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Antibacterial and cytotoxic activities of extracts from (Tunisian) *Rhamnus alaternus* (Rhamnaceae)

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Abstract - The petroleum ether, chloroformic, ethyl acetate, methanolic, Total Oligomers Flavonoids (TOF) enriched extracts, water extract as well as its fractions A_1, A_2, A_3 obtained from aerial parts of *Rhamnus alaternus*, a Tunisian-Mediterranean medicinal species, were investigated for the contents of phenolic compounds, cytotoxic activity against the K562 human chronic myelogenous leukaemia cell line and L1210 leukaemia murine cells and for antibacterial activity against Gram positive and Gram negative bacterial reference strains. A pronounced cytotoxic effect on both the cell lines was shown in the TOF, ethyl acetate, methanolic, aqueous extracts and A_2 fraction, with respectively IC_{50} values 75, 232, 298, 606 and 571 µg/ml on K562 cells and 198, 176, 767, 560 and 614 µg/ml on L1210 cell line. Significant activity against bacterial reference strains: *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella enteritidis* and *Salmonella typhimurium* was shown with ethyl acetate, TOF extracts and A2 fraction. The antimicrobial and cytotoxic activities showed by *R. alaternus* depended on the chemical composition of the tested extracts.

Key words: *Rhamnus alaternus*, cytotoxicity, antibacterial effect.

INTRODUCTION

Although the use of drug treatment regimens including antibiotics have been effective in attaining high cure rates, repeated use of these chemical drugs may lead to development of resistance and resistant strains (Becx *et al*., 1990; Matsumoto *et al*., 1997). Such treatments have determined undesirable effects on non target organisms such as intestinal microorganisms (Ahn *et al*., 2000) and have sometimes caused serious side effects such as diarrhoea, nausea, abnormal taste, dyspepsia, abnormal pain/discomfort and headache. Therefore, there is a strong demand for preparations having all of the beneficial properties of current therapy agents but with fewer side effects. Antimicrobial substances other than antibiotics would be very useful in the treatment of microbial infections if they were shown to be effective against both antibiotic-resistant and susceptible bacterial strains. Plants, particularly higher plants, may be an alternative source of antibacterial because they constitute a rich source of bioactive chemicals (Wink, 1993; Park *et al*., 2006).

In addition, epidemiological studies show that the incidence and mortality rates of leukaemia and lymphoma are still ranked high in the worldwide population, and the use of cancer chemopreventive compounds from medicinal herbs have the ability to induce apoptosis of many leukaemic cell lines (Jemal *et al*., 2003; Kim *et al*., 2003; Al-Dabbas *et al*., 2006).

The genus *Rhamnus*, belonging to the family of Rhamnaceae, comprises numerous species widely disseminated in Tunisian-Mediterranean area. Some of these plants have been used in traditional medicine in the Mediterranean countries for a long time, for the treatment of gastric and hepatic complications (Ben Mrabet and Abed, 1986; Bellakhdar, 1999). Rigorous scientific studies have shown several pharmacological properties for some metabolites isolated from *Rhamnus.* In fact, the more interesting pharmacological properties of the species belonging to *Rhamnus* genus are due, in particular, to the presence, among several metabolites, of flavonoids, tannins, coumarins and anthraquinones (Izhaki *et al*., 2002).

In order to evaluate the therapeutic potentials of Tunisian medicinal herbs for the treatment of antimicrobial and cytotoxic related-diseases pathologies, a medicinal herb species traditionally used in Tunisia to treat gastrointestinal and hepatic infections, was studied for antiproliferative and antibiotic properties against leukaemia cell lines and different strains of several pathogenic bacteria respectively. This paper reports the evaluation of the antibacterial and cytotoxic activities of the aerial part extracts from *R. alaternus*, an endemic species from the Tunisian mountains. The total flavonoid and phenol contents of the extracts as well as their preliminary phytochemical study

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were also determined. We report our findings and relate them to the phytochemical studies of the plant as well as its medicinal uses.

MATERIALS AND METHODS

Collection and preparation of plant extracts. *Rhamnus alaternus* aerial parts were collected in the western north mountains of Tunisia, in December 2004. Botanical identification was performed by a taxonomic botanist from the Horticulture Institute of Chott-Mariam, Tunisia, according to the flora of Tunisia (Pottier-Alaptite, 1978). A voucher specimen (Ra-12-004) has been kept in the Laboratory of Pharmacognosy, Faculty of Pharmacy of Monastir-Tunisia, for future reference.

The leaves were air-dried at room temperature under shade, and ground into powder using an electric mill. The powder was packaged and stored in dry and well-ventilated room until use.

Petroleum ether (PE), chloroformic ($CHCL_3$), ethyl acetate (EtOAc), and methanolic (MeOH) extracts with different polarities were obtained by Soxhlet apparatus (6 h). They were concentrated to dryness and kept at 4 °C in the absence of light.

The powdered leaves were also extracted with boiling water for 15 min. After filtration, the extract was filtered to obtain the aqueous extract (A) and lyophilised. A_1, A_2 and A_3 were respectively the chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, obtained by a liquid-liquid separation.

In order to obtain a total oligomers flavonoids (TOF) enriched extract, the powdered leaves were macerated in water:acetone mixture (1:2) for 24 h, under continuous stirring. The extract was filtered and the acetone was evaporated under low pressure to obtain an aqueous phase. The phlobaphènes were removed by precipitation with an excess of NaCl at 5 °C for 24 h. The supernatant was extracted with ethyl acetate, concentrated and precipitated in an excess of chloroform. The precipitate was separated and TOF extract yielded.

Thin layer chromatographic (TLC) analysis of extracts. *Rhamnus alaternus* leaf extracts were screened for the presence of various secondary metabolites such as tannins, flavonoids, coumarins and sterols, using the methods previously described by Tona *et al*. (1998, 2004).

TLC analysis of each extract was carried out on silica gel 60 F254 aluminium sheets (Merck, Germany) as follows. An aliquot of the extract dissolved in the adequate solvent was spotted on TLC sheets. After development with a an appropriate mixture of solvents, the sheets were air-dried, examined under visible and UV light and then sprayed with 1% aluminium chloride solution in ethanol or 10% sulphuric acid, followed by heating at 110 °C. The sheet was examined again under visible and UV light. For flavonoids, TLC was developed in n-butanol:acetic acid:water (4:1:5, top layer), spots were visualised with 1% aluminium chloride solution in methanol under UV 366 nm. Spots of phenolic compounds, particularly flavonoids, yielded yellow to orange colour by the sulphuric acid reagent and yellow fluorescence under long-wave UV light (366 nm) by the aluminium chloride reagent.

Steroids were identified with Libermann-Burchard, as reagent using n-hexanes:CH_2Cl_2 (1:9) as mobile phase. A range of colours are produced after heating sprayed plates for 10 min at 100 °C. The test for tannins was carried out with $FeCl_3$. Each class of tannins gave a specific coloration. Coumarins were detected under UV (366 nm), thanks to their blue fluorescence which becomes intense after spraying 10% potassium hydroxide solution in ethanol.

Determination of total phenolic compounds and flavonoid contents. The amount of phenolic compounds present in the *R. alaternus* leaf extracts was determined by Folin Ciocalteu reagent (Duh and Yen, 1997). Gallic acid was used as a standard for a calibration curve (Yuan *et al*., 2005). Aliquots of each extract (100 µl) were mixed with 2 ml of 2% Na_2CO_3 and incubated at room temperature for 2 min. After the addition of 100 µl 50% Folin-Ciocalteu's phenol reagent, the reaction tube was further incubated for 30 min at room temperature, and finally absorbance was measured at 720 nm (Genesys 10_{UV} scanning spectrophotometer). The total amount of phenolic compounds was determined from the calibration curve, and expressed as percent of gallic acid in the extract (Capecka *et al*., 2005).

The amount of flavonoids was determined by the method of Zhishen *et al*. (1999). Quercetin was used as the standard for a calibration curve. A known volume of the extracts was placed in a 10 ml volumetric flask. Distilled water was added to make the volume to 5 and 0.3 ml $NaNO_2$ (1:20 w/v) was added to this dilution. Three millilitres of 10% aluminium trichloride (1:10 w/v) was added 5 min later. After 6 min, 2 ml of 1 N NaOH was added and the total absorbance was measured at 510 nm (Kumar and Chattopadhyay, 2007). The flavonoid content was determined from the calibration curve, and expressed as percent of quercetin in the extract (Eberhardt *et al*., 2000; Luximon-Ramma *et al*., 2002).

Determination of tannins. The amount of tannin content in extracts was determined according to the method described by Pearson (1976). Extraction of tannins in the tested samples was achieved by dissolving 5 g of extract in 50 ml distilled water in a conical flask, allowing the mixture to stand 30 min, shaking of the flask every 10 min and then centrifuging at 5000 *x g*, to obtain a supernatant (tannin extract). The extract was diluted to 100 ml in a standard flask using distilled water. Five millilitres of the diluted extract and 5 ml of standard tannic acid (0.01 g/l tannic acid) were added into different 50 ml volumetric flasks. Folin-Denis reagent (1 ml) was added to each flask followed by 2.5 ml of saturated sodium carbonate solution. The solutions were made up to 50 ml mark with distilled water and incubated at room temperature (20-30 °C) for 90 min (Nwabueze, 2007). Absorptions of these solutions were measured against that of the blank (containing 5 ml distilled water in place of extract or standard tannic acid solution) in a Genesys 10_{UV} scanning spectrophotometer at 760 nm wavelength. Tannin content was expressed as percent of tannic acid in the studied extract.

Chemicals. 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Sigma Aldrich Co (St. Louis, USA). RPMI-1640, foetal bovine serum, gentamycin and L-glutamine were purchased from GIBCO BRL Life Technologies (Grand Island, NY, USA). Mueller-Hinton agar was purchased from Fluka Biochemika (Spain).

***In vitro* antimicrobial assay.** Antimicrobial activity of *R. alaternus* extracts was tested on the Gram-positive bacteria *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923 and *Enterococcus faecalis* ATCC 29212, and the Gram-negative bacteria *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076 and *Salmonella typhimurium* NRRLB (National Regional Research Laboratory in Peoria, Ill, USA) 4420, using the micro dilution method (Eloff, 1998; Roussis *et al*., 1998; Hayder *et al.*, 2005). Overnight grown microbial suspensions were standardised to approximately 10^5 cells/ml (Cremieux, 1991). The agar dilution method was used to determine the MICs of *R. alaternus* extracts: 100 µl of microbial suspension containing approximately 1 x 10^5 cells/ml, were added to 100 µl of the test extract dilution (concentrations ranging from 50 µg/ml to 6 mg/ml in tubes). A set of tubes containing only agar and microbial suspension served as the negative control. These serially diluted cultures were then incubated at 37 °C for 24 h. Subsequently, 10 µl of each culture were plated on substance-free Mueller-Hinton agar plates and further incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of plant extract that completely suppressed colony growth. Minimal bactericidal concentration (MBC) was defined as the lowest concentration of the tested extract that kills 99.99% of the tested bacteria.

Cytotoxicity studies *in vitro.* *Rhamnus alaternus* extracts were tested *in vitro* for their antiproliferative activity against K562 (human chronic myelogenous leukaemia) and L1210 (murine leukaemia) cell lines.

Culture of cells. K562 and L1210 cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were cultivated in RPMI-1640 medium supplemented with 10% v/v foetal calf serum, 1% gentamycin and 2 mM L-glutamine as a complete growth medium. Cells were maintained in 25 cm^3 flasks with 10 ml of medium and were incubated at 37 °C in an incubator with 5% CO_2 in humidified atmosphere. Every 3 days the cells were subcultured by splitting the culture with fresh medium.

Assay for cytotoxic activity. Cytotoxicity of *R. alaternus* extracts against the two leukaemia cell lines was estimated by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells. The resulting blue formazan product can be measured spectrophotometrically. The MTT colorimetric assay was performed in 96-well plates (Uliasz and Hewett, 2000; Polydoro *et al*., 2004). Cells were seeded in a 96-well plate at a concentration of 5 x 10^4 cells/well and incubated at 37° C for 24 h in a 5% CO_2 enriched atmosphere. The extracts were firstly dissolved in 1% DMSO, then in the cell growth medium. Cells in exponential growth phase were incubated again at 37 °C for 48 h with each of the tested extracts at concentrations ranging from 100 to 800 µg/ml. After that, the medium was removed and cells in each well were treated with 50 µl of MTT solution (5 mg/ml) at 37 °C for 4 h. MTT solution was then discarded and 50 µl of 100% DMSO were added to dissolve insoluble formazan crystal. Optical density was measured at 540 nm. Each drug concentration was tested in triplicate, and repeated three times in separate experiments.

The cytotoxic effects of the extracts were estimated in terms of growth inhibition percentage and expressed as IC_{50} which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells). The IC_{50} values were graphically obtained from the dose-response curves. We determined IC_{50} values when activities resulted more than 50% at screening concentrations.

RESULTS AND DISCUSSION

This study is designed to evaluate the cytotoxic and antibacterial activities of *R. alaternus* extracts against human chronic myelogenous K562 and L1210 murine leukaemia cell lines, and five Gram-positive and Gram-negative human pathogenic bacterial strains. The metabolite contents of the extracts as well as their preliminary phytochemical study were also determined.

***Rhamnus alaternus* extracts: phytochemical study and metabolites content**

TLC analysis results of extracts after sheets charring, spraying and visible/UV light checking showed at least ten distinct yellow to orange spots (indicating the presence of phenolic compounds, particularly flavonoids and anthraquinones) in the ethyl acetate, aqueous, A_2 fraction and TOF extracts. Among them three spots exhibited blue florescence under UV light (366 nm) (indicating the presence of polymethoxylated flavonoids) in the TOF extract only. Five yellow to orange spots and a single red spot (indicating the presence of phenolic compounds, particularly flavonoids) in each of the methanolic extract as in the A_1 and A_3 fractions extracts were observed. Three distinct fluorescent blue spots and two red spots (indicating the presence of coumarins and chlorophyls, respectively) under the UV light (366 nm) were observed with petroleum ether and chloroformic extracts.

The tested extracts showed in the ethyl acetate, methanolic, TOF, aqueous extracts and A_2 fraction, the presence of coumarins, tannins and particularly of flavonoids and anthraquinones (anthraquinones give spots with orange color by the sulphuric acid reagent and yellow fluorescence under long-wave UV light by the aluminium chloride reagent).

As shown in Table 1, the ethyl acetate, the methanolic, the TOF, the aqueous (A) extracts and the A_2 fraction contained: 25, 13.8, 29.5, 22.7 and 20.5% of phenolic compounds (as gallic acid equivalent), respectively, and 33.8, 28.3, 42.3, 21.3 and 18.8% of flavonoids (as quercetin equivalent), respectively; however, the petroleum ether, the chloroformic extracts, the A_1 and A_3 fractions contained 0.6, 8, 7.2 and 5.7% of phenolic compounds (as gallic acid equivalent) and 7.9, 15.4, 8.2 and 4.8% of flavonoids (as quercetin equivalent), respectively.

The content of tannins varied between 0.01 and 16%. The highest amounts were recorded in the ethyl acetate extract, followed by the methanolic and then the aqueous extracts by respective amounts of 16, 14.72 and 12.52%.

TOF, ethyl acetate, methanolic, aqueous extract and A_2 fraction were shown to be rich in flavonoids and phenolic compounds. Previous phytochemical screening of *R. alaternus* evidenced the presence of flavonoids (Rosell, 1980; Marzouk *et al*., 1999), anthraquinones (Abou Chaar *et al*., 1982; Izhaki *et al*., 2002) and tannins (Ben Ammar *et al*.,

TABLE 1 - Total polyphenol, total flavonoid and tannin contents of extracts from *Rhamnus alaternus*

Extracts[a]	Metabolites		
	Polyphenols[b] (%)	Flavonoids[c] (%)	Tannins (%)
PE	0.62 ± 0.07[d]	7.9 ± 0.51	0.01 ± 0.00
$CHCL_3$	8 ± 0.84	15.4 ± 0.57	0.06 ± 0.02
EtOAc	25 ± 0.61	33.8 ± 1.39	16 ± 0.48
MeOH	13.8 ± 0.71	28.3 ± 0.94	14.72 ± 0.38
TOF	29.5 ± 0.93	42.3 ± 1.42	1.56 ± 0.06
A	22.7 ± 0.46	21.3 ± 1.12	12.52 ± 0.32
A_1	7.2 ± 0.31	8.2 ± 0.56	3.50 ± 0.14
A_2	20.5 ± 0.86	18.8 ± 1.02	6.08 ± 0.22
A_3	5.7± 0.28	4.8 ± 0.44	2.48 ± 0.1

[a]PE: Petroleum ether extract, $CHCL_3$: chloroformic extract, EtOAc: ethyl acetate extract, MeOH: methanolic extract, TOF: total oligomers flavonoids enriched extract, A: aqueous extract, A1, A2, A3: chloroformic, ethyl acetate and butanolic fractions of the aqueous extract, respectively.
[b]Polyphenols (%) as gallic acid equivalent.
[c]Flavonoids (%) as quecetin equivalent.
[d]Means ± SD of three independent determinations.

2005).

High total flavonoid and polyphenols content in the active *R. alaternus* extracts may corroborated the traditional use of this plant species in Tunisia and other African and Mediterranean countries as well as its reported use in several antimicrobial and cytotoxic related-disease pathologies since immemorial time (Boukef, 2001).

Antimicrobial activity

The antibacterial activity of the nine *R. alaternus* extracts was evaluated on five pathogenic bacteria. Our results showed that leaf extracts of *R. alaternus* exhibited various levels of antibacterial effect against all bacterial strains tested. MICs values varied from 62.5 µg/ml to over 6 mg/ml and MBCs values varied from 1.75 to over 6 mg/ml (Tables 2 and 3).

Overall, three extracts (3/9) displayed strong activity against both Gram-negative and Gram-positive bacteria. *Salmonella typhimurium* was the most susceptible bacterial species to TOF extract, followed by *S. aureus*, then *S. enteritidis* and finally *E. coli* and *E. faecalis,* with MICs of 62.5, 120, 125, 175 µg/ml and 1.75 mg/ml, respectively. Whereas with ethyl acetate extract, *S. aureus* was the most susceptible bacterial species followed by *S. enteritidis* then *E. faecalis* and *S. typhimurium* with MICs of 70, 100, 150 and 175 µg/ml.

In this study, aqueous extract (A) had weak antibacterial activity even at the highest concentration (6 mg/ml), whereas its A_2 fraction showed higher activity against *S. aureus, S. enteritidis and S. typhimurium* compared to A. In fact, the A_2 MICs towards the cited bacteria were 150, 200 and 250 µg/ml. Against *E. faecalis* and *E. coli*, relatively high concentrations of A_2 (respectively 1.2 and 4.25 mg/ml) were needed to completely suppressed strain growth. The chloformic (A_1) and the butanolic (A_3) fractions of the aqueous extract didn't exhibit any significant

TABLE 2 - Activity of *Rhamnus alaternus* extracts, expressed as Minimum Inhibitory Concentration

Extracts	Gram positive organisms		Gram negative organisms		
	S. aureus ATCC 25923	*E. faecalis* ATCC 25922	*E. coli* ATCC 25922	*S. enteritidis* ATCC 13076	*S. typhimurium* NRRLB 4420
PE	> 6[a]	> 6[a]	> 6[a]	> 6[a]	> 6[a]
$CHCL_3$	> 6[a]	> 6[a]	> 6[a]	> 6[a]	> 6[a]
EtOAc	70[b]	150[b]	3.75[a]	100[b]	175[b]
MeOH	> 6[a]	> 6[a]	> 6[a]	> 6[a]	> 6[a]
TOF	120[b]	175[b]	1.75[a]	125[b]	62.5[b]
A	> 6[a]	> 6[a]	> 6[a]	> 6[a]	> 6[a]
A_1	> 6[a]	> 6[a]	> 6[a]	> 6[a]	> 6[a]
A_2	150[b]	1.2[a]	4.25[a]	200[b]	250[b]
A_3	> 6[a]	> 6[a]	> 6[a]	> 6[a]	> 6[a]

[a]Values in mg/ml, means of three experiments.
[b]Values in µg/ml, means of three experiments.

TABLE 3 - Activity of *Rhamnus alaternus* extracts, expressed as Minimum Bactericidal Concentration

Extracts	Gram positive organisms		Gram negative organisms		
	S. aureus ATCC 25923	*E. faecalis* ATCC 25922	*E. coli* ATCC 25922	*S. enteritidis* ATCC 13076	*S. typhimurium* NRRLB 4420
PE	> 6[a]	> 6	> 6	> 6	> 6
$CHCL_3$	> 6	> 6	> 6	> 6	> 6
EtOAc	2.25	2.00	5.50	3.00	2.40
MeOH	> 6	> 6	> 6	> 6	> 6
TOF	1.75	1.80	3.00	2.50	1.75
A	5.5	> 6	> 6	> 6	> 6
A_1	> 6	> 6	> 6	> 6	> 6
A_2	2.60	2.75	6.00	2.80	4.00
A_3	> 6	> 6	> 6	> 6	> 6

[a] All values in mg/ml, means of three experiments.

inhibitory effect until 6 mg/ml, against neither the Gram-negative nor the Gram-positive bacteria. Same results were shown by petroleum ether, chloroformic and methanolic extracts, while ethyl acetate and TOF solvents (acetone) extracted more antibacterial compounds.

In conclusion, 3 out of the 9 *R. alaternus* extracts tested (TOF, ethyl acetate extracts and A_2 fraction) showed significant antimicrobial activities, whereas no inhibitory effect was produced by the petroleum ether, chloroformic, methanolic extracts, aqueous extract and its A_1 and A_3 fractions. This result is not surprising as far as the active extracts showed high amounts of flavonoids and other polyphenolic compounds in their chemical composition. These families of compounds are reported to play a role in the prevention of colonisation by parasites, bacteria and fungi (Chiang *et al*., 2003).

In our study, the active extracts showed no selective activity towards one group of bacteria. It is interesting to note that *R. alaternus* extracts exhibited antimicrobial activity, particularly towards organisms of interest to the medical field such as *Staphylococci*, *Enterococci* and *Salmonella*. In fact, *Salmonella* remains a primary cause of food poisoning worldwide, and massive outbreaks have been reported in recent years. The Centre for Disease Control and Prevention estimated that approximately 1.4 million cases of salmonellosis were annually reported in the United States (Mead *et al*., 1999), and the European Union also reported more than 100.000 cases to Enter-net (O'Brien and de Valk, 2003). In Tunisia, between 1978 and 1993, 1022 *Salmonella* strains were isolated: 578 in hospitals and 444 from the environment (Dhidah *et al*., 1995). Certain pathogenic *Salmonella* serotypes adapted to man, such as *S. typhimurium*, usually cause severe diseases such as enteric fever in humans. However, some pathogenic *Salmonella* serotypes, such as *S. enteritidis* or *S. typhimurium*, can infect a wide range of hosts and are termed ubiquitous. Again, foodborne illness resulting from consumption of food contaminated with pathogenic bacteria has been of vital concern to public health. *Salmonella* sp. and *E. coli* accounted for the largest number of outbreaks, cases, and deaths. In Canada, the cost to treat the foodborne disease due to meat and meat products contamination is estimated to $500 millions per year (Todd, 1989).

Preliminary screening for antibacterial activities of *R. alaternus* extracts against tested bacteria revealed such activities with ethyl acetate, TOF extracts and A_2 fraction only. This suggests that compounds such as phenols or flavonoids (phenolic compound contents in active extracts higher than in the other extracts) may act as antimicrobial active compounds (Sakanaka *et al*., 2000; Rodriguez Vaquero *et al*., 2007).

TOF, ethyl acetate and A_2 fraction extracts were the only effective in this antibacterial study. This indicates that intermediate polarity compounds are active in the antibacterial assays. This activity can be ascribed to the compounds extracted by solvents with intermediate polarity used in order to obtain each of the corresponding extracts (in this case, TOF, ethyl acetate and A_2 fraction). We believe that extract components of higher polarity are not involved in antibacterial activity. Also, the heating and crushing of plant material during the preparation of extracts or decoction may play a role in liberating active compounds from storage sites in plant tissues.

Our previous studies have found *R. alaternus* extracts to contain anthraquinones as one of the major components, particularly in TOF, ethyl acetate extracts and A_2 fraction (Ben Ammar *et al*., 2005). Thus, it can be deduced that the moderate to good anti-bacterial activity may be attributed to anthraquinones in the active extracts. Furthermore, for other extracts, in which the anthraquinones are absent, there was a drop in the antibacterial activity.

In the literature, antimicrobial properties of anthraquinones from other plant species such as *Morinda elliptica* have been reported (Ismail *et al*., 2000). Also, the anthraquinone emodin has been reported to possess various pharmacological and biological activities including immunostimulation, antiparasitic, anti-inflammatory and analgesic effects (Izhaki *et al*., 2002).

Cytotoxic activity of *Rhamnus alaternus* extracts

The cytotoxic effect of the *R. alaternus* extracts, expressed as cell viability, was assessed on the human chronic myelogenous K562 and murine leukaemia L1210 cell lines. We have examined the effect of different concentrations (from 10 to 800 µg/ml) of each extract on proliferation of leukaemia cells *in vitro* using the MTT assay.

Results of these experiments, shown in Table 4, demonstrated that the tested *R. alaternus* extracts inhibited at various levels (good to moderate), the proliferation of the tested malignant cells. In fact, five of the tested *R. alaternus* extracts (TOF, ethyl acetate, methanolic extracts, aqueous extract A and its A_2 fraction) had cytotoxic activity towards K562 cells, whereas L1210 proliferation was inhibited also by the A_3 fraction.

TABLE 4 - IC_{50} (μg/ml) values for extracts of *Rhamnus alaternus* towards L1210 and K562 leukaemia cell-lines

Extracts	Activity on proliferation of cell line	
	K562	L1210
PE	> 800	> 800
$CHCL_3$	> 800	> 800
EtOAc	232 ± 5.12	176 ± 7.53
MeOH	298 ± 9.25	767 ± 11.88
TOF	75 ± 3.64	198 ± 6.97
A	606 ± 14.53	560 ± 9.13
A_1	> 800	> 800
A_2	571 ± 15.69	614 ± 10.36
A_3	> 800	394 ± 8.74

IC_{50} values, from the *in vitro* data, were calculated by regression analysis. Untreated culture cells were used as the 100% viability value. Results are means ± standard deviation of duplicate analysis of three replications.

The strongest cytotoxic effect was obtained with TOF extract against K562 cells (IC_{50} 75 μg/ml), followed by ethyl acetate, methanolic extracts and A_2 fraction, then aqueous extract, with respective IC_{50s} of 232, 298, 571 and 606 μg/ml.

Similarly, TOF and ethyl acetate extracts were the most active extract towards L1210 cell line, with IC_{50} of 198 and 176 μg/ml, respectively. Aqueous extract, A_3 and A_2 fractions, as well as methanolic extract inhibited L1210 cells proliferation having IC_{50s} of 560, 394, 614 and 767 μg/ml, respectively.

No significant cytotoxic activity was shown towards both the cell lines after treatment of cells with petroleum ether, chloroformic extracts as wells as with A_1 aqueous fraction (IC_{50s} > 800 μg/ml).

The cytotoxic activity seemed to be dose-dependent; in fact, at 100 μg/ml all the tested extracts inhibited the proliferation of L1210 and K562 cells from 2 to 35%, except TOF extract which determined 67% K562 proliferation inhibition. Inhibition percentages of proliferation of cells incubated with 400 and 800 μg/ml of each tested extract, increased to reach values comprised between 38 and 92%. These results show, although the drug-resistant nature of K562 cells (Efthimiadou *et al*., 2007), that the active extracts from *R. alaternus* inhibited the cell proliferation in a concentration-dependent manner. Since it is well known that different cell-lines might exhibit different sensitivities to a cytotoxic compound, the use of more than one cell-line is, therefore, considered necessary in the detection of antiproliferative compounds.

TOF extract was the most active in comparison to the other extracts against both the two cell-lines which differ by their origin, morphology and tumorigenicity. The cell-type specificity observed in the extracts is likely to be due to the presence of different classes of compounds such as flavonoids and total polyphenols, as reported for others classes of compounds (Cragg *et al*., 1994). Besides, some studies have shown that flavonoids are able to influence a variety of cell function by modulating cell signalling (Musonda and Chipman, 1998), altering proliferation and cytotoxicity in cancer cell lines (Kuntz *et al*., 1999). Also, flavonoids show cytotoxic effects on various human cell lines, for example leukaemia cells (Larocca *et al*., 1990; Hirano *et al*., 1994) and ovarian cancer cells (Benavente-Garcia *et al*., 1997).

On the other hand, petroleum ether and chloroformic extracts showed a weak inhibitory activity. Since the plant was extracted successively with petroleum ether, chloroform, ethyl acetate and methanol, a slight cytotoxic activity of the two first extracts indicated that the compounds responsible for the cytotoxic effect were not extracted at first, due to their polar characteristics. The weak cytotoxic activities exhibited by some *R. alaternus* extracts against the two cell lines indicated that the compounds present in the extracts are non cytotoxic. However, since reactive oxygen radicals play an important role in carcinogenesis (Lee *et al*., 1998, Abdelwahed *et al*., 2007; Bouhlel *et al*., 2007; Ben Mansour *et al*., 2007), it is possible to suggest that the presence of antioxidants in the active *R. alaternus* extracts (Chevolleau *et al*., 1992; Ben Ammar *et al*., 2005) may play some role in reducing cell number.

High antibacterial and high cytotoxic activities were observed in the A_2 fraction compared to the aqueous extract A. This result could be explained by the presence of active compounds masked by various components in the whole crude aqueous extract and more accessible in the A_2 fraction.

Finally, the use of natural products as antibacterial compounds (Conner, 1993; Dorman and Deans, 2000) seems to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food.

To our knowledge, this is the first report on the antimicrobial and cytotoxic effects of the *R. alaternus* extracts and these effects may justify the popular use of this plant in the folk medicine in Tunisia.

The emergence of multi-drug resistant strains of bacterial pathogens and the research of natural anticancer remedies is a problem of ever-increasing significance. Consequently, the search of new anticancer and antimicrobial agents will always remain an important and challenging task for us. Due to the potent inhibitory activity of some *R. alaternus* extracts against the two leukaemia cell lines and towards the most susceptible bacteria, the A_2 fraction, TOF and ethyl acetate extracts were selected for further study and, at present, our group is concerned with the isolation of pure compounds and the elucidation of their structures in order to better evaluate their pharmacological activity *in vitro* and *in vivo*.

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REFERENCES

Abdelwahed A., Bouhlel I., Skandrani I., Valenti K., Kadri M., Guiraud P., Steiman R., Mariotte A.M., Ghedira K., Laporte F., Dijoux-Franca M.G., Chekir-Ghedira L. (2007). Study of antimutagenic and antioxidant activities of gallic acid and 1,2,3,4,6-pentagalloylglucose from *Pistacia lentiscus*. Confirmation by microarray expression profiling. Chem. Biol. Inter., 165: 1-13.

Abou-Chaar Ch.I., Kabbara R.A., Shamlian N.S. (1982). A chromatographic study of the anthraquinones of *Rhamnus alaternus* L. Extraction, isolation and chromatographic characterization of the anthraglycosides of the stem bark. Int. J. Crude Drug. Res., 20 (1): 13-18.

Ahn Y.J., Park S.J., Lee S.G., Shin S.C., Choi D.H. (2000). Cordycepin: selective growth inhibitor derived from liquid culture of *Cordyceps militaris* against *Clostridium* spp. J. Agric. Food Chem., 48: 2744-2748.

Al-Dabbas M.M., Suganuma T., Kitahara K., Hou D.-X., Fujii M. (2006). Cytotoxic, antioxidant and antibacterial activities of *Varthemia iphionoides* Boiss. extracts. J. Ethnopharmacol., 108: 287-293.

Becx M.C.J.M., Janssen A.J.H.M., Claaener H.A.L., de Koning R.W. (1990). Metronidazole-resistant *Helicobacter pylori*. Lancet, 335: 539-540.

Bellakhdar J., Ed. (1999). La Pharmacopée Marocaine Traditionnelle, Ibis Press, Paris.

Ben Ammar R., Kilani S., Abdelwahed A., Hayder N., Mahmoud A., Chibani J., Chekir-Ghedira L., Ghedira K. (2005). *In vitro* mutagenicity, antimutagenicity and free radical scavenging activities of *Rhamnus alaternus* L. (*Rhamnaceae*) extracts. Pak. J. Biol. Sci., 8 (3): 439-445.

Ben Mansour H., Boubaker J., Bouhlel I., Mahmoud A., Bernillon S., Ben Chibani J., Ghedira K., Chekir-Ghedira L. (2007). Antigenotoxic activities of crude extracts from *Acacia salicina* leaves. Env. Mol. Mutagen., 48: 1-9.

Ben Mrabet K., Abed L. (1986). Quelques aspects de la pharmacopée traditionnelle algérienne. Pharmacien du Maghreb, spécial 2: 18-282.

Benavente-Garcia O., Casillo O., Marin F., Ortuno A., Del-Rio J. (1997). Uses and properties of *Citrus* flavonoids. J. Agric. Food Chem., 45: 4505-4515.

Bouhlel I., Ben Mansour H., Limem I., Ben Sghaier M., Mahmoud A., Ben Chibani J., Ghedira K., Chekir-Ghedira L. (2007). Screening of antimutagenicity via antioxidant activity in different extracts from the leaves of *Acacia salicina* from the center of Tunisia. Environ. Toxicol. Pharmacol., 23: 56-63.

Boukef K. (2001). *Rhamnus alaternus*. Essaydali, 81: 34-35.

Capecka E., Mareczek A., Leja M. (2005). Antioxidant activity of fresh and dry herbs of some Lamiaceae species. Food Chem., 93: 223-226.

Chevolleau S., Debal A., Ucciani E. (1992). Détermination de l'activité antioxydante d'extraits végétaux. Rev. Fr. Corps Gras, 39 (1/2): 3-8.

Chiang L.C., Chiang W., Liu M.C., Lin C.C. (2003). *In vitro* antiviral activities of *Caesalpinia pulcherrima* and its related flavonoids. J. Antimicrob. Chemother., 52: 194-198.

Conner D.E. (1993). Naturally occurring compounds. In: Davidson P., Branen A.L., Eds, Antimicrobials in Foods, Marcel Dekker Inc., New York, pp. 441-468.

Cragg G.M., Boyd M.R., Cardellina II J.H., Newman D.J., Snader K.M., McCloud T.G. (1994). Ethanobotany and drug discover: the experience of US National Cancer Institute. Ethanobotany and Search for New Drugs, Ciba Foundation Symposium 185, Wiley, Chichester, pp. 178-196.

Cremieux A. (1991). Perspectives méthodologiques dans l'évaluation de l'activité antibactérienne des huiles essentielles. In: Phytothérapie, Aromathérapie, Encycl. Med. Nat., Paris, C-4.

Dhidah M., Trabelsi A., Mzoughi R., Dhidah L., Boujaafar N., Jeddi M. (1995). *Salmonella* and salmonelloses in the region of Sousse (Tunisia). Microbiol. Hyg. Alim., 7 (19): 3-7.

Dorman H.J.D., Deans S.G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol., 88: 308-316.

Duh P.-D., Yen G.C. (1997). Antioxidative activity of three water extracts. Food Chem., 60: 639-645.

Eberhardt M.V., Lee C.Y., Liu R.H. (2000). Antioxidant activity of fresh apples, Nature, 405: 903-904.

Efthimiadou E.K., Thomadaki H., Sanakis Y., Raptopoulou C.P., Katsaros N., Scorilas A., Karaliota A., Psomas G. (2007). Structure and biological properties of the copper (II) complex with the quinolone antibacterial drug N-propyl-norfloxacin and 2,2'-bipyridine. J. Inorg. Biochem., 101 (1): 64-73.

Eloff J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med., 64: 711-713.

Hayder N., Ben Ammar R., Abdelwahed A., Kilani S., Mahmoud A., Ben Chibani J., Mariotte A.M., Ghedira K., Dijoux-Franca M.G., Chekir-Ghedira L. (2005). Antibacterial and antimutagenic activity of extracts and essential oil from (Tunisian) *Pistacia lentiscus*. Toxicol. Env. Chem., 87 (1-4): 567-573.

Hirano T., Gotoh M., Oka K. (1994). Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. Life Sci., 55: 1061-1069.

Ismail N.H., Ali A.M., Aimi N., Kitajima M., Takayama H., Lajis N.H. (1997). Anthraquinones from *Morinda elliptica*. Phytochemistry, 45 (8): 1723-1725.

Izhaki I., Tsahar E., Paluy I., Friedman J. (2002). Within population variation and interrelationships between morphology, nutritional content and secondary compounds of *Rhamnus alaternus* fruits. New Phytol., 156: 217-223.

Jemal A., Murray T., Samuels A., Ghafoor A., Ward E., Thun M. (2003). Cancer statistics, CA Cancer J. Clin., 53: 5-26.

Kim M.S., Lee J., Lee K.M., Yang S.H., Choi S., Chung S.Y., Kim T.Y., Jeong W.H., Park R. (2003). Involvement of hydrogen peroxide in mistletoe lectin-II-induced apoptosis of myeloleukemic U937 cells. J. Life Sci., 73: 1231-1243.

Kumar A., Chattopadhyay S. (2007). DNA damage protecting activity and antioxidant_potential of pudina extract. Food Chem., 100: 1377-1384.

Kuntz S., Wenzel U., Daniel H. (1999). Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. Eur. J. Nutr., 38 (3): 133-142.

Larocca L.M., Piantelli M., Leone G., Sica S., Teofili L., Benedetti-Panici P., Scambia G., Mancuso S., Capelli A., Ranelletti F. (1990). Type II oestrogen binding sites in acute lymphoid and myeloid leukemias: growth inhibitory effect of oestrogen and flavonoids. Br. J. Haematol., 75: 489-495.

Lee R.M., Hirano K.I., Anant S., Baunoch D., Davidson N.O. (1998). An alternatively spliced form of apobec-1 messenger RNA is over expressed in human colon cancer. Gastroenterology, 115 (5): 1096-1103.

Luximon-Ramma A., Bahorun T., Soobrattee M.A., Aruoma O. (2002). Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extract of *Cassia fistula*. J. Agric. Food Chem., 50 (18): 5042-5047.

Marzouk M.S., El-Toumy S.A.A., Merfort I., Nawwar M.A.M. (1999). Polyphenolic metabolites of *Rhamnus disperma*. Phytochemistry, 52 (5): 943-946.

Matsumoto S., Washizuka Y., Matsumoto Y., Tawara S., Ikeda F., Yokota Y., Karita M. (1997). Appearance of a metronidazole-resistant *Helicobacter pylori* strain in an infected-ICR-mouse model and difference in eradication of metronidazole-resistant and -sensitive strains. Antimicrob. Ag. Chemother., 41: 2602-2605.

Mead P.S., Slutsker L., Dietz V., McCaig L.F., Bresee J.S., Shapiro C., Griffin P.M., Tauxe R.V. (1999). Food-related illness and death in the United States. Emerg. Inf. Dis., 5: 607-625.

Musonda C.A., Chipman J.K. (1998). Quercetin inhibits hydrogen peroxide (H_2O_2)-induced NF-kappa B DNA binding activity and DNA damage in HepG2 cells. Carcinogenesis, 19: 1583-1589.

Nwabueze T.U. (2007). Effect of process variables on trypsin inhibitor activity (TIA), phytic acid and tannin content of extruded African bread fruit-corn-soy mixtures: A response surface analysis, LWT-Food Sci. Technol., 40: 21-29.

O'Brien S.J., de Valk H. (2003). *Salmonella* - "old" organism, continued challenges! Eurosurveillance, 8: 29-31.

Park B.S., Lee H.K., Lee S.E., Piao X.L., Takeoka G.R., Wong R.Y., Ahna Y.J., Kima J.H. (2006). Antibacterial activity of *Tabebuia impetiginosa* Martius ex DC (Taheebo) against *Helicobacter pylori*. J. Ethnopharmacol., 105: 255-262.

Pearson D. (1976). The Chemical Analysis of Foods, 7th edn. Churchill Livingstone, London.

Polydoro M., De Souza K.C.B., Andrades M.E., Da Silva E.G., Bonatto F., Heydrich J., Dal-Pizzol F., Schapoval E.E.S., Bassani V.L., Moreira J.C.F. (2004). Antioxidant, a pro-oxidant and cytotoxic effects of *Achyrocline satureioides* extracts. Life Sci., 74: 2815-2826.

Pottier-Alapetite G. (1978). Flore de la Tunisie, Imprimerie Officielle de la République Tunisienne, pp. 138-141.

Rodriguez Vaquero M.J., Alberto M.R., Manca de Nadra M.C. (2007). Antibacterial effect of phenolic compounds from different wines. Food Control, 18 (2): 93-101.

Rosell G. (1980). Aspectos farmacognósticos y farmacodinámicos de *Rhamnus alaternus* ssp. *myrtifolia* Will. II, Cir. Farm., 38 (269): 445-458.

Roussis V., Tsoukatou M., Chinou I., Ortiz A. (1998). Composition and antibacterial activity of the essential oils of *Helichrysum rupestre* and *Helichrysum ambiguum* growing in the Balnearic islands. Planta Med., 64: 675-676.

Sakanaka S., Juneja L.R., Taniguchi M. (2000). Antimicrobial effects of green tea polyphenols on thermophilic spore-forming bacteria. J. Biosci. Bioeng., 90 (1): 81-85.

Todd E.C.D. (1989). Costs of acute bacterial foodborne disease in Canada and the United States. Int. J. Food Microbiol., 9 (4): 313-326.

Tona L., Cimanga R.K., Mesia K., Musuamba C.T., De Bruyne T., Apers S., Hernans N., Von Miert S., Pieters L., Totté J., Vlietinck A.J. (2004). *In vitro* antiplasmodial activity of extracts and fractions from seven medicinal plants used in Democratic Republic of Congo. J. Ethnopharmacol., 93: 27-32.

Tona L., Kambu K., Ngimbi N., Cimanga K., Vlietnick A.J. (1998). Antiamoebic and phytochemical screening of some Congolese medicinal plants. J. Ethnopharmacol., 61: 57-65.

Uliasz T.F., Hewett S.J. (2000). A microtiter trypan blue absorbance assay for the quantitative determination of excytotoxic neuronal injury in cell culture, J. Neurosc. Meth., 100: 157-163.

Wink M. (1993). Production and application of phytochemicals from an agricultural perspective. In: van Beek T.A., Breteler H., Eds, Phytochemistry and Agriculture, Proc. Phytochem. Soc. Europe, Vol. 34, Clarendon, Oxford University, Press, pp. 171-213.

Yuan V.Y., Bone D.E., Carrington F. (2005). Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated *in vitro*. Food Chem., 91 (3): 485-494.

Zhishen J., Mengcheng T., Jianming W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64 (4): 555-559.