Contribution to the Ethnobotanical, Phytochemical, Antimicrobial and Antioxidant Study of the Leaves' Aqueous Extract of the Common Walnut "*Juglans regia* L."

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Abstract. This work has focused on phytochemical screening of the Common Walnut *Juglans regia* L. leaves, and biological tests: antioxidant and antimicrobial effects of the aqueous extracts (infusion, crude) of the common walnuts leaves that we've picked, dried and preserved. An ethnobotanical survey in Blida region has been achieved, where it has been concluded that the inhabitants of this region use the common walnuts leaves in their daily care, and the various organs of the Common walnut are widely used as well. The phytochemical screening based on specific tests helped to highlight the presence of the main secondary metabolites such as tannins, flavonoids, alkaloids, free quinones, anthocyanins and saponins, which could be responsible for the biological properties. The antimicrobial study was conducted on bacterial strains and a yeast showed that both aqueous (infusion, crude) extracts have antibacterial effect against certain bacterial strains: *Staphylococcus aureus* (40.43 mm), *Staphylococcus epidermidis* (35.36 mm), *Sarcina lutea* (31, 17 mm), respectively, and an inhibitory effect only for the crude aqueous extract of *Escherichia coli* (12 mm), *Klebsiella pneumoniae* (12 mm), for against these two extracts were proved to be inactive for *Candida albicans*. In addition, the study of antioxidant activity by the method of reduction of iron (FRAP) and the method of reduction of free radical (DPPH) has revealed an average rate of aqueous extract (infusion) compared with that of ascorbic acid and rutin respectively. With an EC₅₀ of the aqueous extract and rutin which equal to 0.18 and 0.017 mg/ml respectively. According to these results we confirm some information obtained during the ethnobotanical survey and that *Juglans regia* L. is endowed with an antimicrobial and antioxidant power.

1. Introduction

Plants have always been used by humans for curative purposes. They have even learned to appreciate their soothing and analgesic properties [1].

Natural remedies and especially medicinal plants were the main recourse of the medicine of our grandparents, despite the important development of the pharmaceutical industry which enabled modern medicine to treat a large number of diseases often fatal. About 80% of the world's population benefits from traditional herbal medicine [2].

In Algeria, medicinal plants and herbal remedies have never been totally abandoned and people have never stopped using traditional medicine, which has led to maintaining a therapeutic tradition alive despite the spectacular development of modern medicine [3].

Among these medicinal plants strongly used in Algerian traditional medicine [4], Iranian, Chinese or Turkish, *Juglans regia* L as a natural remedy [5-8]

Juglans regia L., a species belonging to the *Juglandaceae* family [9], is a medicinal plant known more particularly for its active ingredient, Juglone, the latter confer to the plant several therapeutic virtues [6,10,11].

Our work therefore involves initially conducting an ethnobotanical survey in order to obtain as much information as possible on the traditional use of *Juglans regia* L. by a random population in the Blida region. Then, in a second step, identify the main chemical components of this plant by carrying out phytochemical tests.

And finally, the verification of the antimicrobial effect and the antioxidant power of this plant.

2. Material and Methods

2.1. Material

2.1.1. Plant material: *Juglans regia* L. leaves were collected from a garden tree Fig1, in May 2014, in the wilaya of Blida r, region of "Bab Khouikha".

Figure 1. Common walnuts (*Juglans regia* L.).

The freshly harvested leaves were dried away from light and moisture for one week. Once dried, they were crushed using a "Moulinex" house grinder. More or less fine flour was obtained. After weighing using an analytical balance (KERN mark), 350 g of walnut leaf powder was obtained. This one was stored in a sealed jar protected from light and moisture.

2.1.2. Microbiological material: The strains used are presented in the table below:

Table 1. List of the stains that have been used in the antimicrobial test.

	Stains	References
Gram-	<i>Escherichia coli</i>	ATCC 10536
	<i>Escherichia coli</i>	ATCC 25922
	<i>Klebseilla pneumoniae</i>	ATCC 4352
	<i>Pseudomonas aeruginosa</i>	ATCC 27853
Gram+	<i>Bacillus subtilus</i>	ATCC 6633
	<i>Sarcina lutea</i>	Pasteur Institut
	<i>Staphylococcus aureus</i>	ATCC 6538
	<i>Staphylococcus epidermidis</i>	ATCC 12228
Yeast	<i>Candida albicans</i>	ATCC 10231

(-) : Negative

2.2. Methods

2.2.1. Ethnobotanical survey:

We have sought to delimit and prospect the maximum number of areas in the Blida region. For this, a field companion was programmed during the year 2014 (March-May).

In this study, an ethnobotanical field survey was conducted using 100 questionnaires (prepared and rectified) and used to investigate populations like: local citizens, herbalists,

phytotherapists, doctors, pharmacists and biologists (who were handed over to them) in order to collect knowledge in herbal medicine, information on a medicinal plant: The common walnut "*Juglans regia* L." of their regions, local traditional uses, information about the plant's efficiency and whether it is toxic or not, what are the main parts that are used in the treatments.

The information was obtained through ethnobotanical interviews with people born and / or have lived long in the region.

This information was complimented by a thorough literature review and experimental trials in order to know the chemical composition of the plant and confirm some biological activities.

2.2.2. Preparation of infusion and crude aqueous extract:

- **Preparation of the infusion:** this method was proposed by Bruneton [12] 20 g of powder are left infused for 15 min in 200 ml of boiling distilled water. The infused was filtered to produce the aqueous extract.
- **Preparation of the crude aqueous extract:** 10 g of powder in 100 ml of boiling physiological water at 0.9% and with the help of a mortar we tried to mix well the powder with physiological water

The two extracts were placed in a shaded flask and kept at a low temperature.

2.2.3. Determination of water content: The determination of the water content was carried out according to the method monitored by Simpson and Zerrad [13,14]

2.2.4. Phytochemical Screening: The purpose of these tests is to know the chemical composition of *Juglans regia*. They are carried out on the powder and infusion of the plant.

We have characterized the different chemical groups by referring to the techniques described by Bruneton [12].

2.2.5. Study of the antimicrobial activity of the two aqueous extracts of the leaves of *Juglans regia*:

The technique in solid medium is based on a technique used in medical bacteriology, called an antibiogram. It has the advantage of being very flexible in the choice of the products to be tested and applied to a large number of bacterial species [15].

We use filter discs of 9 mm diameter, impregnated with both aqueous extract (infusion, crude). We have deposited these discs on the surface of an agar medium seeded on the surface of a bacterial suspension with the aid of a sterile forceps.

The incubation is carried out in an oven at 37 ° C. for 24 h for the bacteria and at 25 ° C. for 48 h for the yeasts

The absence of microbial growth results in a translucent halo around the disk whose diameter is measured and expressed in millimeters.

The control box has a disk not impregnated with the extract. Another diskless box is seeded under the conditions of the experiment. It informs us about the homogeneity of the bacterial carpet.

- We performed two repetitions for each extract
- Reading of the zones of inhibition around the disk is done with the aid of a caliper.
- The scale of estimation of antimicrobial activity is given below [16]:
 - ✓ Very strongly inhibitory: $D \geq 30mm$
 - ✓ Highly inhibitory: $20mm \leq D \leq 29mm$
 - ✓ Moderately inhibitory: $16mm \leq D \leq 20mm$
 - ✓ Slightly inhibitory: $11mm \leq D \leq 16mm$
 - ✓ Non-inhibitory: $D < 10mm$

2.2.6. Anti-oxidant activity of the aqueous extract (infusion):

- **Determination of the reduction power by the FRAP method:**

Reducing power measures the ability of an antioxidant to give an electron [17].

The absorbance of the reaction medium is read at 700 nm against a similarly prepared white, replacing the extract with distilled water which makes it possible to calibrate the apparatus (UV-VIS spectrophotometer).

The positive control is represented by a solution of a standard antioxidant: ascorbic acid, the absorbance of which was measured under the same conditions as the samples. An increase in the absorbance corresponds to an increase in the reducing power of the extract tested.

➤ **Estimation of the antioxidant power by the reduction of the free radicals (DPPH) method:**

The DPPH test is carried out according to the method described by Cuendet and Burits[18,19]. A methanolic solution of synthetic antioxidant rutin was used as a reference. The results are read in a visible UV spectrophotometer at 515 nm.

❖ **IC₅₀:** It is the 50% inhibitory concentration, also known as EC₅₀ (Efficient concentration 50), it is the concentration of the test sample needed to reduce 50% DPPH.

The IC₅₀s are calculated graphically by percent inhibition in function of the different concentrations of the extracts tested[20].

3. Results and Discussion

3.1. Results of the Ethnobotanical Survey:

The ethnobotanical survey carried out in the Blida region was carried out among 100 people, including 38 men and 62 women from the local population, doctors, pharmacists, herbalists and phytotherapists.

This study allowed us to gather information on the importance of phytotherapy as well as on the traditional uses of the Common Walnut (*Juglans regia* L.). The persons surveyed are of different levels and different backgrounds and all of them agreed that plants have great efficiency if the dose is respected.

- **Used parts and collect period:** All the persons surveyed have great knowledge of common walnuts; they all agreed that all parts (leaves, fruits, husks, brunch, bark) of the plant can be used as treatment for several diseases and the harvest period was mentioned to be in Fall, Spring and Summer.
- **Method of use:**
The persons surveyed mentioned that *Juglans regia* has different ways of use, 38% said that they used to use the plant alone, 22% said that they used to use it mixed with other medicinal plants, 19% said that they use it mixed with olive oil and honey, and 21% combined all the three previous ways of use
- **Diseases treated by *Juglans regia*:**
21% among the persons surveyed use *Juglans regia* for nutrition for instance they use it to make traditional pastry such as: Baklawas, Makrout ...ect, while the others(79%) use it against: Cancer, hypercholesterolemia, pain or aches, hemorrhoids, diabetes, eczema, Digestive diseases, Intestinal worms and acute respiratory infections, dental caries, Aphthous, teeth whitening, Gingivitis, dermatosis, skin lesions, healing wounds and Foot bands. It is used also for its aphrodisiac effects and to improve the condition of Alzheimer's patients.
- **Side effects:** Almost all of the surveyed persons agreed that common walnuts has side effects (72%) and the rest of them said (28%) said that it has no side effects. The side effects that they have been noticed are: Aphthous, Gingival burns, Allergies, Constipation and nausea.
- **Some recipes collected during the ethnobotanical study,** Presented in the table below:

Table 2. Recipes collected during the ethnobotanical survey.

The recipe	The use
Walnut + other dried fruits "almonds, cashew, nuts" ground + honey	To improve concentration and memory, to improve cases of patients with Alzheimer's, aphrodisiac, Anemia
Leaves powder + Henna	Healing foot cracks and tinting hair
Walnut leaves (infusion) used as Herbal tea for 40 days	Against hypercholesterolemia
Decoction of leaves	Against eczema
Leaves mixed with horsetail(plant)	Hair loss
Leaves infusion	• Against hair loss, hair care, anti-inflammatory (back pain) bath of seat (cracks of feet), respiratory infection "gargle"
• Infusion: nuts + Anise Or with cumin + anise (ground + olive oil or honey)	Digestive
• Nuts + nigelle seeds (ground) + honey	Respiratory infections
Vegetable oil of the fruit	Cosmetology: skin care
Walnuts + pomegranate	Anti-inflammatory
• Chewing bark or the husk (Souak)	Gingivitis and oral infections
Fruits grounded with honey	Skin care
• ½ walnut leaf powder + ¼ Marjoram (leaves) + ¼ laurel noble + vegetable oil "olive oil for example"	Applied on the head against migraine or head aches
Dry leaves + olive oil	Massage on the back

Our results are in agreement with those found by [21] Idolo et al. (2010), whose ethnobotanical study on medicinal plants used in Italy proves that the leaves of *Juglans regia* are widely used by the Italian population by using them in the care And for the treatment of foot cracks.

According to an ethnobotanical study in the Ecuador, the leaves of a plant of the same genus *Juglans neotropica* are used to relieve hepatic pain and rheumatic pain [22].

The results of an investigation of medicinal plants used in Pakistan, revealed that the leaves, fruit, bark, and husk are used against skin conditions and eczema [23], Which is perfectly in line with our results.

3.2. Results of the Phytochemical Study:

3.2.1. Water content: The analyzes of our samples revealed a high water content (68.94%), which means that more than two thirds of the weight of the fresh plant is water, and that this rate is comparable with the standards of the **French pharmacopoeia** which recommends a percentage of 65% [24].

3.2.2. Result of phytochemical screening:

These tests were carried out to demonstrate the presence of certain chemical groups which may be responsible for the biological activities studied. The results are presented in the table 3 below:

Table 3. Results of the phytochemical screening.

Component	Result	Reaction
Anthocyanins	+	Blue
Leucoanthocyanins	-	No red coloration
Tanins	+	Black blue coloration.
Gallic tanins	+	Dark blue coloration
Catechic tanins	+	Red coloration
alkaloids	+	Red precipitate
Free Quinones	+	Red coloration
Combined Quinones	-	No red coloration
Saponosides	+	White precipitate
Coumarines	+	Formation of commotion
Flavonoïds	+	Orange red Coloration
Glycosides	-	No red coloration
Starch	-	No violet coloration

(+): Positive Reaction / (-): Negative Reaction

The phytochemical test carried out on the powder and the infused *Juglans regia* reveals the presence of several families of chemical compounds.

Our results are in agreement with Fukuda's work that revealed the presence of a large quantity of tannins [5].

The phytochemical analyzes carried out in India of the leaves extracts of *Juglans regia* L. revealed the presence of many bioactive compounds: carbohydrates, cardiac glycosides, flavonoids, steroids and tannins [25].

Another Indian study reports that the extract of the *Juglans regia* bark contains reducing sugars, alkaloids, tannins, phenols and saponins [26] .

3.3. Results of the antimicrobial activity of the two extracts "infusion and crude" of *Juglans regia* L:

The estimation of the antimicrobial activity is based on a specific scale of measurement [16] . They classified the antimicrobial power, according to the diameters of the zones of inhibition of the microbial growth.

The table 4 and Fig 2 extrapolate the results obtained concerning the diameters of the zones of inhibition revealed by the two aqueous extracts.

Table 4. Results of the antimicrobial activity of the two aqueous extracts "infusion and crude".

	Stains	References	Diameter (mm)	
			EAI	EAB
Gram-	<i>Escherichia coli</i>	ATCC 10536	-	-
	<i>Escherichia coli</i>	ATCC 25922	12	-
	<i>Klebseilla pneumoniae</i>	ATCC 4352	12	-
	<i>Pseudomonas aeruginosa</i>	ATCC 27853	-	-
Gram+	<i>Bacillus subtilis</i>	ATCC 6633	31	17
	<i>Sarcina lutea</i>	Institut pasteur	-	-
	<i>Staphylococcus aureus</i>	ATCC 6538	40	43
	<i>Staphylococcus epidermidis</i>	ATCC 12228	35	36
Yeast	<i>Candida albicans</i>	ATCC 10231	-	-

(-): Negative

Figure 2. Results of the antimicrobial activity of the two aqueous extracts "infusion and crude" (↔ : inhibition zone).

From this table we notice that the infusion of leaves of *Juglans regia* L. had an effect on *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Saccharomyces cerevisiae* and *Escherichia coli* with zones of inhibition: 31mm, 40mm, 35mm, 12mm, 17mm and 12mm, respectively.

The crude aqueous extract of leaves of *Juglans regia* L. had an effect on *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Saccharomyces cerevisiae* with an area of inhibition equal to: 17mm, 47mm, 37mm and 35mm, respectively.

On the other hand, we noticed no effect for the two extracts on *Pseudomonas aeruginosa* and *Escherichia coli* (ATCC 10536).

The crude aqueous extract had no effect on *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae*.

From the results obtained, it can be seen that regardless of the nature of the two aqueous extracts (Infusion, Crude), Gram negative bacteria possess strong resistance. According to Faucher and Avril, this resistance is related to the nature of their outer membranes (impermeable to most biocidal agents) [27].

Our results are in agreement with those found in the study of an antibacterial activity of the essential oil from the leaves of the Common Walnut *Juglans regia* collected in India which found an antimicrobial effect on *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Showed no effect on *Escherichia coli* and *Klebsiella pneumonia* [28].

Other studies have shown that alcohol extracts of the green husk of *Juglans regia* L. from Portugal and India possess antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* [29,30], which is in agreement with our results.

3.4. Results of the Antioxidant Activity of the Infusion:

3.4.1. Results of the antioxidant activity by the method of Ferric reducing antioxidant power (FRAP):

The antioxidant activity of the aqueous extract (infusion) of the studied plant was evaluated by the FRAP method. The latter is a simple, rapid and reproducible test [31]

The presence of the reducing agents in the extracts of the plant causes the reduction of Fe^{+3} / ferricyanide complex to the ferrous form. Therefore, Fe^{+2} can be evaluated by measuring and monitoring the increase in the density of the blue color in the reaction medium at 700 nm [32]

The figure below represents the reducing power of ascorbic acid and aqueous extract (infusion), at different concentrations.

Figure 3. Reduction power of aqueous extract (infusion) and ascorbic acid by the FRAP method.

At a concentration of 0.1 mg / ml, the reducing power of the aqueous extract (infusion) has an optical density (DO = 0.0299) much lower than that of ascorbic acid (OD = 0.3133).

At a concentration of 4 mg / ml, the aqueous extract appears to be more powerful with respect to iron reduction, with an optical density equal to 0.688, but it is always lower than that of ascorbic acid (OD = 2.4473).

The simultaneous increase in the reducing power of the aqueous extract of *Juglans regia* L. demonstrates the presence of the antioxidant activity.

3.4.2. Results of the antioxidant activity by the method of inhibition of the DPPH radical (2,2-Diphenyl-1-picrylhydrazyl):

The antioxidant activity of the aqueous extract (infusion). Of the studied plant was evaluated by the method of inhibition of the DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical.

The results obtained in the measurement of the percentage inhibition of the DPPH radical for the aqueous extract (infusion) and for the rutin taken as reference are shown in figure below

Figure 4. Percentage of inhibition of the aqueous extract as a function of the different concentrations.

Our results, expressed as a percentage of the anti-free radical activity (Fig 4), reveal that the extract tested as well as the Rutin taken as reference are anti-free radicals.

It seems that the percentage of inhibition of the free radical increases with increasing concentration either for the standard or for the aqueous extract (infusion) of *Juglans regia* L.

It is noted that the percentages of inhibition of the free radical for rutin is slightly higher than that of the aqueous extract (infusion) for all the concentrations used.

We note that at a concentration of 0.05 mg / ml, the percentage inhibition of the aqueous extract (infusion) is equal to 21.34%, which is considerably lower than that of rutin which is equal to 71.20%.

At the concentration of 0.4 mg / ml, the percentage inhibition is higher with respect to the DPPH free radical inhibition which is equal to 73.14%, but it is always lower than that of the rutin which is Equal to 88.11%.

The simultaneous increase in the percent inhibition of DPPH free radicals of the aqueous extract (infusion) of *Juglans regia* L. demonstrates the presence of the antioxidant activity.

The calculation of the concentration which inhibits 50% of the free radicals is made graphically, we obtained the following results:

- EC50 of the aqueous extract (infusion) and rutin is equal to 0.18, 0.017 mg / ml respectively.

The results of the antioxidant activity are in agreement with those done in Japan that The latter found that the extracts of the fruit of *Juglans regia* L. possess antioxidant activity[5]. This activity could be linked, To their wealth of phenolic compounds found in the fruit.

The antioxidant capacity of phenolic compounds is based on their ability to trap free radicals, pro-oxidant chelating agents of metal ions and inhibitors of certain enzymes [33. Nevertheless, the contribution of organic acids cannot be ignored.

Our results are in line with those obtained in the study of common walnut's (*Juglans regia*) antioxidant power of Pakistan[34], and those obtained by Carvalho and Almeida *Juglans regia* L. of Portugal [7,35], and also those obtained in the study of the antioxidant activity of the essential oil of the leaves of the Common Walnut of India[28].

Conclusion

This work allowed us to carry out on a small scale an ethnobotanical study in the wilaya of Blida on the one hand, and on the other hand, a phytochemical and biological study of the aqueous extracts of the leaves of *Juglans regia* L.

During this investigation, we found that the people surveyed consume the fruits of the common walnut tree and consider it as food and also use it in large variety of traditional pastry

(Baklaw, Makrout ... etc.). In addition, it is indicated in the treatment of diseases related to the central nervous system (Alzheimer's), while leaves, husks, bark are used for the treatment of gingivitis (such as Souak), and also for the treatment of eczema, hemorrhoids, diabetes, dermatoses and hypercholesterolemia. We've also been able to collect several recipes about the common walnuts.

The phytochemical screening based on specific tests identified tannins, flavonoids, alkaloids, free quinones, anthocyanins and saponosides. These secondary metabolites are known for their high therapeutic values.

Concerning the antimicrobial activity, we found that the two aqueous extracts (infusion, crude) revealed a good inhibitory effect, the study of antioxidant activity by the method of reduction of iron (FRAP) and the method of reduction of the free radicals (DPPH) proved average for the aqueous extract (infusion) compared with that of the ascorbic acid and the rutin, this is largely due to the chemical composition of these two extracts.

According to these results, we confirm some information obtained during the ethnobotanical survey and that *Juglans regia* L. is endowed with an antimicrobial and antioxidant power.

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