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# THE BIOLOGICAL ACTIVITY OF AQUEOUS AND METHANOLIC EXTRACTS OF *JUGLANS REGIA* ON YEASTS AND PATHOLOGIC BACTERIA

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## Abstract

In this study *Juglans regia* extracts showed activities against *Malassezia furfur* yeast, Methanolic extract exhibits more activity than aqueous extract. The Minimum Inhibition Concentrated rate (MIC) for *Malassezia furfur* methanolic extract was 75µg/ml. The extracts also examined on *Candida albicans* opportunistic yeast in the mouth) and found to be more active than M. furfur were MIC 50 µg/ml. The biological activity of aqueous and methanolic extracts were also examined against six pathogenic bacteria isolated from patients suffering from different diseases from several hospitals in Basrah, including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi, Proteus mirabilis, pseudomonas aeruginosa* and *Vibrio parahaemolyticus*. The Methanolic extract showed an effective effect in inhibiting all bacterial species compared with the aqueous extract. This study provides the possibility of using a natural plant such as *Juglans regia* antimicrobial agent for many pathogenic yeasts and bacteria.

***Key words:*** *Juglans regia*, activity, antifungal, antibacterial

## Introduction

Some fungi found on human skin and scalp, such as *Malassezia* and are found in some human cavities such in the mouth and vagina such as *Candida*. *Malassezia* is yeast that is naturally found on the skin of healthy human and warm-blooded animals, and can become a pathogen and cause skin diseases such as Ptryasis versicolor (Yarrow and Ahearn, 1984; Cunningham and Midgley; 1993; Ingham *et al*., 1997; Dismukes *et al*., 2003).

This yeast has a tendency to fat, therefore called lipophilic and all sex-related species of the seven species considered to be lipophilic, except *M. pachydermdis*. Therefore, the medium used to isolate should contain fatty acids (Gueho *et a.l*, 1996; Midgley *et al*., 1997; Gueho *et al*., 1998). Olive oil is one of the most common fats used to isolate this yeast. The most important characteristic of this yeast is the possession of the enzyme Lipase (Ran *et al*., 1993). *Candida* is bivalent yeast that is co-existent in warm-blooded animals, including humans. It colonizes the mucous surfaces of the mouth, vagina and digestive tract. It is capable of causing various infections, according to the host. The infection caused by *Candida* is called Candidiasis and was two types oral (Thrash) and vaginal candidiasis. *Candida* is dimorphic yeast it has two phases, yeast phase, and the filament phase and is transformed from one phase to another depending on PH and temperature and the presence of other compounds such as Acetylglycosamine (Leberer; 1991, Culture *et al*., 1997; Lo *et al*., 1997; Ryley, 1990).

Some primitive methods of decoration are used, such as the use of *Juglans regia* to whiten the teeth and apply dye to the lips (AL-Khawaja, 1997).

The walnut trees live in northern Iran, the Caucasus, Baluchistan, and Armenia, and these perennial trees date back to 500 years (Chopra and Jamaatah, 1958). It was used as an antifungal agent especially in the treatment of ringworm infections for the first time in 1950 by the world Dorlan. The extract successfully used in inhibiting yeast growth *Candida albicans* (Sytykiewicz *et al*., 2015).

The plant extract can be used as a natural alternative to control of pathogenic bacteria in general and to reduce the risk of antibiotic-resistant pathogens in particular (Tiwari and Tiwari, 2011).

In a study carried out by Sharafati-Chaleshtori *et al*., 2011, the *Juglans regia* extract showed a wide spectrum effect against bacteria such as *Staphylococcus*

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*aureus, Streptococcus mutans, Escherichia coli, Pseudomonas aeruginosa*.

The discovery of antibiotics in the last century led to a decrease in disease rates and deaths due to infectious diseases, but the inappropriate use of these antibiotics resulted from the emergence of groups of resistant bacteria that threaten human health, especially with immunocompromised patients, creating the need to research new types of natural anti-microbial substances to control on different diseases and has led to an increase in interest in medicinal plants where 25-50% of the current pharmaceuticals derived from plants because chemically manufactured drugs have side effects, while there is no such effects in medical alternatives such as plant extracts (Gupta and Birdi, 2017).

Therefore, the aim of this study was to study the antimicrobial effect of *Juglans regia* extract (aqueous and Methanolic) on yeasts and pathogenic bacteria isolated from patients suffering from different diseases in Basrah hospitals.

## Materials and Methods

Preparation of aqueous and methanolic *Juglans regia* extracts: 20gm of the powdered dry bark of *Juglans regia* were weight separately in different flasks, one of them percolated with 200ml of methanol this flask was proper sealed with aluminum foil and left with stirring magnetic stirrer for 24 hours at room temperature. The solution was then filtered using a funnel filled in a filter paper and the methanol extracts collected in Petri dishes and dried at room temperature. The above procedure was repeated with the use of 200 ml of distilled water to prepare the aqueous extract (Handa *et al*., 2008).

**Isolation of yeasts**

*Malassezia* yeast isolated from person with pityriasis versicolor by used of skin scraping that cultured in Sabouraud Dextrose Agar (SDA )medium supplemented with 20ml of olive oil per liter ,0.01 cyclohexamide and 10 drops of tween 20. the isolated Malassezia classified as *Malassezia furfur* While *Candida* yeast isolated from person with mouth candidiasis infection.

Oral swab used and cultured in (SDA) both yeasts incubated in $32^0$C the isolated candida classified as *Candida albicans* by using of germ tube test.

**Study the effect of alternative oils on the *Malassezia* yeast growth**

Appropriate medium for the growth of Malassezia is the added to some oils communally olive oil and Tween 80 as mentioned in the introduction, but in this experiment has been replaced olive oil with some oils such as sunflower oil and castor oil and olive oil treatment compared to add to use a combination of the three types as an independent treatment, the previous oils were added by 20ml per liter of SDA media. Each of the cultivars was poured into the dishes. The yeast was then planted on the medium in a streaking manner, taking into consideration three replicates per treatment and incubated at 32°C.

**Antifungal activity test**

Diffusion agar method was used to examine the antifungal activity of both aqueous and methanolic extracts toward *Malassezia furfur* and *Candida albicans* using (SDA) medium , 10µl of each yeast suspension add on the surface of the medium then spread with sterile L-shape glass rod, after drying, two 5mm diameter pores was made in the center of plates , 100µl of both crud extracts was add separately in the pores of plates with used of control treatment for each yeast , cultures were incubated at $32^0$C for 2 days after incubation period the diameter of the inhibition zones were evaluated in millimeters. (Sykes, spooner, 1972).

**The minimum inhibitory concentration (MIC) test for yeast**

The MIC values were determined by the standard serial dilution assay using serial dilutions of the aqueous and methanolic extracts (300,200,100, 50, 25), µg/L). The suspensions of yeasts was prepared and standardized to a turbidity equivalent to that of 0.5 MacFerland scale (1×106 cfu/ml). 100µl of yeast suspension add on the surface of the media then media incubated in $32^0$C, The MIC values in this assay were indicated by the absence of yeast growth at the minimal concentration of the extract.

**Antibacterial activity test**

The Muller Hinton agar was used for the purpose of measuring the biological efficacy of six types of pathogenic bacteria *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Proteus mirabilis, pseudomonas aeruginosa* and *Vibrio parahaemolyticus* were isolated from patients suffering from different diseases admitted to Al Fayhaa General Hospital and Al Shifa General Hospital in Basrah. The well-diffusion methods were used by Bansode and Chavan (2012). 0.1 ml of the bacteria was distributed in the Muller Hinton agar dish using a sterile swab and then holes were made in the center of the plates using a cork borer. 100µl of both aqueous and methanolic crude extracts were placed separately in each hole. One distilled water was placed in a single hole as a control. The dishes were then left for half an hour after being placed in the incubator for 24 hours at 37°C. The

effectiveness of the two extracts was determined by measuring the diameter of the inhibition zone formed around the hole mm.

**The Minimum Inhibitory Concentration (MIC) test for bacteria**

The MIC of different concentrations of Methanolic and aqueous *Juglans regia* extracts against tested bacterial isolates were done using Muller-Hinton agar. The concentrations of the extracts tested were (500, 250, 100, 50) µg/ml, the assays were performed three times for each bacterium. Plates were inoculated with 0.1 ml of the bacterial suspension was distributed in the Muller Hinton agar dish using a sterile swab and then holes were made using a cork borer. Different extract were placed in each hole. The plates then left for half an hour after being placed in the incubator for 24 hours at 37°C. The highest dilution of extract that showed no visible bacterial growth per hole was considered as MIC Standards.

## Results

**Preparation of aqueous and methanolic *Juglans regia* extracts**

After the preparation of the extract it was observed that the aqueous extract is characterized by viscous nature while the methanolic extract appeared in the crystalline nature due to the difference in the type of chemical compounds extracted from each one.

**Isolation and Diagnosis of Yeast**

After the *Malassezia* Yeast isolate from the Skin infection pityriasis versicolor the colony was raised and smooth initially and get dry and wirnkled in time the color of colony was creamy to a slightly yellowish-colored. The microscopic examination indicated that it represented the *M. furfur.*

Species *M. furfur* is an ellipsoids yeast with small collarets and contains a bud or scar from one side as a characteristic feature.

The other yeast isolate was *Candida albicans*, which had a cream glistening and sometime waxy surface is smooth The microscopic examination of *Candida albicans* was small oval with single budding sometime this yeast was give positive germ tube test. (Midgley *et al*., 1997).

**Study the effect of alternative oils on the *Malassezia* yeast growth**

Experiment showed that the best growth of *Malassezia furfur* was in the medium containing olive oil and sunflower oil. Either the medium with other treatment of castor oil appeared weak growing of *Malassezia furfur*, mixing oils as results of castor oil, Fig. 1.

**Antifungal activity Test**

Both aqueous and methanolic extracts showed effectiveness in inhibiting the growth of both yeasts. However, the effect of the extracts was higher with respect to the yeast *C. albicans* compared with the yeast *M. furfur*. The diameters of the inhibitory zone were 20mm and 25 mm respectively for the aqueous and methanolic extracts. *C. albicans* (45) mm for aqueous extract and (3) mm for methanolic extracts. Table 1, Fig. 2.

**The MIC test for yeast:**

The concentrations of 200 and 300 µg / ml showed a lack of growth for both extracts, MIC for *C. albicans* yeast 50 µg/ ml in the aqueous extract and 75 µg/ ml in the Methanolic extract.

MIC for *M. furfur* yeast in the treatment of the aqueous extract was 100µg/ml and 75µg/ ml in methanolic extract obtained. Table (2) shows this.

**Antibacterial activity Test:**

Two positive and negative Gram bacteria were selected in order to determine the effect of *Juglans regia* extracts on them. Methanolic extract showed a clear and almost fatal effect on most bacterial species (Table 3). *Staphylococcus aureus* showed the most sensitive germs, followed by *Salmonella typhi.*

**The MIC for pathogenic bacteria:**

Concentrations showed 50 micrograms / ml growth loss for all bacterial pathogens in both extracts. MIC for *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa* 500 µg / ml in the water extract were 250 µg / ml in the alcohol extract.

MIC for *Staphylococcus aureus* showed a concentration of 50 µg/ ml growth in the treatment of aqueous and methanolic extract and Table 4 shows this.

## Discussion

The experiment demonstrated that olive oil and sunflower oil were comparable in their effectiveness to support the growth of *M. furfur* compared to castor oil (Ran *et al.*, 1993) because it is high in density and complex structure which is difficult to digest into simple fatty acids by Lipase enzyme and contains a protein called Resin inhibits protein building in the cell.

The extract of *Juglans regia* was tested against *M. furfur* as tested for yeast *C. albicans*, and extract is used as a cosmetic to beautify the lips and whiten teeth in the mouth (Al-khawaja, 1997).

The use of plant extracts is still common due to the resistance of many types of yeast to antifungal agents. The extract of *Artemisia abrotanum* has been successfully used to inhibit the growth of yeast *M. furfur* and *C. albicans* (Brodink *et al*., 2007).

Essential oils derived from orange and lemon has been found to be effective against the fungus *M. furfur*, which causes varicose veins (Sharma *et al*., 2012).

*Juglans regia* is used as an extract in the inhibition of the growth of many skin fungi and with high efficiency such as *Epidermophyton flocosum* and *Trichophyton mantagrophtes a*nd *C. albicans*. Al-Khawaja, 1997 noted that the use of *Juglans regia* plant in the mouth to clean the teeth and adornment increases the acidic function of the PH saliva, which leads to an antimicrobial action. The experiment proved superior to the aqueous than methanolic extract in the antimicrobial activity experiment on *C. albicans* and has a higher MIC, this is due to the ability of water to pull many compounds and active ingredients (alkaloids, tannins and amino acids) from their plant source (Grimshow, 1976). While the result was reflected in the yeast of *M. furfur* in the effectiveness of

**Table 1:** shows the diameter of the inhibition zones of extracts toward yeasts.

| Yeast | Inhibition zone diameter(mm) | |
|---|---|---|
| | Aqueous extract | Methanolic extract |
| *M. furfur* | 20 | 25 |
| *C. albicans* | 45 | 30 |

**Table 2:** Shows MIC of extracts toward yeasts

| yeast | MIC value( µg / ml ) | |
|---|---|---|
| | Aqueous extracts | Methanolic extracts |
| *M. furfur* | 100 | 75 |
| *C. albicans* | 50 | 75 |

**Table 3:** Results of the inhibitory effect and diameters of the inhibition zones in (mm) for the aqueous and Methanolic extract against 6 bacterial isolates.

| Pathogenic bacteria | Diameters of mm inhibition zones | |
|---|---|---|
| | Alcoholic Extract Mm / mg | Water extract Mm / mg |
| *Staphylococcus aureus* | 22.5 | 15.5 |
| *Pseudomonas aeruginosa* | 7 | 8 |
| *Vibrio parahaemolyticus* | 12.5 | R |
| *Salmonella typhi* | 20 | 10 |
| *Proteus mirabilis* | 11 | 10 |
| *Escherichia coli* | 17.5 | 15.5 |

**Table 4:** The MIC of the pathogenic bacteria under study towards aqueous and alcohol extract.

| Bacteria | 500 | | 250 | | 100 | | 50 | |
|---|---|---|---|---|---|---|---|---|
| | C | W | C | W | C | W | C | W |
| *Staphylococcus aureus* | + | + | + | + | + | + | - | - |
| *Pseudomonas aeruginosa* | + | - | - | - | - | - | - | - |
| *Escherichia coli* | + | + | + | + | + | - | - | - |
| *Vibrio parahaemolyticus* | + | - | - | - | - | - | - | - |
| *Salmonella typhi* | + | + | + | - | - | - | - | - |
| *Proteus mirabilis* | + | + | + | - | - | - | - | - |

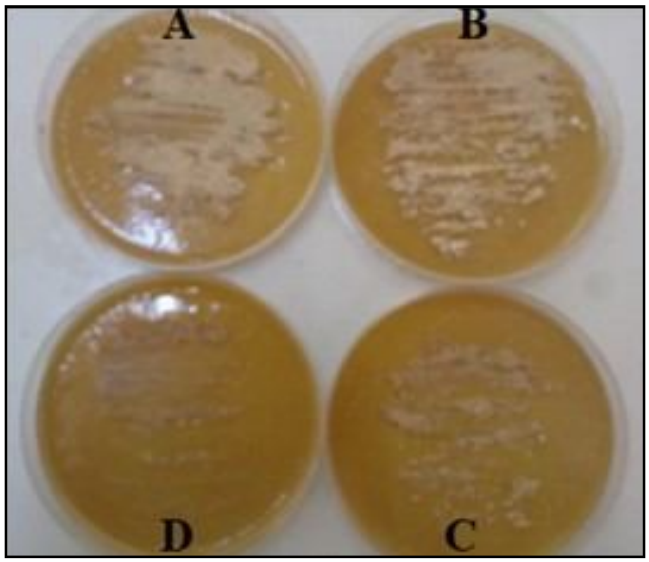


**Fig. 1:** Shows the growth of *Malassezia furfur*, on the medium containing different oils A. Olive oil B. Sunflower oil C. Castor oil D. Oil mixture

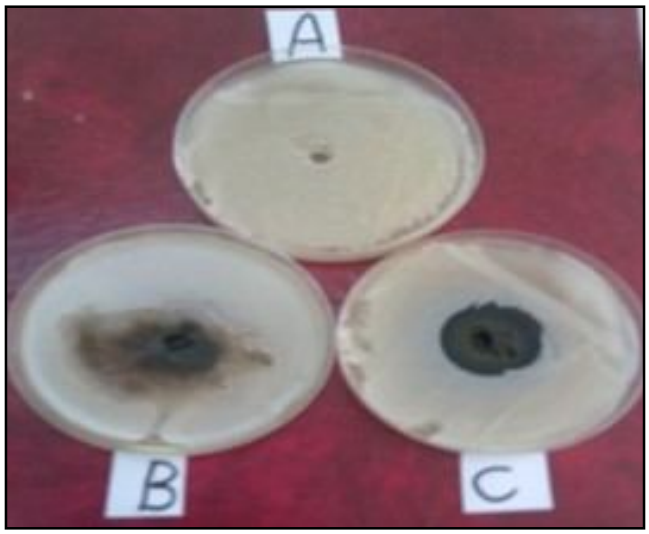


**Fig. 2:** Indicate the diameters of the inhibition zone of *C. albicans* in the effect of the extracts A. Control B. Aqueous extract C. Methanolic extract.

antifungal activity and MIC, the methanolic extract was more effective in inhibition of the growth this is because to the fact that mehanol is able to extract flavonoids that may affect this yeast (Harborn, 1984). This is agreement with Qadan *et al.,* (2005) found that phenolic compounds such as flavonoids have anti-fungal effects. Buttery noted that *Juglans regia* is containing terpenes and hydrocarbons Esters that have antifungal and antioxidants action such as phenolic compounds.

It is recognized that antibacterial resistance to antibiotics has been rising to dangerous levels around the world. Antibiotic resistance leads to prolonged hospital stay, high medical costs and increased mortality. New resistance mechanisms are emerging and spreading globally and threaten our ability to treat common infectious diseases (Rossiter and his group 2012).

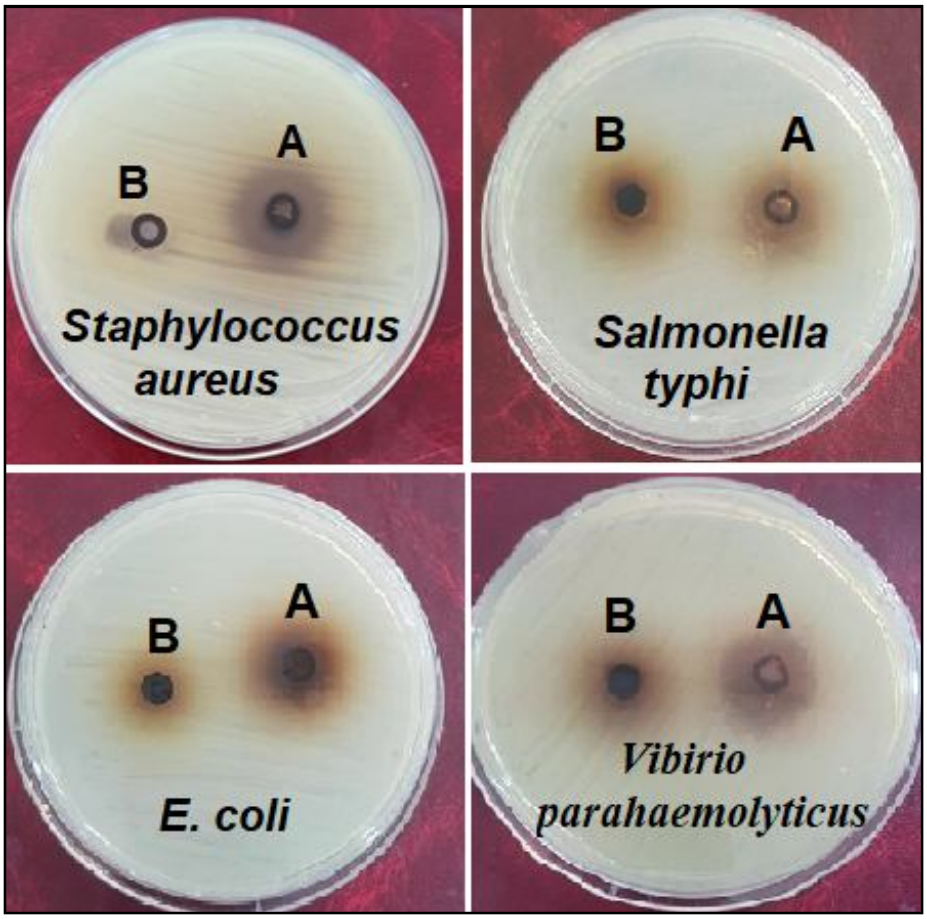


**Fig. 3:** The diameters of the 4 type bacteria growth inhibitors A. Methanolic extract B. Aqueous extract

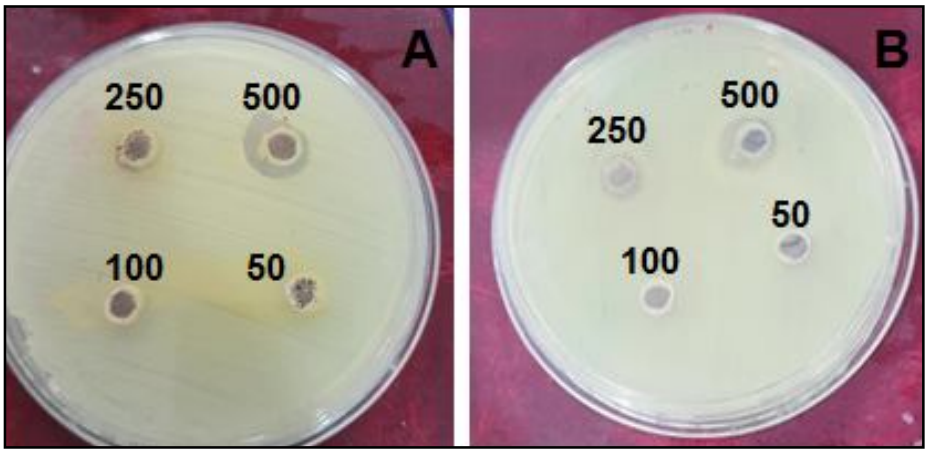


**Fig. 4:** shows MIC of *Vibrio parahaemolyticus* bacteria with the effect of the extracts A. Methanolic extract B. Aqueous extract

Antibiotic resistance increases and spreads in cases where the over-the-counter purchase of antibiotics is considered for human or animal use. All these things called for rethinking the return to natural resources.

The results of the test of the efficacy of aqueous and methanolic extracts of *Juglans regia* as an anti-bacterial agent showed that gram-negative bacteria and gram-positive bacteria were affected by the both extracts used.

*Juglans regia* methanolic extract showed zone of inhibition against all tested bacterial strains, while the aqueous extract was active with relatively smaller inhibition zones, which is consistent with Zakavi and its group in 2013. *S. aureus* and *Salmonella typhi* showed that they were the most sensitive species for the effect of methanolic extracts for the extract of water of the *Juglans regia*, bacteria *S. aureus* showed high sensitivity while *Salmonella* appeared less sensitive.

The results showed that bacteria *Vibrio parahaemolyticus* were sensitive to the alcohol extract, while the aqueous dermis showed no efficacy against them (Alkhawajah, 2012).

## Conflicts of Interest

There are no conflicts of interest.

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