Int. J. Plant Bas. Pharm. (2022) 2(2), 210-219

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

https://ijpbp.com

RESEARCH ARTICLE

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Antihemolytic activity of hydroalcoholic leaves and bark extracts from *Rhamnus alaternus* against AAPH induced hemolysis on human erythrocytes

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ARTICLE INFO

Article History:

Received: 20 May 2022
Revised: 04 June 2022
Accepted: 04 June 2022
Available online: 06 June 2022

Edited by: B. Tepe

Keywords:
Rhamnus alaternus
Polyphenols
AAPH
Hemolysis
Antioxidant
Erythrocytes

ABSTRACT

Rhamnus alaternus is a Mediterranean shrub that has been used in traditional medicine to treat various diseases. This study aimed to determine the phenolic composition, as well as antioxidant and antihemolytic activities of *R. alaternus* leaves (LRA) and bark (BRA), extracts against AAPH-induced hemolysis. The extraction yields were 19.8% and 18.2% for leaves and bark. Total polyphenols (88.1 ± 1.83 mg GAE/g) and condensed tannins (36.24 ± 5.44 mg CE/g) were higher in BRA extract than in LRA extract (80.22 ± 1.4 mg GAE/g and 23.48 ± 0.25 mg CE/g, respectively). However, LRA extract was found to be richer in total flavonoids (64.6 ± 2.6 mg QE/g) and flavones/flavonol (18.34 ± 1.65 mg QE/g) than BRA extract (39.87 ±0.58 mg QE/g and 10.08 ± 0.35 mg QE/g), respectively. The IC_{50} of DPPH and ABTS radical scavenging activity were 86.59 ± 2 µg/ml and 12.49 ± 0.29 µg/ml for LRA extract and 69.23 ± 2.14 µg/ml and 12.83 ± 0.13 µg/ml for BRA extract, respectively. Also, both extracts showed good reducing power with 157.09 ± 5.53 mg Asc E/g for LRA extract and 194.97 ± 1.46 mg Asc E/g for BRA extract. The hemolytic effect was tested on human erythrocytes, and both extracts did not have cytotoxic effects at low doses. To induce hemolysis, AAPH was used at a concentration of 200 mM with an incubation time of 4h. The antihemolytic activity of the two extracts showed that pretreatment of human erythrocytes with various doses significantly reduced AAPH-induced hemolysis in a dose-dependent manner. Indeed, at 200 µg/ml, the percentages of hemolysis inhibition were 99.41 ± 1.17% and 76.26 ± 12.03% for BRA and LRA extracts, respectively. BRA extract was more effective (IC_{50} = 106.70 ± 1.48 µg/ml) compared to LRA extract (IC_{50} = 148.64 ± 7.04 µg/ml). Our results demonstrate for the first time that *R. alaternus* attenuates AAPH-induced hemolysis and can be used to prevent and treat hemolytic anemias.

1. Introduction

Medicinal plants are the primary reservoir of indigenous medical systems. Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve plant extracts (Abate, 2019). Nowadays, plants are of increasing interest due to their content of secondary metabolites known to have antioxidant activity and potency that can protect the body's cells from oxidative stress and free radical damage (Chansiw et al., 2018). Indeed, cells contain antioxidant systems that protect them from deleterious effects and regulate the generation of free radicals through diverse mechanisms (Fibach and Rachmilewitz, 2008). However, these systems can be overwhelmed in case of excess free radicals.

Oxidative stress is involved in apoptosis and cellular aging but also hemolytic anemia. The latter is an indicator of free radical damage to the red blood cell membrane (RBCs), which antioxidants can help prevent (Chansiw et al., 2018). Anemia can result from physiological hemolysis, defined as the destruction of senescent RBCs after chemical and mechanical stresses that progressively damage them during their 120 days life span. This phenomenon increased hemoglobin catabolism, loss of membrane proteins, and altered ion transport. Macrophages remove old RBCs from the bloodstream (Badior and Casey, 2018). Moreover, anemia most commonly presents as pathological hemolysis due to alterations in the quantity

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e-ISSN: 2791-7509
doi: https://doi.org/10.29228/ijpbp.3

Please cite this article as: Kherbachi, S., Kheniche, M., Tacherfiout, M., 2022. Antihemolytic activity of hydroalcoholic leaves and bark extracts from Rhamnus alaternus against AAPH induced hemolysis on human erythrocytes. International Journal of Plant Based Pharmaceuticals, 2(2), 210-219, https://doi.org/10.29228/ijpbp.3.

and/or quality of proteins involved in maintaining the RBC membrane's properties, resulting in their increased and premature destruction. It may have a corpuscular or extracorporeal origin due to chronic, hereditary, or acquired destructions (Phillips and Henderson, 2018; Rai et al., 2020).

The polyunsaturated fatty acids of the membrane, the oxygen-rich environment, and the iron-rich hemoglobin make RBCs susceptible to oxidative damage. In RBC, hemoglobin is a major source of superoxide production. There is an electron transfer in the interaction between the heme iron and oxygen in oxygenated hemoglobin. The heme iron generally stays in the Fe(II) ferrous form when hemoglobin oxygenate. Alterations in this exchange, hemoglobin auto-oxidizes, resulting in methemoglobin and superoxide production (Fibach and Rachmilewitz, 2008). The denaturation of hemoglobin induced the formation of the Heinz body responsible for the rigidity of erythrocytes and thus their destruction (Drobatz et al., 2018). Furthermore, superoxide radical ($O_2^{\cdot}$) can dismutase spontaneously or enzymatically to hydrogen peroxide (H_2O_2), generating reactive hydroxyl radical ($OH^{\cdot}$). Consequently, ROS leads to the loss of membrane erythrocytes integrity by initiating lipid peroxidation (Fibach and Dana, 2019). Different aldehydes are formed as secondary oxidation products of lipid peroxidation, among them malondialdehyde (MDA) which leads to cross-links of phospholipids and proteins in the erythrocyte membrane. Indeed, MDA accumulation can affect anion transport and the functions of some enzymes. The processes alter membrane-related functions that ultimately lead to RBC hemolysis (Çimen, 2008; Ayala et al., 2014).

Rhamnus alaternus, commonly known as Buckthorn, belongs to the Rhamnaceae family. It is generally distributed throughout the Mediterranean countries. In North Africa, it grows in Algeria, Morocco, and northern Tunisia (Nekkaa et al., 2021). This plant is one species used in reforestation programs due to its ability to survive in xeric environments. In traditional medicine, the leaves are used as a purgative, laxative, and to treat jaundice (Moussi et al., 2015). In North African countries, the bark is used to cure various dermatological problems and treat diabetes (Nekkaa et al., 2021). *R. alaternus* crude extracts had powerful antimutagenic action as well as strong inhibition of xanthine oxidase. In addition, antioxidant, antiradical, antiproliferative, and cytotoxic properties were also demonstrated (Ben Ammar et al., 2007; Ben Ammar et al., 2008). Kosalec et al. (2013) reported that *R. alaternus* bark extracts contain anthraquinones and other phenols that act as multifunctional antioxidants with antimicrobial activity. While Benchiha et al. (2017) demonstrated that *R. alaternus* leaves extract has potent hepatoprotective properties against the xenobiotic carbon tetrachloride (CCl_4). According to our previous study, flavonoids obtained from *R. alaternus* leaves have an antihyperlipidemic effect on rats induced hyperlipidemic by Triton WR-1339 and on HepG2 cells culture (Tacherfiout et al., 2018).

Leaves and bark of *R. alaternus* are commonly used in an infusion to cure anemia in Algeria's Kabylia region. To our knowledge, no research has been conducted to determine whether *R. alaternus* possesses anti-hemolytic properties. Therefore, this work aimed to investigate the antihemolytic activity of *R. alaternus* leaves and bark extracts on human erythrocytes in a model of AAPH-induced hemolysis.

2. Materials and methods

2.1. Plant material

R. alaternus leaves and bark were harvested in May 2021 from Amizour, Bejaia, Algeria (GPS coordinates: 36°39'50'' North, 4°55'19'' East). They were separated, cleaned, rinsed, and dried for three days in the shade at room temperature before placing a 40 °C for 48 hours to enhance the drying process. Each part was ground in an electric grinder to have homogeneous powder (Ø = 125µm) kept in tightly sealed containers in the dark until extraction.

2.2. Preparation of the crude hydroalcoholic extracts

Fifty grams of each powder was mixed with 500 ml of an ethanol-water mixture (70/30: v/v) and kept under magnetic stirring at room temperature for 24 hours. Then, both mixtures were filtered three times through Whatman paper. The ethanol was completely evaporated using a rotary evaporator, and both extracts were placed in a laboratory oven at 40 °C for 48h to remove water traces. The extraction yield was calculated relative to the initial catch.

2.3. Phytochemical analysis

2.3.1. Determination of total polyphenol content

The total phenolic content of crude leaves and bark hydroalcoholic extracts was determined by Folin-Ciocalteu, which is reduced in the blue complex in the presence of phenols (Wolfe et al., 2003). A volume of 1.25 ml of Folin-Ciocalteu solution (0.1 N in distilled water) was mixed with 250 µl of each extract and then allowed to stand for 5 minutes. Afterward, 1 ml of 7.5% (w/v) sodium carbonate solution was added, and the absorbance was read at 720 nm in a spectrophotometer after 90 minutes of incubation in the dark. The concentration of total phenolic components in both extracts was expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g extract) using gallic acid as a calibration standard.

2.3.2. Determination of total flavonoid content

The total flavonoid content was determined using the aluminum trichloride ($AlCl_3$) method (Quettier-Deleu et al., 2000). Briefly, 1 ml of 2% aluminum trichloride solution (w/v) was mixed with the same volume of each extract. Absorption readings at 435 nm were taken after 15 minutes of incubation in the dark. Quercetin was used to prepare a standard curve, and the total flavonoid contents were expressed as milligram quercetin equivalent per gram of extract (mg QE/g).

2.3.3. Determination of flavone and flavonol content

The content of flavones and flavonols was determined according to the method described by Kosalec et al. (2004). 250 µl of the two hydroalcoholic extract solutions were mixed with 1.4 ml of distilled water, 750 µl of ethanol (95%), 50 µl of 10% $AlCl_3$ solution (w/v), and 50 µl of sodium acetate (1 M). The mixture was homogenized, and the absorbance was read at 438 nm after incubation for 15 minutes in the dark. In a calibration curve containing quercetin, the flavones and flavonol concentrations in the hydroalcoholic extracts were measured as milligram equivalents of quercetin per gram of extract (mg EQ/g).

2.3.4. Determination of condensed tannins content

The vanillin method described by Chaouche et al. (2020) was used to evaluate the content of condensed tannins in each extract. In brief, 1.5 ml of 4% (w/v) vanillin solution in methanol was mixed with 250 µl of each extract, and then 750 µl of sulfuric acid was added. The reaction mixture was allowed for 15 minutes, and the absorbance was recorded at 500 nm. A dilution series of catechin was prepared for a standard curve, and the results were expressed as milligram equivalent of catechin per gram of extract (mg EC/g).

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging activity

The free radical scavenging activity of *R. alaternus* leaves and bark hydroalcoholic extracts was determined by the 2,2-diphényl-1-picrylhydrazyl (DPPH) method described by Athamena et al. (2010). A volume of 50 µl of DPPH solution (5 mM in methanol) was combined with 2.45 ml of each extract at different concentrations. The mixture was shaken and then incubated for 30 minutes in the dark, and the absorbance was then measured at 517 nm. Ascorbic acid was used as standard, and the percentages of DPPH radical scavenging activity were calculated using the following formula:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [Ac - As) / Ac] \times 100$$

Ac: Absorbance of control containing only DPPH

As: Absorbance of the sample containing DPPH and extracts or standard

2.4.2. ABTS radical scavenging activity

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical cation ($ABTS^{\cdot+}$) was produced by dissolving 7 mM ABTS in 2.45 mM potassium persulfate and keeping the solution in the dark for 16 hours. The ABTS stock solution was then diluted with ethanol to get an absorbance of 0.700 ± 0.02 at 734 nm. A volume of 950 µl of this diluted $ABTS^{\cdot+}$ solution is mixed with 50 µl of each extract at different doses. The absorbance reading was registered at 734 nm after 7 minutes of incubation in the dark (Re et al., 1999). Trolox was used as standard, and the following formula was used to determine the percentages of ABTS radical scavenging activity:

$$ABTS\ radical\ scavenging\ activity\ (\%) = [Ac - As) / Ac] \times 100$$

Ac: Absorbance of control containing only ABTS solution

As: Absorbance of the sample containing ABTS solution and extracts or standard

2.4.3. Reducing power

The reducing ability of the extracts was evaluated according to the method described by Kosalec et al. (2013). A volume of 500 µl of phosphate buffer (0.2 M, pH 6.6) and 500 µl of potassium ferricyanide 1% (w/v) were mixed with 250 µl of each extract. This mixture was kept at 50 °C in bath water for 20 minutes before cooling and adding 500 µl of 10% (w/v) trichloroacetic acid solution. After shaking, 500 µl was taken and combined with 500 µl of distilled water and 100 µl of 0.1% (w/v) ferric chloride solution. Absorbance was immediately read at 700 nm, and ascorbic acid was used to generate the calibration curve. Reducing power was expressed as milligram ascorbic acid equivalent per gram of extract (mg Asc E/g).

2.5. Antihemolytic activity on human erythrocytes

2.5.1. Preparation of erythrocyte suspension

Blood samples were obtained from healthy volunteers provided by the CTS (Centre de Transfusion Sanguine = Blood Transfusion Center, Bejaia, Algeria). Erythrocyte suspension was prepared according to the protocol described by Yang et al. (2017). Blood was centrifuged in heparinized tubes at 3000 rpm for 10 minutes. The supernatant was removed, and the pellet containing red blood cells was washed three times with phosphate buffer saline pH 7.4 (PBS) and centrifuged at 3000 rpm for 10 minutes. The erythrocyte pellet was resuspended at 10% hematocrit in PBS buffer and immediately used.

2.5.2. Hemolytic activity of extracts on human erythrocyte suspension

The hemolytic activity of leaves and bark extracts was assessed by mixing 250 µl of the freshly prepared erythrocyte suspension (10% hematocrit) with a volume of 500 µl of PBS (negative control), or 500 µl of each extract at different doses (25-1500 µg/ml). The samples were incubated for 4 hours at 37 °C in a water bath. The tubes were gently mixed every hour. A positive control was made by incubating the erythrocytes in 500 µl of distilled water simultaneously and under the same conditions (considered as 100% hemolysis). The volume of all tubes was then adjusted to 4.5 ml with PBS and centrifuged for 10 minutes at 2000 rpm. Hemolysis was determined by measuring absorbance at 540 nm, corresponding to hemoglobin release (Rafat et al., 2010). Hemolysis rates were calculated using the following formula:

$$Hemolysise\ rate\ (\%) = [(A_{extract} - A_{negative\ control} / A_{positive\ control}] \times 100$$

$A_{extract}$: Absorbance of hemoglobin at 540 nm in tubes treated with leaves and bark extracts

$A_{negative\ control}$: Absorbance of hemoglobin at 540 nm in tubes treated only with PBS

$A_{positive\ control}$: Absorbance of hemoglobin at 540 nm in tubes treated with distilled water

2.5.3. Optimization of hemolysis conditions of human erythrocytes by AAPH

AAPH was used in the present work to generate free radicals and oxidative stress, which could damage the RBC membrane and cause hemolysis. Briefly, 250 µl of erythrocyte suspension (10% hematocrit) was incubated at 37 °C in a water bath with 500 µl of AAPH dissolved in PBS at different final concentrations (100, 200, and 300 mM) for 4 hours. Every 60 minutes, the reaction mixture was gently stirred, and aliquots of the reaction mixture (100 µl) were removed and diluted with 500 µl of PBS. After centrifugation at 2000 rpm for 10 minutes, the absorbance of the supernatant was measured at 540 nm, and the percentages of hemolysis were then calculated as the previous formula (Phrueksanan et al., 2014).

2.5.4. Antihemolytic effects of the extract on AAPH induced hemolysis

The antihemolytic activity of the *R. alaternus* leaves and bark extracts was evaluated according to a previously published method (Yang et al., 2017). The erythrocyte suspension (hematocrit 10%) was pre-treated with different concentrations of the two extracts (50 - 200 µg/ml) for 30 minutes in a water bath at 37 °C. After that, a volume of 500 µl of an AAPH solution was added to have a final concentration of 200 mM, and the reaction mixture was incubated for 4 hours under the same conditions. Then, the volume of all tubes was adjusted to 4.5 ml with PBS and centrifuged at 2000 rpm for 10 minutes. The absorbance of the supernatant was read at 540 nm. Under the same conditions and simultaneously, two controls (negative and positive) were performed. Ascorbic acid was used as standard. The hemolysis inhibition percentages of extracts were calculated as follows.

$$Hemolysis\ inhibition\ (\%) = [(A_{AAPH} - A_{sample}) / A_{AAPH}] \times 100$$

A_{AAPH}: Absorbance at 540 nm in tubes treated only with 200 mM of AAPH

A_{sample}: Absorbance at 540 nm in tubes treated with 200 mM of AAPH and extracts or standard

2.6. Statistical analysis

All data values were expressed as mean ± standard deviation. All measurements were replicated three times. Statistical analyses were performed using Student's t-test or One-way ANOVA test followed by Tukey's post hoc test using Graphpad Prism 6.0 software. The results were considered statistically significant at $p < 0.05$. Determination of IC_{50} was performed using software Origin 9.5.

3. Results and discussion

3.1. Quantification of phytochemical compounds

The extraction yields of leaves and bark of *R. alaternus* using 70% ethanol were calculated concerning the initial dry matter. The results showed extraction yields of 19.8% for the leaf extract and 18.2% for the bark extract. The quantification of phytochemicals was assessed by colorimetric methods, and the results are reported in Table 1.

The results showed that leaves (LRA) were less rich in total phenolic contents than bark (BRA), with respective amounts of 80.22 ± 1.4 and 88.15 ± 1.83 mg GAE/g dry extract. These results were reported in mg gallic acid equivalent from the calibration curve equation (y = 0.0561x + 0.0093 with R^2=0.9916). There is a significant difference ($p < 0.05$) between the total polyphenol contents of the two extracts.

Table 1. Quantification of various phenolic compounds contents in hydroalcoholic leaves (LRA) and bark (BRA) extracts of *R. alaternus*

Phenolic compounds	LRA extract	BRA extract
Total polyphenols (mg GAE/g)	80.22 ± 1.4^a	88.15 ± 1.83^b
Total flavonoids (mg QE/g)	64.6 ± 2.6^a	39.87 ± 0.58^b
Flavones and flavonols (mg QE/g)	18.34 ± 1.65^a	10.08 ± 0.35^b
Condensed tannins (mg CE/g)	23.48 ± 0.25^a	36.24 ± 5.44^b

The mean and standard deviation (SD) are used to express the data ($n = 3$). The statistical analysis was determined using the Student's t-test. Values not sharing a common letter for the same type of compound were statistically different ($p < 0.05$).

In contrast, for the total flavonoid content of the two hydroalcoholic extracts, a higher level was obtained in leaves extract (LRA) (64.6 ± 2.6 mg QE/g dry extract) compared to the bark extract (BRA) (39.87 ± 0.58 mg QE/g dry extract). The results were expressed as mg quercetin equivalent based on a calibration curve that follows an equation y = 0.0428x - 0.028, with R^2 = 0.9895. From these findings, it was shown that there is a significant difference ($p < 0.05$) between the amount of total flavonoid content in the two extracts.

The flavone and flavonol content of leaves extract (LRA) was almost twice as high as that of the bark extract (BRA), with 18.34 ± 1.65 mg QE/g dry extract compared to 10.08 ± 0.35 mg EQ/g dry extract. The calibration curve was created using quercetin and yielded the following equation: y = 0.0712x + 0.0345 with R^2 = 0.9938. Statistical analysis of flavone and flavonol contents between the two extracts reveals a significant difference ($p < 0.05$).

Finally, the quantification of condensed tannins from the calibration curve performed using different concentrations of catechin (y = 0.0771x + 0.0528, with R^2 = 0.99633) showed that leaves extract (LRA) has a lower amount with a value of 23.48 ± 0.25 mg CE/g dry extract, than the bark extract (BRA) which had given a rate of 36.24 ± 5.44 mg CE/g dry extract. There is a significant difference ($p < 0.05$) in the condensed tannins contents between the two extracts.

3.2. Antioxidant activities

3.2.1. DPPH free radical scavenging activity

Leaves and bark extracts of *R. alaternus* were evaluated for their ability to quench DPPH radicals. As shown in Figure 1, both extracts' DPPH radical scavenging activity is dose-dependent. In fact, at the highest concentration tested (125 µg/ml), leaves (LRA) and bark (BRA) extracts exhibited potent scavenging activities with percentages of 73.18 ± 0.65% and 81.21 ± 1.5%, respectively. The DPPH free radical scavenging activity of the two extracts was statistically different ($p < 0.05$). As can be seen in Table 2, the bark extract showed the highest antioxidant activity with an IC_{50} = 69.23 ± 2.14 µg/ml, compared to 86.59 ± 2 µg/ml for leaves extract. Ascorbic acid used as standard gave IC_{50} = 1.04 ± 0.10 µg/ml.

3.2.2. ABTS free radical scavenging activity

The antioxidant ability of both extracts toward the ABTS radical demonstrates that leaves and bark hydroalcoholic extracts are effective even at low doses (Figure 2). A considerable scavenging effect of the ABTS radical was observed at 22.5 µg/ml, with percentages of 70 ± 1.39% for leaves extract and 75.88 ± 0.72% for the bark extract. The ABTS radical scavenging activities of the two extracts were significantly different ($p < 0.05$), except at the 12.5 µg/ml concentration, where the difference was not significant. Moreover, this high scavenging activity of the ABTS radical of both

extracts is illustrated by the IC_{50} values obtained; 12.49 ± 0.29 µg/ml for leaves extract and 12.83 ± 0.13 µg/ml for bark extract (Table 2). Trolox used as a standard in this test gave an IC_{50} of 8.38 ± 0.08 µg/ml.

3.2.3. Reducing power

Ascorbic acid was used as the standard to create the calibration curve following an equation: y = 0.044x + 0.3494 with R^2 = 0.9984. Thus, ascorbic acid equivalents in mg/g extract were used to represent the reducing capacity of *R. alaternus* leaves and bark extracts. Table 2 showed that both extracts have a good reducing capacity, with a slight advantage for the bark extract, which gave a reducing power of 194.97 ± 1.46 mg Asc E/g, compared to leaves extract (157.09 ± 5.53 mg Asc E/g). There was a significant difference between the reducing powers of the two extracts (p < 0.05).

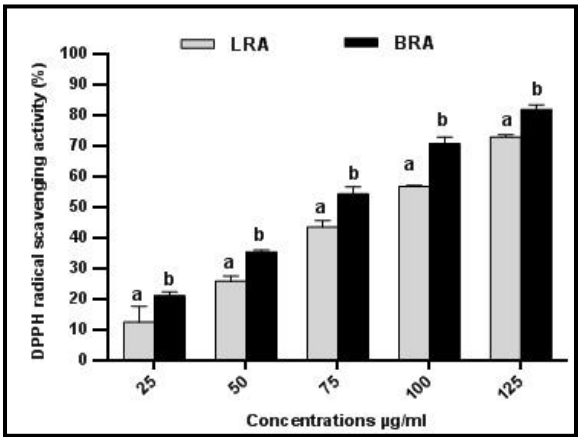

Figure 1. DPPH radical scavenging activity of leaves (LRA) and bark (BRA) extracts (25-125 µg/ml) of *R. alaternus*
Values are mean ± SD (n = 3). The Student's t-test was used to determine the statistical significance of the data. Values not sharing a common letter for the same concentration were statistically different (p < 0.05).

Table 2. Reducing capacity of *R. alaternus* leaves (LRA) and bark (BRA) hydroalcoholic extracts and IC_{50} values for DPPH and ABTS tests

Extract	Reducing power (mg Asc E/g)	DPPH (µg/ml)	ABTS (µg/ml)
LRA	157.09 ± 5.53^a	86.59 ± 2.00^a	12.49 ± 0.29^a
BRA	194.97 ± 1.46^b	69.23 ± 2.14^b	12.83 ± 0.13^a
Ascorbic acid	-	1.04 ± 0.10^c	-
Trolox	-	-	8.38 ± 0.08^b

The mean and standard deviation (SD) are used to express the data (n = 3). The statistical analysis was determined using the Student's t-test. Values not sharing a common letter for the same type of compound were statistically different (p < 0.05).

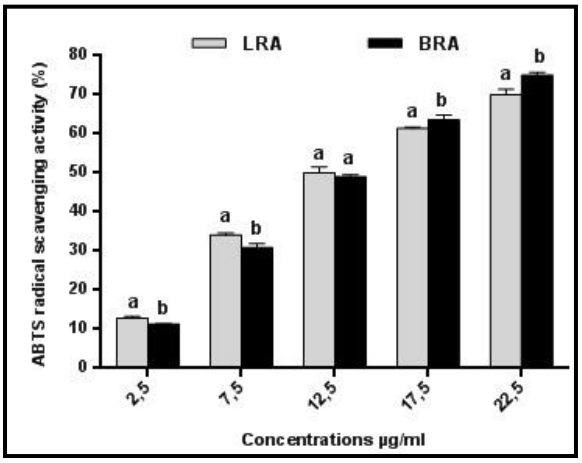

Figure 2. ABTS radical scavenging activity of leaves (LRA) and bark (BRA) extracts (2.5-22.5 µg/ml) of *R. alaternus*
Values are mean ± SD (n = 3). The Student's t-test was used to determine the statistical significance of the data. Values not sharing a common letter for the same concentration were statistically different (p < 0.05).

3.3. Cytotoxicity of R. alaternus leaves and bark extracts on human erythrocytes

The hemolytic activity of different concentrations of leaves and bark extracts was tested on human erythrocytes (Figure 3). Statistical analysis showed a significant difference (p < 0.05) between hemolysis rates induced by the two extracts and complete hemolysis induced by H_2O. The results demonstrate that leaves extract (LRA) induced a very low cytotoxicity potential at concentrations ranging from 25 to 1000 µg/ml. Indeed, hemolysis rates were 1.06 ± 0.98% at 25µg/ml and 0.71 ± 0.75% at 1000 µg/ml. However, at a 1500 µg/ml concentration, the hemolysis rate rises to roughly 5%. On the other hand, hemolysis rates for the bark extract (BRA) were very low at doses ranging from 25 µg/ml (0.09 ±

0.28%) to 500 µg/ml (3.18 ± 0.24%), but increased to 5.5 ± 1.18% at 750 µg/ml, 7.65 ± 0.81% at 1000 µg/ml and 14.9 ± 0.18% at 1500 µg/ml.

3.4. Optimization of hemolysis conditions of human erythrocytes by AAPH

Human erythrocytes were incubated at 37 °C as a 10% hematocrit suspension with AAPH at different final concentrations of 100, 200, and 300 mM. Hemolysis rates were then monitored hourly by measurement at 540 nm of hemoglobin released into the incubation medium. Figure 4 showed that AAPH induced hemolysis in a time and concentration-dependent manner. The onset of hemolysis occurred after 1 h of incubation (16.26 ± 2.75%) for the 300 mM concentration, after 2 h of incubation (31.41 ± 1.52%) for the 200 mM concentration, and after 3 h of incubation (11.42 ± 7.02%) for the 100 mM concentration. In these experimental conditions, the hemolysis was markedly induced after 4 h of incubation at 200 mM and 300 mM with respective percentages of 45 ± 1.25% and 49.24 ± 4.15%. Therefore, AAPH at 200 mM and the incubation period of 4 h were selected for hemolysis conditions in this study.

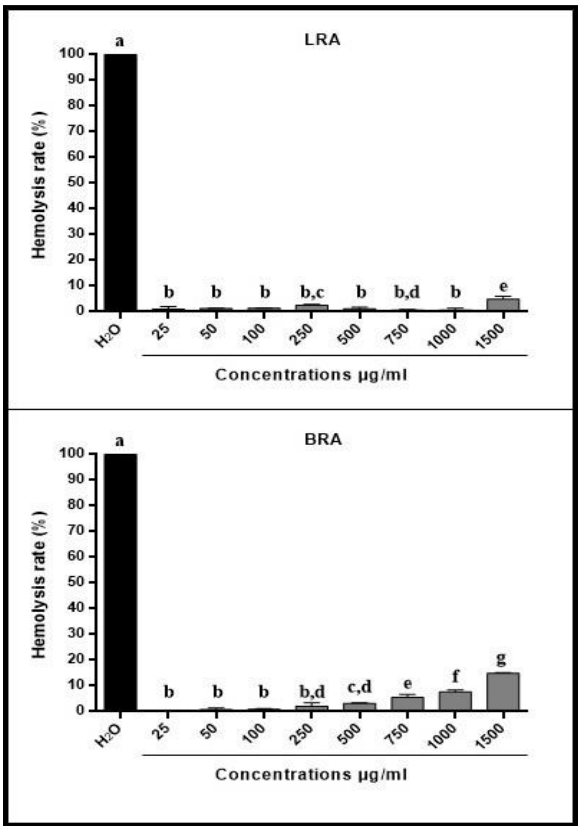

Figure 3. Hemolysis rates induced on human red blood cells (10% hematocrit) by various doses of *R. alaternus* leaves and bark extracts (25–1500 µg/ml)

Values are expressed as mean ± standard deviation (n = 3). Differences between means were statistically tested by one-way ANOVA test, followed by Tukey's post hoc test. Values not sharing a common letter were statistically different ($p < 0.05$).

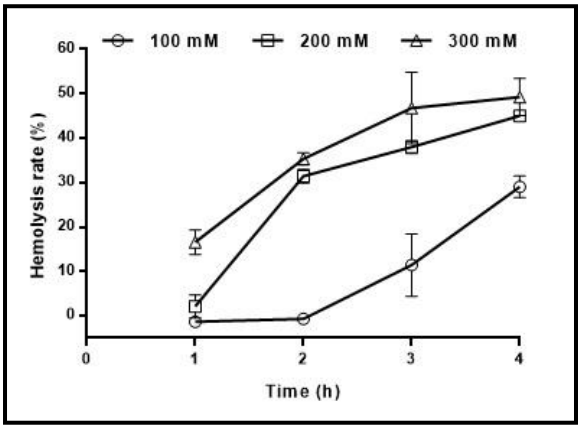

Figure 4. Time-dependent variations in hemolysis rates induced by different final concentrations of AAPH (100, 200, and 300 mM) on human red blood cells

Results are expressed as mean ± standard deviation (n = 3).

3.5. Antihemolytic activity of leaves and bark extracts against AAPH-induced hemolysis on human erythrocytes

The antihemolytic activity of LRA and BRA was assessed by measuring the hemolysis degree induced in human erythrocytes by AAPH-generated free radicals. Pretreatment of human red blood cells by different doses (50-200 µg/ml) of the two extracts of *R. alaternus* significantly attenuated the AAPH-induced hemolysis concentration-dependent (Figure 5). The results clearly show that the BRA was more efficient than LRA extract in reducing AAPH-induced hemolysis, especially at concentrations of 150 and 200 µg/ml. Indeed, the hemolysis percentages inhibition for the bark extract were 91.91 ± 1.77% and 99.4 ± 1.17% (almost 100% inhibition) at the concentrations of 150 and 200 µg/ml, respectively, against 52.69 ± 1.75% and 76.26 ± 12.02% for leaves extract at the same concentrations. Whereas 100 µg/ml of ascorbic acid was used as a standard, giving a percentage inhibition of 34.75 ± 3.7 % (Table 3). This strongest antihemolytic activity of the bark extract compared to leaves extract is illustrated by the IC_{50} values obtained (concentrations inducing 50% inhibition of AAPH-induced hemolysis). The bark extract had an IC_{50} = 106.70 ± 1.48 µg/ml, while leaves extract had an IC_{50} = 148.64 ± 7.04 µg/ml. Data statistical analysis revealed no significant variations in hemolysis rates between leaves extract concentrations of 50, 100 µg/ml, and ascorbic acid. However, in the presence of different doses of bark extract, ascorbic acid, APPH, and positive control (100% hemolysis), there is a statistically significant difference in hemolysis rates (p < 0.05).

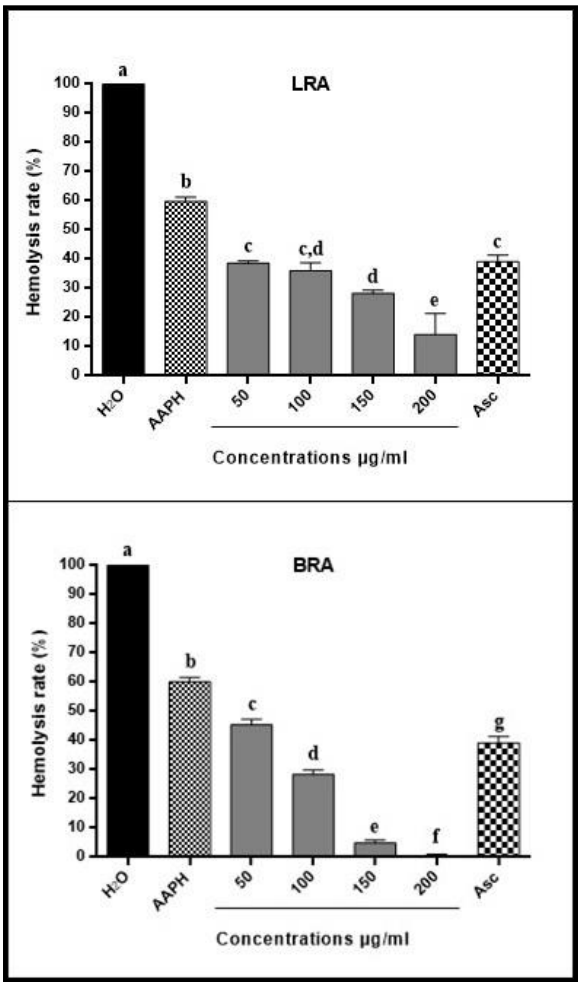

Figure 5. Antihemolytic effect of leaves (LRA) and bark (BRA) extracts from *R. alaternus* at different concentrations against AAPH-induced hemolysis on human erythrocytes

Values are expressed as mean ± standard deviation (n = 3). Differences between means were statistically tested by one-way ANOVA test, followed by Tukey's post hoc test. Values not sharing a common letter were statistically different (p < 0.05).

Table 3. Inhibition rates and IC_{50} values of AAPH induced hemolysis after pretreatment of human erythrocytes with different concentrations of leaves (LRA) and bark (BRA) extracts of *R. alaternus*

Concentration (µg/ml)	LRA hemolysis inhibition (%)	BRA hemolysis inhibition (%)
50	35.4 ± 1.32^{a}	24.49 ± 3.11^{b}
100	39.76 ± 4.59^{a}	52.78 ± 2.51^{b}
150	52.69 ± 1.75^{a}	91.91 ± 1.77^{b}
200	76.26 ± 12.02^{a}	99.4 ± 1.17^{b}
Ascorbic acid 100 µg/ml	34.75 ± 3.7	
IC_{50}	148.64 ± 7.04^{a}	106.70 ± 1.48^{b}

Data are expressed as mean ± standard deviation (n = 3). IC_{50}: Concentration of test substances reducing the cell viability by 50%. The statistical analysis was determined using the Student's t-test. Values not sharing a common letter for the same concentration were statistically different (p < 0.05).

Plants include various bioactive natural compounds used in traditional medicine for their healing power (Kumar et al., 2011). In this context, the purpose of this study was to assess the phytochemicals content, antihemolytic and antioxidant activities of *R. alaternus* hydroalcoholic leaves and bark extracts. Our findings demonstrate that different extraction yields and variable contents of various phenolic compounds were obtained compared to earlier investigations. The harvesting period, geographical location, and meteorological and experimental circumstances all play a role in these variances (Bouchenak et al., 2020). Furthermore, various parameters such as pH, temperature, maceration time, solid/liquid ratio, powder granulometry, the chemical structure of the compounds, and solvent nature might influence the extraction. Indeed, for the extraction of usually polar phenolic compounds, various polar solvents such as ethanol, methanol, and acetone are frequently utilized (Benmeziane et al., 2014; Bouchenak et al., 2020). In addition, the solvent can influence the permeability of plant cells by chemical and biophysical changes, and its efficiency is mostly determined by its capacity to dissolve phenolic groups (Oreopoulou et al., 2019). In this study ethanol was used because it solubilizes medium polar phenolic compounds, and the addition of water to the extraction system improves the yield of glycosylated and highly polymerized phenolic compounds (Nga et al., 2019). It is also preferred since it is less toxic and environmentally friendly (Cheok et al., 2014).

Results showed that bark extract of *R. alaternus* contains more total polyphenols than leaf extract. This may be due to the part of the plant studied, as phenolic compounds are distributed differently throughout the different plant organs (Bouchenak et al., 2020). Ben Ammar et al. (2007) and Chaouche et al. (2020) reported that total polyphenol levels respectively of 138 mg GAE/g extract for methanolic extract and 1.68 mg GAE/g extract for methanol/acetone extract of *R. alaternus* leaves. However, total polyphenol contents reported by Chaouche et al. (2020) for methanol/acetone extract and Kosalec et al. (2013) for the methanolic extract from the bark of *R. alaternus* were ranged between 0.64 mg EAG/g extract and 38.4 mg EAG/g extract, respectively. These variations can be explained by the origin and harvesting period of the plant, the type of solvent used, drying duration as well as the extraction method (Benchaachoua et al., 2018; Chaouche et al., 2020). This also could be due to the Folin-Ciocalteu reagent's limitation, which provides a crude assessment of all phenolic chemicals and can be reduced by other molecules such as reducing sugars and proteins (Bessada et al., 2015).

Flavonoids are a class of polyphenols that are widely distributed throughout the plant kingdom and in all parts of higher plants (Benchaachoua et al., 2018). They have a specific structure that includes the positions of hydroxyl and carbonyl groups as well as a double bond, which results in flavone and flavonol derivatives (Ahmed et al., 2019). In this study, the contents of total flavonoids, flavones, and flavonols were predominantly higher in the leaves extract than in the bark extract. These results are confirmed by Ben Ammar et al. (2007) and Kosalec et al. (2013), who had found flavonoid concentrations of 283 mg QE/g and 33.6 mg QE/g, respectively, in methanolic leaves and bark extracts. This can be explained by the difference in the distribution of flavonoids during plant development and because flavones and flavonols are mainly concentrated in the plant's leaves due to their high exposure to sunlight (Benchaachoua et al., 2018; Ahmed et al., 2019).

Moreover, both leaves and bark extracts contain a significant amount of condensed tannins, with the bark extract having a higher level than the leaves (36.24 ± 5.44 and 23.48 ± 0.25 mg CE/g extract, respectively). However, Ben Ammar et al. (2007) found 736 mg TAE/g extract for the methanolic leaves extract, and Chaouche et al. (2020) found 3 mg CE/g extract for the methanol/acetone bark extract. This discrepancy could be related to the structure of condensed tannins and their solubility, which is influenced by several factors, including the plant's developmental region and the solvent utilized (Elgailani and Ishak, 2016; Chaouche et al., 2020).

Three methods were used to assess the antioxidant activities of the two extracts: DPPH and ABTS radical scavenging activities, as well as reducing power. For the first two procedures, the data are expressed in IC_{50}, the concentration of extracts required to quench 50% of the DPPH and ABTS radicals, and in equivalent ascorbic acid per gram of extract for the reducing power method. DPPH assay showed that bark extract (IC_{50} = 69.23 ± 2.14 µg/ml) had a higher ability to quench the DPPH radical than leaves extract (IC_{50} = 86.59 ± 2.00 µg/ml). In comparison, previous studies showed higher DPPH radical scavenging activity with an IC_{50} = 58 ± 0.007 µg/ml for the ethanolic leaves extract (Zeouk et al., 2020) and lower activity with an IC_{50} = 78.7 ± 3.16 µg/ml for the methanolic bark extract (Kosalec et al., 2013). The difference in DPPH radical quenching between the two extracts could be due to the nature of the phenolic compounds contained in each extract, which can interact in various ways to reduce free radicals (Abate, 2019). The diversity of our results compared to those reported later could be due to the nature of the solvent used and the solvent/water ratio, which significantly impact the total antioxidant activity of the extracts and favor the extraction of potent antioxidants (Benchaachoua et al., 2018). Furthermore, leaves and bark extracts were found to have similar powers to quench the ABTS free radical (IC_{50} = 12.49 ± 0.29 and 12.83 ± 0.13 µg/ml for leaves and bark extracts, respectively). This can be explained by the low sensitivity of the ABTS assay, which cannot distinguish between various bioactive compounds found in plants (Chaves et al., 2020). The difference in results between the DPPH and ABTS assays can be explained by the variety of antioxidant chemicals found in both extracts, which respond differently depending on the radical present. One downside of ABTS is that the produced free radicals are not very stable (Shah and Modi, 2015).

The bark extract (194.97 ± 1.46 mg Asc E/g) was shown to be more powerful than the leaves extract (157.09 ± 5.53 mg Asc E/g) in the reducing power test. The amount of electron-donating phenolic compounds capable of inhibiting the free radical chain reaction may be proportionate to the extracts' reducing capacity (Kosalec et al., 2013). The reducing ability of bioactive compounds is also influenced by their chemical structures, such as the number and placement of hydroxyl groups concerning electron-withdrawing carboxyl groups (Enneb et al., 2015).

The erythrocytes are frequently used as a biological model to assess the antihemolytic and antioxidant potential of different compounds and plant extracts because of their high sensitivity to oxidative stress due to their membrane lipids rich in polyunsaturated fatty acids. Indeed, they are the first target of free radical attack due to their potential as a producer of reactive oxygen species (ROS), as well as the redox reactions of hemoglobin associated with oxygen transfer (Hmidani et al., 2021; Derouich et al., 2020). Many bioactive molecules present in plants can affect biological membranes due to their cytotoxicity (Ramchoun et al., 2015). In the present case, the hemolytic activity of *R. alaternus* leaves and bark extracts was evaluated on human red blood cells. Leaves extract showed no cytotoxic effect at concentrations ranging from 25 to 1000 µg/ml. In contrast, the bark extract caused hemolysis at concentrations greater than 500 µg/ml. At a 1500 µg/ml concentration, this hemolysis rate was around 15%. Our findings suggest that the two

extracts did not have cytotoxic effects at low doses on human red blood cells.

In this study, an assessment was also performed on the ability of leaves and bark extracts to protect red blood cells from damage caused by AAPH-induced hemolysis. Indeed, the AAPH-induced hemolysis model is widely used to estimate plant extracts' antioxidant and antihemolytic capacity. At 37 °C, AAPH generates a low but constant flux of peroxyl radicals, producing free radicals that cause erythrocytes, membrane lipids, and proteins to undergo chain oxidation. Thus, the disruption of the erythrocytes membrane caused by free radical excess ultimately leads to hemolysis or death of healthy erythrocyte cells (Yuan et al., 2005; Ramchoun et al., 2015). The significant increase in hemolysis rate in erythrocytes treated only with 200 mM APPH and incubated for 4 hours can be explained because continuous exposure of erythrocytes to APPH resulted in maximal utilization of endogenous antioxidants (Yang et al., 2017).

The anti-hemolytic activity demonstrated that treating erythrocytes with *R. alaternus* leaves and bark extracts enhanced their resistance to AAPH damage in a dose-dependent manner. Bark extract (BRA) showed a better protective effect on the erythrocyte membrane than leaves extract (LRA). In fact, at a 200 µg/ml concentration, the bark extract almost completely inhibited AAPH-induced hemolysis with an inhibition rate of 99.41 ± 1.17%, which was much higher than the leaves extract inhibition rate of 76.26 ± 12.03%. This antihemolytic activity is probably due to the various phenolic compounds in both extracts, known as phenolic hydrogen atom donors. They help stabilize free radicals through their antioxidant activity, increasing erythrocytes' oxidative stress resistance. Moreover, the phenolic components present in both extracts could donate one or more electrons to neutralize the AAPH radical while inhibiting hemolysis at the same time. In addition, polyphenols have also been shown to have the ability to interact with the hydrophilic section of the lipid membrane, causing changes in the packing arrangement of the polar heads of the lipids (Balderrama-Carmona et al., 2020).

Furthermore, several previous studies on *R. alaternus* leaves and bark extracts revealed the presence of multiple compounds, mainly glycosylated and non-glycosylated flavonoids such as kaempferol, quercetin, kaempferol hexoside, kaempferol 3-*O*-acetyl-rhamnoside, rhamnetin hexoside, and rhamnocitrin hexoside, etc. (Ben Ammar et al., 2009; Boussahel et al., 2015; Moussi et al., 2015; Tacherfiout et al., 2018). These various flavonoids, as well as other polyphenols found in both extracts, may protect erythrocyte cell membranes by interacting with membrane phospholipids, which protect them from lipid deterioration (Derouich et al., 2020). Flavonoids also promote Van der Waals contacts within the lipid bilayer and may be a source of membrane stability, protecting erythrocytes from free radical damage (Ramchoun et al., 2015).

Kaempferol, one of the flavonoids found in *R. alaternus*, has already demonstrated anti-hemolytic action. Indeed, according to Liao et al. (2016), the hydroxyl group at the C3 position plays a critical role in flavonoids' high antioxidant action via controlling heme oxygenase. In addition, kaempferol protects erythrocyte membrane architecture against AAPH-induced structural damage, lowers ROS-induced hemolysis in a dose-dependent manner, and has excellent anti-lipid peroxidation.

4. Conclusions

In summary, *R. alaternus* has a variety of phenolic compounds with good antioxidant properties. The results of this study appear to back up the traditional usage of *R. alaternus* to treat hemolytic anemia. We demonstrated that *R. alaternus* attenuates AAPH-induced hemolysis on human red blood cells for the first time. Leaves and hydroalcoholic bark extracts significantly reduced AAPH-induced hemolysis. *R. alaternus*' antihemolytic action could be attributed to its antioxidant status, which could protect erythrocytes from oxidative damage and hemolysis. *R. alaternus*' bioactive potential could be highly useful in preventing and treating hemolytic anemias and disorders involving excessive oxidative stress generation.

Acknowledgments

None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

CRediT authorship contribution statement

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Supplementary File

None.

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