ISOLATION AND ANTIFUNGAL EVALUATION OF *JUGLANS REGIA L* EXTRACTS

Ahmad I. Husein[1, 2*], Mohammed A. Al-Nuri[1], Nidal A. Zatar[1], Waheed Jondi[1], Mohammed S. Ali-Shtayeh[2,3] & Ismail Warad[*1, 4]

[1]Department of Chemistry, Faculty of Science, An-Najah National University, Nablus, Palestine.
[2]Biodiversity & Environmental Research Center (BERC), Til, Nablus, POB 696, Palestine, West Bank (The Palestinian Authority)
[3]Department of Biology and Biotechnology, Faculty of Science, An-Najah National University, Nablus. Palestine.
[4]Department of Chemistry, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia
*E-Mail: hamaydah2500@yahoo.com ,tel: 00972-9-2680685, mobile: 00972-0-599877286

ABSTRACT

The potential of antifungal effects of *Walnut (Juglans regia L., Juglandaceae*) ethanolic and chloroform extracts was reported against four pathogenic fungi: three dermatophytes, *Microsporum canis* (MC), *Trichophyton mentagrophytes* (TM) and *T. Rubrum* (TR), and the causative agent of chalk brood disease of bees, *Ascophaera apis* (AsA). Both extracts had considerable activity against the studied fungi. The main antifungal component of walnut was isolated and tested for its antimycotic activity individually. Based on its IR, ^{13}C and ^{1}H NMR analysis the main significant activity against was indentifying to be 5-hydroxy- 1, 4-naphthoquinone (Juglone).

Keywords: *Medicinal plants, Antifungal activity, Juglans regia, 5-hydroxy -1, 4-Naphthoquinone.*

1. INTRODUCTION

Although the last century has been characterized by a drastic decreasing in the mortality caused by infectious diseases, some diseases still represent a dreadful menace to plant, animal and human health and therefore, for a more efficient control, require the steady development or isolate of new, more powerful and inexpensive drugs [1-2]. The evaluation of phytochemical constitution and their biological activities in medicinal plants is necessary for the development of new therapeutic agents. Novel chemicals isolated from such plants with some biological activities may be used by the chemist as a guideline for the synthesis of useful drugs. [1-10]

The herbal medicines are recognized as most reliable, and most effective than any other system of medicinal practice. In addition, the use of higher plants and their preparation to treat infectious and non-infectious diseases is an age old practices and are the only method available in the past. Plants which constitute an active part of the ecosystem have been found to be useful to man both as sources of foods and medicine [1-4].

The use of medicinal herbs in the treatment of skin diseases, as example including mycotic infections is an old-age practice in many parts of the world, which currently developed to isolate and characterize the effective materials to be used as medicines [2-8]. Fungal infections remain a therapeutic problem despite the availability of a number of treatments. Being largely synthetic and non-biodegradable, these agents used in treating fungal infections can cause adverse effects and may have residual toxicity [9-12].

Many Species of Juglandaceae have been traditionally used by different peoples for treating several diseases. Cubans use *Juglans insularis* as herb decoction in baths for treating children's skin diseases.

Green walnuts, shells, kernels, bark and leaves have been used in the pharmaceutical and cosmetic products [13]. The leaves and pericarp of *J. regia* have been used as extracts in traditional medicine and pharmacologically demonstrated to be *anti*-helmintic, astringent, antifungal, hypoglycaemic, antidiarrhoeal and more recently, sedative.1 Phenolic compounds are secondary metabolites, which are reported to occur in abundance in fresh *J. regia* leaves. Flavonoids and naphthoquinones are the main phenolic compounds in walnut leaves [14-16].

The fleshy green covering (mesocarp and epicarp) of walnut fruit has been used in Traditional Arabic Palestinian Herbal Medicine to treat several human ailments including, skin diseases caused by fungi, syphilis, antihelmintic, astringent, stomachache, and nerve tonic [5-6]. Ethanolic extract of *Juglans nigra* fruit husks were found to have considerable antifungal activity active against dermatophytic fungi [6]

In this work ethanolic and chloroform as well as the expected active component 5-hydroxy- 1,4-Naphthoquinone extracted and isolated from *Walnut (Juglans regia L*) which grow up in Nablus Mountains, found to be active when it was investigated as antifungal agents using four harmful fungal sets.

2. RESULTS AND DISCUSSION

Few antifungal substances are known or available in the market compared to antibacterial substances. Antimycotic substances are also relatively unsatisfactory in the control of dermatophytes. New, more powerful and specific antimycotic agents are needed to combat these infections. The discovery of active components exhibiting a broad spectrum of antifungal activity may prove useful for the development of antifungal agents.

Figure 1, **2**, and **3** show the nature of fungi static activities encountered in walnut extracts and the pure component '**C**' compared with econazole (percent inhibition range 2.5-5.0 µg/ml) as a reference. The ethanolic extract '**E1**' the chloroform fraction '**E2**' and the pure component '**C**' all showed antimycotic activity against all tested fungi. Fungal toxicity (% mycelial inhibition) ranged from 45% in *AsA* to 100% inhibition in *TM* at concentration of 15 mg/ml of extract 'E1'.

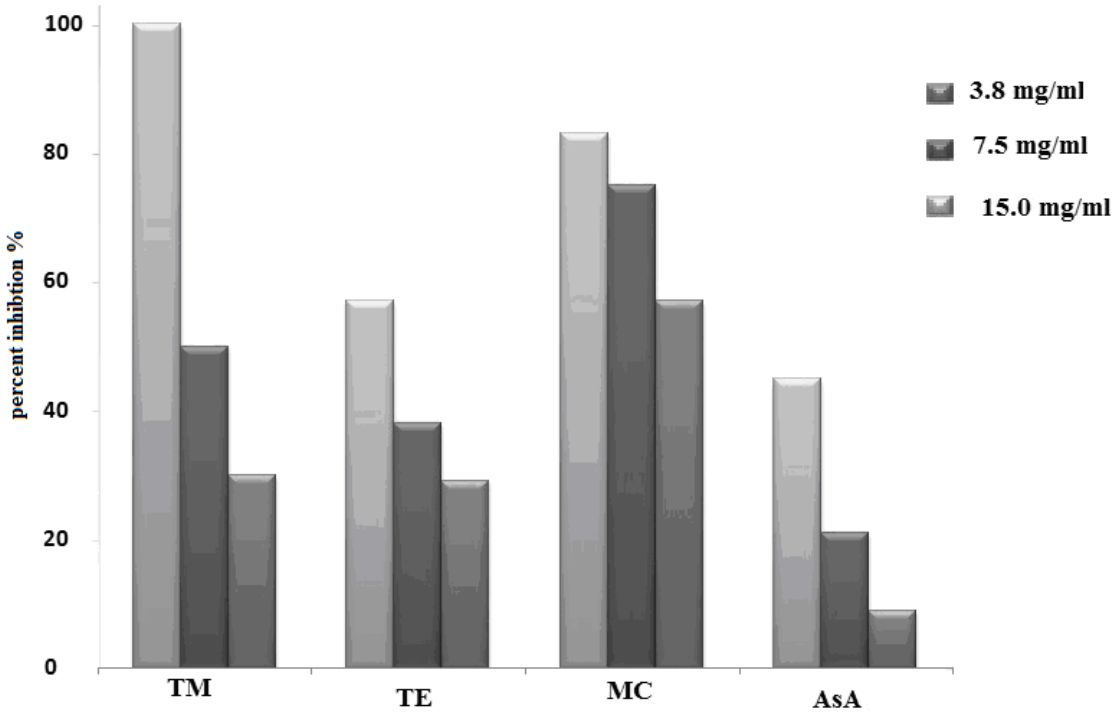

Figure 1. Antifungal activity of the ethanolic extract '**E1**' of walnut.
*****AsA, *A. apis;* MC, *M. canis;* TR, *T. rubrum*; TM, *T. mentagrophytes*.

Sufficient increased in the antifungal activity was observed in cases of ASA and TM compared by the other when the concentration of the extracts was increased.

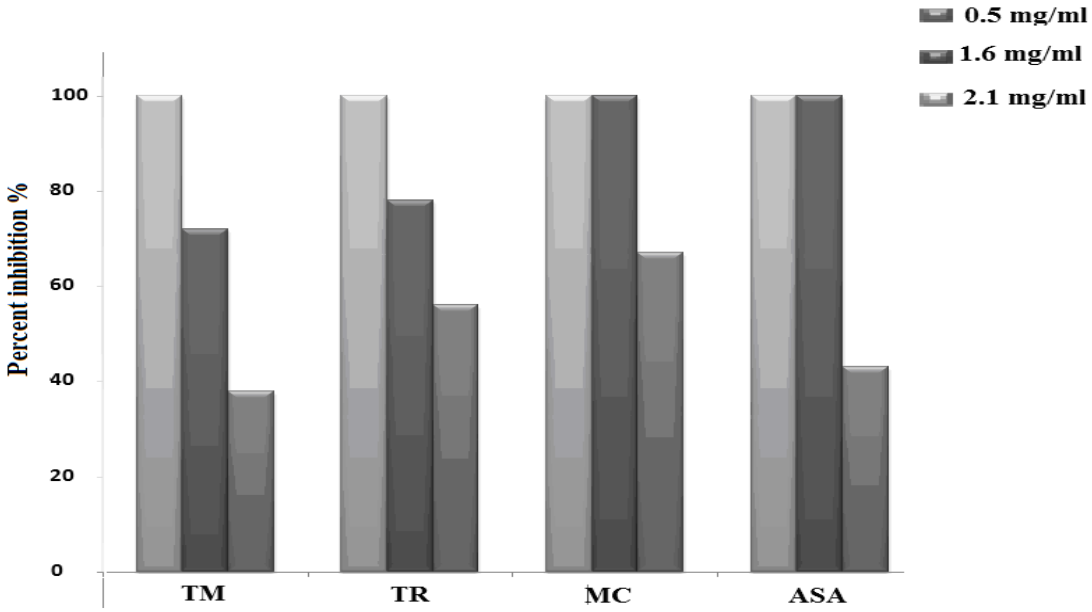

Figure 2. Antifungal activity of the chloroform extract '**E2**'of walnut.
*****AsA, *A. apis;* MC, *M. canis;* TR, *T. rubrum*; TM, *T. mentagrophytes.*

Fraction '**E2**' was clearly more efficient than extract '**E1**' inhibition test fungi; complete inhibition was achieved in all fungi at concentration of 2.1 mg/ml of fraction '**E2**'.

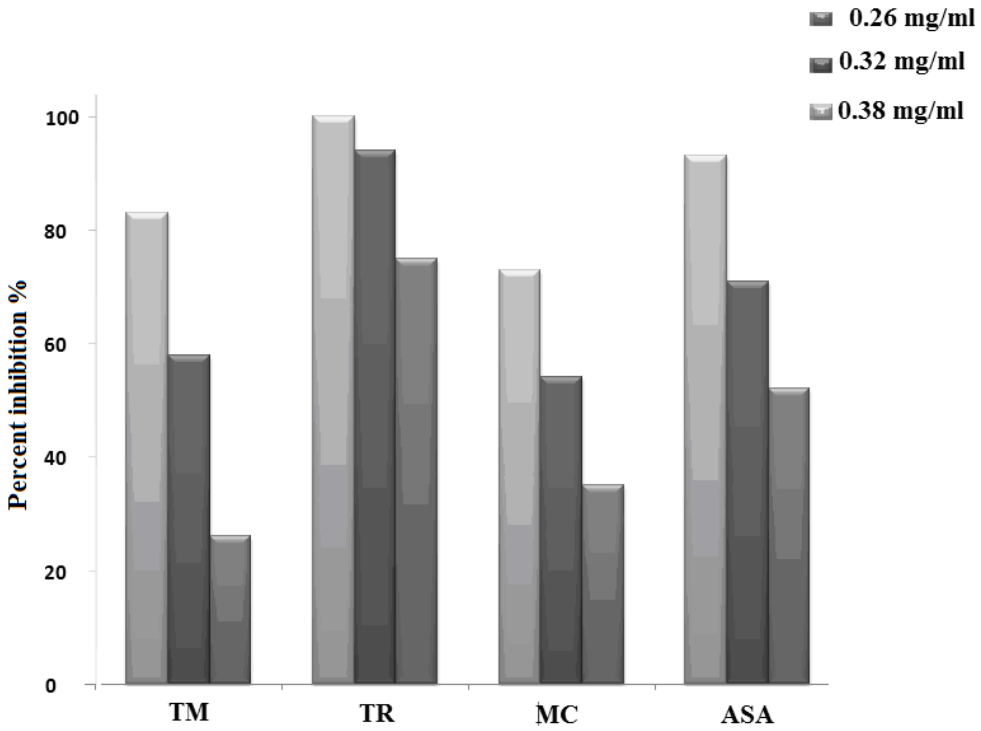

Figure 3. Antifungal activity of component '**C**' of walnut. *****AsA, *A. apis;* MC, *M. canis;* TR, *T. rubrum*; TM, *T. mentagrophytes.*

One way ANOVA test was performed to test for statistical difference among the three extracts: E1, E2, and E3. The results show that the test is statistically significant (F=10.277, P<0.005). We concluded that at least one extract mean is different than the others. A one-way ANOVA table (below) shows the means to differ significantly (P < 0.0005):

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13564.042	2	6782.021	10.277	.000
Within Groups	93051.958	141	659.943		
Total	106616.000	143			

The overall one-way ANOVA results are significant, so we concluded that not all the population means are equal. So, we compared means two at a time in the form of the post hoc analysis test (Least Square Difference (LSD) test. The results show that the only extracts that do not differ at p = 0.05 are extracts E1 and E3 (P = 0.414). This can be explained by the fact that we did not include the concentration of the extracts as part of the analysis.

(I) Extract	(J) Extract	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-22.396*	5.244	.000	-32.76	-12.03
	3	-4.292	5.244	.414	-14.66	6.08
2	1	22.396*	5.244	.000	12.03	32.76
	3	18.104*	5.244	.001	7.74	28.47
3	1	4.292	5.244	.414	-6.08	14.66
	2	-18.104*	5.244	.001	-28.47	-7.74
*. The mean difference is significant at the 0.05 level.						

The results show that the test is statistically significant (F=10.277, P<0.005). So at least one extract is different than the others, and the third one (component C) is the most different one, and has the largest effect when compared to the first two extracts (Tables 1 &2).

These results confirm that the use of walnut in combating fungal diseases of skin in folk medicine may be justified. Antifungal component(s) of walnut may also prove useful for the control of the calkboared disease; an economically important disease.

Our results are in agreement with those of Ali-Shtayeh et al (1992) and Heisey & Gorham (1992) who found the extracts of *Juglans nigra* fruit husks to possess antifungal activity against dertmatophtic fungi and *Candida albicans.* The pure component '**C**' has also been shown to possess antimycotic activity; a mycelial inhibition percentage of 83-100 % was obtained at a concentration of 0.38 mg/ml (Figure 3).

The chemical structure of the pure substance '**C**' was found to be 5-hydroxy- 1,4-naphthoquinone (Figure 4) based on its elemental analysis and IR, ^{13}C NMR and ^{1}H NMR spectra .This was consistent with that of standard 5-hydroxy- 1,4-naphthoquinone obtained from commercial source (Aldrich Chemical Company). Binder [7] separated eight 1,4-naphthoquinones from acetone extracts of unripe black walnut (*J. nigra*) fruit. Other monomeric naphthoquinones have been found in different plant organs in juglandaceae [10]. Also, juglone oligomers and symmetrical cyclotrijuglone were found to occur in root bark of this plant [17].

3. EXPERIMENTAL

3.1. General remarks, materials, and instrumentation

The pure sample of 5-hydroxy- 1, 4-naphthoquinone was purchased from Sigma Aldrich GmbH, Sternheim, Germany. Melting point (mp) was taken on a BUCHI 530 apparatus. Column chromatography (cc) was carried out on a silica gel 60 (230—400 mesh; Merck). Thin-layer chromatography (TLC) was performed on pre-coated silica gel F254 plates (Merck) using a 254-nm UV lamp to visualize the compounds. IR spectra were recorded on a Shimadzu Fourier Transform Infrared Spectrophotometer FTIR-8700 using Nujol as mulling agent or performed neat; only the most significant absorption bands are reported (n_{max} cm^{-1}). ^{1}H and ^{13}C NMR data were recorded at room temperature on a Varian 300 NMR spectrometer at 300 and 75 MHz, respectively. Mass spectra were recorded on a JEOL JMS-300 spectrometer. Elemental analyses were obtained using a Perkin-Elmer 2400 Elemental Analyzer.

3.2 Plant Material

Green nearly ripe fruits of walnut were obtained from walnut trees growing in Til village located 5km south-west of Nablus in the West Bank in 2009. The plant was identified by Dr. Ali-Shtayeh, M. and a voucher specimen was deposited in the Biodiversity and Environmental Research Center (BERC) Til village-Nablus.

3.2 Extraction and Isolation

The fleshy green covering of walnut (50g) was soaked in 200 mL 95% ethanol for 7 days. The extract was filtered by Whatman filter paper no. 4 and divided into two parts. The solvent of the first part was removed from the extract under reduced pressure at 0°C? This fraction is denoted as "**E1**". The second one was extracted with Chloroform and separated using a separatory funnel. The chloroform portion was freed of the solvent and the solid extract obtained is denoted by "**E2**". The solid E2 was further subjected to column chromatography using silica gel petroleum ether (60-80°) - diethyl ether (2:1) as eluent. A component "**C**" was obtained in a pure form as a yellow crystalline substance with an R_f value of 0.61 and melting point (mp) of 163-165°C.

IR Spectrum: n (KBr) 3400, 3058, 1662, 1641, 1590, 1448, 1289, 1225, 1151, 1098, 1081, 863, 827, 762 and 703 cm^{-1}.

^{1}H NMR Spectra: (400 MHz; CDCl3): 6.94 (2 H, s, Hquin.), 7.27 (1 H, dd, J 2.2 and 7.5 Hz, Harom.), 7.60-7.65 (2 H, m, Harom.) and 11.90 ppm (1 H, s, OH).

^{13} C NMR (100 MHz, CDCl3): 114.0, 118.1, 123.5, 130.8, 135.5, 137.6, 138.6, 160.6, 183.2 and 189.3 ppm.

Mass Spectrometry: MS: m/z (EI, 70 eV) 174 (M+, 100%), 146, 118, 90, 63 and 39.

Elemental Analysis: Found: C, 68.25; H, 3.70. Calc. for $C_{10}H_6O_2$: C, 68.97; H, 3.47.

3.3 Test Organisms

The following fungi were used in this study: *Microsporum canis* Bodin (BERC-MC101); *Trichophyton rubrum* (castellani) Sabouraud (BERC-TR201); *T. mentagrophyte*s (Robin) Blanchard (BERC-TM301); and *Ascosphaera apis* (Olive Spiltoir) Maassan ex Claussen (BERC-AsA711). The first three fungi are human pathogens causing dermatophytoses. The fourth fungus is animal pathogens that cause chalkboard disease of bees. These fungal isolates were obtained from BERC's Fungal Culture Collection, Til village, Nablus, Palestine. The isolates have been maintained under mineral oil in tubes on oat agar at room temperature.

3.4 Preparation of Samples for Testing:

Extracts E1, E2 and C were dissolved in hot sterile distilled water and the solution sterilized using membrane filtration (0.45µm Millipore filters).

3.5 Antifungal Testing:

The plant extracts were tested at different concentrations (**Figure 1**, **2** and **3**) for their fungi toxicity against the test pathogens by a modified 'poisoned food' technique [8]. The required amounts of each extract were mixed in requisite amount of pre-sterilized Sabouraud dextrose agar (SDA) medium. A mycelial disk of 5 mm diameter, cut out from the periphery of 7-days old culture, was aseptically inoculated on to the medium. In controls, sterile distilled water was used in place of test extract. Three replicate agar plates were used for each treatment. The inoculated plates were used for each treatment. The inoculated plates were incubated at 24°C and the observations were recorded after 7 days. Percentage of mycelial inhibition was calculated on the basis of the colony diameter using the following formula:

% of mycelial inhibition = [(colony diameter of control-colony diameter of treatment)/ colony diameter of control] 100 [12].

3.6 Statistical analysis:

We performed one way ANOVA test, and the post hoc analysis test (Least Square Difference (LSD) for statistical difference among the three extracts on the different types of the studied fungi.

CONCLUSION

Ethanolic and chloroform extracts of Walnut plant which grow up in Palestinian mounts was investigated in this study. The extracts and one of their constituents were studied against four pathogenic fungi: three dermatophytes, (MC), (TM) and (TR), and the causative agent of chalk brood disease of bees, (AsA). They

revealed a promising result against the studied fungi. Based on IR, ^{13}C and ^{1}H NMR analysis the main component was identified to be 5-hydroxy- 1, 4-naphthoquinone.

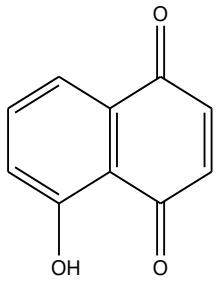

Figure 4 :Structure of component c

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Sample Availability: Samples of the compounds are available from the authors.