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Research article

Wound healing; antimicrobial and anti-oxidant activity for Jordanian *Juglans Regia L.* unripe fruits

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Key words: *Juglans regia L*, Unripe fruit, Wound healing, Microwave extraction, Antioxidant.

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Abstract

The present study, attempts to evaluate, extraction efficiency of unripe fruit of *J. regia* in Jordan using different solvents with soxhlet and microwave extraction methods. We used five different types of solvents according to their increase in polarity to cover a wide range of phytochemical products. We examined the presence of some secondary metabolites as Tannins, Flavonoids, Saponins, and Alkaloids, as well as total phenols. Our results showed a higher efficiency for the microwave extraction method over the soxhlet extraction method in terms of higher yield amount and less time and solvents consuming. Water usage as an extraction solvent has generated the highest yield amount of 1.3 g in the defatted *J. regia* using microwave methods, whereas, the highest concentration of total phenols was demonstrated in the ethyl acetate extract of *J. regia*. In the antimicrobial susceptibility test, the ethyl acetate extract of *J. regia* showed a significant MIC inhibition concentration of 0.85 mg/ml against *S. aureus, P. aerogenosa,* and *S. epidermidis*. The healing activity of the ethyl acetate extract was as good as the reference drug (Baneocin) and even the ethyl acetate had an increase of 5% ($P< 0.05$) than reference drug.

Introduction

Wounds type of injury occurs as a result of skin: cut, open, or force trauma. Pathologically, it refers to a sharp injury which damages the dermis of the skin. Micro-organisms rapidly infect injured skin to seriously delay healing process [1]. Moreover, wound hypoxia, and vessels occlusion delayed the healing process [1]. Wounds divide On the basis of condition in to: one is chronic; burn and sepsis. Wound healing is a complex and dynamic process of replacing devitalized and missing cellular structures and tissue layers [2].

The process of wound healing is ordered cascade of events, which can be divided into four distant but over lapping phase of homeostasis, inflammation, proliferation and maturation [2]. The utilization of medicinal plants in curing various ailments was the sole source of ensuring human welfare until the development of chemistry and organic compound [3]. Traditional herbal healing is widely practiced throughout the world generally and in Jordan spesicifically.

Juglans regia, (walnut) is widely cultivated across Europe, Turkey and in some areas of the Middle East [4]. There is a great genetic diversity for Juglans species cultivated all over the world, in particular, ancestral forms with lateral fruiting, hence, during the migration of *Juglans regia* to Western Europe some common characteristics were lost and became large trees with terminal fruiting [4].

The biochemical composition of walnut gave it significant economic value and medicinal importance for human health [5]. People consume it in large quantities, therefore, it has a very important place in public nutritional habits, it also has a high-calorie level [6]. Walnut (*Juglans regia L.)* is a medicinal plant that has been widely used in traditional medicine. Various parts have been used as a remedy in folk medicine such as leaves, roots, seeds, barks and fruits including their husk [7].

Several studies suggest that regular consumption of walnuts may have beneficial effects against oxidative stress-mediated diseases such as cardiovascular disease and cancer [8-9], due to the antioxidant potential of walnut products including: nuts (green or dry) and green walnuts mesocarp [10-11], leaves [12], bark [4] or flowers [13].

Various extracts of walnuts have *in vitro* antioxidant and anti-proliferative activity due to a high phenolic content [14]. *Juglans regia* is used to treat diabetes mellitus symptoms in traditional medicine, whereby air-dried leaves are used as aqueous decoction or liquor preparation and are consumed on a daily basis [15].

Traditionally, medicinal plants have been used for many for many years as topical preparations to promote wound

healing. Some of these plants owe their effects to direct effect on the wound healing processes for their tannin content and some to their anti-inflammatory and anti-microbial properties. A combination of these properties is also possible in some of the medicinal plants used in wound care [16].

Wound healing is a natural process of regenerating dermal and epidermal tissue. Whenever there is a wound, a set of overlapping events takes place in predictable fashions to repair the damage [16].

In our present work we evaluated the optimal extract from the unripe fruits of *Juglans regia* using microwave-assisted extraction technique as well as the conventional soxhlet method, for their wound healing capability. Comparison will depend on their phytochemical analysis; anti-oxidant as well as antimicrobial activity.

Materials and methods

Materials

Juglans regia L. unripe fruit were collected from Shafabadran in Amman-Jordan, all the chemicals and reagents used were of analytical reagent grade and were purchased from Sigma Aldrich Company.

Plant material

The immature fruit of walnut plant (*Juglans regia L.)* were picked in 1st May, 2015 from the fields of Shafabadran in Amman, Jordan and the plant was authenticated by Dr. Fahmi Shatat, Faculty of Agriculture; University of Jordan. The voucher specimen was deposited in a drug discovery laboratory along with a given specimen number R001.

Extract preparation

The walnut fruits were cleaned and stored at –80°C in the deep freezer. Later on, the walnut fruits were sliced and ground to a fine powder using a kitchen-type blender (Braun) finally the samples weighed for the extraction process. Half of the grinded samples were defatted by refluxing with Petroleum ether at 50°C for 4 hours, filtered and allowed to dry in shade to be subjected to the extraction.

Extraction methods

The plant samples (15 gm) were extracted in soxhlet apparatus using various solvents separately according to the gradual increase of polarity, these solvents are: ethyl acetate, acetone, ethanol, methanol, and water. The extraction processes were done for 2-3 hours, solid to solvent ratio was 1:7 and the number of extraction cycles using soxhlet apparatus was considered according to the separation of components using TLC technique.

For Microwave-assisted extraction; extraction process was performed using a laboratory scale microwave oven (Milestone). For each extract, the sample was weighed (15 gm) and put with the solvent into the round bottom flask then placed into the oven. The power was 500 watt, the temperature was 50°C, solid to solvent ratio 1:5 and the time was15 min, and these extraction parameters were adjusted using the control panel of the microwave oven. When each extraction was completed, it was filtered under vacuum, followed by evaporation in the fume hood. Water extracts were frozen overnight then lyophilized to give the dry extract. The yield of extracted plant was collected, weighed accurately and kept in dark colored bottles at refrigerator to be used for the analysis.

Preliminary phytochemical analysis

Qualitative phytochemical analysis of was tested as follows: Kumar test for flavonoids detection; ferric chloride test for tannins detection; testing the presence of saponins using formation of persistent frothing and finally Wagner test for alkaloids detection

Estimation of total phenolics

The Folin-Ciocalteu method [17] was used in order to determine the total phenolic content of the extracts. This method is based on the principle that phenolic substances reduce Folin-Ciocalteu reagent in the presence of sodium carbonate causing a color change.

According to this method, diluted samples were pipetted into test tubes and Folin- Ciocalteu reagent is added and mixed for a minute, then they were allowed to rest for 5 min in a dark place at room temperature for incubation. After that, the relevant amount of sodium carbonate was added. After stirring again, the mixture is kept for 1 h in the dark place at room temperature. The absorbance was measured at 760 nm using a UV/VIS spectrophotometer (Thermo, USA). The results were expressed as mg gallic acid equivalent per (g) of dry material. The calibration curve was prepared using gallic acid solution range from 0.0125 to 0.2 mg/ml. All of the spectrometric measurements were taken in three replicates and the average value is used in the calculations of total phenolic content.

Determination of antioxidant activity

The antioxidant activity of the walnuts extracts was measured in term of radical scavenging ability using DPPH method [18-19].

This method is based on the principle that the DPPH radicals are reduced by antioxidants. This reduction causes a color change. According to this method, 0.025 g DPPH reagent, which is a dark purple radical, is dissolved in 1 L methanol. Then 3.9 mL of this solution is added to 0.1 mL of each sample that was diluted in a pure solvent of extraction at different concentrations. The mixtures were incubated for 90 min. in a dark place at room temperature and then absorbance for each sample was measured at 517 nm using a UV/VIS spectrophotometer. The antiradical activity was expressed as IC_{50} ($\mu g.mL^{-1}$).

The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $((A_0-A_1)/A_0)*100$

Where A_0 is the absorbance of the control at 90 min, and A_1 is the absorbance of the sample at 90 min.

All samples were analyzed in triplicate.

Antimicrobial susceptibility assays

For the determination of the MIC, we applied the standard protocols of Antibacterial Susceptibility Testing and Antifungal Susceptibility Testing of Yeasts according to the EUCAST reference method with modifications [20, 21] to our strain collection, we used *C. albicans* ATCC 90028*, S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922, and *P. aerogenosa* ATCC 27853 as reference control strains confirming that the MIC values were within the limits of the EUCAST procedure.

Our procedure: In contrast to the classical EUCAST protocol, prior to the test, bacterial strains were grown at 37°C for 24 hours on solid LB (Lauria-Bertiani) plates. *C. albicans* strains were grown at 25 °C for 24 hours on solid sabouraud (Sab) plates. On each micro dilution plate row number 1 was used as a growth control for the viability of microbial cells. Rows 2 to 12 contained decreasing amount of the test compound in a 2-fold titration scheme resulting in a range of final concentrations from 0.85 mg/mL to 100 mg/mL. Ciprofloxacin was used as a reference drug for antibacterial activity of the test compounds whereas Fluconazole was used for the antifungal activity. Microtiter plates were incubated at 35°C and the growth of microbial cells was evaluated after 22 ± 2 hours by measuring the optical density at 450 nm using a TECAN microtiter plate reader and analyzed with Magellan software. MICs were defined as the lowest drug concentration giving rise to an inhibition of growth of more than 100% of that of the drug-free control. The average MIC (MICav) of *J. regia* extract for a subset of test strains was calculated as the geometric mean of the MIC test results of strains included in the subset.

Animals

Animal care and use were conducted in accordance to standard ethical guidelines, and all of the experimental protocols were approved by the Research and Ethical Committee at the Faculty of Pharmacy-Applied Science University y (Ethics Certificate no:26/10/2015. No.1). Healthy Sprague-Dawley rats of either sex, weighing 180-200 gm and aged 8-12 weeks were used in this study. Separate cages with wooden shaving were used to keep the rats. The environmental parameters in the animal room were: 50–60% humidity, 25°C temperature with continuous ventilation.

Drug formulation

Four types of the extracts were chosen depending on the results of the analysis which are: Defatted Ethyl acetate (Microwave); defatted Acetone (Soxhlet); fresh Ethanol (Soxhlet) and fresh Methanol (Soxhlet). These extracts were formulated as 5% (w/w) ointment using paraffin base.

Wound healing activity

Incision wound model was used for the assessment of wound healing activity [16]. The rats were anesthetized using Chloral hydrate. Paravertebral incisions of 1 cm length were made through the entire thickness of the rat's skin. The wound was closed by means of interrupted sutures (figure 3). The formulated extracts along with the paraffin (control) and a reference drug (Baneocin ointment) (results in six groups and six animals for each case) were applied on the wound for ten days. Complete healing as no leaving of the wound was considered as the end point of complete epithelization. The wound contraction in term of skin tensile strength was the measured parameter after ten days (10 days) of applying the formulas on the wound incision [16].

Experimental procedure: In the experiment a total of 36 normal rats were used. The rats were divided into eight groups of 6 rats each and the groups were as follows:

Group 1: control treated with paraffin

Group 2: treated with Baneocin ointment; reference drug

Group 3: treated with 5% w/w ethyl acetate extract ointment.

Group 4: treated with 5% w/w acetone extract ointment.

Group 5: treated with 5% w/w ethanol extract ointment.

Group 6: treated with 5% w/w methanol extract ointment.

Statistical analysis

Statistical analysis was done using SPSS software version 18. Data are presented using mean ± SEM (Standard Error of Mean). The statistical significance among groups was determined using one-way ANOVA and paired sample T-test in SPSS. A p-value < 0.05 was considered significant.

Results

Extraction yield

The unripe fruit of *J. regia* was extracted whether in the fresh form or after treating with Pet. Ether for defatting purposes by two different methods; soxhlet and microwave-assisted methods using different solvents of increasing polarity, so that the yield in each extraction process was vary from one sample to another depending on the method, solvent used or whether the plant was in fresh or defatted form as shown in (Table 1).

Table 1. Effects of extracting technique / solvent type on the extract yield (w /w) *J. regia* unripe fruit extraction.

	Soxhlet method		Microwave method	
	Defatted	Fresh	Defatted	Fresh
Ethyl acetate	0.16	0.13	0.25	0.2
Acetone	0.65	0.64	0.7	0.72
Ethanol	0.73	0.46	0.94	0.63
Methanol	0.7	0.53	1.2	0.88
Water	0.8	0.6	1.3	0.7

15 gm dry material
For microwave extraction; 500 W, 15 min and 1: 5 solid to solvent ratio were used.
For soxhlet extraction; 1:7 solid to solvent ratio was used.

Total phenolic contents

The total phenolic content of each extract was determined by the folin-Ciocalteu method which based on changing the color, all the concentrations were expressed as mg gallic acid equivalent per (g) of dry material, and the concentrations were ranged between (0.013-0.165) mg/g in which M.W defatted ethyl acetate had the highest concentration whereas Pet. Ether had the lowest one as shown in (Table 2). The calibration curve was prepared using gallic acid solution range from 0.0125 to 0.2 mg/ml.

Table 2. Total phenolic concentration of *J. regia* plant extracts using different solvents.*

	Soxhlet method		Microwave method	
	Defatted	Fresh	Defatted	Fresh
Ethyl acetate	0.048	0.118	0.165	0.126
Acetone	0.086	0.075	0.096	0.092
Ethanol	0.1	0.059	0.053	0.085
Methanol	0.048	0.054	0.053	0.052
Water	0.048	0.023	0.05	0.046
Pet. Ether	0.013			

The DPPH free radical scavenging activity

The antioxidant activity of the *J. regia* unripe fruit extracts was assessed using spectrophotometry for the presence of the DPPH radical. Samples with a plant extract concentration range of 0.10–5.0 g/mL were analyzed.

For comparison, we also measured the radical scavenging activity of juglone in the same conditions. Table 3 shows the antioxidant activity of the tested extracts and the positive control (juglone) expressed as the percentage of deactivation of the DPPH free radicals. The quality of the antioxidants in the extracts was determined by the IC_{50} values, denoting the concentration of the sample required to scavenge 50% of the DPPH free radicals (Table 3).

The effective concentration of sample required to scavenge DPPH radical by 50% (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

Preliminary phytochemical investigations

All extracts were subjected to qualitative analysis to detect the presence of some important secondary metabolites such as flavonoids, tannins, alkaloids and saponins and the results were shown in (Table 4).

Antimicrobial activity

In order to determine the antimicrobial activity of *J. regia* extracts against a set of bacterial and fungal strains, we performed a standard MIC determination of *J. regia* extracts using the protocol according to EUCAST. In addition, we compared their activity to a reference drugs (Ciprofloxacin, and Fluconazole, respectively). A summary of the MIC distribution for test strains is shown in (Table 5, Figure 1).

Wound healing activity

According to the antimicrobial activity and total phenolic contents, four types of extracts were chosen to be formulated as an ointment and were applied on an induced rat's injury to assess the wound healing activity, and the wound contraction was measured. Results were shown in (Table 6, Figure 2-3).

Table 3. DPPH radical scavenging activity expressed as IC_{50} values (µg/ml) of various extracts from *J. regia*.

	Soxhlet method		Microwave method	
	Defatted IC_{50}	Fresh IC_{50}	Defatted IC_{50}	Fresh IC_{50}
Ethyl acetate	0.62 (0.993)*	0.26 (0.958)	0.19 (0.998)	0.24 (0.998)
Acetone	0.37 (0.98)	0.36 (0.98)	0.29 (0.99)	0.30 (0.99)
Ethanol	0.69 (1.00)	0.7 (1.00)	0.84 (0.98)	0.33 (0.99)
Methanol	0.63 (0.99)	0.66 (0.99)	0.60 (0.96)	0.57 (0.95)
Water	0.70 (0.99)	0.93 (0.96)	0.91 (0.96)	0.86 (0.99)
Pet. Ether	1.51 (0.99)			
Juglone	10.21 (0.99)			

* R^2: Correlation factor.

Table 4. Qualitative analysis for phytochemical metabolites in *J. regia* extracts.

Solvent	Conditions	Tannins	Flavonoids	Saponins	Alkaloids[a]
Ethyl acetate	Sox defatted	4+	2+	-ve	-ve
	Sox fresh	4+	4+	-ve	+ve
	M.W defatted	5+	4+	-ve	+ve
	M.W fresh	4+	5+	-ve	+ve
Acetone	Sox defatted	3+	3+	-ve	+ve
	Sox fresh	3+	3+	+ve	+ve
	M.W defatted	3+	3+	-ve	+ve
	M.W fresh	3+	3+	-ve	+ve
Ethanol	Sox defatted	3+	2+	+ve	-ve
	Sox fresh	1+	3+	-ve	+ve
	M.W defatted	2+	2+	+ve	-ve
	M.W fresh	3+	4+	-ve	+ve
Methanol	Sox defatted	2+	2+	+ve	-ve
	Sox fresh	3+	3+	-ve	+ve
	M.W defatted	2+	2+	-ve	-ve
	M.W fresh	2+	3+	+ve	+ve
Water	Sox defatted	1+	2+	+ve	-ve
	Sox fresh	0	2+	-ve	-ve
	M.W defatted	1+	2+	-ve	-ve
	M.W fresh	2+	2+	-ve	-ve
Pet. Ether		0	0	-ve	-ve

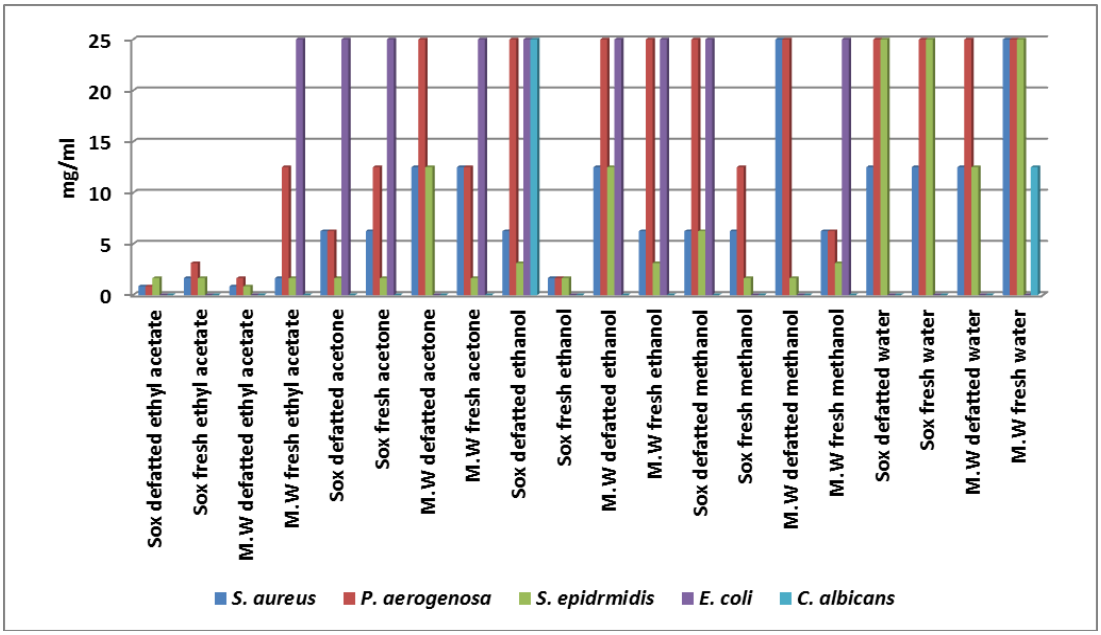
Table 5. Antimicrobial susceptibility test.

		MIC mg/ml				
Solvent	**Conditions**	***S. aureus***	***P. aerogenosa***	***S. epidrmidis***	***E. coli***	***C. albicans***
Ethyl acetate	Sox defatted	0.85	0.85	1.65	NA[a]	NA
	Sox fresh	1.65	3.125	1.65	NA	NA
	M.W defatted	0.85	1.65	0.85	NA	NA
	M.W fresh	1.65	12.5	1.65	25	NA
Acetone	Sox defatted	6.25	6.25	1.65	100	NA
	Sox fresh	6.25	12.5	1.65	100	NA
	M.W defatted	12.5	25	12.5	NA	NA
	M.W fresh	12.5	12.5	1.65	100	NA
Ethanol	Sox defatted	6.25	25	3.125	100	100
	Sox fresh	1.65	1.65	1.65	NA	NA
	M.W defatted	12.5	25	12.5	100	NA
	M.W fresh	6.25	25	3.125	100	NA
Methanol	Sox defatted	6.25	25	6.25	100	NA
	Sox fresh	6.25	12.5	1.65	NA	NA
	M.W defatted	25	25	1.65	NA	NA
	M.W fresh	6.25	6.25	3.125	100	NA
Water	Sox defatted	12.5	25	25	NA	NA
	Sox fresh	12.5	25	25	NA	NA
	M.W defatted	12.5	25	12.5	NA	NA
	M.W fresh	25	25	25	NA	12.5
Pet. Ether		12.5	25	6.25	12.5	6.5
Ciprofloxacin[b]		1	0.5	2	0.25	NA
Fluconazole[c]		NA	NA	NA	NA	18

[a]NA: Not active

[b]Ciprofloxacin (µg/mL) as antibacterial reference drug

[c]Fluconazole (µg/mL) as antifungal reference drug



A

B C

Figure 1. A) MIC comparison of solvent extracts of *J. regia* against *S. aureus, P. aerogenosa, S. epidrmidis, E. coli, and C. albicans*; B) MIC plate method and C) MIC- Micro-broth dilution method.

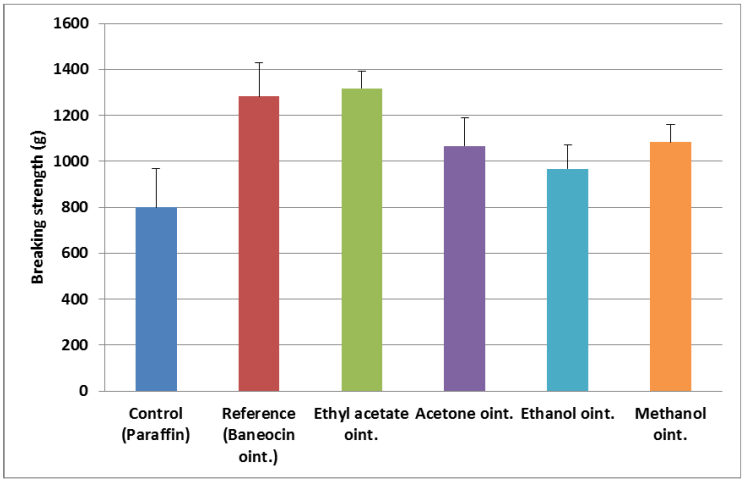


Figure 2. Comparison for skin tensile strength for formulas of *J. regia* extracts in grams.

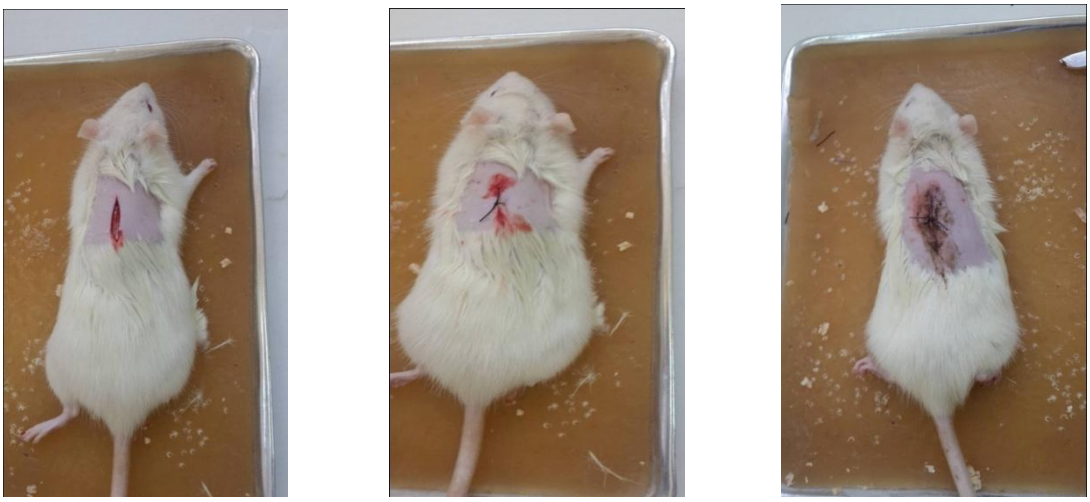


Figure 3. Procedure of wound healing activity.

Table 6. Breaking strength of experimental groups on the 10th-day post-wounding.

Groups	Breaking strength (g)
Control (Paraffin)	800 ± 167
Reference (Baneocin oint.)	1283 ± 147
Ethyl acetate oint.	1317 ± 75
Acetone oint.	1067 ± 121
Ethanol oint.	967 ± 103
Methanol oint.	1083 ± 75

Values are mean ±SEM of six animals in each group on the 10th-day post-wounding.
*p =1.10*10^{-5} as compared to the vehicle group.

Discussion

Extraction is a process by which substances and active constituents are separated from the original matrix; it depends on many physical and chemical characteristics. The process is divided into two types liquid phase extraction and solid phase extraction depending on the physical phase of extraction agent [22]. In our study, we have adopted liquid phase extraction for several reasons: Firstly, simple, inexpensive, relatively precise and wide range methods for separation, identification, and quantification of drugs, poisons, and herbal medicine. Secondly, an equilibrium distribution is established by the ratio between two phases will be influenced by the choice of the extracting solvent, the pH value of the aqueous phase and the ratio of the volumes of the organic to aqueous phases [23].

In this study, we have used five solvents for the extraction process from fresh and defatted *J. regia* unripe fruit using two extraction methods (microwave and soxhlet methods). The selection of solvents was based on the gradual increase in the polarity, ethyl acetate, acetone, ethanol, methanol, and water. The highest efficient solvent for extraction was water as the total yield weight of the extracts was 1.3 g in the defatted *J. regia* using microwave methods. Whereas, the lowest efficient solvent for extraction was ethyl acetate as the extraction yield was only 0.13 g in the fresh form of the unripe fruit by soxhlet method. The increase in the polarity of the extraction solvent has conferred a significant increase in yield amount as there were 9 folds increases in the amount of yield between the lowest polarity solvent (ethyl acetate) and the highest polarity solvent (water) regardless to the extraction method.

On the level of methods, the microwave method was more efficient than soxhlet method with a proportional increase of 40% as the yield of extraction for fresh and defeated *J. regia* in the two methods using the five solvents was 7.52 g in microwave method and 5.4 g in soxhlet method.

Substantially, we found there is a proportional increase of 34% in the yield amount of extracts using defatted plant instead of fresh plant regardless to the solvent or the method applied.

Total phenolic content

The process of total phenols determination using gallic acid standard curve is a widely applicable protocol [17]. In our study, the highest total phenols concentration was found in extracts of ethyl acetate 0.457 mg/g. whereas, the lowest total phenol concentration was found in extracts of water 0.167 mg/g. The increase in the polarity of the extraction solvent has conferred a significant decrease in the concentration of total phenols as there were 2.75 fold decreases in the concentration of phenols between the lowest polarity solvent (ethyl acetate) and the highest polarity solvent (water) regardless to the extraction method.

Despite the highest extraction yield been revealed using the water solvent, the total phenolic analysis showed most of the weight was not photochemically active substances. Inconsistent with the results of the extraction yield, the using of microwave extraction has conferred an increase

of 1.72 fold than the soxhlet method. Microwave extraction using ethyl acetate solvent has revealed 0.291 mg/g of total phenolic concentration, whereas, soxhlet extraction using ethyl acetate solvent has revealed 0.169 mg/g of total phenolic concentration. In addition, the usage of microwave extraction method was more applicable when compared with soxhlet method in the manner of time saving and lower usage of solvents amount.

On the level of using the fresh or defatted plant, there was no significant difference in the total phenolic compounds, the usage of defatted *J. regia* in extraction has revealed 0.213 mg/g, whereas, the usage of fresh *J. regia* revealed 0.244 mg/g.

The DPPH free radical scavenging activity

The antioxidant activity of the tested extracts range was (IC_{50}=0.19-1.51 mg/mL). For all the tested unripe fruit extracts, the activity at a given concentration was highest for the M.W defatted ethyl acetate extracts (IC_{50}= 0.19mg/mL). It was noticed that MW technique enhanced the free radical scavenging activity three times compared to soxhlet one as shown in table 4 (from 0.19 to 0.62 mg/mL) in case of defatted ethyl acetate extract, while it was two times in case of ethanol solvent (from 0.33 for MW fresh to 0.7 for soxhlet fresh (Table 4). As shown in table 4 this effect cannot be noticed in the case of methanol, acetone and water extracts, there was no effect to extraction technique or fat content.

Also the antioxidant activity of the juglone (as a reference) was tested (IC_{50}=10.21 mg/mL) and when compared with that of ethyl acetate extract (IC_{50}=0.19 mg/ml) as shown in table 4, the ethyl acetate extract showed better activity than the juglone which indicates the presence of mixture of phenolic compounds in the ethyl acetate extract.

Antimicrobial susceptibility test

To determine the activity of *J. regia* unripe fruit extracts against a set of microbial strains, we performed MIC-assays according to the EUCAST guidelines [20-21]. The MIC determination was performed for a relevant collection of five reference strains. Our results showed that ethyl acetate extract of *J. regia* has a good antibacterial activity as compared to the ciprofloxacin, a well-known antibacterial drug. It acts against a broad spectrum of reference strains with an MIC value of 0.85 mg/mL for *S. aureus*, *P. aerogenosa* and *S. epidermidis*. In the contrary, *E. coli* and *C. albicans* showed no susceptibility for ethyl acetate extracts of *J. regia*. Petroleum ether extract of *J. regia* has showed a significant antibacterial and antifungal effect against *E. coli* and *C. albicans* with an MIC value of 12.5 mg/mL and 6.5 mg/mL, respectively. This result points to the loss of active antimicrobial substances in the process of defatting of *J. regia*, hence, we have used petroleum ether as defatting agent and not as an extracting solvent. In addition, as a control, we decided to figure out the effect of the defatting process on the pharmacological activity of *J. regia* we made one more extraction process using petroleum ether.

Wound healing activity

In our study we have chosen four types of extracts formulated in Paraffin beside the control (Paraffin) and the reference drug (Baneocin ointment) to be tested on Sprague-Dawley rats following the induction of a longitudinal incision in the rats skin and after 10 days of treatment using set of the extracts formulations, the control and the reference drug; the wound was tested against weight force required to tear up the healing process (Figure 3). Unexpectedly, we found that the healing activity of the ethyl acetate extract was as good as the reference drug (Baneocin) and even the ethyl acetate had an increase of 5% than reference drug. Ethyl acetate extract showed an exponential increase of 65% in the healing activity when compared to paraffin (control group).

In addition, using one-way ANOVA and paired sample T- test showed statistically significant activity (P=1.10*10^{-5}) for all *J. regia* extracts when compared with the control group. Ethanol *J. regia* extract showed the lowest wound healing activity in the group of extracts but still it showed an increase of 20% in the healing activity when compared with control group.

These results could not be concluded as a potential active constituent in the wound healing agents since we did not purify an active substance but even this highlight on a promising core for further research in this field.

Conclusion

Unripe fruit of *J. regia* has been extracted via two important techniques: soxhlet and MW using fresh and defatted material. Five different solvents with different polarity have been used, to cover a wide range of phytochemical products. In addition, we examined qualitatively the presence of Tannins, Flavonoids, Saponins, and Alkaloids, as well as we measured quantitatively the concentration of total phenols yield of the extraction processes. Their chemical and pharmacological activity such as antioxidant, antimicrobial and wound healing were evaluated. Our results showed a higher efficiency for the microwave extraction method over the soxhlet extraction method in terms of higher yield amount and less time and solvents consuming. Water usage as extraction solvent has generated the highest yield amount. Whereas, the highest concentration of total phenols and DPPH scavenging activity were demonstrated in the ethyl acetate extract of *J. regia*. In the antimicrobial susceptibility test, the ethyl acetate extract of *J. regia* showed a significant MIC inhibition concentration of 0.85 mg/mL against *S. aureus,*

P. aerogenosa, and *S. epidermidis*. Unexpectedly, we found that the healing activity of the ethyl acetate extract was as good as the reference drug (Baneocin) and even the ethyl acetate had an increase of 5% ($P< 0.05$) than reference drug.

In conclusion, despite the numerous pharmacological activities of *J. regia* we highly recommend further investigation on the wound healing activity of *J. regia* unripe fruit.

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Conflict of interest

We wish to confirm that "there are no known conflicts of interest" associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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