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ANTIMICROBIAL ACTIVITY OF CHINABERRY *Melia Azedarach* EXTRACT AGAINST *Pseudomonas syringae* PV. *syringae* IN VITRO

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ABSTRACT

Using hot aqueous, cold aqueous and ethanol, the antimicrobial activity of both fresh leaves and chinaberry fruits extract were evaluated at 50, 100, 150, and 200 mg ml^{-1} against *Pseudomonas syringae* pv. *syringae* (*Pss*) growth on nutrient agar medium. Fruit extract was inhibited bacterial growth evidently with 8.84 mm in diameter, the hot aqueous and ethanol extracts showed a high antimicrobial potential when the inhibition zones reached to 8.68mm and 8.85mm, respectively. The results exhibited the imperceptible augmentation of inhibition zones of *Pss* growth with increasing extract doses (50, 100, 150, and 200) mg ml^{-1} and attained (6.72, 7.46, 8.89, and 10.37) mm, respectively. The highest and considerable inhibition zone 9.32 mm appeared using ethanol extract of fruit, the latter was more obvious on the bacterial development and inhibited its growth to 11.2mm when used at 200 mg ml-1, followed by leaf extract at the same concentration. Extracts at 200 mg ml^{-1} revealed substantial inhibition zone ranged between 10.37mm and 10.95mm using different methods. Therefore, the combination of ethanol extract of fruit at 200 mg ml^{-1} gave the maximum inhibition 11.7mm in diameter, followed by 10.97mm and 10.93mm when used hot and cold aqueous extracts.

KEY *WORD*: P. syringae pv. syringae, Chainberry, M. Azedarach

INTRODUCTION

Phytopathogenic bacteria of *Pseudomonas syringae* pv. *syringae* (*Pss*) is one of worldwide dispersal characterized with fluorescent; gram-negative, aerobic, motile with one or several polar flagella, straight or slightly curved rod bacteria (Holt *et al.*, 1994; Kersters *et al.*, 1996; Palleroni, 2005).

Spraying bactericides or antibiotics considered the famous and universal approach for controlling bacterial canker caused by *Pss*. These mainly include copper compounds or additional heavy metals, which may be combined with fungicides.

Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens. It is expected that plant extracts showing target sites other than those used by antibiotics (Ahmad and Beg, 2001). Recently, application of antibiotics created substantially resistance to *Pss* strains thus; using inhibiters of such plants as chinaberry trees thrives to reducing the risk of this problem (Kim *et al.,* 1995 and Alagesaboopathi, 2011).

Derivatives of Chinaberry (*Melia azedarach* L.) plants of the family Meliaceae are known for their insecticidal and antimicrobial activity (Nicoletti *et al.,* 2012). Chemical composition reveals the presence of alkaloids, tannins, meliotannic acid, benzoic acid, vanillic acid, and others (Khalid *et al.*, 1981 and Baquar, 1989).

The extracts of *M. azedarach* foliage recognized substantial antibacterial activity against several strains of *P. syringae* pv. *syringae*, *Xanthomonas campestris* pv. *campestris*, *Rathayibacter tritici* and *Escherichia coli* (Neycee *et al.*,2012). Conversely, Ethanolic fruit extract showed bacteriostatic / bactericidal activities vs. different bacterial isolates (Marino *et al.,* 2011).

In particular, a leaf aqueous extract of *M. azedarach* showed variable antimicrobial activities against phytopathogenic bacteria and fungi (Gaggia *et al.,* 2008 and Zhou *et al.,* 2004). If added to culture media, it was effective in eliminating some *Bacillus* spp (Marino *et al.,* 2009).

After scrutiny of published literature, this work aimed to estimate the antimicrobial potential of *M.*

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azedarach L. for both leaves and fruit extracts against *Pseudomonas syringae* pv. s*yringae* in vitro.

MATERIALS AND METHODS

Sampling of Plant Materials-

The fresh leaves and mature fruit of *M. Azedarach* were collected from plantation of College of Agriculture, University of Duhok, Iraq during Oct. to Dec. 2015.

The materials were washed with tap water followed by 2% sodium hypochlorite solution and rinsed again with distilled water. After adequate drying at room temperature for 2 days and in oven 40˚c for overnight, they crushed into a crude powder using mechanical grinder and kept in a container in cool, dark and dry place to avoid any possible fungal attacks.

Preparation of *M. Azedarach* Extracts

Three methods of extraction were used:

1. Ethanol Extraction: twenty nine g of powdered material (leaves and fruit) were soaked in 100 ml of 95%ethanol. The content was preserved for 4 days, the whole mixture filtered with cheesecloth followed by filter paper. Finally, the ethanol was evaporated at room temperature to find the dried crude extract.

2. Cold water Extraction: The obtained powdered material 10g/100ml for each fruit and leaves respectively was soaked in cold distilled water and shaked with Magnetic stirrer for 20 min after that lifted for 4 days. The soaked material was filtered using cheesecloth and filter paper. The filtered solution put in oven at 45ºC (Harborne, 1973).

3. Hot water Extraction: Cold distilled water was replaced with hot distilled water with the same steps of cold extraction method. All extracts were stored in vials at room temperature for further investigations.

The chemical analysis of *M. Azedarach* fruit and leaf extracts was presented in (Table 1).

Table(1): Percentage chemical analysis of *M. azedarach* fruit and leaf extract according to stage of maturity

Analysis	Fruit Extract%		Leaf Extract%	
	Green	Mature	Juvenile	Mature
Ashes	5.12	4.40	31.14	11.92
Protein	6.87	5.98	6.60	6.10
lipids	5.23	5.17	2.80	1.08
Raw fiber	35.70	38.68	10.94	10.33
*NNA	47.08	45.77	66.52	70.57

NNA: Non nitrogenated extract

Culture and Maintenance of *P. syringae* pv. *syringae*

Stock culture of PCR identified *Pseudomonas syringae* pv. *syringae* (Hassan and Al-doski, 2014) was obtained from the Plant Protection Department, College of Agriculture and stored at 4˚C for further studies.

Antimicrobial Activity Assay

Antibacterial activity was investigated using modified disc-diffusion method with four concentrations for each extract (50, 100, 150, 200 mg/ml).

Disc-diffusion method, sterile disc of filter paper (6mm) impregnated with different concentrations of plant solvent extracts were placed on the cultured plates and lifted at room temperature for 1hrs. Distilled water was set up as control. The plates were incubated at 27±2˚C for 24-72 hrs (Ncube *et al.*, 2008).

The inhibition zone in diameter (mm) was measured in four crossing directions and the average values were recorded. The experiment was repeated thrice.

Data Analysis

Statistical Analysis of data carried out using (version 9.4; SAS Institute Inc., 2000-2002) and subjected to analysis of variance (ANOVA) at $P \leq 0.05$. Means of the treatments were compared by Duncan Multiple Range.

RESULTS AND DISCUSSION

The antibacterial potential of *M. azedarach* leaf and fruit extracts were evaluated according to their zones of inhibition against phytobacterium *pseudomonas syringae* pv. *syringae*.

Our preliminary investigation showed that hot aqueous and ethanol extracts were active in suppression pathogens development.

Fruit extract was inhibited bacterial growth considerably with 8.84mm in diameter compared to 8.06mm of the leaves extract (Fig.1).

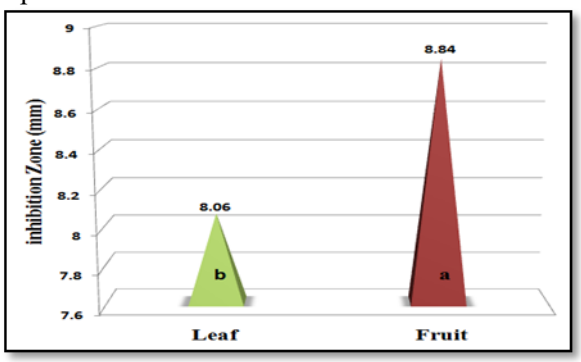

Fig. (1): Effect of leaf and fruit extracts on the *Pss* growth**.**

The hot aqueous and ethanol extracts were exhibited relatively a high antibacterial activity, since their inhibition zones attained to 8.68m and 8.85mm for both extraction methods, respectively versus 7.82mm when used cold aqueous (Fig.2). The action of most medicinal plants constituents is not yet fully known, it's not clear that the efficiency of the extracts largely depend on the solvent used. The organic extracts of alcohols provided more powerful antimicrobial activity as compared to aqueous one.

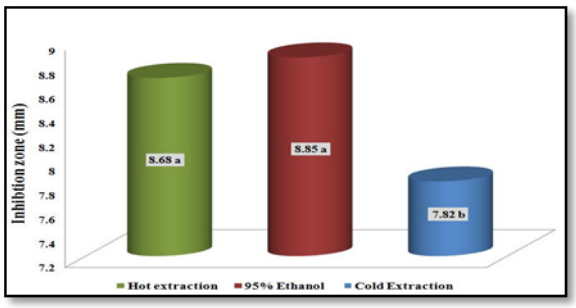

Fig. (2): Effect of extract methods on the inhibition zones of *Pss*

The difference in extracts activity may due to the variance between extract compounds and the solvents, since, the most of antibiotic compounds already identified in plants as aromatic or saturated organic molecules which can easily solubilized in organic solvents (Seyydnejad *et al.*, 2010). In this aspect, existences of non-polar residues such as hydrocarbons in the extracts support the bactericidal and bacteristatic abilities. Cowan (1999).

The differences of inhibition zone also depend primarily upon such factors as diffusion capacity of substances present in the extracts in the agar media, antimicrobial activity of diffused substances, growth and metabolic activity of microorganisms in the medium (Banderia *et al* .,2006 and Majeed, 2013). This suppression can further associated with polarities of substances which make up the tested extracts, and also with cell wall composition of tested organisms, since gram-positive bacteria present cell walls with lower lipid levels than gram negative bacteria including *Pseudomonas* spp. Several authors reported that the higher resistance of gram negative bacteria to plant extracts attributed to thick murein layer in their outer membrane, which prevents the entry of inhibitor substances into the cell (Brantner *et al.*, 1996, Palombo and semple, 2001; Matu and Van Staden, 2003).

The mureiu layer composed of peptidoglycan made up of sugars and amino acids and many molechules of peptidoglycan.

The results in (Fig.3) exhibit that graduation increment of extract concentrations (50,100,150 and 200) mg ml^{-1} led to increasing of inhibition zones of *Pss* growth progressively and amounted (6.72, 7.46, 8.89 and 10.73) mm, respectively. However, litterature have shown that extracts repressed the development of microorganism gradually with increasing of their doses (Akujobi *et al.*, and Nweze *et al.*, 2004).

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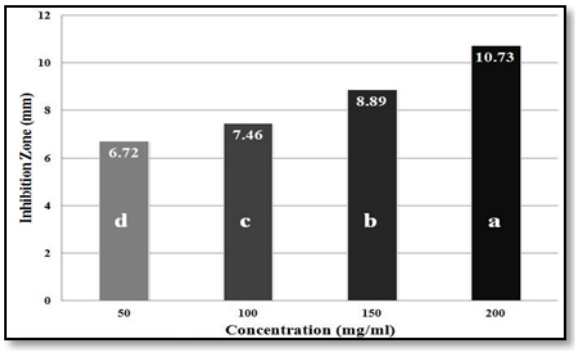

Fig. (3): Effect of extract concentration on the inhibition zones of *Pss*

Data analysis of extraction method and plant parts revealed that the highest and considerable inhibition zone 9.32mm was obtained using ethanol extract of fruits followed by 8.89mm using hot aqueous extracts, the lowest suppression resulted using cold aqueous of leaf extract (Fig.4).

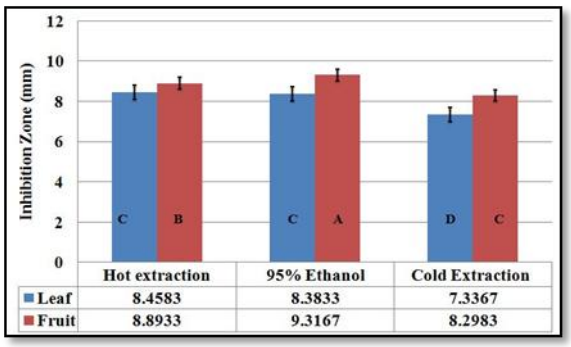

Fig. (4): Effect of extraction methods and plant part on the *Pss* growth**.**

The fruit extract at 200 mg ml^{-1} was more pronounced on the pathogen and inhibited its growth to 11.2 mm in diameter followed by leaf extract at the same dose. Thus, the lower concentrations at 50 mg ml^{-1} for both leaf and fruit extract resulted of the lowest inhibition 6.51mm and 6.93mm for both plant parts, respectively (Fig.5).

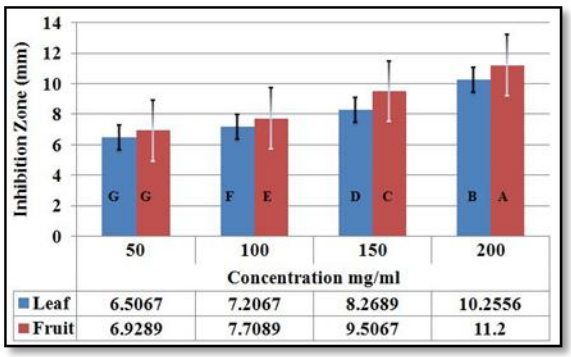

Fig. (5): Effect of plant part and their concentration on the *Pss* growth.

The high concentration of extracts 200 mg ml^{-1} reveled remarkable inhibition zones that ranged between 10.37 and 10.95mm using cold and hot extraction methods. Extract dose at 150mg ml^{-1} prevented *Pss* growth with 9.0-9.68 mm in diameter using hot extraction and 95% Ethanol methods. The lowest dose of extract at 50 mg ml^{-1} exhibited non-significant inhibition zones using different extraction methods (Fig.6).

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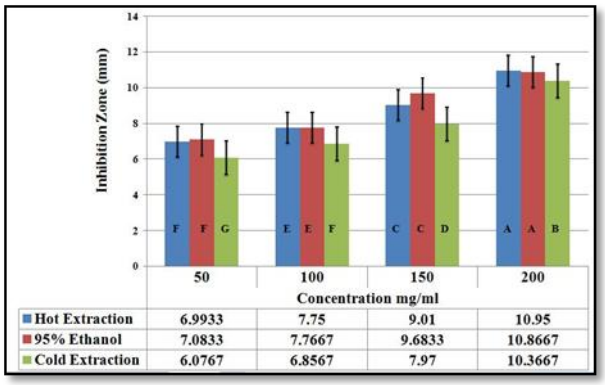

Fig. (6): Combined Effect of plant parts, extraction and their concentration on the *Pss* growth.

The results of combined plant parts, extraction methods, and extract concentration illustrate that ethanol extract of fruits at 200 mg ml^{-1} resulted in the maximum inhibition and attained to 11.7 mm in diameter followed by 10.97mm and 10.93mm when used both of hot and cold aqueous extracts at the same concentration, the similar inhibitory effect observed using hot aqueous for leaf extract. The lowest dose of leaf extract at 50 mg ml^{-1} gave a minimum inhibition that ranging between 5.85mm and 6.83mm for different methods (Table 2).

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Table (2):- Growth inhibition of *Pss* by combination of plant parts, concentration and methods of extraction for *M. azedarach in vitro*.

Methods of Extractions	Concentration mg/ml							
	Leaves				Fruits			
	50	100	150	200	50	100	150	200
Hot Extraction	6.83^{l}	7.5^{j}	8.57^{h}	10.93^{b}	7.15^{k}	8^{i}	9.45^{e}	10.97^{b}
95% Ethanol	6.83^{l}	7.7^{i}	8.97^{f}	10.03^{d}	7.33^{k}	7.83^{i}	10.4^{c}	11.7^{a}
Cold Extraction	5.85^{l}	6.42^{l}	7.27^{k}	9.8^{d}	6.3^{l}	7.29^{k}	8.67^{g}	10.93^{b}

The preliminary phytochemical screening of *M. azedarach* extracts ascertained the presence of bioactive compounds (Table 3), and their antibacterial effects supposed to be accrue to their chemical components of tannins, alkaloids, and flavonoids, steroids phenols, and saponins (Cowan, 1999; Esimone *et l.*, 1998 and Draughon, 2004).

Table (3): Qualitative analysis of phytochemical constituent of leaves and fruits for *M. azedarach*

Phytochemical Group	Leaves	Fruits
Alkaloids	+	+
Anthroquinone	+	-
Catachols	-	+
Coumarins	-	+
Flavonides	+	+
Glycosides	-	+
Phenolic compounds	+	+
Phytosteroids	+	+
resucing sugars	-	-
Saponins	+	+
Tannins	+	+
Triterpenoids	+	+

+: Presence ; -: Absence

According to the results of recent work we conclude that medicinal plants of *M. azedarach* origin of their chemical composition to replace synthetic bactericides , since phytochemical products are less toxic and more effective pesticides and drugs (Kelmanson *et l.*, 2000; Ahmed and Beg, 2001). Furthermore, *M. azedarach* extracts consist potent phytochemicals against phytobacterical pathogen *Pss,* the cause of bacterial canker and gummosis on stone fruits. Finally, the hot aqueous and ethanol extracts possessed significant and substantially inhibitory effects.

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چالاکییا نه میکروبی ژ بو ڤەگرتیا شیراڤا رووەکێ میلیایێ دژی بەکتیریایا Pseudomonas syringae pv. syringae د لابورێدا.

پوختە

هەلسەنگاندن ژبو چالاکییا میکروبیا هەڤدژ ژ بو شیراڤا بەلك و فێقیێ رووەکێ میلیایێ ب رێکێن ڤەگرتنا ئاڤێ یا سار و گەرم و ئیتانولی هاتبونە کرن، دناستێن)05 ، 055 ، 005 ، و 055) میلیگرام/ مل دژی Pss (Pseudomonas syringea pv. syringae)یا شینبوی لسەر میدیایێ Nutrient Agar. ڤەگرتیا فێقی شینبونا بەکتیریایێ خاڤ کربو ب تیرەیا 8.8. مم و د ڤەگرتین ئاڤی ین گەرم و ئیتانولی شیانێن مەزن ژ هەڤدژیا میکروبی دیاربو و دگەهشتە ل جهێن خاڤبونێ .8.. و 0.. . ملم ئێك لدویڤ ئێك. ئەنجاما زێدەبونەکا پلەبەندی د جهێن خاڤبونا گەشەکرنێ Pss دگەل زێدەکرنا قورچێن ڤەگرتیان (05 ، 055 ، 005 ، و 055) میلیگرام/مل دیارکرن.بلندترین جهێن خاڤبونێ بریکا ڤەگرتیا فێقی ژ ئیتانولێ) 8.0. ، .88. ، 8.8. ، 0580.) ملم ئێك لدویڤ ئێك ب دەستخوڤە ئینا کو د گەهشتە 8800 مم، ویا دوماهیکێ گەلەك یا روهن وئاشکرا بو لسەر وەرارا گەشەکرنا بەکتیری و خاڤبونێ تا 0080 ملم دەمێ د هێتە ب کارئینان د 055 میلیگرام/ مل و لدویڤدا دگەل ڤەگرتیێ بەلکان د هەمان تیراتیێ دا. هەمی ڤەگرتیان د 055 میلیگرام/ مل خاڤبونەکا پێشچاڤ دناڤبەرا .0580مم و 0588 مم بریکێن ڤەگرتنێن جوراجور تومارکرن. و ژبەر ڤێ چەندێ ڤەگرتیێ ئیتانولی ژ فێقی د 055 میلیگرام/ مل مەزنترین خاڤبون ب تیرەیا .008ملم ، لدویڤدا 0588. و05880ملم دەمێ ب کارئینانا ڤەگرتیێن ئاڤی ین گەرم وسار دانە مە.

النشاط الميكروبي المضاد لمستخلص نباتالسبحح *Melia* azedarach ضد بكتريا Pseudomonas syringae pv. syringae **في المختبر**

الخلاصة

تم تقييم النشاط الميكروبي المضاد لمستخلصات أوراق وثمار نبات السبحبح بطرق الإستخلاص المائي، البارد والحار والايثانول ، عند(05 ، 055 ، 005 ، و 055) **ملغم/مل**ضد(Pss) *Pseupmpnas* syringae pv. syringae **النامي على الوسط** Nutrient Agar. **وثبط مستخلص الثمار النمو البكتيري بقطر** 848 **مم ، وأظهرت المستخلصات المائية الساخنة والإيثانول إمكانات عالية من التضاد الميكروبي عندما وصلت مناطق التثبيط إلى** 84.8 **و** 8.80 **ملم على التوالي. أظهرت النتائج زيادة تدريجية في مناطق التثبيط لنمو** Pss **مع زيادة جرعات المستخلصات** (05 ، 055 ، 005 ، و 055) **ملغم/مل وحققت**) 4.0. ، ..4. ، 8488 ، .0540(**ملم على التوالي. ظهرت أعلى منطقة تثبيط بإستخدام مستخلص الايثانول للثمار حيث بلغت** 8400 **مم ، وكان الأخير أكثر وضوحا على تطورالنمو البكتيري وثبطه لحد**0040 **ملم عند استخدامه في** 055 **ملغم/مل ، متبوعا بمستخلص الأوراق عند نفس التركيز. أثبتت المستخلصات عند** 055 **ملغم/مل تثبيطاً معنوياً تراوحت بين** .0540 **مم و** 0548 **مم باستخدام طرق الأستخلاص المختلفة. ولذلك فإن مستخلص الإيثانول للثمار عند** 055 **ملغم/مل أعطى أقصى تثبيط بقطر** .004 **ملم ، يليه** .0548 **و**05480 **ملم عند استخدام المستخلصات المائية الساخنة والباردة.**