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PHYTOCHEMICAL SCREENING AND FREE RADICAL SCAVENGING ACTIVITY OF THE AQUEOUS EXTRACT OF *JUGLANS REGIA* (WALNUTS)

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ABSTRACT

The world of plants is full of resources and virtues from which people take not only his food but also active substances that often provide a benefit to his body. This research aimed to the valorization of a medicinal plant known by their traditional use; *Juglans regia* L (*Juglandaceae*) by phytochemical screening of bioactive compounds and evaluation of the antioxidant activity of leaves and bark aqueous extracts. The extraction yield indicated that the aqueous extract decocted from the leaves presented high yield (28.23 ± 0.63%) compared to the other extracts. Qualitative phytochemical tests demonstrated a richness of extracts in bioactive compounds by the presence of total and Gallic tannins, saponosids and coumarins in all extracts. Quantitative determination revealed that total phenolic content in the aqueous leaf decoction extract (553 mg GAE / g) was higher than in the other extracts. For the determination of flavonoids, the aqueous decoction extract of the leaves and even the bark was found to be the richest (254 and 226 mg QE/g respectively). The results of the antioxidant activity showed that the totality of the extracts possess advantageous antiradical properties, in particular the aqueous extract of the bark which has an IC_{50} value (0.02 mg / ml) close to that of ascorbic acid (0.019 mg / ml). The study of the antioxidant activity of extracts of Juglans regia L. suggests that this plant represents a natural source of chemical molecules that has very important biological activities.

1.Introduction

Medicinal plants are considered as a source of essential material for the discovery of new molecules necessary for the development of future drugs (Maurice, 1997). The therapeutic use of the extraordinary virtues of plants for the treatment of all diseases of the human is very old and evolves with the history of humanity (Gurib-Fakim, 2006). These natural products contain a large number of molecules that have multiple interests put to use in the food industry, cosmetology and pharmacy; these compounds include coumarins, alkaloids, phenolic acids, tannins, terpenes and flavonoids (Bahorun *et al.*, 1997). Oxidative stress is involved in many diseases as a triggering factor or associated with complications. Most oxidative stress-induced diseases appear with age as aging decreases antioxidant defenses, increasing mitochondrial multiplication of radicals (Girodon *et al.*, 2010). Among the biological activities of medicinal plants, in recent years attention has been focused on antioxidant activity because of the role it plays in the prevention of chronic diseases such as heart disease, cancer, diabetes, hypertension, and Alzheimer's disease by combating oxidative stress (Meddour *et al.*, 2013). The evaluation of phytopharmaceutical and antioxidant properties remains a very interesting and useful task, especially for plants that are less frequently used or are not known in traditional medicine

(Teixeira Da Silva, 2004).

Juglans regia (walnut) is a species that belongs to the Juglandaceae family. It is rich in vitamins (B1, B2, B3, B5, B6) and minerals (selenium and manganese). It also provides an excellent intake of omega 3 fatty acids (Weir *et al.*, 2004). Green leaves and nut husks contain oxygenated aromatic compounds (naphthoquinones), the most specific of which are juglone, flavonols (hyperoside, juglanoside) and gallic and ellagic tannins. *Juglans regia* has been used extensively in medicine; the leaves have been used traditionally in the treatment of cutaneous inflammations, ulcerations. They have an anti-diarrheal, antiseptic and astringent effect (Almeida, 2008). Walnut leaves are also used against rickets, anemia because they have fortifying and toning actions (Ait Youssef, 2006).

To this end, our work aims at contributing by a phytochemical study, as well as the evaluation of the antioxidant activity of the aqueous extracts of the leaves and the bark of *Juglans regia* L. (walnut).

2.Materials and methods

2.1. Plant material

It consists of two parts (leaves and bark) of the species *Juglans Regia.* They were collected during July 2017. Collection and identification of the bark and leaves was carried out in the El Qoll area (Skikda), Algeria. The different parts (leaves, bark) were dried in a dry place at room temperature for a few days. Once dried, the parts of the plant were reduced to powder and subjected to extraction.

2.2. Preparation of aqueous extract of *Juglans regia*

The extraction was carried out according to the methods of Romani et al (2006). For maceration, 10 g of powder of the leaves (or bark) are macerated in a volume of 100 ml of the solvent (distilled water) at room temperature, protected from light and with magnetic stirring for 48 hours. After filtration, the extracts obtained were then placed in a desiccator to completely remove the solvent.

In other hand, for decoction, 10 g of leaf powder and bark was added to 100 ml of the solvent (distilled water) in a reflux system. After extraction, the mixture was filtered to obtain the decocted. The concentrated filtrate was dried in the oven and was collected in sterile flasks.

The yield of the various extracts obtained was defined as being the ratio between the mass of the dry extract obtained and the mass of the plant material used (Harborne, 1980). This yield was calculated by the equation:

$$R\ (\%) = (Me / Mp) \times 100. \qquad (1)$$

R (%): Yield in%.
Me: Mass of the extract.
Mp: Mass of the plant.

2.3. Qualitative analysis

This was a qualitative study aimed at the search for the main chemical groups (alkaloids, flavonoids, tannins, saponosides, coumarins ...). The characterization tests were based on precipitation and complexation reactions with formation of insoluble and colored complexes. The observed staining was caused by the use of a suitable reagent and is generally due to the formation of a conjugation into a molecule (Harborne *et al.,* 1980, Rai and Carpinella, 2006).

2.4. Quantitative analysis

Colorimetric methods based on the use of the UV-visible spectrophotometer were used to evaluate the amount of polyphenolic compounds in the plant material.

2.4.1. Total Phenol Content

The total polyphenols were assayed according to the method of Folin-Ciocalteu (FC) (Singleton and Ross 1965, Benhammou 2009). The FC reagent consisted of a mixture of phosphotungstic acid (H3PW12O40) and phosphomolybdic acid (H3PMO12O40). The oxidation in an alkaline medium of this reagent by the oxidable groups of the phenolic compounds led to the formation of a mixture of blue oxide. The intensity of the coloration produced was proportional to the amount of polyphenols present in the analyzed extract. 1.25 ml of Folin-Ciocalteu was added to 1 ml of

sodium carbonate Na_2CO_3 at 2% and 0.25 ml of diluted extract (or standard substance of gallic acid and its dilutions), incubated at room temperature for 90 min. Absorbance was measured at 765 nm. The results were expressed in mg gallic acid equivalent / g dry plant (mg GAE / g dry) by referring to the gallic acid calibration curve.

2.4.2. Total Flavonoid Content

The determination of total flavonoids was carried out by the aluminum trichloride ($AlCl_3$) method according to the protocol of Dewanto et al. (2002). The formation of a covalent bond between the aluminum trichloride and the hydroxyl (OH) groups of the flavonoids produced a yellow complex. 1 ml of diluted extract (or quercetin solution and its dilution) was added to 0.3 ml of sodium nitrite (5% $NaNO_2$). After 5 min, 0.3 ml of aluminum trichloride (10% $AlCl_3$) and 2 ml of sodium hydroxide (4% NaOH) were added to the mixture. The volume was completed to 10 ml. Absorbance was measured at 510 nm. The results were expressed in mg quercetin equivalent / g dry plant by referring to the quercetin calibration curve.

2.4.3. DPPH Radical Scavenging Assay

To evaluate the antioxidant activity, the DPPH method was used according to the protocol described by (Sanchez-Moreno *et al.*, 2002). 50 µl of different concentration or standard (ascorbic acid) were added to 1.95 ml of the methanolic solution of DPPH (0.0025 g / l). In parallel, a negative control was prepared by mixing 50 µl of methanol with 1.95 ml of the methanolic solution of DPPH. The absorbance was read against a control prepared for each concentration at 517 nm after 30 minutes of incubation in the dark and at room temperature. The results were expressed as anti-free radical activity or the inhibition of free radicals in percentages (I %) using the following formula:

I% = [1 - (Abs Sample - Abs Control)] x 100

I %: Percentage of antiradical activity.

Abs Sample: Absorbance of the sample.

Abs control: Absorbance of the negative control.

2.4.5. Calculation of IC_{50}

IC50 or 50% inhibitory concentration (also called EC50 for Efficient Concentration 50) was the concentration of the tested sample required to reduce 50% of DPPH radical. IC50 was calculated graphically by linear regression of the graphs, inhibition percentages as a function of different concentrations (Benhammou *et al.*, 2008). For the entire experiment, each test was performed in triplicate and the results were calculated by the average of three trials.

3.Results and discussion

3.1. Extraction yield

Based on these results, it was found that the aqueous leaf decoction and maceration extract (28.23 ± 0.63, 22.13 ± 0.43% respectively) showed higher yields than the bark (Figure 1).

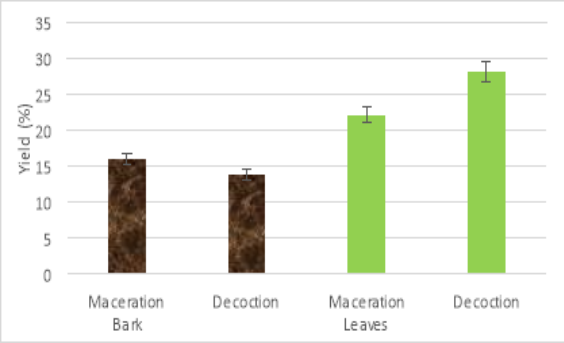

Figure 1. Extraction Yields of aqueous extracts of *J. regia.*

The contents of extracts vary not only from one plant to another of the same family but also according to the parameters of the solid-liquid extraction of the polyphenols, the temperature, the extraction solvent, the particle size and the coefficient of diffusion. Extraction with polar solvents and at elevated temperatures has been shown to provide higher yields of extract (Majhenic *et al.,* 2007). In general, plant diversity is responsible for the wide variability in the physicochemical properties that influence polyphenol extraction (Koffi *et al.,* 2010, Mahmoudi *et al.,* 2013).

3.2. Qualitative analysis

The qualitative analysis of plant extracts was based on color reactions or precipitation by specific chemical reagents, performed on extracts reconstituted from a plant sample.

The analysis of these experimental results leads us to the presence of total and Gallic tannins, coumarins and saponosids in all extracts of leaves and bark of *J. regia*. Anthocyanins were present in small amounts in the aqueous extract decocted and macerated of leaves and absent in aqueous extracts of bark. It was noticed that Irridoids were present in the aqueous extracts of leaves (Table 1). Flavonoids were present only in the aqueous extract macerated of leaves. Nirmala *et al.* (2011) reported the presence of the same classes of chemical families found in the leaves and barks of *J. regia.* This difference in the results would be due to the choice of the part to extract and the extraction method, as stated by Mamyrbekova - Bekro *et al.,* (2013).

3.3. Quantitative analysis

3.3.1. Total phenolic content

The total phenolic content was determined according to the calibration curve of gallic acid. The results were expressed in mg gallic acid equivalent / g of dry plant (mg GAE / g) (Figure 2).

The results obtained revealed that the level of these phenolic compounds in the various extracts was interesting. It was noted that the level of total phenolic content in the aqueous leaf decoction extract (553 mg GAE / g) was higher than in the other extracts. Even the aqueous extract of maceration bark showed considerable levels of total polyphenols (330.5 mg GAE / g). Like our results, Wu (2004) reported high levels of total phenolic content ranging from 680 to 2016 mg GAE / g for 10 varieties of Walnut. Kornsteiner *et al.,* (2006) also reported higher values for walnut (from 1020 to 2052 mg GAE / g). Ogunmoyole *et al.,* (2011) estimated a total polyphenol level equal to 200.2 mg GAE / g for the aqueous extract of Walnut, which confirmed the richness of the bark and the leaves of *J. regia* in phenolic compounds. Levels of phenolic compounds in Walnut were influenced by environmental factors, soil composition, and level of ripening (Wakeling, 2001).

Table 1. Phytochemical tests of the aqueous extracts of *J. regia.*

	Phytochemical tests						
Extracts	Total tannins	Irridiods	Gallic tannins	Saponosides	Flavonoids	Coumarins	Anthocyans
B-maceration	+++	-	+++	++	-	+	-
B-decoction	+++	-	+++	++	-	++	-
L-maceration	+++	+	+++	+++	+	++	+
L-decoction	+++	+	+++	++	-	++	+

B : Bark, L : Leaves

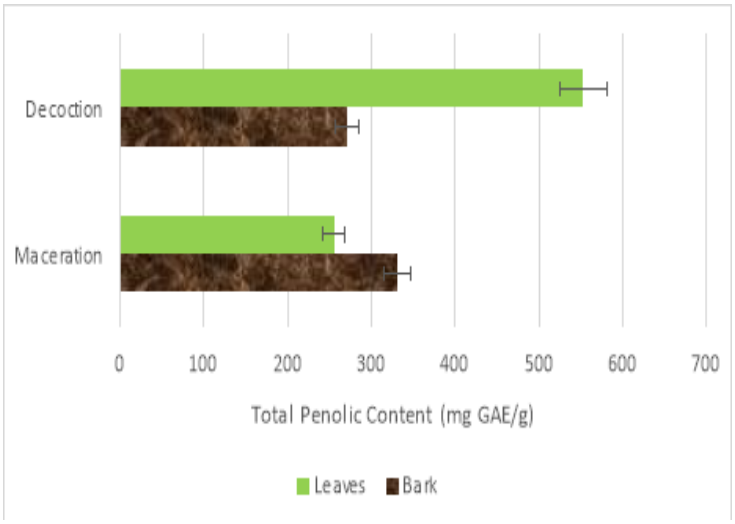

Figure 2. The total phenolic content of *Juglans regia* extracts.

3.3.2. Total flavonoid content

In return, the flavonoid content was calculated in mg quercetin equivalent / g dry plant (mg QE / g dry) while referring to the calibration curve of quercetin.

For the determination of flavonoids, the aqueous decoction extract of the leaves and even the bark was found to be the richest (254 and 226 mg QE/g respectively) followed by the aqueous extract macerated leaves (127 mg QE/g). On the other hand, the aqueous extract macerated from the bark had minimal flavonoid contents (91 mg QE/g dry) (Figure 3). Our results appeared in agreement with other studies. Referring to the results of Ogunmoyole *et al.*, (2011) who estimated a total flavonoid level equal to 148.2 mg QE/g for the aqueous extract of Walnut.

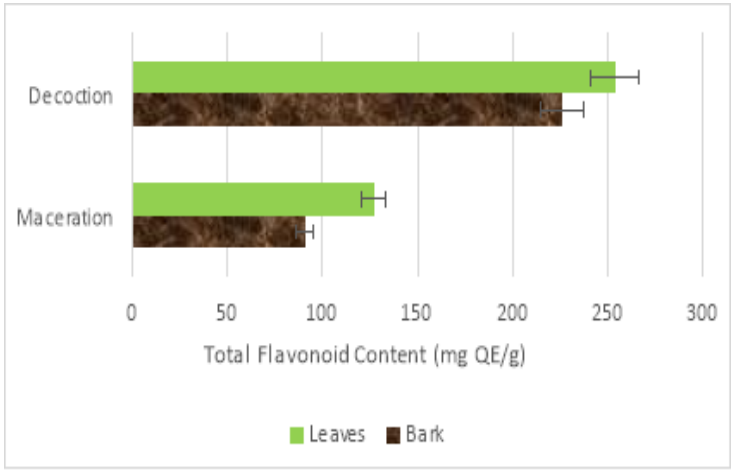

Figure 3. The total flavonoids content of *Juglans regia* extracts.

3.3.3. DPPH Radical Scavenging Assay

Different concentrations ranging from 0 to 1000 µg / ml of *J. regia* extracts were tested for their antioxidant activity *in vitro*. In order to compare their antioxidant activity with that of ascorbic acid, a calibration curve carried out by ascorbic acid was plotted (Figure 4).

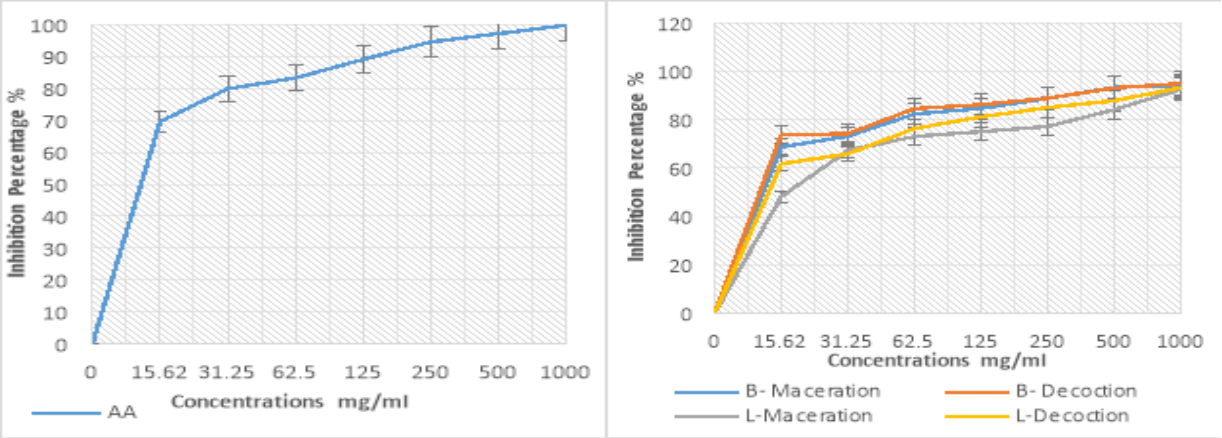

Figure 4. Inhibition Percentage (%) according to different concentrations (AA: Ascorbic Acid, B: Bark, L: Leaves).

In general, all four extracts tested caused a greater decrease in absorbance at 515 nm depending on their concentration (Figure 4). For walnut bark extracts, the DPPH free radical scavenging capacities by the macerated and decocted aqueous extract are 93.4 and 93.2% respectively in the concentration of 500 mg / ml. Whereas for extracts of walnut leaves, DPPH free radical scavenging capacities by the macerated and decocted aqueous extract are 84.2 and 87.9% respectively in the concentration of 500 mg / ml.

To better characterize the antioxidant power, it was necessary to introduce the IC_{50} parameter. It was defined by the effective concentration of substance that causes the reduction of 50% of the DPPH radical in solution. Therefore, the lower value of IC_{50} indicated a higher antiradical activity of the extract (Pokorny and Korczac, 2001).

The comparative study showed that the IC_{50} value of the macerated aqueous leaf extract has an IC_{50} value (0.03 mg / ml) greater than the ascorbic acid (0.019 mg / ml). While, the aqueous extract of the bark has an IC_{50} value close to that of ascorbic acid (0.02 mg / ml) (Figure 5). This indicated that this extract has a high antioxidant capacity because the very low IC_{50} value.

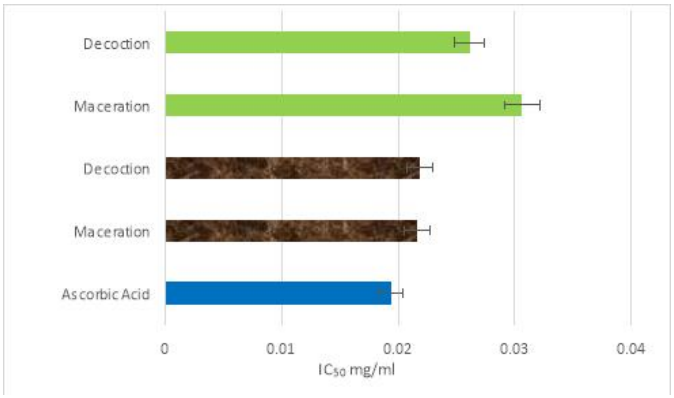

Figure 5. Values of IC_{50} of *J. regia* extracts.

According to the study by Agarwal *et al.*, (2012), the aqueous and alcoholic extract of *J. regia* showed an IC_{50} value of 0.182 mg / ml and 0.238 mg / ml, respectively. While it was found that the aqueous extracts have a lower IC_{50} value. This strong antioxidant activity of walnut could be attributed to the presence of phytochemicals such as phenolic compounds. Recently, Zhang *et al.*, (2009) identified seven phenolic compounds in *J. regia* by spectroscopic methods, which were pyrogallol, p-hydroxybenzoic acid, vanillic acid, ethyl gallate, proto-catechic acid, gallic acid and 3, 4, 8, 9, 10 penta-hydroxy-di-benzo (b, d) pyran-6-one, containing important antioxidant activities. Several studies have shown that walnut consumption can improve human antioxidant capacity (Ma *et al.*, 2010, Mckay *et al.*, 2010).

4.Conclusions

The qualitative and quantitative phytochemical study demonstrated a richness of *J. Regia* species in bioactive compounds by the presence of total and Gallic tannins, saponosides and coumarins. The results of DPPH Radical Scavenging Assay showed that all the extracts have interesting antiradical properties including the macerated extract and even decoction of the bark. It was manifested by low IC_{50} values that were very close to those of standard compounds (ascorbic acid).

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