**University of Szeged**
**Faculty of Pharmacy**
**Department of Pharmacognosy**

# Studies on industrial *Cyanotis arachnoidea* extracts and semi-synthetic ecdysteroid derivatives

**Dávid Laczkó, Pharm. D.**

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**University of Szeged**
**Faculty of Pharmacy**

**Doctoral School of Pharmaceutical Sciences**
**Program director:** Prof. Dr. Judit Hohmann, DSc
Department of Pharmacognosy

**Supervisors:**
Prof. Dr. Attila Hunyadi, DSc
Dr. Máté Vágvölgyi, PhD

**Dávid Laczkó, Pharm. D.**

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**Complex exam committee:**
**Head:**
Prof. Dr. Judit Hohmann, DSc
**Members:**
Prof. Dr. István Ilisz, DSc
Dr. Gábor Janicsák, PhD

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# INTRODUCTION

Ecdysteroids are a special group of natural steroids discovered in 1954 that can be found in invertebrates (arthropods, corals, worms) where they regulate the molting and development, and in plants, where they likely serve as pesticide defense. Among the many natural ecdysteroids, the most abundant one is 20-hydroxyecdysone (20E, **39**), which induces protein synthesis in both invertebrates and vertebrates, manifesting anabolic effects along with blood glucose and cholesterol lowering, immunomodulatory, anti-inflammatory, and antioxidant activities. Due to their versatile pharmacological properties and significant anabolic effects that interest athletes, World Anti-Doping Agency (WADA) included 20E in their monitoring program in 2020. In addition, ecdysteroids have also attracted considerable interest from medicinal, agricultural, and cosmetic perspectives.

Ecdysteroids have been shown to exhibit very low toxicity, with the toxic dose found to be approximately 9g/body weight kg in mice, contributing to the growing interest towards 20E as a dietary supplement. These compounds can increase protein synthesis, stimulate liver function, and in combination with chemotherapeutic agents, exhibit significant anti-tumor effects by reducing the drug resistance of both MDR and drug-susceptible tumor cells. Furthermore, these compounds can reduce hyperglycemic effects by inhibiting gluconeogenesis. Ecdysteroids are known to exert beneficial immunomodulatory and adaptogenic effects, enabling organs to adapt to external stressors, and have antioxidant properties that inhibit lipid oxidation. Recently, advancement was made in investigation of their mechanism of action, suggesting an interaction between the MAS receptor and membrane-bound palmitoylated estrogen receptor, with clinical trials demonstrating more than 40% decreased incidence of respiratory failure in COVID-19 patients and clinically relevant improvements in the physical performance of sarcopenic patients, when using 20E as an active ingredient.

Subsequent extensive screening has led to the present registration of 590 natural ecdysteroids in the online ecdysteroid database, which is updated on a regular basis. Although industry is often focused on *Cyanotis arachnoidea* due to its high ecdysteroid content, phytoecdysteroids are found in many different plant species in various families (like *Microsorium membranifolium, Achyranthes bidentata* and *Chenopodium).*

The literature on the isolation of ecdysteroids dates back to the 1960s, and various liquid-liquid chromatographic methods such as droplet counter-current chromatography (DCCC), high-speed countercurrent chromatograph (HSCCC) and centrifugal partition

chromatography (CPC) can be used to isolate these compounds from their mixtures. Among these techniques, CPC deserves particular attention due to its loading capacity, scalability, flexibility and cost-effectiveness. This study focuses on the scale-up possibilities of the chromatographic isolation of calonysterone from its natural source using laboratory-scale CPC (250 mL rotor volume) and semi-pilot-scale CPC (2100 mL rotor volume).

Calonysterone is a naturally occurring phytoecdysteroid with an unusual chemical structure that contains several unique structural features including a 14α-hydroxy-7-en-6-one chromophore, an intact sterol side chain, a rare o-quinol B-ring, and its isolation has been reported from several plants including *Cyanotis arachnoidea*. Although calonysterone itself has been less studied pharmacologically compared to other widely investigated ecdysteroids, it may be a promising candidate for various clinical therapeutic uses.

The autoxidation of ecdysteroids under alkaline conditions has attracted research interest, as this process often yields structurally diverse derivatives, the nature of which depends on the reaction conditions and workup procedures. Furthermore, modifying the polarity of ecdysteroids offers an effective means of altering their action and metabolism, with low-polarity derivatives readily accessible as ether or dioxolane compounds derived from carbonyl precursors. The oxime function belongs to the group of imines and can be described with the formula RR'C=N-OH. The synthetic production of oximes is widely studied, and aldoxime and ketoxime functions can be straightforwardly produced by the treatment of aldehyde and ketone functional groups with hydroxylamine analogues.

The preparation of the oxime function is a popular medicinal chemical transformation as the oxime function can significantly influence pharmacological properties, contributing to the observed bioactivity of the molecule, such as its anti-fungal, antioxidant, anti-inflammatory, antibacterial and anti-cancer effects. Oximes from ecdysteroids can be prepared in a regioselective way, as the reactivity of the ecdysteroid B-ring's keto group is much lower compared to the keto group formed at C-20 as a result of an oxidate side-chain cleavage on the derivative.

# OBJECTIVES

**Analytical evaluation of *Cyanotis arachnoidea* extracts:** Develop a high-resolution LC-MS/MS technique that can identify up to 100 components and provide detailed characterization of both naturally occurring and industrially processed plant extracts containing ecdysteroids. Conduct a comparative analysis using the method at hand to identify possible artifacts with bioactivity relevance.

**Scale up the preparation and isolation of calonysterone:** develop a method suitable for producing large quantities of calonysterone and establish a scalable and economical CPC purification method for its isolation.

**Preparation and investigation of semi-synthetic calonysterone derivatives:** semi-synthesize a set of sidechain-cleaved oxime ether-containing derivatives from calonysterone and develop methods for their chromatographic purification. Examine the compounds' pharmacological potential as blood-brain barrier protecting agents and establish possible structure-activity relationships.

# MATERIALS AND METHODS

## Analytical evaluation of *Cyanotis arachnoidea* extracts

Sample preparation and analytical method development: The ecdysteroid standards were provided by the Institute of Pharmacognosy, University of Szeged. Two *Cyanotis arachnoidea* extracts—CAPR1 (50% 20E, Xi'an Olin Biological Technology, methanol-percolated) and CAPR2 (10.85 % 20E, Kingherbs Limited)—together with authentic methanolic root (CARO) and leaf (CALF) extracts, and 20E autoxidized sample (20EOX) were examined. RP-HPLC on an Agilent 1260 Infinity II/6420 QQQ-ESI-MS equipped with a Kinetex F5 column and an ammonium-formate/formic-acid–acetonitrile gradient at 40 °C, and full-scan positive-ion LC-ESI-MS (m/z 200–800) in DDA or DIA mode, with UV detection (210, 248, 360 nm).

## Preparation and isolation of calonysterone

Oxidative preparation of calonysterone: To accomplish the autoxidation of 20E, a 3 g aliquot of 20E was dissolved in a methanol-water mixture and treated with sodium hydroxide for 6 hours. Subsequently, it was acidified with hydrochloric acid and was left to stir for overnight. Following this, the solution was neutralized, evaporated under reduced pressure at 40 °C, and the resulting calonysterone was purified by centrifugal partition chromatography with fractions analyzed by TLC and HPLC before a final evaporation.

Solvent system selection, CPC methods: The selection of suitable solvent systems for liquid-liquid chromatography requires the determination of partition coefficients (KD values) for the main ecdysteroid component and impurities in two-phase solvent systems, obtained by mixing crude extract with pre-equilibrated biphasic solvent systems, allowing phase separation, and analyzing peak areas by HPLC. CPC separations were conducted using laboratory scale and semi-pilot scale rotors with preparative LC systems. The biphasic liquid systems were

prepared in separation funnels with vigorous shaking and settling. These separated phases became the stationary and the mobile phases.

### Preparation and investigation of semi-synthetic calonysterone derivatives

Oxidative side chain cleavage of calonysterone and oxime formation (**99a–99g**): Calonysterone was oxidized with PIDA in methanol, followed by a neutralization and flash chromatographic purification using a dichloromethane-methanol gradient system to yield the desired product (**99a**). The purified compound was then derivatized with hydroxylamine or alkoxyamine hydrochloride in ethanol under reflux conditions, and the resulting ecdysteroid oxime products (**99b–99g**) were isolated through extraction and preparative RP-HPLC purification. Structures were evaluated using HR-MS and NMR measurements.

Procedures for biological investigation: Human brain microvascular endothelial cells (hCMEC/D3) were cultured and treated with test compounds at various concentrations, with cell viability assessed using real-time cell electronic sensing analysis (RTCA) and impedance measurements. Barrier integrity was evaluated through TEER measurements and paracellular permeability assays using fluorescent markers, while total ROS generation was measured by fluorometric detection, with statistical analysis performed using mean ± SD values and significance determined at $p<0.05$.

## RESULTS AND DISCUSSION

### Analytical evaluation of *Cyanotis arachnoidea* extracts

Analyzed ecdysteroid-containing samples: Within this thesis framework, five ecdysteroid-containing samples were analyzed. Two commercial *Cyanotis arachnoidea* extracts (CAPR1 and CAPR2) were purchased from independent Chinese companies, as China is a major distributor of *C. arachnoidea* extracts since the plant is native to Asia. An authentic *C. arachnoidea* sample was collected in Taiwan, with roots and leaves separated and extracted with methanol, referred to as CARO and CALF, respectively. Preliminary experiments indicated CAPR1 contained significantly more components than authentic extracts, suggesting artifacts formed during industrial processing. The base-catalyzed autoxidation product mixture of 20E (20EOX) served as the fifth analyte to evaluate this hypothesis. Pure 20E was used rather than *Cyanotis* extracts, as 20E is the main phytoecdysteroid component in these samples.

Reversed-phase HPLC with tandem mass spectrometry analyzed the ecdysteroid fingerprints using a Kinetex F5 column. This column's superficially porous pentafluorophenyl

propyl stationary phase provides an alternative to traditional C18 columns with similar selectivity. Separation parameters balanced the large number of sample components between longer run times producing broader peaks and shorter run times causing co-eluting components that complicate mass spectrometric evaluation (**Fig. 1**)

LC-MS data of identified ecdysteroids: The analytical evaluation was greatly aided by compounds isolated during previous works. Several ecdysteroids were identified using authentic reference materials from earlier research (**Table 2** and **Fig. 3**). We unambiguously identified 25 substances in the extracts. Artifacts were identified in autoxidized and industrial samples but not in authentic extracts.

Comparison of ecdysteroid containing samples: Based on the fingerprints, we compared the extracts in pairs and counted the exact number of common peaks at the LOD level. We then expressed this as a percentage of components in one sample that were also present in the other, where 100% indicates that all peaks in each extract were found in the other extract. Results of this analysis are summarized in **Table 1**.

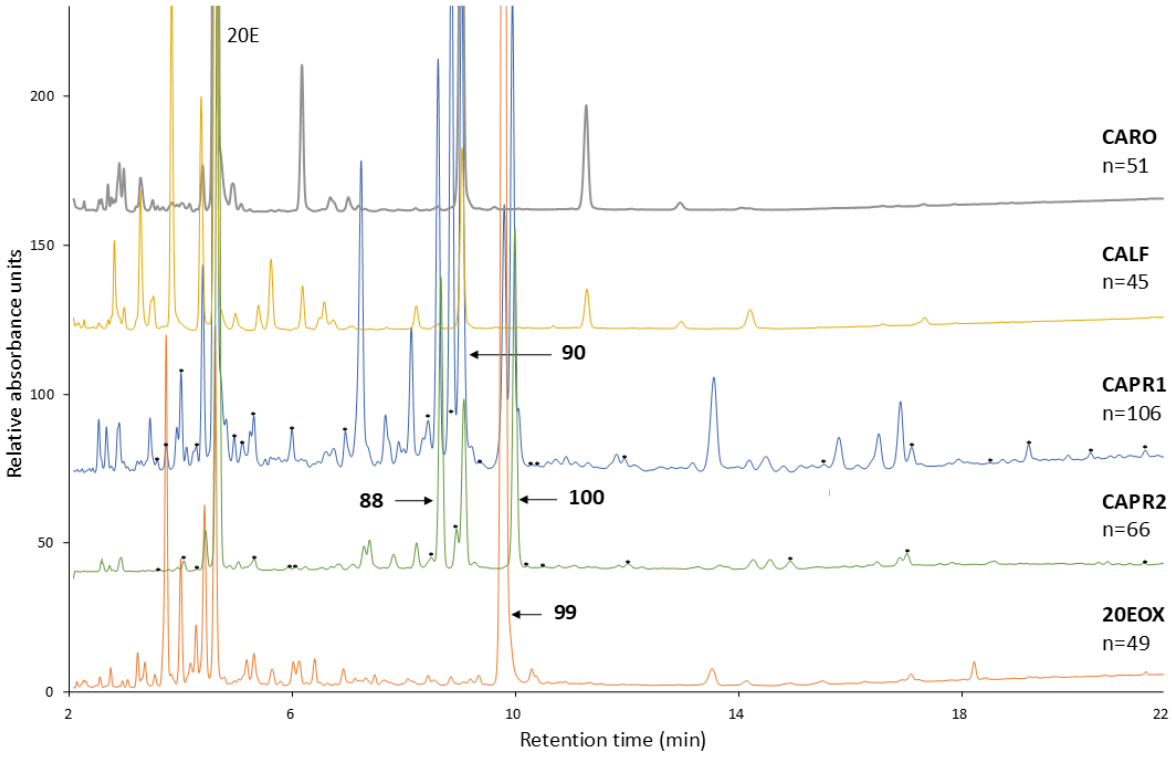



**Figure 1** Analytical chromatograms of CARO, CALF, CAPR1, CAPR2 and 20EOX samples. UV detection was carried out at 248±4 nm. Peaks present only in commercial extracts and absent from authentic natural extracts are marked with asterisks (*) on the chromatograms of CAPR1 and CAPR2.

**Table 1.** Fingerprint comparison of the extracts. Intersection: the number of common compounds identified. Similarity 1st to 2nd, and 2nd to 1st are expressed as the percentage of detectable peaks in the 1st sample that are also present in the 2nd, and vice versa, respectively.

| 1st | 2nd | Intersection | Similarity (%) | |
|---|---|---|---|---|
| | | | 1st to 2nd | 2nd to 1st |
| CAPR1 | CAPR2 | 52 | 49 | 79 |
| CAPR1 | CARO | 41 | 39 | 80 |
| CAPR1 | CALF | 32 | 30 | 71 |
| CAPR1 | 20EOX | 33 | 31 | 67 |
| CAPR2 | CARO | 30 | 45 | 58 |
| CAPR2 | CALF | 21 | 32 | 46 |
| CAPR2 | 20EOX | 15 | 23 | 31 |
| CARO | CALF | 37 | 73 | 82 |
| CARO | 20EOX | 2 | 4 | 4 |
| CALF | 20EOX | 1 | 2 | 2 |

We aimed to compare authentic extracts of wild-collected *C. arachnoidea* roots and leaves (CARO and CALF) to industrially processed extracts. Approximately 50–60 components were detectable in both authentic extracts using HPLC analysis. 20E was the main component in both extracts, which also contained significant amounts of ajugasterone C. Their qualitative composition is very similar, however the root extract's ecdysteroid content is significantly higher than the leaf extract. After comparing CAPR1 and 20EOX, nearly 70% of the alkaline autoxidation products of 20E were present in CAPR1, implying that oxidative artifacts were formed during industrial processing. Alkaline autoxidation may occur for every ecdysteroid with a 7-ene-6-one molecule in its B-ring and requires a strong alkaline medium. Since 20EOX originated from a single compound (20E), it is less complex than expected from autoxidation of a crude *C. arachnoidea* extract containing various ecdysteroids. No such artifacts were detectable in the authentic extracts CARO or CALF.

**Table 2.** HPLC-MS characteristics of 25 ecdysteroids identified by fully characterized reference standards and their semiquantitative presence in the samples. Color codes for the suggested origin: *green*, genuine compound; *yellow*, oxidized artifact; *violet*, genuine compound present in quantities likely altered by oxidation. The genuine or artifact nature of compounds without a highlighting color could not be decided based on the available data. For a complete overview of all detected compounds, see publication **No II**. supplementary information (**Table S2**).

| Comp. No. | Trivial name | Retention time (min) | $[M+H]^+$ | Presence | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | CAPR1 | CAPR2 | CARO | CALF | 20EOX |
| **17** | Oxycalonysterone C | 3.28 | 493.3 | + | - | - | - | ++ |
| **39** | 20-Hydroxyecdysone | 4.53 | 481.2 | ++++ | ++++ | ++++ | ++++ | +++ |
| **70** | Isovitexirone | 6.93 | 479.1 | + | + | ++ | + | - |
| **71** | Poststerone | 7.10 | 363.1 | + | + | + | - | - |
| **72** | 14-Deoxy-20-hydroxyecdysone | 7.15 | 465.3 | +++ | +++ | - | - | - |
| **73** | 14-Deoxy-25-hydroxydacryhainansterone | 7.20 | 463.3 | + | + | - | - | - |
| **78** | 5α-14-Deoxy-20-hydroxyecdysone | 7.82 | 465.2 | + | - | - | - | - |
| **79** | 5α-Stachysterone B | 7.91 | 463.3 | + | - | + | - | - |
| **80** | 14-Epi-14-deoxy-20-hydroxyecdysone | 8.05 | 463.2 | ++ | ++ | - | - | - |
| **88** | 20-Hydroxyecdysone 3-acetate | 8.53 | 523.3 | +++ | +++ | + | + | - |
| **90** | Ajugasterone C | 8.95 | 481.3 | +++ | +++ | +++ | +++ | - |
| **91** | 14-Epi-14,15-dihydrocalonysterone | 9.00 | 461.2 | + | - | - | - | - |
| **97** | Oxycalonysterone A | 9.61 | 491.2 | + | - | - | - | + |
| **98** | 14-Epi-14-deoxy-20(*S*)-dihydropoststerone | 9.66 | 349.2 | + | - | - | - | - |
| **99** | Calonysterone | 9.72 | 477.2 | +++ | + | + | + | ++++ |
| **100** | 20-Hydroxyecdysone 2-acetate | 9.86 | 523.3 | +++ | +++ | + | + | - |
| **106** | 14β(H)17β(H)-14-deoxypoststerone | 11.73 | 347.2 | + | | - | - | - |
| **111** | 5α-Stachysterone B 2-acetate | 13.09 | 505.3 | + | + | - | - | - |
| **112** | Dacryhainansterone | 13.46 | 463.3 | +++ | ++ | + | - | ++ |
| **124** | Oxycalonysterone B | 17.17 | 475.2 | + | - | - | - | - |
| **131** | Dacryhainansterone 3-acetate | 19.82 | 505.2 | + | - | - | - | - |
| **133** | 14-Deoxydacryhainansterone | 21.21 | 447.3 | + | - | - | - | - |
| **135** | 5α-2-Deoxyponasterone A | 21.46 | 449.2 | + | + | - | - | - |
| **136** | Dacryhainansterone-2-acetate | 21.61 | 505.3 | + | - | - | - | - |
| **140** | 22-Oxodacryhainansterone | 22.60 | 461.2 | + | - | - | - | + |

CAPR2 extract composition was more like authentic CARO sample than CAPR1. However, CAPR2 also contained compounds indicative of possible artifact formation and was particularly rich in acetates, suggesting acetic acid use during processing (**Fig. 2**).

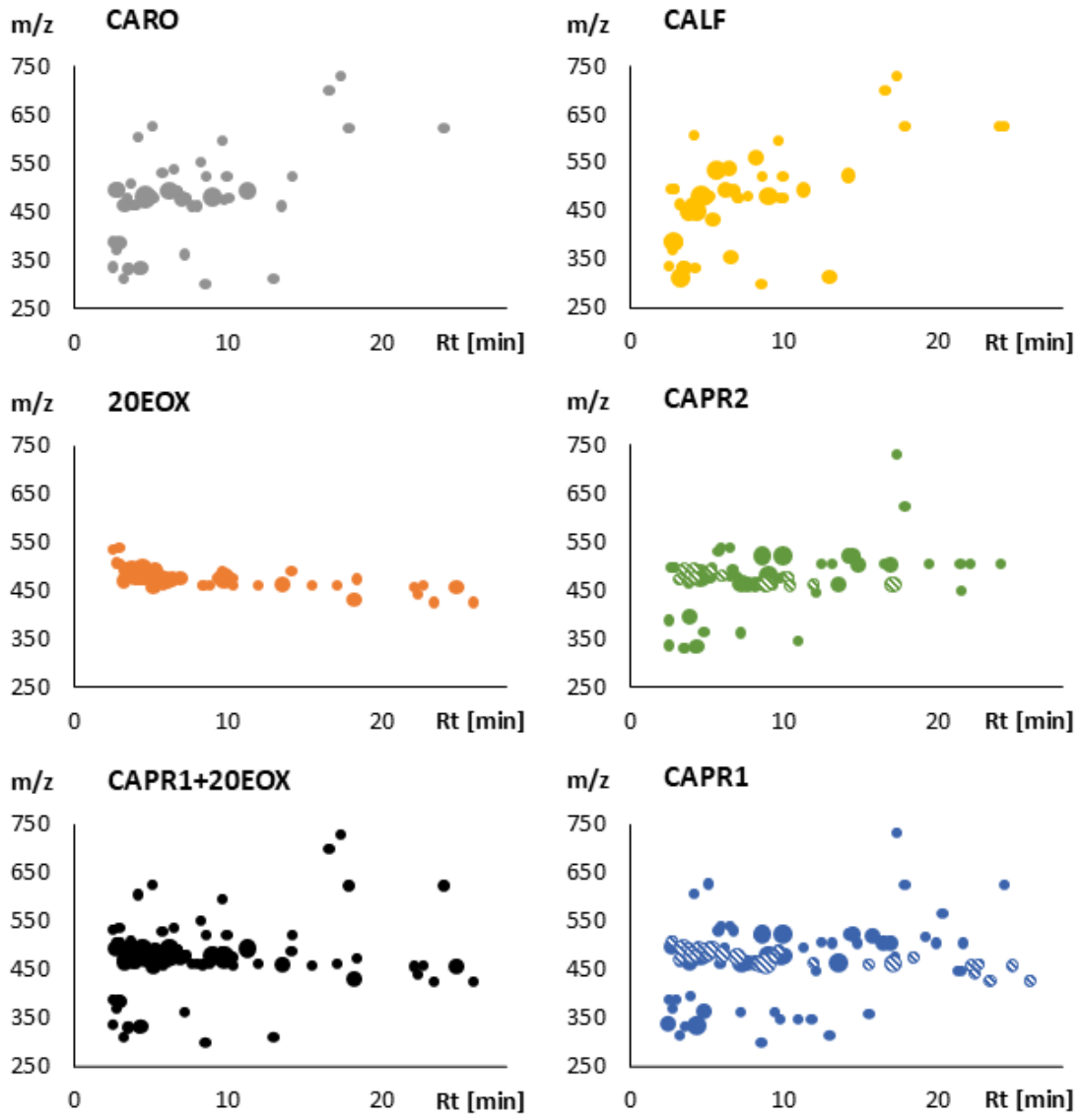



**Figure 2** Overview of the LC-MS fingerprints of the samples analyzed: CARO, CALF, 20EOX, CAPR1 and CAPR2; CARO+20EOX: mathematical sum of CARO and 20EOX. The size of the bullets represents the relative peak area values of each peak. Striped bullets represent the presumable artifacts.

Very little is known about the pharmacology of minor phytoecdysteroids in mammals, even though the numerous beneficial health advantages associated with 20E. In our earlier research, we demonstrated that less polar ecdysteroids, including acetonides, have the opposite effect on cancer cell resistance as intact 20E. Compared to their parent molecule, autoxidized

derivatives of 20E, such as compound **99**, have a significantly greater effect in activating protein kinase B (Akt). Other oxidized analogues of 20E likewise had stronger effects on two important pathways that control cell survival and death, AMPK and Akt. These examples demonstrate that the significant alteration in the ecdysteroid composition of a plant extract has a high potential to significantly change the overall bioactivity profile.

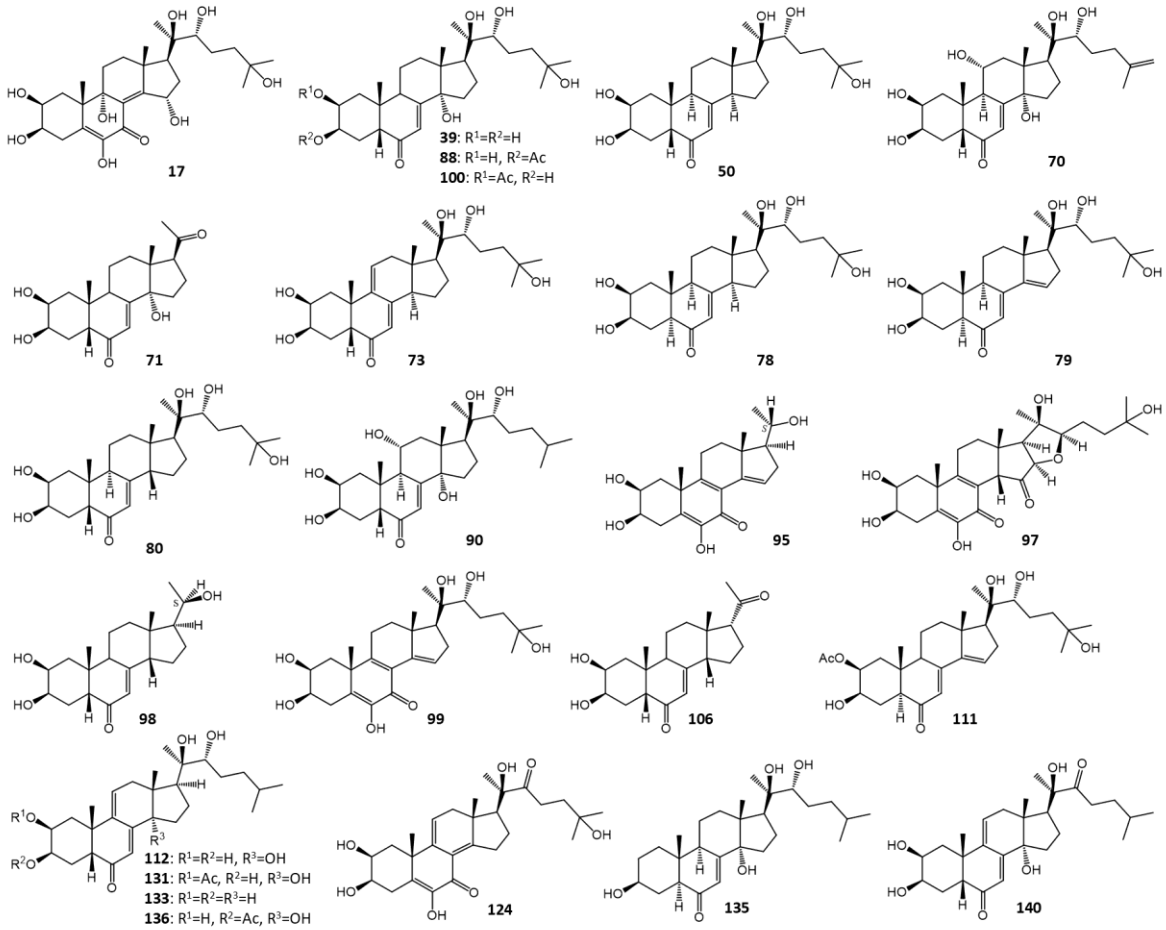



**Figure 3** Chemical structures of ecdysteroids categorically identified in the extracts using fully characterized reference standards.

## Preparation and isolation of calonysterone

Synthetic preparation of calonysterone: Calonysterone synthesis implementation was based on previous work by monitoring 20E autoxidation composition changes in alkaline environment through capillary electrophoresis. The key intermediate, 14,15-dihydro-14α-hydroxycalonysterone (**HC**) yields isocalonysterone through base-catalyzed elimination of 14-OH, or calonysterone through acid catalysis. Time dependence study showed precursor maximum (~80%) at 5.5-6 h (**Fig. 4**). Autoxidation was stopped by acidification, achieving 77% calonysterone content by HPLC at 248±4 nm.

Measurements demonstrate that highly acidic environment (pH=1) is required for proper water elimination reaction after autoxidation. This pH must be maintained for approximately 16 hours, as longer exposure increases degradation products. Neutralization should not exceed pH 7 as calonysterone converts to desmotropic pair in slightly alkaline conditions, impairing recovery. Subsequent isolation steps are optimal at pH 6.

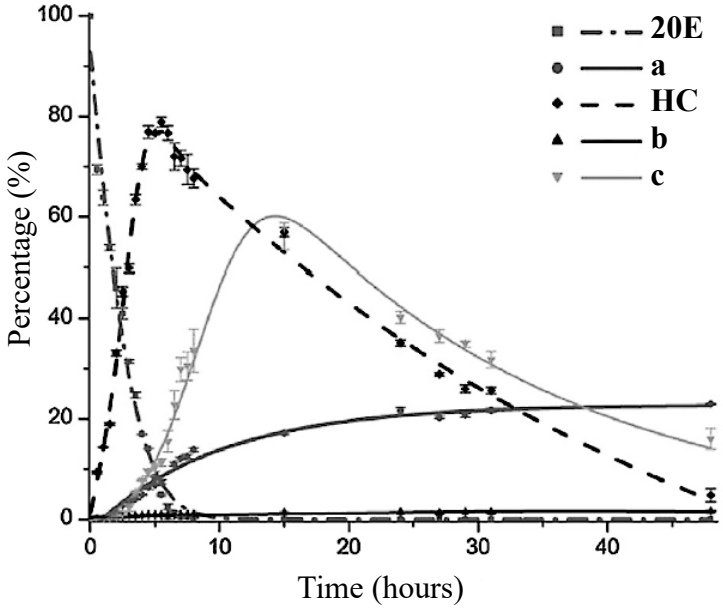



**Figure 4** Composition changes of **20E**, **a**, **HC**, **b** and **c** in the reaction mixture during the autoxidation process of 20E.

Solvent system selection: The principal of successful CPC separation is finding the solvent system with most ideal component distribution. In evaluating partition, solubility, and selectivity data, special emphasis was made on sustainability. General requirements for two-phase solvent system: optimal distribution for target components ($K_D$=0.5–2.0); high selectivity ($\alpha$>1.5) between compounds; short settling time (<30 sec) and desirable solvents with low cost, low toxicity and high solubility. Solvent system choice involved literature survey and database of previous ecdysteroid measurements. $K_D$ measurements showed ketone/ester – alcohol – water systems have best solubility and optimum distribution for ecdysteroid components.

During method development, we considered industrial factors and used the greenest possible solvents and sustainable alternatives, (e.g. limonene instead of hexane). FDA's ICH Q3C guideline was considered for solvent residuals. We kept separation time under 30 min, including equilibration. Ascending mode was prioritized as it makes fraction processing easier and cheaper. We strived to set the $K_D$ value of the compound of interest (calonysterone) to around 1, as the resolution of the separation is the highest at this point. Considering this, the

following solvent systems were selected for further investigation: **labCPC No1.** MIBK – acetone – $H_2O$ (3:4:3, *v/v*); **labCPC No2.** EtOAc – MeOH – $H_2O$ (4:2:4, *v/v*); **labCPC No3.** EtOAc – MeOH – $H_2O$ (4:1,5:4, *v/v*); **labCPC No4.** MEK – EtOAc – $H_2O$ (5:1:4, *v/v*); **labCPC No5.** MIBK – MeOH – $H_2O$ (1:1:1, *v/v*).

Centrifugal partition chromatographic isolation of calonysterone: Promising solvent systems from screening are tested on laboratory scale CPC using generic parameters: 2000 rpm rotation speed, 15 mL/min flow rate, and 20 mL fraction collection from the upper organic phase. Ascending mode was employed due to calonysterone's partition coefficients, resulting in faster elution, narrower peaks, and shorter run times. This mode also facilitates upper phase evaporation and efficient salt removal, as the aqueous stationary phase retains NaCl on the rotor, eliminating costly desalting steps.

For laboratory measurements, 1 gram of crude sample was dissolved in 10 mL of the lower phase and injected. Due to high salt content, dissolution in the lower aqueous phase was the only viable option. Comparative analysis revealed that solvent systems containing EtOAc and MeOH (**labCPC No2.** and **No3.**) provided superior peak shapes compared to MEK and MIBK systems (**labCPC No1., No4.,** and **No5.**). Both EtOAc/MeOH systems yielded similar separation and purity results. The system with higher MeOH content was selected for scale-up due to reduced run time and enhanced solubility (**Fig. 5A** and **5B**).

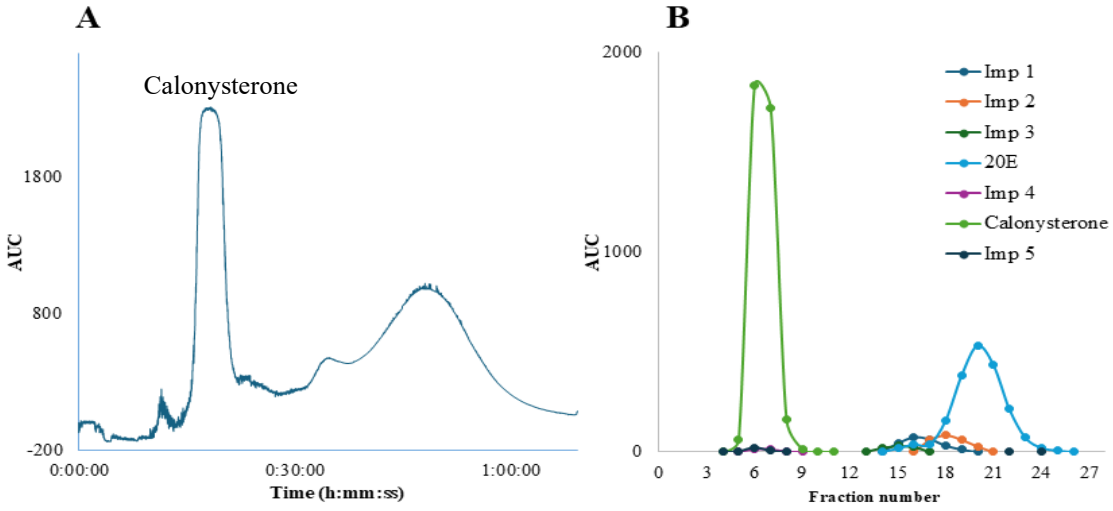



**Figure 5 A:** Chromatogram of **labCPC No2**. UV detection was carried out on a wavelength of 248±4 nm. **B:** Fractogram of **labCPC No2.** is compiled by the analytical measures of fractions.

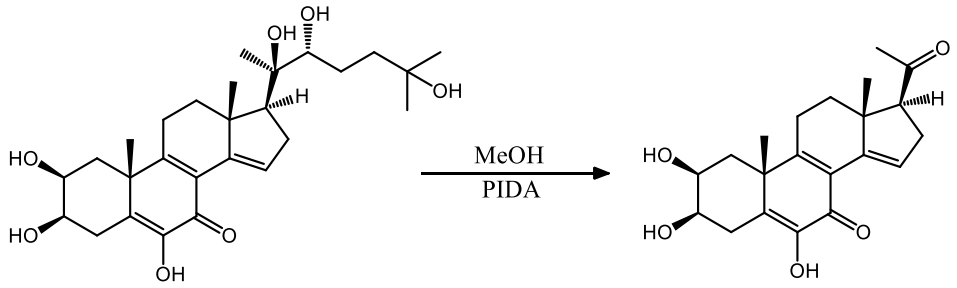
The EtOAc - MeOH - $H_2O$ (4:2:4, v/v) solvent system was chosen for scale-up in ascending mode. An 8 g sample on a 2100 ml rotor resulted in a 20-minute method with wider

peaks due to higher loading. The achieved 95% purity, 85% calculated yield, and 63.1% isolated yield, are demonstrating scalability.

## Preparation and investigation of calonysterone semi-synthetic derivatives

Oxidative cleavage of the side chain: The regioselective cleavage of the calonysterone side chain at the C-17 position between the 20,22-diol was performed with PIDA (**Scheme 1**), which achieves better yields than PIFA with fewer minor by-products. The reaction completes in 1 hour based on TLC tracking. After completion, the solution was neutralized with 10% aqueous $NaHCO_3$. The solvent was removed using rotary vacuum evaporator. The material was purified by normal phase flash chromatography (**Fig. 6**), which proved more practical than reverse phase separation due to higher chromatographic load capacity and easier solvent removal. From 4.86 g starting material, 1.79 g of product was isolated after purification, corresponding to a 45.5% yield for **99a**.

Experiment to produce the C-7-oxime function on the B ring of calonysterone: Previous studies revealed that the C-6-enone function of poststerone is comparatively less reactive toward oxime formation than its C-17-oxo group. Subsequent preliminary experiments with calonysterone failed to form an oxime at position C-7 under various tested conditions, including multiple solvents. This led to the postulation that the oxo group of a C-17-acetyl function, remaining after oxidative side-chain cleavage, could undergo regioselective oxime formation in a manner similar to poststerone.




**99** **99a**

**Scheme 1** Side chain cleavage reaction of calonysterone.

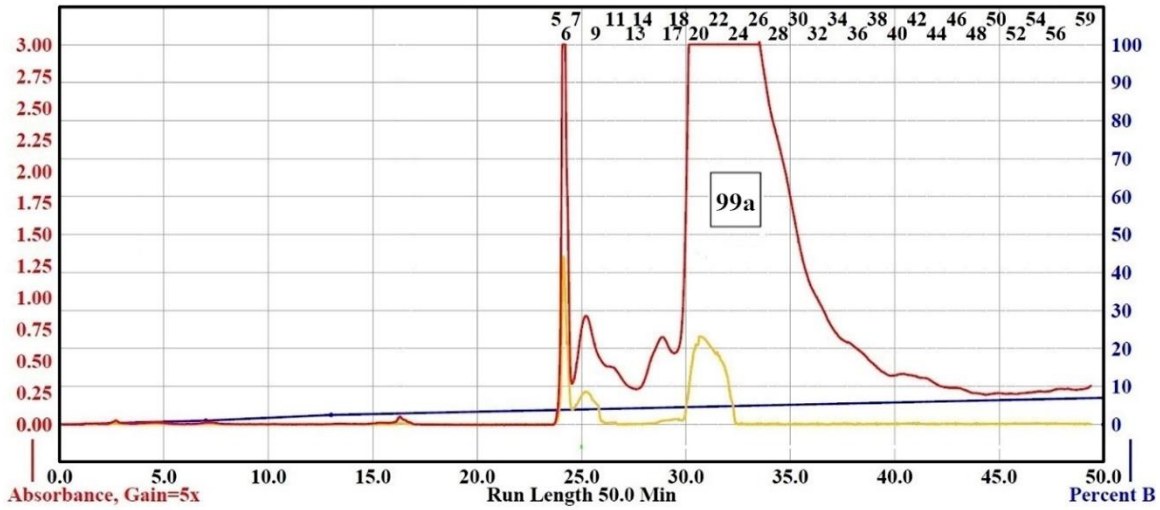
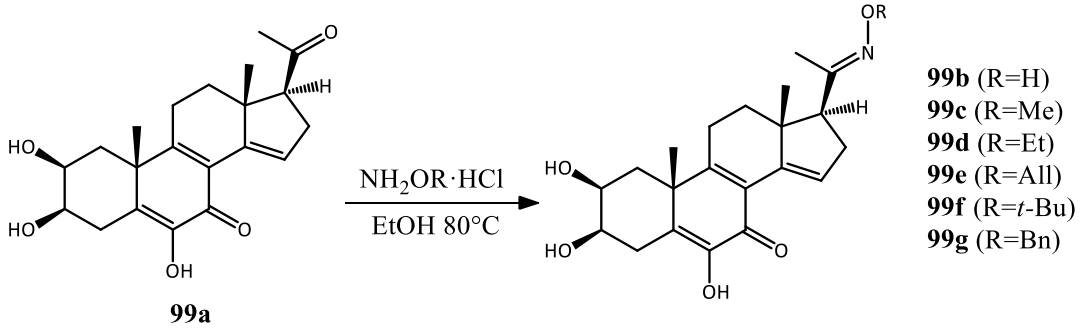



**Figure 6** Chromatogram of **99a** purification by normal phase flash chromatography. UV detection was carried out on a wavelength of 248±4 nm (red).

Regioselective preparation of oxime derivatives: The carbonyl group at position C-7 remains inert in oxime-forming reactions under a variety of experimental conditions, and the absence of the 14α-hydroxyl group typical of ecdysteroids in calonysterone was advantageous during reaction planning since this hydroxyl group is sensitive to both acid and alkali, making synthetic oxime formation in non-neutral pH media circumstantial (**Scheme 2**). TLC monitoring showed all derivatives reacted within 40 min, with ethanol selected as the preferred solvent over pyridine due to its lower boiling point facilitating easier evaporation during work-up, followed by rotary vacuum evaporation and liquid-liquid extraction with water and ethyl acetate to separate products from remaining salts.




**Scheme 2.** Synthesis of **99b**–**99g**.

After extraction, HPLC chromatograms were recorded and products were purified using preparative RP-HPLC with a DMSO–acetonitrile solvent mixtures, achieving at least 97% purity at 254±4 nm detection wavelength. The observed tailing peaks were attributed to solubility issues rather than impurities, as confirmed by spectral purity analysis showing

>99.9% purity, and all compounds prepared from 120 mg of starting material **99a** were dried under nitrogen with yields presented in **Table 3.**

**Table 3** Isolated yields and purities of **99b**–**99g**.

| C20-NO*R* | Isolated product | Purity | Isolated yield |
|---|---|---|---|
| Comp. **99b** (R=H) | 49.4 mg | 98.1% | 39.5% |
| Comp. **99c** (R=Me) | 33.1 mg | 99.1% | 25.5% |
| Comp. **99d** (R=Et) | 50.6 mg | 98.0% | 37.7% |
| Comp. **99e** (R=All) | 52.1 mg | 98.8% | 37.7% |
| Comp. **99f** (R=tBu) | 27.8 mg | 97.4% | 19.4% |
| Comp. **99g** (R=Bn) | 54.9 mg | 97.1% | 35.3% |

Structure elucidation: Structure elucidation covers HRMS and NMR, complete $^{1}$H and $^{13}$C signal assignment of compounds **99b–99g**. Regioselectivity and newly formed functions were determined by one- and two-dimensional NMR methods including $^{1}$H NMR, $^{13}$C DeptQ, edHSQC, HMBC, selective ROESY spectra and HRMS. Only one set of signals appeared in NMR spectra of each compound, indicating regioselective oximation. The Δδ 55 ppm diamagnetic change of δC-20 supported C═O→C═NOR conversion. Selective ROESY experiments differentiated (*Z*/*E*) isomers on CH3-21 signals, and steric responses unequivocally proved E-configuration of the oxime moiety.

Biological evaluation of the products: The biological evaluation assessed compound effects on hCMEC/D3 cell viability using impedance measurements at concentration ranges from 0.01–10 μM, with compound **99g** exhibiting the highest and most significant activity by demonstrating positive effects on barrier integrity at all tested concentrations (**Fig. 7**). When evaluated as a ROS anti-agent against 350 μM *tert*-butyl hydroperoxide (tBHP)-induced oxidative damage, 10 μM of compound **99g** efficiently protected cells from harmful effects, while at smaller concentrations (10 nM, 100 nM, and 1 μM) it surprisingly increased tBHP-induced toxicity, leading to cellular layer disruption. The herein reported compounds are semi-synthetic ecdysteroids containing oxime ether moieties in their sidechain, a functional group not expectable to occur in natural ecdysteroids or their metabolites, therefore these results do not directly imply any risk connected to phytoecdysteroid consumption.

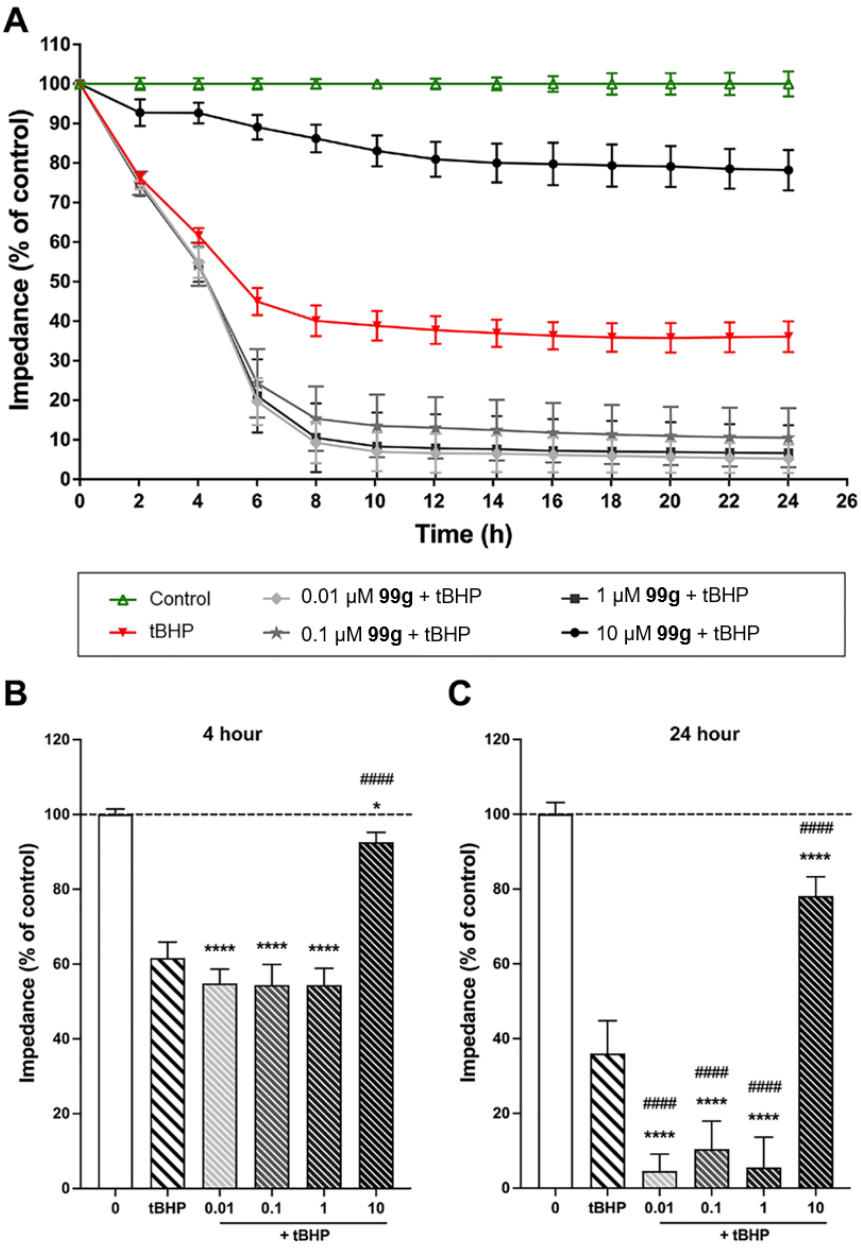



**Figure 7** The effects of compound **99g** at concentrations of 0.01, 0.1, 1, and 10 μM treatment on human brain microvascular endothelial cells (hCMEC/D3) were evaluated using impedance-based assays to assess cell viability and barrier integrity in the absence and presence of oxidative stress promoted by *tert*-butyl hydroperoxide (tBHP). **A:** Time-dependent impact of **99g** on cell viability following co-treatment with tBHP (350 μM). **B:** Impact of **99g** on cell viability at 4 hours co-treatment with tBHP (350 μM). **C:** Impact of **99g** on cell viability at 24 hours co-treatment with tBHP (350 μM). The data are presented as the mean ± standard deviation (SD) and were obtained from a minimum of two independent experiments (n = 2−3) with 3−9 technical replicates. Data analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The results were statistically significant with $^{*}p < 0.05$, $^{****}p < 0.0001$, compared to the control group, and $^{\#\#\#\#}p < 0.0001$, compared to the tBHP group.

Barrier integrity tests: Compound **99g** at 10 μM concentration demonstrated protective effects against tBHP-induced ROS damage by maintaining transendothelial electrical resistance (TEER), reducing paracellular permeability for both 4 kDa FITC-dextran (FD4) and Evans blue labeled albumin (EBA), and preserving brain endothelial cell morphology in the blood-brain barrier model (**Fig. 8**).

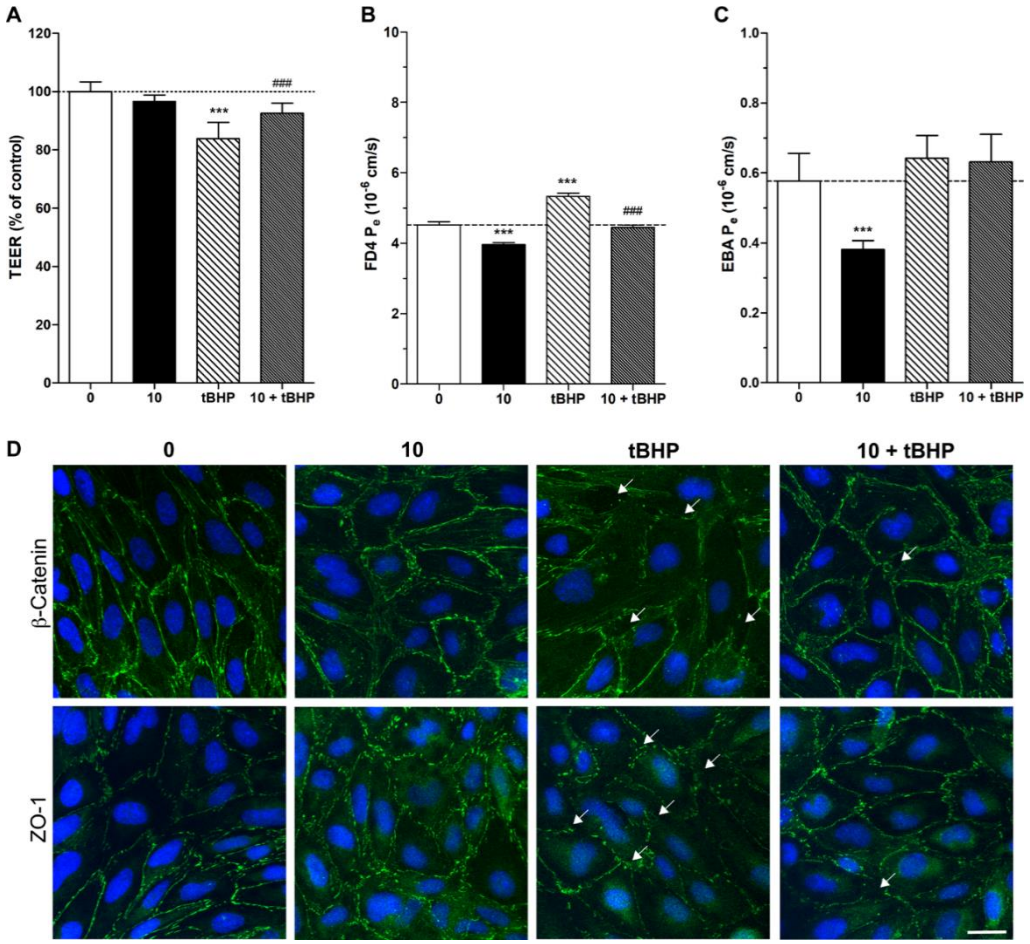



**Figure 7** Effects of compound **99g** (10 μM) on the barrier integrity of human brain microvascular endothelial cells (hCMEC/D3) **A:** Transendothelial electrical resistance (TEER) measurement. **B:** Permeability measurement, FITC-dextran 4 kDa (FD4). **C:** Permeability measurement, Evans blue labeled albumin (EBA, 67 kDa). **D:** Immunocytochemistry for β-catenin and zonula occludens-1 (ZO-1) junctional associated molecules.

# CONCLUSIONS

In this study, we performed the extensive analytical investigation of industrial *Cyanotis arachnoidea* extracts, prepared and processed by unknown methods, to reveal the presence of a wide range of oxidized ecdysteroid artifacts. This may pose both threats and opportunities in both chemical and biological terms in the use of ecdysteroid containing extract. The intensively

growing food supplement market and the ever-wide distribution of plant extracts is an explicit cause for concern, as little is known about the toxicological characteristics of these new, chemically diverse process-related artifacts. On the other hand, some of these newly revealed artifacts may serve as potential leads themselves, and their drug discovery value could be exploited by a suitable and well-designed industrial processing strategy. The implications for our findings highlight the need for further studies on the safety and efficacy of minor ecdysteroids that are unintentionally taken by people worldwide, including, but not limited to, bodybuilders and athletes.

Based on our expanding scientific knowledge of minor ecdysteroids, it is clear that their structural diversity translates into complex biological profiles. To expand this knowledge by relevant *in vivo* and possibly even clinical studies, simple, economic, and scalable semi-synthesis, and/or isolation strategy is required. We have previously found several promising bioactivities for calonysterone. Within the framework of this thesis, we have successfully developed a semi-synthetic method for its production using 20E as a starting material, which is an inexpensive and widely available substance. Following the synthesis of calonysterone, we have successfully developed a scalable centrifugal partition chromatographic purification method that fully adapts to both the organic and inorganic impurity profile of the crude reaction mixture. This will ensure the basis for wide availability of the compound at near industrial scale. Hopefully, the developed method may provide significant support for meeting the quantitative requirements of future studies involving the compound.

For the reason of confirmed neuroprotective activity of 20E, the aim of this thesis was also to prepare new ecdysteroid derivatives with potential BBB protecting activity. Inspired by our preliminary results, we aimed at a regioselective modification of the sidechain of calonysterone to prepare structurally diverse oxime and oxime ether derivatives. After the optimization of reaction conditions, the oxidative cleavage of the sidechain was achieved at C-17, followed by regioselective oxime ether formation at the C-20 position. In the framework of this thesis, we have successfully prepared 7 new ecdysteroid derivatives and tested their BBB protective potential. While at high concentrations, **99g** acted as a neurovascular protective agent, at low concentrations, it increased stress-induced cellular damage. This indicates that certain ecdysteroid derivatives may have unexpected and unwanted harmful (side) effects. Our results highlight the importance of further detailed studies of semi-synthetic ecdysteroid derivatives through the example of this unexpected bioactivity profile change.

**List of publications related to the thesis**

I. *17-Oxime ethers of oxidized ecdysteroid derivatives modulate oxidative stress in human brain endothelial cells and dose-dependently might protect or damage the blood-brain barrier.* Máté Vágvölgyi, Dávid Laczkó, Ana Raquel Santa-Maria, Judit P Vigh, Fruzsina R Walter, Róbert Berkecz, Mária A Deli, Gábor Tóth, Attila Hunyadi **PLoS One** 2024; 19 (2): e0290526.

**IF: 2.6**, SJR: 0.803, Q1

II. *LC-MS/MS fingerprinting analysis of Cyanotis arachnoidea extracts: process-related artifacts in anabolic food supplements.* Dávid Laczkó, En-Liang Chu, Ching-Chia Chang, Fang-Rong Chang, Gábor Girst, Tamás Gáti, Gábor Tóth, Árpád Könczöl, Attila Hunyadi **ACS Omega** 2025; 10 (18): 18605-18614.

**IF: 4.3**, SJR: 0.773, Q1

III. *Investigation of calonysterone and 20-hydroxyecdysone effects in high-fat, high-sugar diet-induced obesity rat model.* Alaa AM Osman, Dávid Laczkó, Máté Vágvölgyi, Adrien Seres-Bokor, Anita Sztojkov-Ivanov, Kata Kira Kemény, Attila Hunyadi, Eszter Ducza **Heliyon** 2025; 11 (3): e42435.

**IF: 3.6**, SJR: 0.644, Q1