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Antioxidant, antibacterial, antibiofilm, and antihemolytic activities of the aqueous extract of *Juglans regia* bark: a natural and ancestral approach to oral and cosmetic care.

Sebbane Hillal[1], Almi-Sebbane Dalila[1], Lahcene Souad[1], Saci Sarah[1], Moualek Idir[1], Bariz Karim[1] & Houali Karim[1].*

Corresponding author: *hillalmicrobio@yahoo.com*

[1] *Laboratory of analytic biochemistry and biotechnology (LABAB). Department of Biochemistry and Microbiology, Faculty of Biological Sciences and Agronomic Sciences. Mouloud Mammeri University, Tizi-Ouzou, 15000, Algeria.*

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Abstract

The aim of this study was to evaluate the safety, antioxidant potential, and antimicrobial properties of the aqueous extract (AE) of *Juglans regia* L. bark collected in the Tizi-Ouzou region in Algeria. The richness of AE in polyphenols was assessed by RP-HPLC. The biological activities of AE were expressed through their antioxidant, antibacterial, antibiofilm, and antihemolytic potentials. 18 phytochemical molecules (12 flavonoid molecules and 6 phenolic acid molecules) were identified by RP-HPLC. The antioxidant activity expressed by TAC was 119.08± meq AA/g of DW, while the results of DPPH and FRAP expressed by IC50 and EC50, were 57±1.02 µg/ml and 65±0.67 µg/ml, respectively. AE showed antibacterial activities against all tested strains: *E. coli* ATCC 25922, *B. cereus* ATCC27853, *S. aureus* ATCC25923, *E. faecalis* WDCM0009, and *P. aeruginosa* ATCC 27853. The biofilm of *S. aureus* ATCC25923 was inhibited by 93% at a biofilm inhibitory concentration of 56.25 mg/ml. AE was non-hemolytic and protected the erythrocyte membrane against hemolysis caused by saponin. The results of this study demonstrate that walnut bark can be integrated into pharmaceutical formulations, especially those intended for oral hygiene and cosmetic products.

Keywords: biological activities, biological product, *Juglans regia* bark, polyphenols components.

Introduction

Juglans regia Linn (walnut), commonly known as walnut, is a deciduous tree that grows in temperate regions[1]. Ethnobotanical studies have reported that various parts of *Juglans regia* are used in the treatment of numerous diseases, such as digestive disorders, hypertension, diabetes, gout, and oral cavity diseases[2-5]. The traditional medicine-related products industry generates annual profits of up to 62 billion USD[6].The treatment of oral cavity infections with walnut bark has been reported in several scientific works[7-8]. In Algeria, the use of walnut bark as a traditional remedy forrelieving oral infections, tooth decay, gingivitis, and dental pies, has been reported by Souilah *et al*[9] and in the Kabylie region by Meddour *et al*[10]. Walnut bark has shown a high richness in bioactive molecules[11,12]such as polyphenols, flavonoids and phenolic acids, which

are responsible for its biological activities[13,14] : antioxidant, antibiofilm, antibacterial and anti-inflammatory.

The purpose of this study is to investigate the medicinal properties of walnut bark collected in Algeria's Tizi-Ouzou region by evaluating its biological activities: antioxidant, antibacterial, antibiofilm, and antihemolytic, with the goal of incorporating them into pharmaceutical formulations for oral cavity hygiene while increasing local and national production of *Juglans regia L*.

Materials and methods

Plant material

Samples of Persian walnut (*Juglan sregia*Linn) bark were collected with the permission of local authoritiesin March 2022 at the Maatkas district, located at an altitude of 620 m and 20 km southwest of Tizi-Ouzou, Algeria (latitude 36° 43' 0.00"N, longitude 4° 2' 60.00"E). Botanical identification was carried out by Dr. Hocine Abbaci from the University of Bejaia (Algeria). A sample was deposited at the herbarium of Mouloud Mammeri University (UMMTO) under the number 2022/UMMTO/26.The barks were washed with distilled water, dried, and then powdered. The powders are stored in darkness and protected from light and moisture.

Preparation of extracts

The aqueous extract of bark (AE) was obtained after 24 hours of maceration of a mixture of distilled water and bark powder at a concentration of 10% (w/v). After filtration of the macerate, the filtrate is lyophilized and stored for later use, as described by Ingle *et al*[15].

Phytochemical screening

Phytochemical screening of saponins, reducing compounds, flavonoids, phenols, and terpenes was carried out according to the protocol described by Yadav and Agarwala[16]. Alkaloids and free quinones were revealed by the method of Dohou*et al*[17]. While anthocyanins, glycosides, and tannins (condensed and gallic) were revealed according to the respective protocols described by Rohit[18],Daira *et al*[19]and Nnanga*et al*[20].

Total polyphenol content determination

The polyphenol content of the AE was quantified using the Folin-Ciocalteu method[21]. Referring to the calibration curve of gallic acid, the results were expressed in mg gallic acid equivalents per gram of dry matter (meq GA/g DM), following the method described by Ghedadba *et al*[22]. 0.2 ml of sample was mixed with 1 ml of diluted Folin-Ciocalteu reagent, and then 800 µl of

7.5% sodium carbonate solution was added. The reaction mixture was left to stand for an hour, and the absorbance was measured at 760 nm using a spectrophotometer.

Estimation of flavonoid content

The concentration of flavonoids was measured by the colorimetric method described by Kosalec *et al*[23]. A reaction mixture was prepared with 1 ml of sample, 3 ml of ethanol (95%), 5.6 ml of distilled water, 0.2 ml of sodium acetate (1M), and 0.2 ml of $AlCl_3$ (10%). After 30 minutes of incubation in darkness, the absorbance was measured at 420 nm. The results were expressed in mg quercetin equivalents per gram of dry matter (meq Q/g DM).

Condensed tannin content

Tannin contents were measured by spectrophotometry following the protocol of Rakitikul[24]. 1 mL of sample was mixed with 20 mL of distilled water, to which 2 mL of Folin-Denis reagent and 20 mL of Na_2CO_3 were added. After 30 minutes of incubation in the dark, the absorbance was measured at 760 nm. The results were expressed in mg of catechin equivalents per gram of dry matter (meqC/gMS) using the catechin calibration curve.

Evaluation of antioxidant activities

Total Antioxidant Capacity (TAC) Test

Total antioxidant capacity (TAC) was assessed according to the method of described Mašković *et al*[25]. 100 µL of AE was mixed with 1 ml of molybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After incubation at 95 °C for 90 min, the absorbance was measured at 695 nm. The antioxidant capacity was expressed in milligrams of ascorbic acid equivalent per gram of extracts (meqAA/g DM) using an ascorbic acid standard curve.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Test

The principle of this method is based on the reduction of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by AE using ascorbic acid as a standard[26]. The DPPH radical scavenging activity was assessed by the IC_{50} value (the concentration at which 50% of radicals are reduced). The absorbance at 517 nm was used to quantify the percentage of radical scavenging inhibition according to the following formula:

$$DPPH\ (\%) = \frac{Abs\ control\ -Abs\ sample}{Abs\ control} \times 100$$

Control Abs: Absorbance of DPPH solution mixtures without extract.

Sample Abs: Absorbance of DPPH mixtures containing the extract.

Ferric Reducing Antioxidant Power (FRAP) Test

The antioxidant capacity was also determined using the Ferric Reducing Antioxidant Power (FRAP) assay. The FRAP test was conducted according to the methods of Oyaizu[27]. The increase in optical density of the sample measured at 700 nm was used to calculate the reducing power, with ascorbic acid serving as the standard. The results are expressed in EC_{50}, which represents the concentration of antioxidant required to achieve an absorbance of 0.5 nm[28].

Antibacterial Activity

The antibacterial activity and Minimum Inhibitory Concentration (MIC) of AE (0.9 g/mL) were determined by the disc diffusion method on agar[29] against the following strains: *E. coli* ATTCC 25922, *S. aureus* ATTCC 25923, *B. cereus* ATCC 14579, and *E. faecalis* WDCM 009. All strains were standardized to an OD of 0.08–0.1 (λ = 625 nm). Distilled water and antibiotics (Pefloxacin 5µg (PEF5), Ceftriaxone 30µg (CRO30), Cephalothin 30µg (KF30), and Pipemidic Acid 20µg (PI20)) were used as negative and positive controls, respectively. After incubation (37°C/24 hours), the MICs were determined following the method of Mbengui*et al*[30]. The antibacterial potential of AE was classified based on the diameter of inhibition zones established by Saraiva*et al*[31]: no activity (ϕ < 9 mm); weak activity (ϕ: 9 to 12 mm); active activity (ϕ: 13 to 18 mm); and very strong activity (ϕ > 18 mm).

Antibiofilm Activity

The antibiofilm activity of AE against *S. aureus* ATTCC 25923, known for its ability to form biofilms, was evaluated using the crystal violet method[32] in microplates according to the protocol described by Islam *et al*[33]with some modifications. Briefly, each well contains 100 µl of BHIB-saccharose, 40 µL of a bacterial suspension (OD = 0.08–0.1 at λ = 625 nm), and 40 µL of BHIB-saccharose at different extract concentrations (0.9 to 0.00044 g/mL) obtained following a series of dilutions in ½. The positive control contains, instead of the extract, a solution of antibiotic (Ceftriaxone) at 750 µg/mL, while in the negative control well, the aqueous extract is replaced by 40 µl of BHIB-saccharose. After incubation of the microplate (24H/37°C), the wells are emptied, and the remaining planktonic cells are removed by gentle washing three times with PBS (0.1M, pH 7.4). The formed biofilms are fixed with 200 µl of a fixing solution (37% formaldehyde, diluted to 1/10, and 2% sodium acetate) for 15 min. A second PBS rinse is perf $\mathbf{I\ (\%)=\frac{OD_C-OD_S}{DO_c}\times 100}$ ıtion, and then each well is stained with 200 µl of 0.1% Crystal Violet (CV) for 15 min. The excess staining solution is removed after three washes with PBS. After 15 minutes of drying at room temperature, 200 µl of ethanol

(95%) is added to the wells to recover the CV retained in the biofilm. After 30 minutes of contact with ethanol, the optical density (OD) is measured (λ = 560 nm). The percentage inhibition of the biofilm is estimated according to the formula of Merghni*et al*[34]mentioned below. The Minimum Biofilm Inhibitory Concentration (MBIC) corresponds to the first well with a density lower than the OD of sterile BHIB[35].

I (%): Percentage of inhibition.

OD_c: optical density of the negative control, OD_S: optical density of the samples

Chromatographic Analysis

RP-HPLC was performed using an AGILENT Ultimate-1100 liquid chromatography system equipped with a degasser, a quaternary pump, an automatic sampler, a column oven, and a UV detector. Separation was carried out using a Hypersil BDS C18 column (250 mm×4.6 mm). The column temperature was maintained at 30°C, and the injection volume was 5 µL (0.16 g/mL) of the extract dissolved in HPLC gradient methanol. The gradient mobile phase used consisted of acetonitrile (A) and 0.2% acetic acid in water (B). The flow rate was maintained at 1.5 mL/min. Retention times of different standards were used to identify the composition of the aqueous extract. The percentage of each chemical compound in the extract is determined from the peak areas obtained by comparing them to standard reference compounds.

Toxicity Evaluation

Preparation of Hematocrit Suspension

The blood used comes from consenting, healthy, non-smoking, and non-alcoholic volunteers. The experimental protocol was approved by the ethics committee of our university (N°Ethi/UMMTO/23-JAN-2023). Blood was collected in heparin tubes (anticoagulant). After centrifuging the blood samples (2500 rpm/10 min/4°C), the plasma was removed. The PBS buffer ($NaH_2PO_4 \cdot 2H_2O$: 123 mmol/L, Na_2HPO_4: 27 mmol/L, NaCl: 123 mmol/L; pH 7.4), prepared as described by Arbos*et al*[36], was used to wash the blood cells three times at 2500 rpm (10 min/4 °C) and to prepare the hematocrit solution (HS) at a concentration of 2%.

Aqueous Extract Safety Test

The toxicity test was evaluated by studying the hemolytic effect of AE on red blood cells, following the protocol described by Hebbani*et al*[37] with some modifications. 1 mL of SH (2%) was mixed with 80 µL of AE solution at different concentrations (0 to 6000 µg/mL) diluted in PBS. In parallel, a standard range made with saponin (0 to 6000 µg/mL) was prepared to serve as a reference for the hemolysis study. Saponin at a concentration of 6 mg/mL causes 100% hemolysis, while PBS buffer was used as a negative control for hemolysis. The solution mixtures were incubated at 37°C for 1 hour with gentle agitation, then centrifuged (2500 rpm/10 min/4°C) to sediment the red blood cells. The measurement of absorbance at λ of 540 nm of the supernatant from the different solutions, corresponding to the amount of hemoglobin released by the red blood cells following their lysis, allows us to assess the degree of hemolysis according to the following formula:

$$\% \text{ Hemolysis} = (A/B) * 100$$

A: Absorbance of the supernatant of AEsolutions; B: Absorbance of saponin(6mg/mL)

Antihemolytic effect of AE

The protective effect of AE against saponin-induced hemolysis on HS was estimated following the protocol of Iratni*et al*[38], by mixing 1 mL of SH (2%) with 40 µL of saponin solution (1000 µg/mL) and 40 µL of AE at different concentrations (500, 1000, and 2000 µg/mL). The assessment of hemolysis described previously informs us about the degree of protection of our extract.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) and Tukey's post-hoc test were performed using SPSS software version 25.0 with a 5% error risk level.

Results

Extraction

The extraction yield of AE obtained was 13.25 ± 0.4%. This yield is close to the yield of the bark infusion (11.8%) obtained by Amirou*et al*[39], but lower than those of the hydroalcoholic leaf extract (20.16%) and the aqueous infusion of green walnut husks (31.63%), obtained by Almeida*et al*[40] and Oliveira *et al*[41], respectively.

Phytochemical screening

The results of the phytochemical screening mentioned in table 1 revealed several compounds, including flavonoids, terpenoids, phenols, gallic tannins, saponins, alkaloids, reducing compounds, glycosides, and free quinones. However, anthocyanins and catechin tannins were not detected. These results are consistent with the chemical compounds of walnut reported in several studies[42-44].

RP-HPLC

The results of the RP-HPLC analysis (Table 2) of the aqueous extract identified 18 molecules. The analysis revealed a richness in polyphenols, particularly flavonoids (Catechin, Vitexin, Aesculetin, Orientin, Rutin, Naringenin-7-glucoside, Luteolin-7-glycoside, Spiracoside, Naringenin-7-glucoside, Vitexin 2.0 rhamnoside, Quercetin, and Hesperidin) and phenolic acids (3.4.5. Tri-methoxybenzoic acid, Cinnamic acid, Resorcylic acid, Isovanillic acid, Resorcinol, and Tannic acid). These molecules are known to have biological activities, including antioxidant, antimicrobial, and antibiofilm activities. Studies have demonstrated that these biological activities (antioxidant, antimicrobial, antibiofilm, etc.) are attributable to the polyphenols found in various walnut extracts, such as resorcinol, catechin, vanillic acid, resorcylic acid, aesculetin, cinnamic acid, and quercetin[42,45-48].

Total contents of polyphenols, flavonoids, and tannins

The total polyphenols, flavonoids and condensed tannins are mentioned in table 3. These results are lower than those reported by Noumi*et al*[46]and Rusu[49]. Biotic and abiotic factors significantly influence the quantity and quality of phenolic compounds synthesized by the plants (Ashraf *et al*[50]).

Antioxidant activities

The TAC(Table 3) of AE was 119.08 ± 0.16 meq AA/g DM. The antioxidant activities of AE, expressed as FRAP (EC_{50} = 65 ±0.67 µg/ml) and DPPH (IC_{50} = 57.02 ±1.02 µg/ml), shown in figures 1 and 2, respectively, are better than those obtained with methanolic extracts of walnut bark: DPPH 123.42±3.71 and FRAP: 34.51 ±0.64 µg/ml Bourais*et al*[48].The antioxidant activity is influenced by the nature of the solvent; indeed, the DPPH (36 µg/ml) is higher than that of the aqueous extract (DPPH = 582 µg/ml), reported respectively by Noumi *et al*[46] and Bhatia *et al*[51].

Antibacterial Activity

The results of the antimicrobial activities of walnut ethanolic extract are represented in table 4. Referring to the diameters of the inhibition zones, as reported by Saraiva*et al*[31], the extracts exhibit activity against all tested strains, both Gram-positive and Gram-negative, except for *B. cereus* ATCC 14575 ($\phi = 8 \pm 1.46$ mm), for which the ethanolic extract showed low activity. However, the ethanolic extract showed variable activities against different strains: *P. aeruginosa* ATCC 27853 ($\phi = 20 \pm 1.35$ mm) was the most sensitive, followed by *E. coli* ATCC 25922 ($\phi = 18.66 \pm 1.41$ mm), *E. faecalis* WDCM 00009 ($\phi = 18 \pm 1.28$ mm), and *S. aureus* ATCC 25923 ($\phi = 13.33 \pm 0.7$ mm). Indeed, the analysis of variance (ANOVA) reveals statistically significant differences between the means of the inhibition zones induced by the extract on the different bacterial strains tested ($p < 0.05$). The post-hoc test specifically reveals that the inhibition zones are significantly larger for *E. coli*, *E. faecalis,* and *P. aeruginosa* ($p < 0.05$) compared to the other strains ($p > 0.05$). The results obtained are consistent with those reported in the scientific literature[52,53]. These results also show that the antibacterial potential of the ethanolic extract is not affected by the nature of the bacterial wall, contrary to what has been reported in the works of Auer *et al*[54] and Caporarello *et al*[55] on the resistance of Gram-negative bacteria to the extracts.

Antibiofilm Activity

The antibiofilm activity of AE against *S. aureus*, as shown in table 4, demonstrates that AE at a concentration of 0.9 g/ml inhibits 98.12% of the biofilm, while it inhibits 93% of the biofilm at the minimum biofilm inhibitory concentration (MBIC = 56.25 mg/ml). These results indicate that biofilm inhibition is a dose-dependent factor, as described by Quave *et al*[56] and Acquaviva *et al*[57]. The results obtained are lower compared to those reported by Milek *et al*[58], who achieved 92% inhibition of *S. aureus* biofilm with only 1.25 mg/ml. This difference may be explained by the nature of the solvents used. These solvents influence the presence of organic compounds that alter the characteristics of the biofilm surface, preventing bacterial adhesion to the biofilm[59].

Study of the cytotoxicity of AE

The results of the hemolytic activity, represented in the figure 03, clearly show that the AE exhibits low hemolysis, not exceeding 4.94 ±0.65% hemolysis at the maximum tested concentration (6000 µg/ml), compared to saponin. This indicates that the AE at the tested concentrations exhibits low toxicity towards blood cells. In addition to its low hemolysis, it

protects against hemolysis induced by saponin. 100% hemolysis was achieved by saponin at a concentration of 6000 µg/mL, indicating that the hemolytic power is a dose-dependent factor. The ANOVA test indicates that there is a statistically significant difference ($p<0.05$) between the means of the 4 tested groups: saponin alone and saponin combined with 3 concentrations of extract (500, 1000, and 2000 µg/mL).The Tukey post-hoc test shows that the significant difference ($p<0.05$) lies specifically between saponin and saponin+ 1000 µg/ml of the extract, implying that the plant extract at a concentration of 1000 µg/mL is more effective in protecting cells against hemolysis compared to the other tested concentrations. Thus, the AE (1000 µg/mL) reduces saponin-induced hemolysis by 8.34%, whereas it only reduces by 2.4% at a concentration of 500 µg/ml. However, in the presence of AE at 2000 µg/mL, saponin-induced hemolysis is increased by 1.62% (Figure 04). These results are consistent with those obtained by Iratni*et al*[38], who reported that the anti-hemolytic effect of saponin at 100 µg/mL of aqueous leaf extract of Pistacialentiscus provides better erythrocyte protection at 1000 µg/mL than at 500 µg/mL. Other studies have reported that the methanolic extract of Juglansregia flowers exhibited good anti-hemolytic activity caused by H_2O_2 andCuOOH[60].

Discussion

The extraction yield is a difficult parameter to define as it is multifactorial, depending on the plant material to be used (the part used, geographical origin, etc.), as well as the solvents and extraction techniques used for extracting secondary metabolites[61-63]. The extraction yield directly affects the quality and quantity of extracted polyphenols. Indeed, the quantitative and qualitative analysis of polyphenols depends on the plant's origin, the nature of the extraction solvent, and the method used[64,65]. Biotic and abiotic stress increase the biosynthesis of polyphenols, particularly flavonoids, as a defense mechanism against environmental aggressions[66,67].The results of the screening, quantification, and nature of phytochemical compounds obtained by RP-HPLC explain the antioxidant, antimicrobial, antibiofilm, and anti-inflammatory potential of AE obtained in this study. It has been reported that the richness of walnut crude extracts in polyphenols and flavonoids confers excellent biological activity[68,69].

The antioxidant power of polyphenols, as scavengers of free radicals, depends on the number and position of OH groups on their benzene ring[70,71]. This has been confirmed by the study of Veiko *et al*[72], who demonstrated that quercetin (flavanols) exhibits a higher antiradical power (DPPH) than catechin (flavan-3-ols), while naringenin (flavanones) does not reduce DPPH. Furthermore, the glycosylation of polyphenols reduces their antiradical power. Indeed, the study conducted by Zhu *et al*[73] highlighted all these factors on the IC_{50} values of the tested

polyphenols, namely quercetin (flavanols), catechin (flavan-3-ols), hyperoside (flavonols), rutin (flavones), quercitrin (quercetin-3-O-rhamnoside), luteolin (flavones), and luteolin-7-O-β-glucoside (glycosylated flavones), with respective IC50 values of 4.36, 5.06, 6.34, 6.36, 7.12, 7.29, and 8.12 µM.Flavonoids, especially flavones and flavonols, are the most common polyphenols in the plant. Flavonoids trap free radicals either by a simple hydrogen atom transfer from the OH group or by an electrochemical oxidation reaction with proton or electron donation[74,75]. In addition to neutralizing free radicals, polyphenols act as chelators of transition metal ions in the Fenton reaction, such as Fe^{2+}, which prevents oxidation caused by highly reactive hydroxyl radicals[7,76,77].The antibacterial and antibiofilm potentials of polyphenols have been demonstrated in the scientific literature[78,79].

Phenolic acids and flavonoids are known for their antimicrobial activity by forming a complex with the bacterial wall or membrane. Polyphenols thus disrupt membrane fluidity and cause leakage of cytoplasmic content[80]. According to Cowan[81], the antimicrobial activity of flavonoids with fewer free hydroxyl groups is more significant because the chemical affinity for membrane lipids is increased compared to flavonoids rich in free OH groups. The lipophilic nature of polyphenols enhances their antibacterial activity, as it facilitates their penetration through the bacterial membrane, subsequently inducing irreversible damage to the membrane structure and inhibition of membrane and intracellular enzymes[82,83].

Thus, the antibacterial potential of polyphenols has been attributed: to their interactions with the cell wall and cytoplasmic membrane of bacterial cells, the disruption of membrane fluidity, inhibition of nucleic acid, protein, and cell wall synthesis, as well as affecting energy metabolism[84,85].

The combined effect of the position of the OH group and the double bond in the structure of polyphenols affects the antibacterial efficacy of polyphenols[86]. Indeed, the presence of OH groups, which through hydrogen bonding interactions affect the membrane permeability of bacterial cells, results in leakage of cytoplasmic contents[87,88]. Furthermore, the ionic strength exerted by the double bonds in the aromatic ring of polyphenols causes electron delocalization, resulting in bacterial depolarization. This affects the proton motive force across the membrane, causing a decrease in pH gradient and ATP levels[89,90].

A biofilm is a bacterial growth on solid surfaces or air-liquid interfaces, regulated by quorum sensing (QS), allowing bacteria to survive and resist antimicrobial agents[91]. The antibiofilm activity of polyphenols is attributed either to their direct effect on bacteria or to the disruption

of quorum-sensing regulatory mechanisms without affecting bacterial growth[68,92]. Studies have shown that the antibiofilm effect is also exerted either by inhibiting quorum sensing regulatory genes (LasR, LasI, HhlR, and RhlI), as reported byLima *et al*[93] and Higuera-Ciapara *et al*[94], or by inactivating genes responsible for biofilm detachment and dissemination (flhD, fimA, fimH, and motB), as described byLee *et al*[95] and Didehdar *et al*[96].

Erythrocyte cells are used as a study model because the red blood cell membrane is very close to the lysosomal membrane, the lysis of which leads to an inflammatory reaction[97,98].

The reduction of hemolysis in the presence of AE is an indicator of protection against lysosomal lysis. The antihemolytic effect of *Juglans regia* flower extracts has been attributed to their richness in polyphenols and flavonoids that stabilize erythrocyte membranes to prevent their hemolysis[11]. The reduction of saponin-induced hemolysis in the presence of AE at the studied concentrations can be explained by the stabilization of erythrocyte membranes through the reduction of interactions between saponin and membrane lipids (sterol) in the presence of AE, as reported by Istvan *et al*[99] and Dawid *et al*[100].According to Fidelis *et al*[101], the antihemolytic effect of plant extracts is attributed to the formation of hydrogen bonds between the OH radicals of polyphenols and the hydrophilic part of membrane phospholipids, leading to increased membrane rigidity and making them less susceptible to hemolysis.

Conclusion

In conclusion, the study highlights the importance of quantitative and qualitative analysis of polyphenols, shedding light on their variability depending on the plant origin, extraction solvent, and methods used. The results obtained by RP-HPLC reveal the antioxidant, antimicrobial, antibiofilm, and anti-inflammatory potential of *Juglansregia* extracts, attributable to the richness in polyphenols and flavonoids. The antioxidant properties of polyphenols depend on their molecular structure, with the position and number of OH groups influencing their effectiveness. The antibacterial and antibiofilm activity of polyphenols stems from their interaction with bacterial walls and membranes, inducing cellular disturbances. Furthermore, flavonoids show increased antimicrobial activity by forming complexes with the bacterial membrane. The study also highlights the anti-inflammatory activity of the extracts, demonstrated by hemolytic and antihemolytic tests, attributed to the ability of polyphenols to stabilize erythrocyte membranes. These results contribute to understanding the mechanisms of action of polyphenols, emphasizing their potential in the development of therapeutic and nutraceutical solutions.

Conflicts of Interest

The authors declare no conflict on interest.

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Table 1: Phytochemical screening of *Juglansregia L* extracts

Compounds	Reactions	Results
Anthocyanins	Absence of blue-violet color	-
Catechin tannins	Absence of light pink precipitate	-
Gallic tannins	Darkblue coloration	+
Flavonoids	Red or pink coloration	+
Saponins	Formation of a stable foam	+
Reducing compounds	Formation of a brick-red precipitate	+
Phenols	Dark blue-black or dark green coloration	+
Terpenoids	Reddish-brown coloration	+
Alkaloids	Yellow precipitate	+

Free quinones	Red coloration	+
Glycosides	Presence of violet-red coloration	+

Table 2: Compounds identified in the extract of *Juglansregia* L by RP-HPLC

Polyphenol Classes	Components	RT (min)	Peak Area (%)
Phenolicacids	Tannicacid	3.398	4.8292
	Resorcinol	5.926	0.6425
	Isovanillicacid	6.879	3.3812
	Resorcylicacid	7.090	2.6425
	Cinnamicacid	8.567	2.8488
	3.4.5. Tri- methoxybenzoicacid	10.945	4.5880
Flavonoids	Catechin	6.345	1.6775
	Vitexin	7.251	0.6722
	Aesculetin	7.657	1.8522
	Orientin	8.062	0.9745
	Rutin	9.072	1.1139
	Apigenin-7-glucoside	9.644	15.1330
	Luteolin-7-glycoside	10.295	12.2054
	Spiracoside	10.757	4.4842
	Naringenin-7-glucoside	10.754	4.5514
	Vitexin 2.0 rhamnoside	11.341	3.6005
	Quercetin	13.211	2.2990
	Hesperidin	16.515	1.2030

Table 3-Contents in polyphenols and TAC of Juglans regia aqueous extract

Polyphenols (meq GA/g DM)	24.37 ± 1.06
Flavonoids (meqQ/g DM)	16.89 ± 0.26
Condensed tannins (meq TA)	7.797 ± 0.82
TAC (meqAA)	119.08 ± 0.16

AA: ascorbic acid, DM: dry Mater, GA: gallic acid, meq: milligram equivalent

TA: tannic acid and

Table 4-Antibacterial and antibiofilm activities of aqueuos of walnut

Strains	Antibacterial activity		Antibiofilm activity against *S.aureus* ATCC 25923		
	Inhibition diameter (ϕ :mm)	MIC (mg/mL)	Concentration	0.9 g/mL	52 mg/mL (MBIC)
S. aureus ATCC25923	13.33±0.7	14.06	Inhibition percentage	98.12	93
E. coli ATCC 25922	18.66±1.41	7.03	not a biofilm forming		
E. faecalis WDCM 00009	18±1.28	3.51	not a biofilm forming		
B. cereus ATCC 14575	8±1.46	56.25	not a biofilm forming		
P. aeruginosa ATCC 27853	20±1.35	14.06	not a biofilm forming		

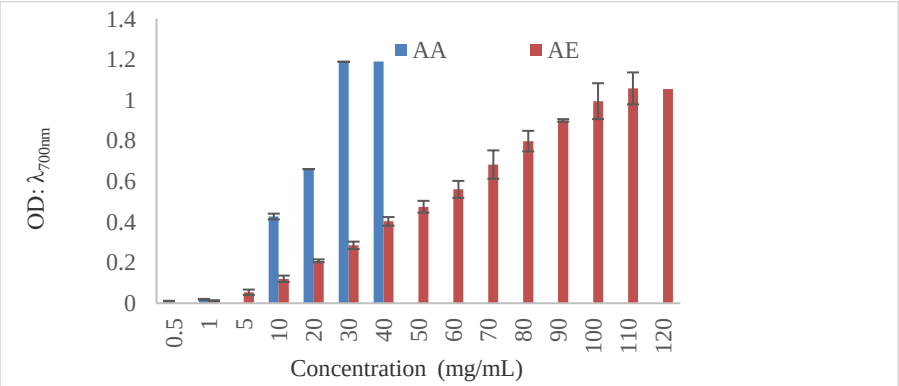

Figure 01: Ferric reduccing antioxydant power (FRAP) assay

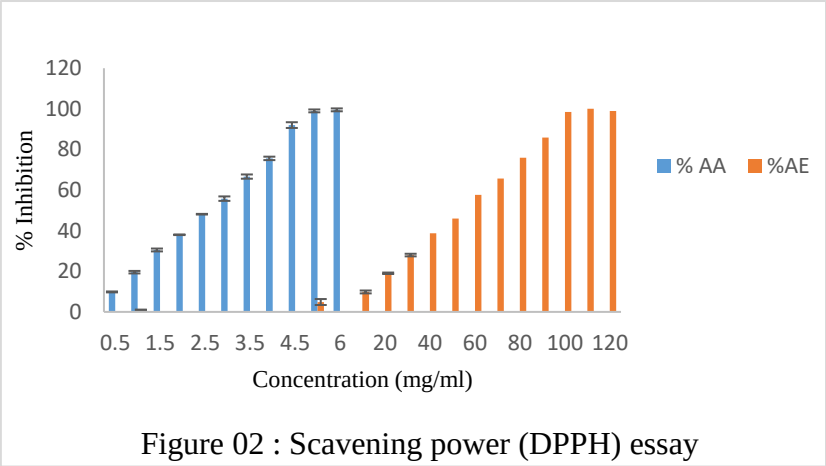

Figure 02 : Scavening power (DPPH) essay

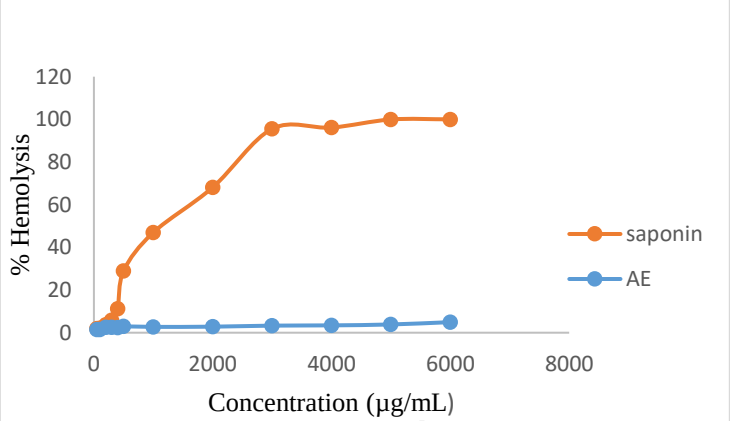

Figure 03: Hemolysis activity

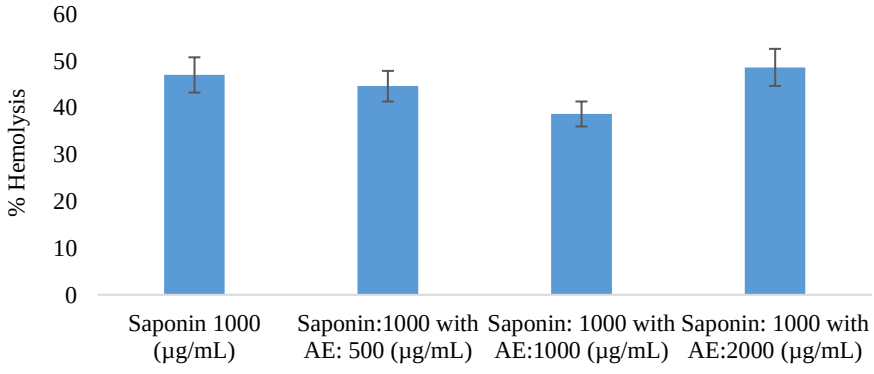

Figure 04: Antihemolytic activity of AE