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Bioactivity of Walnut: Investigating the Triterpenoid Saponin Extracts of *Juglans regia* Kernels for Antioxidant, Anti-diabetic, and Antimicrobial Properties

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Abstract: The increasing demand for plant-based medicines makes plant research attractive to find natural alternatives that can replace synthetic products or at least reduce their use. Saponins are a group of natural phytochemical compounds in most herbs, vegetables, and legumes. There are two different types of saponin compounds, which can be classified based on the nature of the aglycone bound to the oligosaccharide fragments, whether it is a steroid or triterpenoid. This paper is devoted to saponins with triterpenoid aglycone and aims to study the relationship between their content and several biological activities in different *Juglans regia* kernel extracts, including antioxidant, antimicrobial and antibacterial activities. Antioxidant activity was assessed against two free radicals: DPPH and ABTS. Oleanolic acid

was used as a standard to quantify triterpenoid saponins, measured by the vanillin method, whereas Acarbose and Tetracycline were used as positive controls to evaluate the α-glucosidase inhibitory and antimicrobial activities, respectively, of walnut kernel extracts. Findings show that hydroalcoholic extract (EtOH 70%) has a considerable content of phenolic compounds, with 59.51 ± 0.26 mg GAE/g in total polyphenol contents, while the content of total flavonoids and condensed tannins were 3.14 ± 0.27 mg QE/g and 85.65 ± 0.37 mg CE/g of crude extract, respectively. In terms of antioxidant activity, the butanolic extract proved to be the most effective against DPPH with IC_{50} = 7.74 ± 1.49 µg/mL, while the precipitated extract showed the highest scavenging activity against the ABTS free radical with IC_{50} = 33.14 ± 2.96 µg/mL. In addition, butanolic and hydroalcoholic extracts of *Juglans regia* kernels showed an α-glucosidase inhibitory activity even more efficiently than synthetic drugs such as Acarbose, with IC_{50} values of 11.66 µg/mL and 16.17 µg/mL, respectively, versus 18.01 µg/mL in Acarbose. The evaluation of the antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* strains revealed that some extracts could inhibit these bacteria. This study successfully assessed the bioactivities of the extracts, suggesting that the extracts of medicinal plants can demonstrate stronger bioactivity than pure synthetic compounds.

Keywords: *Juglans regia* kernels; triterpenoid saponin; antioxidant activity; DPPH; ABTS; antidiabetic activity; α-glucosidase; antimicrobial activity; agar well diffusion method

1. Introduction

Nowadays, walnut consumption is considered a key element that can improve human health [1,2]. Several clinical trials have been devoted to highlighting the benefits of walnut seed consumption on human health and, in particular, on the brain and cardiovascular health [3–7], blood lipids and pressure [1,8–11], along with its effect on improving insulin resistance [12]. All these properties related to the consumption of walnut seeds are related to their composition in unsaturated fatty acids and bioactive compounds.

Currently, infectious diseases caused by multidrug-resistant strains (MDR) constitute a major problem for health. Some predictions show the major future health damage that humanity could have due to the re-emergence of these infectious diseases. Moreover, the search for antimicrobial molecules has become an essential approach to replace antibiotics which are undergoing elimination because of the inefficiency rendered by resistance [13–15]. Among the most sought-after products, natural substances occupy the first place because they have already shown promising antibacterial effects [16–18].

On the other hand, oxidative stress and its related diseases, such as diabetes and cancer also constitute another group of complex pathologies which affect human health massively [19]. The treatment of these pathologies is based on the use of chemical and biochemical compounds targeting different pathways involved. However, these therapeutical approaches always remain inefficient because of the multiple risk factors associated with

these pathologies as well as the stochasticity of mechanistic events leading to their major phenotypes. Therefore, the identification and evaluation of natural compounds as drugs candidate [20] for these diseases is based on, particularly, the evaluation of anti-oxidative properties of these bioactive compounds [21,22].

In fact, walnuts (*Juglans regia*) are a great source of unsaturated fatty acids [23–25], fiber, minerals (potassium, calcium, and magnesium) [26,27], amino acids and proteins [28,29], phenolic compounds and saponins [30–32], as well as a variety of other compounds that make *J. regia* has a vast diversity of medicinal properties [33]. In addition, the phytochemicals present in plants like *J. regia* have been shown to have therapeutic effects, including anti-cancer, anti-inflammatory, and anti-bacterial properties [34].

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 [35]. Various studies have been devoted to investigating the anticancer activity of saponin molecules isolated from different species of plants such as *Platycodon grandiflorum*, *Kalopanax pictus*, *Glochidion eriocarpum*, *Entada phaseoloides*, *Dipsacus asperoides*, *Bupleurum falcatum*, and *Acacia victoriae* [36–43]. Other studies have proven the anti-inflammatory activity of glycyrrhizin [44–46], which is a natural triterpenoid saponin isolated from *Glycyrrhiza glabra* root [47]. Furthermore, other authors have focused on hepatoprotective activity [48–50], and the possibility of employing saponins as anti-diabetic agents in type II diabetes [51].

Considering all these reasons and in order to contribute to the valorization of walnut kernels, and also to see whether plant extracts can possess biological activities better than pure synthetic compounds, this paper will present an assessment of the antioxidant, antimicrobial and anti-diabetic activities of triterpenoid saponins extracted from walnut kernels, through the quantification of phenolics, polysaccharides and saponins, as well as the examination of the free radical scavenging activity against DPPH and ABTS.

2. Materials and Methods

2.1. Plant materials

Walnut *Juglans regia* fruits were harvested in June 2022, in Demnate - Azilal province (31° 43′ 52″ **N**, 7° 02′ 10″ **W**), Morocco, peeled and then manually dehulled to obtain only the kernels, which were ground to a fine powder.

2.2. Triterpenoid saponins fractionation process.

Figure 1 shows the extraction protocol of triterpenoid saponins from *Juglans regia* kernels which was performed according to Chua's *et al*. protocol with some modifications [52].

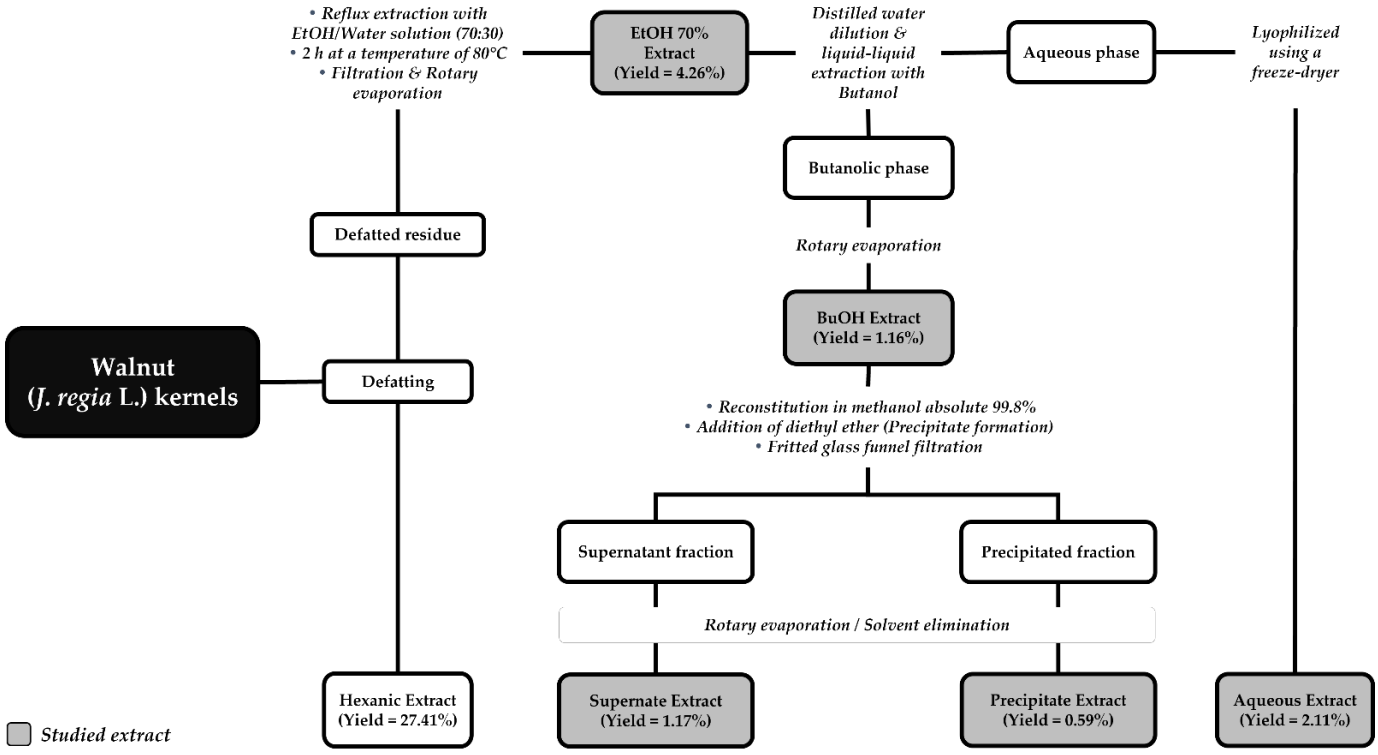

Figure 1. Diagram of Triterpenoid saponins fractionation process from *Juglans regia* kernels.

The ground kernel powder was firstly defatted in a soxhlet cartridge using *n*-Hexane as a delipidating solvent to delimit the non-polar fraction of our kernels. After defatting, the cartridge was dried overnight in an oven (T = 25°C) 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°C. 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 hydroalcoholic extract (EtOH 70%) 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. Total polyphenols quantification

Quantification of the total phenols content (TPC) of the crude ethanolic 70% extract was performed by the Folin-Ciocalteu method according to the protocol described by Soto-Maldonado in 2022, with minor modifications [53]. Shortly, 2500 µL of 10% Folin-Ciocalteu in distilled water was added to 2000 µL of Na_2CO_3 (7.5%) and 500 µL of the ethanolic extract prepared previously at a concentration of 1000 µg/mL. After 15 min of incubation at 45°C, 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 contains the same volume of Folin-Ciocalteu and Na_2CO_3 and we replace the volume of ethanolic extract of *J. regia* kernels with 500 µL of EtOH/H_2O (70:30). The results were expressed as mg equivalent of gallic acid per gram of crude extract

2.4. Total flavonoids quantification

Quantification of the total flavonoid content (TFC) of the crude ethanolic 70% extract was performed by the aluminium trichloride method according to the protocol described by El-Guezzane *et al*., in 2021, with minor modifications [54]. 1 mL of the ethanolic extract (70%) at a concentration of 1000 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 (1M) 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 per gram of crude extract.

2.5. Total condensed tannin quantification

The transformation of condensed tannin into anthocyanidols using hydrochloric acid and vanillin is used to quantify the total condensed tannin concentration (TCT) according to Cesprini *et al*. protocol [55]. Briefly, 25 µL of our 70% ethanolic extract solution (1000/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 presented in mg equivalent of catechin per gram of crude extract.

2.6. Total Polysaccharides quantification

Quantification of the total polysaccharides content of the five extracts was performed by the phenolic sulfuric acid method according to the protocol described by Zeng *et al*., in 2019 [56]. Briefly, 1000 µL of each sample (1000 µg/mL) was added to 1000 µL of phenol (5%) and 5000 µL of concentrated sulfuric acid. The mixture was left for 10 min and then incubated for 20 min in a water bath at 30°C. The results were presented in mg glucose equivalent per gram of crude extract.

2.7. Triterpenoid saponin quantification

The content of triterpenoid saponins was measured by the vanillin method [52]. 250 µL of each sample was added to 250 µL of 8% ethanolic vanillin solution and 2500 µL of concentrated sulfuric acid. The mixture was incubated for 10 min in a water bath at 60°C 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 presented in mg of oleanolic acid equivalent per gram of extract.

2.8. DPPH free radical scavenging activity

Assessment of antioxidant activity by DPPH (1,1-diphenyl-2-picrylhydrazyl) was performed according to the Nounah protocol described in 2017 [57]. 2500 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 our DPPH solution and 2500 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. ABTS free radical scavenging activity

Equal volumes of ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and Potassium persulfate solutions at the concentration of 7mM and 2.4mM, respectively, were vortexed and left in the dark for 16h at room temperature. The resulting solution was adjusted with Ethanol Absolute to achieve an optical density of 0.700 ± 0.02 at 734nm. Afterward, 1800 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 [58]. 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 assessment of α-glucosidase inhibitory activity was carried out following a protocol previously described [59–61]. 100 µL of 0.1M 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°C for 10 min, afterwards, 200 µL of 4-Nitrophenyl-α-d-glucopyranoside (*p*NPG) 1mM, prepared in the same buffer solution, the mixture was incubated at 37°C for 30min. 1000 µL of Na_2CO_3 was added to stop the reaction and the optical densities were measured at 405 nm. Acarbose has been used as a positive control.

2.11. Antimicrobial activity evaluation

The antimicrobial activity of *Juglans regia* kernel extracts is assessed using the agar well diffusion method [16,62,63]. 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 is aseptically drilled with a sterile tip, and a volume

(10 µL) of the antimicrobial agent (*Juglans regia* kernel extract) is introduced into the well. The plates were allowed to diffuse and incubated for 24 hours at 37°C. Antimicrobial agent extracts from *Juglans regia* kernels will diffuse in Mueller Hinton agar medium, and the size of the inhibition zone was measured in millimeters. The assay was performed in triplicate, whereas Tetracycline (TET 30 µg) control disks (Sigma) were used as the 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 *Juglans regia* kernels against a Gram-positive bacterium *Bacillus subtilis* (MW471619) and two Gram-negative bacteria, *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* (MW524112). All materials were steam-sterilized at 120°C for 20 min. A 20 ml sterile culture medium was inoculated with tested bacteria.

3. Results and Discussion

3.1. Total polyphenol, flavonoid, and condensed tannin content

Juglans regia kernels contain a considerable content of phenolic compounds such as polyphenols, flavonoids, and tannins [64–67], which may contribute to the prevention of a wide range of oxidative stresses [68], leading to reduce the risk of cardiovascular and degenerative illnesses [69–71]. Those secondary metabolites exhibit many other pharmacological properties, including antioxidant, antimicrobial, and anticancer activities [72–74].

Figure 2 represents the TPC, TFC and TCT contents exhibited in the ethanolic extract of 70% of the walnut kernels (before the fractionation process). The results show that the walnut kernels have a total polyphenol content of 59.51 ± 0.26 mg GAE/g, while the content of total flavonoids and condensed tannins were 3.14 ± 0.27 mg QE/g and 85.65 ± 0.37 mg CE/g crude extract, respectively, ($p < 0.0001$).

These results are higher than those found in several Polish walnut cultivars, which did not exceed 20.9 mg GAE/g in terms of TPT [75]. However, these results are generally lower than the levels found in the leaves and flowers of walnut, which are considered an excellent source of phenolic compounds when compared to other parts of this plant [76,77].

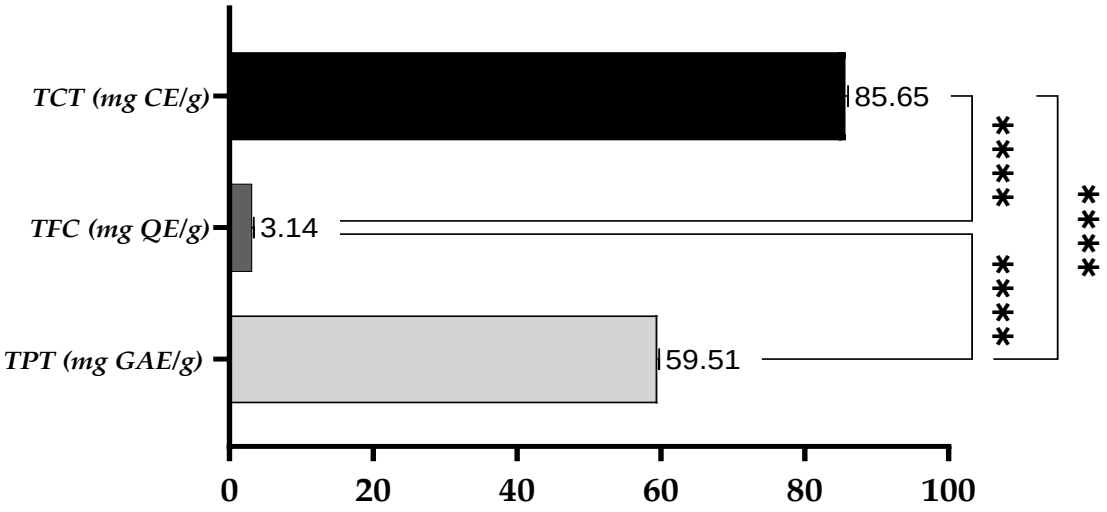

Figure 2. Polyphenols, flavonoids, and condensed tannins content of 70% ethanolic extract of *Juglans regia* kernels. TPT: Total polyphenol content. TFT: Total flavonoid content. TCT: Total condensed tannin. ****$p < 0.0001$.

3.2. Polysaccharides quantification

Polysaccharides are natural polymers which generally are made up of more than 10 monosaccharides linked by O-glycosidic bonds [78], which have an essential and primary role in the growth and development of organisms [79], and also in the reinforcement of the immune function [80,81], as well as having numerous pharmacological properties such as antioxidant [82,83], and anti-inflammatory activities [84,85].

The polysaccharide contents of the different *Juglans regia* kernel extracts are summarized in Table 1. The results show that all the contents are significantly different at $p < 0.0001$, indicating that the fractionation solvent may have an impact on the polysaccharide contents.

Table 1. Polysaccharides and triterpenoid saponin contents of *Juglans regia* kernel extracts.

	EtOH 70%	BuOH	Aqueous	Supernate	Precipitate
PSC (mg GE/g)	95.02 ± 1.35 [a]	68.58 ± 0.80 [b]	79.02 ± 1.02 [c]	103.47 ± 1.13 [d]	170.13 ± 2.09 [e]
TSC (mg OA/g)	94.94 ± 1.14 [a]	106.66 ± 1.98 [b]	10.09 ± 0.98 [c]	94.66 ± 1.47 [a]	140.94 ± 2.15 [d]

PSC: Polysaccharides Content; TSC: Triterpenoid saponin content
Data with the same superscript numbers in the same row are not significantly different ($p > 0.05$).

The highest polysaccharide content was recorded in the precipitate extract with 170.13 ± 2.09 mg GE/g, indicating that this fraction may contain a significant amount of saponins, since polysaccharides constitute the hydrophilic part of saponin molecules [86]. Second, we found the supernate and hydroalcoholic extract with 103.47 ± 1.13 mg GE/g and 95.02 ± 1.35 mg GE/g, respectively ($p < 0.0001$). Concerning the aqueous and butanolic

extracts showed the lowest contents compared to the other walnut kernel extracts, with 79.02 ± 1.02 mg GE/g in the aqueous extract and 68.58 ± 0.80 mg GE/g. Overall, it can be seen that walnut kernels have respectable levels of polysaccharide compounds that give them the possibility of being valued in many pharmaceutical and nutratherapeutic fields [80,83,84].

3.3. Triterpenoid saponin quantification

As previously mentioned, this study aimed to evaluate the impact of solvent fractionation on triterpenoid saponins and quantify their content in *Juglans regia* kernels. We were interested in this group of compounds (triterpenoid saponin) because of their ability to provide a variety of pharmacological properties, which help to prevent a wide range of cancers and cardiovascular diseases [87–92], and also have potential anti-inflammatory activity as evidenced by numerous research [93–96].

The quantification results (Table 1) showed that all investigated extracts had significantly different contents ($p < 0.05$) except for the hydroalcoholic and aqueous extracts which contained similar concentrations of triterpenoid saponins with 95.16 ± 1.14 mg OA/g and 94.88 ± 1.47 mg OA/g, respectively ($p > 0.05$). These significant differences mean that the triterpenoid contents have been influenced by polarity and type of fractionation solvent. The highest triterpenoid saponin content was found in the precipitated extract with 141.16 ± 2.15 mg OA/g, followed by the butanolic extract with 106.81 ± 1.98 mg OA/g ($p < 0.0001$), and these results indicate that the fractionation protocol applied in this study was effective in extracting the maximum content of the target compound in the last extraction step as a precipitate fraction. The fractionation process is based on the fact that the highest polar compound transfers to the aqueous phase while the less polar compounds (such as triterpenoid saponin in this case) remain in the organic phase, which may explain the lower saponin concentration in the aqueous extract 10.14 ± 0.98 mg OA/g.

However, it is important to note that these results are similar to those reported by Chen *et al.* in 2022, who quantified the saponin content of *Camellia sinensis* seeds and reported concentrations above 140 mg/g in different collection periods [97]. Furthermore, *Juglans regia* kernel extracts show considerable saponin content compared to *Xanthoceras sorbifolium* and *Tetracarpidium ionophore* (black walnut) seeds with 6.7 mg/100g and 0.08 mg/100g, respectively [98,99]. This increases the possibility of being exploited for their pharmacological properties.

3.4. DPPH and ABTS radical scavenging activity

It is recommended to use several radicals to evaluate the antioxidant activity, since the bioactive compounds have varying sensitivity and activity towards different free radicals, and for this reason the antioxidant activity of our extract has been evaluated using two free radicals : DPPH and ABTS.

According to the results presented in Table 2, it can be clearly seen that the hydroalcoholic and butanolic extracts showed stronger activity against DPPH compared to

ABTS, opposite to the other extracts that showed more activity against the ABTS free radical than DPPH.

Table 2. IC_{50} values (µg/mL) of the DPPH, ABTS, and α-glucosidase assays of *J. regia* kernel extracts.

	EtOH 70%	BuOH	Aqueous	Supernate	Precipitate	Std
DPPH	9.16 ± 0.56^{a}	7.74 ± 1.49^{a}	43.63 ± 2.33^{b}	88.51 ± 2.68^{c}	63.93 ± 0.23^{d}	$1.75^{\bullet} \pm 0.05^{e}$
ABTS	51.36 ± 1.10^{a}	43.73 ± 0.61^{b}	36.42 ± 1.79^{c}	61.37 ± 1.29^{d}	33.14 ± 2.96^{c}	$2.32^{\bullet} \pm 0.01^{e}$
α-Glu	16.17 ± 0.24^{a}	11.66 ± 1.21^{b}	42.08 ± 0.92^{c}	55.89 ± 1.04^{d}	29.63 ± 1.31^{e}	$18.01^{\bullet\bullet} \pm 2.00^{a}$

Data with the same superscript numbers in the same row are not significantly different ($p > 0.05$).
α-Glu: α-glucosidase. • Acid ascorbic IC_{50} value; •• Acarbose IC_{50} value.

Starting with DPPH, our findings showed that the butanolic extract has the strongest antioxidant activity compared to the other extracts studied, with an IC_{50} value equal to 7.74 ± 1.49 µg/mL, indicating that *Juglans regia* has potent antioxidant activity. This is well supported by the study of Zhang *et al*, that also reported a higher activity in the butanolic fraction compared to ethyl acetate and aqueous fractions [100]. The 70% ethanolic extract also showed strong antioxidant activity similar to the butanolic extract ($p > 0.05$) with 9.16 ± 0.56 mg/mL in the IC_{50} value, which may be related to the considerable content of triterpenoid saponins and phenolic compounds in this fraction. All remaining fractions showed considerable antioxidant activity against DPPH with IC_{50} values of 43.63 ± 2.33 mg/mL in the aqueous fraction, 63.93 ± 1.39 mg/mL in the precipitated fraction, and 88.51 ± 2.68 mg/mL in the supernatant fraction, which indicates that the fractionation protocol applied in this study was able to extract the target compounds and preserve their biological activity.

As mentioned above, the bioactive compounds had different activity against different free radicals, and this was demonstrated in the present study. The precipitate extract which came third on antioxidant activity against DPPH, was the most effective extract against ABTS free radical compared to other fractions of *Juglans regia*, with IC_{50} = 33.14 ± 2.96 mg/mL. The aqueous extract showed a very similar result to that of the precipitate with an IC_{50} of 36.42 ± 1.79 mg/mL ($p > 0.05$), and both fractions were recorded the highest contents of triterpenoid saponins, which may justify their effectiveness as a source of antioxidant molecules. Moreover, all other extracts showed significant antioxidant activity with IC_{50} values of 51.36 ± 1.10 mg/mL, 43.73 ± 0.61 mg/mL, and 61.37 ± 1.29 mg/mL, in hydroalcoholic (EtOH 70%), butanolic and supernate extracts, respectively ($p < 0.05$).

These findings support and confirm the potent antioxidant activity of *Juglans regia* kernels reported in several studies [75,101–104].

3.5. α-glucosidase inhibitory activity

Diabetes is a chronic illness that happens when a patient's body can't produce sufficient insulin or can't use it [105]. The International Diabetes Federation (IDF) ranks hyperglycemia as the third most severe risk that leads to death worldwide, after high blood pressure and smoking [106,107]. In Africa, 14.2 million people were diagnosed with diabetes in 2015, and that number is expected to jump to 34.2 million by 2040, according to the IDF [106], which makes diabetes one of the most prevalent diseases in Africa and the world.

On the other hand, herbal medicines are largely used in Morocco to treat various diseases, including diabetes [108], and this is due to the fact that these medicinal plants have a strong ability to inhibit enzymes that are involved in carbohydrate digestion. α-amylase and α-glucosidase are important enzymes involved in carbohydrate metabolism [109], the first one is responsible for the hydrolysis of long-chain carbohydrates into oligosaccharides and then, α-glucosidase intervenes to hydrolyze oligosaccharides into glucose which is then absorbed by the small intestine [110], and increase blood glucose levels [111,112].

The glucosidase assay is essentially based on the inhibition of the enzyme glucosidase prepared in a sodium phosphate buffer solution (pH = 6.4), which is able to hydrolyze pNPG. 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 efficiency of the inhibition capacity of the extract.

Table 2 showed that all our extracts significantly inhibited α-glucosidase enzyme, in vitro, with all IC_{50} values not exceeding 60 µg/mL. The butanolic and hydroalcoholic extracts with the most potent antioxidant activity against DPPH also show the strongest inhibition of α-glucosidase better than acarbose, with IC_{50} values equal to 11.66 ± 1.21 µg/mL and 16.17 ± 0.24 µg/mL, respectively, ($p < 0.05$), and this means that the antioxidant activity can impact the anti-diabetic property of our extract. In their study, Tan *et al.* report that the compound isolated from EtOH 70% walnut extract, especially flavonoids, shows a significant α-glucosidase inhibitory activity (α-GIA), which can justify and support our results [113].

The precipitated extract also showed significant inhibition of α-glucosidase enzyme with IC_{50} = 29.63 ± 1.31 µg/mL, this extract is previously reported with the highest triterpenoid saponin content, which may correlate and impact the anti-diabetic property of this extract. Several triterpenoid saponins were isolated from *Gypsophila oldhamiana* roots and the results shows that all active compounds exhibited better α-GIA than acarbose with IC_{50s} between 15.2 and 98.2 µM, whereas the IC_{50} of acarbose was 388.0 µM [114]. Furthermore, in 2015, a study by Nguyena *et al.* revealed that triterpenoid saponins isolated from *Schefflera sessiliflora* leaves showed greater α-GIA (IC_{50} = 5.99 - 76.58 µM) than acarbose (IC_{50} = 214.5 µM) [115], and this may support our hypothesis of saponin content are compounds responsible for α-GIA from the precipitate extract.

The remaining extracts (aqueous and supernate) showed the lowest α-GIA compared to other studied extracts, but still have significant anti-diabetic activity with IC_{50} values of 42.08 ± 0.92 µg/mL and 55.89 ± 1.04 µg/mL, respectively ($p < 0.0001$).

Overall, *Juglans regia* kernels showed enhanced anti-diabetic activity, which was positively correlated with the DPPH results ($r = 0.900$) (Figure 3) and this result affirms that walnut kernels are a great source of bioactive compounds for various pharmacological purposes.

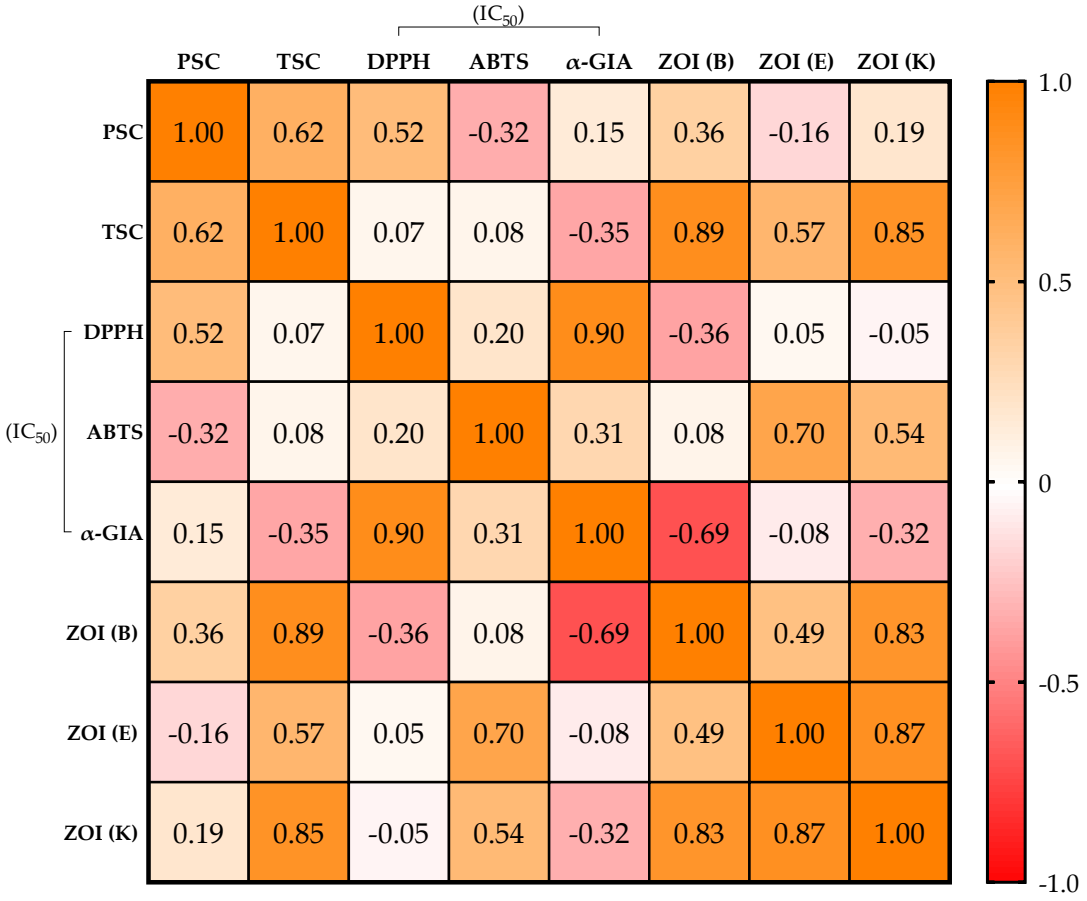

Figure 3. Pearson's matrix correlation coefficient between antioxidant, anti-diabetic and antimicrobial activities and contents of saponin and sugar. PSC: Polysaccharides contents. TSC: Triterpenoids saponin contents. ZOI (B) : Zone of inhibition of *B. subtilis*. ZOI (E) : Zone of inhibition of *E. coli*. ZOI (K) : Zone of inhibition of *K. pneumoniae*.

3.6. Antimicrobial activity

The results of the antimicrobial activity of the studied walnut kernel extracts (zone of inhibition and MIC in µg/mL) are summarized in Table 3. The results showed that all extracts (except the aqueous extract) exhibited antimicrobial activity against the bacteria tested. Discussing the MICs based on Table 3, it can be seen that our active extracts (EtOH 70%, BuOH, supernate and precipitate) inhibits gram-negative bacteria (MICs ≤ 1000 µg/mL) more effectively than gram-positive bacteria, (5000 µg/mL ≤ MICs ≤ 1000 µg/mL). The

butanol extract has the lowest MICs, which is to say the highest antimicrobial activity against the three bacteria, with MIC = 1000 µg/mL in *B. subtilis* and 500 µg/mL in both *E. coli* and *K. pneumoniae*.

Table 3. Inhibition zone halos corresponding to the MICs (µg/mL) of *Juglans regia* kernel extracts.

	EtOH 70%	**BuOH**	**Aqueous**	**Supernate**	**Precipitate**	**TET**
B. subtilis	5000 (+ + + +)	1000 (+ + +)	ND (-)	2000 (+ +)	5000 (+ + +)	(+ + +)
E. coli	500 (+ +)	500 (+ + + +)	ND (-)	500 (+ + + +)	500 (+ + +)	(+ + + +)
K. pneumoniae	500 (+ + +)	500 (+ + +)	ND (-)	1000 (+ + +)	500 (+ + +)	(+ + + +)

TET: Tetracycline. 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.

These results were stronger compared to the finding in Pereira *et al.* study [116], which was dedicated to evaluating the bioactive properties (including antimicrobial activity) of six different cultivars of *Juglans regia* L. The authors report MICs of 100 mg/mL against the same bacteria studied in our investigation [116], suggesting that the fractionation protocol used in this paper can preserve the bioactive compound and retain its activity.

Regarding the zones of inhibition, Figure 4 shows that both butanolic and supernate extracts inhibit *E. coli* better than the control with ZIO = 6.33 mm and 6.67 mm respectively, versus 6.00 mm in the control. Similarly, the EtOH 70%, butanolic and precipitated extracts inhibit B better than the control with ZIO = 6.00 mm, 5.33 mm, and 5.67 mm respectively, while TET have a ZIO = 5.00 mm. To explain these results, we adhere to the authors' interpretation which states that antimicrobial activity is strongly related to the content of phenolic compounds [117–119].

It is noteworthy that Figure 4 shows that zones of inhibition of *B. subtilis* and *K. pneumoniae* are highly correlated with triterpenoid content (r = 0.890 and 0.850, respectively) which may explain the efficacy of our extracts against this bacterium and may also explain the inactivity of the aqueous extract (with the lowest triterpenoid saponin content 10.09 ± 0.98 mg OA/g) against the studied bacterium.

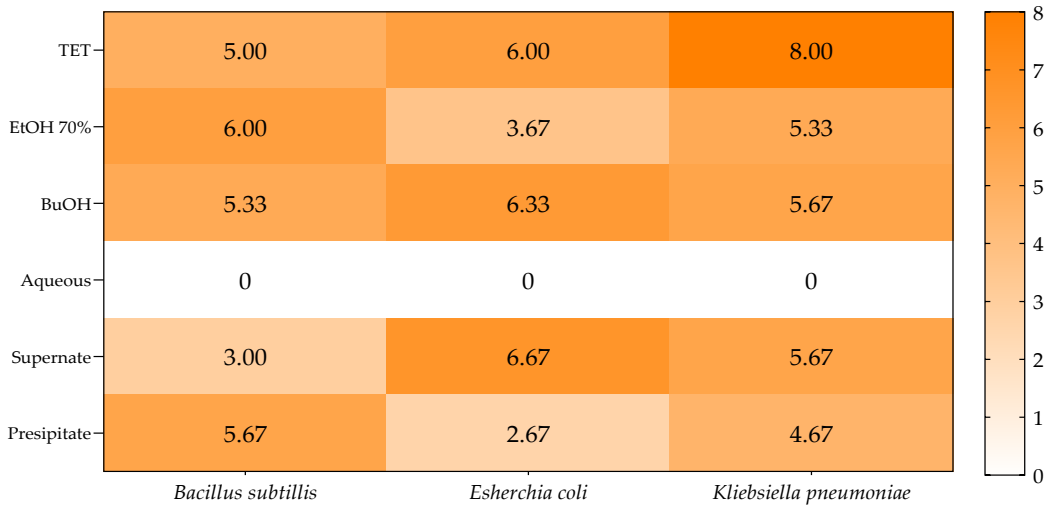

Figure 4. Heatmap diagrams representing zones of inhibition diameter (mm) of different extracts of *Juglans regia* kernels compared to Tetracycline (TET) as reference.

4. Conclusions

This study has provided an evaluation of the antioxidant, antimicrobial, and α-glucosidase inhibitory activities of crude walnut extracts fractionated by a specific protocol in which triterpenoid molecules were precipitated in the last phase. The precipitated extract was the richest in polysaccharides, which was a positive indication of its richness in triterpenoid saponin compounds. Indeed, the precipitated extract recorded the highest content of triterpenoid saponins compared to all the extracts investigated, which may explain its potent antioxidant activity against ABTS free radical that had the lowest IC_{50} value of 33.14 ± 2.96 µg/mL. The DPPH radical scavenging activity reveals different activity results of the extracts compared to ABTS assay. The butanolic and ethanolic extracts showed the highest activity against DPPH free radical, and this may also explain their α-GIA potency which were better than acarbose. All extracts (excluding the aqueous extract) demonstrated antimicrobial activity against the studied bacteria. The most potent extract was the BuOH extract, with a minimum inhibitory concentration (MIC) of 1000 µg/mL against *B. subtilis*, and 500 µg/mL against *E. coli* and *K. pneumoniae*. These results indicate that crude extract may reveal more activity than pure compounds.

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