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Investigating the Antibacterial Potential of Ethanolic and Methanolic Extracts of the *Schinus molle* L Tree

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Abstract

Schinus molle L., an ornamental plant of the Jordanian environment, was acknowledged recently for its therapeutic properties against many microbes. This study aimed at evaluating the antibacterial potential of the ethanolic and methanolic extracts obtained from different parts of the *Schinus moll*e L. tree against four strains of bacteria. Data in this study showed that, the ethanolic and methanolic extracts from all experimented plant parts inhibited the growth of *Bacillus subtilis* successfully. However, the best results were obtained from the ethanolic extract of leaves as it resulted in a growth inhibition zone of (22.0 ±0.06 mm), while growth was completely inhibited at MIC value of (1.563 mg/mL). Moreover, growth of *Enterobacter aerogenes* and *Klebsiella pneumoniae* were mostly inhibited after exposure to the methanolic extract of the leaves as it resulted in inhibition zones of (18.0 ±0.086 and 17.0±0.12 mm) respectively, while full growth inhibition was obtained at MIC being (1.563 mg/mL). Moreover, results of disc diffusion assay indicated that *Micrococcus luteus* growth was slightly affected by the leaves' ethanolic extract, while all other types of either ethanolic or methanolic extracts failed to inhibit growth of these bacteria. However, the results of microdilution assay revealed a full growth inhibition of *Micrococcus luteus* when exposed to either fruit or leaves' ethanolic extracts at level of (6.25 mg/mL). However, methanolic extracts from all invistigated plant parts faild to prevent the growth of *Micrococcus luteus*.

Keywords: Antibacterial activity, *Bacillus subtilis, Enterobacter aerogenes*, Extract type, *Klebsiella pneumoniae, Micrococcus luteus*.

1. Introduction

Synthetic drugs are routinely used as therapeutic agents against microbial diseases. But, recently it has been proven that such compounds might have dangerous or even lethal side effects on humans. Many records have revealed serious numbers of victims of synthetic drugs (Mehani and. Segni, 2013). For example, it was reported that in the United States, 100,000 people are direct victims of synthetic drugs; their deaths were related to the drugs' toxicities (Karimi *et al*., 2015). Also, the high cost of many of these drugs has increased the burden on people especially those who can't even afford the cost of living (Iserin *et al*., 2001; Mehani and. Segni, 2013). In addition to all this, the appearance of new microbial stains with resistant properties against synthetic drugs poses another major threat that highly concerns most scientists in the medical field.

Searching for other new and effective weapons against microbes has been on top of man's priorities for many years. The growing fears regarding the side effects of synthetic drugs on human health have triggered phyto-mining researches to look for other antimicrobial options with minimal hazards on health. Many medicinal plants were acknowledged recently for their remarkable therapeutic properties against many microbes (Jaganthan *et al.,* 2015). Such healing properties of some the plants can be attributed to the production of many active ingredients known as natural products (NP) made by the plant cell machinery (Ochoa-Villarreal *et al.,* 2016). Using medicinal plants for medical purposes was rarely reported to have serious side effects (Hajian, 2013), but in some cases, some remedies of medicinal plants were found to be highly toxic possibly because of the misidentification of these plants, or the incorrect preparation and administration by unprofessional people (Karimi *et al.,* 2015).

Schinus molle L. (also known as American pepper, Peruvian peppertree, escobilla and false pepper) is an evergreen tree which originated in South America, and widely spread to most tropical and subtropical areas in addition to the Mediterranean region (Marongiu *et al.*, 2004; Belhamel, *et al.*, 2008). *Schinus molle* L. belongs to the *Anacardiaceae* family and has thin, long leaves, and is mostly distinguished by its bright pink fruits *(Blood, 2001). Schinus molle* L. is commonly grown as an ornamental tree in addition to its usage in the spice production and the beverage industry (Hayouni *et al.*, 2008).

Due to the presence of a high oil content in most of the plant parts that is being rich in monoterpene hydrocarbons and some sesquiterpenes (Bernhard *et al.*, 1983) *Schinus moll*e L. has been used in folk medicine (Erazo *et al.*, 2006; Deveci *et al.*, 2010) for the treatment of diuretic, digestive toothache, rheumatism and menstrual pains

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(Daniele *et al.*, 2007). Moreover, it was reported that the extracts of *S. molle* had showed promising activities against several strains of bacteria and fungi (Belhamel, *et al.*, 2008; Rocha *et al*., 2012; Mehani and Segni, 2013). Additionally, (Ibrahim and Al-Naser, 2014) stated that that the oil extracted from the fruit berries of *Schinus moll*e L. showed a promising antifungal potential, as it inhibited the growth of *Botrytis cinerea* fungus. Most of the studies searched the antimicrobial potential of *Schinus moll* L. using essential oils extracted from several plant parts such as the leaves and fruits (berries) after hydrodistillation in a Clevenger-type apparatus (Hayouni *et al*., 2008; Belhamel, *et al*., 2008; Rocha *et al.*, 2012; Mehani and Segni, 2013). However, Deveci *et al.* (2010) used hexanic extracts in his experiment against several bacterial strains and oriental cockroach, where the hexanic extracts from the leaves were found to be very promising against *Escherichia coli* and oriental cockroach. In another study, Ibrahim and Al-Naser (2014) used the petroleum ether and n- hexane extracts of *Schinus molle* L. fruits against *Botrytis cinerea* fungus and found that the inhibition of fungi growth varied with the type and dose of the extract. The current study is aimed at evaluating the antibacterial potential of the extracts obtained from different parts of the *Schinus moll*e L. tree (leaves, fruits, Bark and flowers) growing in Jordan against several strains of bacteria using other extraction techniques (namely ethanolic and methanolic extracts).

2. Materials and Methods

2.1. Plant Material and Extract Preparation:

Leaves, ripe fruits, flowers and bark samples were collected directly from a single ten- year old *Schinus molle* L. tree growing on the campus of Jordan University in Amman, Jordan (Latitude: 32° 00' 30.00" N, Longitude: 35° 52' 13.19" E). The leaves and bark samples were collected in May, 2017, whereas the flower samples were collected in July, 2017, and the ripe fruits were collected in November, 2017. The samples were then oven dried at 45°C before being grounded into fine powder. The extraction from each sample was performed by adding either ethanol or methanol to the powder obtained from each plant part with the ratio of 1:10. Next, the extracts were filtered and evaporated to dryness using a rotary evaporator before being dissolved in DMSO to obtain a final concentration of 100 mg/mL.

2.2. Antibacterial Activity

Antibacterial activity of each extract type was evaluated against four bacterial strains (*Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 31488 and *Micrococcus luteus* ATCC 10240) using Disc diffusion and microdilution assays.

2.2.1. Disc Diffusion Assay:

6mm diameter discs on Muller Hinton agar (Mast Group Ltd. U.K) plates streaked with 100 µL of each bacterial strain suspension were adjusted to 0.5 McFarland (Karlsmose, 2010). Then, 10 mg of each extract type were loaded into the discs. A standard antibiotic (Tetracycline 10 mg/mL) was used as reference. The antibacterial activity was determined by measuring the zone of inhibition after incubation at 37C° for twenty-four hours.

2.2.2. Microdilution Assay

The minimum inhibitory concentration (MIC) was determined using the microdilution method; the extracts were serially diluted according to NCCLS (2000). Ten dilutions were prepared from each extract by being serially diluted (2-fold) with Muller Hinton, potato dextrose broth. Well number eleven consisted of nutrient broth plus Tetracycline (10 mg/mL) and was used as a control. The resulted ten concentrations of the methanolic and ethanolic extracts were from (50) to (0.098) mg/mL. Bacteria inoculate were adjusted to contain approximately 10^5 CFU/mL. The results were visually recorded after incubation in a plate shaker incubator at 37 °C for twenty-four hours.

2.3. Experimental Design

For the disc diffusion assay, the treatments were arranged in a completely randomized design (CRD), where each treatment was replicated five times. The data recorded for the measurement of inhibition zones were analyzed according to the analysis of variance (ANOVA) using (SPSS, version 17) analysis system, while means separation was performed and standard errors (SE) were extracted according to the Tukey's HSD test (Honest Significant Difference) at a probability level of 0.05.

3. Results and Discussion

3.1. Disc Diffusion Assay

The data revealed that the ethanolic extract of all types of the plant material has an antibacterial potential against *Bacillus subtilis* (Table 1, Figure 1). Meanwhile, the antibacterial efficacy of the ethanolic extracts varied significantly according to the type of plant part. The strongest antibacterial activity was obtained from the leaves' extract as it resulted in a maximum inhibition zone of (22.0 mm) which was very close to the results obtained by Tetracycline (24.0 mm) (Table 1). Variation in the antibacterial potential according to the parts of the *Schinus molle* L. tree was also reported by Deveci *et al.* (2010) who showed that the leaves' hexanic extract was more effective against some selected types of bacteria than the fruit hexanic extract. However, according to Deveci *et al.* (2010), the strong antimicrobial activity found in the leaves' extract can be referred to the action of the major chemical components of the leaves extract namely Germacrene D at 20.77 % and Beta-ceryophyllene at 13.48 %. However, these two ingredients were found in very much lower levels in the *Schinus molle* L. fruit extract (Germacrene D: 0.32 % and only traces of Beta-ceryophyllene) (Hayouni *et al*., 2008). This can explain the reason for the superior antibacterial potential of the leaves methanolic and ethanolic extracts as investigated in the current study.

Moreover, in the methanolic extract experiments, all types of the extracts had inhibited the growth of *Bacillus subtilis* (Table 1), while maximum inhibition zones (18.0 and 16.0 mm) were recorded from the fruit and the leaves' extracts compared to the extracts of other plant parts. Tetracycline was significantly the best as it resulted in an inhibition zone of (22.0 mm) (Table 1). The current results regarding *Bacillus subtilis* were consistent with the results reported by Padin *et al.* (2007) in their study of the efficacy of the ethanolic fruit extract of *Schinus molle* L. of Argentina, recording an inhibition zone of (8.0 mm).

Moreover, the present results showed that only the extracts obtained from either the fruits or leaves have showed antibacterial activity against *Enterobacter aerogenes* in the ethanolic extract experiment resulting in the inhibition zone diameters of (12.0 and 14.0 mm) which were significantly similar to the Tetracycline results (Table 1). On the other hand, the methanolic extract showed antibacterial activity in more parts of *Schinus molle* L. For example, the *Enterobacter aerogenes* growth was hindered when exposed to the extracts of the fruits, bark, or leaves (Table 1), with the maximum inhibition effect obtained from the leaves' methanolic extract (18.0 mm) (Table 1).

Moreover, only the leaves ethanolic extracts had showed antibacterial potential against *Klebsiella pneumoniae* and resulted in an inhibition zone with a diameter of (13.0 mm), while the other plant extracts failed to influence *Klebsiella pneumoniae* (Table 1). On the other hand, fruits and leaves were found to limit the growth of this bacterium by the methalolic extract (Table 1) resulting in a maximum inhibition zone with a diameter of (17.0 mm) (Table 1), even though, this is still significantly lower than the value recorded by Tetracycline (26 mm) (Table 1). However, the current results show that the *Schinus molle* L. leaves ethanolic and methanolic extracts were effective on the *Klebsiella pneumoniae* growth better that what was reported in the findings of Hayouni *et al.* (2008) and Mehani and Segni (2013) who experimented the effectiveness of leaves essential oil of *Schinus moll* L. against *Klebsiella pneumoniae,* and recorded an inhibition zone of (11.0 and 15.0 mm) respectively.

Moreover, the data of Disc diffusion assay showed that *Micrococcus luteus* was slightly affected by the *Schinus molle* L. leaves ethanolic extract, while the ethanolic extracts of the other plant parts did not have any activity against *Micrococcus luteus* (Table 1). On the other hand, all *Schinus molle* L. methanolic extracts failed to ihnibit the growth of this bacterium in the Disc diffusion assay experiment (Table 1).

3.2. Microdilution Assay

Results of the microdilution assay showed that a complete inhibition of *Bacillus subtilis* growth was obtained from the ethanolic extracts of fruits and leaves at MIC value of 1.563 mg/mL, which is similar to the Tetracycline results (Table 2). On the other hand, the fruit methanolic extract was found to be the most effective against *Bacillus subtilis* recording MIC value of 1.563 mg/mL compared to the methanolic extracts of other plant parts (Table 2). Padin *et al*. (2007) studied the effect of the ethanolic extract of *Schinus molle* L. fruits against the growth of *Bacillus subtilis* recording MIC value at 15 mg/mL. However, the current results regarding the fruit ethanolic extract proved to be more effective than what reported by the Padin's *et al*. findings (2007); the current results determined an inhibition zone of (18.0 mm) as well as a less MIC value of 1.563 mg/mL (Tables 1, 2).

Table 1. Diameters of inhibition zones (mm) obtained from disc diffusion assay for ethanolic and methanolic *Schinus molle* extracts against selected strains of bacteria.

Bacillus subtilis				
Ethanolic extract				
Fruit*	Flower	Bark	Leaves	Tetracycline
18.0±0.24 b	7.0±0.07 c	5.0±0.02 c	22.0 ±0.06 ab	24.0 ±0.13 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
18.0±0.05 b	5.0 ±0.06 c	2.0 ±0.03 d	16.0 ±0.07 b	22.0±0.07 a
Enterobacter aerogenes				
Ethanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
12.0 ±0.06 a	-	-	15.0 ±0.17 a	20.0 ±0.46 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
15.0 ±0.16 b	-	6.0 ±0.07 c	18.0 ±0.086 b	22.0 ±0.10 a
Klebsiella pneumoniae				
Ethanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	13.0 ±0.067 b	20.0 ±0.32 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
6.0 ±0.03 c	-	-	17.0 ±0.12 b	26.0 ±0.25 a
Micrococcus luteus				
Ethanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	2.0 ±0.05 b	20.0 ±0.12 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	-	12.0 ±0.08 a

*Each value represents mean ± standard error (SE) of five replicates. Means within each raw were analyzed separately, and values with different letters are significantly different according to Tukey HSD at $P<0.05$.

Also, the MIC values of *Enterobacter aerogenes* revealed that growth was totally inhibited when treated with fruit, bark or leaves' ethanolic extracts. The best results were obtained from the fruit and leaves' extract at MIC value of 3.125 mg/mL; however, Tetracycline was most efficient against *Enterobacter aerogenes* at MIC value of (1.563 mg/mL) (Table 2). On other hand, the methanolic extracts from all studied plant parts were found to possess antibacterial activities against *Enterobacter aerogenes*, while extract from leaves was the most promising as it resulted in full inhibition of *Enterobacter aerogenes* at MIC level of (1.563 mg/mL), which is similar to Tetracycline results (Table 2). The antibacterial potential of other *Schinus* species against *Enterobacter aerogenes* was also experimented by few researchers, for example Gehrke *et al.* (2013) reported a promising antibacterial potential of the methanolic extract of *Schinus lentiscifolius* aerial parts against this bacterium at MIC value of (100 µg/mL).

Table 2. Minimal inhibition concentration (MIC) values (mg/mL) of ethanolic and methanolic *Schinus molle* extracts against selected strains of bacteria.

Bacillus subtilis				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
1.563	3.125	6. 25	1.563	1.563
Methanolic extract (mg/mL)				
Fruit	flower	Bark	leaves	Tetracycline
1.563	6. 25	-	3.125	1.563
Enterobacter aerogenes				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
3.125	-	6. 25	3.125	1.563
Methanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
3.125	6.25	6.25	1.563	1.563
Klebsiella pneumoniae				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	3.125	1.563
Methanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	1.563	1.563
Micrococcus luteus				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
6. 25	-	-	6. 25	1.563
Methanolic extract (mg/mL)				
Fruit	flower	Bark	leaves	Tetracycline
-	-	-	-	3.125

Moreover, according to the current results, only leaves' ethanolic extract was able to inhibit growth of *Klebsiella pneumoniae* at MIC of (3.125 mg/mL) (Table 2), which contrasted with Padin *et al.* (2007) findings, as they reported that only fruit ethanolic extract against this bacterium was effective at MIC of (14 mg/mL). The results of this study indicated that leaves' methanolic extract was found to be very promising at MIC value of (1.563 mg/mL) which is similar to the MIC value of the Tetracycline treatments (Table 2). On the other hand, Hayouni *et al.* (2008) applied essential oil extracted from the leaves of *Schinus moll* L. against *Klebsiella pneumoniae* and found it to be effective only at MIC levels that exceeded 72 mg/mL. This indicates that, methanolic and ethanolic extracts of leaves were more effective against this bacterium than leaves' essential oil as proven in the present study.

Additionally, the current results showed that growth of *Micrococcus luteus* was totally inhibited when exposed to either fruit or leaves' extracts at level of (6.25 mg/mL), whereas growth was totally inhibited at the Tetracycline level of (1.563 mg/mL) (Table 2). Moreover, the methanolic extracts from all the invistigated plant parts faild to prevent the growth of *Micrococcus luteus* (Table 2). In another related study, Deveci *et al*. (2010) reported a succesfful inhibition of *Micrococcus luteus* growth after exposure to hexanic extracts of either leaves or fruits of *Schinus molle* L. at MIC values of (1 and 2 mg/mL) respectively.

It was noted from the current data that the antibacterial potential has also varied with the type of the extacting solvent (methaol or ethanol), (Tables 1, 2). Methanol was reported to be more commonly used as an extacting solvent than ethanol, because it is more polar (0.762) (ethanol relative polarity is 0.654), which allows methanol to dissolve more polar compounds, in addition to the fact that methanol is less expensive, relatively easily-evaporated, and is free of regulation compared to ethanol, (Eloff, 1998). Also, in another related study, it was found that the differences in chemical composition, and the availability of the extractable compund in each plant part can contribute to the diffirence in the antioxidant potential between the methaolic and ethanolic extracts (Sultana *et al.* 2007).

Conclusions

The obtained results revealed presence of antibacterial activity in *Schinus molle* L. tree against the tested bacterial strains. Moreover, data showed a variation in the antibacterial potential of this tree against each tested bacterial strain according to plant part and type of extract. The ethanolic extract of leaves was the best to cause growth inhibition of *Bacillus subtilis*, while the growth of *Enterobacter aerogenes* and *Klebsiella pneumoniae* were mostly inhibited after exposure to the methanolic extract of leaves. Moreover, the results of Disc diffusion assay indicated that *Micrococcus luteus* growth was slightly affected by the leaves ethanolic extract, while all other types of either ethanolic or methanolic extracts failed to inhibit the growth of this bacterium.

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