ANTIBACTERIAL ACTIVITY OF FUROQUINOLONE ALKALOIDS AGAINST POTATO SOFT ROT BACTERIUM, *Erwinia carotovora* FROM *Ruta chalepensis* LEAVES

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ABSTRACT

Soft rot bacterium; *Erwinia carotovora* is one of the most severe post-harvest diseases of potatoes worldwide. This bacterium affects tubers during storage, transit and marketing. The aqueous ethanolic extract (80%) of *Ruta chalepensis* leaves (Rutaceae) showed antibacterial activity against *Erwinia carotovora* (MIC = 625 µg/ml). Bioassay-guided isolation of this aqueous extract by using a combination of different chromatographic methods (TLC and column chromatography) yielded two furoquinolone alkaloids for the first time from this plant. Their structures were characterized as 2, (hydroxy isopropyl) – 6, hydroxy –9, methyl-dihydrofuro [2,3] –quinol-4-one (**I**) and 3, (hydroxy methyl)-6, methoxy-2, 2, 9-trimethyl-dihydrofuro [2,3] quinol-4-one (**II**) by ^{1}H, ^{13}C NMR and MS spectral data. The isolated alkaloids **I** and **II** showed antibacterial activity against *Erwinia carotovora* with MIC at 20 and 35 µg/ml respectively.

Key Words: Furoquinolone alkaloids, Soft rot bacterium, *Ruta chalepensis*, Rutaceae and *Erwinia carotovora.*

INTRODUCTION

Soft rot bacterium; *Erwinia carotovora* is one of the most severe post-harvest diseases of potatoes worldwide. This bacterium affects tubers during storage, transit and marketing, causing a major problem to the potato industry worldwide.

In Egypt, potato is one of the most important vegetable crops. Its important is not due only to local consumption but also for exportation to European community, which represents about 42.7% of Egypt's agricultural exports. Synthetic bactericides like Tecto 5% D (1.25 kg/Ton tuber) have been used for control the soft rot disease. However, their use is restricted due to their harmful effects on human beings and environment, in addition to the chemical residues of potato diminished the potato exportation quantity to European community **(Food and Veterinary Office, 2000).**

Therefore, the replacement of synthetic by natural pesticides for pest control application has increased interest in the potential use of natural products in general.

Ruta chalepensis L. (Rutaceae) is a small shrub originating in southern Europe, but now spread over North America and some other places [**Fischer *et al*. 1988**] The leaves and roots are used in folk medicine against intestinal colic, spasmodic atonic amenorrhoea, rhematic diseases, headaches and wounds [**Mahran, 1967**]. This plant exhibited many activities such as anti-inflammatory activity [**Al-Said *et al*. 1990**], antifertility activity [**Ulubelen *et***

***al*. 1994**], anticonvulsant activity [**Aguilar-Santamaria and Totoriello 1996**], molluscicidal activity [**Hmamouchi *et al*. 2000**], antimicrobial activity [**El-Sayed *et al*. 2000**] larvicidal activity [**Mookey *et al*. 2002**] and repellent activity [**Hadis *et al*. 2003**]. Previous phytochemical research on this plant has resulted in the isolation of several acridone, quinoline and quinlone alkaloids and coumarines from the aerial parts [**Mohr *et al*. 1982; Ulubelen *et al*. 1986; Ulubelen and Guner 1988 and Zobel *et al*. 1990**] from the cellcultures [**Fischer *et al*. 1988 and Baumert *et al*. 1992**] and from the roots [**Ulubelen and Terem 1988; Ulubelen *et al*. 1988; Ulubelen and Tan 1990 and El-sayed *et al*. 2000**].

In this paper we report for the first time the isolation and structural elucidation of two furoquinolone alkaloids responsible for the bactericidal activity of *Ruta chalepensis* leaves against the Potato soft rot; *Erwinia carotovora.*

MATERIALS AND METHODS

Plant material

The leaves of *Ruta chalepensis* L. (Rutaceae) were collected from the experimental farm of the Faculty of Agriculture, Cairo University, Giza, Egypt and identified by the Botany Department, Faculty of Science, Cairo University. A voucher specimen deposited in the Biochemistry Department, Faculty of Agriculture, Cairo University, Giza.

Extraction

Ground air dried leaves (350 g) was extracted three times with 80% ethanol (each 700 ml) at room temperature (25 $\pm$ 2ºC). After filtration, the combined extract was evaporated under reduced pressure to afford 55.2 g of dry extract.

Antibacterial test

Tester strain of *Erwinia caratovora* was obtained from Department of Plant Pathology, Faculty of Agriculture, Ain Shams University.

The in vitro antibacterial activity of the aqueous ethanolic extract (80%) and the isolated compounds were determined by bacterial broth dilution methods described by **Ellen *et al* 1994** against the soft rot bacterium; *Erwinia carotovera.* Minimum Inhibitory Concentrations (MICs) were determined as the lowest concentrations preventing visible growth.

Analytical Thin Layer Chromatography (TLC)

TLC analysis was carried out on precoated silica gel plates (F_{245} 0.25 mm and F_{245} 2.0 mm Merck) using the following solvent systems:

1) Chloroform – Methanol – Water (70:30:5).
2) Chloroform – Methanol (80:20).
3) Chloroform – Methanol (90:10).
4) Ethylacetate- Acetic acid- Formic acid- Water (100:11:11:27)
5) n-Butanol- Acetic acid – Water (4:1:5) upper layer

Zones were detected under UV light (255 and 365 nm) and by spraying with: concentrated H_2SO_4 followed by heating at 105^{o}C for 5 min or with modified Dragendorff reagent (**Farnsworth 1966**).

Isolation of the bioactive constituent (s)

A portion of the aqueous ethanolic extract (40 g) was suspended in water (150 ml) and extracted with $CHCl_3$ (3 x 50 ml) to give $CHCl_3$ soluble components (Fraction A, 6.5 g). The aqueous layer was freeze dried (33.5 g) and were then extracted with $CHCl_3$: MeOH: H_2O (70 : 30: 5; 150 ml). After centrifugation both the supernatant and the precipitate were dried under reduced pressure to afford 4.6 g (Fraction B) and 28.8 g (Fraction C) respectively. The three fractions A, B and C were tested for their antibacterial activity against the soft rot bacterium; *Erwinia carotovora.* The bioactive fraction B (4.5 g) was subjected to the isolation of the bioactive component (s) as follows:-

Fraction B (4.5 g) was chromatographed over silica gel column (100 g, 230-200 mesh, Merck) and eluted with the solvent mixtures of $CHCl_3$: MeOH : H_2O (80:20:0 and 70:30:5, 200 ml for each eluent). Twenty fractions of each eluent were collected. The eluates were combined on the basis of similarity of TLC profiles to afford 7 fractions and were then tested for antibacterial activity.

The bioactive fractions No. 1 and 2 were further purified several times over Sephadex LH-20, silica gel column and PTLC as shown in Fig. (1) yielded two active compounds I and II. The purity of these two compounds were established by the resolution of each one as a single spot in four different TLC systems.

Structure identification of the isolated compounds:

The isolated compounds were characterized by detection tests and spectroscopic methods.

Detection tests:

The preliminary screening of the isolated compounds for saponins, flavonoids alkaloids and phenolic compounds were performed according to the methods described by **Farnsworth (1966)**.

Spectroscopic methods

Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H and ^{13}C-NMR spectra were recorded in CD_3OD on a varion Mercury VXP 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C). Chemical shifts (ppm) were related to that of the solvent.

Mass spectrometry (MS)

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

Ultraviolet spectrometry (UV)

The UV-spectra were registered with a spectrophotometer CeCil 3000 series.

RESULTS AND DISCUSSION

The aqueous ethanolic extract (80%) of the air dried leaves of *Ruta chalepensis* exhibited antibacterial activity against the soft rot bacterium, *Erwinia carotovora* (MIC: 625 µg/ml). Bioassy- guided isolation of this aqueous extract by using chromatographic methods (see Malerials and Methods) yielded two pure compounds **I** (157 mg) and **II** (89 mg). The isolated compounds **I** and **II** exerted bactericidal activity against *Erwinia carotovora* with MIC values of 20 and 35 µg/ml respectively. Thus, These compounds were in part responsible for the antibacterial activity of *Ruta chalepensis* leaves. The structure of these compounds (**I** and **II**) were characterized as follows:-

Compound I

It was obtained as colorless neddles, which gave positive reaction with modified dragendorff reagent on TLC suggesting it is an alkaloid compound. Its structure was characterized as dihydrofuroquinolone alkaloid in accordance with the following considerations:-

The mass spectrum (Fig. 2) showed molecular ion peak (M^+) at m/z 275 corresponding to molecular formula $C_{15}H_{17}NO_4$.

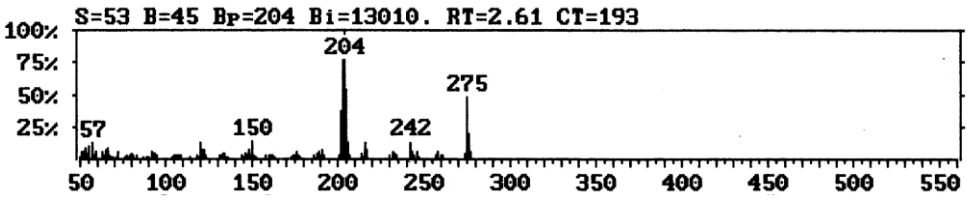

Fig. (2) Mass spectrum of compound I

The ^{13}C–NMR spectrum (Fig. 3 and Table 1) showed 15 carbon atom signals out of which six carbon signals accounted for the aromatic group (between δ 97.88 to δ 154. 91 ppm). The remaining nine carbon atom signals were identified as three methyl groups (δ 22.03, δ 25.53 and δ 31.20 ppm; assigned to C-11,12 and 13 respectively), one methylene group (δ 26. 87 ppm; assigned to C-3), Oxymethine group (δ 84. 01 ppm; assigned to C-2) and four quaternary carbon atom signals including carbonyl group (δ 177.65 ppm; assigned to C-4), Two olefinic carbons (δ 109. 51 and δ 156. 15 ppm; assigned to C-3a and 9a) and Carbinol group (δ 69.11 ppm; assigned to C-10).

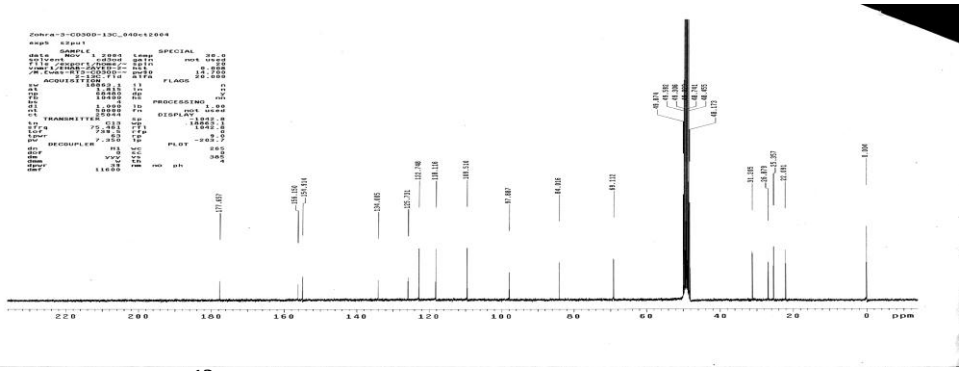

Fig.(3) ^{13}C-NMR spectrum of compound I in CD_3OD

Table (1) ^{13} C and ^{1}H-NMR spectral data of compound I.

C- atom No.	δ C	^{13} C	^{1} H
2	CH	84.01	3.87 t
3	CH_2	26.87	2.69, 2.97 dd
3 a	C	109.51	-
4	C	177.65	-
4 a	C	118.11	-
5	CH	97.88	7.63 d
6	C	134.00	-
7	CH	125.73	7.22 dd
8	CH	122.74	7.56 d
8 a	C	154.91	-
9 a	C	156.15	-
10	C	69.11	-
11	CH_3	25.35	1.45 d
12	CH_3	22.09	1.45 d
13	$N.CH_3$	31.20	3.75 s

The ^{1}H-NMR spectrum of this compound (Fig. 4 and Table 1) supported the presence of substituted aromatic ring due to the three aromatic proton signals type ABX at δ 7.63 (1H, d, J = 3.0Hz, H- 5), δ 7.22 (1H, dd, J = 9.0, 3.0 Hz, H- 7) and δ 7.56 ppm (1H, d, J = 9.3 Hz, H- 8). Also the spectrum exhibited methyl proton signal at δ 3.75 ppm (3H, s) assigned to N- methyl group (Noshita *et al* 2001) and two geminal dimethyl signal at δ 1.45 ppm (6H, d, J = 3.3 Hz) assignable to the protons of C-11 and 12.

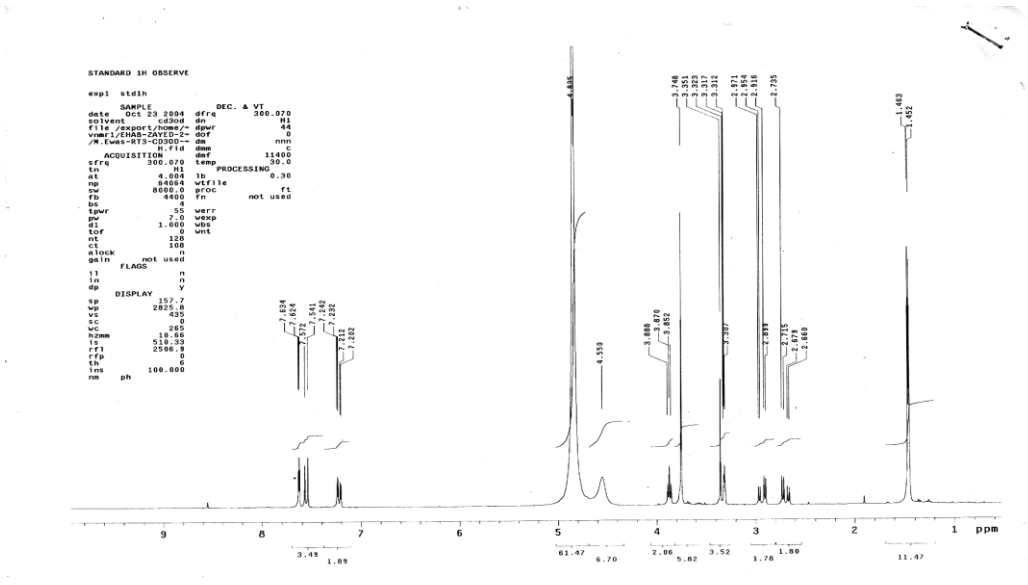

Fig. (4) ^{1}H-NMR spectrum of compound I in CD_3OD

The presence of dihydrofuran ring was established by the appearance of three proton signals in the ^{1}H-NMR spectrum at δ 2.69 (1H, dd, J = 5.7, 16.8 Hz), δ 2.97 (1H, dd, J = 5.1, 16.5 Hz) and δ 3.87 ppm (1H, t) ascribed to the two protons of C-3 and the proton of C-2 (Boyd *et al.* 2000) as well as the carbon atom signals at δ 84.01, δ 26.87, δ 109. 51 and δ 156.15 ppm corresponding to C-2, 3, 3a and 9a.

The presence of hydroxy isopropyl group in the position of C-2 was established by the ^{13}C and ^{1}H-NMR spectral data due to the signals at δ 25.35, δ 22.09 and δ 69.11 ppm in ^{13}C-NMR spectrum and δ 1.45 ppm (6H, d, J = 3.3 Hz) in ^{1}H-NMR spectrum, as well as by comparing these signals with previously reported for this group (Boyd *et al.* 2000 and Noshita *et al.* 2001).

The diagnostic fragment ions of mass spectrum Fig. (2) at m/z 275 (M^{+}; 47.7%), 242 (M- CH_5O; 13.2%), 204 (M- C_4H_7O; 100%), 150 (M- $C_7H_9O_2$; 13.8%), 120 (M- $C_8H_{13}O_2N$; 12.1%) and 57 (M- $C_{12}H_{12}O_3N$) were further supported the above assigned structure.

The ^{1}H and ^{13}C-NMR spectral data of this compound were similar to those of the dihydrofuroquinoline alkaloid (Ribalinium) isolated from the *Ruta graveolens* (Reisch *et al.* 1969 and Szendrei *et al.* 1969 and 1971) except the replacement of methoxyl group at C4 with carbonyl group.

From these data the structure of this compound was deduced to be 2-(hydroxy isopropyl) – 6-hydroxy- 9-methyl- dihydrofuro [2,3] – quinol – 4-one (Fig. 5). This compound was isolated for the first time from this plant.

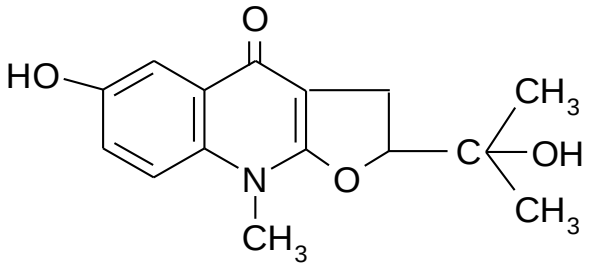

Fig (5) Structural formula of compound I ($C_{15}H_{17}NO_4$).

Compound II

It was obtained as a slightly yellow amorphous powder and gave positive colour with modified dragendorff's reagent on TLC suggesting it is an alkaloid compound. The mass spectrun (Fig. 6) of this compound showed a molecular ion peak (M^{+}) at m/z 289 in accord with the molecular formula $C_{16}H_{19}NO_4$.

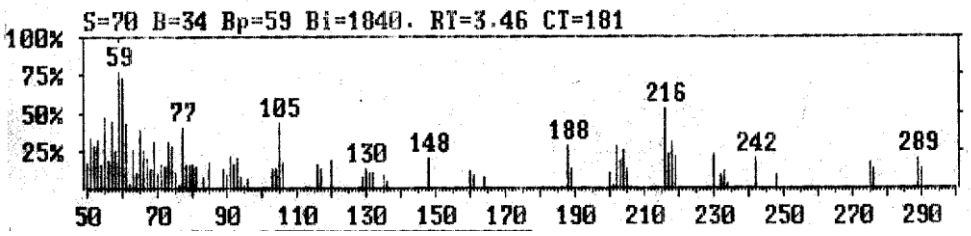

Fig. (6) Mass spectrum of compound II

The ^{1}H- NMR spectrum (Fig. 7 and Table 2) showed signals for three aromatic protons formed an ABX system at δ 7.53 (1H, d, j = 2.4 Hz), δ 7.85 (1H, d, j = 9.0 Hz) and δ 7.45 (1H, d, j = 9.0 Hz) assigned to H 5, 7 and 8. The ^{13}C- NMR spectrum (Fig. 8 and Table 2) confirmed the presence of aromatic ring due to the six carbon signals between δ 95.16 to 163.43 ppm. The remaining ten carbon signals were identified as one methine group (δ 30.07 ppm; C-3), hydroxy methylene group (δ 64.29 ppm; C-12) four methyl groups (two geminal methyl at δ 24.94 and 26.02 ppm, methoxyl group at δ 60.28 ppm and N-methyl at δ 34.44 ppm) and four quaternary carbon atoms including carbonyl group (δ 171.99 ppm; C-4), two olefinic carbons (δ 108.62 and 166.12 ppm; C- 3a and 9a) and oxycarbon (δ 71.94 ppm; C-2). The presence of the four methyl groups were established by the ^{1}H-NMR spectrum due to the signals at δ 1.29 (3H, d, J= 5.7, Hz), δ 1.46 (3H, d, J= 5.7, Hz), δ 3.60 (3H, s) and δ 4.05 ppm (3H, s) assigned to protons of C-10, 11, 13 and 14 respectively.

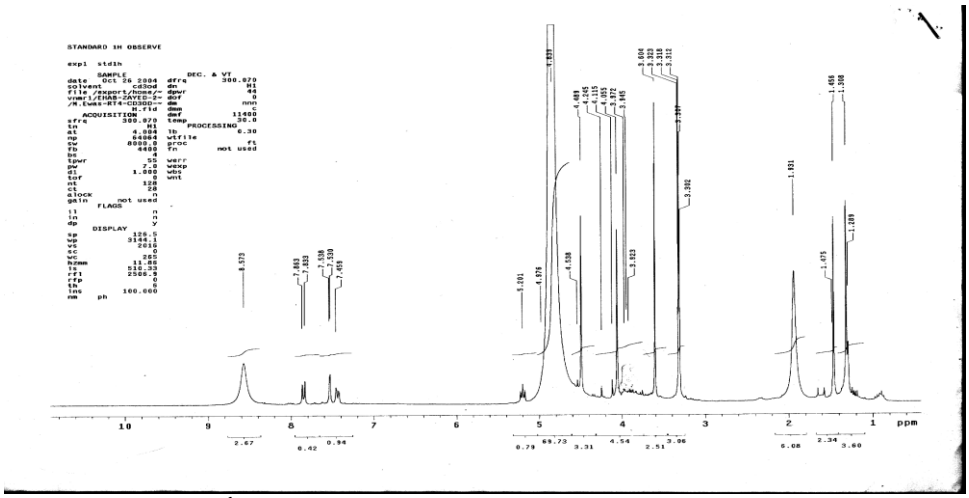

Fig. (7) ^{1}H-NMR spectrum of compound II in CD_3OD

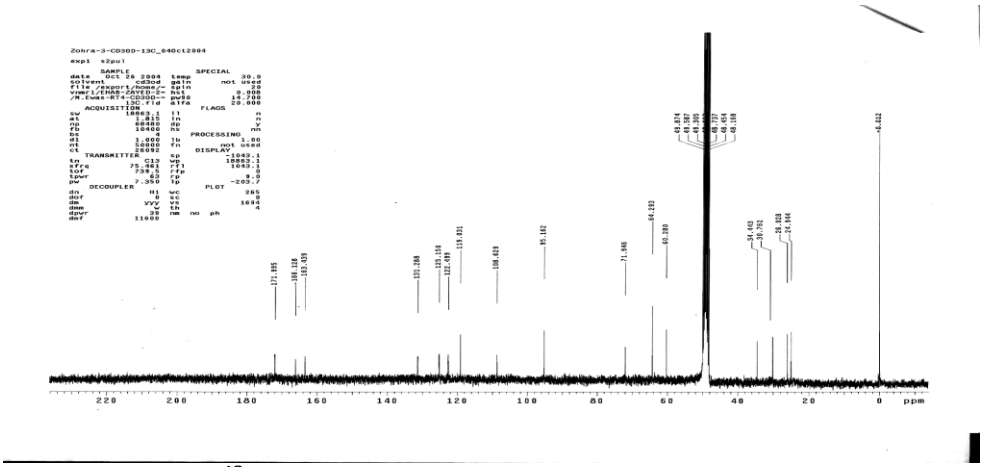

Fig.(8) ^{13}C-NMR spectrum of compound II in CD_3OD

Table (2) ^{13}C and ^{1}H-NMR spectral data of compound II.

C-atom No	δ C	^{13} C	^{1} H
2	C	71.94	-
3	CH	30.07	5.2 t
3 a	C	108.62	-
4	C	171.99	-
4 a	C	119.03	-
5	CH	95.16	7.53 d
6	C	131.28	-
7	CH	125.15	7.85 d
8	CH	122.49	7.45
8 a	C	163.43	-
9 a	C	166.12	-
10	CH_3	26.02	1.29 d
11	CH_3	24.94	1.46 d
12	CH_2	64.29	4.51 d
13	N-CH_3	34.44	3.6 s
14	O-CH_3	60.28	4.05 s

The presence of proton signal at δ 5.2 ppm (1H, t) due to the proton of methine group (C-3) and the signal at δ 4.51 (2H, d, J= 14.7, Hz) assigned to the protons of hydroxy methylene group (C-12) in the ^{1}H–NMR spectrum indicated that the two groups attached to each others.

The position of methoxyl group at C-6 was confirmed by the ^{13}C-NMR spectrum through the difference in the chemical shifts of the C-5 and C-6 in comparing with compound I and spectral data reported in the literature (**Noshita *et al.* 2001 and Biavatti *et al.* 2002)**. Also the lack of a bathochromic shift on the addition of NaOAc in the UV spectrum indicated the absence of free hydroxyl group at the C-6 position. The presence of dihydrofuran ring was established by comparing carbon signals of this ring with previously reported **(Reisch *et al*. 1969, Mohr *et al.* 1982, Boyd *et al*. 2000 and Biavatti *et al.* 2002).** The position of the two geminal methyl groups at the C-2 were established by comparing the ^{13}C- signals with previously reported **(Boyd *et al* 2000).**

The diagnostic fragment ions of mass spectrum (Fig. 6) at m/z 289 (M^{+}; 19.0%), 242 (M- C_2H_7O; 19.0%), 216 (M- C_4H_9O; 52.2%), 188 (M- $C_5H_9O_2$; 27.7%), 148 (M- $C_7H_{11}NO_2$; 19.6%), 130 (M- $C_7H_{13}NO_3$; 12%), 105 (M- $C_9H_{14}NO_3$; 42.4%), 77 (M- $C_{11}H_{18}NO_3$; 39.7%), and 57 (M- C_{13} $H_{14}NO_3$; 100%), were further supported the above assigned structure.

From these data, the structure of this compound was deduced to be 3-(hydroxy methyl)- 6-methoxy- 2, 2, 9-trimethyl-dihydrofuro [2,3] quinol -4-one (Fig. 9). This compound was also isolated for the first time from this plant.

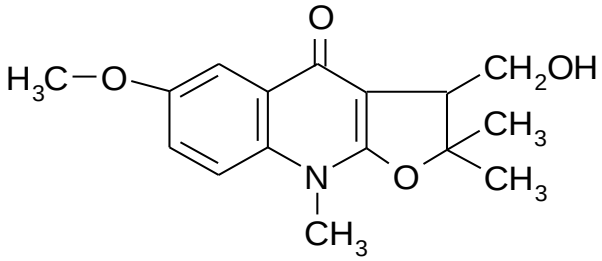

Fig. (9) Structural formula of compound II ($C_{16}H_{19}NO_4$)

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النشاط المضاد لمركبان من قلويدات الفيوروكوينولون المستخلصة من اوراق نبات الروتا كاليبنسس ضد بكتريا العفن الطرى للبطاطس ايرونيا كاروتوفورا

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١- قسم الكيمياء الحيوية – كلية الزراعة – جامعة القاهرة – فرع الفيوم.

٢- قسم النبات "الميكروبيولوجى" كلية العلوم – جامعة القاهرة – الجيزة.

يعتبر العفن الطرى الذى تسببه بكتريا "ايرونيا كاروتوفورا" من اكثر الامراض خطورة على درنات البطاطس بعد حصادها على النطاق العالمى وذلك اثناء عمليات التخزيـن والنقل والتسويق. ونظرا لان استخدام مبيدات البكتريا المخلقة صناعيا في مكافحة هذا المرض تؤدى الى تأثيرات ضارة على الانسان و البيئة علاوة على خفض كمية البطاطس المصدرة للاسواق الاوربية لذا فقد زاد الاهتمام بالمنتجات الطبيعية ذات الفعالية لاستخدامها ضد هذه البكتريا المسببة للمرض. وفى هذه الدراسة اظهر مستخلص الايثانول المائى (٨٠%) لاوراق نبات الروتا كاليبنسس فعالية ضد بكتريا الايرونيا كاروتوفورا وكان أقل تركيز مثبط (MIC) له ٦٢٥ ميكروجرام/مل. ونتجت عن عملية الفصل المقرونة باختبار الفعالية للمكونات المفصولة باستخدام طرق التحليل الكروماتوجرافي (الطبقة الرقيقة و الاعمدة) عن فصل مركبان فعالان من قلويدات الفيوروكونيولون لأول مرة من هذا النبات وتم التعرف على التركيب الكيميائى لكلا منهما باستخدام طرق التحليل الطيفى (الرنين المغناطيسى وتقدير الكتلة) حيث وجد أنهما ٢- (هيدروكسى ايزوبروبيل) ٦- هيدروكسى – ٩-ميثيل - دايهيدروفيورو (٢، ٣) –كوينول-٤- أون (I)، ٣ (هيدروكسى ميثيل) ٦-مثيوكسى –٢، ٢، ٩ ثلاثى ميثيل –دايهيدروفيورو (٢، ٣) –كوينول ٤-اون (II). وكانت فعالية هذان المركبان ضد هذه البكتريا MIC تساوى ٢٠، ٣٥ ميكروجرام/مل على الترتيب.