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EXPERIMENTAL PAPER

Phytochemical composition, antioxidant, cytotoxic, haemolytic and antibacterial activities of the aerial parts of *Rhamnus alaternus*

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Summary

Introduction: *Rhamnus alaternus* L. belongs to the genus *Rhamnus* (*Rhamnaceae*). It is a medicinal plant growing in Algeria. This plant is used worldwide as effective pharmacological remedy.

Objective: This study describes in detail the phytochemical composition and the *in vitro* biological activities of *R. alaternus* L. aerial parts extracts.

Methods: Total contents of bioactive components were estimated and the antioxidant potential was assessed by DPPH and hydroxyl radical scavenging assays, ferric reducing antioxidant activity and total antioxidant capacity. Additionally, the haemolytic activity was evaluated towards human red blood cells and the cytotoxicity was performed using Brine shrimp lethality test. Furthermore, the antibacterial activity was estimated *in vitro* against five bacteria. Seven known secondary metabolites have been established using different spectroscopic techniques such as 1D- and 2D-NMR experiments and ESI mass spectrometry and by comparison to data reported in the literature.

Results: The chemical investigation of the aerial portions' ethyl acetate extract of *R. alaternus* allowed the characterization of three triterpenoids, two phytosterols and two flavonoids. High levels of phenols and flavonoids were estimated in both ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) extracts. These extracts exhibit strong ferric reducing antioxidant activity and significant hydroxyl and DPPH radicals scavenging

effects. Moreover, the petroleum ether (PE) extract shows a very strong haemolytic effect with 94.03 ± 0.21% at 50 μg/mL and induces the mortality of 78.57 ± 0.17% of Brine Shrimp nauplii at 20 μg/mL. Furthermore, the tested crude extracts (PE, EtOAc and *n*-BuOH) inhibit the growth of all the bacterial strains.

Conclusion: *R. alaternus* constitutes a natural resource of bioactive substances with potential cytotoxic, antioxidant and antibacterial effects.

Keywords: ***Rhamnus alaternus, secondary metabolites, NMR, biological activity***

Słowa kluczowe: ***Rhamnus alaternus, metabolity wtórne, NMR, aktywność biologiczna***

INTRODUCTION

The genus *Rhamnus* belonging to the *Rhamnaceae* family includes about 900 plants, from which 5 species were found in Algeria [1]. Previous chemical studies performed on *Rhamnus* plants revealed a variety of secondary metabolites including steroids-triterpenes [2,3], flavonoids [4,5], anthraquinones [6,7], alkaloids [8,9], sugars [2] and catechin tannins [2].

R. alaternus is an evergreen sub-shrub very ramified and creeping which rises up only barely from the soil 30-50 cm. This species is distributed in the Mediterranean region, particularly in North Africa, the Middle East and the Southern Europe. In Algeria, *R. alaternus* grows in the mountains of Aures region [1].

The species *R. alaternus* is frequently employed in folk medicine as laxative, purgative and hypotensive agent and it is used in the treatment of diverse diseases like gastric and hepatic affections [10-12]. Furthermore, it was previously mentioned that *R. alaternus* possesses a wide spectrum of interesting *in vitro* and *in vivo* biological activities including antimutagenic and antigenotoxic [13], antimicrobial [14,15], antiproliferative and cytotoxic [15,16], antioxidant [7,16], antihyperlipidemic [17], renal and hepatoprotective effects [18,19].

The present work describes the chemical composition and the *in vitro* biological evaluation of various extracts derived from the aerial parts of *R. alaternus*. To the best of our knowledge, this investigation reports the PE, EtOAc and *n*-BuOH extracts' cytotoxicity of larvae for the first time.

MATERIAL AND METHODS

Apparatus

Isolated compounds were characterized by ESI-MS in positive and negative modes (ion trap Bruker Esquire, Waters, Milford, MA, USA) and 1D- (^{1}H and ^{13}C) and 2D-NMR spectral data (COSY, HSQC, HMBC, Bruker Avance Spectrometer, ^{1}H 500 MHz, ^{13}C 125 MHz, Varian, Palo Alto, CA, USA) in $CDCl_3$ or CD_3OD. The signals of the deuterated solvents were taken as references. Column chromatography (CC) was performed on Kieselgel 60 (320-400 mesh), polyamide SC-6 and Sephadex LH-20. Analytical and preparative thin-layer chromatographies (TLCs) were carried out on precoated Kieselgel 60 F_{254} or RP-18 F_{254s} plates (Merck, Darmstadt, Germany). All spectrophotometric measurements were carried out using UV-Vis 7220G spectrophotometer (Rayleigh, BRAIC, China).

Chemicals

All solvents (high purity grade) used in the experiments: petroleum ether (PE), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), chloroform ($CHCl_3$), methanol (MeOH) and ethanol (EtOH)) were purchased from Prolab, Merck Eurolab, France. 2,2-diphenyl-1-picrylhydrazyl (DPPH), anhydrous sodium sulfate (Na_2SO_4), trichloroacetic acid (TCA), potassium ferricyanide ($K_3Fe[CN]_6$), ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, sulfuric acid (H_2SO_4), aluminum chloride ($AlCl_3$), sodium carbonate (Na_2CO_3), dimethyl sulfoxide (DMSO), ammonium molybdate $(NH_4)_2MoO_4$, ferrous sulfate ($FeSO_4$), hydrogen peroxide (H_2O_2), sodium salicylate ($C_7H_5NaO_3$), trisodium phosphate (Na_3PO_4) were purchased from Sigma-Aldrich Chemie and Fluka Chemie (Merck, Darmstadt, Germany). Standard (high quality) quercetin ($C_{15}H_{10}O_7$), butylated hydroxytoluene (BHT) ($C_{15}H_{24}O$), gallic acid ($C_7H_6O_5$), butylate hydroxyanisole (BHA) ($C_{11}H_{16}O_2$), ascorbic acid ($C_6H_8O_6$), tannic acid and α-tocopherol were purchased from Sigma-Aldrich Co., St. Lowis, Mo, USA.

Plant material

R. alaternus aerial parts (leaves, stems and fruits) were harvested in Seriana mountain of Batna-Aures

(Algeria) in May 2019. The plant was determined by Prof. Bachir Oudjehih, Agronomic Institute, University of Batna 1. A voucher specimen was kept in the herbarium (N°827/LCCE).

After harvest, the aerial parts of the plant were dried. Then, the plant material was spread on a paper in a single layer to avoid fermentation and put in a ventilated, shady, dry and warm place at a temperature lower than 30°C for ten days. The plant material was turned over every day for regular and complete drying. Once the aerial parts of the plant were completely dried, afine grinding, using a mechanical grinder was carried out. Then, the obtained powder was stored at ambient temperature in an opaque, clean and airtight container.

Micro-organisms

The strains of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Streptococus pneumoniae* ATCC 49619 and *Klebseilla pneumoniae* ATCC 70063 were obtained from Pasteur Institute (Algiers), Algeria.

Extraction and isolation

The dried powdered (1 kg) of *R. alaternus* aerial parts were exhaustedly extracted with EtOH–H_2O (70:30) (10 l, twice) for 3 days at a room temperature. After filtration and concentration of solvent, the obtained hydroethanolic extract was submitted to liquid-liquid extraction with PE, EtOAc and *n*-BuOH.

The EtOAc extract (7 g) was fractionated over vacuum liquid chromatography VLC on silica gel (SiO_2) eluted with hexane–EtOAc mixture (100:0–0:100) and EtOAc–MeOH (100:0–0:100) to obtain 14 fractions (F1-F14). Fraction F-3 of VLC (550 mg) was separated on silica gel CC using petroleum ether–EtOAc (99:1–0:100) to give 10 fractions (F-3-1 to F-3-10). Fraction F-3-3 was submitted to Sephadex LH-20 CC, using dichloromethane as eluent, to give compound **1** (3.1 mg). Fraction F-3-9 was further purified by washing with methanol to obtain compound **4** (10 mg). Fraction F-4 of VLC (454.2 mg) was fractionated on silica gel CC, employing hexane–EtOAc to get 19 fractions (F-4-1 to F-4-19). F-4-7 (55 mg) was further purified over Sephadex LH-20 CC eluted with dichloromethane–methanol (9:1) to obtain 4 subfractions. TLC RP-18 of sub-fraction 3 using MeOH (100%) allowed the isolation of compound **2** (3.2 mg). The fraction F-4-17 (12.3 mg) was precipitated in EtOAc to furnish compound **3** (3.8 mg). Fraction F-5 of VLC (104.3 mg) was applied to CC (SiO_2, $CHCl_3$–MeOH, 90:10 to20:80) to yield **5** (6.2 mg). Fraction F-8 of VLC (664 mg) was subjected to CC of polyamide SC-6, the elution was performed with toluene–methanol mixture to provide 12 fractions. Fraction F-8-5 was submitted to silica gel CC and eluted with $CHCl_3$–MeOH mixture (99:1–20:80) to yield two compounds **6** (3.4 mg) and **7** (3.7 mg).

Phytochemical screening

Using the techniques of Fransworth NR (1966) [20], the presence of many phytochemicals in *R. alaternus* extracts has been estimated.

Determination of total bioactive contents

The quantification of phenols and flavonoids was determined by Folin–Ciocalteu and trichloroaluminum methods, respectively [21]. For total phenolic content, 1 ml of the diluted Folin–Ciocalteu reagent (1:10) was mixed with 200 μl of the tested samples, and the obtained solutions were agitated and left for 4 min. Then, 800 μl of Na_2CO_3 solution (75 g/l) were included. The tubes were incubated for 2 hours at room temperature (25°C) and in obscurity. After incubation, the absorbances at 765 nm were recorded. A calibration curve of gallic acid was used to calculate the concentration of phenolic compounds. For the total amount of flavonoids, 500 μl of $AlCl_3$ solution (2%) were transferred to tubes containing 500 μl of the tested extract. The mixture was agitated and incubated at 25°C for 10 min. Then, the absorbances were reported at 430 nm. The concentration of flavonoids was estimated using quercetin calibration curve.

Antioxidant activity

DPPH and hydroxyl radical scavenging assays

Scavenging effects of the extracts obtained from *R. alaternus* were evaluated on DPPH free radical (2,2-diphenyl-1-picrylhydrazyl)[21]. To 100 μl of sample dilutions, 900 μl of DPPH solution (25 μg/ml) was added. The mixtures were agitated and incubated in obscurity for 30 min at 25°C. Then, the absorbances were recorded at 517 nm. The percentage of DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{(A_{\text{Control}} - A_{\text{Sample}})}{A_{\text{Control}}}\right] \times 100,$$

where A_{Control} is the absorbance of negative control and A_{Sample} is the absorbance of standards or sample.

In addition, the hydroxyl radical scavenging activity of *R. alaternus* was assessed [22]. To 1.5 ml of reaction mixture containing (500 µl of 1.5 mM $FeSO_4$, 700 µl of 6 mM hydrogen peroxide, 300 µl of 20 mM sodium salicylate), 500 µl of diluted samples at different concentrations were added. The obtained mixtures were agitated and incubated at 37°C for 1 hour, and the absorbances were measured at 562 nm. The hydroxyl radical scavenging activity was computed using the following formula:

$$OH^{\bullet} \text{ scavenging effect (\%)}: = [\frac{1-(A-A')}{A^*}] \times 100,$$

where A_* is absorbance of the control and A is the absorbance of the extract, A' is the absorbance without sodium salicylate.

Ferric reducing antioxidant activity (FRAP)

The ferric reducing capacity of *R. alaternus* extracts was tested. Firstly, 100 µl of the examined solutions were mixed with 500 µl of phosphate buffer (pH 6.6; 0.2 M) and 500 µl of ferrocyanidesolution (1%). The tubes were kept for 20 min at 50°C. Then, 500 µl of trichloroacetic acid solution (10%) were added, and the obtained mixtures were centrifuged (3000 rpm/10 min). 0.5 ml of the supernatant was diluted with 0.5 ml of double distilled water, then 100 µl of $FeCl_3$ (0.1%) were added. The absorbances of all the samples were read at 700 nm and the reducing potential was calculated using ascorbic acid calibration curve [23].

Total antioxidant capacity (TAC)

To 100 µl of *R. alaternus* extracts, 900 µl of phosphomolybdenum reagent containing: 0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM $(NH_4)_2MoO_4$ were added. The obtained mixtures were incubated in a water bath (95°C) for 90 min. After total cooling of the reactive mixture (30 min), the absorbance was recorded at 695 nm. The total antioxidant capacity was calculated according to the calibration curve prepared from ascorbic acid [23].

Antibacterial activity

The *in vitro* assessment of the antibacterial activity of *R. alaternus* extracts was conducted by agar disk diffusion method [24] against five bacterial strains: two Gram positive (*Staphylococcus aureus* ATCC 25923 and *Streptococus pneumoniae* ATCC 49619) and three Gram-negative (*Escherichiacoli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebseilla pneumoniae* ATCC 70063). The types of bacteria were isolated one or few settlements and were prepared by 9 ml of nutrient solution. The bacterial suspension is used turbidity standard tubes to match the 0.5 McFarland standard and were incubated at 37°C for 24 h by inoculation into Muller-Hinton agar. The extracts were dissolved in DMSO solutions in different concentrations. Sterile empty disks (6 mm in diameter) were soaked in 10 µl of the extracts. Muller-Hinton agar were poured in Petri dishes homogeneously and bacteria distribution in Petri dishes and were incubated at 37°C for 24 h. The assays were carried out three times and the results were based on diameter in mm of inhibition zone formed within the discs.

***In vitro* cytotoxic effect**

The cytotoxicity of *R. alaternus* extracts was tested according to brine shrimp lethality assay [25]. 50 mg of shrimp eggs were sprinkled into a shallow rectangular dish divided into two unequal compartments (the large compartment was darkened, and the small one was illuminated) and filled with a saline solution (38 g marine salts /1 l of distilled water). After two days, phototropic nauplii were collected. Each 10 nauplii were transferred into vial tubes containing 4.9 ml of sea water. 100 µl of each extract prepared at different concentrations (0.5, 1, 2, 4, 8 mg/ml) were added into the vial tubes containing the larvae. The vials were then kept under illumination for one day. After that the survived nauplii were counted, and the percentage of mortality of each concentration was computed.

Haemolytic activity

The haemolytic effect of *R. alaternus* extracts was tested *in vitro* [26]. Firstly, blood was collected from a healthy female in heparin tubes. The collected blood was centrifuged (1500 rpm/3 min) and the supernatant was discarded. The pellet was clarified three times using a sterile phosphate buffer saline solution (PBS) (pH=7.2) and then the redblood cells were resuspended in normal saline solution (5%). 500 µl of erythrocytes solution were added to 500 µl of the extracts prepared at different concentrations. The vial tubes were incubated at 37°C for 30 min and centrifuged (1500 rpm/5 min). The absorbances of the supernatants, phosphate buffer salineand distilled water used as controls were measured at 540 nm. The percentage of hemolysis was computed as follows:

$$\text{Hemolysis (\%)}: [\frac{(A_s-A_p)}{(A_d-A_p)}] \times 100,$$

where A_s is the absorbance of samples, A_p is the absorbance of PBS and A_d is the absorbance of distilled water.

The experimental protocol used to evaluate the haemolytic activity was conducted under the framework of best ethical practices, where, human Rights Related to Research Involving Human Subjects were respected and the volunteers signed the informed consent for this study. The experience was assessed after a prior approval from the local Ethics Committee of Microbiology and Biochemistry department of University of Batna-2, Algeria (approval n.30/DMB/FSNV/UB2/2020).

Statistical analysis

The results were given as values of means± standard deviation (SD) for three measurements for each sample. Significant differences between means were established by one-way ANOVA, and then the Graph Pad prisme 5.04 was used to carry out the Duncan test and all statistics.

RESULTS AND DISCUSSION

Phytochemical screening

The results of the qualitative analysis of the crude extracts prepared from *R. alaternus* were grouped in table 1.

According to the results (tab. 1), *R. alaternus* extracts are rich in secondary metabolites. Terpenoids, phenols, flavonoids and quinones were detected in all the tested extracts. Indeed, it is well reported that *Rhamnus* species (*R. alaternus*, *R. fallax*, *R. intermedia*, *R. pumila R. catharticus*, *R. orbiculatus*, *R. saxatilis*, *R. frangula* and *R. alpinus*) produce high amounts of flavonoids, anthraquinones and phenolic compounds with numerous biological and pharmacological activities [27, 28].

Chemical constituents of *R. alaternus*

Purification of *R. alaternus* EtOAc extract by repetitive chromatographic separations, such as silica gel, polyamide SC-6 and Sephadex LH-20 column chromatographies, precipitation, and preparative TLCs (Silica gel and RP-18), provided seven known compounds including three triterpenoids named lupeol (**1**) [29], oleanolic acid (**2**) [30], 3-acetoxyolean-12-en-28-oic acid (**3**) [29], two phytosterols; *β*-sitosterol (**4**) [30] and daucosterol (**5**) [31] as well as two flavonoids: apigenin (**6**) [32] and quercetin (**7**) [33]. The isolates have been characterized mainly by spectroscopic techniques, particularly 1D- and 2D-NMR experiments (^{1}H, ^{13}C, COSY, HSQC and HMBC) and mass spectrometry ESI-MS, and by comparison with the data reported in literature (fig. 1).

In present work, three isolated compounds namely lupeol (**1**), oleanolic acid (**2**) and 3

Table 1.

Phytochemical constituents of crude extracts from *R. alaternus*

Metabolites	Extracts		
	PE	EtOAc	*n*-BuOH
Tannins	-	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Anthocyanins	-	-	+
Alkaloids	+	-	+
Terpenoids	+	+	+
Steroids	-	+	-
Quinones	+	+	+
Lipids	-	-	-
Glycosides	+	-	-

(+) presence of phytochemical, (–) absence of phytochemical

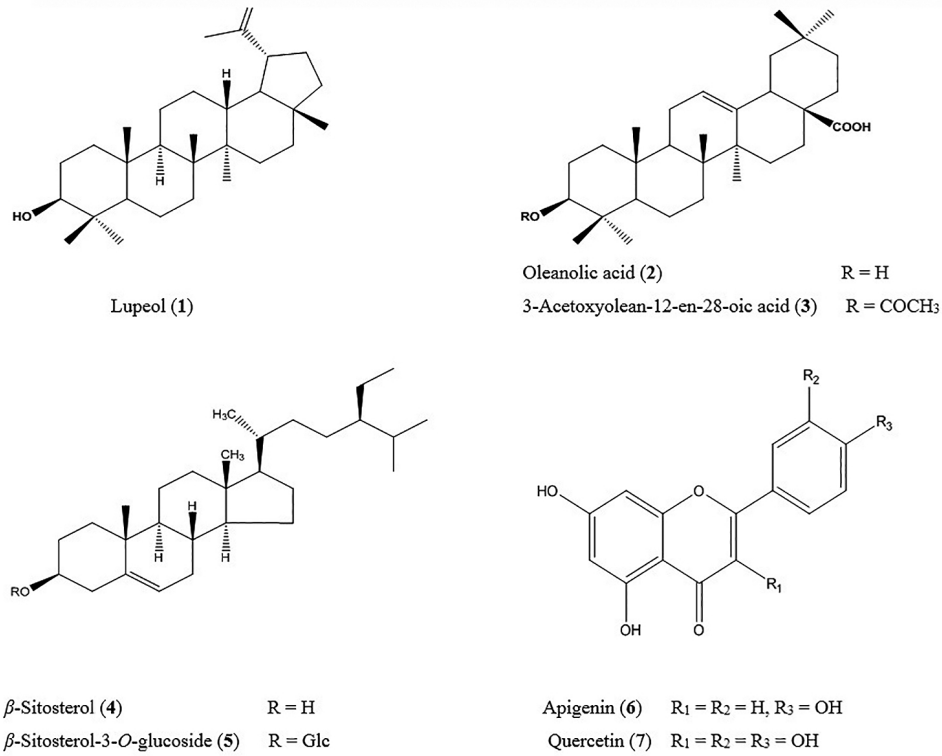

Figure 1.

Chemical compounds isolated from *R. alaternus*

acetoxyolean-12-en-28-oic acid (**3**) are found for the first time in *R. alaternus*. Lupeol (**1**) was previously isolated from two species of the family *Rhamnaceae* like *Gouania ulmifolia* [34] and *Ventilago leiocarpa* [35]. Compounds *β*-sitosterol (**4**) and daucosterol (**5**) were isolated previously from many plants of the family *Rhamnaceae*, such *Gouania ulmifolia* [34], *Ventila gobombaiensis* [36] and *R. alaternus* [37]. Compound apigenin (**6**) was already found in *Rhamnus alaternus* [5, 37]. Quercetin (**7**) was previously isolated from numerous *Rhamnus* species such as *R. alaternus* [4, 37], *R. saxatilis*and *R. catharticus* [4] and *R. lycoides* subsp. *Oleoide* [38].

It is noteworthy to mention that some other phytochemicals were not abundant in *R. alaternus* crude extracts growing in Algeria (tab. 1). Indeed, glycosides were found only in the PE fraction, anthocyanins were detected in *n*-BuOH extract and steroids were present only in the ethyl acetate extract of this species. Lipids were totally absent in

Table 2.

Total bioactive content of the crude extracts obtained from *R. alaternus*

Extracts	Total bioactive contents	
	Phenolic content*	Flavonoid content**
n-BuOH	149.81±0.52	78.48 ± 0.76
EtOAc	138.87 ± 0.66	70.89 ± 0.22
PE	105.36 ± 0.35	27.78 ± 0.18

* Total phenolic contentexpressed as *μ*g equivalents of gallic acid per mg of plant extract (*μ*g EGA/mg extract); ** Total flavonoid content expressed as *μ*g equivalents of quercetin per mg of plant extract (*μ*g EQ/mg extract).

every fraction examined. However, it has already been documented that these types of compounds occurred in *R. alaternus*. Indeed, Longo and his collaborators (2005) [39] purified six common anthocyanidins from the berries of *R. alaternus,* and several nonpolar anthraglycosides were obtained previously from the bark of *R. alaternus* [40].

Total bioactive contents

The results of total bioactive contents of crude extracts obtained from *R. alaternus* were presented in table 2.

According to the results, high levels of phenols and flavonoids were estimated both in ethyl acetate and *n*-butanolic extracts. However, moderate levels have been found in PE extract. This finding is expected since many phenolic compounds and flavonoids were previously identified in *R. alaternus* in cluding luteolin, quercetin-3-rhamnoside, *p*-coumaric acid, ferulic acid, gallic acid, kaempferol, rutin, quercetin-3-*O*-neohesperidoside, kaempferol-3-*O*-(2G-*α*-L-rhamnosyl) -rutinoside, rhamnetin hexoside, kaempferol-3-*O*-rutinoside, rhamno-citrinhexoside, pilosinhexoside, apigenin, apigenin glucoside, kaempferol-3-*O*-glucoside; kaempferol 3-*O*-isorhamninoside, rhamnocitrin-3-*O*-isorhamninoside and rhamnetin-3-*O*-isorhamninoside[4, 5, 37, 41-44]. Boussahel and his collaborators (2015) estimated simiilar levels of flavonoids and phenols in *R. alaternus* leaves extract [42]. In contrast, Nekkaa and his collaborators (2021) found highest levels of bioactive contents in *R. alaternus* crude extract after the optimization of many extraction factors, including the ratio of solid material plant to solvent, the period of extraction and the stirring speed [45].

Antioxidant activities

The antioxidant potential *R. alaternus* crude extracts was tested and the findings are grouped in table 3.

Results demonstrated that all crude extracts prepared from *R. alaternus* exhibit strong antioxidant activities in different tested systems. This result was highly expected, since it is well documented that *Rhamnus* species (*R. yoshinoi*, *R. fallax, R. pumila, R. triquetra, R. intermedia*, *R. pallasii, R. purpurea* and *R. prinoides*) possess strong reducing and radical scavenging activities associated to their richness in phenolic compounds, especially in anthraquinones [28, 46-50]. Indeed, *R. alaternus* extracts with different polarities *(n*-BuOH, EtOAc and PE) had a strong capability to scavenge DPPH radicals better than that of all the tested reference molecules. In addition, the ethyl acetate and the *n*-butanolic extracts showed strong hydroxyl radical scavenging effects. This radical is extremely reactive in biological systems and considered as the main cause of oxidative stress. Its deleterious effects on many macromolecules, including nucleic acids, lipids and proteins are well-known. This interesting radical scavenging potential could be related to the chemical composition of this plant. Many compounds isolated previously from the root bark and leaves of *R. alaternus*, including 1,6-dihydroxy-3-methyl-6-[2'-Me (heptoxy)]anthraquinone, anthraquinone glycosides, physcion-8-*O*-rutinoside, kaempferol-7-

Table 3.

Antioxidant activities of the plant *R. alaternus*

Extract and standards	DPPH assay[a] IC_{50} [*μ*g/mL]	Hydroxyl radical scavenging assay[a] IC_{50} [*μ*g/mL]	FRAP assay[a] *μ*g EAA/mg ex	TAC assay[a] *μ*g EAA/mg ex
n-BuOH	0.01±0.13	7.76±0.07	100.86±0.02	74.29±0.004
EtOAc	0.53±0.04	6.84±0.62	105.42±0.01	77.08±0.005
PE	1.68±0.23	16.38±0.12	53.52±0.2	76.41±0.005
BHA[b]	6.82±0.49	NT	NT	NT
BHT[b]	22.32±0.02	NT	NT	NT
Tannicacid[b]	7.74±0.19	NT	NT	NT
Ascorbicacid[b]	3.1±0.002	7.43±0.09	NT	NT
α-Tocopherol[b]	13.02±0.17	NT	NT	NT

[a]Values expressed are means ± SD of a triplicate ($p < 0.05$); [b]Reference molecule; NT: not tested

methylether, rhamnetin-3-*O*-isorhamninoside, kaempferol 3-*O*-*β*-isorhamninoside, emodin, kaempferoland rhamnocitrin 3-*O*-*β*-isorhamninosideexhibit strong radical scavenging activities [7, 29, 37, 41].

Also, a strong ferric reducing antioxidant power was revealed by the EtOAc and *n*-BuOH extracts. However, these crude extracts showed very close total antioxidant capacities. Previous studies realized on this species displayed that *R. alaternus* had potential free-radical scavenging effects and exhibited a strong reducing iron power. Additionally, *R. alaternus* extracts prevent $AlCl_3$-induced liver oxidative damage, decrease lipid peroxidation and the levels of many oxidative stress markers, and restore the antioxidant activity of many antioxidant enzymes [5, 28, 29, 42, 45, 51, 52].

Haemolytic activity

The haemolytic activity of *R. alaternus* extracts was assessed through the measurement of the liberated free haemoglobin after the lysis of human erythrocytes. The results presented in figure 2 show that all the extracts induce haemolysis. The PE extract exhibits a very strong haemolytic effect with 94.03±0.21% at the concentration of 50 *μ*g/ml. The haemolysis of red blood cells induced by PE extract could be explained by its richness in glycosides (tab. 1). Indeed, it has been reported that high concentration of glucose causes oxidative stress and lipid peroxidation resulting in the loss of cell membranes integrity. In addition, the height level of glycosides may facilitate the advanced glycosylation of cell membrane proteins which increases the membrane osmotic fragility and induces the loss of elasticity resulting in haemolysis [53, 54]. Also, the presence of alkaloids in this extract may be used as a tool to justify the interesting haemolytic effect. In fact, the toxicity of alkaloids and their poisoning properties are highly linked to their ability to induce haemolysis [55]. However, the EtOAc and the *n*-butanolic extracts have a very low haemolytic activity at the same concentration with percentages of 26.69±0.38 and 14.18±0.43%, respectively. This low hemolytic effect could be associated to the richness of EtOAc and *n*-BuOH extracts in phenolic compounds especially flavonoids and anthraquinones known for their preventive effects against lysis and their capability to preserve the membranes integrity [56, 57].

The obtained results are in good agreement with those of recent studies realized in this species. Indeed, it was reported that organic extracts prepared from *R. alaternus* leaves and bark had a very low *in vitro* haemolytic effect (lower than 3.5%) [58]. Also, the pretreatment of human erythrocytes with various doses of *R. alaternus* leaves and bark extracts significantly reduced the haemolysis induced by AAPH in a concentration-dependent manner [52].

Brine shrimp lethality bioassay

The results of the cytotoxicity of *R. alaternus* crude extracts are given in figure 3. All the brine

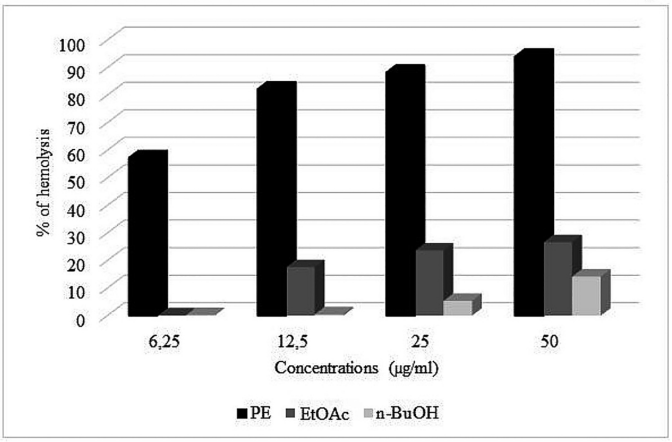

Figure 2.

Haemolytic effect of crude extracts prepared from *R. alaternus*

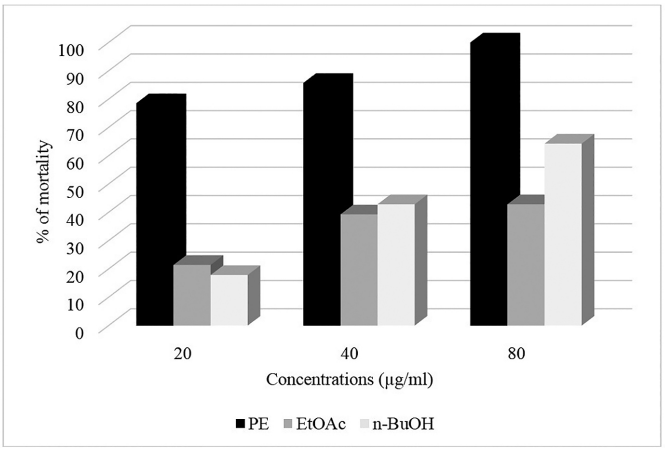

Figure 3.

Percentage of mortality observed after treatment with crude extracts of *R. alaternus* in brine shrimp lethality bioassay

Table 4.

Antibacterial activity of crude extracts from *R. alaternus*

Extracts/ antibiotics	Dilution [μg/ml]	Inhibition zone [mm]				
		E.coli (ATCC 25922)	*S. aureus* (ATCC 25923)	*P. aeruginosa* (ATCC 27853)	*S. pneumoniae* (ATCC 49619)	*K. pneumoniae* (ATCC 70063)
PE	100	12±1.0	11.±1.0	-	13.33±1.04	14.66±1.32
	50	9.33±0.58	9.0±1.0	-	8±1.0	12±1.0
	25	8±1.0	7.33±0.58	-	-	11.33±0.74
	12.5	7.0±1.0	-	-	-	9±1.0
	6.25	-	-	-	-	7±1.0
	MIC (μg/ml)	12.5	25	-	50	6.25
EtOAc	100	12±1.0	12±1.0	14.33±0.58	15.5±1.0	27.66±2.08
	50	10.33±0.58	9.66±1.15	11±1.0	12.2±0.1	24±1.0
	25	8.66±1.15	8.4±1.15	9.15±0.05	9.63±0.05	17±1.0
	12.5	7±1.0	7±1.0	8±1.0	8±1.0	9.33±0.57
	6.25	-		7±1.0	7.33±0.58	7.5±0.1
	MIC (μg/ml)	12.5	12.5	6.25	6.25	6.25
n-BuOH	100	17±1.0	-	8.66±0.58	14±1.0	-
	50	15.66±1.15	-	7±1.0	11.33±0.35	-
	25	11±1.0	-	-	9±1.0	-
	12.5	9.33±0.58	-	-	8±1.0	-
	6.25	7.5±.0.86	-	-	7.4±0.4	-
	MIC (μg/ml)	6.25	-	50	6.25	-
Cefotaxime		15.66±0.58	NF	28.66±1.15	29.3±0.1	NF
Cefalexin		20.33±0.58	19±1.0	15±1.0	21.66±0.11	-
Amoxicillin		20.66±1.15	NF	18.33±0.58	26.2±0.1	-

Values are means ± SD of three measurements. (-) There are no restriction zones surrounding the discs. MIC: the minimum inhibitory concentration. NF: not found

shrimp nauplii are survived in the control group. However, the crude extracts of *R. alaternus* induce the mortality of the larvae at the concentration-dependent manner. The petroleum ether (PE) extract causes the mortality of all the brine shrimp nauplii at the concentration of 80 μg/ml. While the EtOAc and *n*-BuOH extracts induce the mortality of 42.86 and 64.28%, respectively at the same tested concentration. The significant cytotoxicity of PE extract could be associated by the presence of alkaloids and to its lytic properties and its ability to induce the destabilization of cells membranes.

In fact, the cytotoxic effects of the species *R. alaternus* were previously documented. The methanolic extract of *R. alaternus* exerts an excellent cytotoxic activity against human monocytic leukaemia cells. As stated by the authors, this activity is attributed to the existence of rhamnocitrin glycosides and kaempferol [59]. Extracts prepared from *R. alaternus* display a remarkable anti-proliferative effect on human leukaemia cells due to their proapoptotic properties and protective effects against oxidative stress and free radicals [15, 16, 60]. Moreover, *R. alaternus* en riched flavones extract inhibits melanoma cell proliferation, induces apoptosis and delays the cell migration. In addition, this extract possesses *in vivo* cytotoxic effects. It was proven that the pretreatment with this extract significantly reduces tumour volume and weight, modulates the level of several pro-inflammatory cytokine, enhances the levels of TH1 cytokine, and induces macrophage activation [43, 61].

Antibacterial activity

R. alaternus extracts (PE, EtOAc and *n*-BuOH) exhibited antibacterial activity against both Gram-negative and Gram-positive bacterial strains (tab. 4). The ethyl acetate showed strong antibacterial effect on all the tested bacterial species, with value of MIC equal to 12.5 μg/ml. The Gram-negative bacteria *Klebsiella pneumonia* had the highest susceptibility towards PE extract, followed by *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Moreover, the *n*-BuOH extract presented antibacterial effect against the Gram-negative bacteria *E. coli* and the Gram-positive bacteria *S. pneumoniae*.

Phytoscreening study on the antibacterial activity of *R. alaternus* harvested in Tunisia [15] revealed that the EtOAc extract tested against several bacterial strains displayed strong antibacterial activity comparable to the results of this research.

Phytoconstituents examination of the crude extracts (PE, EtOAc and *n*-BuOH) demonstrated the presence of several classes of biomolecules like flavonoids, anthocyanins, alkaloids, terpenoids, sterols and quinones. It appears that these bioactive compounds were necessary for the antibacterial activity noticed in the matching extracts [62].

CONCLUSION

In this paper, we present the outcome of the phytochemical investigation of the ethyl acetate extract derived from the aerial parts of *R. alaternus* L. growing in Algeria and the *in vitro* evaluation of antioxidant, haemolytic, cytotoxic and antibacterial activities of the PE, EtOAc and *n*-BuOH extracts.

The phytochemical study allowed the isolation and structural characterization of seven secondary metabolites, including triterpenoids and flavonoids. The phytochemical screening of the extracts indicatedits richness in several chemical classes of biomolecules known for their interesting bioactivities. All the tested crude extracts exhibited antibacterial effects. Both EtOAc and *n*-BuOH extracts contained high levels of phenols and flavonoids, had the ability to scavenge hydroxyl and DPPH radicals and possessed a strong ferric-reducing antioxidant activity. Moreover, the PE extract displayed very strong *in vitro* cytotoxic and haemolytic activities.

Furthermore, we can conclude that *R. alaternus* growing in Algeria is a natural source of phenolic compounds with potential antioxidant, antibacterial and cytotoxic effects. However, the haemolytic effects induced by this species require a careful use. For this reason, more in-depth *in vivo* investigations are needed to confirm the safety of this plant and to elucidate the possible cytotoxic mechanisms.

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