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Natural products with dual anti-SARS-CoV-2 activity and selective cytotoxicity against leukemia cells from *Rhaponticum carthamoides*

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Abstract

Rhaponticum carthamoides (*Leuzea carthamoides*) is a Siberian medicinal plant known for a variety of beneficial effects and traditionally used in Russia and Eastern Europe. However, its potential as a source of antiviral agents and compounds with selective cytotoxicity against cancer cells remains underexplored. In this study, nine flavonoids, including (*2R*)-eriodictyol 5-O-β-glucopyranoside, (*2S*)-eriodictyol 5-O-β-glucopyranoside, eriodictyol 7-O-β-glucopyranoside, eriodictyol, quercetagetin 7-O-β-glucopyranoside, 6-hydroxykaempferol 7-O-β-glucopyranoside, 6-methoxyquercetin 7-O-β-glucopyranoside, spinacetin 7-O-β-glucopyranoside and 6-methoxykaempferol 3-O-β-glucopyranoside were isolated and characterized from the plant's leaves. Their individual structures were determined by 2D NMR spectroscopy techniques and high-resolution mass spectrometry. The compounds were tested for cytotoxic activity against an acute myeloid leukemia (AML) cell line (MOLM-13), and two non-malignant cell lines representing kidney epithelium (NRK) and cardiomyocyte (H9c2). Three compounds, quercetagetin 7-O-β-glucopyranoside, 6-methoxyquercetin 7-O-β-glucopyranoside, and spinacetin 7-O-β-glucopyranoside showed selective cytotoxicity towards MOLM-13 cells at 28-50 μM concentrations with little or no effect on the non-malignant cells. Notably, spinacetin

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7-O-β-glucopyranoside was effective against MOLM-13 cells without affecting the viability of the non-malignant NRK and H9c2 cells, and it showed synergistic activity with the bench-mark AML drug daunorubicin. In SARS-CoV-2 pseudovirus neutralization assays, spinacetin 7-O-β-glucopyranoside, 6-methoxykaempferol and 3-O-β-glucopyranoside demonstrated significant antiviral activity against variant Omicron (XBB 1.5) and some antiviral activity against Delta (B.1.617.2.) in A549$^{ACE2/TMPRSS2}$ cells. These findings identify spinacetin 7-O-β-glucopyranoside as a promising dual-acting compound with both anti-leukemic and antiviral properties, emphasizing the relevance of natural products in modern drug discovery.

Keywords: Rhaponticum carthamoides; polyoxygenated flavonoids; Spinacetin 7-O-β-glucopyranoside; cytotoxicity; acute myeloid leukemia; 2D NMR; SARS-CoV-2

Introduction

Maral root, (*Rhaponticum carthamoides* (Willd.) syn. *Leuzea carthamoides* (Willd.) Iljin, also known as Russian root or Russian leuzea, is an herbaceous perennial plant in the Asteraceae family (Fig. S1).[1]. This plant is native to southern Siberia, east Kazakhstan and the Altai region, up to 2300 m above sea level and it is named after the maral deer (*Cervus elaphus sibiricus*), which excavate and consume plant roots during rutting season[2]. Based on observations by local hunters long time ago, maral deer regain their strength after consuming the roots, inspiring its medicinal use for recovery following illness or physical overstrain. Maral root has for centuries been used by the Siberian population as a medical plant, particularly because of its significant adaptogenic effect[3]. In traditional Chinese, Tibetan and Mongol medicine, its beneficial effects have been known for millennia[4].

The plant extract has been commonly used by elite Soviet and Russian athletes, and recently it was shown in a study by Roumanille et al. that Maral root supplement stimulates protein synthesis after resistance training in rats[5]. Currently, compounds from roots and rhizomes are used for their tonic and adaptogenic properties in different dietary supplements to eliminate exhaustion, promote muscle growth, treat impotence, as well as for recovery after illnesses, surgery, or chemical intoxication[3,6].

Maral root produces several biologically active constituents. Approximately 200 chemical constituents belonging to several groups of natural products, including ecdysteroids, and phenolics (flavonoids and phenolic acids) in addition to polyacetylenes, sesquiterpene lactones and triterpenoid glycosides have been found in the plant[7, 8]. Flavonoids are notably found in all

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higher plants and plant-derived products, including fruits, vegetables, propolis and honey. In addition to being potent antioxidants and free radical scavengers, several flavonoids exhibit antiviral activity and selective cytotoxic activity against cancer cell lines[9,10, 11, 12].

The primary objective of this study was to characterize the phenolic compounds present in the leaves of Maral root, to determine their molecular structures, and their biological activity in the context of both acute myeloid leukemia (AML) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) cellular models. Patients with AML, whose immune systems are weakened by both the disease and its therapy, are at increased risk of experiencing severe outcomes from infections, including COVID-19. The respiratory viruses, such as SARS-CoV-2 or influenza, induce both local and systemic inflammation, facilitating metastasis in cancer patients[13, 14]. Given the known therapeutic potential of Maral root, we evaluated these compounds' potential as multifunctional anti-viral and anti-leukemic therapeutic agents.

Results

Isolated and elucidated natural products of Maral root leaves

Nine flavonoids were characterised from the leaves of *Rhaponticum carthamoides*, including (*2R*)-eriodictyol 5-O-β-glucopyranoside (**1**), (*2S*)-eriodictyol 5-O-β-glucopyranoside (**2**), eriodictyol 7-O-β-glucopyranoside (**3**), eriodictyol (**4**), quercetagetin 7-O-β-glucopyranoside (**5**), 6-hydroxykaempferol 7-O-β-glucopyranoside (**6**), 6-methoxyquercetin 7-O-β-glucopyranoside (**7**), spinacetin 7-O-β-glucopyranoside (**8**) and 6-methoxykaempferol 3-O-β-glucopyranoside (**9**) (Fig. 1.). The structures of the individual compounds were determined by 2D NMR spectroscopy and mass spectrometry. ^{1}H and ^{13}C NMR chemical shift values of these compounds were completely assigned by a combination of several 1D and 2D NMR experiments including 1D ^{1}H, 1D ^{13}C CAPT, 2D ^{1}H-^{13}C HMBC, 2D ^{1}H-^{13}C HSQC, 2D ^{1}H-^{13}C H2BC, 2D ^{1}H-^{13}C HSQC-TOCSY, 2D ^{1}H-^{1}H COSY and 2D ^{1}H-^{1}H ROESY (Tables S1-S3). In addition, the flavonoids 6-OH-kaempferol 7-O-β-(6-O-acetyl-glucopyranoside) (**10**), 6-OMe-quercetin 7-O-β-(6-O-acetyl-glucopyranoside) (**11**), the phenolic compounds chlorogenic acid (**12**) and neochlorogenic acid (**13**), as well as the ecdysteroid hormone 20-OH-ecdysone (**14**) were identified by a combination of their mass spectra, HPLC retention times and UV spectra (table S4) and 1D ^{1}H selective TOCSY NMR (fig. S10).

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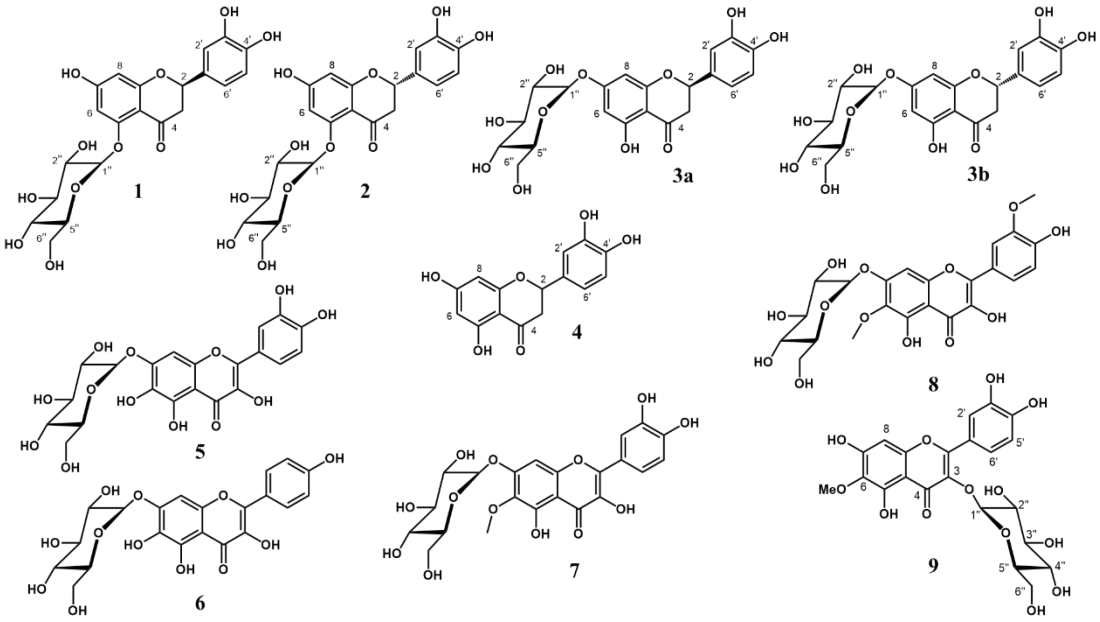
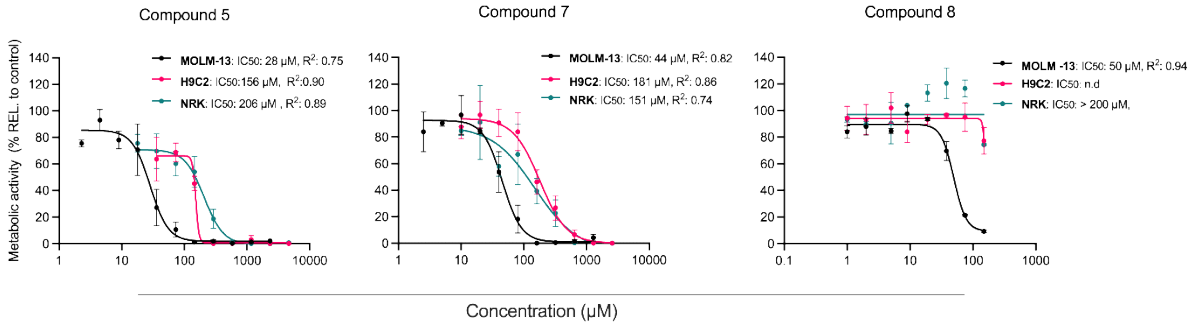


Fig. 1. Molecular structures of the isolated natural compounds from leaves of *Rhaponticum carthamoides.* (*2R*)-eriodictyol 5-O-β-glucopyranoside (**1**), (*2S*)-eriodictyol 5-O-β-glucopyranoside (**2**), eriodictyol 7-O-β-glucopyranoside (mixture of **3a** and **3b**), eriodictyol (**4**), quercetagetin 7-O-β-glucopyranoside (**5**), 6-hydroxykaempferol 7-O-β-glucopyranoside (**6**), 6-methoxyquercetin 7-O-β-glucopyranoside (**7**), spinacetin 7-O-β-glucopyranoside (**8**) and 6-methoxykaempferol 3-O-β-glucopyranoside (**9**) isolated from leaves of *R. carthamoides*.

Compounds 5, 7 and 8 exhibit cytotoxic selectivity towards the AML cell line MOLM-13

The AML cell line MOLM-13 was used for the anti-leukemic activity assessment of the nine compounds isolated from Maral root. NRK and H9c2 cells were used as control cells, representing cardiomyocyte and kidney epithelial cells, both tissues that are sensitive to traditional anti-cancer drugs. First, a dose-response curve was generated from each of the nine compounds (Fig. 2. and supplementary Fig. S2.). Three compounds displayed high cytotoxic activity (**5**, **7**, **8**), two intermediate activity (**1** and **9**), and three low (**2**, **3**, **4** ,**6**) activity towards MOLM-13 cells. Low activity was defined at EC_{50} >500 µM, intermediate activity was defined as EC_{50} in 51-500 µM range. All $EC_{50} \leq 50$ µM was defined as high activity.



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Fig. 2. Compounds were tested on MOLM-13 AML cells and H9C2 cardiomyoblastic and NRK kidney epithelial cells and cytotoxic activity determined by metabolic activity using the WST-1 assay. The EC_{50} values were determined by non-linear regression analysis as described in the methods section. The data are presented as average of 3-4 independent experiments, and presented as mean ± SEM.

Compounds **5**, **7** and **8** showed high cytotoxicity towards MOLM-13 cells. To assess whether these compounds demonstrate selective cytotoxicity against leukemia cells, they were subsequently tested on NRK and H9c2 cell lines (Fig. 2.). Compounds **5** and **7** showed cytotoxic activity against both NRK and H9c2 cells; Compound **5** was intermediate cytotoxic towards both NRK and H9c2 cells with EC_{50} of 206 µM and 156 µM, respectively. Even so, this activity was considerably lower than its cytotoxic activity against MOLM-13 cells with an EC_{50} of 28 µM. Compound **7** exhibited EC_{50} of 151 µM against NRK and 181 µM against H9c2, respectively, which was notably lower than that against MOLM-13 cells (EC_{50}: 44 µM). Compound **8** showed low cytotoxic activity towards H9c2 or NRK cells while being highly cytotoxic to MOLM-13 cells (IC_{50}: 50 µM) and was thus considered the most promising drug for further cytotoxicity studies.

Compound 8 and daunorubicin combination reveals model-specific differences in additivity

To investigate whether natural compounds could potentiate the anti-leukemic activity of currently used drugs, the most promising compounds, compounds **7** and **8** were tested in combination with either of the three AML drugs: etoposide, daunorubicin (DNR), and cytarabine (AraC). To validate the drug interactions, Bliss, Loewe, HSA, and ZIP calculations were employed by using SynergyFinder Plus (Fig. 3.)[15]. According to the LOEWE and HSA model, compound **8** showed positive interaction with DNR, exceeding the effect of the stronger single agent in concentrations of 120 µM of compound **8** with 0.0075 or 0.01 µM DNR. However, under the Bliss independence model, the interaction was less than additive, suggesting the effect is model-dependent. Synergy score details of interactions of compound **7** and **8** in combination with the AML drugs are provided in supplementary data (Fig. S3-S7.).

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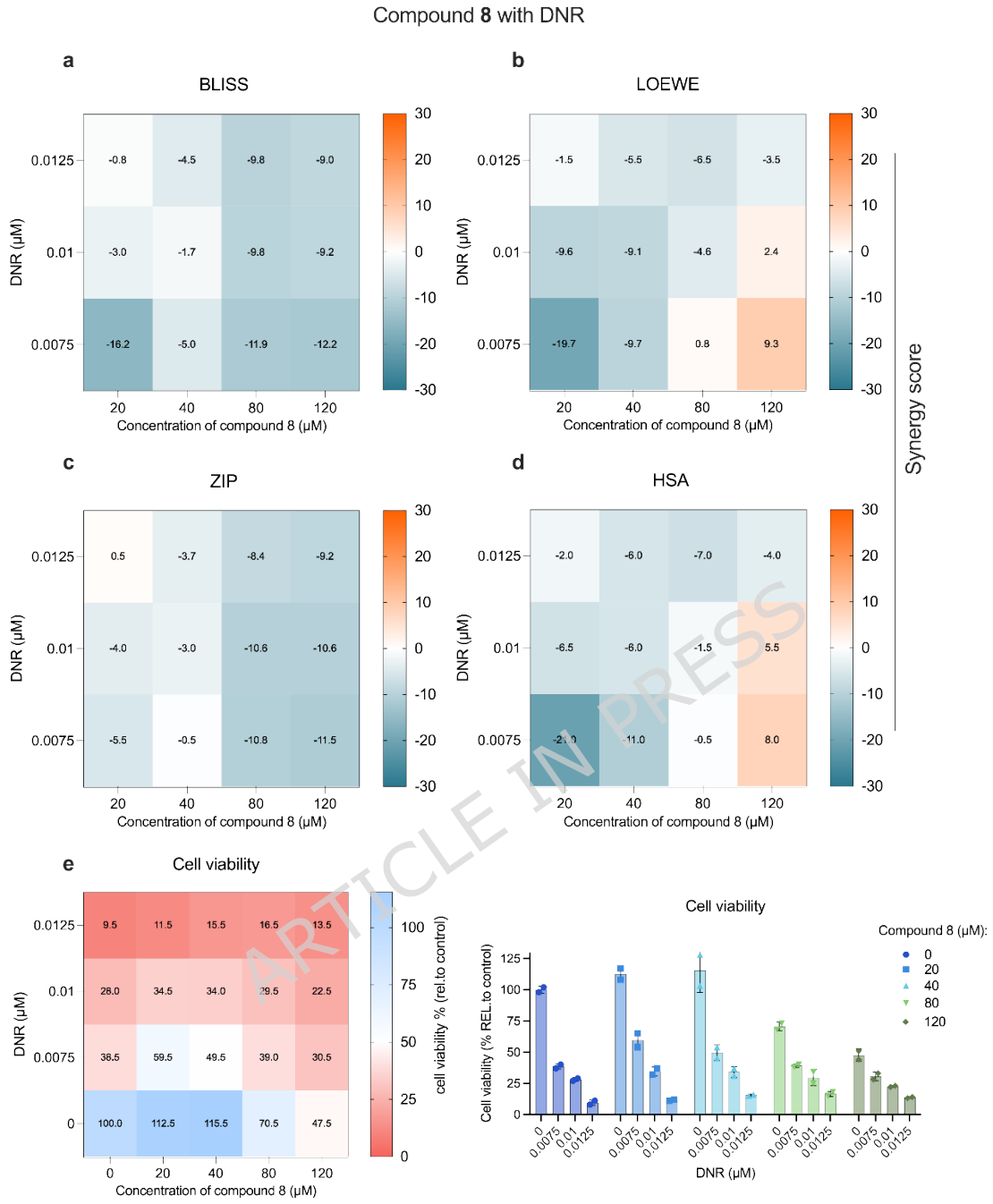


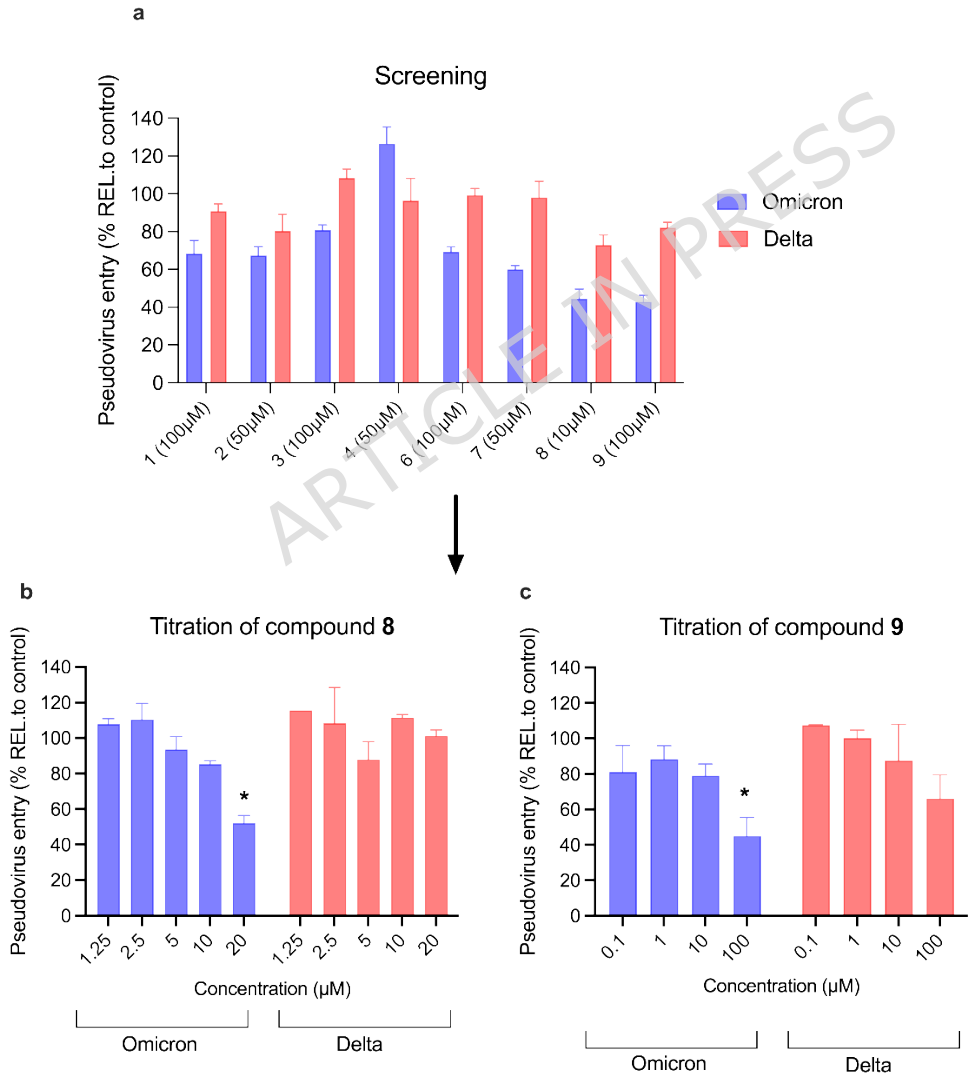
Fig. 3. Additive effect of compound **8** in combination with the AML drug daunorubicin (DNR) in MOLM-13 cells after 72 hours of co-treatment. Interactions were evaluated **a**. Bliss **b**. Loewe **c**. ZIP **d**. HSA reference models in SynergyFinder Plus. Color intensity indicates the magnitude and direction of synergy, with orange representing synergy (positive scores) and blue indicating antagonism (negative scores). Strongest additive effects were observed in LOEWE and HAS models. **e.** The cell viability matrix is used to calculate the additive effects. n=2.

Selective inhibition of Omicron and Delta SARS-CoV-2 pseudoviruses by isolated natural compounds

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We first assessed the cytotoxicity in both the A549$^{ACE2/TMPRSS2}$ cells and the parental cell line A549 and observed no viability reduction within the tested concentrations (Supplementary Fig. S8.). Based on this data, in the following pseudovirus neutralization assay experiments, the highest achievable concentrations of compounds were utilized (Fig. 4., a).

The compounds were screened for inhibitory effects on Omicron (XBB 1.5) and Delta (B.1.617.2.) pseudoviruses, viruses that contain the spike protein of SARS-CoV-2 but not requing high containment laboratories. Overall, all tested compounds exhibited more substantial inhibitory activity against the Omicron pseudoviruses, with compounds **8** inhibiting entry of 67% of pseudoviral particles at only 10 µM. Similar degrees of inhibition was achieved with compound 9, but at higher concentration (100 µM). Maral root compounds showed limited potential against the Delta variant, with compound **8** as the most efficient (inhibition of entry of 27%).



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Fig. 4. Screening and titration of the compounds of interest on A549$^{ACE2/TMPRSS2}$ cell line. **a**. Percentage pseudovirus entry in the host cells relative to untreated was evaluated in the isolated compounds from Maral root **1**, **2**, **3**, **4**, **6**, **7**, **8** and **9** after 72 h. Screenings of both Omicron (XBB 1.5) and Delta (B.1.617.2.) variant were tested. One biological replicate was conducted with three technical replicates. **b**. Titration of compound **8** with Omicron (XBB 1.5) and Delta (B.1.617.2.) variant of the SARS-CoV-2 in concentrations ranging from 1.25 to 25 µM. n=3 **c.** Titration of compound **9** with Omicron (XBB 1.5) and Delta (B.1.617.2.) variant of the Sars-CoV-2 in pseudovirus particle in concentrations ranging from 0.1 to 100 µM. n=2. One-way ANOVA with Dunnett's multiple comparisons test was used to establish statistically significant differences when compared to uninhibited control sample. $p<0.05$ (*). Data are presented as mean ± SEM

To further investigate the antiviral potential of compounds **8** and **9**, titration experiments were conducted. The titrations (Fig. 4., b and c) showed a dose dependent Omicron inhibition for compound **8** with a 48% reduction in neutralizing activity against the Omicron variant (from 100% to 52%, $p = 0.0189$), while only an 11% reduction was observed for the Delta variant (from 100% to 89%). Compound **9** demonstrated a 55% neutralization activity against the Omicron variant (100% to 45.0% pseudoviral entry rate, $p = 0.0255$) and for the Delta variant a 34% reduction (100% to 66% pseudoviral entry rate). No toxicity was observed towards the host cells as established by LDH assays (Supplementary Fig. S9.).

Discussion

This study aimed to investigate flavonoids isolated from *Rhaponticum carthamoides* (Maral root) for their potential as multifunctional therapeutics, supported by reports of both antiviral properties and selective cytotoxicity against cancer cells of flavonoids[16, 17]. Considering the decades-old AML chemotherapy regimen and its associated toxicity, resistance, and relapse, these flavonoids may offer promising alternatives. The goal was to contribute to the development towards new therapeutic options and enhancing the efficacy of existing treatments[18]. We identified one compound (compound **8**) with notable activity against AML cells and also significant inhibition of SARS-CoV-2 pseudoviruses. Such dual activity is particularly valuable in the context of AML, where patients are often immunocompromised and highly susceptible to severe viral infections[19].

A total of 14 compounds, including 11 flavonoids, two caffeoylquinic acid derivatives and a steroid were identified from the leaves of Maral root harvested in Bosheimsmarken, Ulnes, Norway. The ^{1}H and ^{13}C chemical shift values of these compounds were in agreement with NMR data available in current literature[20]. Quercetagetin 7-O-β-glucopyranoside (**5**) has previously been identified by Erzsebet et al.[21] and eriodictyol 7-O-β-glucopyranoside (**3**) along with eriodictyol were earlier reported in the leaves by Koleckar et al[22]. In contrast, two diastereomers (*2S*)-eriodictyol 5-O-β-glucopyranoside and (*2R*)-eriodictyol 5-O-β-glucopyranoside (**1** and **2**)

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were isolated from *R. carthamoides* for the first time. Compounds **1-9** were chosen for further investigation of their biological activity.

Investigation of the anti-leukemia properties of the nine flavonoids (**1-9**) revealed that compounds **5**, **7**, and **8** exhibited EC_{50} values between 28 and 50 μM towards the AML cells line MOLM-13, with no/low toxicity towards the non-cancerous cell lines (Fig. 2.). Although standard AML drugs demonstrate higher potency[23,24], it is important to note that compounds **1–9** are structurally unmodified, naturally derived compounds, and that structural modifications could enhance their properties and increase potency[25]. Although the inseparable mixture of compounds **3a** and **3b** was inactive, it may not be overlooked that a mixture containing a biologically active diastereomer and an inactive (or less active) diastereomer can be, or appear to be, biologically inactive overall. While diastereomers have different physical and chemical properties, their biological activity is highly dependent on how they interact with receptors, for instance by competitive binding to the target receptor. The inactive diastereomer may still bind to the target receptor but fail to initiate a response, physically blocking the active diastereomer from binding. If the inactive form has higher affinity, it can turn an active mixture inactive. This cannot be excluded to occur with respect to the observed inactivity of the mixture of diastereomers **3a** and **3b**. However, the fact that both diastereomers of the structurally related compound eriodictyol 5-O-β-glucopyranoside, namely (*2R*)-eriodictyol 5-O-β-glucopyranoside (**1**), (*2S*)-eriodictyol 5-O-β-glucopyranoside (**2**), as well as the core structure eriodictyol were inactive, indicate that any significant activity of compounds **3a** and **3b** may not be expectable. The selective toxicity of compounds **5**, **7**, and **8** toward MOLM-13 cells compared with non-malignant NRK and H9c2 cells suggests they may target vulnerabilities specific to leukemic cells while sparing healthy tissues. AML cells are characterized by a high proliferation rate, genomic instability, and altered redox balance, making them more susceptible to agents that interfere with cell cycle progression, DNA integrity, or mitochondrial function[26].

We next evaluated whether the isolated flavonoids in combination with standard cytotoxic drugs could potentiate the effect of the latter. Combination therapy is of particular benefit to cancer patients, as it has potential to reduce the side effects and avoid chemoresistance[27]. The combination of compound **8** and DNR showed positive interactions under the HSA and Loewe models, indicating that the combined effect exceeded the strongest single agent and was greater than dose additivity (Fig.3., a-d). However, no synergy was detected under the Bliss or ZIP

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models, suggesting that the observed benefit does not surpass the expectation of independent action. Thus, the apparent interaction is model-dependent and may reflect modest or context-specific synergy rather than a consistent synergistic effect across definitions. The additive effect of the compound could be used to reduce the necessary concentration of DNR, potentially minimizing its well-known cardiotoxic side effects[28].

Given the heightened susceptibility of AML patients to severe viral infections[29], we also evaluated the antiviral activity of the compounds to the SARS-CoV-2 Delta and Omicron variant where the SARS-CoV-2 spike protein interaction was evaluated. Compounds were screened for the potential cell entry inhibition against two SARS-CoV-2 variants: Omicron XBB 1.5 and Delta B.1.617.2. Overall, the viral entry inhibiting effects of the compounds were slightly more pronounced against the Omicron variant compared to the Delta variant (Fig. 4., a), suggesting a strain-specific activity. The known biological differences between these variants are relevant in interpreting these results. The older Delta variant prefers the TMPRSS2-mediated plasma-membrane fusion, while the Omicron has shifted to endosomal, cathepsin-dependent entry[30, 31, 32]. Therefore, the natural compounds utilized in this study may inhibit steps of the endosomal route leading to their higher efficacy against Omicron variants which are currently circulating. This variant specific trend was consistent across all compounds except for compound **4**, the only non-glycosylated isolated flavonoid tested.

Since pseudoviruses are non-replicative and lack the full pathogenic lifecycle, the results might be perceived as less representative of in vivo viral behavior. Nevertheless, studies have demonstrated a correlation between the antiviral efficacy observed in pseudovirus neutralization assays and that observed with the pathogenic virus[33].

In the titration experiments (Fig.4., b and c), neither of the two natural products (**8** and **9**) showed detectable inhibition of the Delta variant at any tested concentration. However, both compounds displayed significant inhibition of Omicron at the highest concentration tested. This selective activity aligns with the proposed mechanism in which Omicron relies more heavily on the cathepsin-dependent endosomal entry pathway, suggesting that the compounds may target this route. Notably, the concentrations required to observe significant inhibition were substantially higher than those reported for known entry inhibitors such as camostat or nafamostat [34,35,36]. This indicates that, while the compounds exhibit measurable antiviral activity, their potency is modest, and further structural optimization may be needed to enhance their efficacy.

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Interestingly, three out of four compounds of interest in this study were O-methylated. Compound **5** is unmethylated, compounds **7** and **9** contain one O-methyl group, and compound **8** has two. Their potency and selectivity towards cytotoxicity against MOLM-13 cells appear to follow this methylation gradient to some extent (**5**<**7**<**8**). The same potency is followed in the SARS-CoV-2 anti-viral assays where compound **8** is more potent than compound **9**. Indeed, previous studies have reported that O-methylation of flavonoids can enhance metabolic stability, improve membrane permeability, and, in some cases, modulate target binding[37, 38].

Despite these results, limitations must be acknowledged. NRK and H9c2 cell lines were used as controls, but their non-hematopoietic lineage and rat origin limits their relevance when comparing to human myeloid cells. However, their inclusion provides reference for evaluating general cardio- and renotoxicity of drugs, and they are more robust and easier to maintain in culture compared to human monocytes[39,40]. In the pseudovirus neutralization assay, the A549 transfected cell line was derived from one single-cell clone with overexpression of ACE2 and TMPRSS2. This can induce unintended phenotypic alterations and heterogeneity[41], but no unexpected results were observed when comparing to non-transfected parental cell line in cytotoxicity assays.

Taken together, compound **8** exhibited dual activity, demonstrating selective cytotoxicity against MOLM-13 leukemia cells and significant inhibition of SARS-CoV-2 Omicron viral entry at 20 μM concentration. In addition, compound **8** was the only compound of the nine flavonoids investigated that showed additive effects with the established AML drug DNR. The dual activity might be attributed to the compound's capacity for regulating broader cellular pathways involved in immune regulations or stress responses. Elucidating its mechanism of action could provide valuable insights for developing derivatives with even greater potency.

Conclusion

Natural products continue to play a vital role in modern drug discovery by offering structurally unique compounds that synthetic libraries often fail to capture. The compounds investigated in this study highlight the potential within natural sources, not only as direct therapeutics but also as tools to uncover novel mechanisms of disease patterns. For cancer patients that are already vulnerable to infection-related complications and inflammatory stress, a dual anti-viral and anti-leukemia activity could be beneficial. Moreover, the potential for synergistic interactions with existing AML treatments may enhance the therapeutic efficacy and lower the side effects of the

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drugs. Advancing our understanding of these compounds could pave the way for even better variants ultimately leading to more effective and better-tolerated therapies.

Materials and Methods

Extraction of plant material

Dried leaves of Maral root (*Rhaponticum carthamoides*) were harvested in Boheimsmarken, Ulnes, Norway (coordinates 60.998890 N and 9.128230 E). 3.0 kg of dried and ground leaves were extracted with 2 x 25 L 95% MeOH in water for 24 h at room temperature. The combined extracts were concentrated by use of rotary evaporation, yielding a slurry of 4.3 kg extract with a dry matter content of 18.3%.

Compound isolation

100 g of the wet extract was applied to a bed of 0.5 kg polymeric adsorbent (Amberlite XAD-7 HP, Sigma-Aldrich) packed with water in a 5 x 100 cm glass column. The extract was purified by using 5 L distilled water as mobile phase. The purified extract was then eluted from the XAD-7 column by using 3 L MeOH as mobile phase. The eluted methanolic fraction was then concentrated by rotary evaporation. Aliquots of XAD-7 purified extract was thereafter separated by means of Sephadex LH-20 column chromatography by using 400 g Sephadex LH-20 (Sigma-Aldrich) wet-packed in a 5 x 100 cm glass column, using a step-gradient of mobile phase consisting of varying proportions of MeOH in water (from 10 to 100%). Fractions of 100 mL were collected and analysed with UV-Vis spectroscopy (Agilent 8453 spectrophotometer; Agilent Technologies, CA, USA) and UHPLC. Fractions containing more than one major constituent were either re-chromatographed with the same type of Sephadex LH-20 column, or the compounds were separated on MPLC equipped with a RP-18 tri-octadecylsilyl column (PF-C18HP F0080, 113.6 mL, 50 µm; Interchim, France). MPLC separation was performed by using a step-gradient of mobile phase consisting of varying proportions of MeOH in water (from 5 to 100%) to achieve separation and isolation of phenolic compounds. Pure compounds were diluted to create stock solutions with a final concentration of 20 mM in DMSO (Dimethyl Sulfoxide – for molecular biology, Sigma-Aldrich, Saint Louis, USA). From 3.0 kg of dried and ground leaves of *R. carthamoides*, 25 mg of compound **1**, 132 mg of compound **2**, 326 mg of compound **3**, 72 mg of compound **4**, 167 mg of compound **5**, 81 mg of compound **6**, 95 mg of compound **7**, 5.7 mg of compound **8** and 77 mg of compound **9** was isolated.

UHPLC-DAD-MS analysis

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Extracts, purified fractions and isolated compounds were characterized by UHPLC-DAD-MS (Agilent 1290 Infinity II hyphenated with a 6120 single quadrupole (SQ) mass spectrometer, Agilent Technologies, CA, USA). Extract samples (1 g) were dissolved in 100 mL 95% MeOH and further diluted ten times with distilled water. 5 µL of samples were injected. Prior to injection, individual samples were filtered through a 17 mm diameter 0.45 µm nylon syringe filter (Chromacol Ltd, UK). Separation was achieved with an Ascentis Express RP18-column (100 x 2.1 mm, 2 µm; Supelco, PA, USA) by using a solvent gradient of increasing concentrations of acetonitrile (% B) in water (% A): from 5 to 30% B in 31 min, from 30 to 100% B in 9 min, from 100 to 5 in 2 min, and finally equilibration of column with starting conditions for 2 min. The flow rate was 0.3 mL min^{-1}, and separations were obtained at ambient column temperature. UV spectra were obtained for 230 to 450 nm range. The absorptions at individual wavelengths 240, 280, 320 and 360 nm were used to quantify 20-OH-ecdysone, flavanones, chlorogenic acids, and flavonols, respectively. The four groups of compounds were quantified against standards of 20-OH-ecdysone (Tokyo Chemical Industry Co, Ltd , TCI product number H1480), (2R)-eriodictyol 5-O-β-glucopyranoside, chlorogenic acid (Sigma-Aldrich Co, product number 00500590), and 6-hydroxy-kaempferol 7-O-β-glucopyranoside, respectively. Mass spectra of individual compounds were obtained by electrospray SQMS in both positive and negative scan-modes (150-750 m/z). Scan time was 585 ms and fragmentor was set to 40 V and 70 V. The source conditions were gas temperature at 300°C, nitrogen flow at 10.4 L min^{-1}, nebulizer pressure at 35 psi and capillary voltage ±4 kV. Nitrogen was obtained from a N118LA-nitrogen gas generator (Peak Scientific, Inchinnan, UK).

NMR spectroscopy

NMR samples were prepared by dissolving the isolated compound in deuterated dimethyl sulfoxide (DMSO-D_6; 99,96 atom % D, Sigma-Aldrich, Saint Louis, MO, USA). The 1D ^{1}H, 1D ^{1}H selective TOCSY, 1D ^{13}C CAPT and the 2D ^{1}H-^{13}C HMBC, the 2D ^{1}H-^{13}C HSQC, the 2D ^{1}H-^{13}C HSQC-TOCSY, the 2D ^{1}H-^{13}C H2BC, the 2D ^{1}H-^{1}H COSY and 2D ^{1}H-^{1}H ROESY NMR experiments were obtained on a Bruker BioSpin AVANCE III HD 850 MHz equipped with a ^{1}H, ^{13}C, ^{15}N triple resonance cryogenic probe at 298 K.

Cell culture and experimental conditions

Cell culture

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Normal rat kidney epithelial cells (NRK, ATCC no.: CRL-6509) and rat cardiomyoblasts (H9c2, ATCC no.: CRL-1446) were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, Sigma-Aldrich, Merck), enriched with 10% fetal bovine serum (FBS, Sigma-Aldrich, Merck). Once the cells achieved 80% confluence, they were gently detached with trypsin (0.33 mg/mL trypsin for 5 min at 37°C), centrifuged (160 x g, 4 min), and then reseeded in fresh medium at 25% confluence.

The AML cell line MOLM-13 (DSMZ no.: ACC554, [39]) was maintained in RPMI-1640 medium supplemented with 10% FBS (Sigma-Aldrich, Merck). The cells were kept in suspension cultures with a density of 150,000 to 700,000 cells/mL.

All media were further supplemented with 1 IU/mL penicillin and 1 mg/mL streptomycin (Sigma-Aldrich, Merck).

A549 adenocarcinoma human alveolar basal epithelial cells (ATCC, CCL-185) and $A549^{ACE2/TMPRSS2}$ cells, kindly provided by Professor Krzysztof Pyrc[42], were utilized for the study of XTT cytotoxicity assay and pseudovirus neutralization assay. The cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, high glucose, Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS, 1 U/mL penicillin and 1 mg/mL streptomycin. In addition, $A549^{ACE2/TMPRSS2}$ were supplemented with blasticidin S (10 µg/mL, Sigma-Aldrich, Merck), and puromycin (0.5 µg/mL, Sigma-Aldrich, Merck) to maintain the $ACE2^{OE}TMPRSS2^{OE}$ phenotype. Routine subculturing once the cells achieved 80% confluence occurred every 2-3 days. This was done by gently detaching with trypsin (0.33 mg/mL trypsin for 5 min at 37°C) and reseeding at 25% confluency. All cells used in this study were maintained in 37°C, 5% CO_2, and 95% humidity.

WST-1 cytotoxicity assay

Compound cytotoxicity on MOLM-13, NRK and H9c2 cells was evaluated using colorimetric-based cell proliferation WST-1 reagent (11644807001, Roche, Merck). NRK and H9c2 cells were seeded in 96-well tissue culture plates (4000 cells/well, 100 µL) and allowed to adhere for 16 h before treatments. The MOLM13 cells were seeded in 96-well tissue culture plates at 20,000 cells/well in 100 µL on the day of the experiment.

Compounds dissolved in DMSO were added to the cells, and the plates were incubated for 72 h before adding the tetrazolium salt WST-1 according to the manufacturer's instructions. In parallel, for blank subtraction controls, the natural compounds were suspended in medium in

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absence of cells. The maximum concentration of DMSO was 1%, and a control consisting of a dilution series with DMSO was performed to verify the minimal effect of DMSO. The plates were incubated for two hours in the presence of WST-1 reagent before the signal was recorded at 450 nm with reference at 620 nm. Afterwards, the cells were fixed with 2% buffered formaldehyde (pH 7.4) with 0.01 mg/mL Hoechst 333342. The presence of dead (apoptotic or necrotic) cells was verified by differential interference contrast and fluorescence microscopy.

Combination assays for AML treatment

To establish potential additive effects of the natural Maral root compounds with established AML drugs, MOLM-13 cells were cultured with the AML drugs cytarabine (ARA-C), daunorubicin and etoposide together with the natural compounds. Each 96-well plate was arranged with dilution series of a cytostatic drug and an individual flavonoid in combination, and each flavonoid and cytostatic drug alone, using concentrations of drugs and compounds giving between 20 and 80% reduction in viability. The incubation time for all these experiments was 72 hours. The web application SynergyFinder Plus was utilized to calculate the Bliss, Loewe, ZIP, and HAS score[15]. These models provide complementary perspectives on the expected effects of drug combinations under different assumptions of additivity:

The Bliss model assumes that two drugs act independently through non-overlapping mechanisms. The expected combined effect is calculated as

$$y_{BLISS} = y_1 + y_2 - y_1 \cdot y_2$$

Where y_1 and y_2 are the observed effects of drug A and drug B as single agents, expressed as fractional inhibition. Synergy is defined as the deviation of the observed combination effect from this expected value. A positive Bliss score indicates synergism, while a negative score indicates antagonism.

The Loewe model is based on the concept of dose equivalence, assuming that a drug cannot interact with itself. It defines synergy by comparing the combination to the effect that would be achieved if both drugs were the same compound. The Loewe synergy score is calculated as

$$\frac{x_1}{\chi^1_{LOEWE}} + \frac{x_2}{\chi^2_{LOEWE}} = 1$$

where x_1 and x_2 are the doses in the combination, and X^1_{LOEWE} and X^2_{LOEWE} are the doses of the individual drugs needed to achieve the same effect. A positive Loewe score indicates synergism, while a negative score indicates antagonism.

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The ZIP model integrates the principles of both Bliss and Loewe by assuming that drug interactions do not alter the potency or efficacy of individual agents. It models synergy by comparing the observed effect to a calculated expected effect that considers both independence and dose additivity

$$y_{ZIP} = \frac{(\frac{x_1}{m_1})^{\lambda_1}}{(1+\frac{x_1}{m_1})^{\lambda_1}} + \frac{(\frac{x_2}{m_2})^{\lambda_2}}{(1+\frac{x_2}{m_2})^{\lambda_2}} - \frac{(\frac{x_1}{m_1})^{\lambda_1}}{(1+\frac{x_1}{m_1})^{\lambda_1}} \cdot \frac{(\frac{x_2}{m_2})^{\lambda_2}}{(1+\frac{x_2}{m_2})^{\lambda_2}}$$

Positive ZIP scores (zero interaction potency) suggest synergistic enhancement of potency, while negative scores indicate antagonism.

The HSA model (highest single agent) compares the observed effect of the combination to the more effective single agent at the same concentrations

$$y_{HSA} = max(y_1, y_2)$$

Synergy is defined as any effect exceeding the best individual response. This model is conservative and does not assume any mechanistic interaction between compounds. Positive HAS scores suggest synergistic enhancement of potency, while negative scores indicate antagonism.

Pseudovirus production

The SARS-CoV-2 spike plasmids were of Delta (B.1.617.2, Sino Biological) and Omicron (XBB 1.5) variants, where the latter was kindly provided by Dr. Marceline Côté (Faculty of Medicine, University of Ottawa, Canada). The SARS-CoV-2 pseudotype virus was generated by co-transfection lentiviral vectors pHR'CMV-Luc, pCMVRΔ8.2, and pCMV3 construct encoding spike protein into HEK293T cells as previously described[43]. After 48 h of incubation (37 °C, 5% CO_2, and 95% humidity), the cells were harvested and filtered through a 0.45 μM filter to collect the supernatant containing the pseudovirus. The supernatant was stored at -80°C.

Pseudovirus based neutralization assay

To assess the antiviral potential of Maral root-derived compounds, neutralization assays were performed at concentrations well within the non-cytotoxic range, as determined by the XTT assay. The neutralization assay was performed on the stably transfected cell line $A549^{ACE2/TMPRSS2}$. The compounds were diluted in DMEM supplemented with 1% FBS and added in a 50 μL volume at 100 μM at the highest final concentration to 10 μM at the lowest final concentration to a white 96 well plate (136101, Fisher scientific, USA). For compounds with special interest there were

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performed titration between 100 μM to 1 μM range. Due to differences in stock concentrations and compound availability, serial dilutions were prepared with varying schemes. Compound **9** was diluted 1:10 starting at 100 μM over five dilutions, while compound **8** was diluted 1:2 starting at 20 μM over eight dilutions.

Then, pseudovirus in 100 μL volume of DMEM (1% FBS) was added and incubated for 30 minutes. Finally, 1.0 x 10^4 $A549^{ACE2/TMPRSS2}$ cells were added in a 50 μL volume of DMEM (1% FBS) making the total volume 200 μL and incubated for 72 h. All assay conditions were performed in triplicate.

The supernatants were collected for LDH assay (protocol provided in supplementary information). The cells were carefully washed once with 150 μL PBS. The cells were lysed with 50 μL 1x lysis buffer (Luciferase Cell Culture Lysis 5X Reagent, E1531, Promega Corporation). The surface was carefully scarped with pipet tips to ensure total cell lysis and shaken 60 rpm for at least 15 minutes and at most 1 h.

Synergy H1 plate reader (Biotek, VT, USA) was used for the relative light unit (RLU) readout, where the injector function of the instrument added 100 μL luciferase substrate (luciferase assay system, E1500, Promega Corporation) to each well before shaking and read out. Depending on the pseudovirus variant the RLU was expected to be in 100 000 to 1 000 000 range for the positive control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.4.2, GraphPad Software, San Diego, CA, USA). Data are presented as the mean ± standard error of mean (SEM). Curve fitting and determination of IC_{50} values from cytotoxicity data in MOLM-13, NRK and H9c2 cell lines were calculated using non-linear fit, [inhibitor] vs response, four parameters. Comparisons between treated experiments and controls were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). At least two biological replicates were conducted with three technical replicates each if not stated otherwise. Descriptive statistics were used to find top/bottom % of pseudovirus entry.

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Author contributions statement

T.F., D.O., S.A., M.K., L.H. and R.S. planned and designed the research project. R.S isolated the compounds. T.F. and R.M. determined the structures of the isolated compounds. R.M., R.A. and L.H. performed the tests of cytotoxic activity of the isolated compound towards normal and cancer cell lines and the combination assays for AML treatment. D.O., M.K., S.A., S.H., F.Z., and R.C. performed the SARS-CoV-2 pseudovirus neutralization assays and related experiments. T.F., D.O., R.M., L.H., S.A., M.K. and S.H. performed data analysis. T.F., D.O. R.M. S.H. R.S. F.Z. R.C. R.A. M.K. S.A. and L.H. wrote the manuscript.

Data availability statement

Data will be available on request from Torgils.Fossen@uib.no (data for molecular structure determination), Lars.Herfindal@uib.no for cytotoxic activity against acute myeloid leukemia (AML) cell line (MOLM-13), and kidney epithelium (NRK) and cardiomyocyte (H9c2) cell lines, as well as synergistic activity with the bench-mark AML drug daunorubicin, and Silke.Appel@uib.no for data for SARS-CoV-2 pseudovirus neutralization assays.

Additional Information

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The authors declare no competing interests.

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