

Article

Bioassay-Guided Isolation of Antiproliferative Compounds from *Limbarda crithmoides* (L.) Dumort

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Abstract: *Limbarda crithmoides* (L.) Dumort (Asteraceae) *n*-hexane extract displayed high cell proliferation inhibitory activity against acute myeloid leukaemia cells (OCI-AML3) and was therefore subjected to a bioassay-guided multistep separation procedure. Two thymol derivatives, namely 10-acetoxy-8,9-epoxythymol tiglate (**1**) and 10-acetoxy-9-chloro-8,9-dehydrothymol (**2**), were isolated and identified by means of NMR spectroscopy. Both of them exhibited a significant dose-dependent inhibition of cell proliferation.

Keywords: *Inula crithmoides*; *Limbarda crithmoides*; Asteraceae; antiproliferative activity; OCI-AML3

1. Introduction

The genus *Limbarda* (Asteraceae), formerly included in the genus *Inula*, comprises two accepted species: *L. crithmoides* (L.) Dumort., and *L. salsoloides* (Turcz.) Ikonn [1]. *L. crithmoides* is a halophyte plant, commonly known as *Inula crithmoides* (L.), that is widespread across the salt marshes and sea cliffs of the Mediterranean Sea, French Atlantic coasts, English channel and western European seaboard [1]. *L. crithmoides* is a common edible plant. Its leaves, eaten raw or boiled, are a good source of protein, amino acids, fibers, vitamins and other components, and traditionally formed an important part of the Lebanese diet [2]. Young shoots of *L. crithmoides* are also consumed as pickles in vinegar in Spain [3], while its raw tops are added to salads in the Basilicata region of southern Italy [4]. *L. crithmoides* biological activities have been reviewed, along with other eight Asteraceae representative species [5]. Essential oil composition has been widely explored [6–8], and it showed to be dependent on the region where plants grow [8].

Extracts of *L. crithmoides* have been applied to crops and weeds to investigate their properties, which confirmed their herbicidal potency [9]. It was also reported that the extracts of callus cultures show good antifungal activity against *Alternaria solani* and *Phytophthora cryptogea* [10]. In a previous work, methanol and hexane extracts of the aerial parts of this plant were found to reduce the radial growth of *A. solani* and *P. cryptogea*, and they also show weak antifungal activity against fungi of the *Fusarium* genus [11]. The antioxidant activity of *L. crithmoides* has been widely investigated [11–14], and seems to be directly correlated with the presence of phenolic metabolites such as quinic acid derivatives [12,15–17]. For example, the ethyl acetate fraction of a methanolic extract of *L. crithmoides* showed in vitro and in vivo hepatoprotective activity against carbon tetrachloride (CCl₄)-induced liver

injury through antiradical and antioxidant activities [18]. In addition to antifungal activity, antimicrobial as well as antileishmanial activities of *L. crithmoides* extracts have also been reported [15,19–21].

As part of our ongoing research into antiproliferative compounds derived from natural sources [22–26], a phytochemical investigation of *L. crithmoides* was undertaken to isolate active compounds by means of a bioassay-guided fractionation of active extracts against the Ontario Cancer Institute-Acute Myeloid Leukemia-3 (OCI-AML3) acute myeloid leukaemia cell line.

2. Results and Discussion

L. crithmoides (*I. crithmoides*) is a halophyte plant present in areas of high salinity [27,28] that represents an important agricultural crop [29,30] for its use as a food and for biological activities. Proximate composition of different extracts [6,7,31] showed the presence of saccharides [32] and proteins [33]. The most representative metabolites of this plant are phenolics [16,17,20,34–37] that are responsible of the good antioxidant and antiradical activity of extracts [10–13,15,38]. *L. crithmoides* essential oil and extracts were studied for their antimicrobial and antifungal activities [10,15,19,20,33,38]. *Inula* spp. have been investigated in depth for their cytotoxic/antiproliferative activity [39–41]. Despite the large number of biological studies undertaken on *L. crithmoides* [16,18,34,42], nothing has been reported so far about the cytotoxic/antiproliferative activity of this species and this prompted us to investigate the activity of *L. crithmoides* extracts against the OCI-AML3 cell line.

In a preliminary study, 24 h of treatment with 100 and 200 µg/mL of the methanolic extract (M) of *L. crithmoides* induced a significant decrease in OCI-AML3 cell number (Figure S1A, left panel, Supplementary Material). The augmentation observed with 200 µg/mL was accompanied by a significant increase in apoptosis (Figure S1A, right panel and S1B, Supplementary Material), associated with an accumulation of cells in the G0/G1 phase and a consequent decrease of cells in S and G2/M phases of the cell cycle (Figure S2A,B, Supplementary Material). The active M extract was subjected to solvent–solvent partitioning, yielding *n*-hexane (H), methylene chloride (DCM) and aqueous MeOH-soluble extracts. Both H and DCM extracts were able to significantly decrease the OCI-AML3 cell number compared to the vehicle (control) at concentrations of 15 or 10 µg/mL (with both extracts, Figure S3A, left panel, Supplementary Material). Analysis of apoptotic cell death showed a significant increase in apoptosis with 10 and 15 µg/mL of the H extract when compared with DCM extract (Figure S3A, right panel, and S3B, Supplementary Material). An analysis of the cell cycle revealed a significant increase in cells in the G0/G1 phase (Figure S4A, left panel, Supplementary Material) and fewer cells in S and G2/M phases of the cell cycle (Figure S4A, middle and right panels, respectively, Supplementary Material) with concentrations of 10 and 15 µg/mL for both fractions. Figure S4B shows a representative experiment of the experiment series quantified in Figure S4A. Thus, the H and DCM extracts were at least 10-fold more active than the M extract (Figures S3 and S4, Supplementary Material), and the H extract was more potent than the DCM extract in increasing apoptotic cell death. For this reason, we decided to continue only with the H extract.

The serial bioassay-guided chromatographic procedures led to the isolation of two active compounds (1 and 2; Figure 1). Using NMR spectroscopic data, compound 1 was identified as 10-acetoxy-8,9-epoxythymol tiglate, previously isolated from *Athrixia* spp. [43], *Schkuhria multiflora* Hook & Arn. [44] and *Eupatorium cannabinum* L. [45], and compound 2 was characterized as 10-acetoxy-9Z-chloro-8,9-dehydrothymol, previously isolated from *Arnica sachalinensis* (Regel) A. Gray [46] (Figures S5–S8, Supplementary Material). The Z configuration of the double bond was confirmed by NOESY experiment (Figure S9). Thymol derivatives are quite common in different *Inula* spp. [47–49], and it is known that *L. crithmoides* produces chlorinated thymol derivatives [37]. However, as far as we know, this is the first time that compounds 1 and 2 have been isolated from *L. crithmoides* (*I. crithmoides*). The two pure compounds were tested for their biological activity.

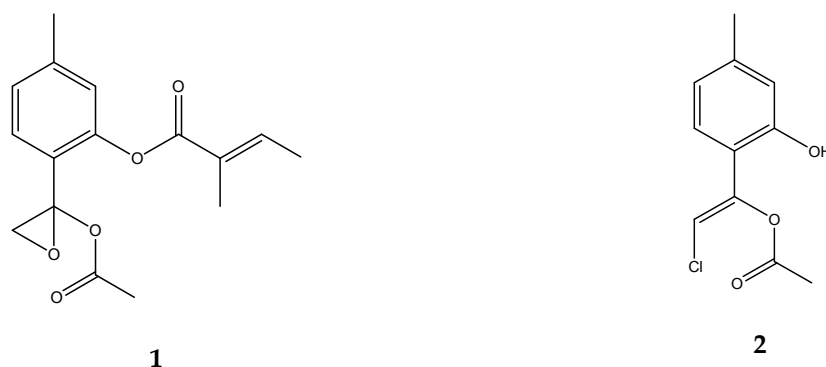


Figure 1. Chemical structures of active compounds 1 and 2.

Compound 1 caused a significant decrease in OCI-AML3 cell number at 5 $\mu\text{g/mL}$ (Figure 2A, left panel). This was possibly the consequence of a significant increase in apoptosis (Figure 2A, right panel, and Figure 2B) and blocking of cell cycle progression, as we observed significant accumulation of cells in the G0/G1 phase (Figure 3A, left panel) and fewer cells in the S (middle panel) and G2/M (right panel) phases of the cell cycle.

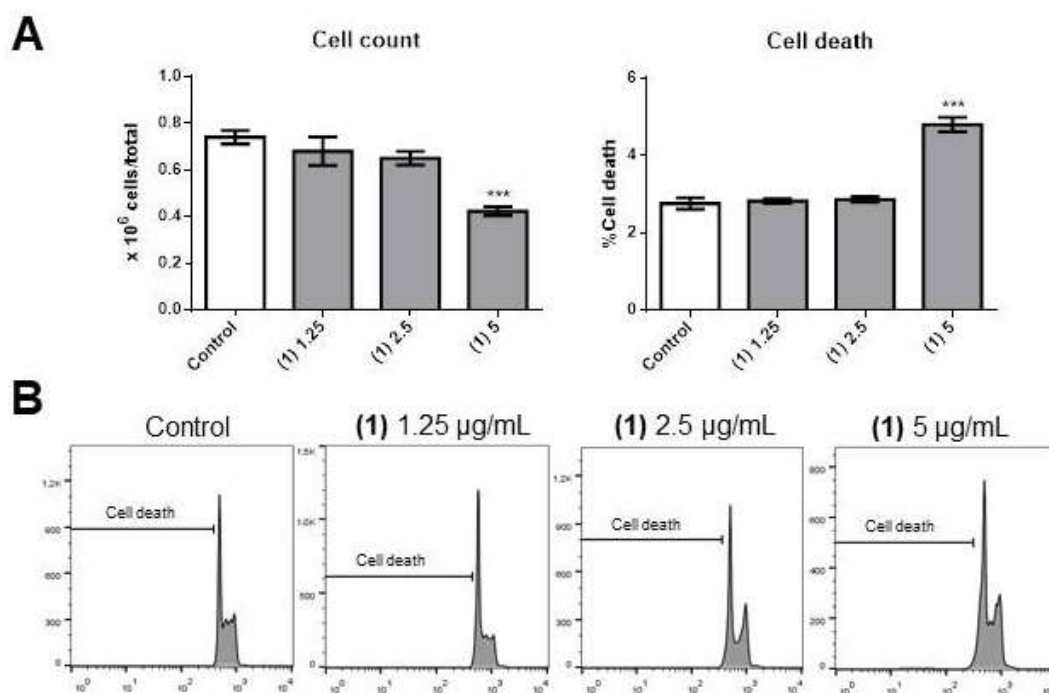


Figure 2. Effects of compound 1 on OCI-AML3 cell number and apoptotic cell death. (A) Bars represent the cell number (left panel) or the percentage of apoptotic cells after 24 h of treatment with control vehicle (Control) or 1,25 [(1) 1.25], 2,5 [(1) 2.5] or 5 [(1) 5] $\mu\text{g/mL}$ of compound 1. (B) Flow cytometry analyses of a representative experiment. Data from three independent experiments are reported as mean \pm SEM. *** $p < 0.001$.

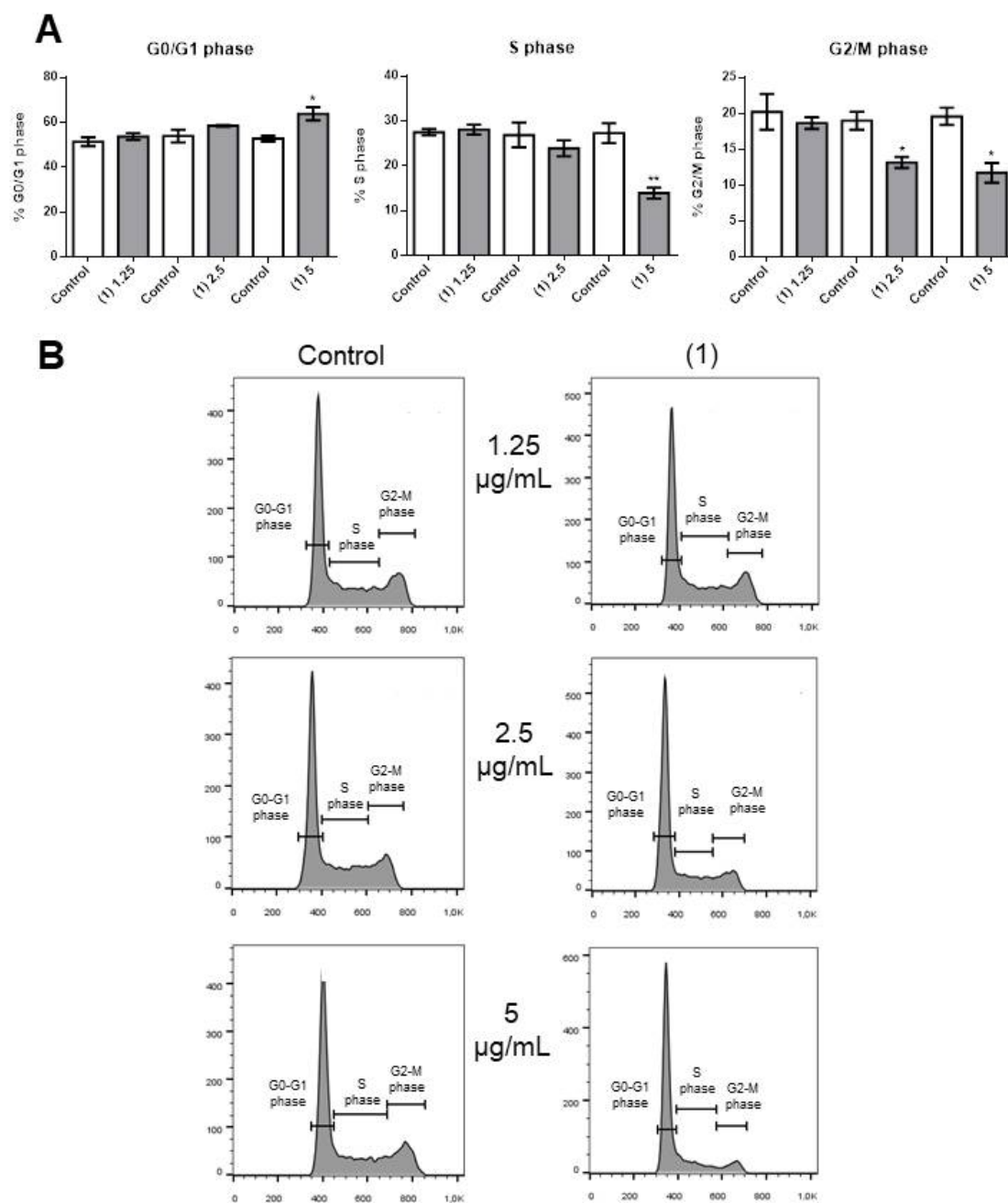


Figure 3. Effects of compound 1 on OCI-AML3 cell cycle progression. (A) Bars represent the percentage of cells in G0/G1 (left panel), S (middle panel), or G2/M (right panel) phases after 24 h of treatment with control vehicle (Control) or 1.25 [(1) 1.25], 2.5 [(1) 2.5] or 5 [(1) 5] µg/mL of compound 1. (B) Flow cytometry analyses of a representative experiment. Data from three independent experiments are reported as mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

Compound 2 caused a significant decrease in cell number at concentrations as low as 1.25 µg/mL (Figure 4A, left panel), and there was a significant increase in apoptosis in cells treated with 2.5 µg/mL (Figure 4A, right panel, and Figure 4B). Compound 2 blocked the cell cycle progression at G0/G1 (Figure 5A, left panel), with 1.25 µg/mL causing a consequent significant decrease in cells in the S (Figure 5A, middle panel, and Figure 5B) and G2/M (Figure 5A, right panel, and Figure 5B) phases of the cell cycle.

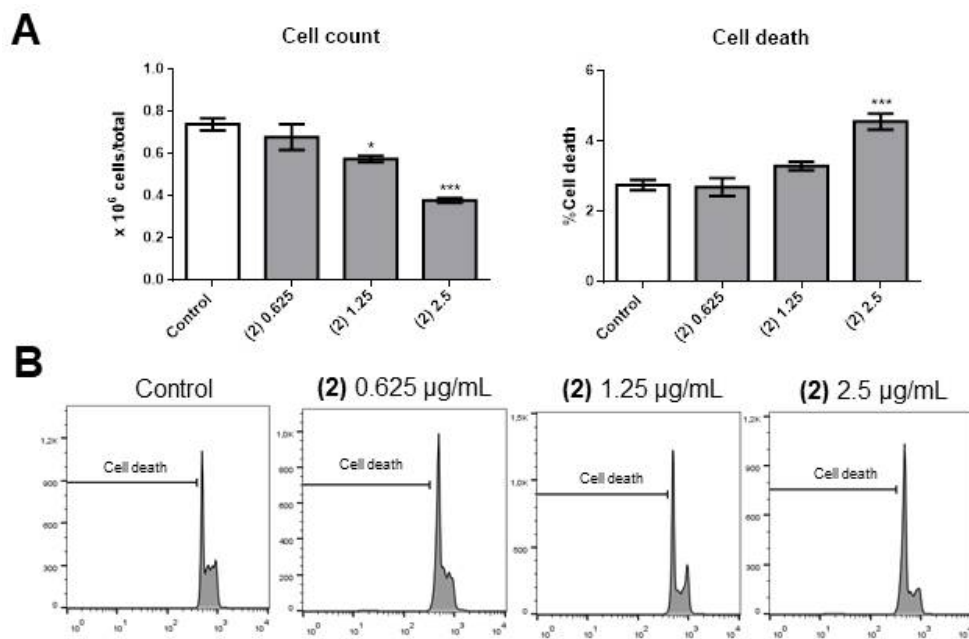


Figure 4. Effects of compound 2 on OCI-AML3 cell number and apoptotic cell death. (A) Bars represent the cell number (left panel) or the percentage of apoptotic cells after 24 h of treatment with control vehicle (Control) or 0.625 [(2) 0.625], 1.25 [(2) 1.25] or 2.5 [(2) 2.5] µg/mL of compound 2. (B) Flow cytometry analyses of a representative experiment. Data from three independent experiments are reported as mean ± SEM. * $p < 0.05$; *** $p < 0.001$.

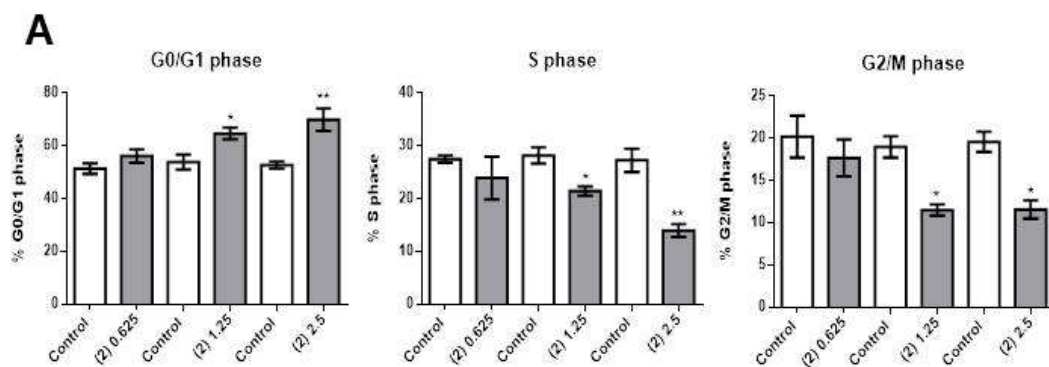


Figure 5. Cont.

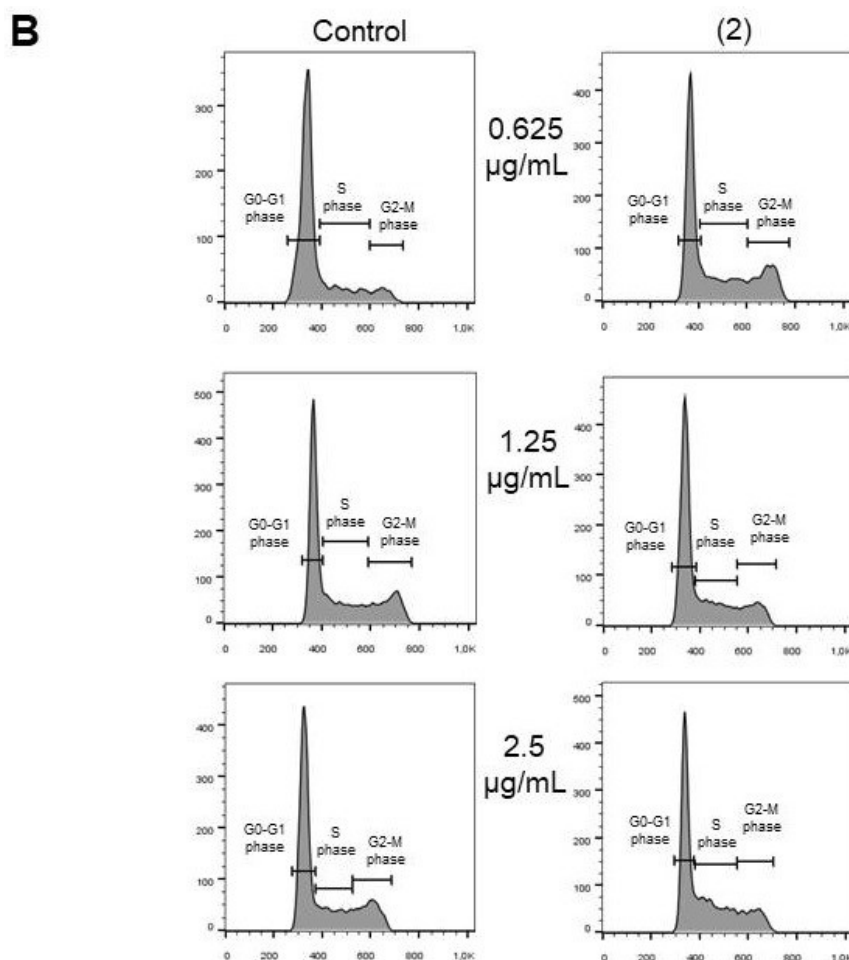


Figure 5. Effects of compound 2 on OCI-AML3 cell cycle progression. (A) Bars represent the percentage of cells in G0/G1 (left panel), S (middle panel), or G2/M (right panel) phases after 24 h of treatment with control vehicle (Control) or 0.625 [(2) 0.625], 1.25 [(2) 2.25] or 2.5 [(2) 2.5] $\mu\text{g}/\text{mL}$ of compound 2. (B) Flow cytometry analyses of a representative experiment. Data from three independent experiments are reported as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

Thus, compounds 1 and 2 were very effective in decreasing the number of OCI-AML3 cells through both increasing apoptosis and blocking cell proliferation. Compound 2 was more potent than compound 1 as it exerted inhibitory effects at a concentration as low as 1.25 $\mu\text{g}/\text{mL}$. The increased expression of p21 is largely associated with the arrest of cell cycle by two different pathways. The first is a p53-dependent and the second is p-53 -independent pathway. This prompted us to examine the expression of both p21 and p53 in OCI-AML3 cells untreated or treated with the M, DCM, H extracts and the compounds 1 and 2 isolated from the latter as a possible mechanism of cell cycle arrest since these pathways regulates mitotic progression and promotes cellular stress response [50]. We used Western blotting to measure expression of p21 in control and treated cells. As shown in Figure 6, upper panel, all the tested extracts and compounds induced an upregulation of p21, but only with the H extract and with compound 2 the increase of p21 was significant compared to controls (Figure 6, lower left panel). Because p21 is regulated through either p53-dependent or -independent pathways, we also measured expression of p53, which did not change after treatment with any of the extracts or compounds (Figure 6, upper and lower right panels).

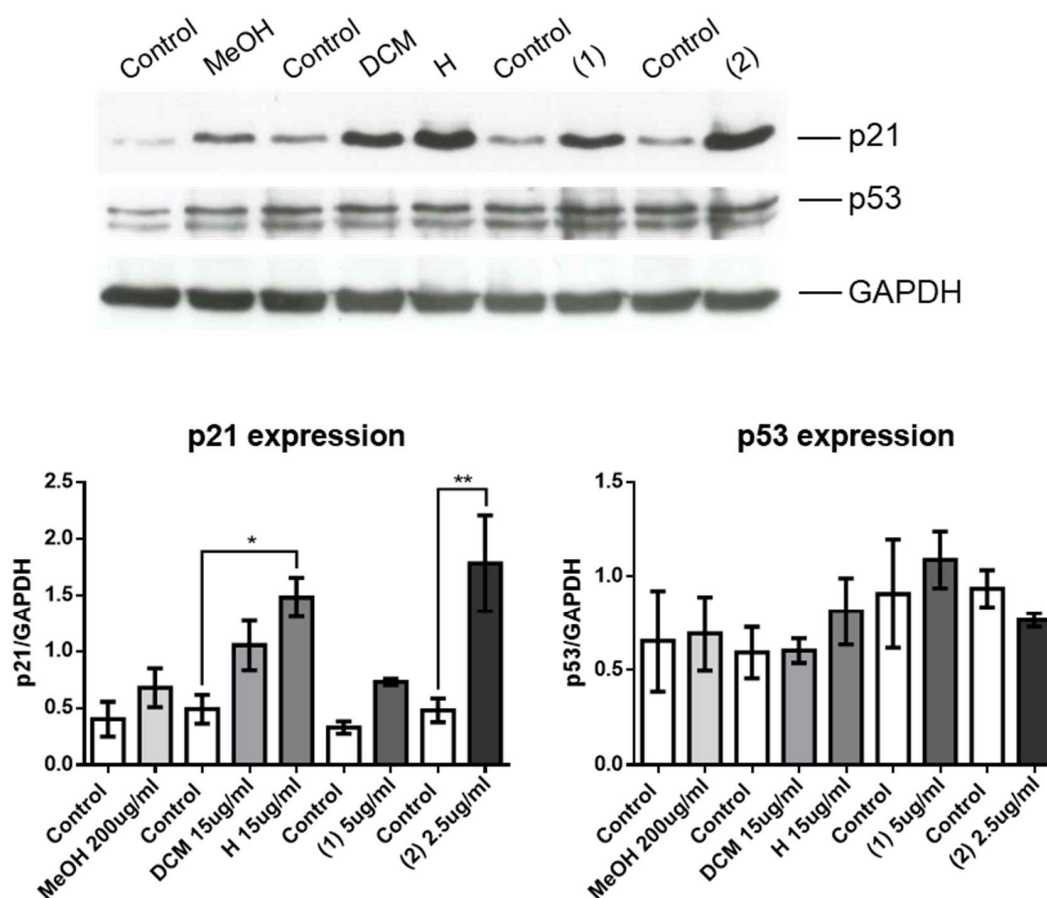


Figure 6. Effects of extracts and compounds on expression of proteins involved in the cell cycle. Upper panel, western blot analysis illustrating expression of p21, p53 and GAPDH using cell lysates extracted from OCI-AML3 cells treated with vehicle (Control), M (MeOH), DCM (DCM), H (H) extracts and compound 1 (1) or 2 (2) for 24 h. Western blots are representative of three independent experiments. Lower panels, Quantification of experiments shown in upper panel with the indication of the concentration used. Data from three independent experiments are reported as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

These results suggest that the H extract induced a significant activation of a p21-dependent, p53-independent pathway in OCI-AML3 cells and that compound 2 was responsible of this activity and the associated cell cycle arrest. Notably, the p21-dependent, p-53-independent pathway has been shown to be associated with an increased apoptosis, as is evident in our system, in contrast to the p21-dependent, p53-dependent pathway that promotes the translocation of p21 from the nucleus to the cytoplasm thus determining an anti-apoptotic effect absent in our study [22].

It is known from previous studies that compounds isolated from *Inula* species may have antiproliferative effect on a different leukaemia cells [51–59] but, as far as we know, only one work describing the activity of *Inula* extract on an acute myeloid leukaemia (KG1a cell line) has been reported [60]. This work highlighted the effect of *Inula* compounds on induction of apoptosis by the mitochondria-dependent pathway. Most of the *Inula* metabolites tested were sesquiterpenolides. On the other hand, thymol and thymol derivatives as well as extracts and essential oils containing these compounds [61–64] are known to be cytotoxic.

3. Materials and Methods

3.1. General Chemical Material

NMR spectra were recorded using Avance DRX-400 and DPX-200 spectrometers (Bruker, Milan, Italy) operating at frequencies of 400 MHz (^1H) and 100 MHz (^{13}C) and 200 MHz (^1H) and 50 MHz (^{13}C), respectively. The spectra were measured in CDCl_3 . The ^1H - and ^{13}C -NMR chemical shifts (δ) are expressed in ppm with reference to the solvent signals (CDCl_3 , δ_{H} 7.26 and δ_{C} 77.1). Coupling constants are given in Hz. NOESY (2D- NOE) experiments were executed on the Bruker Avance DRX-400 instrument. Preparative TLC was performed using pre-coated silica gel 60 F-254 plates (10 × 20 cm, Merck, Sigma-Aldrich, Milan, Italy) using *n*-hexane-acetone 8.5:1.5 as the eluent. Spots were visualized under UV light. Compounds were recovered from the stationary phase by washing five times with CH_2Cl_2 (DCM). Column chromatography was performed using MN Kieselgel 60 (70–230 mesh, Macherey-Nagel, Fisher Scientific, Milan, Italy). Fractions were monitored by TLC (Silica gel 60 F254; Merck), and spots on TLC were visualised under UV light and after staining with *p*-anisaldehyde- H_2SO_4 -EtOH (1:1:98) followed by heating at 110 °C. All solvents used were of analytical grade and were purchased from VWR (VWR, Milan, Italy). Anhydrous Na_2SO_4 was purchased from Scharlau S.L. (Milan, Italy).

3.2. Plant Material

Aerial parts of *L. crithmoides* were collected during the flowering period (August 2016) in Fano, Urbino, Italy. Specimen collection was restricted to coastal habitats, nearly always within the reach of sea spray. Specimens were authenticated by Dr Laura Giamperi (University of Urbino). Voucher specimens have been deposited at the Herbarium of the Botanic Garden of the University of Urbino (GS 203).

3.3. Extraction and Isolation Procedure

Air-dried and finely powdered plant material (50 g) was extracted by maceration in methanol (MeOH) (3 × 500 mL for 24 h each). The combined extracts gave 12.87 g of active MeOH extract (M; yield 26%). The extract was dissolved in 10 mL of MeOH, diluted with H_2O (100 mL), then subjected to solvent-solvent partition between *n*-hexane (3 × 50 mL; H extract; 6.2% yield) and methylene chloride (CH_2Cl_2) (3 × 50 mL; DCM extract; 3.4% yield). The H extract, which was the most active, was subjected to silica gel column chromatography (2 × 40 cm) under conditions of gradient elution using a mixture of CH_2Cl_2 in *n*-hexane (0→100%). Using *n*-hexane (1 L), we obtained four fractions: fraction 1 (IC-H-F1; 13 mg), using *n*-hexane- CH_2Cl_2 (1:1) (1.2 L) we obtained F2 (IC-H-F2, 280 mg), using CH_2Cl_2 (1.25 L) we obtained F3 (IC-H-F3, 120 mg) and F4 (IC-H-F4, 190 mg). Finally, the column was washed with 500 mL of MeOH (200 mg). The obtained fractions were tested for their antiproliferative activity using the same procedure for the extracts. An aliquot of the most active IC-H-F3 (109 mg) was further purified by silica gel column (1 × 20 cm) chromatography using CH_2Cl_2 as a solvent. Fractions (5 mL) were collected, evaluated by TLC and combined as a result of their similar appearance, yielding four pooled fractions: IC-H-F3(1), 36 mg; IC-H-F3(2), 52 mg; IC-H-F3(3), 6 mg; IC-H-F3(4), 6 mg. A solution of the IC-H-F3(1) fraction in 1 mL of DCM was purified by semipreparative TLC using *n*-hexane-acetone 8.5:1.5 as the eluent, resulting in the isolation of compound 1 (8 mg) and compound 2 (12 mg) (Figure S9, Supplementary Material). Each compound was identified by direct comparison of spectral properties, MS, ^1H -NMR and ^{13}C -NMR, with those of the authentic compounds in the literature [19,22].

3.4. Cell Line Culture and Characterisation

The antiproliferative activity of the two compounds under consideration was tested on the acute myeloid leukaemia (AML) cell line OCI-AML3 [65]. OCI-AML3 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% foetal bovine serum (FBS), 100 U/mL penicillin,

and 100 µg/mL streptomycin at 37 °C and 5% CO₂. Cells were purchased from ATCC (LGC Standards S.r.l., Sesto San Giovanni, Milan, Italy), kept at logarithmic growth and cultured in 24-well plates to assess their number and morphology. Cultures kept at 2 × 10⁵ cells/mL were treated with different concentrations of DMSO or the test compounds at the final concentrations reported in the figures. These reported concentrations were chosen based on preliminary experiments. After 24 h, the cell number was quantified using a haemocytometer.

3.5. Analysis of Cell Viability and Cell Cycle Progression

Cell viability and cell cycle progression were examined by flow cytometry to measure the amount of DNA in nuclei stained with propidium iodide (PI; Sigma-Aldrich, Milan, Italy), with the exclusion of necrotic cells by forward light scatter (FSC) [66]. Briefly, cells were harvested by centrifugation and gently resuspended in 1.5 mL hypotonic PI solution (50 µg/mL in 0.1% sodium citrate plus 0.1% Triton X-100). Tubes were kept in the dark at 4 °C for 30 min. PI fluorescence of individual nuclei was measured by flow cytometry using a Coulter Epics XL-MCLTM flow cytometer (Beckman Coulter, Cassina De' Pecchi, Milan, Italy) and analysed using FlowJo_V10 software (BD Biosciences, Milan, Italy).

4. Conclusions

This study showed that the M extract of *L. crithmoides* has cell proliferation inhibitory activity against acute myeloid leukaemia cells (OCI-AML3) and solvent partition showed that the H fraction was more active than the DCM fraction. For this reason, we decided to continue testing the H fraction. Chromatographic purification of fraction H led to the isolation of two active thymol derivatives, 1 and 2. Compound 2 was shown to be highly active. As far as we know, this is the first report of the antiproliferative activity of chlorinated thymol derivatives. The DCM fraction, to a minor extent, was shown to still be active, and for this reason we think it would be interesting to explore its composition and the antiproliferative activity of its fractions and compounds.

Supplementary Materials: NMR spectra of the active compounds and biological activity assay result informations are available online.

Author Contributions: Conceptualization, M.C.M. and D.V.D.; plant material and preparation of extracts, L.G. and A.E.A.B.; chemical investigation, M.C.M.; biological investigation, S.A.; data curation, S.A.; writing—original draft preparation, M.C.M. and D.V.D.; writing—review and editing, M.C.M., D.V.D., L.G. and A.E.A.B.; supervision, M.C.M. and D.V.D.; funding acquisition, D.V.D. All authors have read and agreed to the published version of the manuscript.

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References

1. The Plant List: A Working List of All Plant Species. Available online: <http://www.theplantlist.org> (accessed on 10 October 2019).
2. Zurayk, R.A.; Baalbaki, R. *Inula crithmoides*: A candidate plant for saline agriculture. *Arid Soil Res. Rehabil.* **1996**, *10*, 213–223. [[CrossRef](#)]
3. Tardio, J.; Pardo de Santayana, M.; Morales, R. Ethnobotanical review of wild edible plants in Spain. *Bot. J. Linn. Soc.* **2006**, *152*, 27–71. [[CrossRef](#)]
4. Guarrera, P.M.; Salerno, G.; Caneva, G. Food, flavouring and feed plant traditions in the Tyrrhenian sector of Basilicata, Italy. *J. Ethnobiol. Ethnomed.* **2006**, *2*, 37. [[CrossRef](#)] [[PubMed](#)]

5. Bessada, S.M.F.; Barreira, J.C.M.; Oliveira, M.B.P.P. Asteraceae species with most prominent bioactivity and their potential applications: A review. *Ind. Crop. Prod.* **2015**, *76*, 604–615. [[CrossRef](#)]
6. Harispe-Grandperrin, M.; Harispe, J.V. Proximate principles of *Inula crithmoides* L. family Compositae. II. Extraction and physical constants of the essential oil from the aerial parts. *Bull. Soc. Chim. Biol.* **1943**, *25*, 418–421.
7. Harispe-Grandperrin, M.; Harispe, J.-V. Proximate principles of *Inula crithmoides*. III. Preliminary examination of the essential oil of the flower heads. *Bull. Soc. Chim. Biol.* **1944**, *26*, 192–202.
8. Tsoukatou, M.; Roussis, V. Chemical composition and intra Mediterranean variation of the *Inula crithmoides* m L. oil. *J. Essent. Oil Res.* **1999**, *11*, 199–202. [[CrossRef](#)]
9. Omezzine, F.; Daami-Remadi, M.; Rinez, A.; Ladhari, A.; Haouala, R. In vitro assessment of *Inula* spp. organic extracts for their antifungal activity against some pathogenic and antagonistic fungi. *Afr. J. Microbiol. Res.* **2011**, *5*, 3527–3531.
10. Bucchini, A.; Giamperi, L.; Ricci, D. Total polyphenol content, in vitro antifungal and antioxidant activities of callus cultures from *Inula crithmoides* L. *Nat. Prod. Commun.* **2013**, *8*, 1587–1590. [[CrossRef](#)]
11. Bucchini, A.; Ricci, D.; Messina, F.; Marcotullio, M.C.; Curini, M.; Giamperi, L. Antioxidant and antifungal activity of different extracts obtained from aerial parts of *Inula crithmoides* L. *Nat. Prod. Res.* **2015**, *29*, 1173–1176. [[CrossRef](#)]
12. Jallali, I.; Waffo Teguo, P.; Smaoui, A.; Merillon, J.-M.; Abdelly, C.; Ksouri, R. Bio-guided fractionation and characterization of powerful antioxidant compounds from the halophyte *Inula crithmoides*. *Arab. J. Chem.* **2020**, *13*, 2680–2688. [[CrossRef](#)]
13. Andreani, S.; De Cian, M.-C.; Paolini, J.; Desjobert, J.-M.; Costa, J.; Muselli, A. Chemical variability and antioxidant activity of *Limbarda crithmoides* L. essential oil from Corsica. *Chem. Biodivers.* **2013**, *10*, 2061–2077. [[CrossRef](#)] [[PubMed](#)]
14. Giamperi, L.; Bucchini, A.; Fraternali, D.; Genovese, S.; Curini, M.; Ricci, D. Composition and antioxidant activity of *Inula crithmoides* essential oil grown in central Italy (Marche region). *Nat. Prod. Commun.* **2010**, *5*, 315–318. [[CrossRef](#)] [[PubMed](#)]
15. Jallali, I.; Zaouali, Y.; Missaoui, I.; Smeoui, A.; Abdelly, C.; Ksouri, R. Variability of antioxidant and antibacterial effects of essential oils and acetonetic extracts of two edible halophytes: *Crithmum maritimum* L. and *Inula crithmoides* L. *Food Chem.* **2014**, *145*, 1031–1038. [[CrossRef](#)]
16. Aboul Ela, M.A.; El-Lakany, A.M.; Abdel-Kader, M.S.; Alqasoumi, S.I.; Shams-El-Din, S.M.; Hammada, H.M. New quinic acid derivatives from hepatoprotective *Inula crithmoides* root extract. *Helv. Chim. Acta* **2012**, *95*, 61–66.
17. Males, Z.; Plazibat, M.; Greiner, M. Qualitative and quantitative analysis of flavonoids of golden samphire -*Limbarda crithmoides* (L.) Dumort. *Farm. Glas.* **2004**, *60*, 453–459.
18. Malash, B.N.; Ibrahim, S.M.; Ibrahim, A.-R.S.; Kabbash, A.; El-Aasr, M. In vitro and in vivo hepatoprotective study of *Inula crithmoides* L. *Pluchea dioscoridis* (L.) Desf. and *Phyllanthus reticulatus* Poir. *J. Pharm. Sci. Res.* **2015**, *7*, 987–993.
19. Assi, M.; Aboul-Ela, M.A.; Ellakany, A.M.; Abdul-Ghani, M. A comparative phytochemical and antimicrobial analysis of *Inula viscosa* and *Inula crithmoides* grown in Lebanon. *Acad. J. Med. Plants* **2014**, *2*, 057–067, 11 pp.
20. Aboul Ela, M.A.; El-Lakany, A.M.; Shams-El-Din, S.M.; Hammada, H.M. Phytochemical and antimicrobial investigation of *Inula crithmoides* L. *Alexandria J. Pharm. Sci.* **2011**, *25*, 37–40.
21. Oliveira, M.; Joao Rodrigues, M.; Pereira, C.; Neto, R.L.D.M.; Junior, P.A.S.; Neng, N.D.R.; Nogueira, J.M.F.; Varela, J.; Barreira, L.; Custodio, L. First report of the in vitro antileishmanial properties of extremophile plants from the Algarve Coast. *Nat. Prod. Res.* **2018**, *32*, 600–604. [[CrossRef](#)]
22. Adorasio, S.; Fierabracci, A.; Gigliarelli, G.; Muscari, I.; Cannarile, L.; Liberati, A.M.; Marcotullio, M.C.; Riccardi, C.; Curini, M.; Robles Zepeda, R.E.; et al. The hexane fraction of *Bursera microphylla* A Gray induces p21-mediated antiproliferative and proapoptotic effects in human cancer-derived cell lines. *Integr. Cancer Ther.* **2017**, *16*, 426–435. [[CrossRef](#)] [[PubMed](#)]
23. Gigliarelli, G.; Zadra, C.; Cossignani, L.; Robles Zepeda, R.E.; Rascon-Valenzuela, L.A.; Velazquez-Contreras, C.A.; Marcotullio, M.C. Two new lignans from the resin of *Bursera microphylla* A. Gray and their cytotoxic activity. *Nat. Prod. Res.* **2018**, *32*, 2646–2651. [[CrossRef](#)] [[PubMed](#)]

24. Marcotullio, M.C.; Loizzo, M.R.; Messina, F.; Temperini, A.; Tundis, R.; Menichini, F.; Curini, M. Bioassay-guided fractionation of *Euphrasia pectinata* Ten. and isolation of iridoids with antiproliferative activity. *Phytochem. Lett.* **2015**, *12*, 252–256. [[CrossRef](#)]
25. Messina, F.; Curini, M.; Di Sano, C.; Zadra, C.; Gigliarelli, G.; Rascon-Valenzuela, L.A.; Robles Zepeda, R.E.; Marcotullio, M.C. Diterpenoids and triterpenoids from the Resin of *Bursera microphylla* and their cytotoxic activity. *J. Nat. Prod.* **2015**, *78*, 1184–1188. [[CrossRef](#)] [[PubMed](#)]
26. Torres-Moreno, H.; Velazquez, C.A.; Garibay-Escobar, A.; Curini, M.; Marcotullio, M.C.; Robles-Zepeda, R.E. Antiproliferative and apoptosis induction of cucurbitacin-type triterpenes from *Ibervillea sonorae*. *Ind. Crop. Prod.* **2015**, *77*, 895–900. [[CrossRef](#)]
27. Al Hassan, M.; Chaura, J.; López-Gresa, M.P.; Borsari, O.; Daniso, E.; Donat-Torres, M.P.; Mayoral, O.; Vicente, O.; Boscaiu, M. Native-Invasive Plants vs. Halophytes in Mediterranean Salt Marshes: Stress Tolerance Mechanisms in Two Related Species. *Front. Plant Sci.* **2016**, *7*, 473. [[CrossRef](#)]
28. Gil, R.; Bautista, I.; Boscaiu, M.; Lidón, A.; Wankhade, S.; Sánchez, H.; Llinares, J.; Vicente, O. Responses of five Mediterranean halophytes to seasonal changes in environmental conditions. *AoB Plants* **2014**, *6*, plu049. [[CrossRef](#)] [[PubMed](#)]
29. Ventura, Y.; Eshel, A.; Pasternak, D.; Sagi, M. The development of halophyte-based agriculture: Past and present. *Ann. Bot.* **2015**, *115*, 529–540. [[CrossRef](#)]
30. Fita, A.; Rodríguez-Burruezo, A.; Boscaiu, M.; Prohens, J.; Vicente, O. Breeding and Domesticating Crops Adapted to Drought and Salinity: A New Paradigm for Increasing Food Production. *Front. Plant Sci.* **2015**, *6*, 978. [[CrossRef](#)]
31. Harispe-Grandperrin, M.; Harispe, J.V. Proximate principles of *Inula crithmoides* L. family Compositae. I. Biochemical study of glucide constituents. *Bull. Soc. Chim. Biol.* **1943**, *25*, 416–418.
32. Kacem, I.; Majdoub, H.; Roudesli, S. Fraction of soluble polysaccharides from *Inula crithmoides* by sequential extraction. *J. Appl. Sci.* **2008**, *8*, 2442–2448. [[CrossRef](#)]
33. Attard, E.; Pacioni, P. The Phytochemical and In Vitro Pharmacological Testing of Maltese Medicinal Plants. In *Bioactive Compounds in Phytomedicine*; InTech: London, UK, 2011; pp. 93–112.
34. Selim, M.A.; El-Hefnawy, H.M.; Ibrahim, T.A.; Sleem, A.A. Flavonoids and antihepatotoxic activity of *Inula crithmoides* L. growing in Egypt. *Egypt. J. Biomed. Sci.* **2003**, *12*, 83–95.
35. El-Lakany, A.M.; Aboul Ela, M.A.; Hammada, H.M.; Ghazy, N.M.; Mahmoud, Z.F. New methoxylated flavonols from *Inula crithmoides*. *Pharmazie* **1996**, *51*, 435–436.
36. Metwally, M.A.; Dawidar, A.M. A thymol derivative from *Inula crithmoides*. *Phytochemistry* **1985**, *24*, 1377–1378. [[CrossRef](#)]
37. Marco, J.A.; Sanz-Cervera, J.F.; Manglano, E. Chlorinated thymol derivatives from *Inula crithmoides*. *Phytochemistry* **1993**, *33*, 875–878. [[CrossRef](#)]
38. Jdey, A.; Falleh, H.; Ben Jannet, S.; Mkadmini Hammi, K.; Dauvergne, X.; Ksouri, R.; Magne, C. Phytochemical investigation and antioxidant, antibacterial and anti-tyrosinase performances of six medicinal halophytes. *S. Afr. J. Bot.* **2017**, *112*, 508–514. [[CrossRef](#)]
39. Seca, A.M.L.; Grigore, A.; Pinto, D.C.G.A.; Silva, A.M.S. The genus *Inula* and their metabolites: From ethnopharmacological to medicinal uses. *J. Ethnopharmacol.* **2014**, *154*, 286–310. [[CrossRef](#)]
40. Wang, G.-W.; Qin, J.-J.; Cheng, X.-R.; Shen, Y.-H.; Shan, L.; Jin, H.-Z.; Zhang, W.-D. *Inula* sesquiterpenoids: Structural diversity, cytotoxicity and anti-tumor activity. *Expert Opin. Investig. Drugs* **2014**, *23*, 317–345. [[CrossRef](#)] [[PubMed](#)]
41. Trendafilova, A.; Ivanova, V.; Rangelov, M.; Todorova, M.; Ozek, G.; Yur, S.; Ozek, T.; Aneva, I.; Veleva, R.; Moskova-Doumanova, V.; et al. Caffeoylquinic Acids, Cytotoxic, Antioxidant, Acetylcholinesterase and Tyrosinase Enzyme Inhibitory Activities of Six *Inula* Species from Bulgaria. *Chem. Biodivers.* **2020**. Ahead of Print. [[CrossRef](#)]
42. Abdel-Wahhab, M.A.; Abdel-Azim, S.H.; El-Nekeety, A.A. *Inula crithmoides* extract protects against ochratoxin A-induced oxidative stress, clastogenic and mutagenic alterations in male rats. *Toxicol.* **2008**, *52*, 566–573. [[CrossRef](#)]
43. Bohlmann, F.; Zdero, C. Neue norkauren- und thymol-derivate aus *Athrixia*-arten. *Phytochemistry* **1977**, *16*, 1773–1776. [[CrossRef](#)]

44. Pacciaroni, A.D.V.; Sosa, V.E.; Espinar, L.A.; Oberti, J.C. Acyl glucopyranosides and other constituents from *Schkuhria multiflora*. *An. Asoc. Quim. Argent.* **1998**, *86*, 256–260.
45. Chen, J.-J.; Tsai, Y.-C.; Hwang, T.-L.; Wang, T.-C. Thymol, Benzofuranoid, and Phenylpropanoid Derivatives: Anti-inflammatory Constituents from *Eupatorium cannabinum*. *J. Nat. Prod.* **2011**, *74*, 1021–1027. [[CrossRef](#)] [[PubMed](#)]
46. Passreiter, C.M.; Matthiesen, U.; Willuhn, G. 10-Acetoxy-9-chloro-8,9-dehydrothymol and further thymol derivatives from *Arnica sachalinensis*. *Phytochemistry* **1998**, *49*, 777–778. [[CrossRef](#)]
47. Wang, C.; Zhang, X.; Wei, P.; Cheng, X.; Ren, J.; Yan, S.; Zhang, W.; Jin, H. Chemical constituents from *Inula wissomanniana* and their anti-inflammatory activities. *Arch. Pharm. Res.* **2013**, *36*, 1516–1524. [[CrossRef](#)] [[PubMed](#)]
48. Cheng, X.-R.; Ye, J.; Ren, J.; Zeng, Q.; Zhang, F.; Qin, J.-J.; Shen, Y.-H.; Zhang, W.-D.; Jin, H.-Z. Terpenoids from *Inula sericophylla* Franch. and their chemotaxonomic significance. *Biochem. Syst. Ecol.* **2012**, *42*, 75–78. [[CrossRef](#)]
49. Zhao, J.; Li, Y.; Liu, Q.; Gao, K. Antimicrobial activities of some thymol derivatives from the roots of *Inula hupehensis*. *Food Chem.* **2010**, *120*, 512–516. [[CrossRef](#)]
50. Adoriso, S.; Fierabracci, A.; Muscari, I.; Liberati, A.M.; Cannarile, L.; Thuy, T.T.; Sung, T.V.; Sohrab, H.; Hasan, C.M.; Ayroldi, E.; et al. Fusarubin and Anhydrofusarubin Isolated from A *Cladosporium* Species Inhibit Cell Growth in Human Cancer Cell Lines. *Toxins* **2019**, *11*, 503. [[CrossRef](#)]
51. Yami, A.; Hamzeloo-Moghadam, M.; Darbandi, A.; Karami, A.; Mashati, P.; Takhviji, V.; Gharehbaghian, A. Ergolide, a potent sesquiterpene lactone induces cell cycle arrest along with ROS-dependent apoptosis and potentiates vincristine cytotoxicity in ALL cell lines. *J. Ethnopharmacol.* **2020**, *253*, 112504. [[CrossRef](#)]
52. Karami, A.; Hamzeloo-Moghadam, M.; Yami, A.; Barzegar, M.; Mashati, P.; Gharehbaghian, A. Antiproliferative Effect of Gaillardin from *Inula oculus-christi* in Human Leukemic Cells. *Nutr. Cancer* **2019**, 1–14. [[CrossRef](#)]
53. Xu, X.; Huang, L.; Zhang, Z.; Tong, J.; Mi, J.; Wu, Y.; Zhang, C.; Yan, H. Targeting non-oncogene ROS pathway by alantolactone in B cell acute lymphoblastic leukemia cells. *Life Sci.* **2019**, *227*, 153–165. [[CrossRef](#)] [[PubMed](#)]
54. Cai, H.; Li, L.; Jiang, J.; Zhao, C.; Yang, C. Costunolide enhances sensitivity of K562/ADR chronic myeloid leukemia cells to doxorubicin through PI3K/Akt pathway. *Phytother. Res.* **2019**, *33*, 1683–1688. [[CrossRef](#)] [[PubMed](#)]
55. Cai, H.; Meng, X.; Li, Y.; Yang, C.; Liu, Y. Growth inhibition effects of isoalantolactone on K562/A02 cells: Caspase-dependent apoptotic pathways, S phase arrest, and downregulation of Bcr/Abl. *Phytother. Res.* **2014**, *28*, 1679–1686. [[CrossRef](#)] [[PubMed](#)]
56. Pal, H.C.; Sehar, I.; Bhushan, S.; Gupta, B.D.; Saxena, A.K. Activation of caspases and poly (ADP-ribose) polymerase cleavage to induce apoptosis in leukemia HL-60 cells by *Inula racemosa*. *Toxicol. Vitro.* **2010**, *24*, 1599–1609. [[CrossRef](#)] [[PubMed](#)]
57. Zeng, G.Z.; Tan, N.H.; Ji, C.J.; Fan, J.T.; Huang, H.Q.; Han, H.J.; Zhou, G.B. Apoptosis inducement of bigelovin from *Inula helianthus-aquatica* on human Leukemia U937 cells. *Phytother. Res.* **2009**, *23*, 885–891. [[CrossRef](#)]
58. Pan, M.H.; Chiou, Y.S.; Cheng, A.C.; Bai, N.; Lo, C.Y.; Tan, D.; Ho, C.T. Involvement of MAPK, Bcl-2 family, cytochrome c, and caspases in induction of apoptosis by 1,6-O,O-diacetylbritannilactone in human leukemia cells. *Mol. Nutr. Food Res.* **2007**, *51*, 229–238. [[CrossRef](#)]
59. Yue, G.G.; Chan, B.C.; Kwok, H.F.; Wong, Y.L.; Leung, H.W.; Ji, C.J.; Fung, K.P.; Leung, P.C.; Tan, N.H.; Lau, C.B. Anti-angiogenesis and immunomodulatory activities of an anti-tumor sesquiterpene bigelovin isolated from *Inula helianthus-aquatica*. *Eur. J. Med. Chem.* **2013**, *59*, 243–252. [[CrossRef](#)]
60. Ding, Y.; Pan, W.; Xu, J.; Wang, T.; Chen, T.; Liu, Z.; Xie, C.; Zhang, Q. Sesquiterpenoids from the roots of *Inula helenium* inhibit acute myelogenous leukemia progenitor cells. *Bioorg. Chem.* **2019**, *86*, 363–367. [[CrossRef](#)]
61. Elbe, H.; Yigiturk, G.; Cavusoglu, T.; Uyanikgil, Y.; Ozturk, F. Apoptotic effects of thymol, a novel monoterpene phenol, on different types of cancer. *Bratisl. Lek. Listy* **2020**, *121*, 122–128. [[CrossRef](#)]
62. Yu, Y.; Liu, Y.; Shi, R.; Zhang, D.; Li, C.; Shi, J. New thymol and isothymol derivatives from *Eupatorium fortunei* and their cytotoxic effects. *Bioorg. Chem.* **2020**, 103644. [[CrossRef](#)]
63. De La Chapa, J.J.; Singha, P.K.; Lee, D.R.; Gonzales, C.B. Thymol inhibits oral squamous cell carcinoma growth via mitochondria-mediated apoptosis. *J. Oral Pathol. Med.* **2018**, *47*, 674–682. [[CrossRef](#)] [[PubMed](#)]

64. Gunes-Bayir, A.; Kocyigit, A.; Guler, E.M. In vitro effects of two major phenolic compounds from the family Lamiaceae plants on the human gastric carcinoma cells. *Toxicol. Ind. Health* **2018**, *34*, 525–539. [[CrossRef](#)] [[PubMed](#)]
65. Thuy, T.T.; Huong, N.T.T.; Nhung, L.T.H.; Ninh, P.T.; Delfino, D.V.; Van Sung, T. Isolation, characterization and biological evaluation of a phenoxazine, a natural dyestuff isolated from leaves of *Peristrophe bivalvis*. *Nat. Prod. Res.* **2013**, *27*, 771–774. [[CrossRef](#)]
66. Riccardi, C.; Nicoletti, I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat. Protoc.* **2006**, *1*, 1458–1461. [[CrossRef](#)] [[PubMed](#)]

Sample Availability: Samples of the compounds are not available from the authors.



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