ORIGINAL ARTICLE

New technology acquisition 3α,14α,22*r*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one and biological research anti-inflammatory activities

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Abstract

Aim: Design a new pharmaceutical technology for isolation novel ecdysteroid 3α,14α,*22R*, *25*-tetrahydroxy-5α (*H*)-cholest-7-en-6-one from the epigeal organs of *ComastomatenellaRottb*. Research NMR spectrscopy and biological activity. **Material and Methods:** The isolation procedure involved extraction with aqueous ethanol, partitioning(petroleum ether–ethyl acetate) of the obtained extract to remove non-polar impurities, extraction of the water layer with isobutyl alcohol, and subsequent purification by aluminum oxide column chromatography. According to NMR spectroscopy, mass spectrometry, and microanalysis, the structure of 3α,14α,*22R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1). **Results and Discussion:** During the study of plants *ComastomatenellaRottb* was isolated new phytoecdysteroid.According to NMR spectroscopy, mass spectrometry, and microanalysis, the structure of 3α,14α,*22R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one.Isolated object was examined for anti-inflammatory activity of the compound.Thus, the present study established high anti-inflammatory activity of 3α,14α,*22R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one1 in the dose of 50 mg/kg in an experimental model of an acute exudative reaction. **Conclusion:** From the results of the study on the anti-inflammatory activity, it became clear that object potently and significantly reduced the number of abdominal writhings as compared to the control animals.

Key words: Biological activities, *ComastomatenellaRottb*, ecdysteroid

INTRODUCTION

It is known that plants produce more than 10,000 various substances of different classes serving as cardiac and anti-tumor agents, hormones, diuretics, antibiotics, analgesics, etc. A unique and rich flora of the Republic of Kazakhstan with more than 6000 plant species of which 667 species are endemic, and most of them are scarcely studied can be regarded as a potentially renewable source for the development and production of novel original plant-based medicines with anabolic, adaptogenic, tonic, and other activities.

Ecdysteroids have also been found in some species of the genera *Dianthus* and *Melandrium* [1,2]. To the best of our knowledge, there are no data on ecdysteroids produced by the genus *Comastoma tenella* Rottb. The roots of *C. tenella* Rottb. [3] contain up to 12% of triterpenoid saponins and are widely used together with the roots of another plant, *Saponaria Officinalis* L., as a foamer in production of sparkly drinks and halva, a type of dense sweet confections. The ecdysteroid composition of *C. tenella* Rottb. has not yet been studied. In this work, we focused on isolation, structure elucidation, and biological activities of novel ecdysteroid from *C. tenella* Rottb.

MATERIALS AND METHODS

General

Optical rotations were measured on a JASCO P-1020 polarimeter. Infrared (IR) spectra (KBr) were recorded

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on a Bruker FT-IR-113V spectrometer. Ultraviolet (UV) spectra were determined with a Shimadzu UV-2450 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker AV-600 instrument at 600 MHz (^{1}H) and 125 MHz (^{13}C) with TMS as an internal standard. HRESI mass spectra were acquired on an Agilent 6520B Q-TOF mass instrument. Column chromatography was performed with aluminum oxide (neutral, Brockmann I). Analytical thin layer chromatography (TLC) was carried out on precoated plates Sorbfil. Reversed-phase high-performance liquid chromatography (HPLC) was performed with a Hewlett-Packard Agilent 1100 Series system (Zorbax SB-C_{18} analytical column, 150 mm × 4.6 mm), elution with 10% aqueous propan-2-ol (elution flow rate of 0.75 mL/min), UV-detection at 254 nm, the column temperature was kept at 20°C, volume of the analytical sample was 20 µl.

Plant Material

The epigeal organs of *C. tenella* Rottb. were collected nearby Karakum village in Zaisan river shores of Ust-Kamenogorsk region in May 2016 in the budding phase. Taxonomic identification was done by doctor of Biological Sciences, Professor A. Kupriyanov. The collected plant materials were air dried and powdered.

Extraction and Isolation

Preliminary, we analyzed the amount and composition of extractives obtained by extraction of *C. tenella* Rottb. with 70% aqueous ethyl alcohol (EtOH). Reversed-phase HPLC revealed that *C. tenella* Rottb. is the promising source for ecdysteroids. The main ecdysteroid of the epigeal part of *C. tenella Rottb*. is ecdysterone, 20-hydroxyecdysone, with the content up to 0.19% [Figure 1].

Air-dried powdered epigeal plant organs (leaves, flower buds, stems) (1.0 kg) were extracted four times with 70% aqueous EtOH (10 L) under reflux for 1-1.5 h. The obtained extracts were cooled, separated from plant materials, and the solvent was removed under vacuum maintaining the temperature below 50°C. The resulting thick syrupy brown residue was diluted with ethanol (0.2 L) and water (0.4 L) and then partitioned with petroleum ether–ethyl acetate (0.6 L, 2:1) to remove the nonpolar components. The aqueous phase was extracted with isobutyl alcohol (0.6 L) to give a dense extract. The butanolic extracts were combined, and the solvent was removed under vacuum to give a dense syrupy green residue (81 g) containing a sum of ecdysteroids and other extractives. TLC and qualitative analysis of the crude product revealed the presence of ecdysteroids. Multiple aluminum oxide column chromatography (sorbent weight of 1.6 kg, elution with chloroform-ethanol [90:10]) afforded 600 mg of an individual substance (TLC data). Biological assays were performed in the Laboratory of Experimental and Clinical Pharmacology of the Belarusian Medical University.

Main fractions were recrystallized from ethyl acetate-ethanol. Further aluminum oxide column chromatography yielded novel ecdysteroid 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1).

3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1)

Crystalline solid (MeOH/H_2O); mp 230-232°C, $[\alpha]_D^{27}$ + 99.7 (*c* 0.25, MeCN); UV (MeCN) λ_{max} (log ε) 241 (4.11) nm; IR (KBr) ν_{max} 3314, 2969, 1646, 1040, 1029 cm^{-1}; ^{1}H and ^{13}C NMR spectral data [Tables 1 and 2]; HRESI-MS *m/z* 471.3069 $[M+Na]^+$ (calcd for $C_{27}H_{44}O_5Na$,471.3064).

Absolute Configuration of H-5 of Compound 1

Position of the substituents and the α-orientation of the proton at C-5 was established by modern spectroscopic methods (mass spectrometry, IR and UV spectroscopy, ^{1}H and ^{13}C NMR spectroscopy and two-dimensional [2D] COSY and ROESY NMR technique).

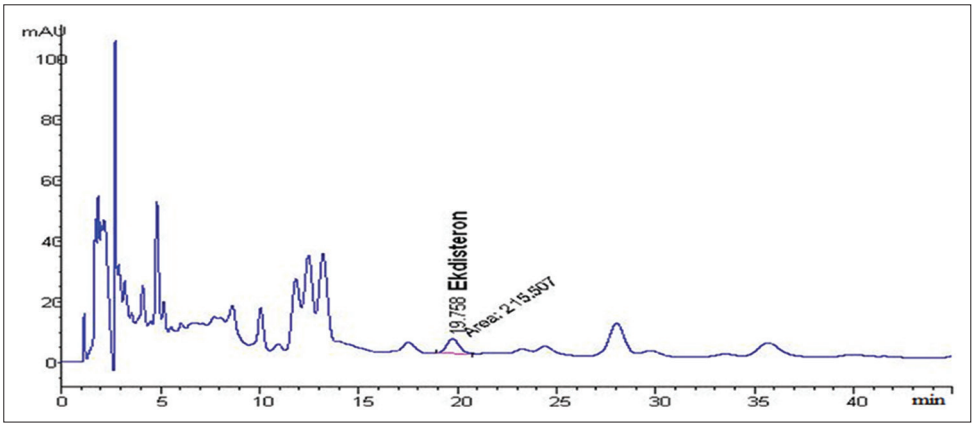

Figure 1: Reversed phase high-performance liquid chromatography profile of the epigeal part of *Comastoma tenella* Rottb.

Table 1: ^{1}H, ^{13}C and HMBC NMR spectroscopic data for3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1) in DMSO

Position	δ_H	δ_C	HMBC
1	1.488 1.375	28.40	C-3, C-5, C-19 C-3
2	1.643 1.529	27.91	C-3 C-10
3	3.827[a]	62.61	
4	1.692 1.405	31.75	C-3 C-10
5	2.274[b]	50.18	C-3, C-4, C-6, C-7, C-10, C-19
6		201.96	
7	5.643	119.93	C-5, C-9, C-14
8		164.84	
9	3.076	33.16	C-1, C-7, C-8, C-11, C-19
10		35.77	
11	1.635 1.503	20.30	C-8, C-13 C-13, C-12
12	2.026 1.623	30.43	C-11, C-13, C-14, C-17, C-18 C-11, C-13, C-14, C-17, C-18
13		46.92	
14		82.75	
15	1.847 1.527	30.16	C-13, C-14, C-16, C-17 C-13, C-14, C-16, C-17
16	1.843 1.383	25.45	C-13, C-14, C-15, C-17 C-13, C-14, C-15, C-17, C-20
17	1.952	46.92	C-12, 13, 16, 18,20, 21
18	0.617	15.03	C-12, C-13, C-14, C-17
19	0.881	23.45	C-1, C-5, C-9, C-10
20	1.616	41.49	C-13, C-17, C-22, C-23
21	0.851	12.60	C-17, C-20, C-22
22	3.430	72.27	C-21
23	1.366 1.177	24.14	C-20, C-22, C-25 C-22, C-24, C-25
24	1.655 1.281	40.96	C-22, C-23, C-25, C-26, C-27 C-22, C-23, C-25, C-26, C-27
25		68.55	
26	1.094	29.49	C-24, C-25, C-27
27	1.080	28.97	C-23, C-24, C-25, C-26
OH-3	4.296		C-2, C-3, C-4
OH-14	4.487		C-8, C-13, C-14, C-15
OH-22	4.024		C-20, C-22, C-23
OH-25	3.928		C-24, C-25, C-26, C-27

[a]Multiplet with the widened components, the peak width at half height is 12 Hz. [b]Doublet of doublets, $J_{5,4(ax)}$=12 Hz, $J_{5,4(eq)}$=4 Hz. DMSO: Dimethyl sulfoxide, HMBC: Heteronuclear multiple bond correlation, NMR: Nuclear magnetic resonance

Table 2: ^{1}H and ^{13}C NMR spectroscopic data for3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one1 andecdysone E20 in CD_3OD

Position	1		E20 [5]	
	δ_H	δ_C	δ_H	δ_C
1	1.46; 1.63	29.92	1.79; 1.43	37.33
2	1.67; 1.81	29.08	3.84	68.69
3	3.97	65.53	3.95	68.50
4	1.56; 1.83	33.24	1.69; 1.76	32.89
5	2.44	52.27	2.38	51.79
6		206.38		206.55
7	5.81	121.82	5.82	122.01
8		168.15; 167.96		167.63
9	3.20	49.42	3.16	35.23
10		37.59		39.24
11	1.75; 1.65	22.11	1.81; 1.67	21.57
12	2.11; 1.76	32.39	2.11; 1.77	32.03
13		48.57		48.11
14		85.39		85.07
15	1.96; 1.60	31.91	1.96; 1.60	32.07
16	1.50; 1.96	27.08	1.50; 1.96	27.00
17	2.03	48.91	2.03	48.79
18	0.73	16.21	0.73	16.18
19	0.97	24.36	0.97	24.46
20	1.75	43.45	1.75	43.46
21	0.95	13.29	0.95	13.22
22	3.60	75.36	3.59	75.24
23	1.53; 1.33	25.58	1.54; 1.32	25.30
24	1.78; 1.44	42.27	1.78; 1.41	42.25
25		71.40		71.41
26	1.19	29.08	1.19	29.05
27	1.20	29.49	1.20	29.61

NMR: Nuclear magnetic resonance

Anti-inflammatory Activity

Anti-inflammatory activity of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one was evaluated in white outbred adult male rats with average weight of 209-246 g; the animals were kept under standard vivarium conditions. The rats were divided into three groups of five animals for each (total 15 animals). Acute exudative reaction (peritonitis) was induced by an intraperitoneal injection of 1% aqueous acetic acid in a dose of 1 ml/100 g of the rat body weight. 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one and diclofenac sodium (comparator drug) were administrated orally 1 h before the acetic acid injections in the single doses of 50 and 25 mg/kg, respectively, with starch mucilage. The rats were sacrificed 3 h after acetic

acid injection, the peritoneal cavities were dissected, and the exudates were collected. The volumes of the peritoneal exudates were estimated following the known procedure [4]. A control group of animals received only starch mucilage in an equivolume amount.

RESULTS AND DISCUSSION

The structure of compound 1 [Figure 2] was established by mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy and confirmed by IR and UV spectroscopy. The ^{13}C NMR spectrum revealed signals of 27 carbon atoms including five signals for the methyl carbon atoms, nine signals for the methylene carbons, seven signals for the methine carbons, and six signals for the quaternary C atoms (data of APT, attached proton test). The ^{1}H NMR spectrum contained *inter alia* signals for five methyl groups: Four of them resonated as singlets and the fifth methyl group appeared as doublet with spin-spin coupling constant of 6 Hz. The other proton signals were located in the region of δ_H 1.1-5.2. The signals in ^{1}H and ^{13}C NMR spectra were assigned using 2D homonuclear $^{1}H/^{1}H$ COSY, TOCSY, ROESY and heteronuclear $^{1}H/^{13}C$ HSQC and heteronuclear multiple bond correlation (HMBC) NMR experiments [Table 1].

Analysis of the COSY and TOCSY spectra allowed assignment of all proton signals belonging to four ring systems (A-D), two angular methyl groups, and the protons of the C-20-C-27 opened chain. The analysis of HSQC and HMBC spectra led to full assignment of the signals in ^{13}C NMR spectra [Table 1] and revealed double bond at C-7-C-8

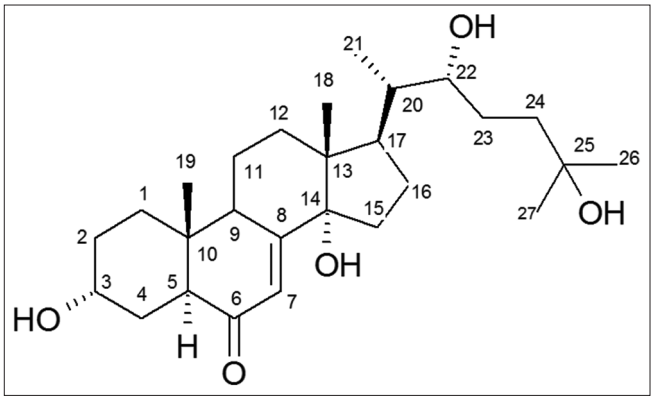

Figure 2: Structure of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1)

and the carbonyl group at C-6, as well as location of the hydroxy groups at C-3, C-14, C-22, and C-25. Type of the A and B ring fusing (*trans*) was obvious from the H-5 signal splitting (*dd*, $J_{5,4(ax)}$ = 12 Hz, $J_{5,4(eq)}$ = 4 Hz). The axial orientation of the hydroxy group at C-3 is followed from the shape of the H-3 signal, which is a broadened singlet with peak width at half height of 12 Hz, that is the axial-axial coupling constants are absent in the signal, and the H-3 proton is an equatorial position in the A ring. Agreement between the chemical shifts for the atoms in the C and D rings and the C-20-C-27 chains in ^{1}H and ^{13}C NMR spectra of compound 1 and ecdysone E20 [5] suggests the same stereochemistry for these parts of the molecules of both compounds [Table 2].

Thus, we isolated novel ecdysteroid, 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one 99.9% purity, molecular formula $C_{27}H_{44}O_5$, R_f 0.4 from *C. tenella* Rottb. According to the spectral data have a structure of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1).

Plant ecdysteroids exert a wide range of biological activities on various biological objects. The most important effects of ecdysteroids on mammals are adaptogenic and tonic actions; several ecdysteroids also show anabolic activity. It is known that the anabolic activity level depends on both the number and positions of the hydroxy groups in ecdysteroid skelenon. The presence of the 2,3-diol system and the hydroxy group at C-20 is the most significant for the anabolic activity. Hence, the anabolic activity of the known ecdysteroids decreases in the following order: Ecdysterone > 2-deoxyecdysterone > α-ecdysone > 2-deoxy-α-ecdysone [6]. Taking this issue in account, the screening for the new biological activities and study of the structure-activity relationship in the ecdysteroid family are of great importance for the clear understanding of the ecdysteroid functions in plants. Specific pharmacological activities found during these researches can further be used for the development of novel ecdysteroid-containing preparations.

In the present work, we reported on the evaluation of anti-inflammatory activities of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1) isolated from *C. tenella* Rottb.

The results of the examining of anti-inflammatory activity in a rat model are given in Table 3. The data from Table 3 revealed that the pretreatment of the experimental rats with

Table 3: Effect of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1) and diclofenac sodium on acetic acid-induced exudate formation in rats

Parameter	Control	Diclofenac sodium	3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1)
Rat weight (g)	246.60±8.32	224.00±6.00	209.30±33.70
Exudate volume[b] (mL)	6.53±0.37	5.23±0.58	4.96±0.28[a]

[a]*P*<0.05 compared with the control group. [b]Exudate volumes were measured 3 h after acetic acid injection

3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one in a dose of 50 mg/kg reduced the exudate volume by 24% of the control group 3 h after the acetic acid injection. Thus, the present study established high anti-inflammatory activity of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1) in the dose of 50 mg/kg in an experimental model of an acute exudative reaction.

CONCLUSION

During the study of plants, *C. tenella Rottb*. was isolated new phytoecdysteroid. According to NMR spectroscopy, mass spectrometry, and microanalysis, the structure of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1). Isolated object was examined for anti-inflammatory activity of the compound.

Thus, this study established high anti-inflammatory activity of 3α,14α,22*R*,25-tetrahydroxy-5α(*H*)-cholest-7-en-6-one (1) in the dose of 50 mg/kg in an experimental model of an acute exudative reaction.

From the results of the study on the anti-inflammatory activity, it became clear that object potently and significantly reduced the number of abdominal writhings as compared to the control animals.

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