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Genoprotective, antimutagenic, and antioxidant effects of methanolic leaf extract of *Rhamnus alaternus* L. from the Bissa mountains in Algeria

Leila Gadouche[1,2,*], Khayra Zerrouki[2], Azdinia Zidane[2], Adda Ababou[2], Ibtissem Bachir Elazaar[2], Dounya Merabet[2], Wissam Henniche[2], Samah Ikhlef[2]

[1] University of Science and Technology Houari Boumediene, Bab Ezzouar, Algeria
[2] Hassiba Benbouali University of Chlef, Chlef, Algeria

* e-mail: gadoucheleila@gmail.com

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Abstract:
Rhamnus alaternus L. is a *Rhamnaceae* shrub and a popular traditional medicine in Algeria. The present research objective was to investigate the antioxidant, genotoxic, and antigenotoxic properties of *R. alaternus* methanolic leaf extract.
Antiradical scavenging activity was tested by *α*, *α*-diphenyl-*β*-picrylhydrazyl free radical scavenging and *β*-carotene bleaching method. DNA damage and repair were measured by the *Allium cepa* test with sodium azide as a mutagenic agent. Mitotic index and chromosomal aberrations were calculated by microscopy of meristem roots stained with 2% carmine acetic.
The methanolic extract of *R. alaternus* leaves inhibited the free radical DPPH (IC_{50} = 0.74 ± 0.30 mg/mL) and prevented the oxidation of *β*-carotene (50.71 ± 4.17%). The root phenotyping showed that sodium azide changed their color and shape, decreased their stiffness, and significantly reduced their length. The roots treated with both *R. alaternus* leaf extract and sodium azide demonstrated a better root growth. The roots treated with the methanolic extract were much longer than the control roots ($P < 0.001$). The microscopy images of root meristem treated with the sodium azide mitodepressant agent showed significant chromosomal aberrations, which indicated a disruption of the cell cycle.
The *R. alaternus* leaf extract appeared to have a beneficial effect on cytotoxicity. The antioxidant properties of *R. alaternus* L. makes this plant an excellent genoportector.

Keywords: *Rhamnus alaternus* L., antioxidant activity, *Allium cepa*, chromosomal aberrations, antigenotoxicity, mitotic index

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INTRODUCTION

Radical oxygen species lead to cell damage, which can induce genetic instability responsible for many pathological processes. This damage can be repaired by some natural compounds, e.g. radical scavengers and powerful protective antioxidants [1]. Kitagishi *et al.* proved that medicinal herbs could one day become a promising therapeutic means of cancer therapy [2]. According to Dayani *et al.*, antioxidant, anti-inflammatory, and anti-apoptotic properties of plants and their derivatives make them good radioprotectors against the mutagenic action of X-rays [3].

Phytotherapy relies on medicinal plants and their active compounds. *Rhamnus alaternus* L. (*Rhamnaceae* family), also called *imlilesse* or *safir* in the North of Algeria, is well known for its biological properties [4]. Zeouk and Bakheti reported that a decoction of the aerial part of the *R. alatrenus* leaves and branches has been widely used in traditional medicine to lower blood pressure and treat hepatitis, icterus, musculoskeletal disorders, and gastrointestinal diseases. They also serve as a cataplasm for skin infections [5].

Previous findings proved that *R. alaternus* extracts possess potential antioxidant, cytotoxic antimutagenic, antigenotoxic, and antimicrobial activities [5–8]. In their bibliographic review, Nekkaa *et al.* focused on the phytochemical and pharmacological properties of *R. alaternus* [4]. Its leaf extracts were rich in flavonoids,

tannins, and anthocyanins, which explains their potential antigenotoxic and antimutagenic activity.

Bhouri *et al.* isolated kaempferol 3-O-b-isor-hamninoside and rhamnocitrin 3-O-b-isor-hamninoside from *R. alternus* leaves [9]. These flavonoids are effective free radical scavengers and potent antigenotoxics. However, they can induce apoptosis in human lymphoblastoid cells by the extrinsic apoptotic mechanism including DNA fragmentation, PARP cleavage, and active caspase-3 and caspase-8 [10]. Oligomer flavonoid extract from *R. alaternus* leaves proved to have a good potential for alternative antimelanoma therapies [11].

Although some plant remedies have well-documented protective effects and alleviate many diseases, cytotoxicity studies are very important for developing new drugs. Gadouche *et al.* described the toxic effect of *Aristolochia longa* L. and *Calycotome spinosa* L. on the blood cells and concluded that it should be studied on cancer cells [12]. Natural antioxidants can even protect human organism against the cytotoxic and mutagenic effects of xenobiotics.

In this research, we analyzed the genotoxic and DNA damage protecting activity of *R. alaternus* leaf extract by using the *Allium cepa* assay with azide sodium as a mutagen agent.

STUDY OBJECTS AND METHODS

Plant material. The research featured *Rhamnus alaternus eu-alaternus* L., a subspecies of *Rhamnus alaternus* L. The samples were collected in the Bissa forest located in the north of the Chlef province (Algeria). This species of Algerian flora was identified by Dr. Belhacine, a botanist from the Chlef University [13].

The *R. alaternus* leaves were dried in the dark for 10 days. After that, they were ground into a fine powder and kept in an airtight container, and 10 g of the dry powder was macerated in 100 mL of petroleum ether for 24 h with stirring. The mix was filtered on Whatman No. 1 paper. The maceration included 100 mL of methanol. After filtration, the marc was evaporated in a rotary evaporator at 39°C. The extract obtained was stored at 4°C until use [14].

Quantitative analysis and antioxidant activity. The polyphenols were assayed according to the method developed by Raafat and Samy [15]. The amount of total polyphenols was determined spectrophotometrically using the Folin-Ciocalteu reagent and deduced from a calibration curve established with gallic acid (0–1 mg/mL). The results were expressed in mg of gallic acid equivalent per g of dry matter (mg GAE/g of dry matter). The mix included 250 µL of Folin Ciocalteu's phenol reagent, 50 µL of each concentration prepared from stock solution, and 500 µL of 20% Na_2CO_3 aqueous solution. After vortexing, the solution was adjusted with 5 mL of distilled water. After 30 min of incubation, the absorbance was measured at 765 nm. The same procedure was carried out with the extract obtained from *R. alaternus* leaves.

The flavonoid content was assayed according to the method developed by Hmid *et al.* [16]. After 1 mL of extract was added to 1 mL of 2% aluminum chloride, the absorbance was determined at 430 nm after 10 min of incubation. Quercetin served as calibration curve standard and was established from the concentration of 40 µg/mL of stock solution. Total flavonoids content in the extract was expressed as mg quercetin equivalents per g of sample (mg EQ/g of dry matter).

The DPPH assay followed the method described by Burits and Bucar [17]. The *R. alaternus* extract had the following concentrations: 0.2, 0.4, 1.0, and 2.0 mg/mL. We mixed 50 µL of each concentration with 5 mL of 0.004% DPPH. The absorbance was measured at 517 nm after 30 min of incubation. The results were compared to ascorbic acid, which was used as standard antioxidant and handled under the same conditions. The percentage of inhibition and IC_{50} were calculated according to Sharififar *et al.* [18]. The percentage inhibition was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{\text{ODcontrol} - \text{ODsample}}{\text{ODcontrol}} \times 100$$

where OD is optical density.

IC_{50} is the concentration of extract required for 50% inhibition of DPPH. It was calculated using a linear regression analysis.

The *β*-carotene-linoleic acid assay was performed according to the method described by Kartal *et al.* [19]. The emulsion included 0.5 mg of *β*-carotene, 1 mL of chloroform, 25 µL of linoleic acid, and 200 mg of tween 40. The chloroform was eliminated in a rotary evaporator under vacuum, and 100 mL of distilled oxygen-saturated water was added to the emulsion. Subsequently, 350 µL of the extract at a concentration of 2 mg/mL was mixed with 2.5 mL of the emulsion. After 48 h of incubation, the absorbance was registered at 490 nm and compared with that obtained with butylohydroxytoluene (BHT), which served as a standard antioxidant and was prepared under the same conditions. The inhibition percentage of bleaching (I, %) was measured for each assay using the following equation:

$$I = \frac{\text{Abcorbance of a sample at 48 h}}{\text{Abcorbance of BHT at 0 h}}$$

***Allium cepa* assay.** The *A. cepa* assay was performed according to Tedesco and Laughinghouse with some modifications [20]. The onion bulbs were kept in a culture medium that included 60 mg/L of $CaSO_4$, 60 mg/L of $MgSO_4$, 96 mg/L of $NaHCO_3$, and 4 mg/L of KCl. They were incubated at 25°C for 72 h until the roots reached 2 cm. Seven onion bulbs were utilized for each treatment as follows:

Sample 1: culture medium + distilled water;
Sample 2: culture medium + sodium azide (50 mg/mL);
Sample 3: culture medium + sodium azide (100 mg/mL);

Sample 4: culture medium + methanolic extract (50 mg/mL);
Sample 5: culture medium + methanolic extract (100 mg/mL);
Sample 6: culture medium + sodium azide (100 mg/mL) + methanolic extract (50 mg/mL);
Sample 7: culture medium + sodium azide (100 mg/mL) + methanolic extract (100 mg/mL).

The effect of the different treatments on the growth (cm) of the *A. cepa* roots was measured at different time intervals: 0, 24, 48, and 72 h. In parallel, the roots were tested for color, shape, and stiffness. After each time interval, the roots were collected for microscopic observation of the meristem cells and stored in 70% ethanol for later use. The roots were fixed in acetic acid and ethanol solution (1:3) for 24 h. After triple rinsing with distilled water, the roots were hydrolyzed with HCl (1N) and incubated in a hot water bath at 60°C for 10 min. After the hydrolysis, the roots were rinsed once again in distilled water and stained with 2% acetic carmine in a hot water bath at 60°C for 10 min. After incubation, the terminal meristem cells of the colored roots were cut with a scalpel under a binocular magnifier. The meristem regions were crushed manually between blade and coverslip to visualize the chromosomes and the different stages of cell division. Meristem cells were counted for each sample and tested for normal or abnormal cell division in search for mutations. The mitotic index and the rate of aberrant cells of each bulb were calculated by the following formula [21]:

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells scored}} \times 100$$

$$\%\text{ of observed aberration} = \frac{\text{Number of abberant cells}}{\text{Total number of cells scored}} \times 100$$

Statistical analysis. The experimental data were analyzed using the ExcelSTAT software. The research also included the ANOVA variance analysis, followed by the Tukey's test. The statistically highly significant value was $P < 0.001$.

RESULTS AND DISCUSSION

The total phenol content in *Rhamnus alaternus* L. leaves was 32.6 ± 1.82 mg GAE/g DM, and the total flavonoid content was 27.58 ± 0.01 mg EQ/g DM. The methanolic extract of *R. alaternus* leaves demonstrated a moderate efficiency against free radicals emitted by linoleic acid (50.71 ± 4.17%). Its capacity to beat free radical of DPPH (I% = 80.39 ± 2.33%, IC_{50} = 0.74 ± 0.30 mg/mL) was close to that of ascorbic acid, i.e. 96.80 ± 9.98% with IC_{50} = 0.37 ± 1.10 mg/mL (Table 1).

Plants are excellent indicators of the cytotoxic, cytogenetic, and mutagenic effects of environmental chemicals. They can serve as an alternative for detecting possible genetic damage in cells [22]. Genotoxicity studies were carried out by the *Allium cepa* assay. This method provides a convenient *in vivo* model to evaluate cell cycle alterations induced by mutagens [20].

The *A. cepa* roots treated with distilled water had no morphological change: the growth rate was good, the color was whitish, and the roots were rigid and bulky. However, the roots treated with sodium azide (50 and 100 mg/mL) changed the color and shape of the roots, as well as reduced their rigidity (+very brittle) and growth rate.

The methanolic leaf extract of *R. alaternus* had no negative effect on the morphology. The samples demonstrated good growth, strong rigidity, and whitish color. Their morphology was comparable to the control roots. The roots incubated in both methanolic extract and sodium azide had a phenotype close to the control roots. They were better preserved than the roots treated only with sodium azide. The roots of this sample showed good growth, and the color was comparable to that of the control roots (Fig. 1).

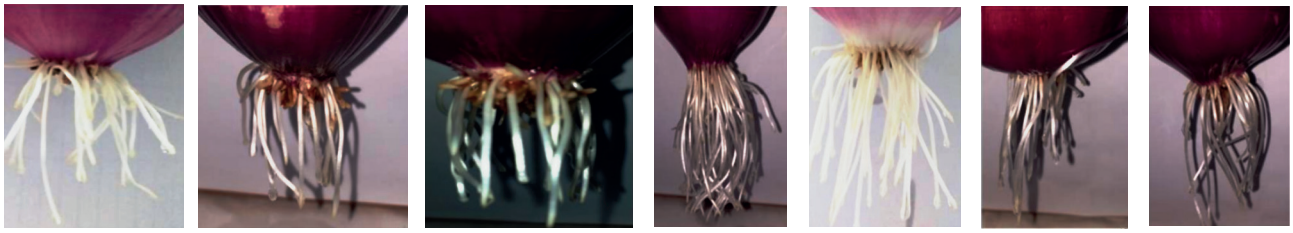
Table 2 shows a highly significant decrease in the growth of the *A. cepa* roots treated with sodium azide at two concentrations (50 and 100 mg/mL) at three time intervals. The data obtained from the sample treated with 50 mg/mL of sodium azide after 48 h was found insignificant ($P < 0.001$).

The roots treated with the methanolic extract of *R. alaternus* leaves showed highly significant growth ($P < 0.001$) after 24 and 48 h. The roots reached 8 cm after 72 h ($P < 0.001$) and were longer than those treated with distilled water (7 cm).

The difference in length for the antigenotoxicity test was highly significant after 48 and 72 h and not significant after 24 h. The roots demonstrated a clearly significant improvement in the diameter after 72 h.

Table 1 Total phenolic content and total flavonoid content of *Rhamnus alaternus* leaves, DPPH inhibition, IC_{50}, and % bleaching of *β*-carotene

Parameter	Leaves of *R. alaternus*	Ascorbic acid	Butylated hydroxytoluene
Polyphenol, mg GAE/g dry matter	32.60 ± 1.82	–	–
Flavonoids, mg QE/g dry matter	27.58 ± 0.01	–	–
DPPH, % (*R. alaternus* extract concentration = 1 mg/mL)	80.39 ± 2.33	96.80 ± 9.98	–
IC_{50}, mg/mL	0.74 ± 0.30	0.37 ± 1.10	–
β-carotene bleaching, % (*R. alaternus* extract concentration = 2 mg/mL)	50.71 ± 4.17	–	98.84 ± 1.69



a b c d e f g

Figure 1 Morphological aspects of *Allium cepa* roots: (a) control group; (b) sodium azide (50 mg/mL); (c) sodium azide (100 mg/mL); (d) methanolic extract (50 mg/mL); (e) methanolic extract (100 mg/mL); (f) sodium azide (100 mg/mL) + methanolic extract (50 mg/mL); (g) sodium azide (100 mg/mL) + methanolic extract (100 mg/mL)

Table 2 ΔL – differences in length of the *Allium cepa* roots before and after each treatment, % mitotic index, and chromosomal aberrations

Treatment	Time, h	ΔL, cm	Mitotic index, %	Chromosomal aberrations, %
Control	0	2.66 ± 0.56		
	24	0.57 ± 0.05		
	48	0.84 ± 0.09		
	72	0.70 ± 0.24	69.76 ± 7.01	0
Sodium azide (50 mg/mL)	0	2.36 ± 0.48		
	24	0.50 ± 0.11**		
	48	0.94 ± 0.10		
	72	–0.54 ± 0.31**	34.48 ± 10.50	5.03 ± 1.51**
Sodium azide (100 mg/mL)	0	2.84 ± 0.47		
	24	–1.12 ± 0.18**		
	48	–0.40 ± 0.16**		
	72	–0.03 ± 0.12**	29.25 ± 8.50	7.84 ± 2.41**
Methanolic extract (50 mg/mL)	0	2.93 ± 0.19		
	24	1.09 ± 0.21**		
	48	0.90 ± 0.07**		
	72	0.46 ± 0.15	69.54 ± 14.5	0.43 ± 0.53
Methanolic extract (100 mg/mL)	0	2.80 ± 0.47		
	24	1.87 ± 0.04**		
	48	1.37 ± 0.09**		
	72	1.05 ± 0.02	73.66 ± 9.41	0.29 ± 0.49
Sodium azide (100 mg/mL) + methanolic extract (50 mg/mL)	0	2.71 ± 0.38		
	24	0.11 ± 0.05		
	48	–0.33 ± 0.12**		
	72	0.26 ± 0.18**	51.77 ± 14.44	2.55 ± 1.98
Sodium azide (100 mg/mL) + methanolic extract (100 mg/mL)	0	2.47 ± 0.57		
	24	0.53 ± 0.08		
	48	–0.10 ± 0.07**		
	72	0.13 ± 0.09**	52.34 ± 8.12	3.34 ± 2.82

ΔL is mean difference in length of *Allium cepa* roots before and after treatment

**$P \leq 0.001$

It was 0.26 and 0.13 cm, respectively, for the two extract concentrations.

Microscopy revealed no abnormalities or disturbances in mitotic division: chromosome integrity maintained its high mitotic index (69.76 ± 7.01%), and no chromosomal aberrations were registered (Fig. 2, Table 2).

The microscopy of the roots stained with 2% acetic carmine after treatment with two concentrations of sodium azide revealed several chromosomal anomalies with disruption of all the stages of cell division (Figs. 3 and 4, Table 2). Several cells contained C-mitosis, S-mitosis, chromosomal breaks, bridges, and uneven distribution of chromosomes, which led to disturbed anaphases, metaphases, and telophases. These anomalies were caused by both concentrations of azide; however, they were much more severe at 100 mg/mL of sodium azide.

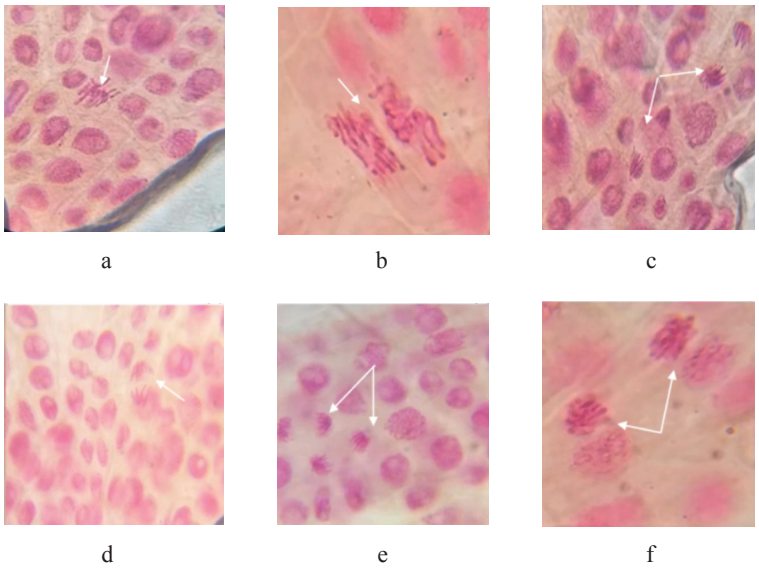


Figure 2 Normal mitotic divisions of *Allium cepa* meristem cells (100×): (a) interphase, prophase, and metaphase; (b) start of anaphase; (c), (e), (f) anaphase; (d) telophase

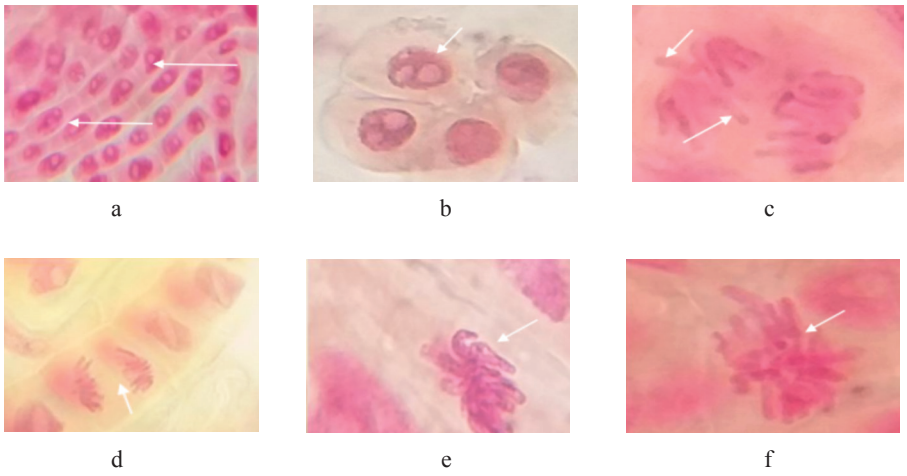


Figure 3 Various anomalies caused by sodium azide at 50 mg/mL (100×): (a) binucleated cell; (b) disturbed telophase; (c) disturbed anaphase; (d) normal anaphase; (e), (f) disturbed metaphase

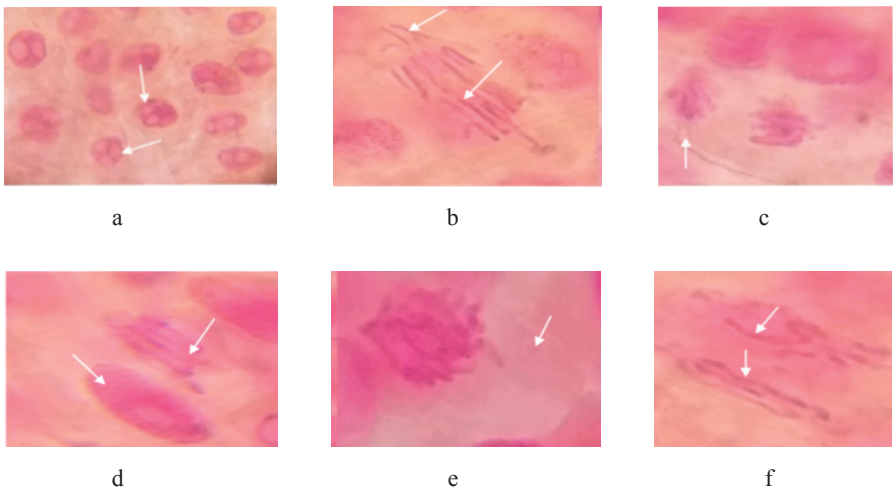


Figure 4 Chromosomes of *Allium cepa* roots treated with 100 mg/mL of sodium azide (100×): (a) binucleated cells; (b) chromosomal break, chromosomal bridge; (c), (e) disrupted (uneven) anaphase; (d) prophase, chromosomal bridge; (f) C-mitosis

Chromosomal aberrations increased together with the concentration of sodium azide. The genotoxic effect was most severe at 100 mg/mL. Both concentrations of sodium azide reduced the mitotic index, which meant that sodium azide blocked cell division. On the other hand, the number of chromosomal aberrations grew together with sodium azide concentration. They were represented mainly by C-mitosis, chromosomal bridges and breaks, and nuclear lesions of binucleate types. Therefore, sodium azide was an aberration inducer (Figs. 3 and 4, Table 2).

Sodium azide produced a cytotoxic effect which led to poor growth and length narrowing. Its mitodepressive effect decreased mitotic activity and increased chromosomic abnormality incidence. Indeed, chemical agents are recognized as factors involved in the structural and numerical modifications of chromosomes. As a result, they cause defects in chromosome segregation, abnormal DNA replication, and DNA breaks. These chromosomal aberrations result from clastogenic and aneugenic effects [23]. This study confirmed the genotoxic effect of sodium azide. According to Al-Qurainy *et al.*, sodium azide is a mutagenic metabolite that damages DNA by substituting one base pair with another [24]. Indeed, the shorter length of *A. cepa* roots treated with sodium azide could be explained by the mitodepressive effect caused by the apoptosis of meristem cells. Other samples demonstrated evolution of the normal length, probably, due to the resumption of mitosis.

Sodium azide induced the development of chromosome bridges in the meristem cells of *A. cepa* roots. According to Neelamkavil and Thoppil, the chromosomal aberrations and nuclear lesions in *A. cepa* root meristems treated with bleaching powder indicated a genotoxic effect, which confirms that sodium azide is genotoxic [25]. The clastogenic effects suggest that bleaching powder caused chromosome and chromatin breaks, which, in return, led to abnormal chromosome number, stickiness, breakage, and reunion of chromosome, as well as to bridges during mitotic division [26, 27].

The mitotic index was higher in the roots treated with two concentrations of the *R. alaternus* extract than in those treated with distilled water. Therefore, the extract induced cell division and, subsequently, produced a genoprotective effect. Moreover, the number of cells in division was high with traces chromosomal aberrations also proven by a marked root length. The samples treated with 50 mg/mL of *R. alaternus* methanolic extract had pycnotic nuclei and chromosomal breaks (Figs. 5 and 6, Table 2). This finding confirms the conclusion made by Ben Ammar *et al.*, who experimented with methanolic, petroleum ether, chloroform, and aqueous extracts of *R. alaternus* leaves and registered no mutagenicity, which means that *R. alaternus* is a promising antimutagenic [28].

The antigentoxic effect showed that the mitotic index was close to that of the control. It had a moderate chromosomal aberration percentage, chromosomal bridges and breaks, and a lower C-mitosis (Figs. 7 and 8, Table 2).

A quantitative analysis of the *R. alaternus* methanolic extract revealed a lot of polyphenols and flavonoids and thus a prominent antioxidant effect. This antioxidant effect might be the cause of the continuous cell division, mitoprotective activity, and a good DNA protection. Perron *et al.* tested 12 polyphenolic compounds, which demonstrated a 100% ability to inhibit DNA damage [29]. The polyphenolic compounds had hydroxyl radicals in their chemical structures, which prevented oxidative DNA damage.

On the other hand, Silva *et al.* showed that flavonoids have special DNA repair mechanisms that enable them to reduce and repair DNA strand breaks induced by oxidative stress [30]. Therefore, polyphenols are effective protectors against oxidative DNA damage.

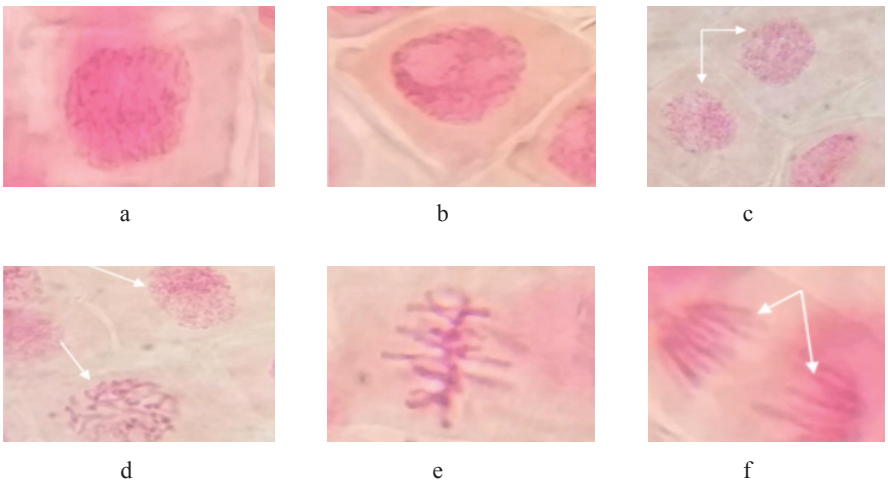


Figure 5 Chromosomes of *Allium cepa* roots treated with 50 mg/mL of *Rhamnus alaternus* leaf extract (100×): (a) prophase; (b) end of interphase; (c) telophase; (d) pycnotic nucleus; (e) metaphase; (f) anaphase

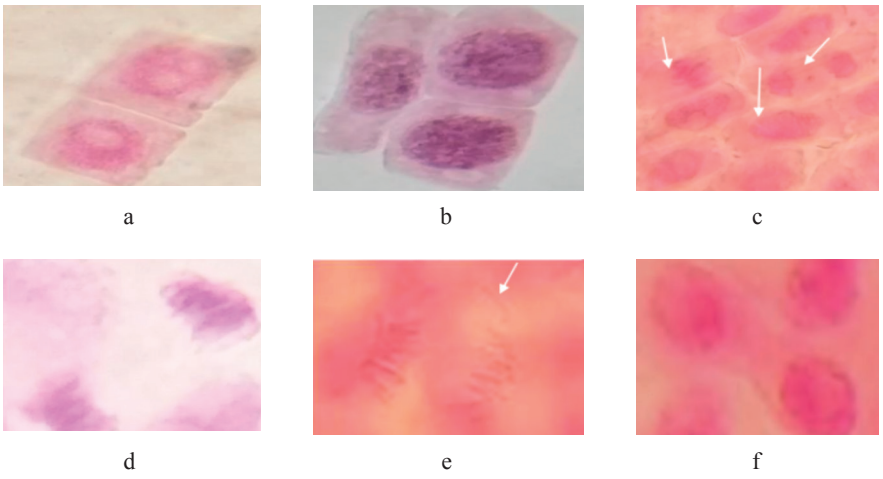


Figure 6 Chromosomes of *Allium cepa* roots treated with 100 mg/mL of *Rhamnus alaternus* leaf extract (100×): (a), (b), (f) telophase/cytodiuresis; (c) anaphase, prophase, and metaphase; (d) anaphase with chromosome breaks; (e) chromosome bridge with an isolated chromosome

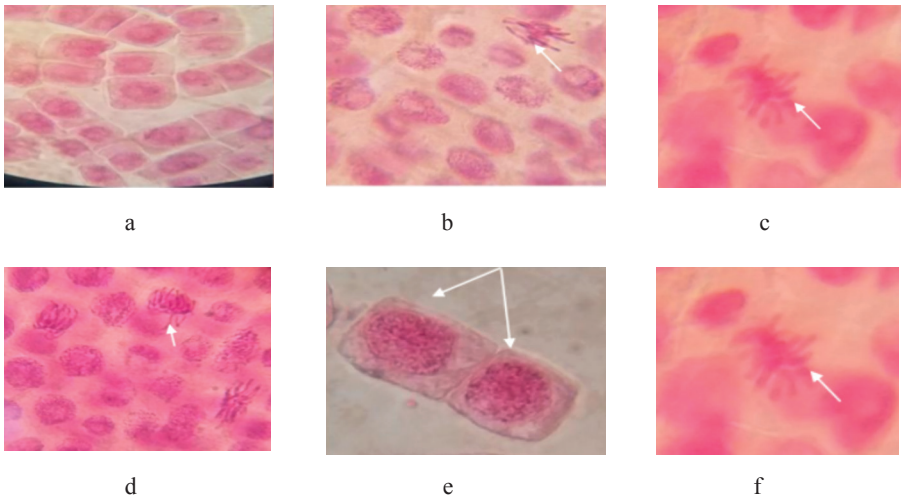


Figure 7 Chromosomes of *Allium cepa* roots treated with 50 mg/mL of methanolic extract and 100 mg/mL of sodium azide (100×): (a) several prophases; (b) several prophases; (c), (f) metaphase; (d) anaphase; (e) telophase start of prophase

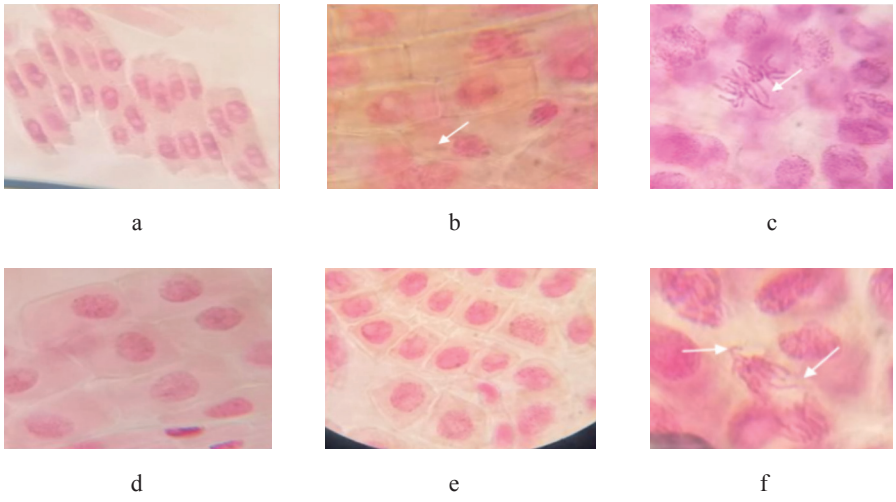


Figure 8 Chromosomes of *Allium cepa* roots treated with 50 mg/mL of methanolic extract and 100 mg/mL of sodium azide (100×): (a) binucleated; (b) metaphase, anaphase; (c) several prophases; (d) prophase; (e) prophase, metaphase, and anaphase.

Polyphenols have a powerful potency to donate electrons or hydrogen atoms, thus hampering oxidative stress, cell damage, and inflammation. They create a defensive obstacle against free radicals and reactive oxygen species. These protective effects might be explained by their antioxidant capacity [31].

Probably, the high content of flavonoids and polyphenols in *R. alaternus* protected the DNA as they opposed to the attack of free radicals emitted by sodium azide. Previous studies attributed the antigenotoxic activity of plant extracts to their numerous phenolic and flavonoid compounds, as well as to their ability to combat oxidative stress. The results imply that the extract managed to protect the genome of

the roots against mutagen because division anomalies were very scarce. These findings confirm those made by Ben Amar *et al.*, who proved that the extract of *R. alaternus* leaves and roots had anti-free-radical, anti-mutagenic, and antiproliferative properties as they trapped mutagenic free radicals [32]. According to Ben Sghaier *et al.*, medicinal plants contain phytochemicals that may have potential chemopreventive activity since they protect DNA from attack of free radicals [33].

CONCLUSION

Medicinal plants contain a lot of secondary metabolites with beneficial therapeutic and pharmacological properties, which deserve extensive research. *Rhamnus alaternus* L. proved to be an effective antioxidant and mitoprotector that can boost the development of pharmacognosy and produce new herbal drugs for the pharmaceutical industry. The genoportective effect of *R. alaternus* leaf extract could be a source of new cancer drugs and protect human genome from the side effects of chemical treatment.

CONTRIBUTION

L. Gadouche conceived and designed the analysis, performed the biological experiments, and wrote the paper. A. Zidane and K. Zerrouki contributed to the data analysis and revised the paper. A. Ababou performed the statistical analysis. I. Bachir Elazaar, D. Merabet, W. Henniche, and S. Ikhlel performed the biological experiments. All the authors revised the manuscript for publication.

CONFLICT OF INTEREST

The authors declare that there was no potential conflict of interests regarding the publication of this article.

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ORCID IDs
Leila Gadouche https://orcid.org/0000-0003-1757-1988
Khayra Zerrouki https://orcid.org/0000-0002-0217-3138
Azdinia Zidane https://orcid.org/0000-0003-1575-5994
Adda Ababou https://orcid.org/0000-0002-2056-0553
Ibtissem Bachir Elazaar https://orcid.org/0000-0002-8637-637X
Dounya Merabet https://orcid.org/0000-0001-6019-444X
Wissam Henniche https://orcid.org/0000-0002-7460-7417
Samah Ikhlef https://orcid.org/0000-0003-1370-9206