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Antioxidant, antimicrobial, and α-glucosidase inhibitory activities of saponin extracts from walnut (*Juglans regia* L.) leaves

Youssef Elouafy[1], Adil El Yadini[1], Salma Mortada[2], Mohamed Hnini[3], Hicham Harhar[1], Asaad Khalid[4,5], Ashraf N. Abdalla[6], Abdelhakim Bouyahya[7✉], Khang Wen Goh[8], Long Chiau Ming[9✉], My El Abbes Faouzi[2], Mohamed Tabyaoui[1]

[1]*Laboratory of Materials, Nanotechnology and Environment, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco*

[2]*Laboratories of Pharmacology and Toxicology, Pharmaceutical and Toxicological Analysis Research Team, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Rabat, Morocco*

[3]*Center of Plant and Microbial Biotechnology, Biodiversity and Environment, Microbiology and Molecular Biology Team, Faculty of Sciences, Mohammed V University in Rabat, Rabat, Morocco*

[4]*Substance Abuse and Toxicology Research Center, Jazan University, Jazan 45142, Saudi Arabia*

[5]*Medicinal and Aromatic Plants and Traditional Medicine Research Institute, National Center for Research, Khartoum, Sudan*

[6]*Department of Pharmacology and Toxicology, College of Pharmacy, Umm Al−Qura University, Makkah, Saudi Arabia*

[7]*Laboratory of Human Pathologies Biology, Faculty of Sciences, Mohammed V University in Rabat, Morocco*

[8]*Faculty of Data Science and Information Technology, INTI International University, Nilai, Malaysia*

[9]*School of Medical and Life Sciences, Sunway University, Sunway City, Malaysia*

ABSTRACT

Objective: To investigate the relationship between triterpenoid saponin content and antioxidant, antimicrobial, and α-glucosidase inhibitory activities of 70% ethanolic, butanolic, aqueous, supernate and precipitate extracts of *Juglans regia* leaves.

Methods: Triterpenoid saponins of different *Juglans regia* leaf extracts were measured by the vanillin method. Antioxidant activity was evaluated against DPPH and ABTS free radicals. We also assessed α-glucosidase inhibitory and antimicrobial activities of the leaf extracts. Pearson's correlation coefficient was evaluated to determine the correlation between the saponin content and biological activities.

Results: The butanolic extract was most effective against DPPH with an IC_{50} of 6.63 μg/mL, while the aqueous extract showed the highest scavenging activity against ABTS free radical with an IC_{50} of 42.27 μg/mL. Pearson's correlation analysis indicated a strong negative correlation ($r = −0.956$) between DPPH radical scavenging activity (IC_{50}) and the saponin content in the samples examined. In addition, the aqueous extract showed the best α-glucosidase inhibitory activity compared with other extracts. All the extracts had fair antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, and *Klebsiella pneumoniae* except for the aqueous extract.

Conclusions: *Juglans regia* extracts show potent antioxidant,

Significance

Triterpenoid saponins are glycosides with a wide range of bioactive structures and biological activities, and can be found in many medicinal plants. However, no studies have been done on *Juglans regia* to identify or even quantify the content of these molecules in this plant. This study shows the relationship between triterpenoid saponin content and various biological activities in different leaf extracts of *Juglans regia*. It provides evidence for the potential medicinal properties of *Juglans regia* leaves, including antioxidant, antimicrobial, and α-glucosidase inhibitory properties, and a solid foundation for further research on the potential uses of *Juglans regia* leaves as a natural source of medicinal compounds.

✉To whom correspondence may be addressed. E-mail: a.bouyahya@um5r.ac.ma (A Bouyahya); longchiauming@gmail.com (LC. Ming)

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antimicrobial, and α-glucosidase inhibitory activities. There is a correlation between saponin levels in *Juglans regia* leaf extracts and the studied activities. However, additional research is required to establish these relationships by identifying the specific saponin molecules responsible for these activities and elucidating their mechanisms of action.

KEYWORDS: *Juglans regia* leaves; Triterpenoid saponin; Antioxidant activity; DPPH; ABTS; Antidiabetic activity; α-Glucosidase; Antimicrobial activity

1. Introduction

Morocco's flora is abundant with many medicinal plants which are largely employed as traditional medicines to combat numerous illnesses, including cardiovascular and hypertension problems, diabetes, rheumatism, stomach pain, as well as many other diseases[1,2].

Juglans regia (*J. regia*) is among the commonest plants in Morocco, covering more than 7 600 hectares[3]. This plant is composed of several families of bioactive molecules, such as polyphenols, flavonoids, saponins, phytosterols, tocopherols, and various other molecules that make *J. regia* a vast diversity of medicinal properties[4–7].

On the other hand, saponin compounds have attracted huge interest over the past few decades, as they have proven their ability to treat several health problems[8]. Various studies have been devoted to investigating the anticancer activity of saponin molecules isolated from different species of plants[9–11]. Other studies have proven the anti-inflammatory activity of glycyrrhizin[12,13], which is a natural triterpenoid saponin isolated from *Glycyrrhiza glabra* root[14]. Furthermore, other authors have focused on hepatoprotective[15], and antidiabetic activities of saponins[16].

Given the relationship between *J. regia* and the pharmacological properties of saponins, as well as the valorization of this plant, this article will quantify triterpenoid saponin contents, polyphenols, flavonoids, condensed tannins, as well as the total sugar contents of different extracts of *J. regia* leaves. Additionally, we aimed to investigate the antioxidant, α-glucosidase inhibitory, and antimicrobial activity of the plant extracts.

2. Materials and methods

2.1. Plant materials

J. regia leaves were harvested in July 2022 at Demnate (31° 43′ 52″ N, 7° 02′ 10″ W) in Azilal province, Morocco. The leaves were cut into tiny pieces and dried in light-proof conditions at room temperature (22 ± 1) ℃ for 72-96 h. Afterward, the leaves were ground until obtaining a fine powder.

2.2. Saponins extraction process

Figure 1 illustrates the extraction protocol of saponins from *J. regia* leaves which was performed according to Chua's protocol with some modifications[17].

The dried leaf powder was firstly delipidated in a Soxhlet cartridge using *n*-hexane as a delipidating solvent to delimit the apolar part of the plant. After delipidation, the cartridge was dried overnight in an oven at 25 ℃ to remove traces of hexane. On the following day, the residue was removed from the cartridge and underwent reflux extraction with EtOH/water solution (70∶30) for 2 h at a temperature of 80 ℃. After that, the solution was filtered and concentrated using a rotary evaporator (Heidolph Hei-VAP Precision motor, Germany) to obtain a crude extract of 70% EtOH, which was used afterward in the fractionation procedure. The crude ethanolic extract was diluted in distilled water which was then fractionated using the liquid-liquid extraction technique with butanol, and this process was repeated three times.

After the liquid-liquid extraction and completion of the separation process, the aqueous phase was lyophilized using a freeze-dryer (VaCo 2, Zirbus technology GmbH, Germany), while the butanol phase was concentrated using a rotary evaporator (Heidolph Hei-VAP Precision motor, Germany). The crude butanol extract was reconstituted in 99.8% methanol, and upon addition of diethyl ether, a precipitate was formed carrying all compounds non-soluble in diethyl ether, this precipitated fraction was filtered using a Fritted glass funnel, and then rotavaporized to remove any trace of solvent.

2.3. Determination of the total polyphenols content

Quantification of the total phenols content of the crude ethanolic 70% extract was performed by the Folin-Ciocalteu method according to the protocol described by Soto-Maldonado *et al.* with minor modifications[18]. Shortly, 2 500 µL of 10% Folin-Ciocalteu in distilled water was added to 2 000 µL of Na_2CO_3 (7.5%) and 500 µL of the ethanolic extract prepared previously at a concentration of 1 000 µg/mL. After 15 min of incubation at 45 ℃, the absorbance was measured at 756 nm against a blank solution, using a UV-visible spectrophotometer (Model UV-5800PC UV/VIS Spectrophotometer, manufactured by Shanghai Metash Instruments CO., LTD). The blank contained the same volume of Folin-Ciocalteu and Na_2CO_3 and we replaced the volume of ethanolic extract of *J. regia* leaves with 500 µL of EtOH/H_2O (70∶30). The results were expressed as mg equivalent of gallic acid (GAE) per gram of crude extract.

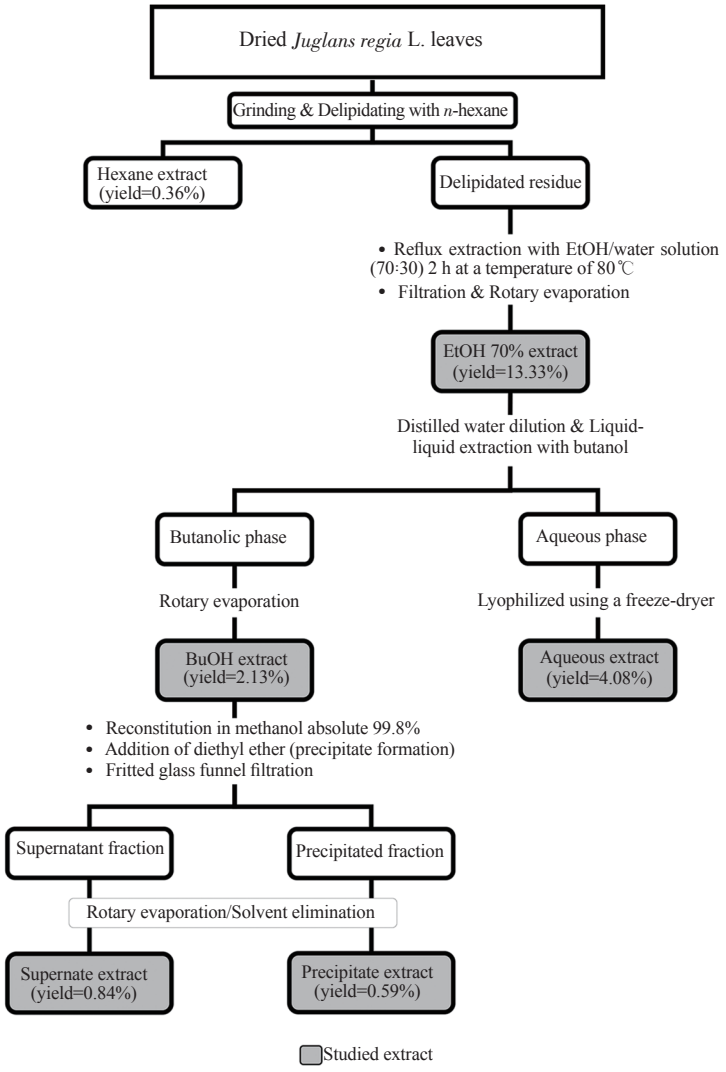

Figure 1. A diagram of saponins extraction from *Juglans regia* leaves.

2.4. Determination of the total flavonoids content

Quantification of the total flavonoid content of the crude ethanolic 70% extract was performed by the aluminum trichloride method according to the protocol described by El-Guezzane *et al.* with minor modifications[19]. Briefly, 1 mL of the ethanolic extract (70%) at a concentration of 1 000 μg/mL was diluted with 6.4 mL of distilled water, and then 0.3 mL of $NaNO_2$ solution (5%) was added. Afterward, 0.3 mL of $AlCl_3$ (10%) was added to the mixture after 5 min, then 2 mL of NaOH (1 M) was also added after another 5 min. The absorbance was measured at 510 nm against a blank solution and the results were expressed as mg equivalent of quercetin (QE) per gram of crude extract.

2.5. Determination of the total condensed tannin content

The transformation of condensed tannin into anthocyanidols using hydrochloric acid and vanillin was used to quantify the total condensed tannin concentration according to the protocol described by Cesprini *et al*[20]. Briefly, 25 μL of the 70% ethanolic extract solution (1 000 μg/mL) was added to 1.5 mL of 4% methanol vanillin solution and 750 μL of concentrated hydrochloric acid. Thereafter, the absorbance was measured at 500 nm after 15 min against a blank solution, and the results were expressed as mg equivalent of catechin (CE) per gram of crude extract.

2.6. Determination of the total sugar content

Quantification of the total sugar content of the five extracts was performed by the phenolic sulfuric acid method according to the protocol described by El Moudden *et al*[21]. Briefly, 1 000 μL of each sample (1 000 μg/mL) was added to 1 000 μL of phenol (5%) and 5 000 μL of concentrated sulfuric acid. The mixture was left for 10 min and then incubated for 20 min in a water bath at 30℃. The results were expressed as mg glucose equivalent (GE) per gram of crude extract.

2.7. Determination of the triterpenoid saponin content

The content of triterpenoid saponins was measured by the vanillin method[17]. Briefly, 250 µL of each sample was added to 250 µL of 8% ethanolic vanillin solution and 2 500 µL of concentrated sulfuric acid. The mixture was incubated for 10 min in a water bath at 60℃ and then placed in ice water for 5 min in order to stop the reaction. Thereafter, the mixture absorbances were measured at 544 nm and the results were expressed as mg of oleanolic acid equivalent (OA) per gram of extract.

2.8. 1,1–diphenyl–2–picrylhydrazyl (DPPH) free radical scavenging activity

The antioxidant activity was evaluated by DPPH assay according to the protocol described by Nounah *et al*[22]. Briefly, 500 µL of different concentrations of each extract (5-100 µg/mL) were added to 500 µL of 0.2 mM DPPH ethanolic solution, vortexed, and incubated in the dark at room temperature for 30 min, and then the absorbance values were measured at 517 nm against a blank containing 500 µL of DPPH solution and 2 500 µL of pure ethanol. The results were expressed as the amount of concentration required (µg/mL) to reduce 50% of the free DPPH radical (IC_{50}).

2.9. 2,2–azino–bis(3–ethylbenzothiazoline–6–sulfonic acid) (ABTS) free radical scavenging activity

Equal volumes of 7 mM ABTS and 2.4 mM potassium persulfate solutions were vortexed and left in the dark for 16 h at room temperature. The resulting solution was adjusted with ethanol absolute to achieve an optical density of 0.700 ± 0.020 at 734 nm. Afterward, 1 800 µL of the adjusted solution was then added to 200 µL of different concentrations (5-100 µg/mL) of each extract and the absorbances were measured after 30 min of incubation at 734 nm[23]. The results were expressed as the amount of concentration required (µg/mL) to reduce 50% of the free ABTS radical (IC_{50}).

2.10. α–Glucosidase inhibitory activity

The glucosidase assay is essentially based on the inhibition of the enzyme glucosidase prepared in a sodium phosphate buffer solution (pH = 6.4), which can hydrolyze 4-nitrophenyl-α-*D*-glucopyranoside. The formation of a yellow solution is a sign of the hydrolysis reaction. It is inversely proportional to the inhibition capacity. The appearance of a light yellow or transparent mixture indicates a higher inhibition capacity of the extract. The assessment of α-glucosidase inhibitory activity was performed following a protocol previously described[24]. Briefly, 100 µL of 0.1 M sodium phosphate buffer solution (pH = 6.4) that contains the α-glucosidase enzyme solution, was incubated along with 150 µL of each sample at 37℃ for 10 min. Afterward, 200 µL of 1 mM 4-nitrophenyl-α-*D*-glucopyranoside, prepared in the same buffer solution, was incubated at 37℃ for 30 min. Then 1 000 µL of Na_2CO_3 was added to stop the reaction and the optical densities were measured at 405 nm. Acarbose was used as a positive control.

2.11. Antimicrobial activity evaluation

The antimicrobial activity of *J. regia* leaf extracts was assessed using the agar well diffusion method[25]. Similar to the procedure used in the disk diffusion method, the Mueller Hinton agar plate surface was inoculated by spreading a volume of the microbial inoculum over the whole agar surface (20-100 µL) depending on the growth of each strain. Then, a well with a diameter of 6 to 8 mm was aseptically drilled with a sterile tip, and a volume (10 µL) of *J. regia* leaf extract was introduced into the well. The plates were allowed to diffuse and incubated for 24 h at 37℃. The inhibition zone was measured and the assay was performed in triplicate. Tetracycline (30 µg, Sigma) was used as a reference antibiotic.

Microbial susceptibility tests using agar dilution and minimum inhibitory concentration (MIC) were performed to evaluate the antibacterial activity of the prepared extracts of *J. regia* leaves against a Gram-positive bacterium *Bacillus subtilis* (*B. subtilis*) (MW471619) and two Gram-negative bacteria, *Escherichia coli* (*E. coli*) ATCC 11775, and *Klebsiella pneumoniae* (*K. pneumoniae*) (MW524112). All materials were steam-sterilized at 120℃ for 20 min. A 20 mL sterile culture medium was inoculated with tested bacteria. These strains in this study have been determined to be the most representative bacteria. Considering their antibiotic resistance, these are the most common bacteria detected in a clinical setting in health care institutes. They are usually used to evaluate antimicrobial capacity and provide an appropriate evaluation grid to determine the efficacy of collected plant extracts.

2.12. Statistical analysis

The statistical significance of the data was verified using a two-way ANOVA test with the aid of GraphPad Prism 9 software. The Bonferroni's multiple comparisons test was applied at a confidence level of 95.0% to determine the significance of the results. The data were expressed as mean±standard error of the mean of triplicate experiments (n = 3). Statistically significant differences were considered at P-values less than 0.05.

3. Results

3.1. Total polyphenol, flavonoid, and condensed tannin content

The secondary metabolites were quantified before the fractionation protocol, therefore, the assays were performed for the 70% ethanolic

leaf extract. The total polyphenol content was (147.15 ± 0.34) mg GAE/g while the total flavonoid and condensed tannin content were (22.17 ± 0.15) mg QE/g and (201.02 ± 0.26) mg CE/g crude extract, respectively.

3.2. Total sugar content

The total sugar contents of different *J. regia* leaf extracts are summarized in Table 1. The study found that the total sugar content of different *J. regia* leaf extracts is dependent on the fractionation solvent used. The highest amount of total sugar was recorded in the precipitate extract with (304.47 ± 2.11) mg GE/g, followed by the ethanolic, aqueous and butanolic extracts ($P < 0.001$). In contrast, the supernate extract had the lowest sugar content of (92.13 ± 0.93) mg GE/g.

3.3. Triterpenoid saponin of J. regia leaf extracts

The total triterpenoid saponin contents of different *J. regia* leaf extracts are reported in Table 1. The butanolic, precipitate, and supernate extract had relatively higher triterpenoid saponin content of (214.49 ± 8.91), (205.46 ± 4.11), and (200.41 ± 7.99) mg OA/g, respectively, with no significant difference ($P > 0.05$). The aqueous extract showed the lowest triterpenoid saponin content. These results indicate that less polar compounds, including saponins, may disperse in organic phases while the polar compounds remain in the aqueous extract.

3.4. Antioxidant activity of J. regia leaf extracts against DPPH and ABTS

Table 2 shows the IC_{50} values of *J. regia* extracts against DPPH and ABTS free radicals, and it could be seen that all these extracts had excellent antioxidant activity with IC_{50} values not exceeding 75 μg/mL in both DPPH and ABTS assays.

The butanolic, supernate, and precipitate extracts showed significant antioxidant activity among all extracts while the aqueous extract presented the weakest antioxidant activity. The results of the ABTS test showed that the fractionation process increased the antioxidant capacity of *J. regia* leaves. All extracts after the fractionation process recorded lower IC_{50} values than the 70% ethanolic extract with an IC_{50} of (72.26 ± 1.80) μg/mL. The aqueous extract showed the highest scavenging activity in the ABTS assay with an IC_{50} of (42.27 ± 0.80) μg/mL. The precipitate, butanolic, and supernate extracts also showed high IC_{50} values compared to those recorded in the DPPH assay, but still presented considerable antioxidant activity towards the ABTS radical.

3.5. α-Glucosidase inhibitory activity of J. regia leaf extracts

IC_{50} values of different extracts against α-glucosidase are reported in Table 2, and the percentages of inhibition of each extract at different concentrations are illustrated in Figure 2. *J. regia* leaf extracts showed excellent antidiabetic activity, even at low concentrations, less than 250 μg/mL, with IC_{50} values extremely close to the positive control acarbose [IC_{50}= (18.01 ± 2.00) μg/mL], except for the supernate extract which had the highest IC_{50} value of (81.99 ± 1.12) μg/mL. Both the aqueous and precipitate extracts exhibited strong α-glucosidase inhibitory activity with IC_{50} values of (11.00 ± 1.03) μg/mL and (13.66 ± 1.18) μg/mL, respectively, with no significant difference ($P > 0.05$).

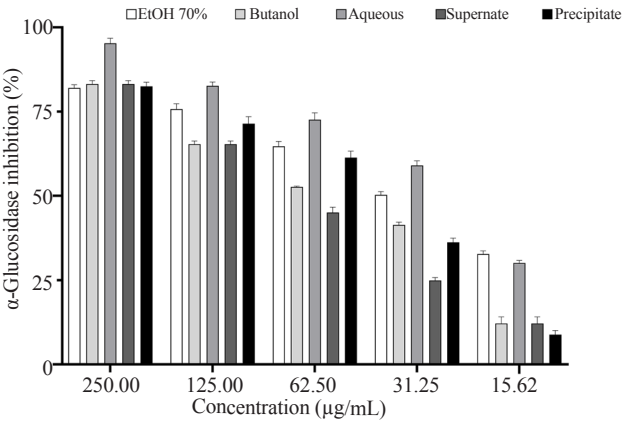

Figure 2. Percentage of α-glucosidase inhibition of different concentrations of *Juglans regia* leaf extracts.

Table 1. Triterpenoid saponin and sugar contents of *Juglans regia* leaf extracts.

Content	EtOH 70% extract	Butanolic extract	Aqueous extract	Supernate extract	Precipitate extract
Sugar content (mg glucose equivalent/g)	229.24 ± 1.02[a]	203.91 ± 2.33[b]	219.58 ± 1.25[c]	92.13 ± 0.93[d]	304.47 ± 2.11[e]
Triterpenoid saponin content (mg oleanolic acid/g)	110.27 ± 4.90[a]	214.49 ± 8.91[b]	27.70 ± 2.21[c]	200.41 ± 7.99[b]	205.46 ± 4.11[b]

Data with different superscripts in the same row are significantly different ($P<0.05$).

Table 2. IC_{50} values of the antioxidant and antidiabetic activities of *Juglans regia* leaf extracts (μg/mL).

Items	EtOH 70% extract	Butanolic extract	Aqueous extract	Supernate extract	Precipitate extract	Std
DPPH	27.74 ± 0.94[a]	6.63 ± 0.11[b]	74.29 ± 0.46[c]	11.92 ± 0.13[d]	16.49 ± 0.23[e]	1.75 ± 0.05[f]
ABTS	72.26 ± 1.80[a]	56.40 ± 2.08[b,d]	42.27 ± 0.80[c]	60.89 ± 2.20[b]	50.82 ± 2.12[d]	2.32 ± 0.01[e]
α-Glucosidase	20.27 ± 0.89[a,d]	23.32 ± 0.77[a]	11.00 ± 1.03[b]	81.99 ± 1.12[c]	13.66 ± 1.18[b]	18.01 ± 2.00[d]

Std DPPH & ABTS (ascorbic acid); Std α-glucosidase (acarbose). Data with different superscripts in the same row are significantly different ($P<0.05$).

3.6. Antimicrobial activity of J. regia leaf extracts

Table 3 depicts the zones of inhibition (ZOI) corresponding to the MICs of *J. regia* leaf extracts. Our findings revealed that the antimicrobial activity of *J. regia* leaves was relatively high compared to the reference antibiotics (Tetracycline 30 µg). All our extracts showed antimicrobial activity against the investigated strains, except for the aqueous extract which had no antimicrobial activity in the

Table 3. Inhibition zones corresponding to minimum inhibitory concentrations (µg/mL) of *Juglans regia* leaf extracts.

Strain	EtOH 70% extract	Butanolic extract	Aqueous extract	Supernate extract	Precipitate extract	Tetracycline
Bacillus subtilis	1 000 (+ + +)	500 (+)	ND (-)	250 (+ +)	1 000 (+ +)	(+ + +)
Escherichia coli	500 (+ + +)	500 (+ + +)	ND (-)	250 (+ + +)	250 (+ + +)	(-)
Klebsiella pneumoniae	250 (+ + + +)	100 (+ + +)	ND (-)	250 (+ + + +)	100 (+ + +)	(+)

ND: No antimicrobial activity detected, (-) inhibition zone < 1 mm, (+) inhibition zone 2-3 mm, (+ +) inhibition zone 3-4 mm, (+ + +) inhibition zone 4-6 mm, (+ + + +) inhibition zone > 6 mm. Standard error of the mean of triplicate readings of inhibition zones ± 0.33.

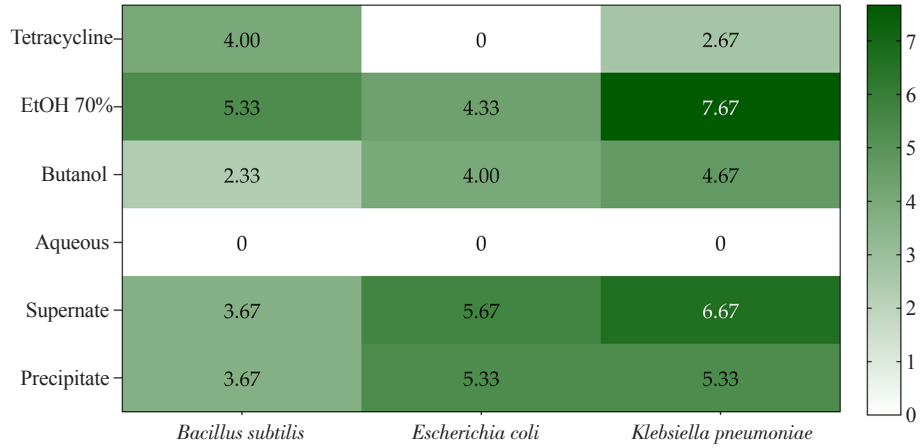

Figure 3. Heatmap diagrams representing zones of inhibition diameter (mm) of different extracts of *Juglans regia* leaves compared to tetracycline as a reference.

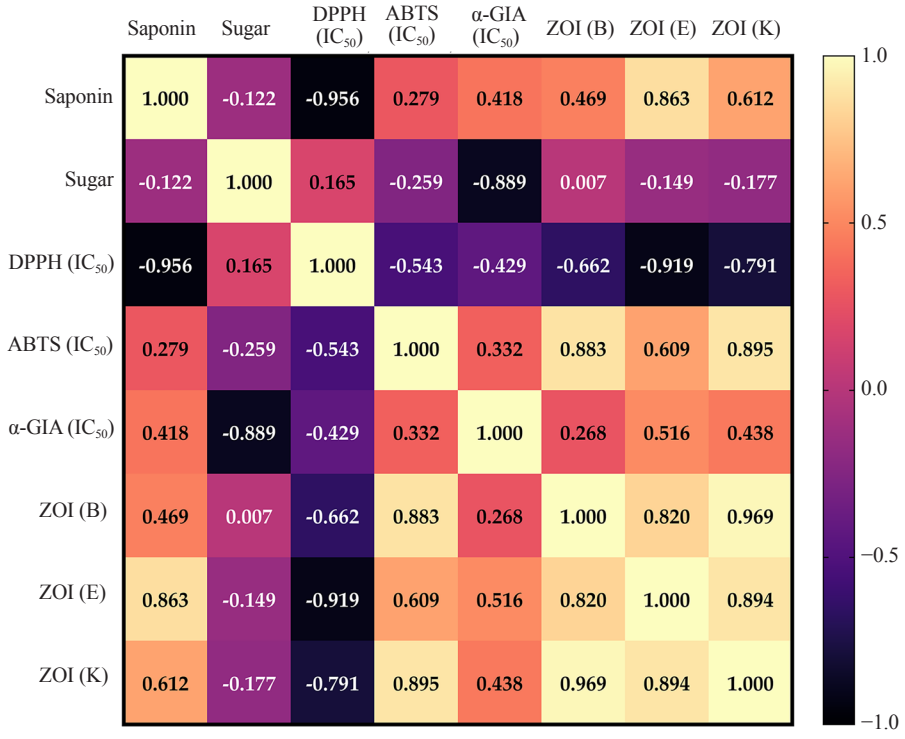

Figure 4. Pearson's correlation coefficient between antioxidant, antidiabetic, and antimicrobial activities and contents of saponin and sugar. α-GIA: α-glucosidase inhibitory activity; ZOI (B): zone of inhibition of *Bacillus subtilis*; ZOI (E): zone of inhibition of *Escherichia coli*; ZOI (K): zone of inhibition of *Klebsiella pneumoniae.*

studied concentration range (100-5 000 μg/mL).

The 70% ethanolic extract showed the largest ZOI of 5.33 mm against *B. subtilis* compared with the standard control (4 mm), with an MIC of 1 000 μg/mL, followed by the precipitate extract (ZOI = 3.67 mm/MIC = 1 000 μg/mL). All the extracts (except for the aqueous extract) inhibited *E. coli* better than the standard control. The precipitate and the supernate extracts were most effective against *E. coli* with ZOI of 5.33 and 5.67 mm, respectively, while the butanolic and 70% ethanolic extracts recorded ZOI of 4.00 and 4.33 mm, respectively. In addition, the 70% ethanolic and supernate extracts showed the most significant ZOI of 7.67 and 6.67 mm, respectively against *K. pneumoniae* (Table 3 and Figure 3).

3.7. Pearson's correlation coefficient

The Pearson's correlation coefficient is a metric utilized to quantify the linear association between two variables. As shown in Figure 4, a heat map displays the correlation coefficients between antioxidant, antidiabetic, and antimicrobial activities, and the contents of saponin and sugar. Of particular significance was the strong negative correlation ($r = -0.956$) between DPPH IC_{50} and saponin contents, which demonstrates that an increase in saponin content leads to a decrease in IC_{50} against DPPH, thereby enhancing antioxidant activity. Another noteworthy correlation was evident between DPPH IC_{50} and the zone of inhibition of *E. coli*, with an r value of −0.919, signifying a robust relationship between antioxidant and antimicrobial activities. Furthermore, the correlation between saponin content and the zone of inhibition of *E. coli*, with an r value of 0.863, underscores the impact of saponin content on antimicrobial activity.

4. Discussion

The polyphenols, flavonoids, and tannins of *J. regia* are widely investigated for their medicinal properties[26]. *J. regia* leaf extracts are rich in secondary metabolites[27,28], which gives them therapeutic potential against a wide range of diseases, including antihypertensive activity, lipid-lowering effect, protection of the liver and kidneys, and anti-cancer activity[4].

In terms of the total sugar content, the results of the study indicate that *J. regia* leaves contain a considerable quantity of total sugars in all different extracts, which can be explained by the fact that the sugars (carbohydrates) are formed through photosynthesis of the leaves[29]. The dependence of the total sugar content upon the fractionation solvent can be attributed to the different solubility properties of the saponin molecules in different solvents, leading to varying levels of sugar extraction. The high total sugar content in the precipitate extract can also suggest that this extract may have a higher saponin content than the other extracts, and the lowest sugar content at the supernatant can be explained by the insolubility of the saponin molecules in diethyl ether taken with them a considerable amount of sugar linked by glycosidic bonds. This study also suggested that the total sugar content could be used as an indication of the saponin content, since these sugar molecules are the hydrophilic part of the saponins, and they can be built up from one or more glycosidic bonds at different places of the saponin aglycone[30].

Recently, triterpenoid saponins have attracted remarkable interest due to their bioactivity and structural diversity[8,31]. They represent a large family of amphiphilic glycosides with lipophilic (triterpenoid-aglycone) and hydrophilic (sugar) parts[8] and offer a diverse range of pharmacological properties including cardiovascular effects[32], anti-inflammatory activity[33] and potential anticancer properties[34,35]. The result of this study suggests that the butanolic, precipitate, and supernate extracts could be a good source of triterpenoid saponins. Although a few studies have focused on phytochemical screening of *J. regia* saponin[36,37], none have been conducted to characterize or identify these molecules. This opens the door for further research to develop new approaches for the purification and characterization of saponins from *J. regia* leaf extracts.

Concerning the antioxidant activity, the results of this study indicate that the antioxidant activity of *J. regia* leaf extracts is influenced by the choice of fractionation solvent used. The butanolic, supernate, and precipitate extracts were found to have the highest antioxidant activity against the DPPH radical, which is consistent with the higher content of triterpenoid saponins in these extracts, the same applies to the aqueous extract which has the lowest triterpenoid saponin value, and the weakest antioxidant activity [IC_{50} = (74.29 ± 0.46) μg/mL]. These results suggest that there is a strong correlation between saponin content and antioxidant activity by DPPH. There is also a strong negative correlation between the IC_{50} value of the DPPH assay and saponin content ($r = -0.956$), proving that higher saponin content correlates with the lowest IC_{50} values, indicating higher antioxidant activity.

The results of the ABTS test showed that the fractionation process increases the antioxidant capacity of *J. regia* leaves, and all extracts after the fractionation process showed lower IC_{50} values than the 70% ethanolic extract. However, the aqueous extract, which had the weakest activity against the DPPH radical, showed the highest scavenging activity in the ABTS assay, which confirms that we cannot assert the inactivity of an extract based on a single free radical test.

However, there are no similar studies that applied the same fractionation protocol on *J. regia* leaves to compare our results with them, but there are several studies that investigate the antioxidant activity of *J. regia* leaves that our results support[4].

α-Glucosidase is an acidic enzyme responsible for the hydrolysis of oligosaccharides into glucose within the intestine leading to increase

blood glucose levels[38]. The results of this investigation indicate that the aqueous extract with the highest antioxidant activity against ABTS radical, also shows the highest α-glucosidase inhibitory activity, meaning that the antioxidant activity may influence the antidiabetic behavior of our extract. Furthermore, both extracts (aqueous and precipitate) exhibited stronger activity than the positive control, acarbose. The same finding has been reported by several researchers in the phytochemical area[39,40]. Zhang *et al.* found that the hydroalcoholic extract (EtOH/Water) of propolis shows more potent α-glucosidase inhibitory activity than acarbose[41]. A recent study in 2020 also revealed that walnut septum acetone extract has a stronger α-glucosidase inhibitory activity than acarbose (IC_{50} = 0.14 mg/mL versus 0.80 mg/mL)[42]. There is a strong negative correlation between IC_{50} values of α-glucosidase inhibitory activity and sugar content (r = −0.889), suggesting that natural sugars from *J. regia* may have the capacity to increase α-glucosidase inhibitory activity by interacting with the α-glucosidase enzyme, inhibit them, and consequently decrease blood glucose levels, but further in-depth studies are required to validate this assumption. Overall, *J. regia* leaves showed outstanding antidiabetic activity as evidenced by excellent α-glucosidase inhibitory activity, making this walnut by-product a promising source of bioactive compounds for pharmacological purposes.

This paper proposes the use of *J. regia* leaves, a by-product of walnut cultivation, as an antimicrobial material. *J. regia* extracts showed interesting antimicrobial potency against a wide variety of microbial pathogens[4]. According to the study by Boulfia *et al*, it can be said that the leaves of *J. regia* have potent antibacterial activity against *B. subtilis*, better than that of the bark extracts with MICs of 5 000 µg/mL in the ethanolic and diethyl ether extracts, and 2 500 µg/mL in the acetone extract[43] and these MICs are significantly higher than those found in *J. regia* leaf extracts. In the case of Gram-negative bacteria, *E. coli* and *K. pneumoniae*, they were found to be more sensitive to *J. regia* extract than *B. subtilis* with MICs not exceeding 500 µg/mL. The 70% ethanolic and supernate extracts showed the most significant ZOI of 7.67 and 6.67 mm, respectively against *K. pneumoniae*. These findings are very important since *K. pneumoniae* is one of the major contributors to healthcare contamination in humans, including pneumonia, urinary tract infections, sepsis, and meningitis[44]. Our results were better than what Oliveira *et al*., reported in their study on the green husk of *J. regia* from different cultivars[45], which were incapable to inhibit the growth of *E. coli* at the high concentration studied (100 mg/mL). The results of the present study provide evidence for the potential use of *J. regia* leaves as an antimicrobial agent. Pearson's correlation coefficient demonstrated the bacterial strains studied are all positively correlated between *B. subtilis* and *E. coli* (r = 0.820), between *B. subtilis* and *K. pneumoniae* (r = 0.969), and between *K. pneumoniae* and *E. coli* (r = 0.894). It also showed a strong positive correlation between triterpenoid saponin content and *E. coli* ZOI (r = 0.863), as well as a strong negative correlation between DPPH IC_{50} and *E. coli* and *K. pneumoniae* ZOI (r = −0.919 and −0.791 respectively), suggesting that triterpenoid saponin compounds may influence antimicrobial activity as they did with antioxidant activity. However, further studies are needed to identify the molecules present in our extract and test them individually to prove this assumption.

In conclusion, the butanol, supernate, and precipitate extracts showed the highest levels of triterpenoid saponins with the most significant antioxidant activity against the DPPH free radicals. All our extracts exhibited considerable antioxidant activity with IC_{50} values not exceeding 75 µg/mL in both DPPH and ABTS tests. Furthermore, the aqueous and precipitate extracts had a potent ability to inhibit α-glucosidase enzyme better than acarbose. *J. regia* leaves also showed a relatively high antibacterial activity compared with a reference antibiotic tetracycline. Overall, the results of this investigation showed that the triterpenoid saponins of *J. regia* leaves have both biochemical and pharmacologically potent activities.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

MT and LCM were responsible for conceptualization, as well as project administration, and AEY provided supervision. YE, SM, and MH were responsible for data curation and formal analysis, while AEY, HH, and AB conducted investigations. AB and LCM also contributed to the drafting and critical revision of the manuscript. MEAF, AB, KWG, and AK were responsible for validation, and YE, AK, ANA, and KWG contributed to visualization. YE, and MH were responsible for writing the original draft, while AB, HH, AK, ANA, KWG, and MEAF contributed to the review and editing of

the manuscript. All authors have read and agreed to the published version of the manuscript.

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