



## Phytochemistry and antimicrobial compounds of *Hymenocrater calycinus*

Ahmad Reza Gohari<sup>1\*</sup>, Soodabeh Saeidnia<sup>1</sup>, Ahmad Reza Shahverdi<sup>2</sup>,  
Narguess Yassa<sup>3</sup>, Maryam Malmir<sup>1</sup>, Kamyar Mollazade<sup>2</sup>, Ali Reza Naghinejad<sup>4</sup>

<sup>1</sup>Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Pharmaceutical Biotechnology and Pharmaceutical Biotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

<sup>3</sup>Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

<sup>4</sup>Department of Botany, Faculty of Sciences, University of Tehran, Tehran, Iran

\*Corresponding Author: goharii\_a@sina.tums.ac.ir

### Abstract

The genus, *Hymenocrater*, belongs to the plant family Lamiaceae which contains eleven shrub species, of which *H. calycinus* belongs and grows wildly in Northeastern Iran. From the ethyl acetate and methanol extracts of the flowered aerial parts of *Hymenocrater calycinus*, four compounds were isolated using chromatographic methods and identified by spectroscopic data (MS, <sup>1</sup>H- and <sup>13</sup>C-NMR, HMQC, HMBC and <sup>1</sup>H-<sup>1</sup>H COSY). The effect of rosmarinic acid, as the main component in our study was applied to *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger* by the broth dilution method. Isolated compounds were identified as  $\beta$ -sitosterol (1), ursolic acid (2), rosmarinic acid (3) and quercetin-3-O- rutoside (4). The results of our assay against bacteria and fungi show that, rosmarinic acid has an antifungal property against *Candida albicans* (MIC, 250  $\mu$ g mL<sup>-1</sup>).

**Keywords:** Antifungal activity, *Hymenocrater calycinus*, quercetin-3-O- rutoside, rosmarinic acid, ursolic acid,  $\beta$ -sitosterol.

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### INTRODUCTION

The genus, *Hymenocrater* Fisch.et Mey., belongs to the plant family Lamiaceae and named Gol-e-Arvaneh in the Persian language. It contains eleven shrub species with colorful calyxes based on Flora Iranica. Some of those species are endemic to Iran such as; *H. incanus* Bunge, *H. oxyodontus* Rech. f., and *H. yazdianus* Rech. f. Among the *Hymenocrater* species, *H. calycinus* (Boiss.) Benth. is growing wildly in the north east of Iran and some parts of Turkmania as an endemic plant (Rechinger 1982, Mozaffarian 1996).

Literature reviews show that there are a few reports on phytochemical investigation of the *Hymenocrater* genus. The antibacterial and antifungal activity of the methanol extract of *H. sessilifolius* Benth., growing widely in

the Balochistan area of Pakistan, has been determined using 12 fungal and 12 bacterial strains by agar well diffusion and disk diffusion assays (Zaidi and Crow 2005). Chemical constituents of the essential oil of *H. incanus* (from Iran), has been studied and the main components reported as 1, 8-cineole, and  $\beta$ -caryophyllene (Mirza et al. 2001). Essential oils from the aerial parts of *H. calycinus*, collected from three different locations in Iran (at Khorasan and Golestan provinces), were analyzed by GC and GC/MS. The compounds  $\beta$ -pinene, sabinene, spathulenol, abietatriene,  $\beta$ -caryophyllene, and caryophyllene oxide were the most

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abundant compounds (Firouznia et al. 2005). Here, we report the separation and identification of some phenolic and triterpene compounds from the aerial parts of *H. calycinus* which has not been previously reported, together with the evaluation of the main phenolic acid constituent effect against some bacteria and fungi.

## MATERIAL AND METHODS

### Plant material

The aerial parts of *Hymenocrater calycinus* (Boiss.) Benth. were collected from Firuzkuh near the village of Delichae, during the flowering stage in July 2005. The Herbarium specimen was identified by Dr. Ali Reza Naghinejad from the Faculty of Sciences, University of Tehran and deposited at the mentioned Herbarium.

### Experimental

The  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were measured on a Bruker Avance 500 DRX (500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ ) spectrometer with tetramethylsilane as an internal standard and chemical shifts are given in  $\delta$  (ppm). The MS data was recorded on a Agilent Technology (HP) instrument with a 5973 Network Mass Selective Detector (MS model). Silica gel 60F<sub>254</sub> pre-coated plates (Merck) were used for the TLC. The spots on the plates, were detected by spraying an anisaldehyde-H<sub>2</sub>SO<sub>4</sub> reagent followed by heating the plates to 120°C using a hot plate for 5 min.

### Isolation process

The flowered aerial parts of *H. calycinus* (400 g) were cut into small pieces and extracted with ethyl acetate and methanol at room temperature, for 48 h. The ethyl acetate extract (4.6 g) was subjected to silica gel column chromatography (CC) with hexane: CHCl<sub>3</sub> (19:1, 4:1), CHCl<sub>3</sub>: AcOEt (2:8) and AcOEt as an eluent to give eight fractions (E<sub>1</sub>-E<sub>8</sub>). The fraction E<sub>5</sub> (670 mg) was added to silica gel CC with hexane: CHCl<sub>3</sub>: AcOEt (5:14:1) to obtain E<sub>51</sub>-E<sub>56</sub>. The fraction E<sub>55</sub> was chromatographed on sephadex LH20 with AcOEt: MeOH (3:1) resulting in compound 1 (42 mg). The fraction E<sub>7</sub> was submitted twice to sephadex LH<sub>20</sub> with AcOEt: MeOH (2:1), to resulting in compound 2 (12 mg).

The MeOH extract (7.4 g) was successively subjected to Reverse Phase (RP) silica gel column chromatography with MeOH: H<sub>2</sub>O (4:6, 7:3 and 1:0) as an eluent to give five fractions (A-E). Fraction A (3.3 g) was fractionated by a silica gel CC with AcOEt: MeOH (6:4) to yield five parts (A<sub>1</sub>-A<sub>5</sub>). Compound 3 (134 mg) was obtained from fraction A<sub>2</sub> (1.2 g) using sephadex CC with MeOH.

Another part of the MeOH extract (4 g) was subjected to silica gel CC with CHCl<sub>3</sub>: AcOEt (4:6, 6:4, 0:1) and MeOH as an eluent to obtain five fractions (M<sub>1</sub>-M<sub>5</sub>). Fraction M<sub>5</sub> (2.72 g) was subjected twice to sephadex LH<sub>20</sub> with MeOH, and then paper chromatography with BAW (n-butanol: acetic acid: water, 4: 1: 5) to obtain compound 4 (11 mg).

### Biological activity

The antibacterial and antifungal activities of compound 3 (rosmarinic acid) were determined using *Staphylococcus aureus* (ATCC 29737), *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 14053) and *Aspergillus niger* (ATCC 16404) by the broth dilution method (Momen-Roknabadi et al. 2008). The bacteria and *C. albicans* were maintained on Nutrient broth (Difco) and adjusted to  $1 \times 10^6$  and  $1 \times 10^4$  organism/mL, respectively. Inoculum for *A. niger* was prepared as a spore suspension from a 5 days old agar-surface culture and adjusted to a concentration of approximately  $1 \times 10^4$  in a final volume of cultures.

The susceptibility of the strains to compound 3 was performed by determining of the minimum inhibitory concentrations (MICs) of the isolates using the broth dilution method. First stock solution of the sample at a concentration of 20 mg/mL was prepared in DMSO (4ul). The test sample was further diluted in sterile water to obtain serial dilution concentrations from 12.5  $\mu\text{g/mL}$  to 800  $\mu\text{g/mL}$ . Then 0.5 mL of each concentration was added to 0.5 mL of double strength medium broth for a test strain. Inoculum which was prepared as described above was introduced to each test tube. The cultured tubes were incubated at 37°C for bacteria for 24 h, at 30°C for 24 h for *C. albicans* and at 25°C for 3 days for *A. niger*. The lowest concentration at which no growth was observed, were

recorded as MICs. A culture media with different concentration of Gentamycin and Fluconazole were used as a control and DMSO (4  $\mu$ L) was used as a negative control. All experiments were performed in triplicate.

## RESULTS AND DISCUSSION

Isolated compounds (Fig. 1) from the ethyl acetate and MeOH extracts of *H. calycinus* were identified as,  $\beta$ -sitosterol (1), ursolic acid (2), rosmarinic acid (3) and quercetin-3-O-rutinoside (4) by comparison of their NMR and MS spectral data with those reported in literature (Agrawal 1989, Goad and Akihisa 1997, Gohari et al. 2003, 2005).

**$\beta$ -sitosterol (1).**  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ ):  $\delta_{\text{H}}$  0.68 (3H, s, H-18), 0.81 (3H, br s, H-26), 0.82 (3H, br s, H-27), 0.84 (3H, br s, H-24b), 0.92 (3H, d,  $J = 6.7$  Hz, H-21), 1.01 (3H, s, H-19), 3.52 (1H, m, H-3), 5.35 (1H, m, H-6).  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CDCl}_3$ ):  $\delta_{\text{C}}$  (from C-1 to C-27) 37.3, 31.7, 71.8, 42.3, 140.8, 121.7, 31.9, 31.9, 50.2, 36.5, 21.1, 39.8, 42.3, 56.8, 24.3, 28.3, 56.1, 11.9, 19.8, 36.2, 18.8, 34.0, 26.1, 45.8, 29.2, 19.0, 19.4, 23.1 (C-24a), 12.0 (C-24b).

**Ursolic acid (2).**  $^1\text{H-NMR}$  (500 MHz, Pyridine- $d_5$ ):  $\delta_{\text{H}}$  0.88 (3H, s, H-25), 0.95 (3H, d,  $J = 6.4$  Hz, H-30), 1.00 (3H, d,  $J = 6.4$  Hz, H-29), 1.02 (3H, s, H-24), 1.05 (3H, s, H-26), 1.22 (3H, s, H-27), 1.24 (3H, s, H-23), 2.12 (1H, dt,  $J = 12.3, 4.2$  Hz, H-16a), 2.33 (1H, dt,  $J = 12.2, 4.2$  Hz, H-16b), 2.63 (1H, d,  $J = 11.3$  Hz, H-18), 3.45 (1H, dd,  $J = 10.1, 5.8$  Hz, H-3), 5.48 (1H, br s, H-12).  $^{13}\text{C-NMR}$  (125 MHz, Pyridine- $d_5$ ):  $\delta_{\text{C}}$  (from C-1 to C-30) 39.4, 28.1, 78.1, 39.1, 55.8, 18.8, 33.6, 40.0, 48.0, 37.3, 23.6, 125.7, 139.3, 42.5, 28.7, 24.9, 48.0, 53.6, 39.5, 39.4, 31.1, 37.5, 28.8, 16.6, 15.7, 17.5, 23.9, 179.9, 17.5, 21.4.

**Rosmarinic acid (3).** See Table 1 for nmr data.

**Quercetin-3-O-rutinoside (4).**  $^1\text{H-NMR}$  (500 MHz,  $\text{DMSO}-d_6$ ):  $\delta_{\text{H}}$  1.02 (3H, d,  $J = 6.1$  Hz,  $\text{H}_{\text{Rha-6}}$ ), 4.40 (1H, brs,  $\text{H}_{\text{Rha-1}}$ ), 5.27 (1H, d,  $J = 6.8$  Hz,  $\text{H}_{\text{Glc-1}}$ ), 6.08 (1H, brs, H-6), 6.27 (1H, brs, H-8), 6.81 (1H, d,  $J = 8.4$  Hz, H-5'), 7.51 (1H, brs, H-2'), 7.55 (1H, d,  $J = 8.4$ , H-6'), 12.97 (1H, s, OH-5).  $^{13}\text{C-NMR}$  (125 MHz,  $\text{DMSO}-d_6$ ):  $\delta_{\text{C}}$  157.0 (C-2), 133.9 (C-3), 177.6 (C-4), 161.8 (C-5), 100.4 (C-6),

164.4 (C-7), 94.9 (C-8), 157.5 (C-9), 104.1 (C-10), 121.3 (C-1'), 116.5 (C-2'), 146.0 (C-3'), 150.7 (C-4'), 116.7 (C-5'), 122.5 (C-6'), 102.7 ( $\text{H}_{\text{Glc-1}}$ ), 75.0 ( $\text{H}_{\text{Glc-2}}$ ), 77.4 ( $\text{H}_{\text{Glc-3}}$ ), 70.8 ( $\text{H}_{\text{Glc-4}}$ ), 76.7 ( $\text{H}_{\text{Glc-5}}$ ), 67.9 ( $\text{H}_{\text{Glc-6}}$ ), 101.7 ( $\text{H}_{\text{Rha-1}}$ ), 71.2 ( $\text{H}_{\text{Rha-2}}$ ), 71.5 ( $\text{H}_{\text{Rha-3}}$ ), 72.8 ( $\text{H}_{\text{Rha-4}}$ ), 69.1 ( $\text{H}_{\text{Rha-5}}$ ), 18.6 ( $\text{H}_{\text{Rha-6}}$ ).

Here, we have been focused on the main constituent, compound 3, isolated from the methanol extract of *H. calycinus* by using sephadex and silica gel column chromatography. HMBC, HMQC, and  $^1\text{H}-^1\text{H}$  COSY data of the compound 3 is given in Table. 1.

The manner in which the caffeic acid and  $\beta$ -(3,4-dihydroxyphenyl) glyceric acid were linked together in compound 3 was established by application of a long-range coupling experiment (HMBC) which showed coupling between H-8' ( $\delta$  4.92) and C-9 ( $\delta$  167.1). The HMBC correlations and full

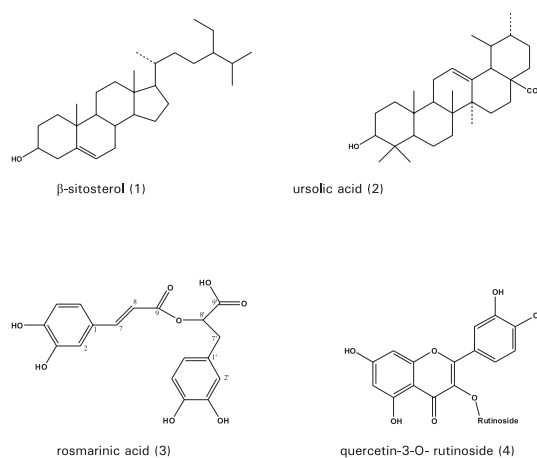


Fig. 1. The structures of isolated compounds from *H. calycinus*.

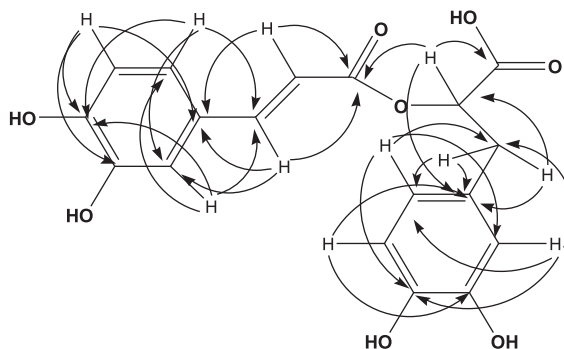


Fig. 2. The HMBC correlations and full assignments of rosmarinic acid.

**Table 1.** HMBC, HMQC, and  $^1\text{H}$ - $^1\text{H}$  COSY data of rosmarinic acid in DMSO- $d_6$ .

C	HMQC		HMBC	H-H COSY
	$\delta_c$	$\delta_H$		
1	126.3		H-5, H-7, H-8	
2	115.8	7.05(d, J = 1.4, 1H)	H-6, H-7	H-6
3	146.7		H-5	
4	149.4		H-2, H-5, H-6	
5	116.8	6.77(d, J = 8.1, 1H)	H-6	
6	121.8	6.94(dd, J = 8.2, 1.5, 1H)	H-2, H-7	H-2, H-5
7	145.4	7.40(d, J = 15.8, 1H)	H-2, H-6	H-8
8	115.5	6.20(d, J = 15.9, 1H)		H-7
9	167.1		H-7, H-8, H-8'	
10	130.4		H-5', H-7'a, H-7'b, H-8'	
2'	117.5	6.69(d, J = 1.5, 1H)	H-6'	H-6'
3'	145.8		H-5'	
4'	144.5		H-2', H-6'	
5'	116.3	6.60(d, J = 8.0, 1H)		H-6'
6'	120.5	6.50(dd, J = 8.0, 1.5, 1H)	H-2', H-7'a, H-7'b	H-2', H-5'
7'	37.9	2.81(dd, J = 10.1, 10, 1H), 3.05(d, J = 10, 1H)	H-2', H-6', H-8' H-7'a, H-8'	H-7'b, H-8'
8'	74.4	4.92(dd, J = 10, 2.8, 1H)	H-7'a	H-7'a, H-7'
9'	174.0		H-8'	

**Table 2.** The results of anti-bacterial and anti-fungal assays for rosmarinic acid.

Compounds	Minimum inhibitory concentrations ( $\mu\text{g}/\text{mL}$ )			
	<i>Staphylococcus aureus</i> (ATCC 29737)	<i>Escherichia coli</i> (ATCC 8739)	<i>Aspergillus niger</i> (ATCC 16404)	<i>Candida albicans</i> (ATCC 14053)
Rosmarinic acid	500	500	1000	250
Gentamycin	16	8	ND*	ND
Fluconazole	ND	ND	8	16

\*ND = not detected

assignments of compound 3 are presented in Fig. 2. Compound 3 was therefore identified as rosmarinic acid which has been reported to be present in the aerial parts of *Dracocephalum kotschyi* Boiss. and *Salvia officinalis* L. (Lu and Foo 1999, Gohari et al. 2003).

Rosmarinic acid occurs throughout the Boraginaceae, whereas within the Lamiaceae it is restricted to the subfamily Nepetoideae. A multitude of biological activities have been described for rosmarinic acid. The main activities are astringent, anti-oxidative, anti-inflammatory, anti-mutagen, antibacterial, and anti-viral. The latter activity is used in the treatment of *Herpes simplex* infections (Petersen and Simmonds 2003).

In conclusion, the results of our experiments with bacteria and fungi (Table 2) indicates that rosmarinic acid has an antifungal property against *Candida albicans* (MIC, 250  $\mu\text{g mL}^{-1}$ ). Rosmarinic acid is rapidly eliminated from the blood and shows a very low toxicity (Petersen and Simmonds 2003) so, rosmarinic acid could find a position in human therapies using further pharmacological investigations.

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## ***Hymenocrater calycinus* Özütünün Fitokimyası ve Antimikrobiyal Bilesikleri**

### **Özet**

*Hymenocrater* cinsi, Lamiaceae familyasına dahil olup, onbir çalı türünü barındırır. Bunların içinden *H. calycinus* kuzeydoğu İran'da doğal olarak yetişir. *Hymenocrater calycinus*'un çiçekli kısımlarından elde edilen etil asetat ve metanol özütlerinden, kromatografik yöntemlerle dört bileşik izole edildi ve spektroskopik data aracılığı ile tanımlandılar (MS, <sup>1</sup>H- ve <sup>13</sup>C-NMR, HMQC, HMBC ve <sup>1</sup>H-<sup>1</sup>H COSY). Çalışmamızın ana bileşimi olan rosmarinik asitin etkisi, broth seyreltme metodu kullanılarak, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* ve *Aspergillus niger*'a uygulandı. İzole edilen bileşimler; β-sitosterol (1), ursolic acid (2), rosmarinic acid (3) ve quercetin-3-O- rutinoside (4) şeklinde belirlendi. Bakteri ve mantarlarla yaptığımız çalışma, rosmarinik asitin *Candida albicans* (MIC, 250 µg mL<sup>-1</sup>)'a karşı antifungal bir etkisi olduğunu göstermektedir.

**Anahtar Kelimeler:** Antifungal aktivite, *Hymenocrater calycinus*, quercetin-3-O- rutinosit, rosmarinik asit, ursolik asit, β-sitosterol.