



RESEARCH ARTICLE

In vitro antimitotic and cytotoxic potentials of aqueous and organic extracts of *Rhamnus alaternus*: Correlation with chemical composition and *in vivo* acute oral toxicity evaluation

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Abstract

Rhamnus alaternus L. is a shrub known for its beneficial digestive, hepatoprotective, antioxidant, cytotoxic and antihyperglycemic properties. The aim of this study was to evaluate, *in vitro*, the antimitotic and cytotoxic activities of aqueous (decocted, infused and macerated) and organic (methanolic, methanolic macerated, chloroform, ethyl acetate and petroleum ether) extracts of *R. alaternus* using two assays: the *Lepidium sativum* phytotest and the brine shrimp lethality test. Principal component analysis (PCA) was employed to analyze correlations between chemical composition and the two biological activities. An *in vivo* study of the acute oral toxicity of the most *in vitro* active plant extracts was carried out on mice. The results of the antimitotic activity study showed that decocted and ethyl acetate extract were the most active, with IC_{50} values of 25.403 ± 0.153 mg/mL and 6.050 ± 0.037 mg/mL, respectively. Similarly, these two extracts possess the most potent cytotoxic effect with LC_{50} values of 7.420 ± 0.135 mg/mL and 0.355 ± 0.004 mg/mL, respectively. Correlation analysis between chemical composition and the two *in vitro* activities revealed a significantly positive correlation. The acute toxicity study of the decocted, methanolic extract and ethyl acetate extract revealed that all three extracts caused no mortality in mice and consequently, the LD_{50} was estimated to be ≥ 5000 mg/kg. The results obtained suggest that *R. alaternus* leaves may have anticancer properties, particularly the decocted, methanolic and ethyl acetate extracts, in addition to demonstrating safety.

Keywords: acute toxicity; antimitotic activity; cytotoxic effect; principal component analysis (PCA); *Rhamnus alaternus*

Introduction

Cancer remains a leading global public health challenge. It is a universal disease that affects all population categories, regardless of gender, age or socio-economic level (1, 2). Global cancer statistics for 2022 reported nearly 20 million new cases and 9.7 million deaths linked to this disease (3). Strategies adopted by the World Health Organization (WHO) to combat this disease have succeeded in improving survival rates over the past two decades, but the incidence of cancer continues to rise worldwide (4). By 2040, forecasts estimate that the number of new cancer cases will reach between 29-37 million (5). According to the 2025 WHO recommendations, cancer control is based on screening, surveillance, prevention, early diagnosis, treatment and palliative care. Today, these strategies remain the key public health pillars in the fight against this disease (6). These various components tend to reduce new cases of cancer through prevention of risk factors such as poor diet, smoking, exposure to carcinogens and lack of physical activity, while treatment of this disease requires screening and early diagnosis (5, 7).

According to 2020 statistics of the National Cancer Prevention and Control Plan, cancer continues to be the leading

cause of death after cardiovascular disease in Morocco. Several lifestyle factors (sedentary lifestyle, obesity), risk behaviours (unbalanced diet, smoking, etc.) and environmental factors (pollution, occupational exposure, etc.) have contributed to the increasing cancer incidence in the Moroccan population (8). Morocco, along with two other Maghreb countries (Tunisia and Algeria), has established a strategy to reduce morbidity and mortality caused by cancer and to improve quality of life. This strategy is inspired by the WHO's global strategy to combat non-communicable diseases and cancer (6).

Late diagnosis of cancer is a major problem in Maghreb countries (9). Morocco, as a developing country, has made significant advances in cancer screening and treatment. However, several factors are attributed to the diagnosis of cancer at advanced stages, primarily the difficulty of access to care and lack of resources in specialized health facilities (9, 10). These limitations have prompted people to seek alternative treatments, such as herbal medicine, which are more affordable than conventional care and are often the only available option given the limited access to modern health services. In addition, the growing number of research on medicinal plants with anticancer properties underlines the relevance of these resources as natural therapeutic alternatives (11-13).

R. alaternus, also known as *Nerprun alaternus*, is a plant widely used in traditional medicine in various parts of the world, notably Morocco, Algeria and Tunisia. The leaves of this plant are traditionally used as a decoction or infusion to treat certain liver and dermatological diseases and are also used for their gastric, laxative, antihypertensive, purgative and hepatoprotective effects (14, 15). Numerous experimental studies have highlighted the richness of this species in bioactive compounds such as phenolics, flavonoids, tannins, coumarins, saponins and anthraquinones, potentially responsible for cytotoxic, antioxidant and antiproliferative activities (16-18).

The ability of plants to produce bioactive compounds with cytotoxic and antiproliferative effects is attracting growing interest in pharmaceutical research. Thus, in the present study, we evaluated *in vitro* the antimitotic and cytotoxic activities of *R. alaternus* through two tests: the *Lepidium sativum* phytotest and the brine shrimp (*Artemia salina*) lethality test. We also assessed the acute oral toxicity of the aqueous and organic extracts of the plant that showed the best *in vitro* activity.

Materials and Methods

Plant material

The leaves of the *R. alaternus* plant used in this study were collected in April 2022 about 33 km west of Taza on the road from Douar Bab El Harcha in Bni Lent, GPS coordinates are 34° 19.821'N 004°13.178'W.

Botanical identification of *R. alaternus* L. was carried out by Prof. Abdelilah Rahou of the Faculty of Science, Moulay Ismail University, Meknes, Morocco. A sample of the reference plant (RA 2022/04) was deposited in the herbarium of the MPCAE Laboratory - Materials, Natural Substances, Environment and Modeling Team (MSNEM), Polydisciplinary Faculty of Taza (FPT), Sidi Mohamed Ben Abdellah University (USMBA) of Fez, Morocco.

Preparation of aqueous and organic extracts

The leaves of the *R. alaternus* plant used in this study were collected, dried and then subjected to two types of extraction: aqueous and organic. The protocol followed for the preparation of aqueous and organic extracts is described in our previous work (18).

Aqueous extraction

Aqueous extracts were obtained following extraction by three methods (decoction, infusion and maceration). For each method, 30 g of *R. alaternus* leaf powder was mixed with 300 mL of distilled water, with variations in temperature and extraction time. The procedure for this extraction is described in previous work carried out by our laboratory (19-22).

Organic extraction

Organic extraction with Soxhlet

Hot Soxhlet extraction was performed on 100 g of *R. alaternus* leaves with a volume of 1000 mL of four solvents of different polarity namely, petroleum ether, chloroform, ethyl acetate and methanol. Extraction time can be as long as 6 hr, depending on our experimental conditions (18, 23-25).

Methanolic maceration

Maceration extraction was carried out cold by placing 100 g of *R. alaternus* leaves in 1000 mL methanol for 48 hr (18, 23-25).

Biological and pharmacological activities

Phytotest *L. sativum*

L. sativum seeds are treated with solutions of the various extracts at doses of 1, 10, 25, 50, 100, 200 and 300 mg/mL for aqueous extracts and 0.5, 2, 6, 10, 14, 18 and 20 mg/mL for organic extracts, while the reference drug (colchicine) was used at doses of 20, 60, 333, 500, 2000, 4000 and 5000 µg/mL to determine the anti-germinative and antimutagenic efficacy of the tested substances. This evaluation is carried out over 3 days (26). The protocol followed is described below, as mentioned in previous laboratory work (21, 25).

Day 1

A total of 10, *L. sativum* seeds are placed in petri dishes (55 mm) containing a disc of filter paper soaked in 1 mL distilled water, then incubated for 24 hr at 25 °C.

Day 2

After 24 hr incubation, 1 mL of test extract or reference drug (colchicine) is added to the dishes. For control dishes, 1 mL of distilled water is added. For each concentration, 3 replicates were used.

Day 3

Results are read out by determining the length of the seed rootlet in mm.

The percentage inhibition (%) of seed germination is calculated according to the formula:

$$\% \text{ inhibition} = (LT - LC) / LT \times 100 \quad (\text{Eqn. 1})$$

Where, LT: length of control rootlets (mm) and LC: length of treated rootlets (mm)

Brine shrimp lethality test

The *in vitro* cytotoxicity of the various extracts obtained from the plant's leaves was assessed by the brine shrimp lethality test on *A. salina* larvae (27). *A. salina* eggs were hatched in a stirred crystallizer containing 500 mL of salt water with a concentration of around 33 g/L, for 48 to 72 hr at 25 °C, with lighting and continuous aeration to promote homogenization of the hatching medium.

The principle consists in bringing the extract to be tested into contact with nauplii. A series of solutions of each extract was prepared at variable and progressive concentrations; then 1 mL of extract solutions at different concentrations was added to tubes containing 4 mL of saline water so that each tube had a final volume of 5 mL (4 mL of saline solution and 1 mL of extract), then 10 larvae were placed in each tube. As a negative control, a series of tubes was prepared containing the dimethyl sulfoxide at 1% (DSMO, 1 %) used for extract solubilization. The test was performed in triplicate.

Tubes were examined after 24 hr and the number of survivors in each was counted and recorded. If the larvae showed no movement during the 10 sec of observation, they were considered dead. The following equation is used to calculate the percentage mortality at each concentration:

Mortality rate (%) =

Number of dead larvae/initial number of larvae x 100

(Eqn. 2)

Acute toxicity

The toxicity study was carried out in accordance with Organisation for Economic Co-operation and Development guidelines No. 423 (OECD) (28). To perform this test, 24 adult Swiss albino mice (12 non-pregnant females and 12 males) weighing between 25 and 35 g were selected for toxicity evaluation of the three extracts such as decocted, methanolic extract and ethyl acetate extract, which proved the most active in the two tests previously described (phytotest *L. sativum* and the brine shrimp lethality). Previous work from our laboratory, provides a detailed description of the procedures used to perform this test (21, 25). Selected mice were marked and kept in cages with three mice per cage to acclimatize them to laboratory conditions for at least five days prior to the experiment. Mice were fasted 4 hr before the experiment, with food but no water eliminated. In the first stage, the mice received the extract by gavage, using a gastric tube, a dose of 2000 mg/kg and a volume of 0.5 mL per 20 g of mouse body weight. In this step, each extract is tested by three mice, while the three control mice were treated with distilled water. One to two hr after administration of the extracts and distilled water, the mice recovered their food. For the first four hr after gavage, each mouse was carefully observed, paying particular attention to how each mouse behaved after extract administration and of course whether any mice died. If there is no mortality, the second step is a repeat of the first. Following the recommendations of guideline 423, we reduce the dose to 300 mg/kg if two or more mice die. After the experiment, the body weight of the mice was monitored daily for 14 days with permanent access to food and water and under lighting conditions of 12 hr light and 12 hr dark. According to the globally harmonized system of classification (GHS), we can estimate the LD₅₀ of the plant studied, as well as the category of the three plant extracts tested, based on the doses administered and the number of mouse deaths.

Statistical analysis

Test results are represented by the mean ± standard error (SEM). Statistical analysis is based on the ANOVA (analysis of variance) variance test, followed by Tukey's test, performed with GraphPad Prism 5 software. The difference is considered statistically significant with a $p < 0.05$. Microsoft Office Excel was used to calculate the LC₅₀ of the extracts tested. Dose-response data were log-transformed and the LC₅₀ were determined by linear regression.

Principal component analysis (PCA) was performed using Addinsoft XLSTAT version 14 software to determine the correlation between phenolic compound contents in aqueous and organic extracts (total polyphenols, total flavonoids and catechic tannins) determined in our previous work (18) with the results of the *L. sativum* phytotest and the cytotoxicity study using the brine shrimp lethality bioassay.

Results

Phytotest *L. sativum*

The aqueous and organic extracts of *R. alaternus* leaves have a dose-dependent effect on cell growth (Fig. 1 & 2). The decocted extract shows the highest percentage of inhibition among the aqueous extracts with 96.45 %, followed by the infused and macerated extracts with 94.72 % and 91.81 %, respectively (Fig. 1). Comparative statistical evaluation revealed that the differences between these three aqueous extracts were statistically significant. All organic extracts show a significant antimitotic effect on *L. sativum* seeds (Fig. 2). Thus, the methanolic extract showed the strongest antimitotic power with a percentage inhibition of 96.30 %, followed by the methanolic macerated extract with a percentage inhibition of 95.17 %, while the two extracts ethyl acetate and chloroform ranked third and fourth with inhibition percentages of 94.64 % and 94.32 %, respectively. Petroleum ether extract had the lowest activity (91.88 %). Statistical treatment of the inhibition percentages of the organic extracts showed that only the difference between the two extracts such as chloroform and ethyl acetate was statistically insignificant, although the inhibition percentages of the five extracts varied between 92.92 and 96.30 %. Colchicine exhibited the highest inhibition (92.92 %) at 5000 µg/mL, significantly higher than all plant extracts tested (Fig. 3).

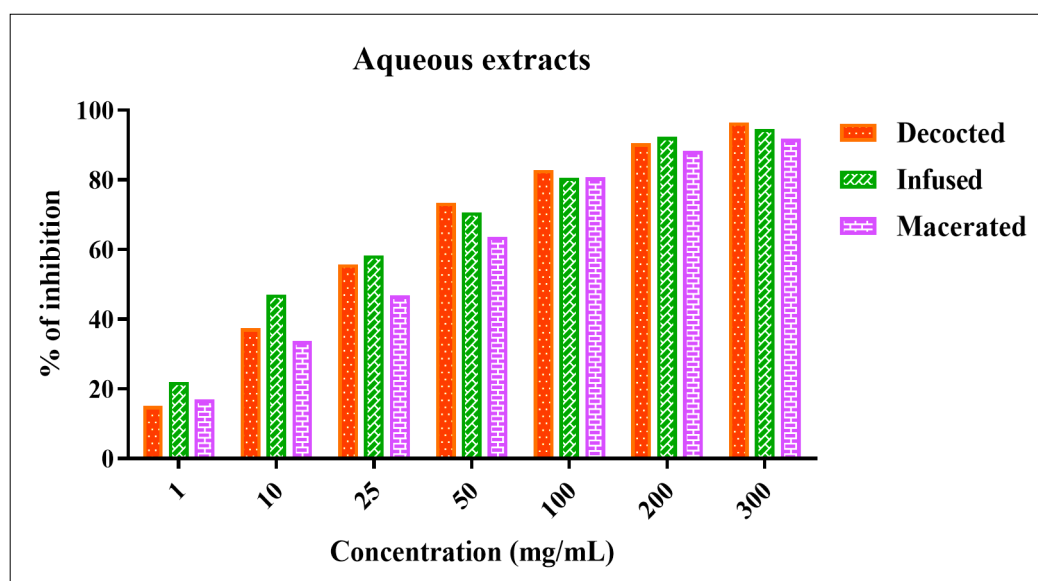


Fig. 1. Percentage inhibition of cell growth by aqueous extracts of *R. alaternus* leaves.

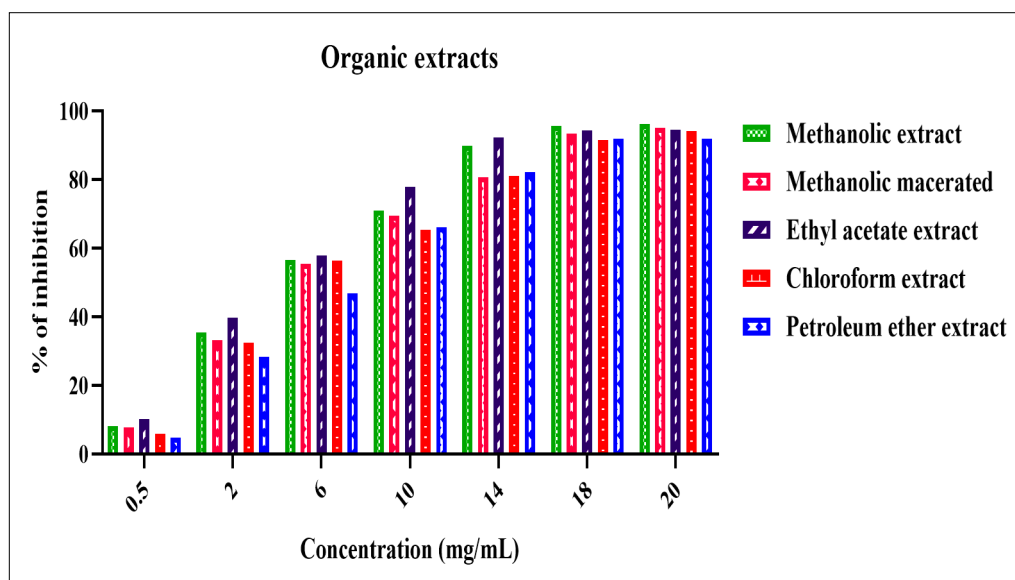


Fig. 2. Percentage inhibition of cell growth by organic extracts of *R. alaternus* leaves.

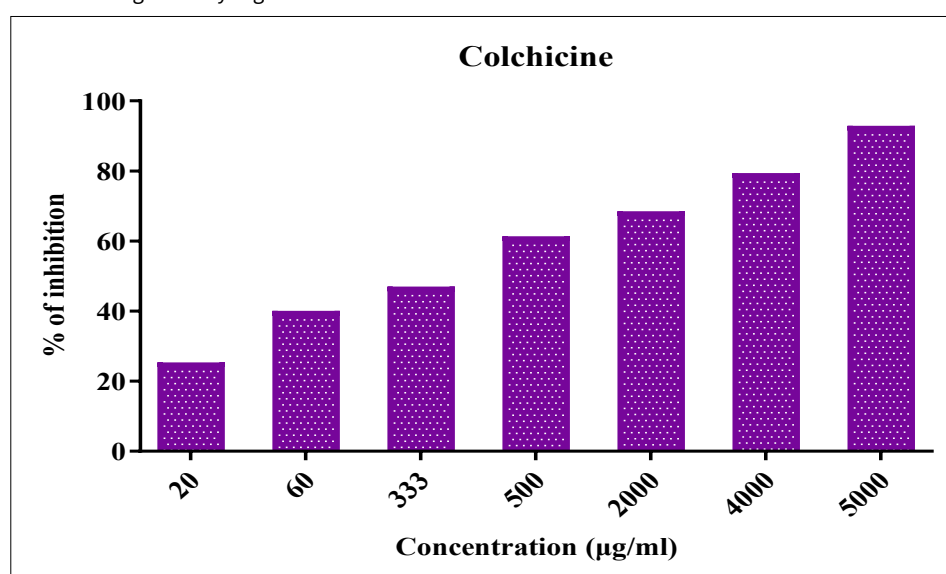


Fig. 3. Percentage inhibition of cell growth by colchicine.

The results of the inhibitory activity of the plant extracts are presented as median inhibitory concentration (IC_{50}) values (Table 1). The IC_{50} of the extract indicates the concentration at which it can stop or inhibit 50 % of *L. sativum* rootlet development. The lowest IC_{50} correlates with the highest inhibition.

Decocted extract showed the most potent activity among aqueous extracts ($IC_{50} = 25.403 \pm 0.153$ mg/mL), followed by infused (26.730 ± 0.100 mg/mL) and aqueous macerated (39.853 ± 0.053 mg/mL), with a significant difference between these three extracts. For IC_{50} analysis of organic extracts, ethyl acetate extract was the most potent with the lowest IC_{50} value (6.050 ± 0.037 mg/mL). The chloroform and methanolic extracts came second and third with IC_{50} values of 8.563 ± 0.108 and 8.596 ± 0.080 mg/mL respectively and there was a statistically non-significant difference between these two extracts. The methanolic macerated extract occupies fourth position with an IC_{50} value equal to 9.126 ± 0.153 mg/mL and a significant difference with all extracts. Petroleum ether extract, which has the lowest antimitotic activity of all organic extracts with an IC_{50} of 12.857 ± 0.098 mg/mL. This value is significantly different from the other extracts. Furthermore, colchicine had a high inhibitory effect compared with the various aqueous and organic extracts of the plant, as indicated by the lowest IC_{50} (0.474 ± 0.001 mg/mL), which was statistically different of all the extracts tested (Table 1).

Table 1. Median inhibitory concentration (IC_{50}) for cell growth of aqueous and organic extracts of *R. alaternus* leaves.

Extracts	IC_{50} (mg/mL)
Aqueous	
Decocted	25.403 ± 0.153^a
Infused	26.730 ± 0.100^b
Macerated	39.853 ± 0.053^c
Organics	
Methanolic extract	8.596 ± 0.080^d
Methanolic macerated	9.126 ± 0.153^e
Ethyl acetate extract	6.050 ± 0.037^f
Chloroform extract	8.563 ± 0.108^d
Petroleum ether extract	12.857 ± 0.098^j
Standard	
Colchicine	0.474 ± 0.001^h

Values are mean $\pm$ SE (n = 3). Different letters in a column indicate significant differences ($p < 0.05$).

Brine shrimp lethality test

The lethality test on *A. salina* is a simple, rapid, inexpensive, practical and generally easy-to-perform bioassay for demonstrating the general cytotoxicity of a natural substance. The percentage mortality as a function of concentration, show that larval mortality follows a dose-response relationship, since the percentage of larval mortality is proportional to concentration (Fig. 4 & 5). The decocted extract exhibited the highest larval mortality (96.67 %), followed by the infused and macerated extracts with a mortality rate of 90 % (Fig. 4). From the results of the organic extracts, the ethyl acetate extract showed the highest percentage mortality of 100 %, followed by the methanolic extract and methanolic macerated with a percentage of 93.33 %, while the two chloroform and petroleum ether extracts induced a mortality of around 90 and 83.33 %, respectively (Fig. 5). For the negative control group (DMSO), no mortality was observed.

The organic extracts have a significant cytotoxic potency compared with aqueous extracts. Decocted extracts showed an LC_{50} of 7.420 ± 0.135 mg/mL, statistically significant to the other extracts. Aqueous macerated showed an LC_{50} of 9.510 ± 0.243 mg/mL, while infused revealed an LC_{50} equal to 9.848 ± 0.074 mg/mL, the latter two aqueous extracts not differing statistically. Among the organic extracts, ethyl acetate extract had the lowest LC_{50} (0.355 ± 0.004 mg/mL), followed by the two methanolic and methanolic macerated extracts with LC_{50} values of 0.516 ± 0.038 and 0.532 ± 0.073 mg/mL, respectively and the two extracts chloroform and petroleum ether with LC_{50} values of the order of 0.753 ± 0.021 and 0.870 ± 0.046 mg/

mL, respectively. No statistically significant differences were observed among the five organic extracts (Table 2).

Table 2. Median lethal concentration (LC_{50}) of aqueous and organic extracts of *R. alaternus* leaves against *A. salina* larvae.

	Extracts	LC_{50} (mg/mL)
Aqueous extracts	Decocted	7.420 ± 0.135^a
	Infused	9.848 ± 0.074^b
	Macerated	9.510 ± 0.243^b
Organic extracts	Methanolic extract	0.516 ± 0.038^c
	Methanolic macerated	0.532 ± 0.073^c
	Ethyl acetate extract	0.355 ± 0.004^c
	Chloroform extract	0.753 ± 0.021^c
	Petroleum ether extract	0.870 ± 0.046^c

Values are mean $\pm$ SE ($n = 3$). Different letters in a column indicate significant differences ($p < 0.05$).

Principal component analysis (PCA)

PCA analysis was used to describe the relationships existing within the data. In our study, we tried to highlight the coexisting relationships between the chemical composition (total polyphenols, total flavonoids and catechic tannins) of the different aqueous and organic extracts of the leaves of the *R. alaternus* plant determined in our previous work as well as the correlation with the antimitotic and cytotoxic activities of the different extracts presented in the present study (18).

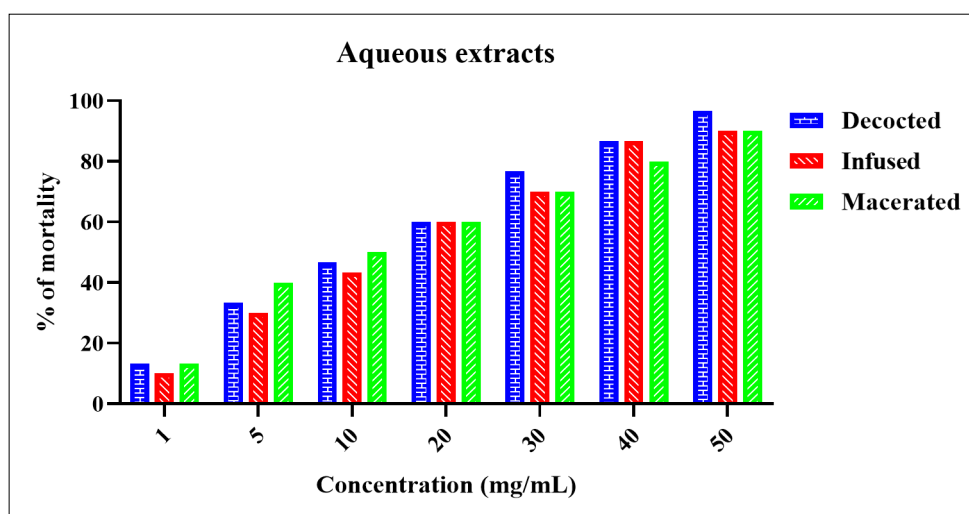


Fig. 4. Percentage of mortality observed after treatment of nauplii with aqueous extracts of *R. alaternus* at different concentrations.

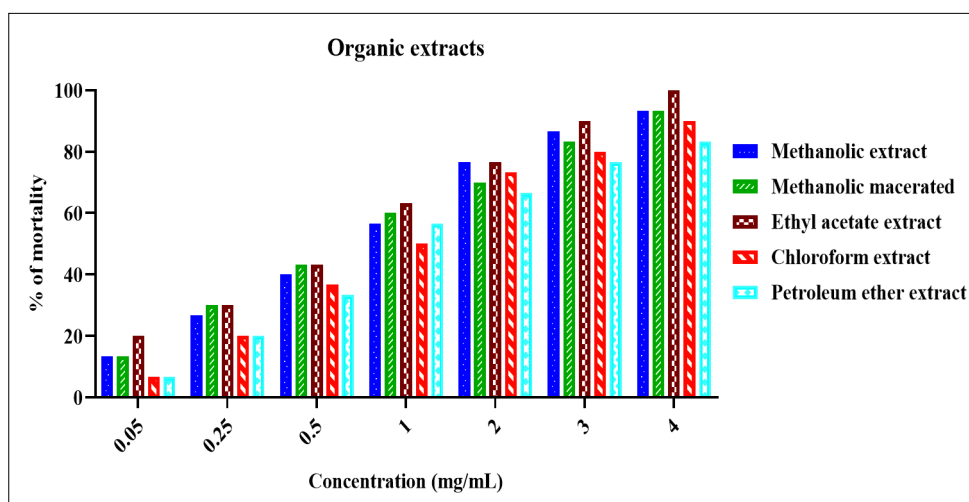


Fig. 5. Percentage of mortality observed after treatment of nauplii with organic extracts of *R. alaternus* at different concentrations.

Correlation matrix

The correlation matrix showed the correlation coefficients, which measure the degree of linear relationship between each pair of variables. Table 3 shows the results of calculating the correlation matrix between the chemical composition (total polyphenols, total flavonoids and catechic tannins) and the antimitotic and cytotoxic activity of aqueous and organic extracts of *R. alaternus* leaves.

PCA revealed 3 groups of extracts, differing in chemical compound concentration and antimitotic and cytotoxic activity. The PCA results showed a highly significant interpretation, with the two axes F1 and F2 accounting for 96.05 % of the total variance of the observations (Fig. 6).

Group 1

It includes decocted, infused and aqueous macerated extracts, which have low levels of total polyphenols, total flavonoids and catechic tannins, as well as minimal antimitotic activity and cytotoxic effect.

Group 2

It includes methanolic, methanolic macerated and petroleum ether extracts, which are rich in total polyphenols. These extracts showed both potent antimitotic and cytotoxic activities.

Group 3

It includes chloroform extract and ethyl acetate extract. According to this analysis, these two extracts are characterized by high concentrations of total flavonoids and catechic tannins with strong antimitotic and cytotoxic activity.

Acute toxicity

The results of 14-day body weight monitoring of the groups of mice treated with the three extracts and the group of mice treated with distilled water are shown in Fig. 7. The treatments of mice with aqueous, decocted and two organic extracts, such as methanolic extract and ethyl acetate extract, caused no mortality in mice in any stage. On the first day of treatment, mainly the first four hours, we observed clinical signs such as increased respiration, increased heart rate and lack of appetite. These signs were maintained throughout the follow-up period, particularly during the first week and a marked reduction in mouse movement was observed during the first day of treatment. Notably, mice treated with ethyl acetate extract exhibited prolonged reduced mobility that extended into the second day.

Body weight monitoring of the mice showed a general weight loss for both the decocted- and ethyl acetate extract-treated groups compared with the control group. The methanolic extract-treated group did show some weight gain, but this was small compared with the distilled water-treated group (Fig. 7).

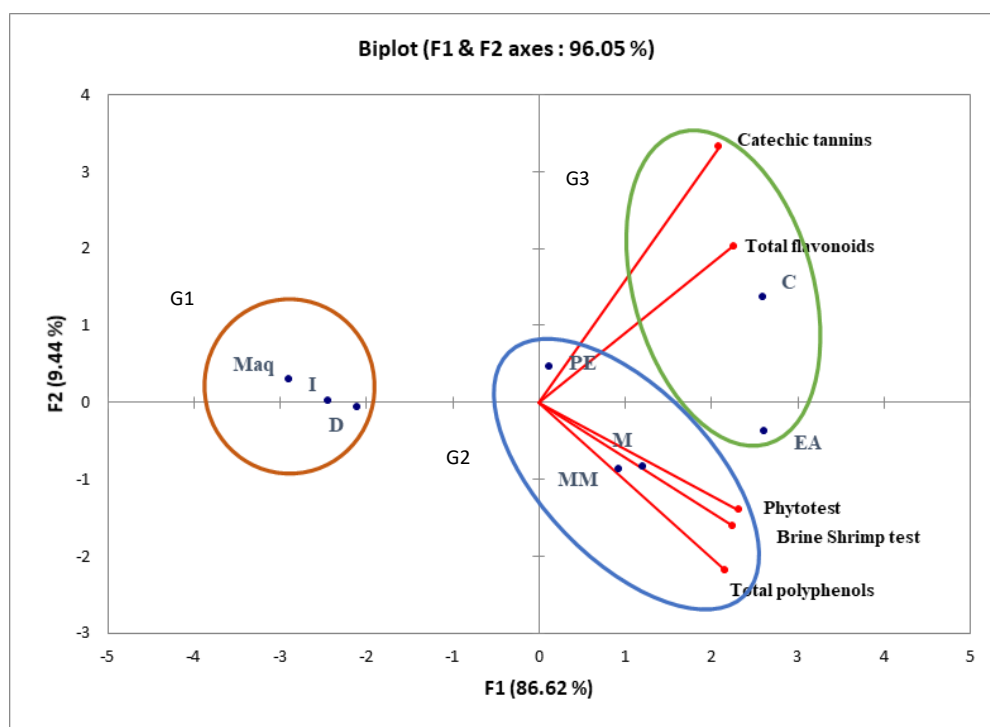


Fig. 6. Principal component analysis (PCA) of the chemical composition and antimitotic and cytotoxic activities of aqueous and organic extracts of *R. alaternus* leaves. **D:** decocted; **I:** infused; **Maq:** aqueous macerated; **M:** methanolic extract; **MM:** methanolic macerated; **EA:** ethyl acetate extract; **C:** chloroform extract; **PE:** petroleum ether extract; **G1:** group 1; **G2:** group 2; **G3:** group 3.

Table 3. Correlation matrix between chemical composition (total polyphenols, total flavonoids and catechic tannins) and the antimitotic and cytotoxic activity of aqueous and organic extracts of *R. alaternus* leaves.

Variables	Total polyphenols	Total flavonoids	Catechic tannins	Phytotest (IC ₅₀)	Brine shrimp test (LC ₅₀)
Total polyphenols	1				
Total flavonoids	0.753	1			
Catechic tannins	0.684	0.948	1		
Phytotest (IC ₅₀)	0.918	0.877	0.757	1	
Brine Shrimp test (LC ₅₀)	0.854	0.846	0.706	0.971	1

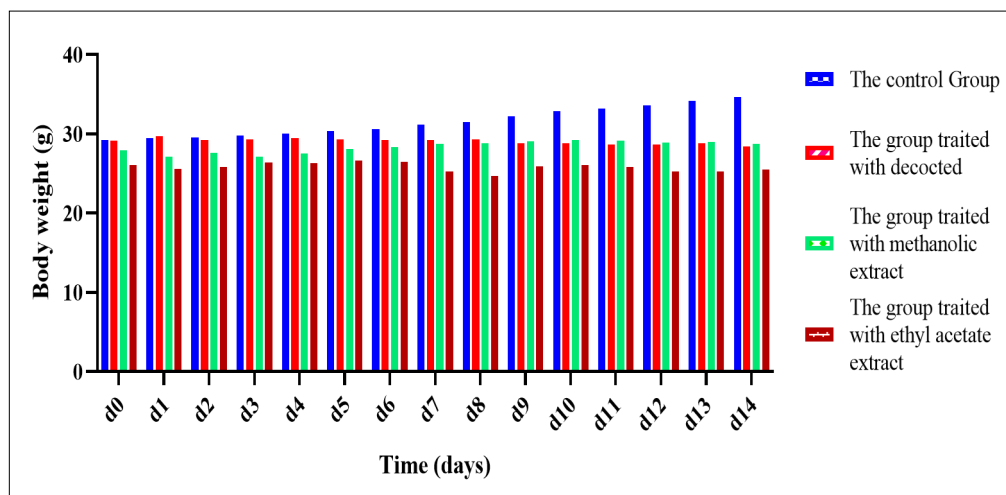


Fig. 7. Body weight trends over 14 days in the control group and groups treated with decocted, methanolic and ethyl acetate extracts of *R. alaternus* leaves.

Discussion

Phytotest *L. sativum*

Investigation of the antimutagenic activity of aqueous and organic extracts of *R. alaternus* leaves revealed that the decocted among the aqueous extracts and the two organic extracts of ethyl acetate and methanolic possess the most potent antimutagenic power among the organic extracts studied, in addition to the chloroform extract which showed a non-significant difference with the ethyl acetate extract. Furthermore, comparison of the antimutagenic activity results of aqueous and organic extracts showed that organic extracts have a significant cell growth inhibitory activity compared to aqueous extracts, this variation may be related to the difference in extraction solvent, extraction modality, polarity and temperature (25).

The germination phase is marked by an increase in rootlet length, leading to a significant increase in the number of cells resulting from mitoses, which are more active during this period, as in the case of seeds treated with distilled water. Conversely, the inhibition of rootlet development in seeds treated with colchicine and high concentration extracts is an indication of the cell division inhibitory activity of the various plant extracts. The cytotoxic or antimutagenic effect of plant extracts may be linked to the presence of certain chemical compounds, notably flavonoids, polyphenols and catechic tannins, which are abundant in the leaves of this plant, as confirmed by our previous study (18). Indeed, alkaloids and their derivatives are the natural compounds best known for their cytotoxic power and their use to treat cancer (29). However, the leaves of the plant in our study are devoid of alkaloids despite their remarkable antimutagenic potential, leading us to deduce that other compounds may exert their power as anticancer agents. Indeed, numerous studies have confirmed that phenolic compounds and flavonoids have effective antimutagenic activity through their ability to prevent and block cell division (30, 31).

To assess the cytotoxic activity of the *R. alaternus* plant, a study was carried out on human monocytic leukemia cells (U937) and the results showed that the methanolic extract exerted excellent cytotoxicity on the U937 line (17). According to this research, the plant's cytotoxic activity could be attributed to the glycosides of the two flavonoid types kaempferol and rhamnocitrin. Similarly, in a study carried out on *R. alaternus* roots and leaves to assess the antiproliferative activity of extracts on K562 human leukemia cells, this research demonstrated that K562 cells responded to flavonoid-

rich extracts of *R. alaternus* roots and leaves, with IC_{50} values of 165 and 210.73 $\mu\text{g/mL}$, respectively (16).

A series of studies carried out under the same experimental conditions as the present study confirm the results obtained and demonstrate the antimutagenic impact of plant extracts (21, 24, 25). Most phytotoxicity studies show a toxic effect on cell growth. A study conducted on *Haloxylon scoparium* revealed a greater antimutagenic effect of decocted with an IC_{50} value of around $1359.00 \pm 106.69 \mu\text{g/mL}$. This effect was also observed for the three organic extracts such as methanolic extract, methanolic macerated and ethyl acetate extracts, with IC_{50} values of 128.16 ± 3.89 , 164.50 ± 8.88 and $752.20 \pm 25.58 \mu\text{g/mL}$ respectively, which are not statistically different from the reference drug colchicine ($474.66 \pm 1.86 \mu\text{g/mL}$) (25). Another study, on *Chamaerops humilis* possesses the ability to inhibit cell growth in a dose-dependent manner and among the aqueous extracts, the decocted has the best antimutagenic potency expressed by an IC_{50} equal to $9.624 \times 10^3 \pm 95.97 \mu\text{g/mL}$ (21). For organic extracts, the two extracts, ethanolic macerated and ethanolic extract, have the most potent inhibitory activity expressed by IC_{50} values of $5.599 \times 10^3 \pm 45.51$ and $5.638 \times 10^3 \pm 22.61 \mu\text{g/mL}$, respectively. In the same context, a study on the plant *Ajuga iva* revealed strong antimutagenic potency in all extracts tested, particularly organic extracts with IC_{50} of 320.43 ± 8.96 , 375.77 ± 17.53 , 493.03 ± 5.08 , 427.10 ± 4.31 and $581.23 \pm 50.41 \mu\text{g/mL}$, respectively for methanolic macerated, methanolic, chloroform, ethyl acetate and petroleum ether extracts (24).

The results of another study evaluating antimutagenic activity using the *Allium cepa* test, by exposing meristematic cells of onion roots to methanolic extracts of three plants, showed a significant reduction in mitotic indices compared with the control, which were 60.78 ± 1.25 , 43.96 ± 1.87 and $51.89 \pm 1.22 \%$, respectively for *Mucuna pruriens*, *Asteracantha longifolia* and *Sphaeranthus indicus* (32). Using the same test (*A. cepa*), the results of the antimutagenic activity of the methanolic extract of *Peganum harmala* leaves revealed a potent antimutagenic potency of this extract which decreases with increasing concentration and is $35.93 \pm 0.62 \%$ at a concentration of 1 mg/mL and $1.25 \pm 0.20 \%$ at a dose of 16 mg/mL (33).

Based on a study of the germination rate of *Sorghum* sp. seeds, a study carried out on 41 plant species of the Euphorbiaceae family assessed the antimutagenic activity of these species. The results of this research showed that aqueous extracts and methanolic extracts of these species exhibited potent antimutagenic power on

Sorghum sp seed germination with a mitosis index below 1 and with a distinction of aqueous extracts that strongly inhibited seed germination (34).

Brine shrimp lethality test

Cytotoxicity on *A. salina* larvae is considered a good indicator for preliminary evaluation of the cytotoxicity of plant extracts (27). According to our research, the decocted extract had the highest cytotoxic effect (7.420 ± 0.135 mg/mL) compared with the infused and macerated extracts, while the extract obtained with ethyl acetate showed the most potent cytotoxic power (0.355 ± 0.004 mg/mL) compared with the other organic extracts.

Phytochemical screening in our previous research showed the absence of a group of secondary metabolites known for their cytotoxic power, alkaloids, which are generally toxic compounds, suggesting the possibility of the presence of other compounds in the leaves of this plant which are at the origin of this activity, such as tannins and flavonoids. The results of the previously described study recorded the richness of the aqueous extract such as decocted by flavonoids and tannins with contents of 58.40 ± 0.33 µg GAE/mg E and 5.43 ± 0.01 µg CE/mg E, respectively. Whereas for organic extracts, the chloroform extract contained the highest concentrations with 470.79 ± 1.70 µg GAE/mg E and 51.75 ± 0.14 µg CE/mg E, respectively for flavonoids and tannins (18). In addition, the way in which solvent polarity affects the quantity and quality of extracts in secondary metabolites and consequently their biological activities have been demonstrated. This may help explain why the majority of chemicals from the same family are found in various extracts, yet cytotoxic potency varies between extracts (35).

Brine shrimp lethality test is a bioassay considered as a preliminary screening of phytochemical compounds present in plants and having cytotoxic properties (36). Furthermore, it was found that cytotoxic compounds generally have a remarkable effect in the Brine shrimp assay, so this assay could serve as a guide for the detection of antitumor and pesticidal compounds due to its ease of use and low cost (37).

According to our results, organic extracts of the plant have a maximum cytotoxic effect compared with aqueous extracts, since the LC_{50} values for organic extracts range from 0.355 to 0.870 mg/mL, whereas these values for aqueous extracts are in the range 7.420 to 9.848 mg/mL. A study conducted on a species of the same family (*R. prinoides*) showed that the aqueous extract of this plant using the same assay presented an LC_{50} of 6921.05 µg/mL, the hydromethanolic extract presented an LC_{50} of 214.33 µg/mL, while the chloroform extract presented the maximum cytotoxic effect with an LC_{50} of 133.33 µg/mL (38). According to this study, not all extracts with $LC_{50} \geq 1000$ µg/mL have cytotoxic potency, whereas extracts with $LC_{50} < 1000$ may be a source of future anticancer and cytotoxic drugs and thus require further study (27, 38).

Principal component analysis (PCA)

The PCA results showed a higher level of correlation between the phenolic compound content of extracts and their antimitotic and cytotoxic activity. Indeed, analysis of the correlation coefficients shows that antimitotic activity depends firstly on total polyphenols ($r = 0.918$), which show a highly positive correlation, then on total flavonoids ($r = 0.877$) and tannins ($r = 0.757$). The inhibitory activity of extracts on cell growth could be explained by variation in the composition of chemical families, this is in agreement with a study carried out on *Helichrysum gymnocephalum* essential oil which

showed a significant correlation of anticancer activity against MCF-7 cells and between the contents of three compounds aromatendrene, α -terpinolene and β -selinene with correlation coefficients of 0.90, 0.88 and 0.76, respectively (39). Total flavonoids and total polyphenols present in the *Haloxylon scoparium* plant show a highly significant correlation with antimitotic activity using the *L. sativum* phytotest, with correlation coefficients of 0.9884 and 0.9453, respectively, but this activity is not linked to the presence of catechins tannins, since there is a very weak correlation ($r = 0.0112$) between tannin content and the plant's antimitotic activity (25). The results of the analysis of the correlation of phenolic compounds with the antimitotic activity of aqueous and organic extracts of *Chamaerops humilis* leaves showed a strong correlation of catechins tannins, total flavonoids and total polyphenols with correlation coefficients of 0.9370, 0.9153 and 0.7612, respectively (21). Indeed, the results of PCA analysis of cytotoxic activity using the brine shrimp lethality test and phenolic compounds showed that there was a highly significantly positive correlation with correlation coefficients of 0.854, 0.846 and 0.706, respectively for total polyphenols, total flavonoids and catechins tannins. A strong association between cytotoxicity of MCF-7 cells and flavonoid concentration and a weak correlation between flavonoid content and cytotoxicity towards Hela, SKOV3 cancer lines (40).

The correlation coefficient between brine shrimp lethality test and *L. sativum* phytotest is 0.971, indicating that the two tests are highly correlated or concordant and also reinforcing the validity of the results obtained from the plant studied as a species with cytotoxic properties. The use of PCA to illustrate the correlation between the results of brine shrimp lethality test and other cytotoxicity tests is limited in the literature. A significant correlation between brine shrimp lethality test and the MTT cytotoxicity test on L929 cells, with a correlation coefficient of 0.72 between the LC_{50} and IC_{50} results obtained by the two tests (41). According to this study, brine shrimp test may be a reliable alternative for studying cytotoxicity (41).

Acute toxicity

The results of the acute toxicity study on *R. alaternus* leaves were characterized by the absence of mortality in all three groups of mice treated with decocted, methanolic extract and ethyl acetate extract. Based on these results and according to OECD guideline No. 423, the lethal dose (LD_{50}) is estimated to be greater than or equal to 5000 mg/kg. All three extracts are classified as category 5 or as unclassified under the GHS (28).

Clinical signs related to body weight revealed slight changes, notably a loss of weight in mice treated with decocted and ethyl acetate extracts, which may be due to the lack of appetite observed during the follow-up period and by disruption in carbohydrate, protein or fat metabolism (42, 43). However, the increase in body weight of mice treated with methanolic extract can be attributed to plant nutrients extracted by methanol (44).

Similarly, the acute toxicity study of the decocted extract of this plant on Wistar rats allowed the determination of the LD_{50} which is 5000 mg/kg (5 g/kg) which is in agreement with our results (45). The methanolic extract of the plant's leaves caused no mortality in mice treated with the extract at the four concentrations such as 150, 300, 450 and 600 mg/kg (46). Thus, the lethal dose was estimated to be 680.79 mg/kg.

Following the same approach and under the same experimental conditions, treatment of mice with the aqueous macerated and methanolic macerated of the plant *Atractylis gummifera* induced no signs of toxicity in the mice (47). Similarly, treatment of mice with the decocted and ethanolic extract of *Chamaerops humilis* leaves did not cause mortality in treated mice; however, a lack of appetite was observed during the first day of treatment with reduced movement for 72 hr in mice treated with the ethanolic extract (21).

Conclusion

The present study involved the evaluation of the antimitotic and cytotoxic potencies of aqueous and organic extracts of *R. alaternus* leaves, in addition to the *in vivo* assessment of the acute toxicity of the most *in vitro* active extracts. The cell growth inhibition test using the *L. sativum* phytotest revealed that the aqueous extract (decocted) and the two organic extracts, methanolic and ethyl acetate, were the most potent antimitotic agents. In the cytotoxicity test on *A. salina* larvae, the cytotoxic potency varied according to extract, with decocted and ethyl acetate extracts being the most cytotoxic. For acute toxicity, administration of a single dose of 2000 mg/kg of the three extracts such as decocted, methanolic extract and ethyl acetate extract produced no signs of toxicity in mice. The lethal dose was estimated to be ≥ 5000 mg/kg. According to the OECD, the three extracts tested are class V or unclassified under the globally harmonized system of classification (GHS). Thus, we can conclude that *R. alaternus* is a promising candidate for anticancer research for its antimitotic and cytotoxic effects, in addition to its safe therapeutic use. Further studies are needed to extract and identify the bioactive molecules responsible for this activity. In addition, further research is needed to confirm its long-term safety.

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Authors' contributions

HB performed the experimental study, statistical analysis of the data and preparation of the manuscript. FL ensured the scientific follow-up of the experimental study, guided the preparation of the manuscript, revised and corrected the final version, then submitted it for publication. HT provided consistent advice, preparation and revision of the manuscript. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: The methods used to conduct the *in vivo* experiment comply with International guidelines for the use and treatment of laboratory animals (OECD Guideline No. 423). In addition, the authors have taken significant steps to mitigate animal suffering and

limit the number of animals used.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used DeepL & Google Translate in order to translate. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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