

**PHYTOECDYSTEROIDS FROM
SILENE CLAVIFORMIS AND THEIR ANTIBACTERIAL
AND ANTIFUNGAL ACTIVITIES**

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Abstract

Phytoecdysteroid compounds, such as makisterone A (**1**), polypodine B (**2**), 20-hydroxyecdysone (**3**), 2,3,20,22-diasetanide 20-hydroxyecdysone (**4**), integristeron A (**5**), cyasterone (**6**), 5 α -2-deoxy- α -ecdysone (**7**), α -ecdysone (**8**) were isolated from *Silene claviformis* plant and their structures were confirmed by NMR, ¹H and IR spectroscopy. In addition, an antibacterial and antifungal potential of each pure compounds and plant extracts were assessed against different microorganisms using the agar-discs diffusion assay. Results revealed that *S. claviformis* extracts and individual phytoecdysteroids did not exhibit antimicrobial activity against tested strains of microorganisms.

Introduction

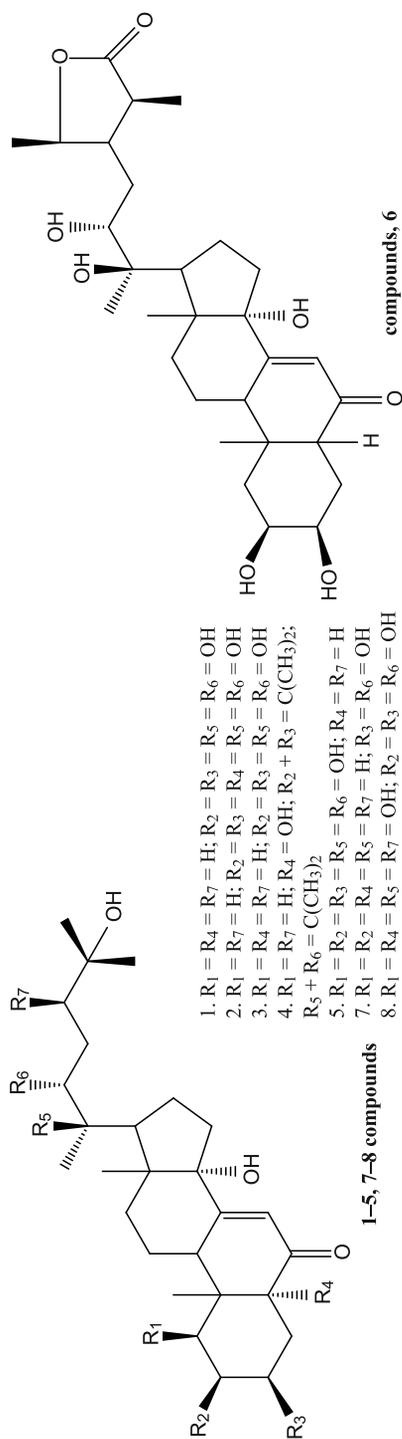
The pharmaceutical industry has come to consider traditional medicine as a source for the identification of bio-active agents that can be used in the preparation of synthetic medicine. Plants are used worldwide in medicine and agriculture. Novel drugs are developed through research of

plants (USMANOV et al. 2019a, 2019b). In Uzbekistan and other parts of Central Asia, the widespread use of medicinal plants has been traditional for centuries (MAMADALIEVA et al. 2014).

Ecdysteroids represent a particularly interesting group of natural compounds from several aspects, with functions in all kingdoms of nature: in insects, they play a crucial hormonal role in controlling molting and development (KARLSON 1974); in plants, they appear to serve as part of the chemical defense against non-adapted herbivores (SCHMELZ et al. 1998). Phytoecdysteroids (ecdysteroids of plants) are a large group of polyhydroxylated steroid compounds identical or structurally similar to insects' hormones response for molting and metamorphosis (KOOLMAN 1990). The absence of the toxic action of ecdysteroids and their strong pharmacological activity served as a stimulus to search for plants producing these substances in various ecological geographic regions of Russia and abroad (MUNKHZHARGA et al. 2010). At present, more than 450 ecdysteroids have been isolated, and their structures have been established. In particular, these compounds were isolated from the plants of the *Caryophyllaceae*. The representatives of the numerous *Silene* genus are distinguished by the diversity of structures and the high level of ecdysteroids. *The Silene genus (Caryophyllaceae) comprises more than 700 species widely distributed in temperate zones of the world* (GOLEA et al. 2017).

There are a few reports about the antimicrobial activity of ecdysteroids. However, AHMAD et al. (1996) reported the antifungal and antibacterial activity of 20E at rather high concentrations (between 100 and 400 µg/ml, i.e. $2 \cdot 8 \times 10^{-4}$ M). Antimicrobial activity of 20E and its acetates was also observed by VOLODIN et al. (1999) and MAMADALIEVA et al. (2013). Toxic effects on protozoa have also been reported; rabbits receiving 20E per os (5 mg/g/day for 3 months) showed a reduced infection with *Lambliia duodenalis* (SYROV et al. 1990), and the improvement of ruminant productivity by ecdysone was also interpreted by its toxicity towards rumen protozoa (PURSER and BAKER 1994).

The present paper deals with the isolation and structure elucidation of eight PE (Figure 1) from the butanol extract of aerial parts of *Silene claviformis*, and its antimicrobial properties.

Fig. 1. Structure of compounds 1-8 from *Silene claviformis*

Material and Methods

Plant Material

Silene claviformis is growing on the slopes of the lower belt of Tashkent and Samarkand district mountains. It is widespread in Central Asia. *S. claviformis* was collected in May, 2015 from the mountains Samarkand region and the plant materials were identified by Dr. Nigmatullayev A.M. at the Institute of the Chemistry of Plant Substances (ICPS), Uzbekistan. A voucher specimen (No. 2615) has been deposited in the herbarium Department of Herbal Plants in the ICPS, Tashkent, Uzbekistan.

Experimental Chemical Part

Extraction and Isolation

The freshly collected whole plant material (1 kg) was cut into small pieces and extracted three times with CH_3OH (3*5L) (each for tree days) at room temperature. The combined CH_3OH extract was evaporated under reduced pressure to yield a residue (105 g). The crude extract was suspended in water and extracted successively with CHCl_3 , EtOAc, and BuOH. The EtOAc extract (18 g) was subjected to column chromatography (CC) on silica gel, eluting with CHCl_3 - CH_3OH (50:1, 40:1, 30:1, 20:1, 12:1, 4:1, 2:1, 1:1), to yield four fractions. Fraction 1 (1.7 g) was further subjected to CC, eluting with CH_3OH -EtOAc (30:1), to yield compounds as Makisterone A (**1**) (0.014 g), 20-hydroxyecdysone (**3**) (0.041 g). Repeated chromatography of Fraction 2 (1.5 g) over a silica gel column (CH_3OH -EtOAc, 30:1, 12:1, CH_3OH - CHCl_3 , 4:1) yielded pure integristeron A (**5**) (0.01 g). Fraction 3 (2.1 g) was subjected to CC, eluting with CH_3OH - CHCl_3 (20:1), and CH_3OH - CHCl_3 (15:1), (12:1) to obtain 5 α -2-deoxy- α -ecdysone (**7**) (0.012 g). α -ecdysone (0.018 g), compound (**8**) was obtained from Fraction 4 (4.3 g), which was separated through repeated column chromatography (CH_3OH - CHCl_3 6:1, 4:1).

The Fraction 5 (2.1 g) was subjected to column chromatography (CC) on silica gel, eluting with CHCl_3 - CH_3OH (100:1, 80:1, 60:1, 50:1, 30:1, 20:1, 15:1, 12:1, 9:1, 4:1, 1:1), to yield fractions which eluted with chloroform-methanol re-chromatographed and eluted with CHCl_3 - CH_3OH increasing order of polarity. Polypodine B (**2**) (0.021 g) and 2,3,20,22-diaetoneid-20-hydroxyecdysone (**4**) (0.015 g). Fraction 6 (1.2 g) was applied on a Sephadex LH-20 column with the solvent system CHCl_3 - CH_3OH (1:1) to give compound (**6**): cyasterone (0.035 g) from this fraction (Table 1).

Table 1
Physical and chemical properties of phytoecdysteroids isolated from *Silene claviformis*

| Compound No. | Compound name | Composition | T [°C] | Yield [% of plant mass] |
|--------------|--|--|---------|-------------------------|
| 1 | Makisterone A | C ₂₈ H ₄₆ O ₇ | 263–264 | 0,0014% |
| 2 | Polypodine B | C ₂₇ H ₄₄ O ₈ | 253–254 | 0.0021% |
| 3 | 20-Hydroxyecdysone | C ₂₇ H ₄₄ O ₇ | 242–243 | 0.0041% |
| 4 | 20-hydroxyecdysone 2,3,20,22-diacetonide | C ₃₃ H ₅₂ O ₇ | 220–221 | 0.0015% |
| 5 | Integristerone A | C ₂₇ H ₄₄ O ₈ | 247–248 | 0.001% |
| 6 | Cyasterone | C ₂₉ H ₄₄ O ₈ | 163–164 | 0.0035% |
| 7 | 5 α -2-Deoxy- α -ecdysone | C ₂₇ H ₄₄ O ₅ | | 0.0012% |
| 8 | α -Ecdysone | C ₂₇ H ₄₄ O ₅ | 233–234 | 0.0018% |

Experimental Biological Part

Evaluation of Antibacterial and Antifungal Activity

Test microorganisms: the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (RKMUZ – 5); the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27879), *Escherichia coli* (RKMUZ – 221); and the fungi *Candida albicans* (RKMUZ – 247). The RKMUZ microorganism cultures were obtained from the strain collection of the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan.

The antibacterial activity of extracts was determined by using the modified agar-disks diffusion method (WAYNE 2009, TASCHENBUCH 2004, ISMAILOVA et al. 2019). The bacterial cell suspension was prepared from a 24 h culture and adjusted to inoculation of $1 \cdot 10^6$ colony forming units per ml. Sterile nutrient agar (LB Agar, Invitrogen, USA, 25 g agar/l distilled water) was inoculated with bacterial cells (200 μ l of bacterial cell in 2 ml 0.9% NaCl suspension and 20 ml medium) and poured into Petri dishes to give a solid medium. *Candida maltosa* ($1 \cdot 10^6$ colony forming units per ml) was inoculated into sterile Mueller-Hinton-agar according to CLSI and DIN E 58940-3 for the agar disks-diffusion assay (TASCHENBUCH 2004, ISMAILOVA et al. 2019). Forty microliters of test material (equivalent to 2 mg of the dried extract or 0.2 mg individual substances), dissolved in the same solvent used for extraction, were applied on sterile paper discs (6 mm diameter, Whatman no. 1). Ampicillin (for Gram-positive bacteria), ceftriaxone (for Gramnegative bacteria), and fluconazole

(for fungi) (Himedia Laboratories Pvt. Limited) were used as positive controls and the solvents as negative controls. The solvents were allowed to evaporate in a stream of air. The discs were deposited on the surface of inoculated agar plates. Plates were kept for 3 h in the refrigerator to enable the prediffusion of the substances into the agar. Plates with bacteria were incubated for 24 h at 37°C and plates with yeasts for 48 h at 26°C. The inhibition zone (including the disc diameter) was measured and recorded after the incubation time. An average zone of inhibition was calculated for the three replicates in independent assays.

Results and Discussion

A preliminary investigation of *S. claviformis* plant has confirmed the presence of phytoecdysteroids in its composition, and allowed to isolate and identify its main ecdysteroids (Figure 1), such as Makisterone A (**1**) (0,0014%), polypodine B (**2**) (0.0021%), 20-hydroxyecdysone (**3**) (0.0041% of dry plant's weight), (YUSUPOVA et al. 2019), 2,3,20,22-diasetonid-20-hydroxyecdysone (**4**) (0.0015%), integristeron A (**5**) (0.001%) cyasterone (**6**) (0.0035%) (YUSUPOVA et al. 2019), 5 α -2-deoxy- α -ecdysone (**7**) (0.0012%), α -ecdysone (**8**) (0.0018%) (GIRAULT et al. 1990)

The isolated each one of ecdysteroids has been identified on the basis of the IR spectroscopy, and ¹H NMR spectroscopy, R_f and melting point on the A. Kruss Optronic Germany, M 5000; 90-264 VIAC, as well as by comparison with reference compounds. Table 1 provides the physicochemical data for the individual substances and ecdysteroids yield from *S. claviformis* plant. The NMR ¹H and ¹³C spectra were recorded by VN MRS-400 (Varian) NMR spectrometer with an operating frequency of 400 MHz.

The *Silene claviformis* extracts were screened for their antibacterial and antifungal activities by using a modified agar diffusion method. The results of the tests showed that the extracts and individual compounds did not exhibit antibacterial and antifungal activity against tested strains of microorganisms (Table 2). This finding was consistent with the previous results, which claimed that most likely such compounds are not responsible for the antibacterial activity of the plant extracts (SHIRSHOVA et al. 2006, AHMAD et al. 1996).

Table 2

Antimicrobial effect evaluated by the diameter of inhibition zone [mm] for *Silene claviformis* plant extracts using the agar disc diffusion assay

| Samples | Gram-positive bacteria | | Gram-negative bacteria | | Fungi |
|--|------------------------|------------------|------------------------|----------------|--------------------|
| | <i>B. subtilis</i> | <i>S. aureus</i> | <i>P. aeruginosa</i> | <i>E. coli</i> | <i>C. albicans</i> |
| <i>S. claviformis</i> (CHCl ₃ extract) [2000 µg/disc] | na* | na | 6 | 6 | na |
| <i>S. claviformis</i> (MeOH extract) [2000 µg/disc] | 6 | 6 | 6 | 6 | na |
| <i>S. claviformis</i> (BuOH extract) [2000 µg/disc] | na | na | 6 | 6 | na |
| Makisterone A [2000 µg/disc] | na | na | na | na | na |
| Polypodine B [200 µg/disc] | na | na | na | na | na |
| 20-Hydroxyecdysone [200 µg/disc] | na | na | na | na | na |
| 20-hydroxyecdysone 2,3,20,22-diacetonide [200 µg/disc] | na | na | na | na | 6 |
| Integristerone A [200 µg/disc] | na | na | na | na | na |
| Cyasterone [200 µg/disc] | na | na | na | na | na |
| 5α-2-Deoxy-α-ecdysone [200 µg/disc] | na | na | na | na | na |
| α-Ecdysone [200 µg/disc] | na | na | na | na | na |
| Ampicillin [10 µg/disc] | 26 | 27 | nt* | nt | nt |
| Ceftriaxone [30 µg/disc] | nt | nt | 25 | 27 | nt |
| Fluconazole [25 µg/disc] | nt | nt | nt | nt | 27 |

* na – not active; nt – not tested

Conclusion

In this study, we revealed that the compounds of *S. claviformis* contains makisterone A (1), polypodine B (2), 20-hydroxyecdysone (3), 2,3,20,22-diasetanide 20-hydroxyecdysone (4), integristeron A (5), cyasterone (6), 5α-2-deoxy-α-ecdysone (7), α-ecdysone (8). Compounds (1) and (6) were isolated from the *Caryophyllaceae* family for the first time; compounds (2), (4), (7) and (8) were obtained for the first time from this plant. The *Silene claviformis* extracts were screened for their antibacterial and antifungal activities by using a modified agar diffusion method. The results of the tests showed that the extracts and individual compounds did not exhibit antibacterial and antifungal activity against tested strains of microorganisms.

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