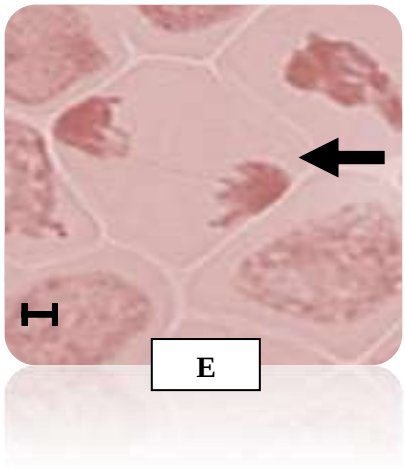


Figure 2-Cytological aberrations in the root tip of meristematic cells in *Allium cepa* treated with different concentrations of aqueous extract of *R. sativus* L. leave for four hours, **A, B**: C- metaphase, **C**: sticky metaphase, **D***:* bridges in anaphase, **E**: polar deviation. X=1000.
H = 5 Micron

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