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Effect of henna (lawsonia inermis) and juglans regia bark extract on some pathogenic microorganisms

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Abstract:

Six types of extracts were prepared for henna leaves (Lawsonia inermis) and Juglans Regia bark, by using distilled water, 1 M phosphate buffer (pH 7.0), 1 M sodium acetate buffer (pH 6.0), 1 M Tris-HCl buffer (pH 8.0), ethanol and methanol alcohol. The antibacterial activities of the henna and J. regia extracts were determined using the agar disc diffusion on nutrient agar for each one of Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli and Candida albicans. The results of the phytochemical screening of henna extract revealed the presence of alkaloids, glycosides, terpens, anthraquinones, tannins and phenols, whilst flavonoids, saponins, steroids, resins and coumarines gave negative test, on the other hand, result indicate the presence of alkaloids, glycosides, flavonoids, tannins, saponins, phenols, steroids and anthraquinones, whereas the terpens, resins, and coumarines gave negative test in J. Regia bark extract. The highest antimicrobial activity was observed upon using 10 gm % (w:v) of henna extracted by 1M phosphate buffer (pH 7.0), and 10 gm % (w:v) of J. regia bark extracted with 1M sodium acetate buffer (pH 6.0), after mixing for 4 hours. The best concentration of henna and J. regia bark extract on discs that gave the high antimicrobial activity against the tested microorganisms was 10 mg/ml.

Key words: henna, Juglans Regia, Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli and Candida albicans, Juglans Regia**,** Lawsonia inermis, antimicrobial.

الخلاصة:

استخدمت ست انواع من المستخلصات لاستخلاص ورق الحنة (Lawsonia inermis) و قلف شجر الجوز Juglans Regia، هذه المستخلصات تضمنت: 1 مولر من بفر الفوسفات (وبأس هيدروجيني 7)، و 1 مولر من بفر الصوديوم اسيتيت (وباس هيدروجيني 6)، و1 مولر من بفر الترس الحامضي (وبأس هايدروجيني 8)، وكحول الايثانول والميثانول. تم تحديد الفعالية المضادة للميكروبات لمستخلص الحنه ومستخلص قلف شجره الجوز باستخدام طريقه انتشار القرص على الاكار وعلى وسط الاكار المغذي ولكل واحده من العزلات Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli and Candida albicans. اظهرت نتائج الكشف باستخدام الكشوفات الكيميائية لمستخلص الحنه عن وجود مركبات القلويدات، والكلايكوسيدات، والتيربين، والانثروكواينون، و التانينات، والفينولات، في حين الفلافونات، والصابونيات، والستيرويدات، والراتنجات و الكومارين، اعطت نتيجة سالبه للكشوفات. اما نتائج الكشوفات الكيميائية لمستخلص قلف شجره الجوز وجود مركبات القلويدات، والكلايكوسيدات، والفلافونات، والتانينات، والصابونيات، والفينولات، والستيرويدات والانثروكواينون، بينما التيربين، والراتنجات و الكومارين اعطت نتيجة سالبه للكشوفات. اظهرت النتائج بأن اعلى فعالية مضادة للميكروبات كانت عند استخدام 10 غرام بالمئة (وزن:حجم) للحنه واستخلاصها بواحد مولر من بفر فوسفات (وبأس هيدروجيني 7)، و10 غرام بالمئة (وزن:حجم) لقلف شجره الجوز والمستخلص بواحد مولر من بفر الصوديوم استيت (وبأس هيدروجيني 6)، بعد المزج مع البفر ولمده 4 ساعات. حدد التركيز 10 ملغرام/مل كأفضل تركيز لمستخلص الحنه و مستخلص قلف شجره الجوز على الاقراص ليعطي اعلى فعالية مضادة للميكروبات ضد الاحياء المجهرية المستخدمة بالاختبار.

مفاتيح الكلمات : Juglans Regia**,** Lawsonia inermis ، مضاد مايكروبي.

Introduction:

Lawsonia inermis (synonym Lawsonia alba), commonly referred to as henna, belongs to the Lythraceae family and is the sole species in the genus [1]. Henna, L. inermis, produces a burgundy dye molecule, lawsone. This molecule has an affinity for bonding with protein and thus has been used to dye skin, hair, fingernails, leather, silk and wool. It is well known that plants have been used in traditional herbal medicine for many years. L. inermis is widely grown in various tropical regions in Asia, America, and Africa [2]. The main uses of henna are as a cooling agent, astringent, antifungal and anti-bacterial herb for the skin and hair. It has also been used as a dye and preservative for hair, skin, and fingernails as well as leather and clothes. Its core chemical components are 2-hydroxynapthoquinone (lawsone), mannite, tannic acid, mucilage and gallic acid. About 0.5-1.5% of henna is made of lawsone. Its bioactive feature is thought to be due to its high protein binding capacity [3]. Many types of bacteria have the ability to produce skin infections. Staphylococcus aureus is the most common cause of skin infections. It is frequently found in the nose and skin [4]. Juglans regia is the most widespread tree nut in the world. The tree belongs to juglandaceae and has the scientific name Juglans regia. The J. regia tree species is native to the old world. It is native in a region stretching from the Balkans eastward to the western Himalayan chain (Fernandez-Lopez et al., 2000) and was cultivated in Europe as early as 1000 BC [5]. Hot and cold solvent and aqueous extract of leaves, barks, fruits and green husks of J. regia from different countries revealed broad spectrum antibacterial activity against gram-positive and gram-negative bacteria, also J. regia bark aqueous and solvents extract exhibited antifungal activity against wide range of fungi using disc diffusion method, agar dilution method, agar streak dilution and Raddish method [6]. The purpose of this study was to determination and evaluates the antibacterial and antifungal properties of henna and J. regia extracts in vitro.

Chemicals and methods:

Chemicals:

Lawsonia inermis leaves and J. regia bark were purchased from markets, nutrient agar and all analytical grad dyes were purchased from Sigma-Aldrich and Hi–Media, respectively.

Extraction of L. inermis leaves and J. regia bark:

Lawsonia inermis leaves were collected from the market of Basrah (or Basra) and J. regia bark was collected from the market of Baghdad. Six types of extracts solvents were prepared in the present study: distilled water, 1M phosphate buffer pH 7, 1M sodium acetate buffer pH 6, 1M Tris-HCl buffer pH 8, ethanol and methanol alcohol. The preparation of the extracts by mixing separately 10, 5 and 2.5 gm of each of L. inermis leaves and J. regia bark powder with 100 mL of extraction solvents for four hours on the magnetic stirrer. The extracts were filtered through filter paper (Whatmann No. 1) and the filtrates were dried and concentrated using the oven at 30°C [7].

Preliminary phytochemical screening for L. inermis leaves and J. regia bark extract:

The preliminary phytochemicals tests were carried out for all the extract as per standard methods.

Detection of alkaloids:

a-Mayer's test:

Few drops of the freshly prepared Mayer's reagent were added to 5 ml of the sample, a white precipitate will appear if alkaloids were present [8].

b- Wagner's test:

Few drops of freshly prepared Wagner's reagent were added to 1 ml of the sample, a brown precipitate will appear if alkaloids were present [9].

Detection of glycosides

Ked's test:

Few drops of Ked's reagent were added to 5 ml of the sample. The presence of

glycosides was indicated by the formation of violet ring color [10].

Detection of flavonoids:

This test was accomplished by adding 4 ml of 95% ethanol to 1 ml of the plant extract sample and then placed in boiling water bath for 25-30 minute. Then few drops of potassium hydroxide 0.5 N were added to 5 ml of the sample. The presence of flavonoids was indicated by the formation of dark yellow color [10].

Detection of tannins:

Two grams of crude plant extract was added to 50 ml of distilled water and boiled then left of to cool then few drops of lead acetate (1%) were added to the 1 ml of the sample, the appearance of white gel precipitate indicating tannins were present [10].

Detection of saponins:

Five milliliters of crude plant extract was added to 3 ml of mercuric chloride (1%) solution, the formation of white precipitate indicating the presence of saponin [9].

Detection of phenols:

The phenol group in the molecule of phenol compounds can be investigated by mixing equal volumes of 1% ferric chloride solution and crude plant extract. The appearances of blue-green color indicated the presence of phenols [11].

Detection of terpenes and Steroid:

Four milliliters of acetic acid anhydride and 1 ml of concentrated sulfuric acid (H_2SO_4) then mixed with 1 ml of crude plant extract. The appearance of pink color indicated the presence of terpenes while the formation of blue color after leaving the sample for 1 minute indicated the presence of steroids [10].

Detection of resins:

Ten milliliters of crude plant extract were added to 50 ml ethanol (95%), and after that, the mixture was left in a water bath for two minutes then filtered and added to 100 ml of acidic distilled water with hydrochloric acid, the formation of turbidity indicates to positive test [10].

Detection of coumarines:

One gram of dried plant extract was dissolved in some drops of alcohol in a test-tube then covered with filter paper, sprayed with NaOH and then placed in a water bath until boiling, then the filter paper was placed under UV light spectrum. The appearance of greenish-yellow color indicated the presence of coumarines [11].

Detection of Anthraquinones:

a-Borntrager's test

About 0.2 g of the extract was boiled with 10 % HCl for few minutes in a water bath. It was filtered and allowed to cool. An equal volume of chloroform was added to the filtrate. Few drops of 10 % NH_3 were added to the mixture and heated. Formation of pink color indicates the presence anthraquinones [10].

Antibacterial and antifungal assay:

The antimicrobial effects of henna and J. regia bark extracts on Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli and Candida albicans, were studied. These bacteria and yeast were isolated from Yarmouk Hospital patients- Baghdad- Iraq. Identification of bacterial types was carried depending on routine laboratory techniques. The nutrient broth was used for growing and diluting of the microorganisms suspension. The antimicrobial activities of the extracts were determined using the agar disc diffusion method described by Khalaphallah and Soliman [12]. The cells density of the inoculum was adjusted with a UV-Visible spectrophotometer in order to obtain a final concentration approximately 105 cells/ml. This suspension was inoculated on nutrient agar except the yeast was inoculated on potato-dextrose agar. After drying, Wattman paper discs containing a concentration of 2.5 mg/ml J. regia and L. inermis separately, were examined and

applied in the petri dish. The activity was determined by the measurement of the inhibitory zone diameter in mm after incubation at 37°C of bacteria, and 28°C of yeast, for 48 hours. The disc without plant extract was used as negative control.

The optimum concentration of L. inermis leaves and J. regia bark on disc:

Phosphate buffer (pH 7.0) and sodium acetate buffer (pH 6.0) were the best buffers for extraction of henna and J. regia bark respectively. The culture media were inoculated with (1 ml) isolate of microorganisms 105 cells/ml. After drying, Wattman paper discs containing 10, 5, 0.1 and 0.05 mg/ml of L. inermis leaves and J. regia bark extract separately were examined and applied in the petri dish. The activity was determined by the measurement of the inhibitory zone diameter in mm after incubation of bacteria and yeast at 37°C and 28°C respectively, for 48 hours. The disc without plant extract was used as negative control.

Optimum extraction time of L. inermis leaves and J. regia bark:

The best extraction time of L. inermis leaves and J. regia bark was prepared by using the phosphate buffer pH 7.0 for L. inermis leaves, and sodium acetate pH 6.0 for J. regia. A ten gram of dried powder of each plant was dissolved in 100 mL of suitable buffer, and mixing for different times; 2 and 8 hours on the magnetic stirrer. The extracts filtered through filter paper (Whatmann No. 1) and the filtrates were dried and concentrated using the oven at 50°C. The dried extracts were collected for further using. The antimicrobial activities of the extracts were determined using the agar disc diffusion method. The prepared culture media were inoculated with a strain of bacteria and yeast 105 cells/ml. After drying, Wattman paper discs containing different concentration (10, 5, 2.5, 0.1 and 0.05 mg/ml of L. inermis leaves and J. regia bark extract separately, which extracted at different times) were examined and applied in the petri dish. The activity was determined by the measurement of the inhibitory zone diameter in mm after incubation of bacteria and yeast at 37°C and 28°C respectively, for 48 hours. The disc without plant extract was used as negative control.

Results and discussions:

Preliminary phytochemical screening:

The crude plant extracts were subjected to a preliminary phytochemical test to determine the presence of functional groups present. The results of these experiments are shown in the table-1.

Table-1: Qualitative phytochemical analysis extracts of *L. inermis* leaves and *J. regia* bark.

Phytochemicals	*L. inermis* leaves extract	*J. regia* bark extract
Alkaloids: mayer's test, wagner's test	+	+
Glycosides	+	+
Flavonoids	-	+
Tannins	+	+
Saponins	-	+
Phenols	+	+
Terpens	+	-
Steroids	-	+
Resins	-	-
Coumarines	-	-
Anthraquinones	+	+

(+) = Positive result, (-) = Negative result

The results of the phytochemical screening of henna extract revealed the presence of alkaloids, glycosides, terpens, anthraquinones, tannins and phenols, while the flavonoids, saponins, steroids, resins and coumarines gave a negative result. Padamanabhan et.al [13], found that hydroalcoholic extract of L. dermis flower revealed the presence of alkaloids, flavonoids, glycosides, saponins, tannins, quinines, resins and sterols. Raja et.al. [14], found that phytochemical screening of the henna extracts by alcohol showed the presence of glycosides, phytosterol, steroids, saponins, tannins, and flavonoids. The result of the phytochemical screening of J. regia bark extract revealed the presence of alkaloids, glycosides, flavonoids, tannins, saponins, phenols, steroids and anthraquinones, while the terpens, resins, and coumarines gave a negative result. Shah et.al [15], found that preliminary phytochemical screening of the J. regia bark extracts revealed the presence of carbohydrates, cardiac glycosides, flavonoids, steroids, and tannins, while Djaalab et.al [16], found that phytochemical screening of the J. regia bark extract revealed the presence of tannins, flavonoids, sterols, terpenoids and saponins.

Antibacterial and antifungal activity:

Dried powder (10%, 5% and 2.5% w:v) of L. inermis leaves and J. regia bark were used to prepare the extracts as it has been reported that dried preparation have more concentrated active phytochemical compounds than fresh plant material [17]. Six different types of extracts were prepared including methanol extract, ethanol extract, distill water extract, phosphate buffer extract (pH 7), sodium acetate buffer extract (pH 6) and Tris-HCl buffer extract (pH 8). The results revealed that extraction of L. inermis leaves by phosphate buffer extract (pH 7), and J. regia bark by sodium acetate (pH6), were best buffers for extraction. The concentration 10 % of each extract, exhibited best antimicrobial activity against all bacterial strains and yeast that used in the present study (Figure 1). However bacterial isolates showed differential sensitivity for some extracts (Table 2 and 3). Antimicrobial activity was not observed with some extracts and controls.

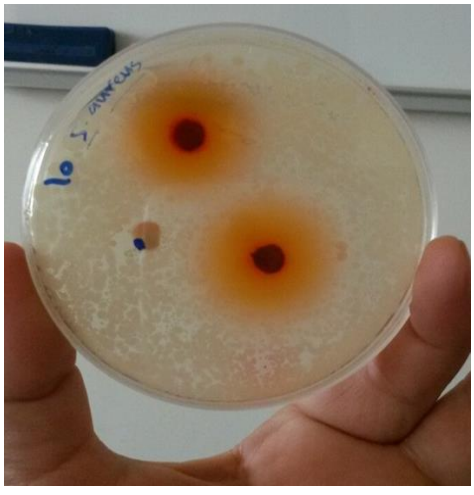

Fig-1: Effect of 10 % L. inermis leaves extracted by phosphate buffer (1 M, pH 7), with 2.5 mg/ml disc concentration against S.aureus.

Table (2): Effect of inhibitor zone of the *L. inermis* leaves extracts against bacterial and yeast growth, with 2.5 mg/ml disc concentrations.

Extraction solution	Inhibitor zone mm				
	P.aeruginosa	***S.aureus***	***S.typhi***	***C.albicans***	***E.coli***
D.w					
10% (w:v)	0	0	0	0	12mm
5% (w:v)	0	0	0	0	9mm
2.5% (w:v)	0	0	0	0	0
Ethanol					
10% (w:v)	0	0	0	11mm	0
5% (w:v)	0	0	0	12mm	0
2.5% (w:v)	0	0	0	0	0
Sodium acetate(pH 6)					
10% (w:v)	0	11mm	0	0	10mm
5% (w:v)	0	9mm	0	0	10mm
2.5% (w:v)	0	0	0	0	9mm
KH2PO4 (pH 7)					
10% (w:v)	10mm	12mm	14mm	11mm	10mm

(+) = Positive result. (-) =

Table-3: Effect of inhibitor zone of the *J. regia* bark extracts against bacterial and yeast growth, with 2.5 mg/ml disc concentrations.Negative result

Extraction solution	**Inhibitor zone mm**				
	P.aeruginosa	***S.aureus***	***S.typhi***	***C.albicans***	***E.coli***
D.w					
10% (w:v)	0	0	0	12 mm	17mm
5% (w:v)	0	0	0	7 mm	11mm
2.5% (w:v)	0	0	0	0	8 mm
Ethanol					
10% (w:v)	0	13 mm	0	0	13 mm
5% (w:v)	0	9 mm	0	0	11 mm
2.5% (w:v)	0	0	0	0	0
Sodium acetate(pH 6)					
10% (w:v)	10 mm	14mm	16 mm	14 mm	22mm
5% (w:v)	9 mm	12mm	14 mm	11 mm	19mm
2.5% (w:v)	-	9 mm	11 mm	9 mm	9mm
KH2PO4 (pH 7)					
10% (w:v)	10mm	11mm	11mm	11mm	17mm
5% (w:v)	10mm	10mm	10mm	10mm	13 mm
2.5% (w:v)	0	0	0	9 mm	9 mm
Tris-HCl (pH 8)					
10% (w:v)	0	0	0	0	14mm
5% (w:v)	0	0	0	0	12mm
2.5% (w:v)	0	0	0	0	9 mm

(+) = Positive result. (-) = Negative result

According to the study of Papageorgiou et al.[18], phytochemical constituents of L. inermis exhibit antimicrobial activity only against gram-positive bacteria while ineffective for gram-negative bacteria. In our study, it was interested to note that L. inermis had antimicrobial activity against both grams positive (S. aureus, B. subtilis, and S. epidermidis, etc.) and gram negative (E. coli, S. typhi, Klebsiella spp. and Shigella, etc.) bacteria. The studies of Hussain et al. [19], support our findings. While Al-kurashy et al. [20], found that chloroform extract of L. inermis was a more promising antimicrobial agent for S. aureus, Staphylococcus epidermidis, S. typhi, Klebsiella spp. and Shigella while its acetone extract for E. coli and Bacillus subtilis at least in the in-vitro antimicrobial assay. The secondary metabolites mainly

attribute the antimicrobial activity of plants [21]. The active constituents of these secondary metabolites include phenolic compounds and tannins [22]. Shah et.al [15], found that the acetone extract was the best extract for extraction of J. regia, and exhibit antimicrobial activity only against E.coli, Klebsiella pneumoneae and S. aureus. Djaalab et.al [16], found that the J. regia bark extract containing tannins, flavonoids, sterols, terpenoids, and saponins, which known for their antimicrobial activities could be responsible for the observed antibacterial and antifungal activities.

Optimum concentration of L. inermis leaves and J. regia bark on disc:

The results in a table (4) showed that the best concentration of henna extract on disc forgave high antimicrobial activity against the tested microorganisms, was 10 mg/ml, extracted by phosphate buffer pH 7.0, at ratio 10:100 (w:v) for four hours. While 5 % mg/ml disc concentration give smaller inhibition zone for bacteria and yeast. A 0.05 and 0.1 mg/ml disc concentration were given negative results of inhibition zone. The results in a table (5) showed that the best concentration of J. regia bark on disc forgave high antimicrobial activity, was 10 mg/ml, extracted by sodium acetate pH 6.0, at ratio 10:100 (w:v) for four hours.

Table-4: Effect of inhibitor zone of 10 % *L. inermis* leaves extracted by phosphate buffer (pH 7.0), with different disc concentrations against bacterial and yeast growth.

Disc con.	Inhibitor zone mm				
	E.coli	***S.typhi***	***S.aureus***	***P.aeruginosa***	***C. albicans***
o.o5 mg\ml	0	0	0	0	0
0.1 mg\ml	0	0	0	0	0
5 mg\ml	14 mm	16 mm	18 mm	17 mm	16 mm
10 mg\ml	17 mm	20 mm	26 mm	21 mm	20 mm

Table (5): Effect of inhibitor zone of 10 % *J. regia* bark extracted by sodium acetate pH 6.0, with different disc concentrations against bacterial and yeast growth.

Disc con.	Inhibitor zone mm				
	E.coli	***S.typhi***	***S.aureus***	***P.aeruginosa***	***C. albicans***
o.o5 mg\ml	6 mm	6 mm	0	0	6 mm
0.1 mg\ml	8 mm	7 mm	5 mm	6 mm	8 mm
5 mg\ml	24 mm	20 mm	17 mm	14 mm	18 mm
10 mg\ml	29 mm	24 mm	23 mm	20 mm	22 mm

Al-Mehna et al. [23], found that alcoholic extract of 5 % L. inermis has high activity in inhibition of Streptococcus pyogens growth at concentration 100 mg\ml. Antimicrobial activity may be due to numerous free hydroxy groups that have the capability to combine with the carbohydrates and proteins in the bacterial cell wall [24]. They may get attached to enzyme sites rendering them inactive. Also, the Tannins which present in the leaves are astringent that either bind and precipitate or shrink proteins and various other organic compounds including amino acids [25]. Topical henna is an extract of the lawsonia plant. Some experimental and clinical studies have reported antibacterial and antifungal effectiveness and wound

healing activity of this product. Henna is an easily accessible, inexpensive antibacterial product. Naphthoquinones are widely distributed in plants, fungi, and some animals. Their biological activities have long been reported to include antibacterial effects on several species of both aerobic and anaerobic organisms. Furthermore, several other biological activities for naphtha quinones have been described such as being anti-inflammatory and bactericidal [26]. Shah et.al. [15], found that alcoholic extract of 40 % J. regia bark has high activity in growth inhibition of E.coli, Klebsiella pneumoneae and S.aureus with 100 µg/ml concentration on the disc. The antibacterial properties of the plant material may be due to the presence of phenolic compounds, terpenoids, alkaloids, flavonoids, and steroids. It is reported that bark from J. regia contains ketones like juglone, regiolone, sterol and flavonoid [27].

Optimum condition for extraction of L. inermis and J. regia:

Four hours was the best time for extraction of L. inermis leaves and J. regia bark used phosphate buffer (1 M, pH 7.0) and sodium acetate (1 M, pH 6.0) respectively (see table 6 and 7). Tan et al. [28], found that best time for L. inermis extraction was 4.5 hours, he showed that TPC (total phenolic content) of henna stem extracts increased gradually with increasing of the extraction time from 30 min up to 4.5 hours, while Noureddini and Joshogan [29], discovered that best time for J. regia extraction was extracted using Soxhlet equipment for one hour. While Jamshid et. al.[30], found that best time for extraction of J. regia with 90% ethanol at room temperature, was 24 hours.

Table-6: The effect of inhibitor zone of 10 % *L. inermis* leaves extracted by phosphate buffer (1 M, pH 7) for 2, 4 and 8 hours, with different disc concentration against bacterial and yeast growth.

Henna extract	Extraction time	Inhibitor zone mm				
		C.albicans	***E.coli***	***S.aureus***	***S.typhi***	***P.aeruginosa***
10 mg\ml	2 h	13 mm	13 mm	17 mm	15 mm	13 mm
5 mg\ml	2 h	9 mm	11 mm	12 mm	11 mm	10 mm
2.5 mg\ml	2 h	8 mm	8 mm	9 mm	9 mm	7 mm
0.1 mg\ml	2 h	0	0	0	0	0
0.05 mg\ml	2 h	0	0	0	0	0
10 mg\ml	4h	20 mm	17 mm	26 mm	20 mm	21 mm
5 mg\ml	4h	16	14 mm	18 mm	16 mm	17 mm
2.5 mg\ml	4h	11	9 mm	12 mm	14 mm	10 mm
0.1 mg\ml	4h	0	0	0	0	0
0.05 mg\ml	4h	0	0	0	0	0
10 mg\ml	8 h	13 mm	15 mm	18 mm	17 mm	17 mm
5 mg\ml	8 h	11 mm	12 mm	14 mm	14 mm	14 mm
2.5 mg\ml	8 h	10 mm	11 mm	13 mm	10 mm	11 mm
0.1 mg\ml	8 h	0	9 mm	0	8 mm	0
0.05 mg\ml	8 h	0	7 mm	0	0	0

Table-7: The effect of 10 % *J. regia* bark extracted by sodium acetate (1 M, pH 6) for 2, 4 and 8 hours, with different disc concentration against bacterial and yeast growth.

J. regia extract	Extraction time	Inhibitor zone mm				
		C.albicans	***E.coli***	***S.aureus***	***S.typhi***	***P.aeruginosa***
10 mg\ml	2 h	20 mm	22 mm	16 mm	17 mm	15 mm
5 mg\ml	2 h	15 mm	19 mm	12 mm	14 mm	11 mm
2.5 mg\ml	2 h	9 mm	16 mm	9 mm	10 mm	7 mm
0.1 mg\ml	2 h	6 mm	8 mm	5 mm	5 mm	6 mm
0.05 mg\ml	2 h	0	0	0	0	0
10 mg\ml	4h	22 mm	29 mm	23 mm	24 mm	20 mm
5 mg\ml	4h	18 mm	24 mm	17 mm	20 mm	14 mm
2.5 mg\ml	4h	14 mm	22 mm	14 mm	16 mm	10 mm
0.1 mg\ml	4h	8 mm	8 mm	5 mm	7 mm	6 mm
0.05 mg\ml	4h	6 mm	6 mm	0	6 mm	0
10 mg\ml	8 h	16 mm	27 mm	17 mm	16 mm	17 mm
5 mg\ml	8 h	15 mm	19 mm	13 mm	13 mm	14 mm
2.5 mg\ml	8 h	10 mm	11 mm	12 mm	10 mm	11 mm
0.1 mg\ml	8 h	7 mm	9 mm	0	6 mm	6 mm
0.05 mg\ml	8 h	6 mm	7 mm	0	0	0

Extraction time was another important parameter influencing the extraction of compounds. These phenomena could be well explained by the Fick's second law of diffusion, predicting that a final equilibrium between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) might be reached after a certain time, leading to a deceleration in the extraction yield [31]. Moreover, prolonged extraction time increases the chance of decomposition and oxidation of phenolics due to their long exposure to unfavorable environmental factors like temperature, light, and oxygen [32]. On the other hand, the increased extraction time is uneconomical and time-consuming from the industrialization point of view, also potentially increasing the loss of solvent by vaporization which directly affects the loss of solvent-to-solid ratio of extraction. The range of time was determined based on the practical and economical aspects. It was probably because longer time will increase cost. However, even at the longer time, there was not much difference in the extraction of phenolic compounds when compared to shorter time. Excessive extraction time is not useful to extract more phenolic antioxidants [31]. Polymers and wall-bound phenolics retained in cells that were extracted out as well as the polymerization reaction that occurs and new components produced probably a reason for the increase of total phenolic contents at a longer extraction time as reported by Spigno & De Faveri [33]. According to Mane et al. [34], short extraction time was aimed to decrease tannin degradation and long ones to maximize extraction, but the concentration of tannins in the extracts tend to fall rather than rise after few hours.

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