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In vitro evaluation of some Egyptian plants against the rot bacteria and spider mite and isolation the active constituent(s) from *Myrtus communis* leaves

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Abstract: The present study investigated activity of 4 different extracts of 25 local plant species belonging to 17 families against two phytopathogenic bacteria, *Erwinia carotovora* and *Ralstonia solanacearum* the pathogens of soft rot and brown rot diseases of many important cultivated plants together with phytophagous mite, twospotted spider mite, *Tetranychus urticae* which feed on numerous food and fiber crops causing serious damage and crop loss worldwide. The biological evaluation results indicated that *Myrtus communis* was the most potent to combat these pests. Bioactivity-guided separation of the bioactive methanol extract of the dried leaves of *Myrtus communis* resulted in the isolation of a chromatographically pure compound. Based on spectroscopic methods (^{1}H, ^{13}C-NMR and MS) as well as chemical methods (detection tests and acidic hydrolysis). The active compound which isolated for the first time from this plant was characterized as 3-methoxy myricetin 7-O- α-L-rhamnopyranoside. This compound exerted a bactericidal activity against both *Erwinia carotovora* and *Ralstonia solanacearum* with MLC values of 200 and 100 $\mu g.ml^{-1}$ respectively, along with an acaricidal activity against the tested mite with LC_{50} value of 67 mg/l.

Keywords: Botanical bactericides, rot bacteria, botanical acaricides, twospotted spider mite, *Myrtus communis*, and flavonol glycosides

Introduction

Nowadays, people are more aware of the potential health risks and environmental hazards of synthetic pesticides. In this light, the growing need to develop pesticides that are not harmful to people, beneficial organisms and the environment has gradually heightened interest in biopesticides. One source of potential new pesticides in natural products produced by plants. Plants constitute a rich source of bioactive compounds, such as terpenoids, alkaloids, flavonoids, tannins and polyacetylenes (Harborne, 1993; Ahn *et al.,* 1998), since these are often active against a limited number of species, including specific target pests, are biodegradable to non-toxic products, and are potentially suitable for use in integrated pest management programs for sustainable agriculture, they could lead to the development of new classes of safer pest control agents (Park *et al.,* 2002; Mansour *et al*., 2004). Therefore, much effort has been focused on plant materials for potentially useful products as commercial pesticides of agricultural interests.

The phytopathogenic bacteria, *Erwinia carotovora* and *Ralstonia solanacearum* and the phytophagous mites, twospotted spider mite, *Tetranychus urticae* are two of the most economically important pests worldwide (Helle and Sabelis 1985; Hayward 1991). These pests affects several significant food, fiber crops and ornamentals plants, leading to serious damage and consequently crop reduction or total loss worldwide (Elphinstone *et al*., 1996; Wegener 2002; Dekeyser and Downer, 1994). Accordingly, a constant search for new bactericidal, acaricidal, and other pesticidal agents from plants species is a necessary strategy in the struggle against these pests, so that problems of selectivity, low activity and environmental pollution can be overcome.

The aim of this study, was to evaluate by *in* vitro bioassay some local plant species for bactericidal activity against both *Erwinia carotovora* and *Ralstonia solanacearum*, and acaricidal activity against the twospotted spider mite, *Tetranychus urticae* Koch., along with the isolation and identification of active constituents from the most promising plants.

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Materials and Methods

Plant materials

Leaf samples of 25 plant species belonging to 17 families as shown in Table 1 were collected locally in April 2000 from Fayoum Faculty of Agriculture garden, to select those which have both high bactericidal and acaricidal activities. The plants were identified by the Botany Department, Faculty of Science, Cairo University. Voucher specimens of each plant species were deposited in the herbarium of Biochemistry Department, Faculty of Agriculture, Fayoum University.

Preliminary phytochemical screening

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

Preparation of the extracts

A portion (100 g) of the fresh leaf samples from each of the plant species collected was air-dried in the shade, grounded into a fine powder and then were consecutively extracted with n-hexane, chloroform, ethylacetate and methanol. The extracts of the leaf samples were evaporated to dryness in a rotary evaporator at 40ºC and subjected to both; antibacterial activity against the brown rot bacterium, *Ralstonia solanacearum* and soft rot bacterium, *Erwinia carotovora* and acaricidal activity against the twospotted spider mite (*Tetranychus urticae*).

Bactericidal activity

The extracts and the isolated compounds were assayed for bactericidal activity against two pure strains of bacterial isolates, namely *Erwinia carotovora*, and *Ralstonia solanacearum* which were obtained from Department of Plant Pathology, Faculty of Agriculture, Ain-Shams University. For bioassays, the disk diffusion technique of Bauer *et al.* (1966) was used. Whatman No.1 filter paper disks (6 mm) were soaked with each methanolic plant extracts (1g/10 ml methanol) and were dried at 40°C. Sterile paper disks were placed onto the inoculated plate surface, then the plates were incubated overnight at 37°C and the diameter of inhibition zone (DIZ) of growth around each disk was measured. The density of the bacterial suspensions was determined by McFarland standard. The agar contained about 10 colony forming units (cfu)/ml. All the determinations were carried out in triplicate and the mean of the diameter of inhibition zones was calculated.

Determination of minimum lethal concentration (MLC)

The minimum lethal concentration of the bactericidal isolated compound was determined by bacterial broth dilution method as described by Ellen *et al.* (1994).

Acaricidal activity

Plant extracts and isolated compound as well, were tested against twospotted mite *Tetranychus urticae* Koch which was obtained from Department of Plant Protection, Faculty of Agriculture, Fayoum University. For bioassays, the slide-dip technique adopted by Voss (1961) and modified by Dittrich (1962) was applied to evaluate the efficacy of the tested extracts and isolated compound. For this purpose, a piece of double face adhesive scotch tape was pressed tightly to the surface of microscopic glass slide. Five concentrations (50, 100, 200, 400 and 800 ppm) of the tested extract of chosen plants and Five concentrations (5, 10, 20, 40 and 80 ppm) of the isolated compound were used to draw the dosage mortality regression line. For each concentration 10 adult females of similar age and size were adhered upside down with legs free to the tape on the glass slide, using moist brush, which was immediately dipped in the extract for 5 sec. Excess solution was dried off on filter paper. Four replicate slides were used for each concentration. The treated mites were kept undisturbed under room conditions (25:30 °C and 60:65%RH) for 24 hours, and then examined for mortality. Females responding to a touch with fine brush were considered alive. Mortality ratios were then recorded and data were plotted on log-dosage probit paper (Abbott's formula 1925). LD_{50} values were determined by Probit analysis (SAS, 1995). Four replicate slides treated with only water were used as control.

Extraction and isolation of the bioactive compound(s) from Myrtus communis *leaves*

Among the tested plants *Myrtus communis* was chosen for the isolation and identification of the bioactive constituent (s) as it exerted the highest bactericidal activity in addition to a good acaricidal activity.

Extraction

Ground air-dried leaves (275 g) of *Myrtus communis* were successively extracted with a series of solvents in order of increasing polarity: n-hexane(1.5L),

chloroform (5L), Ethylacetate (2L) and methanol (5L) at room temperature (25ºC ± 3). The extracts were evaporated to dryness under reduced pressure to offer 9, 13, 3 and 37 g residues respectively.

Isolation of the bioactive compounds

The bioactive methanol extract was subjected to the isolation of the bactericidal and acaricidal compounds as follows: Thirty-five grams of extract residue was fractionated by dissolving in 50 ml distilled water then centrifuged for 10 min. The water soluble fraction (supernatant) added to presaturated butanol 100 ml and left overnight, then separated by separatory funnel to give butanol fraction residue (6.5 g) and water fraction residue (19 g) and then tested for bactericidal and acaricidal activities.

Six grams of the bioactive butanol fraction were subjected to column chromatography over 150 g silica gel (230-400 mesh in column 2.7 i.d. X 60 cm) and eluted with a gradient of chloroform: methanol: water (50:50:5) 1L, (0:100:0) and (0:50:50) 0.5L for each eluent. According to differences in composition monitored by TLC five fractions were obtained and then tested for both bactericidal and acaricidal activities. The bioactive fraction No: 2 eluted with (50:50:5) chloroform:methanol:water between 150 – 400 ml (1.7 g) was further separated using column chromatography on Sephadex LH-20 (20 g in column 1.6 i.d.X 40 cm) with methanol as eluent, the eluates were combined on the basis of similar TLC profiles in chloroform:methanol:water (60:40:5) to afford eight fractions. The bioactive fractions were No: 5 and No: 6 gave extract residues of 215 and 210 mg, respectively.

Further purification of fraction No: 5 was performed on 15 g Sephadex LH-20 with MeOH as eluent in column 1.6 i.d. X 40 cm to yield (107 mg) of semi-purified compound as a main component, Fraction No: 6 was purified using preparative TLC with solvent system chloroform: methanol: water (70: 30: 5) to give (90 mg) semi-purified compound which gave the same band on TLC as main fraction obtained from fraction No: 5. The two fractions obtained from No: 5 and No: 6 were mixed together and purified by preparative TLC with ethylacelate: formic acid: acetic acid: water (100: 11: 11: 27) to give (88 mg ; 0.25%) of purified active compound . The purity of this bactericidal and acaricidal compound was established by its resolution as a single spot in four different TLC systems.

Analytical and preparative Thin Layer Chromatography (TLC)

Analytical and preparative TLC were carried out on Merck precoated silica gel plates (F_{254} thickness: 0.25mm and 2.0 mm respectively) using the following solvent systems: n-Butanol-Acetic acid-Water (4:1:5) upper layer, Chloroform-Methanol-water (50:50:2), (80:20:2) and (70:30:5), Ethylacetate-Acetic acid-Formic acid-Water (100:11:11:27), Dichloromethane-Methanol-Water (50:25:5), Chloroform-Acetone (50:6) and Chloroform-Methanol (75:25). Spots on TLC were detected under UV light (254 and 365 nm) and by spraying with concentrated H_2SO_4 followed by heating at 105ºC for 5 min or by $AlCl_3$. Sugars were detected by spraying with naphthoresorcinol phosphoric acid followed by heating at 105ºC for l0 min.

Structural identification of the purified compound

The purified compound was characterized by detection tests (Farnsworth, 1966), acid hydrolysis and spectroscopic methods.

Acid hydrolysis

The purified compound (2 mg) was heated with aqueous 10% HCL (2 ml) in a sealed tube at 100ºC water-bath for 4 hours. The aglycone was extracted with diethylether and analysed by TLC with chloroform:acetone (50:6). The aqueous layer was neutralized with N,N-dioctylamine (10% in $CHCl_3$). After evaporation to dryness, the sugars were identified by TLC with CH_2Cl_2:MeOH:H_2O (50:25:5) by comparison with authentic samples.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H and ^{13}C-NMR spectra were recorded in deutromethanol (CD_3OD) on a Varion Mercury VXR 300 spectrometer (300 MHz for ^1H and 75 MHz for ^{13}C) chemical shifts (ppm) were related to that of the solvent. The spectroscopic NMR experiments were performed at Central Laboratory in Faculty of Science, Cairo University.

Mass spectrometry (MS)

Mass spectrum was recorded on a GCMS. QP 1000 Ex Shimadzu mass spectrometer at 70 e.v. The MS experiment was carried out at Central Laboratory in Faculty of Science, Cairo University.

Results and Discussion

Evaluation of the bactericidal and acaricidal potential of plant extracts

Among the four organic solvents used, only methanolic extracts of some tested plants gave detectable bactericidal and acaricidal activities. The data pertaining to the bactericidal and acaricidal

potential of the methanol extracts are presented in Table 1. Of the 25 plants tested, 10 leaf methanolic extracts exhibit biological activity at least against one of the three pests tested. From which 8 plant species exhibit antibacterial action against both pathogens tested, i.e., *Erwinia carotovora* and *Ralstonia solanacearum* the pathogens of soft rot and brown rot diseases of many economically important crops such as potato, tomato, tobacco, banana, eggplant and some ornamental plants which lead to economic losses in agriculture worldwide (Hayward, 1991; Elphinstone *et al.,* 1996; Wegener, 2002). Only the leaf extract of *Pittosporum tobira* was active against *Ralstonia solanacearum* but was not active against *Erwinia carotovora*.

The leaf methanolic extract of *Myrtus communis* showed the highest antibacterial activity against both phytopathogenic bacteria tested. The zones of inhibition of *Myrtus communis* extract were 25 mm in diameter in case of *Erwinia carotovora* and 26 mm against *Ralstonia solanacearum*. This plant have high potential use in phytotherapy against the pathogenic bacteria of soft rot and brown rot diseases, since Bonjar (2004) rated antibacterial activities of plants into three classes as : poor (diameter of inhibition zone (DIZ) < 12 mm, medium (DIZ 12 to < 15 mm) and high (DIZ > 15 mm or higher).

The occurrence of antibacterial activity against specific pathogen bacteria of human diseases in leaf extract of *Myrtus communis* was previously reported by Rotstein *et al.* (1974), Mansouri *et al.* (2001) and Bonjar (2004), but no prior reports about the antibacterial activity of plant against phytopathogenic bacteria, *Erwinia carotovora* and *Ralstonia solanacearum* were given.

On the other hand, five plant species only showed acaricidal effect against phytophagous twospotted spider mite, *Tetranychus urticae* (Table 1) which is an economically important pest worldwide (Helle and Sabelis, 1985) and infesting over 200 species of vegetable, fruit, ornamental and fibber crops (Dekeyser and Downer 1994). The LC_{50} values of these five active extracts were between 140 mg/l in case of *Cassia sp*. extract to 250 mg/l for *Acacia saligna* extract. The effectiveness of the five active plants followed the sequence: *Cassia sp*. > *Pittosporum tobira* > *Myrtus communis* > *Lantana camara* > *Acacia saligna.*

The variation of the bactericidal and acaricidal activities of the tested active extracts may be due to the differences in their secondary constituents since natural secondary products have been shown to possess several biological properties. It is interesting to note that among the plant species tested only three extracts of *Myrtus communis, Lantana camara* and *Cassia sp*. were active against both phytopathogenic bacteria, *Erwinia carotovora Ralstonia solanacearum* and against phytophagous twospotted spider mite as well. The current study (Table 1) showed that the best extract for controlling the three tested pests was methanol extract of *Myrtus communis* which exerted the highest antibacterial activity in addition to a good acaricidal activity.

Preliminary phytochemical screening

Screening tests indicated the presence of saponins, sterols, triterpenoids, tannins, flavonoids and glycosides of *Myrtus communis*. It is noteworthy that many plants have been used as botanical pesticides in the agricultural fields because of their bactericidal and acaricidal properties which are due to compounds synthesized in the secondary metabolism of the plant (Harborne, 1993; Ahn *et al.,* 1998; Cseke and Kaufman, 1999). Therefore, the biological activities of *Myrtus communis* leaves may be attributed to the presence of a compound or more belongs to one or more of these classes of plant secondary metabolites.

Biological evaluation of the isolated compound

Bioactivity-guided separation of the methanol extract of the dried leaves of *Myrtus communis* resulted in the isolation of one chromatographically pure compound. The isolated compound exerted a antibacterial activity against the two phytopathogenic bacteria *Erwinia carotovora* and *Ralstonia solanacearum* with MLC values of 200 and 100 µg/ml respectively, and acaricidal activity against adult females of the twospotted spider mite *Tetranychus urticae* with LC_{50} value of 67 mg/l

Therefore, this compound was in part responsible for the bactericidal and acaricidal properties of *Myrtus communis* leaves. No data are available about the isolation of active compound from the leaves of *Myrtus communis* against phytopathogenic bacteria, *Erwinia carotovora* and *Ralstonia solanacearum* as well as phytophagous mite, *Tetranychus urticae.*

Structural identification of the active isolated compound

The pure compound was obtained as a yellow amorphous powder and gave positive reaction with diphenylboryloxyethyl amine test on TLC suggesting that it is a flavonoid compound. The EI mass spectrum of the compound indicated a molecular ion peak at m/z 478 which demonstrated that its molecular formula is $C_{22}H_{22}O_{12}$.

Acid hydrolysis of this compound afforded

Table 1. Evaluation of bactericidal and acaricidal activity of leaf methanol extracts

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

Table 2. ^{13}C and ^{1}H-NMR spectral data of the active isolated compound in CD_3OD

Carbon No.	δ C	^{13}C	^{1}H
Aglycone			
2	C	158.54	
3	C	141.85	
4	CO	177.21	
5	C	165.80	
6	CH	94.74	6.20 d
7	C	165.98	
8	CH	90.32	6.36 d
9	C	156.70	
10	C	103.65	
1`	C	121.98	
2`	CH	109.64	6.95 s
3`	C	146.87	
4`	C	137.21	
5`	C	146.87	
6`	CH	109.64	6.95 s
	OCH_3	52.15	3.6 s
Rhamnose			
1″	CH	99.88	5.32 d
2″	CH	73.41	4.22 dd
3″	CH	72.19	3.98 dd
4″	CH	72.06	3.32 m
5″	CH	71.92	3.51 m
6″	CH_3	17.70	0.96 d

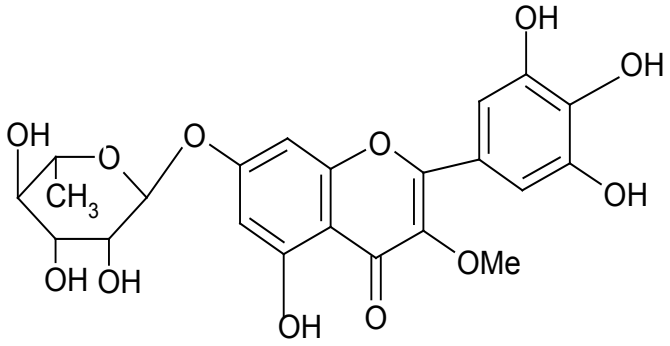

Figure 1. Structural formula of the active isolated compound (3- methoxy myricetin 7-O- α-L-rhamnopyranoside)

L-rhamnose as a sole sugar on TLC by direct comparison with authentic samples. The presence of α-L-rhamnose was confirmed by the ^{1}H-NMR spectrum (Table 2) due to the appearance of only one anomeric proton signal at δ 5.32 (1H, d, j = 1.2 Hz) and methyl group signal at δ 0.96 (3H, d, j = 6.3Hz C-6`), as well as the carbon signals in the ^{13}C- NMR spectrum (Table 2) at δ 99.88 and δ 17.7 ppm for C-1″ and C-6″ of rhamnose respectively.

The aglycone was clearly deduced as 3-methoxy myricetin by the ^{1}H and ^{13}C- NMR spectral data as follows: the presence of a pair of meta coupled doublets of one proton each at δ 6.2 (j = 2.1 Hz) and δ 6.36 (j = 2.1 Hz) assigned to H-6 and H-8 protons of ring A and a signal of two protons at δ 6.95 (2H, s) ascribed to H-2` and H-6` of B ring. In addition to three protons signals at δ 3.6 ppm (OCH_3, s). The methyl etherification at C-3 of the aglycone moiety was established by the characteristic downfield shift of the resonance of this carbon to δ141.85 ppm in the ^{13}C- NMR spectrum as well as by comparing the carbon chemical shifts with previously reported (Horie *et al.,* 1987, 1998; Hussein *et al.,* 2003).

The recognizable upfield shifts of C-6 and C-8 (Table 2) compared with previously reported for these positions (Markham *et al.,* 1978; Hussein *et al.,* 2003) indicating that the C-7 position of this compound must be occupied with the sugar moiety (rhamnose). The position of the rhamnose sugar was established by comparing the ^{13}C- NMR signals of ring A with that previously reported by Markham (1989). On the basis of the above findings compound (Figure 1) was elucidated to be 3- methoxy myricetin 7-O- α-L-rhamnopyranoside.

The antibacterial activity of flavonols and their glycosides has previously been reported by Nishino *et al.* (1987), Yadava and Reddy (1998), Liu *et al.* (1999) and Simin *et al.* (2002) to treat human diseases caused by pathogenic bacteria. However, no previous study on the antibacterial activity of flavonol glycosides particularly, the isolated flavonol glycoside, i.e., 3-methoxy myricetin 7-O-α-L-rhamnopyranoside was carried out to treat phytopathogenic bacteria especially, *Erwinia carotovora* and *Ralstonia solanacearum.* In addition, so far no report about the acaricidal activity of flavonoids was published. Thus, this is the first study on the isolation and identification of an active flavonol glycoside, i.e., 3-methoxy myricetin 7-O- α-L-rhamnopyranoside with both bactericidal actions against phytopathogenic bacteria together with acaricidal effect against phytophagous twospotted spider mite, from *Myrtus communis* leaves.

These results suggest that *Myrtus communis* leaves may be utilized as a potential source of bactericidal and acaricidal compound against the tested phytopathogenic bacteria and phytophagous mite that cause serious damage and crop loss worldwide, but *in vivo* studies are required to support this.

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