# ISOLATION AND IDENTIFICATION OF BACTERICIDE AND MITICIDE COMPOUND FROM *SCHINUS TEREBINTHIFOLIUS* LEAVES AGAINST THE POTATO ROT BACTERIA AND SPIDER MITE

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## ABSTRACT

The MeOH extracts of 26 plant species belonging to 18 plant families were examined against the two spotted spider mite *Tetranychus urticae* and the two pathogenic, brown rot bacterium *Ralstonia solanacearum* and soft rot bacterium *Erwinia carotovora*. The results indicated that *Schinus terebinthifolius* was the most potent plant for controlling these pests as its methanol extract exerted highly activity against the two pathogenic bacteria in addition to moderate activity against the spider mite. The MeOH extract of this plant was purified by using a combination of different chromatographic methods (column chromatography and TLC) to yield an active pure compound. Based on spectroscopic methods ($^{1}H$,$^{13}C$-NMR,UV and MS) the isolated compound was characterized as gallic acid methyl ester, which exerted a miticidal activity against the two spotted spider mite *Tetranychus urticae* ($LC_{50}$=58 $mg1^{-1}$) and antibacterial activity against *Erwinia carotovora* and *Ralstonia solanacearum* (MLC= 250 and 500µg/ml), respectively.

**Key words: Bactericide, Miticide, *Schinus terebinthifolius*, Potato Rot Bacteria, Spider mite and Methyl gallate**

## INTRODUCTION

Potato is one of the most important vegetable crops in Egypt. Its importance is not only due to local consumption but also for potato exportation to European community which represents about 42.7% of Egypt's agricultural exports **(Food and Veterinary Office 2000)**.

There are two specific problems that limit potato production and exportation. Firstly; leaf infestation by the two spotted spider mite, (*Tetranychus urticae*). Secondly; tubers infections in the field by the bacterium, *Ralstonia solanacearum* (brown rot disease) or during the storage by the bacterium *Erwinia carotovora* (soft rot disease) **Barakat *et al.* (1984), Elphinstone (2001) and Toth *et al.* (2003)**.

In the few recent years, Egypt has lost several million dollars as a result of turning back the Egyptian potatoes imported by the European community due to infections with brown rot disease **(Food and Veterinary Office 2000)**.

Synthetic pesticides such as Chalenger (160ml/ Feddan), Rovral (500ml/feddan) and Tecto 5% D(1.25Kg/Ton tuber) have been used for control the mite, brown rot and soft rot diseases, respectively. While these pesticides have done much to improve yields of high quantity of potatoes, the

long term use of synthetic pesticides has harmful effects on human beings, beneficial organisms and environment. The replacement of synthetic by natural pesticides for pest control applications has increased interest in the potential use of natural products in general.

Therefore the present study was undertaken to survey some local plants for both miticidal activity against the two spotted spider mite *Tetranychus urticae* and antibacterial activity against brown rot bacterium *Ralstonia solanacearum* and soft rot bacterium *Erwinia carotovora,* along with the isolation and identification of active constituent(s) from the most active plant(s).

## MATERIAL AND METHODS

### 1- Plant material

Leaf samples of 26 plant species belonging to 18 plant families (Table1) were collected from Fayoum Faculty of Agriculture garden and were identified by the Botany Department, Faculty of Science, Cairo University. A voucher specimen of each plant was deposited in the herbarium of the Department of Biochemistry, Faculty of Agriculture, Fayoum Branch, Cairo University.

A portion (100g) of the leaf samples of each plant species collected was air dried in the shade, ground into a fine powder and then were extracted with methanol. The methanol extracts of the leaf samples were evaporated to dryness and screened for both; miticidal activity against the two spotted spider mite *Tetranychus urticae,* and antibacterial activity against the brown rot bacterium; *Ralstonia solanacearum* and soft rot bacterium; *Erwinia carotovora*.

### 2- Biological evaluation:

### 2.1 Miticidal activity

The miticidal activity of the methanol extracts of plant species was tested according to the slide–dip technique adopted by **Voss (1961)** and modified by **Dittrich (1962)** against adult females of *Tetranychus urticae* isolated locally in Department of Plant Protection, Faculty of Agriculture, Fayoum Branch. For this purpose, a piece of double face adhesive scotch tape was pressed tightly to the surface of a microscopic glass slide. Five aqueous concentrations (50, 100, 200, 400 and 800 $mg1^{-1}$) of each plant extract were used to draw the dosage mortality regression line. Ten adult females were adhered upside down with legs free to the tape on the glass slide and immediately dipped in the aqueous extracts for about 5 seconds. Four replicate slides were used for each aqueous concentration. The mortality ratios were recorded after 24h. The $LC_{50}$ values were determined by computerized probit analysis program.

### 2.2 A**ntibacterial activity**

**2.2.1 Tester strains** : *Ralstonia solanacearum* and *Erwinia carotovora* were obtained from Department of Plant Pathology, Faculty of Agriculture Ain shams University.

**2.2.2. Preliminary test:**

The antibacterial activity of the methanol extract was determined in vitro by the filter paper disc agar diffusion method according to **Bauer *et al*., (1966)** as follows

The sterile whatmann No.1 filterpaper discs (6mm) were soaked with each methanolic plant extract (1g/10 ml MeOH) and dried at 40°C, the sterile discs were placed over the seeded LPN agar plates (LPN agar contained about to colony forming units /ml). The plates were then incubated overnight at 37C°. All the determinations were carried out in triplicate and average zones of inhibition have been recorded in Table (1).

**2.2.3. Determination of Minimum Lethal Concentration (MLC)**

The MLC of the methanolic extract of the most potent plant and the active constituent (s) were determined by bacterial broth dilution method described by **Ellen *et al*. 1994**

The results of preliminary screening (Table l) revealed that *Schinus terebinthifolius* was the most potent plant against both the spider mite and the two pathogenic bacteria, therefore, this plant was subjected to isolation and identification of the active constituent(s) responsible for these activities.

**Table 1: List of plant species used in screening of miticidal and bactericidal activities.**

| Plants scientific name | Family name | Antibacterial activity Inhibition zone of plant extracts (mm) | | Miticidal activity $LC_{50}$ $mg1^{-1}$ |
|---|---|---|---|---|
| | | ***Erwinia carotovora*** | ***Ralstonia solanacearum*** | ***Tetranychus urticae*** |
| *Acacia farnesianal* | Mimosaceae | 00 | 00 | 00 |
| *Lantana camara* | Verbenaceae | 12 | 12 | 225 |
| *Vitex sp.* | Verbenaceae | 00 | 00 | 00 |
| *Clerodendron inerme* | Verbenaceae | 00 | 00 | 00 |
| *Bignonia sp.* | Bignoniaceae | 00 | 00 | 00 |
| *Callistemon chinensis* | Myrtaceae | 12 | 11 | 00 |
| *Myrtus communis* | Myrtaceae | 25 | 26 | 190 |
| *Cassia sp.* | Fabaceae | 11 | 11 | 140 |
| *Sesbania aegyptiaca* | Fabaceae | 00 | 00 | 00 |
| *Parkinsonia sp.* | Fabaceae | 00 | 00 | 00 |
| *Acacia saligna* | Fabaceae | 00 | 00 | 250 |
| *Phyllanthus nivosus* | Euphorbiaceae | 15 | 14 | 00 |
| *Hibiscus sp.* | Malvaceae | 00 | 00 | 00 |
| *Neriam oleander* | Apocynaceae | 00 | 00 | 00 |
| *Thevetia nereifolia* | Apocynaceae | 00 | 00 | 00 |
| *Bouganvillea glabra* | Nyctaginaceae | 18 | 20 | 00 |
| *Schinus terebinthifolius* | Anacardiaceae | 30 | 28 | 230 |
| *Ficus nitida* | Moraceae | 00 | 00 | 00 |
| *Ficus benjamina* | Moraceae | 12 | 11 | 00 |
| *Zebrina pendula* | Commelinaceae | 00 | 00 | 00 |
| *Binus sp.* | Pinaceae | 00 | 00 | 00 |
| *Jasminum grandiflorum* | Oleaceae | 00 | 00 | 00 |
| *Syngonium podophyllum* | Araceae | 00 | 00 | 00 |
| *Melia azadirach* | Meliaceae | 11 | 12 | 00 |
| *Nephrolepis exaltata* | Oleandraceae | 00 | 00 | 00 |
| *Pittosporum tobira* | Pittosporaceae | 00 | 11 | 150 |

**3- Extraction and Isolation of the bioactive constituent (s)**

**3.1 Extraction**

Ground air dried leaves (335g) of S*chinus terebinthifolius* was successively extracted with a series of solvents of increasing polarity: n-Hexan (3L), Chloroform (5L), Ethylacetat (3L) and Methanol (5L) at room temperature (25°C).

The extracts were evaporated to dryness under reduced pressure to offer the following residues, Hexane (10g), $CHCl_3$ (20g) EtOAc (2g) and MeOH (55.6g), then the extracts were tested against both the spider mite and the two pathogenic bacteria.

**3.2.Analytical Thin Layer Chromatography (TLC)**

Analytical TLC was carried out on precoated silica gel plate ($F_{245}$ 0.25 mm and $F_{225}$ 2.00 mm Merck ) using the following solvent systems:

1) n-Butanol- Acetic acid- Water (4:1:5) upper layer .
2) Ethylacetate- Acetic acid- Formic acid- Water (100:11:11:27) .
3) Chloroform- Methanol- Water (80:20:2)
4) Chloroform- Methanol (75:25)

Spots on TLC were detected under UV light (254 and 365 nm) and by spraying with concentrated $H_2 SO_4$ followed by heating at 105C° for 5 min. or by $FeCl_3$ 5%.

**3.3 Isolation of the bioactive component(s)**

The bioactive methanol extract was subjected to the isolation of the bioactive component(s)as fallows:

Thirteen grams of the methanol extract were subjected to column chromatography (CC) over silica gel (230-400 mesh, 500g) and elution with a gradient of $CHCl_3$:MeOH:$H_2O$ ( 70 : 30:5; 2.5L, 60:40:5 ,30:70:0 and 0:100 :0 1.5 L for each eluent). According to differences in composition monitored by TLC, 13 fractions were obtained and then tested for miticidal and antibacterial activities. The bioactive fraction (No: 3 eluted with 70:30:5 between 400-600 ml 1.5g) was further separated by using CC on silica gel (50g) with mixtures of $CHCl_3$:MeOH as eluents (100:0, 95:5, 90:10 and 80:20; 200 ml of each eluent). The eluents were combined on the basis of similar TLC profiles to afford 9 fractions (A-I). The most abundant fraction (No. E= 330 mg eluted with 95:5 between 35:140 ml) which containing the major compound was further purified on Sephadex LH20 column (20g) with MeOH as an eluent followed by preparative TLC with Ethylacetate: Formic acid: Acetic acid: Water (100:11:11:27) to give 230 mg of pure compound. The purity of this active compound was established by its resolution as a single spot in four different TLC systems.

**3.4. Structure identification of the isolated compound**

The isolated compound was characterized by detection test and spectroscopic methods.

**3.4.1 Detection tests**

The preliminary screening of the isolated compound for the following classes of phytoconstituents saponins, flavonoids, alkaloids, glycosides and phenolic compounds was performed according to the methods described by **Farnsworth (1966).**

**3.4.2 Spectroscopic methods**

**3.4.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy**

$^{1}H$ and $^{13}C$-NMR Spectra were recorded in DMSO-$d_6$ on a varion Mercury VXR 300 (300 MHz for $^{1}H$ and 75 MHz for $^{13}C$) chemical shifts were related to that of the solvent .

**3.4.2.2 Mass spectrometry (MS)**

Mass spectrum was recorded on a GCMS. QP 1000 Ex Shimadzu mass spectrometer at 70 e.v.

**3.4.2.3 UV spectrometry**

UV spectrum was recorded on Cecil 3000 series spectrophotometer according to **Mabry *et al*. (1970)**.

## RESULTS AND DISCUSSION

Table (1) showed that the miticidal activity ($LC_{50}$) and the antibacterial activity (average inhibition zones) of the methanolic extracts of the plants examined against the spider mite, *Tetranychus urticae* and the two pathogenic bacteria, *Erwinia carotovora* and *Ralstonia solanacearum*.

The results indicated that 11 methanolic extracts only exhibited biological activity against one or more of the three pests tested. The $LC_{50}$ values of these extracts against the mite were between 140 $mg1^{-1}$ for *Cassia sp* extract to 250 $mg1^{-1}$ for *Acacia saligna* extract, whereas the antibacterial activities (inhibition zones) were between 11 mm for *Cassia sp.* extract to 30 mm for S*chinus terebinthifolius* extract against *Erwinia carotovora* and between 11 mm for C*assia sp.* extract to 28 mm for S*chinus terebinthifolius* extract against *Ralstonia solanacearum*. The results also revealed that only four extracts were found to have both miticidal and antibacterial activities against the three pests tested, these include the extracts of *Lantana camera* , *Myrtus communis* , C*assia sp.* and S*chinus terebinthifolius* .

The S*chinus terebinthifolius* was the most potent plant for controlling the three tested pests as its methanol extract exerted highly activity against the two pathogenic bacteria in addition to moderate activity against the spider mite.

The air dried leaves of S*chinus terebinthifolius* were successively extracted by $C_6H_{14}$, $CHCl_3$, EtOAc and MeOH, then the miticidal and antibacterial activities of each extract were tested. Only the methanol extract showed miticidal activity ($LC_{50}$=200 $mg1^{-1}$) and antibacterial activity against the two pathogenic bacteria, *Erwina carotovara* and *Ralstonia solanacearum* (MLC = 500 and 1000 µg/ml), respectively.

Analytical TLC of the active methanol extract ($CHCl_3$:MeOH:$H_2O$; 80:20:2) showed the presence of a pink major component after spraying with $H_2SO_4$. This component was obtained as white powder (230 mg; 1.77% $R_f$ =0.91 and 0.61 systems l and 3, respectively) after purification through column chromatography and preparative TLC as described in Material and Methods.

The UV spectrum of the pure compound exhibited a distincit maximum at $\lambda$=283nm. Also this compound gave positive reaction with $FeCl_3$ (blue) on TLC indicating that it is a phenolic compound.

The mass spectrum of this compound (Fig.1) showed a molecular ion peak at m/z 184 which indicated that its molecular formula is $C_8H_8O_5$. The presence of phenyl group was established by the appearance of carbon atom signals

between δ109.33 to δ146.51ppm in the $^{13}$C-NMR spectral data (Fig. 2 and Table 2). The $^{13}$C-NMR spectrum also showed the presence of methoxyl group ($OCH_3$) and carbonyl group (CO) due to the carbon atom signals at δ52.15and δ167.09ppm respectively.

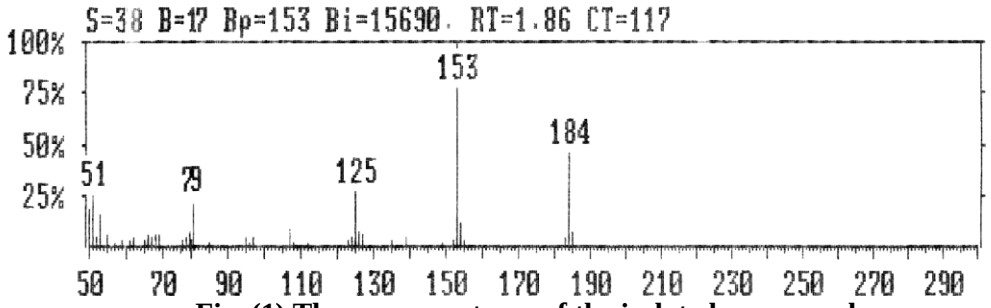


**Fig. (1) The mass spectrum of the isolated compound**

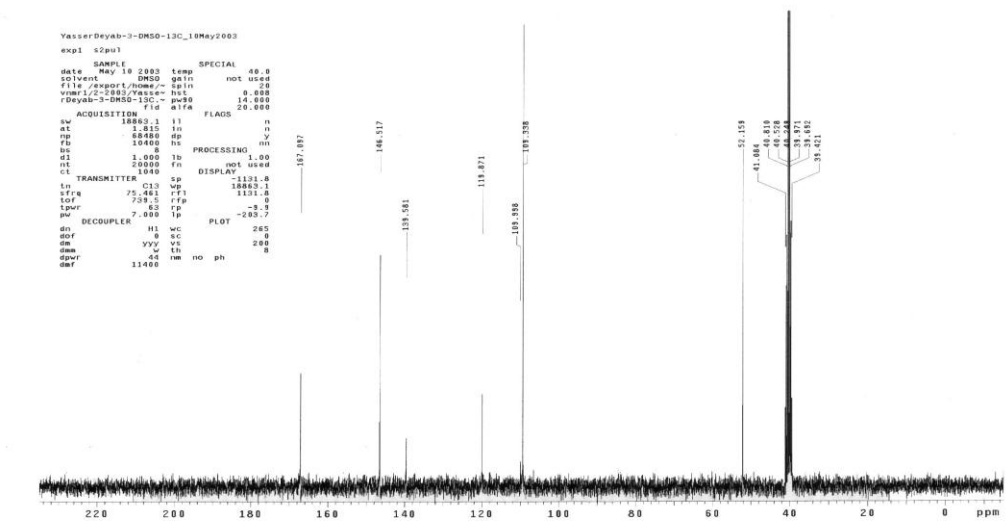


**Fig. (2) $^{13}$C-NMR spectrum of the isolated compound in DMSO-$d_6$**

**Table (2) $^{13}$C and $^{1}$H-NMR spectral data of the isolated compound in DMSO-$d_6$**

| Carbon No. | δC | $^{13}$C | $^{1}$H |
|---|---|---|---|
| 1 | C | 119.87 | - |
| 2 | CH | 109.33 | 6.93 s |
| 3 | C | 146.51 | - |
| 4 | C | 139.58 | - |
| 5 | C | 146.51 | - |
| 6 | CH | 109.33 | 8.40 s |
| 7 | CO | 167.09 | - |
| 8 | $OCH_3$ | 52.15 | 3.79 s |
| - | 3OH | - | 5.39 br,s |

The fragment ions at m/z153(M-$OCH_3$)and 125 (M-$COOCH_3$) were further confirmed the presence of these groups ($OCH_3$ and CO) and also indicated that the methoxyl group attached to the carbonyl group in the form of ester group. The $^1$H-NMR spectrum (Fig.3) showed one methoxyl group (δ3.79ppm, 3H br.s) and three hydroxyl groups (δ5.39ppm, 3H br.s). The appearance of two aromatic proton signals only at δ6.93(1H,s) and δ8.4ppm (1H,s) assignable to the protons of C-2 and C-6 respectively in the $^1$H- NMR spectrum indicating that the other positions on the phenyl group were substituted. The presence of the three hydroxyl groups on the phenyl group were established by the fragment ion peak at m/z 125 (M-$COOCH_3$) and the spectral data ($^1$H and $^{13}$C- NMR).

Thus the structure of this compound was characterized as gallic acid methyl ester (Fig. 4). This methyl gallate which was isolated for the first time from *Schinus terebinthifolius* is a known compound which was previously isolated from A*cacia nilotica* **(Khalid *et al.* 1989)**.

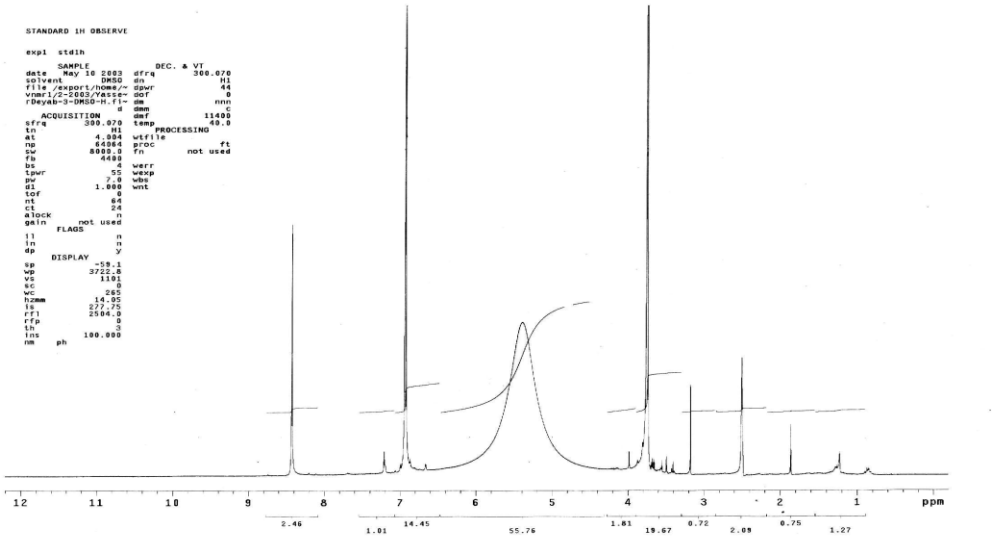


**Fig. (3) $^1$H-NMR spectrum of the isolated compound in DMSO-$d_6$**

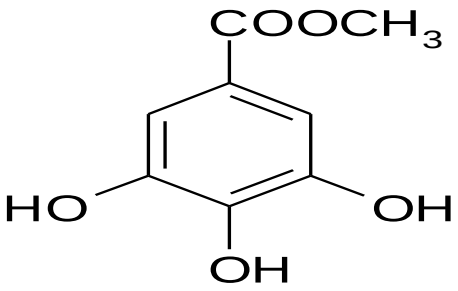


**Fig.(4) Structural formula of the isolated compound ($C_8H_8O_5$)**

The isolated compound (methyl gallate) exerted a miticidal activity against adult females of the two spotted spider mite *Tetranychus urticae*

($LC_{50}$= 58 ppm) and antibacterial activity against the two pathogenic bacteria *Erwinia carotovora* and *Ralstonia solanacearum* (MLC = 250 and 500 µg /ml) respectively.

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**فصل وتعريف مركب ذو أثر مبيد للبكتريا و الاكاروس من أوراق نبات الفلفل العريض ضد البكتريا المسببة لعفن البطاطس و العنكبوت الاحمر**

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**قسم وقاية النبات - كلية الزراعة – جامعة القاهرة – فرع الفيوم***

تم إجراء تجربة استقصائية على فعالية مستخلص الميثانول لأوراق ٢٦ عينة نباتية تنتمى إلى ١٨ عائلة نباتية مختلفة ضد العنكبوت الأحمر وكذلك ضد البكتريا المسببة لكلا من العفن البني والعفن الطري في البطاطس.

وقد أوضحت الدراسة أن مستخلص الميثانول لأوراق نبات الفلفل العريض هو اكثر المستخلصات فعالية ضد الآفات الثلاثة. وقد تم إخضاع هدا النبات للدراسة لكى يتم فصل المركبات المسئولة عن هذه الفعالية وأمكن فصل المركب المسئول عن الفعالية باستخدام طرق التحليل الكروماتوجرافي ثم تم تعريف المركب باستخدام طرق التحليل الطيفي(الأشعة فوق البنفسجية – الرنين المغناطيسى – تقدير الكتلة) وقد أظهر هدا المركب الفعال (استر ميثيل جالات) فعالية ضد أكاروس العنكبوت الاحمر (٥٨ مجم = $LC_{٥٠}$) وضد نوعى البكتريا المسببة لمرض العفن الطري والبنى (٢٥٠ و ٥٠٠ ميكروجرام/ملل ) على الترتيب.