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Posted Date: 30 April 2025

doi: 10.20944/preprints202504.2503.v1

Keywords: caracasine; caracasine acid; leukemia; spheroids

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New Caracasine Acid Derivatives with Anti-Leukemic Activity.

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Abstract: The natural compounds caracasine acid (**1**) and its methyl ester caracasine (**2**), isolated from an extract of *Croton micans* flowers, have proven effective against several tumor cell lines. Five semi-synthetic derivatives (**3**-**7**) were produced upon those structures, and the study aimed to test the cytotoxic activity of these compounds, along with the parental structures. The assays were performed in a panel of twelve human cell lines, consisting of eight cancer and four normal cell lines. Furthermore, the compounds were evaluated on spheroids derived from the HCT116, HCT116 p53 knockout (p53KO), A549, and U2OS cell lines and mixed spheroids from tumor cells and normal fibroblasts. Both the parent compound (**1**), the natural ester (**2**), and two novel derivatives, the anhydride (**3**) and the cyclohexanol ester (**4**), demonstrated cytotoxicity against different leukemic cells and HCT116, HCT116 p53 knockout (p53KO), A549, and U2OS cell lines in conventional two-dimensional cultures. However, these compounds exhibited no activity in spheroid models. These findings indicate potential applications in leukemia treatment, albeit with limited efficacy against solid tumors.

Keywords: caracasine acid derivatives; cytotoxicity; leukemia; spheroids

1. Introduction

Medicinal plants constitute a crucial reservoir of bioactive compounds essential for developing novel therapeutics aimed at addressing various pathologies. The structural diversity of secondary metabolites derived from natural products is paramount in ongoing pharmacological research, highlighting the necessity of their isolation, structural elucidation, and mechanistic evaluation against clinically relevant diseases. Among these plants, the *Croton* genus (*Euphorbiaceae*) has become a significant source of pharmacologically active constituents. Scientific investigations have demonstrated a wide array of multifunctional bioactivities, including, but not limited to, antinociceptive, analgesic, antimicrobial, antidiabetic, anti-HIV, and antineoplastic effects. Systematic reviews and meta-analyses have extensively documented the therapeutic potential of its phytochemicals, underscoring their significance in the drug discovery process [1–9].

The medicinal importance of *Croton* species is increasingly acknowledged due to their remarkable diversity of secondary metabolites, particularly diterpenoids, which are critical to their therapeutic potential. Among these metabolites, clerodane, crotofolan, kaurane, and labdane-type

 doi:10.20944/preprints202504.2503.v1

diterpenoids are particularly noteworthy, as they are commonly found within the phytochemical composition of *Croton* plants [10]. Due to their structural diversity, diterpenoids hold significant potential in developing practical and selective anticancer pharmaceuticals. A prominent example of such a compound is paclitaxel; however, numerous other diterpenoids exhibit substantial bioactivities.

The *ent*-kauranes, classified as a group of diterpenoids, represent a significant category of secondary metabolites that exhibit various biological activities. These activities encompass anti-inflammatory, antiviral (specifically anti-HIV), antibacterial, leishmanicidal, hypotensive, insect antifeedant, and antitumor effects [11-12]. These compounds are found in several plant families but are especially abundant in genera such as *Isodon* (Asteraceae) and *Croton* (Euphorbiaceae). Due to their broad pharmacological potential, *ent*-kauranes have garnered considerable scientific interest, particularly for their antitumor properties as demonstrated by numerous studies [13-19]. Structurally, kauranes are tetracyclic compounds consisting of three six-membered rings and one five-membered ring (Fig. **1**). However, under the *ent*-kaurane classification, significant structural diversity arises due to skeletal rearrangements and metabolic reactions, including oxidations, ring-opening, and the formation of new rings. Among *ent*-kauranes, a relatively small subgroup known as *seco-ent*-kauranes features structures with one of the four constitutive rings opened. These are distinguished by the carbon positions involved in the ring cleavage, such as 2,3-*seco-ent*-kaurane [20], 6,7-*seco-ent*-kaurane [21], 8,9-*seco-ent*-kaurane [22], and 8,15-*seco-ent*-kaurane [23]. To date, the least common are the 3,4-*seco-ent*-kauranes, which nevertheless exhibit notable pharmacological properties [24–26], and recently, a new 8,14-*seco-ent*-kaurane has been reported [27].

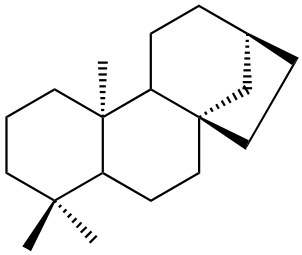


Figure 1. *Ent-kaurane* structure.

In our research, the 3,4-*seco-ent*-kauranes caracasine acid (**1**, Fig. 2) and its methyl ester, caracasine (**2**), were first isolated from *Croton micans* SW (initially misidentified as *Croton caracasana* [26]). Later, we reported a series of dimers derived from these compounds [28]. Both **1** and **2** display broad-spectrum antitumor activity against multiple human cancer cell lines [29]. We have also elucidated their mechanism of action in human leukemia cells [30-31]. As part of this investigation, a small group of caracasine acid derivatives were synthesized and evaluated for cytotoxicity, leishmanicidal, antibacterial, and anti-trypanosomal activity [32].

Building on our previous findings [32], which demonstrated that modifications to the α-β unsaturated system of caracasine acid (**1**) lead to a loss of activity, while esterification yields compounds with enhanced bioactivity, we explored the hemisynthesis of two new esters (**3**, **4**) derived from the parent carboxylic acid. Additionally, we reduced the double bond between C-16 and C-17 to obtain *ent*-3,4-*seco*-15-oxo-kaur-4(19)-en-3-oic acid (**5**), and synthesized two carboxylic anhydrides (**6**, **7**) from compounds **1** and **5**. The results on different human cancer cell lines encourage us to obtain different derivatives from these *seco-ent*-kauranes. As a continuation of our ongoing research, herein we report the synthesis and structural characterization of five new derivatives (**3**-**7**) of the parent compound, caracasine acid (**1**), and the screening of anti-tumoral activities on twelve human cells, including eight cancer cell lines and four normal fibroblasts.

 doi:10.20944/preprints202504.2503.v1

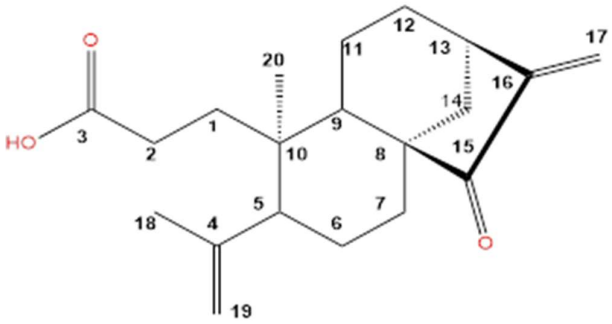


Figure 2. Caracasine acid (**1**), a 3,4-*seco*-*ent*-kaurane.

The present study aimed to elucidate the cytotoxic potential of a select group of caracasine acid derivatives, with consideration for future structural modifications via click chemistry.

2. Materials and Methods

2.1. Biological activity

2.1.1. Cell lines

The cancer cell lines used in the cytotoxic assays were acquired from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA) and maintained as described previously [33]. The MRC-5 and BJ human fibroblasts were used as non-tumoral control cells. The (MRC-5 LD and BJ LD) are also human fibroblast cell lines resistant to doxorubicin. The cell line CCRF-CEM is derived from T-lymphoblastic leukemia; they are cells with high chemosensitivity. Also, the cell line K562 represents cell samples from a patient with acute myeloid leukemia that presents Bcr-Abl translocation. The Raji and Ramos cell lines correspond to B-cell leukemia. Other cells like U2OS represent a child osteosarcoma, HCT116 is a colorectal tumor cell line, and (HCT116p53-/-, Horizon Discovery Ltd., Cambridge, UK) is a similar cell line with its p53 gene knocked down, a model of human cancer frequently associated with poor prognosis. The cells were maintained in Nunc/corning 80 cm2 plastic tissue culture flasks and cultured in a cell culture medium according to ATCC or Horizon recommendations (DMEM/RPMI 1640 with 5g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

2.1.2. Lymphocytes from normal donors.

Leukocytes from 5 normal donors were obtained from the Transfusion Medical Department at the Olomouc University Hospital. The cells were separated by the standard Ficoll-Hypaque method, as described previously [30-31]. The cells were incubated for 72 hours, as described for the standard compound screening method performed at the Institute of Molecular and Translational Medicine.

2.1.3. Cytotoxic MTS assay

In vitro cytotoxicity of compounds (**1-7**) was ascertained using a standard 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(sulfophenyl)-2H-tetrazolium (MTS), and it was performed at the Institute of Molecular and Translational Medicine by a robotic platform (High-ResBiosolutions). Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25.000-35.000 cells/mL based on cell growth characteristics). An automatic pipettor (30 µL) added cells into 384-well microtiter plates. All tested compounds were dissolved in 100% DMSO, and four-fold dilutions of the intended test concentrations were added in 0.15 µL aliquots at time zero to the microtiter plate wells by the echo acoustic non-contact liquid handler Echo550 (Labcyte). The assays were accomplished in technical duplicates and at least three

 doi:10.20944/preprints202504.2503.v1

biological replicates. The cells were incubated with the tested compound for 72 hours at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, cells were assayed using the MTS test. Aliquots (5 µL) of the MTS stock solution were pipetted into each well and incubated for 1-4 h. After this incubation period, the optical density (OD) was measured at 490 nm with an Envision reader (Perkin Elmer). Tumor cell survival (TCS) was calculated using the following equation: TCS = (ODdrug-exposed well/meanODcontrol wells) x 100%. The IC_{50} value, the compound concentration lethal to 50% of the tumor cells, was calculated from the appropriate dose-response curves in Dotmatics software (Updated version 2022, London, UK) [33]. After three days of incubation on the cancer cell lines and normal fibroblasts, the minimum inhibitory concentrations (IC_{50}) were obtained.

As previously described, no significant effect was observed when primary leukocytes were treated with caracasine or caracasine acid [30-31]; the results were confirmed with the different structures.

2.2. Spheroid formation

Spheroid culture aims to analyze the drug's effect on complex structures resembling solid tumors. The spheroids were prepared using the specific nonadherent plates for spheroid formation (Nunc Sphera Thermo Fisher) using the simple protocol outlined by the manufacturer. The cell lines HCT116 parental, HCT116KOTp53, A549, and U2OS were cultured at a density of 2500 cells per well using the medium with 10% fetal calf serum and antibiotics: McCoy for HCT116, F-12K (Kaighn's Modified) for A549, and Dulbecco Modified Eagle Media (DMEM). The cells were grown in culture for 48 hours as recommended by the manufacturer and as described by Das et al. [34] and Muñoz-Garcia et al [35].

Mixed spheroids were made by mixing MRC-5 fibroblasts with HCT116 or HCT116 p53 KO cells at a ratio of 1:1 (1000 cells/well); the cells were added immediately one after the other, and the formation of spheroids was monitored for 48 hrs. The mixed spheroids of both cell types had similar morphological features.

2.2.1. Spheroid viability assay

The cell viability of the treated spheroids was assessed using the kit CellTiter-Glo 3D (Promega Corporation). The kit is designed to measure ATP as an indicator of viability and generates a luminescent readout that is much more sensitive than colorimetric or fluorescence-based methods. The spheroids were cultivated with compounds ranging from 100 pM to 50 µM concentrations in triplicate for 72 hrs, and the luminescence was read with the Enspire apparatus (Perkin Elmer). A standard curve was performed for each analysis as recommended by the manufacturer.

Morphological assessment of spheroids was performed under the inverted microscope using grids every 12 hrs. No changes in morphology were observed during the process.

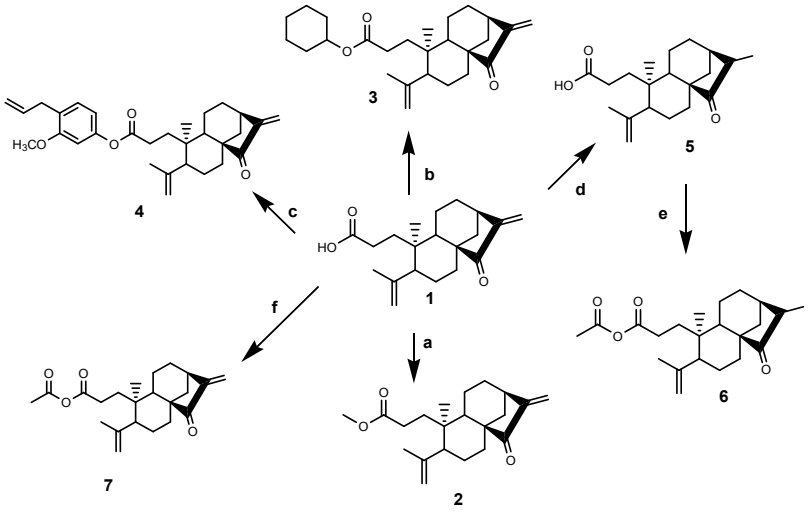
Chemistry

Caracasine acid (**1**), a 3,4-*seco*-*ent*-kaurane diterpenoid, and its methyl ester (**2**) demonstrated promising antitumoral activity across multiple cancer cell lines, prompting further structural optimization. Three key sites on **1** were selected for derivatization: (1) the α,β-unsaturated system (C15–C17), (**2**) the isolated olefin (C4–C19), and (**3**) the carboxylic acid moiety. These modifications yielded 18 new compounds [32], which were screened for antibacterial, antileishmanial, antitrypanosomal, and anticancer activity (MCF-7 and PC-3 cell lines). Structure-activity relationship (SAR) analysis highlighted the essential role of the α,β-unsaturated system in preserving bioactivity.

Building on these findings, which revealed that modifications to the α,β-unsaturated system of 1 diminish activity, while esterification enhances it, we pursued the hemisynthesis of two new esters (**3**–**4**). One incorporated natural eugenol to functionalize the carboxylic acid. Additionally, we

 doi:10.20944/preprints202504.2503.v1

reduced the C16–C17 double bond to obtain *ent*-3,4-*seco*-15-oxo-kaur-4(19)-en-3-oic acid (**5**) and synthesized two carboxylic anhydrides (**6–7**) from **1** and **5**. IR, NMR, and MS characterized all new derivatives.



Scheme 1. Reagents and conditions: a) **1**, CH_3OH, *p*-TsOH, $MgSO_4$, reflux, 6h; b) **1**, cyclohexanol, benzene, *p*-TsOH, $MgSO_4$, reflux, 6h; c) **1**, eugenol, DIC/DMAP/CH2Cl2, 24 h; d) **1**, H_2, Pd/C, THF, 4 h; e) **1**, CH_3COCl/Pyr, THF/N_2, 3h; f) **5**, CH_3COCl/Pyr, THF/N_2, 3h.

3.3.1. Isolation of caracasine acid (1)

The naturally occurring diterpene caracasine acid (**1**) was used as the starting material for the chemical transformations presented in this report. Compound **1** was isolated by water decoction of dry leaves, partitioned with chloroform, and purified by column chromatography on silica gel. Spectroscopic methods characterized compound 1, and the data were compared with those previously reported by us [26].

3.1.2. Synthesis of derivatives

The transformations made from **1** included the hemi-synthesis of five 3,4-*seco*-*ent*-kaurane diterpenes (**3**-**7**) whose structures are depicted in Scheme **1**. Ester derivatives **2** and **3** were synthesized by a Fischer-type esterification reaction using methanol and cyclohexanol, with *p*-TsOH as catalyst, under reflux conditions and anhydrous $MgSO_4$ as desiccant. The eugenol ester was obtained by Steglich esterification using DIC and DMAP in dichloromethane.

Compound **5** was prepared by catalytic hydrogenation on 10% Pd/C of the double bond between carbons 16 and 17 of **1**. This reaction occurs selectively in one of the olefins of the structure, and the compound **5** was obtained in high yields (85.2 %). It is known that the selective hydrogenation of carbon–carbon double bonds of conjugated carbonyl compounds in the presence of other isolated double bonds is a complex reaction, and it is greatly influenced by local structure. In this case, unpredictably, the conjugated olefin was the only one hydrogenated. It is possible that due to the structural arrangement of compound **1**, the reduction occurs more easily and exclusively at the double bond of the α,β-unsaturated system, leaving the other double bond present in the molecule unaffected.

Compound **7** was synthesized by reacting acid **1** with acetyl chloride in the presence of pyridine, and, in a similar procedure, acid **5** was treated with acetyl chloride to obtain anhydride **6**.

 doi:10.20944/preprints202504.2503.v1

The structures of these compounds were confirmed using HREIMS, ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, and HMBC.

3.1.3. Plant material

Plant material of *Croton micans* to obtain caracasine acid **1** was collected in Ocumare de la Costa, Estado Aragua, Venezuela, and identified by the Botanist Giovannina Orsini. A voucher specimen (MYF26071) has been deposited at the Herbario Víctor Manuel Ovalles, Facultad de Farmacia, Universidad Central de Venezuela.

3.1.4. Reagents and equipment

All reagents and solvents were from standard commercial sources and of analytical grade. TLC was used routinely to check all reactions using Merck Kieselgel 60 F254 aluminum plates; spots were examined under UV light at 254 nm and further visualized by sulphuric acid and *p*-anisaldehyde spray. Column chromatography was performed on silica gel (200–400 mm, 60 Å).

Spectroscopic methods elucidated the structures, including 1D and 2D NMR and HREIMS techniques. NMR spectra were recorded in the specified deuterated solvent at 500 MHz on a Brucker DRX-500 Avance spectrometer. Chemical shifts are expressed in ppm (δ) relative to the residual solvent signals, and the coupling constants *J* are given in Hertz (Hz). HREIMS were recorded with a Finnigan Trace mass spectrometer. IR spectra were performed on a FT-IR Thermo Nicolet Nexus 470 spectrophotometer. Melting points (mp) were uncorrected on an Electrothermal apparatus.

4. Synthesis and characterization

4.1.1. Caracasine acid (1)

Compound **1** was isolated from *Croton micans* Sw. by decoction from dried leaves, liquid-liquid extraction with $CHCl_3$, and purification by column chromatography on silica gel using a mixture of Hex/ EtOAc 70:30. White solid, 220–221 °C. IR mmax: 3305, 2933, 2863, 1735, 1720, 1698, 1638, 1120, 936 cm1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 5.95 (s, 1H, H-17b), 5.24 (s, 1H, H-17a), 4.86 (s, 1H, H-19a), 4.60 (s, 1H, H-19b), 3.04 (bs, 1H, H-13), 2.35 (d, *J* = 12.1 Hz, 2H, H-14), 2.06 (m, 2H, H-2), 2.05 (m, 1H, H-5), 1.88 (m, 2H, H-12), 1.72 (s, 3H, H-18), 1.68 (m, 2H, H-1), 1.63 (m, 2H, H-11), 1.50 (m, 2H, H-6), 1.36 (m, 1H, H-9), 1.27 (m, 2H, H-7), 1.06 (s, 3H, H-20). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 210.3(C-15), 179.4 (C-3), 149.3 (C-16), 146.8 (C-4), 114.7 (C-17), 114.1 (C-19), 51.9 (C-8), 49.9 (C-5), 43.4 (C-9), 41.5 (C-10), 38.1 (C-13), 36.4 (C-14), 33.5 (C-1), 32.2 (C-12), 32.1 (C-7), 28.4 (C-2), 24.7 (C-6), 23.4 (C-18), 21.6 (C-20), 18.3 (C-11). EIMS: m/z 316.2034 $[M]^+$ (Calcd for $C_{20}H_{28}O_3$, 316.2038).

4.1.2. Caracasine (2)

This compound was isolated together with **1** from plant material, but with the purpose of obtaining it in larger amounts, it was obtained by a Fisher esterification. To a solution of the acid **1** (101.8 mg, 0.3217 mmol) in methanol (5 mL), *p*-TsOH (0.16 mmol) and anhydrous $MgSO_4$ were added. The reaction mixture was stirred at reflux for 6 h and then filtered. The solvent was removed under reduced pressure and the residue purified by column chromatography on silica (Hex/EtOAc 80:20) to afford **2** as a white solid, 76%, 73-75oC. IR mmax: 2931 m, 2857, 1738, 1723, 1700, 1638, 1406, 1117, 932 cm1. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 5.93 (s, 1H, H-17a), 5.24 (s, 1H, H-17b), 4.87 (s, 1H, H-19a), 4.65 (s, 1H, H-19b), 3.61 (s, 3H, H-10), 3.05 (bs, 1H, H-13), 2.35 (d, J = 11.9 Hz, 2H, H-14), 2.06 (m, 2H, H-2), 2.03 (m, 1H, H-5), 1.87 (m, 2H, H-12), 1.73 (s, 3H, H-18), 1.68 (m, 2H, H- 1), 1.63 (m, 2H, H-11), 1.53 (m, 2H, H-6), 1.36 (m, 1H, H-9), 1.28 290 (m, 2H, H-7), 1.05 (s, 3H, H-20). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 210.0 (C-15), 173.8 (C-3), 149.4 (C-16), 146.9 (C-4), 114.5 (C- 17), 114.0 (C-19), 51.9 (C-8), 51.5 (C-10), 50.0 (C-5), 43.5 (C-9), 41.5 (C-10), 38.2 (C-13), 36.5 (C-14), 33.8 (C-1), 32.3 (C-12), 32.1 (C-7), 28.4 (C-2), 24.8 (C-6), 23.4 (C-18), 21.6 (C-20), 18.4 (C-11). EIMS: m/z 330.2193 $[M]^+$ (Calcd for $C_{21}H_{30}O_3$, 330.2195).

 doi:10.20944/preprints202504.2503.v1

4.1.3. Caracasine acid cyclohexyl ester (3)

To a solution of **1** (0.1002 g, 0.3167 mmol) in benzene (4 mL), cyclohexanol (0.033 mL, 0.3167 mmol), *p*-TsOH (0.16 mmol), and anhydrous $MgSO_4$ were added. The reaction mixture was stirred at reflux for 6 h and then filtered. The solvent was removed under reduced pressure, and the residue purified by column chromatography on silica (Hex/EtOAc 90:10) to afford **7** as a white solid, 72.5 %, m.p. 86-91°C. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 5.93 (s, 1H, H-17a), 5.24 (s, 1H, H-17b), 4.88 (s, 1H, H-19a), 4.67 (s, 1H, H-19b), 4.70 (m, 2H, H-1'), 3.05 (bs, 1H, H-13), 2.35 (m, 2H, H-14), 2.25 (m, 2H, H-2), 2.03 (m, 1H, H-5), 1.91 (m, 2H, H-12), 1.80 (m, 4H, H-2', H-6'), 1.73 (s, 3H, H-18), 1.68 (m, 2H, H-1), 1.61 (m, 2H, H-11), 1.53 (m, 2H, H-6), 1.50 (m, 4H, H-3', H-5'), 1.37 (m, 1H, H-9), 1.27 (m, 2H, H-7), 1.04 (s, 3H, H-20), 0.89 (m, 2H, H-4'). NMR ^{13}C (125 MHz, $CDCl_3$) δ ppm: 210.2 (C-15), 173.2 (C-3), 149.4 (C-16), 146.9 (C-4), 114.4 (C-17), 113.9 (C-19), 72.4 (C-1'), 51.9 (C-8), 49.8 (C-5), 43.4 (C-9), 41.5 (C-10), 38.1 (C-13), 36.4 (C-14), 33.8 (C-1), 32.3 (C-7), 32.1 (C-12), 31.7 (C-2'), 31.7 (C6'), 29.1 (C-2), 25.4 (C-6), 24.8 (C4'), 23.8 (C-3'), 23.8 (C5'), 23.7 (C-18), 21.5 (C-20), 18.4 (C-11). EI:MS: m/z = 398.4 $[M]^+$ for $C_{26}H_{38}O_3$.

4.1.4. Caracasine acid eugenol ester (4)

To a stirred solution of 1 (100.3 mg, 0.32 mmol) in 10 ml of DCM, DIC(43 µL. 0.32 mmol) was added followed by addition of DMAP (15mg, 0.01 mmol) and after 10 min of stirring, eugenol (52.74 mg, 0.32 mmol) was added and allowed to stir the reaction mixture at rt for 24h. Work-up was done with water and the mixture extracted with CHCl3 (3x10 ml), washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude compound was purified by column chromatography using 40% EtOAc: Hexane as the solvent system. The compound was obtained as a pink oil, 79% yield; ^{1}H NMR: $CDCl_3$ (500 MHz): 6.90 (s, 1H, H-3'), 6.87 (m, 2H, H-5'), 6.69 (m, 1H, H-6'), 5.96 (m, 1H, H-8'), 5.92 (s, 1H, H-17b), 5.09 (m, 2H, H-9'), 5.24 (s, 1H, H-17a), 4.85 (brs, 1H, H-19b), 4.65 (brs, 1H, 19a), 3.82 (s, 3H, H-10'), 3.62 (s, 3H, H-21), 3.35 (d, 2H, H-7'), 3.04 (brs, 1H, H-13), 2.35 (d, *J* = 12.3 Hz, 2H, H-14), 2.10 (m, 2H, H-2), 2.05 (dd, *J* = 9.4, 3.5 Hz, 1H, H-5), 1.85 (m, 2H, H-12), 1.72 (s, 3H, H-18), 1.65 (m, 2H, H-11), 1.63 (dd, *J* = 6.7, 3.10 Hz, H-1), 1.50 (m, 2H, H-6), 1.34 (d, *J* = 8.35 Hz, H-9), 1.27 (m, 2H, H-7), 1.06 (s, 3H, H-20). NMR ^{13}C (100 MHz, $CDCl_3$) δ ppm: 210.2 (C-15), 173.2 (C-3), 149.4 (C-16), 148.0 (C-2'), 146.9 (C-4), 144.0 (C-1'), 137.9 (C-8'), 132.3 (C-4'), 121.3 (C-5'), 117.3 (C-6'), 114.5 (C-3'), 114.4 (C-17), 113.9 (C-19), 111.3 (C-9'), 55.9 (C-10'), 51.9 (C-8), 49.8 (C-5), 43.4 (C-9), 41.5 (C-10), 39.8 (C-7'), 38.1 (C-13), 36.4 (C-14), 33.8 (C-1), 32.3 (C-7), 32.1 (C-12), 31.7 (C-2'), 31.7 (C6'), 29.1 (C-2), 25.4 (C-6), 24.8 (C4'), 23.8 (C-3'), 23.8 (C5'), 23.7 (C-18), 21.5 (C-20), 18.4 (C-11). EIMS: m/z = 462.62 $[M]^+$ for $C_{30}H_{38}O_4$.

4.1.5. Ent-3,4-seco-15-oxo-kaur-4(19)-en-3-oic acid (5)

Compound **1** (0.202 g, 0.639 mmol) was dissolved in dry THF (10.0 mL), and then Pd/C 10% (35.6 mg, 31.6 µmol) was added. It was stirred under an H2 atmosphere for 4 h, and the mixture was filtered through celite, and the solvent was evaporated under a vacuum. The residue was purified by column chromatography on silica gel eluted with Hex/EtOAc 80:20, to afford 5 as a white solid, 86.6%, m.p. 169- 171 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 4.87 (s, 1H, H-19a), 4.65 (s, 1H, H-19b), 2.42 (bs, 1H, H-13), 2.39; 1.37 (m, 2H, H-14), 2.29 (m, 2H, H-2), 2.23 (m, 1H, H-16), 2.20 (CH_3O), 2.05 (m, 1H, H-5), 1.78; 1.26 (m, 2H, H-7), 1.72 (s, 3H, H-18), 1.67 (m, 2H, H-6), 1.66 (m, 2H, H-1), 1.56 (m, 2H, H-12), 1.52; 1.23 (m, 2H, H-11), 1.20 (m, 1H, H-9), 1.09 (d, *J* = 6.92 Hz, 3H, H-17), 1.03 (s, 3H, H-20). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 224.6 (C-15), 179.8 (C-3), 146.7 (C-4), 113.9 (C-19), 52.0 (C-8), 49.6 (C-5), 47.7 (C-16), 43.2 (C-9), 41.0 (C-10), 37.1 (C-14), 34.8 (C-13), 33.3 (C-1), 32.7 (C-7), 28.3 (C-2), 24.9 (C-12), 24.5 (C-6), 23.3 (C-18), 21.6 (C-20), 18.1 (C-11), 10.0 (C-17). EIMS: m/z 318.2193 $[M]^+$ (Calcd for $C_{20}H_{30}O_3$, 318.2195).

4.1.6. Acetic-ent-3,4-seco-15-oxo-kaur-4(19)-en-anhydride (6)

 doi:10.20944/preprints202504.2503.v1

To a round-bottom flask containing compound **5** (60 mg, 0.189 mmol) and pyridine (15.26µ µL, 0.189 mmol), under a nitrogen atmosphere, acetyl chloride (13.48µL, 0.189 mmol) was added dropwise. The resulting mixture was stirred for 3h. After complete reaction, workup was done adding 10 mL of water, followed by extraction with CH3Cl (3 × 5 5mL), dried with anhydrous sodium sulphate, and evaporated under vacuum. The crude product was purified by silica gel column chromatography eluted with n-hexane/CH_2Cl_2 (1:1), affording compound **6** as a white amorphous solid with 85% yield.

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 4.87 (s, 1H, H-19a), 4.65 (s, 1H, H-19b), 3.45 (m, 2H, H-17), 3.45 (m, 2H, H-1'), 2.53 (brs, 1H, H-13), 2.25 (CH_3CO), 2.32 (m, 2H, H-14), 2.25 (brs, 2H, H-16), 2.10 (m, 2H, H-2), 2.03 (m, 1H, H-5), 1.72 (s, 3H, H-18), 1.68 (m, 2H, H-1), 1.64 (m, 2H, H-12), 1.51 (m, 1H, H-6), 1.49 (m, 2H, H-11), 1.25 (m, 1H, H-7), 1.23 (bs, 1H, H-9), 1.14 (t, *J* = 10.3 Hz, 3H, H-2'), 1.05 (s, 3H, H-20). ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 221.8 (C-15), 165.7 (C-3), 165.4 (COCH3), 146.6 (C-4), 113.9 (C-19), 69.9 (C-17), 66.7 (C-1'), 53.4 (C-16), 52.8 (C-8), 50.3 (C-5), 43.3 (C-9), 41.8 (C-10), 36.5 (C-14), 34.1 (C-13), 34.0 (C-1), 32.9 (C-7), 30.9 (C-12), 28.6 (C-2), 25.5 (C-6), 23.8 (C-18), 22.0 (C-20), 20.5 (CH3CO), 18.9 (C-11), 15.6 (C-2'). EIMS: m/z = 360.49 $[M]^+$ for $C_{22}H_{31}O_4$.

4.1.7. Acetic/caracasine anhydride (7)

With a similar procedure to that described for compound **6**, the acetyl anhydride of **1** was obtained. To (0.100 mg, 0.32 mmol) of **1** dissolved in 5 mL of THF, 25.8 µL (0.32 mmol) of pyridine was added, the mixture was stirred under N_2 for 15 min, and the acetyl chloride (22.8 µL, 0.32 mmol) was added dropwise. The solution was kept under stirring for 3h and then quenched with 10 mL of water, extracted with dichloromethane, washed with brine, and the organic phase dried over Na_2SO_4. Evaporation under vacuum gave **7** as a white powder. The product was further purified by silica gel chromatography eluted with a mixture of n-hexane/EtOAc (8:2), affording compound (**7**) with 87% yield, m.p. 123- 125 °C.

^{1}H NMR (500 MHz, $CDCl_3$) δ ppm: 1.08 (s, 3H, H-20), 1.27 (m, 2H, H-7), 1.36 (m, 1H, H-9), 1.50 (m, 2H, H-6), 1.64 (dd, 2H, *J* = 6.7, 1.10 Hz, H-1), 1.73 (s, 3H, H-18), 1.86 (m, 2H, H-12), 2.05 (dd, *J* = 9.4, 3.5 Hz, 1H, H-5), 2.12 (m, 2H, H-2), 2.37 (d, *J* =12 Hz, 2H, H-14), 2.22 (s, 3H, CH_3CO), 3.05 (brs, 1H, H-13), 3.60 (s, 3H, H-21), 4.65 (brs, 1H, H-19a), 4.89 (brs, 1H, H-19b), 5.24 (s, 1H, H-17a), 5.94(s, 1H, H-17b). ^{13}C NMR (125.5 MHz, $CDCl_3$) δ ppm: 210.2 (C-15), 165.5 (C-3), 165.4 (CO-CH_3), 149.1 (C-16), 146.3 (C-4), 114.7 (C-17), 114.1 (C-19), 51.9 (C-8), 49.5 (C-5), 43.3 (C-9), 43.5 (C-9), 41.4 (C-10), 37.9 (C-13), 36.4 (C-14), 33.3 (C-1), 32.2 (C-12), 32.0 (C-7), 28.3 (C-2), 24.5 (C-6), 23.4 (C-8), 21.6 (C-20), 20.6 (CH3CO), 18.2 (C-11). ESI-MS: m/z = 358.472 $[M]^+$ for $C_{22}H_{30}O4$.

3. Results

The compounds were not cytotoxic against normal human leukocytes, with viability > 80 % at concentrations up to 50 µM. The results are similar to those described previously for caracasine and caracasine acid [30-31].

Table 1 presents the effects of the compounds under investigation on two fibroblast cell lines. Compounds **7** and **3** had a similar impact to caracasine and caracasine acid against all fibroblast cell lines. Compounds 4, 5, and 6 did not affect the cells.

Table 2 shows that the compounds were highly effective against leukemic cells of different origins. Compounds 3 and 7 retained the cytotoxic activity against the cell lines, and the other structures had no effect.

Table 1. Effect of the different compounds on different cell types and cultures.

HUMAN FIBROBLASTS				
CODE	**BJ**	**BJ LD**	**MRC-5**	**MRC-5 LD**
7	7.68	7.63	6.45	5.53

 doi:10.20944/preprints202504.2503.v1

3	7.38	6.5	5.45	2.69
4	>50	>50	>50	>50
5	>50	>50	>50	>50
6	>50	>50	>50	>50
Caracasine acid (**1**)	6.88	7.04	6.48	6.69
Caracasine (**2**)	7.28	7.42	6.84	6.36

Table 2. Effect of the compounds on leukemic cells.

HUMAN LEUKEMIA CELL LINES				
CODE	**CCRF-CEM**	**K562**	**RAJI**	**RAMOS**
7	0.45	4.6	2.21	0.96
3	0.38	2.21	1.86	0.78
4	>50	>50	>50	>50
5	>50	>50	>50	>50
6	>50	>50	>50	>50
Caracasine acid (**1**)	0.38	4.77	0.85	0.62
Caracasine (**2**)	0.86	3.29	1.45	0.96

The compounds also affected the growth of colon, lung, and osteosarcoma with values lower than 5 μM when the cells were grown in the standard 2D condition; however, the effect is lost when the spheroids are challenged with the compounds (Table 3). Mixed spheroids of different cell types, fibroblasts, and tumor cells that resemble the tumor microenvironment were also resistant to the effect of the compounds, as shown with the monogenic spheroids. There were also no changes in morphology in the spheroids upon treatment.

Table 3. Effects on other cell types.

HUMAN TUMORS 2D				
CODE	**HCT116 par**	**HCT116KO53**	**A549**	**U2OS**
7	2.12	1.91	3.72	2.21
3	1.59	1.63	2.69	1.66
4	>50	>50	>50	>50
5	>50	>50	>50	>50
6	>50	>50	>50	>50
Caracasine acid (**1**)	1.73	1.52	5.98	1.79
Caracasine (**2**)	2.02	1.91	8.39	3.19
HUMAN TUMORS SPHEROIDS				
CODE	**HCT116 par**	**HCT116KO53**	**A549**	**U2OS**
7	>50	>50	>50	>50
3	>50	>50	>50	>50
4	>50	>50	>50	>50
5	>50	>50	>50	>50
6	>50	>50	>50	>50
Caracasine acid (**1**)	>50	>50	>50	>50

 doi:10.20944/preprints202504.2503.v1

Caracasine (**2**)	>50	>50	>50	>50

4. Discussion

Previous investigations into caracasine (**2**) and caracasine acid (**1**) have indicated that these compounds exhibit activity against various leukemic cell lines, with caracasine acid specifically influencing the NFkB signaling pathway. In addition, caracasine acid, the most potent inducer of cytotoxic response, induced apoptosis even at short-term incubations in leukemic cells [30-31]. Compounds **3** and **7** resemble the effect of caracasine acid in the cell cycle, at similar concentrations, compounds **4**, **5**, and **6** do not have any effect. The eugenol ester derivative of caracasine acid (**4**) exhibited no biological activity, likely due to the aromaticity of the ester moiety, which may disrupt the compound's efficacy. Steric hindrance from the bulky substituent could further impede interaction with the target site, a phenomenon consistent with our previous observations for benzyl ester derivatives [32]. In contrast, the loss of activity in compounds **6** and **7** is attributed to the absence of the α,β-unsaturated system, a structural feature critical for their putative mechanism of action.

To examine the structures discussed in this report and explore the potential for a broader impact of these compounds, a series of experiments was conducted utilizing standard two-dimensional (2D) culture and spheroid three-dimensional (3D) culture methodologies. Although the active compounds demonstrated efficacy against different leukemic and other tumor cell lines, they were inactive when tested against the spheroid (3D) cultures. This lack of activity may be attributed to the dense structure of the spheroids, which potentially hampers the compounds' ability to penetrate the cells to induce apoptosis. Furthermore, the spheroids' morphological characteristics remained unchanged compared to the control, despite applying concentrations of up to 50 μM. Similar effects were observed with mixed spheroids.

Notably, none of the compounds were cytotoxic against the primary lymphocyte cells, suggesting that changes in the structure did not affect cell viability. The effect observed on normal fibroblast culture is probably due to the inhibition of NFkB signaling, which is crucial for cell survival, at least in 2D cultures [30, 36-37].

Despite these results, it is conceivable that the most active compounds could influence the tumor microenvironment if an effective delivery system were used to enhance cellular uptake. Future investigations will be directed toward this objective.

These findings reinforce the rationale for further exploring these compounds in the context of cancer, other diseases, and toxicological studies [38-40]. There is considerable potential for new synthetic pathways to modify the carboxylic moiety, providing opportunities to develop new active compounds.

5. Conclusions

The newly synthesized compounds numbered **3** to **7**, which are derived from structural modifications of the parent acid (**1**), provide further confirmation that the α, β-unsaturated system is a critical pharmacophore in these bioactive compounds. Additionally, the findings indicate that substantial structural modifications should prioritize the carboxylic functional group. These results lay the groundwork for developing novel derivatives with improved anticancer activity and optimizing the active compounds presented in this study. Future research endeavors will concentrate on synthesizing innovative hybrid derivatives that link the *ent*-kaurane scaffold with other bioactive natural compounds through triazole rings employing click chemistry. Moreover, the data presented indicate that compounds **1**, **2**, **3**, and **7** stand out as promising therapeutic candidates for advancing leukemia treatment. This study provides compelling evidence regarding the anticancer potential of caracasine acid (**1**) and its derivatives, suggesting that 3,4-*seco*-*ent*-kauranes may serve as promising lead candidates for further exploration in structure-activity relationship (SAR) and quantitative structure-activity relationship (QSAR) studies.

 doi:10.20944/preprints202504.2503.v1

Author Contributions: Conceptualization, A.I.S., and J.B.D.S.; methodology, A.I.S., K.C. J.B.D.S, J.V.G., C.V.D.S, S.G..; validation, A.I.S., K.C., S.G., J.B.D.S., C.V.D.S and P.D.; formal analysis, A.I.S, K.C., J.B.D.S.; investigation, A.I.S., K.C., S.G., J.B.D.S., C.V.D.S and P.D.; resources, A.I.S, M.H. and P.D..; data curation, J.B.D.S..; writing—original draft preparation, A.I.S, J.B.D.S..; writing—review and editing, A.I.S., J.B.D.S..; project administration, A.I.S., P.D. and M.H..; funding acquisition, A.I.S., P.D. and M.H. All authors have read and agreed to the published version of the manuscript

Funding: The Venezuelan Ministry of Science and Technology and FONACIT Project 20230PGP99 funded the chemical part of this research. All biological part of the study was supported in parts by the infrastructural projects (CZ-OPENSCREEN – LM2023052; EATRIS-CZ – LM2023053), the Czech biobank network (BBMRI - LM2023033), and the projects National Institute for Cancer Research (Program EXCELES, ID Project No. LX22NPO5102) Funded by the European Union - Next Generation EU from the Ministry of Education, Youth and Sports of the Czech Republic (MEYS), project TN02000109 (Personalized Medicine: From Translational Research into Biomedical Applications is co-financed with the state support of the Technology Agency of the Czech Republic as part of the National Centers of Competence Program), and project SALVAGE (registration number: CZ.02.01.01/00/22_008/0004644).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The dataset is available upon request from the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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