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Original article

The pro-apoptotic properties of a phytonutrient rich infusion of *A. cherimola* leaf extract on AML cells

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ABSTRACT

Annonaceae family has broad uses in herbal medicine for treatment of several diseases, whether through seeds' or leaves' extracts. The present study investigates the antiproliferative and antitumor activity of *Annona cherimola* aqueous leaf (AAL) extract/infusion in acute myeloid leukemia (AML) cell lines in vitro. High-resolution LC-MS was first used to analyze the composition of the aqueous extract. Cell proliferation assay, Annexin V staining, cell cycle analysis, dual Annexin V/PI staining, cell death quantification by ELISA, ROS level detection and Western Blotting were then performed to elucidate the therapeutic effects of AAL extract. The results obtained revealed a potent antioxidant activity of AAL extract. Moreover, the extract exhibited dose- and time-dependent antiproliferative effects on AML cell lines by decreasing cell viability with an IC_{50} of 5.03% (v/v) at 24 h of treatment of KG-1 cells. This decrease in viability was accompanied with a significant increase in apoptotic cell death with cell cycle arrest and flipping of the phosphatidylserine from the inner to the outer leaflet of the cell membrane. The respective overexpression and downregulation of proapoptotic proteins like cleaved caspase-8, cleaved PARP-1 and Bax and antiapoptotic proteins like Bcl-2 further validated the apoptotic pathway induced by AAL on AML cells. Finally, LC-MS revealed the presence of several compounds like fatty acids, terpenes, phenolics, cinnamic acids and flavonoids that could contribute to the antioxidant and anti-cancer effects of this herbal infusion. In addition to the generally known nutritional effects of the *Annona cherimola* fruit and leaves, the presented data validates the antioxidant and anti-cancerous effects of the leaf infusion on AML cell lines, proposing its potential therapeutic use against acute myeloid leukemia with future in vivo and clinical trials.

1. Introduction

The field of cancer chemoprevention with plant-derived extracts has dramatically increased in importance because of the presence of compounds that reach the desired therapeutic effect with a generally lower toxicity compared to regular drugs [1]. Research on plant-derived compounds has earned extensive attention in the treatment of several human diseases [2]. In fact, the World Health Organization (WHO) has estimated that the use of plant extracts or their active principles is a major component of traditional therapy [3]. Moreover, naturally derived phytochemicals have widely been used for their potential therapeutic effect in various experimental settings, many of which have reached early clinical trials [4]. In addition to that, more studies have been performed to investigate the molecular mechanisms behind the

Abbreviation: AAL, *Annona cherimola* Aqueous Leaf extract; AML, Acute Myeloid Leukemia; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal Bovine Serum; IC 50, Half-maximal Inhibitory Concentration; LC-MS, Liquid Chromatography/Mass Spectrometry; MNCs, Normal Mononuclear Cells; PI, Propidium Iodine; ROS, Reactive Oxygen Species; RPMI, Roswell Park Memorial Institute.

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activity of these phytochemicals [4], such as the inhibition of the growth factor signaling pathway, angiogenesis, and expression of anti-apoptotic proteins, in addition to the induction of apoptosis [5].

Including 112 genera and approximately 2500 species, the *Annonaceae* family has extensive uses in traditional and herbal medicine by indigenous people of different cultures for the treatment of several diseases [6]. Furthermore, the genus of flowering plants *Annona* is the second largest genus in this family [7] and consists of 119 species [6], whose seeds and leaves have shown anxiolytic, antidiabetic, anti-protozoal, anti-inflammatory, hepato-protective, and anticancer activities [6]. Aqueous and organic extracts from defatted *Annona squamosa* seeds displayed an apoptotic effect on rat histiocytic tumor cells, AK-5 through enhanced caspase-3 activity, downregulation of the anti-apoptotic protein Bcl-2 and induction of Reactive Oxygen Species (ROS), confirmed by DNA fragmentation and phosphatidylserine (PS) externalization detected by Annexin-V staining [8]. *Annona reticulata* Linn methanolic leaf extracts exhibited a G2/M cell cycle arrest, as well as mitochondria-mediated apoptosis in human breast cancer cells T-47D through Bax and Bak upregulation, Bcl2 downregulation, and caspases activation [9]. In addition, squamocin isolated from the seeds of the same plant showed a cytotoxic effect on various cancer cell lines, specifically a cell cycle arrest at the G1 phase in T24 bladder cancer cells, through upregulation of the proapoptotic genes Bax and Bad, enhancement of caspase 3 activity, and cleavage of poly (ADP-ribose) polymerase (PARP), indicating apoptosis [10]. Moreover, *Annona muricata* ethanolic leaf extracts caused apoptosis in liver cancer cells through the endoplasmic reticulum stress pathway, by upregulation of HSP70, GRP94 levels, as well as phosphorylation of PERK, eIF2α [11].

Annona cherimola is an edible, semi-evergreen, erect, low branched fruit-bearing tree [12] that belongs to the Annonacea family. Commonly recognized as cherimoya, the large green fruit of the tree was used as a food source by pre-Columbian cultures [13,14], and is mainly cultivated in highlands at sea levels up to 1400 m altitude [12]. It is known for its antiparasitic [15], antihyperglycemic [16], antihyperlipidemic [17], antioxidant [18],and antianxiety [19] effects. Moreover, alkaloids, flavonoids, phytosterols, tannins, and terpenoids are major phytochemicals present in *A. cherimola* that were used in the treatment of nervous disorders, diabetes, and cancer [18,20]. Annonaceous acetogenins rich seeds from *A. cherimola* exhibited antiproliferative as well as intrinsic and extrinsic proapoptotic effects on Acute Myeloid Leukemia (AML) cells [21]. Other studies also showed a selective and proapoptotic activity of A. cherimola ethanolic seed extracts and leaf extracts on gastric adenocarcinoma cell line AGS [22] and triple negative breast cancer cell line MDA-MB-231 [23], respectively. A recent study on *A. cherimola* ethanolic leaf extracts revealed an antitumor activity against acute myeloid leukemia cells in vitro [24], however, the consumption of leaves in human diet is primarily through tea preparations which are aqueous leaf extracts.

Acute myeloid leukemia (AML) is the most common myeloid leukemia in adults, whose incidence increases with age [25]. It starts in the bone marrow, but most often rapidly moves into the blood. It is a genetically heterogeneous disease characterized by an accumulation of genetic changes that would result in a low production of healthy hematopoietic cells, affecting the differentiation of cells into white blood cells other than lymphocytes [26,27]. Treatment of AML is focused on eliminating cancer cells, without harming normal cells, otherwise known as having low toxicity and high specificity [25].

The aim of the present study is to evaluate the antiproliferative activity of *A. cherimola* aqueous leaf extract (AAL) on acute myeloid leukemia cell lines in vitro and elucidate its mechanism of action.

2. Materials and methods

2.1. Plant material

Annona cherimola Mill leaves were taken from a tree located in Awkar-Lebanon (34.4328° N, 35.9169° E, 90 m above sea level), during January 2018 and were confirmed by the botanist Dr. Nisrine Machaka-Houri. A voucher specimen was deposited in Beirut Arab University Herbarium (ID-RCED2019–362).

2.2. Preparation of the leaf extract

Leaves were grinded then shaken in boiling water for 15 min. The aqueous extract was filtered through a cheesecloth and spun at maximum speed to discard the pellet, then filtered using the 0.22 μm pore diameter syringe filters before being applied on the cell lines. The resultant tea or *Annona cherimola* aqueous leaves (AAL) extract was formed of 8% weight of leaves/volume (yield = 9.5 mg/mL). Throughout this paper, various concentrations of the extract were used ranging from 1% to 8% volume/volume.

2.3. AML cell culture

Acute Myeloid Leukemia cell lines KG-1 and Mono-Mac-1 were acquired from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute medium (RPMI-1640, Sigma-Aldrich, St. Louis, MO, USA) enriched with 10% fetal bovine serum (FBS, Gibco, Dublin, Ireland), and 100 U/mL penicillin and 100 μg/mL streptomycin (Lonza, Basel, Switzerland) in a humidified incubator at 5% CO_2 and 37 °C. Cells were observed daily using the ZOE Fluorescent Cell Imager (Abcam, Cambridge, UK), and viability was assessed using the Trypan Blue exclusion method [28].

2.4. Isolation and culture of normal mononuclear cells (MNCs) from human bone marrow (BM)

Normal Mononuclear cells were offered by Dr. Marwan El-Sabban's Lab at the American University of Beirut (AUB) as a kind gift. The Normal MNCs were obtained originally from bone marrow aspirate leftovers of healthy patients attending AUB Medical center (AUB-MC). BM aspirates were centrifuged on Ficoll/Hypaque (GE Healthcare Life Sciences, Uppsala, Sweden), a density gradient step to separate MNCs from red blood cells and neutrophils. Then the buffy coat was aspirated and seeded in petri dishes using Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (FBS, Gibco, Dublin, Ireland) and 100 U/mL penicillin and 100 μg/mL streptomycin (Lonza, Basel, Switzerland) in a humidified incubator at 37 °C and 5% CO_2. One week later, the cells in suspension were collected as a purified MNCs population and cultured in the same conditions mentioned formerly [29].

2.5. Cytotoxicity assay

AML cells were plated in 96-well plates at a density of 0.5×10^5 cells/well and incubated overnight. Triplicates of wells were treated with increasing concentrations of AAL (2–8% AAL). Control cells were treated with RPMI media. The treated cells were incubated for 24 h or 48 h before the addition of MTS cell proliferation reagent (Promega, Wisconsin, USA), as previously described [30]. The color formed by the formation of Formazan was measured at 492 nm by spectrophotometry using the Varioskan™ LUX multimode microplate reader in order to calculate the percent proliferation [31]. MNCs were plated under similar conditions and were treated with either DMEM-low glucose medium or increasing concentrations of AAL (4% and 6%) for 24 h. Percent proliferation was calculated using the MTS cell proliferation assay. Effect of AAL on AML cells and MNCs was compared in order to evaluate selective toxicity.

2.6. Cell cycle analysis

KG-1 cells were plated in 24-well plates at a density of 1×10^5 cells/

well and incubated overnight. Wells were treated with RPMI media (negative control) or increasing concentrations of AAL (3% AAL and 6% AAL) for a duration of 24 h. Cell fixation was then done using ice-cold absolute ethanol and PBS (Lonza, Basel, Switzerland) with overnight storage at −80 °C. Cells were then stained with 50 μg/mL Propidium Iodide (Sigma-Aldrich, St. Louis, MO, USA) in the presence of 0.5 μg/mL RNase (Sigma-Aldrich, St. Louis, MO, USA). The DNA content was analyzed using the Incyte mode on the Guava® easyCyte™ flow cytometer (Luminex Corporation, Austin, TX, USA). The cells were distributed among the different cell cycle phases depending on the extent of PI staining reflecting the variable DNA amount in the cells [32].

2.7. Annexin V-FITC staining and fluorescent microscopy

KG-1 cells were seeded in 24-well plates at a confluency of 1×10^5 cells/well and incubated overnight. Wells were treated with RPMI media (negative control) or increasing concentrations of AAL (3% AAL and 6% AAL) or 50 μM etoposide (Abcam, Cambridge, UK) (positive control) for a duration of 24 h. Cells were then collected and washed with PBS before staining with Annexin V-FITC (Abcam, Cambridge, UK) and visualization using the ZOE Fluorescent Cell Imager. Relative Annexin V extent of binding was measured by quantifying the using the ImageJ software (NIH and LOCI, University of Wisconsin, WI, USA) [23].

2.8. Annexin V/PI staining and flow cytometry

KG-1 cells were seeded in 24-well plates at a confluency of 1×10^5 cells/well and incubated overnight. Wells were treated with RPMI media (negative control) or increasing concentrations of AAL (3% AAL and 6% AAL) or 50 μM etoposide (Abcam, Cambridge, UK) (positive control) for a duration of 24 h. Cells were then collected and washed with PBS before staining with Annexin V-FITC and Propidium Iodide (Abcam, Cambridge, UK). Cells were then analyzed by flow cytometry using the Incyte mode on the Guava® easyCyte™ flow cytometer and distributed into different populations (viable, apoptotic, necrotic) based on their positive or negative staining for Annexin V or PI [33].

2.9. Cell death ELISA

KG-1 cells were seeded in 24-well plates at a confluency of 0.5×10^5 cells/well and incubated overnight. Cells were then treated with RPMI media (negative control) or increasing concentrations of AAL (3% AAL and 6% AAL) or 50 μM etoposide (Abcam, Cambridge, UK) (positive control) for a duration of 24 h. Using the Cell Death ELISA kit (Roche, Basel, Switzerland), cells were lysed cytosolic nucleosomes were collected. After coating microplate wells with anti-histone antibodies, extracted nucleosomes were incubated, then anti-DNA antibodies linked to an enzyme were added creating a Sandwich ELISA [34]. ABTS (2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) colorimetric substrate addition followed by absorbance at 405 nm using the Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) allowed to calculate a DNA fragmentation enrichment factor (absorbance of treated cells/absorbance of non-treated cells) following manufacturers' instructions.

2.10. Western blot

KG-1 cells were seeded in 6-well plates at a confluency of 10×10^5 cells/well and incubated overnight. Wells were treated with RPMI media (negative control) or increasing concentrations of AAL (3% AAL and 6% AAL) for a duration of 24 h. Using the Q-proteome Mammalian Protein kit (Qiagen, Hilden, Germany), cells were lysed, and total protein content was collected and quantified using the DC (Detergent compatible) protein assay (Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS-PAGE and transferred to PVDF (Polyvinylidene fluoride) membranes [35]. After blocking with 5% skimmed milk, membranes were incubated with primary antibodies: anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), anti-cleaved PARP-1, anti-Bax, anti-Bcl2, and anti-caspase-9 (Abcam, Cambridge, UK), and anti-caspase-8 (Elabscience, Houston, TX, USA). After washing and incubation with a secondary antibody (Bio-Rad, Hercules, CA, USA), membranes were washed and images were developed using the Clarity™ Western ECL Substrate (Abcam, Cambridge, UK) on the ChemiDoc machine (BioRad, Hercules, CA, USA) [36]. ImageJ software was used to quantify blot bands and calculate the relative expression of proteins.

2.11. Reactive oxygen species detection

Using the DCFDA Cellular ROS Detection Assay kit (Abcam, Cambridge, UK), levels of Reactive Oxygen Species (ROS) in the cells were quantified. After incubation with the cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), KG-1 cells were plated in duplicates in a 96-well plate and treated with RPMI (Control), increasing concentrations of AAL (3% AAL and 6% AAL), or 50 μM of Tert-Butyl Hydrogen Peroxide (TBHP) (potent ROS inducer used as a positive control). Contact of H_2DCFDA with ROS oxidatively converts it to the highly fluorescent 2′,7′-dichlorofluorescein (DCF), which was by itself quantified by fluorescent spectroscopy on the Varioskan™ LUX multimode microplate reader [37].

2.12. Liquid chromatography/mass spectrometry

As previously described by El Khoury et al., 2.5 μg sample was injected into C18 Gravity-SB Nucleodur (300 Å, 1.8 μm, 2 × 100 mm, Macherey-Nagel, Düren, Germany) using a Dionex Ultimate 3000 analytical RSLC system (Dionex, Germering, Germany) coupled to a heated electrospray source HESI source (Thermo Fisher Scientific, Bremen, Germany). The separation was performed, followed by column washing and re-equilibration steps. Eluting compounds were analyzed on a QExactive HF-HT-Orbitrap-FT-MS benchtop instrument (Thermo Fisher Scientific, Bremen, Germany). Raw data files in positive and negative ionization modes were processed by Compound Discoverer™ 3.1 software for metabolomics data analysis. A blank sample was used for background subtraction and noise removal during the pre-processing step. Custom designed workflow was established for spectra alignment, compound detection, grouping, metabolite identification using mzVault, mzClaud and ChemSpider [38].

3. Results

3.1. Anti-proliferative effect of A. cherimola aqueous leaf extract on AML cell line

In order to study the effect of AAL on cell proliferation of AML cell lines, namely KG-1 and Monomac-1 cell lines, MTS reagent was used. This colorimetric reagent can detect the formation of formazan from MTS tetrazolium salt, reflecting cell viability. A significant decrease in AML cell proliferation was observed with increasing AAL concentrations and incubation times (Supplementary Table 1). Upon treating KG-1 with the highest concentration of AAL (8% v/v), cell proliferation was assessed to be 27.43% and 18.6% at 24 and 48 h respectively, reaching a half-maximal inhibitory concentration (IC_{50}) of 5.03% (v/v) at 24 h (Fig. 1a). However, the decrease in cell viability when Monomac-1 cells were treated with 8% AAL was 67.99% at 24 h and 18.74% at 48 h, showing an IC_{50} of 3.25% at 48 h (Fig. 1b). On the other hand, when Normal Mononuclear Cells (MNCs) from Human Bone Marrow (BM) were treated with AAL, a significantly lower effect on the proliferation was observed, indicating the selective effect of the extract on AML cell lines. In fact, MNCs treated with 6% AAL exhibited 99.28% and 82.63% proliferation after 24 and 48 h respectively (Fig. 1c). All the following

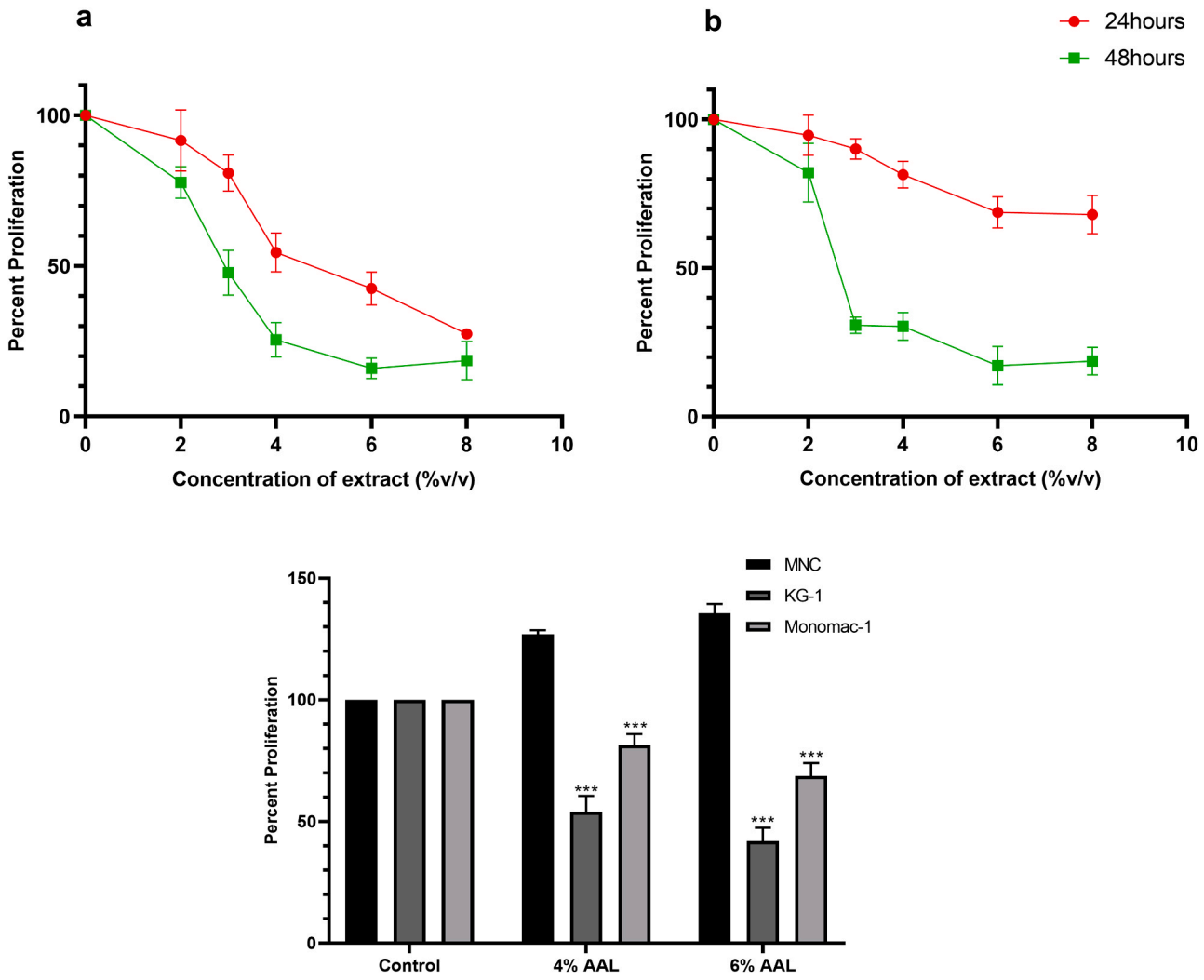

Fig. 1. Percent proliferation of cells upon increasing AAL treatment (2%, 3%, 4%, 6%, and 8%). (a) Line graph showing the percent proliferation of KG-1 cells treated with AAL at 24 and 48 h; (b) Line graph showing the percent proliferation of Monomac-1 cells treated with AAL at 24 and 48 h; (c) Bar graph representing the percent proliferation of MNCs at different concentrations of AAL in comparison to AML cell lines. A significant dose- and time- dependent inhibition of AML cell proliferation is observed upon treatment with increasing concentrations of AAL. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

experiments were conducted on KG-1 cell line at 24 h using the corresponding concentration of AAL extract before and after IC_{50} (3% and 6% AAL).

3.2. *Proapoptotic effect of A. cherimola aqueous leaf extract on KG-1 cells*

After reporting the significant effect of AAL extract on AML cell proliferation in both cell lines used, further experiments were performed on KG-1 cells to elucidate the mechanism of death induced. After fixation, cells were stained with PI that can bind to DNA and quantified using flow cytometry in order to study DNA content and cell cycle distribution. DNA content of cells was defined as follows: cells in the preG contain less than 2 n, in the G0-G1 cells are 2 n, those in the S phase are between 2 n and 4 n whereas in the G2-M phase cells are 4 n (Supplementary Fig. 1). KG-1 cells treated with 3% AAL and 6% AAL showed a significant increase in the preG phase going from 7.28% to 36.48% and 65.30% respectively, accompanied with a significant decrease in the G0-G1 and S phase cells, indicating cellular fragmentation (Fig. 2).

In order to elucidate any proapoptotic effect of AAL extract, Annexin V staining using fluorescent microscopy followed by quantitative analysis on ImageJ was performed. During apoptosis, the phosphatidylserine flips from the inner to the outer leaflet of the membrane, which is detected through the binding of Annexin V. When KG-1 cells were treated with increasing concentrations of AAL at 24 h, a significant increase in Annexin V binding was observed (Supplementary Fig. 2). Annexin V binding is 2.3 times stronger when treated with 3% AAL compared to the control, and 6.19 times stronger when treated with 6% AAL (Fig. 3).

Another approach to study the cell death mechanism is through dual Annexin V/PI staining followed by flow cytometry. The distribution of cells is defined by four quadrants numbered from I to IV in an anticlockwise direction starting from the lower left quadrant, where the x-axis represents the binding of Annexin V and the y-axis that of PI. Cells in quadrant I are defined to be viable cells where they stain negative for both Annexin V and PI. KG-1 cells treated at 24 h with 3% and 6% AAL showed a decrease in the percentage of viable cells going from 91.48% for the control cells to 78.12% and 58.49% respectively (Fig. 4). Apoptotic cells are distributed between early apoptosis in quadrant II (staining positive for Annexin V and negative for PI) and late apoptosis in quadrant III (staining positive for both Annexin V and PI). A significant increase in apoptotic cells, from 8.12% for control cells to 21.22% at 3% AAL and 42.21% at 6% AAL was noticed. This provides further evidence that the mechanism of apoptosis is responsible for the

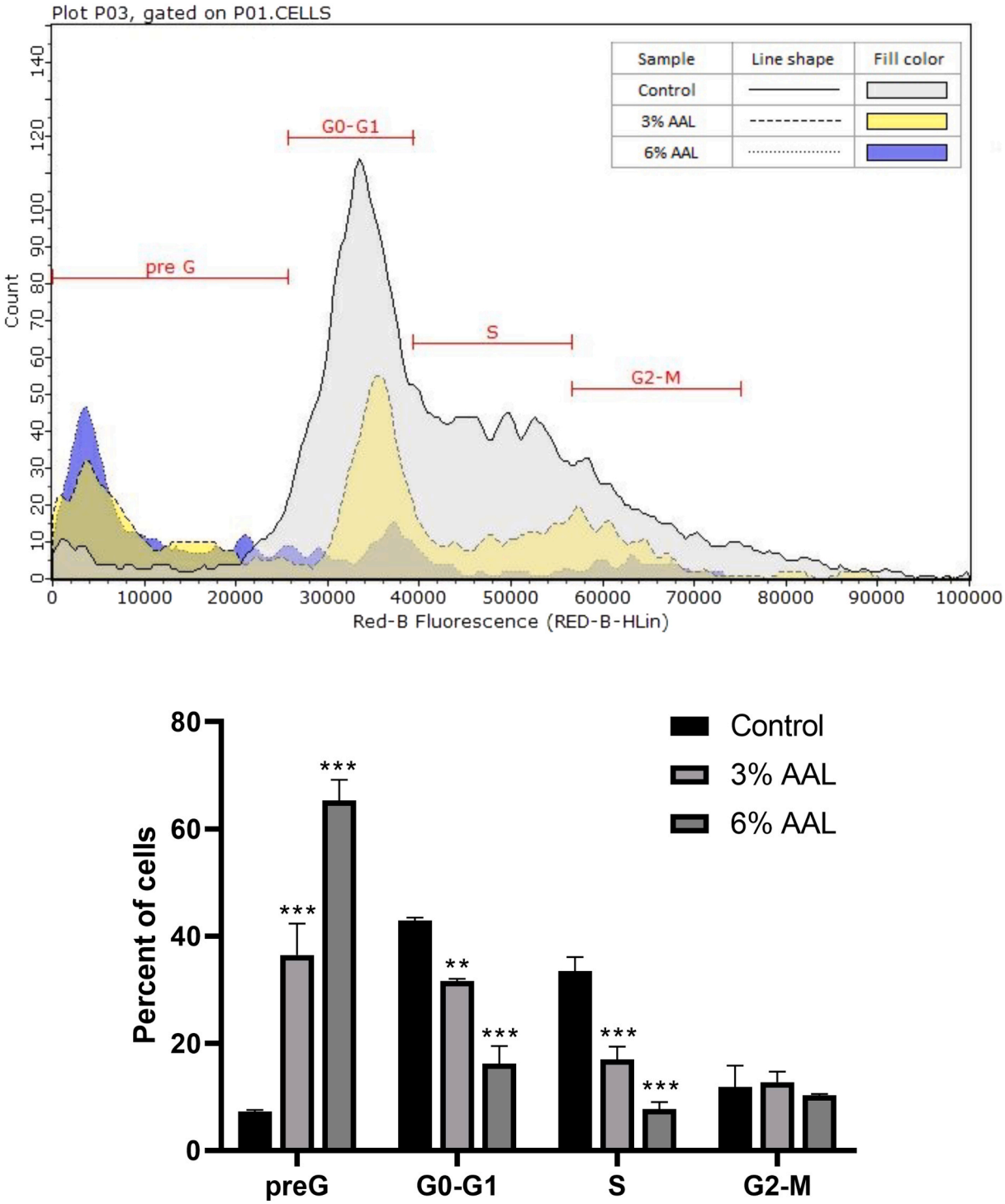

Fig. 2. Cell cycle analysis of KG-1 cells treated with increasing concentrations of AAL (3% and 6%) for 24 h. A dose-dependent increase shown in the pre-G phase with increasing AAL concentrations. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

antiproliferative effect previously observed in KG-1 cells.

Furthermore, DNA fragmentation, an earlier stage of apoptosis, can be assessed by Cell Death detection ELISA. In this sandwich assay, the quantification of anti-DNA peroxidase reflects an increase in DNA fragmentation. When KG-1 cells were treated with 3% and 6% AAL, the enrichment factor, which is the ratio of absorbance compared to the control, reached 3.37 and 4.50 at 24 h respectively (Fig. 5). After all these results, the apoptotic effect of AAL extract on AML can be confirmed being in a dose and time-dependent manner.

3.3. Apoptotic pathway induced by A. cherimola aqueous leaf extract on KG-1

The pathway of AAL-induced apoptosis was determined by Western blot analysis of KG-1 cells treated for 24 h with the AAL concentrations before and after IC_{50} (3% and 6% AAL). The expression of the pro-apoptotic protein, Bax, was constant at the different doses of extract applied (Fig. 6), whereas the anti-apoptotic protein, Bcl-2, was significantly downregulated, revealing an increase in the ratio of Bax/Bcl-2.

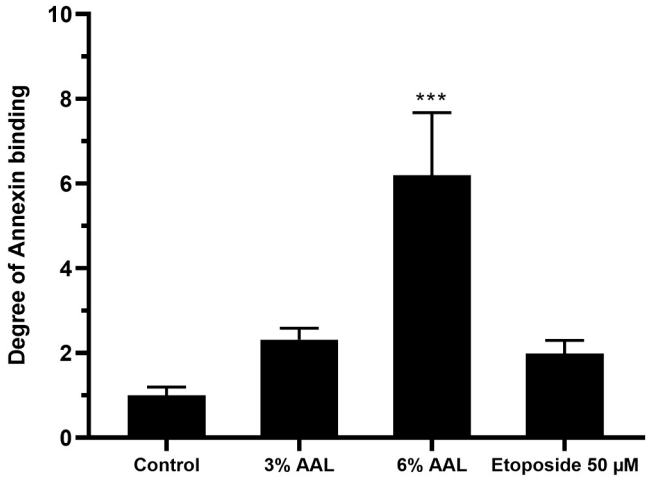

Fig. 3. Quantified Annexin V staining of KG-1 cells treated with increasing concentrations of AAL (3% and 6%); increased Annexin-V binding with increased AAL concentrations. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

Moreover, a significant increase of cleaved poly (ADP-ribose) polymerase (PARP) was observed, in addition to an upregulation of the apoptotic proteins cleaved caspase-8 (c-cas-8) and cleaved caspase-9 (c-cas-9) (Fig. 6). These alterations in the levels of expression confirm the conclusion of the dose-dependent apoptotic effect of the extract in KG-1 cells.

3.4. Antioxidant properties of A. cherimola aqueous leaf extract

The level of ROS (Reactive Oxygen Species) was measured in KG-1 cells by quantifying the degree of fluorescein which is reduced from its non-fluorescent form to its fluorescent form upon oxidant binding. 2.7-dichlorofluorescin diacetate (DCFDA) Cellular ROS Detection Assay kit was used to measure the antioxidant effect of AAL upon increasing concentration. A significant decrease in ROS production was observed upon treating the cells with both concentrations of AAL extract (Fig. 7). Therefore, this extract possesses prominent antioxidant activity on KG-1 cells.

3.5. Chemical characterization of Annona cherimola aqueous leaf extract using LC-MS

Liquid Chromatography coupled to Mass Spectrometry was conducted to elucidate the chemical composition of the extract. Many fatty acids were identified such as 12-Oxo-phytodienoic acid (Retention time = 39.788 min). Quinic acid and derivatives were also determined

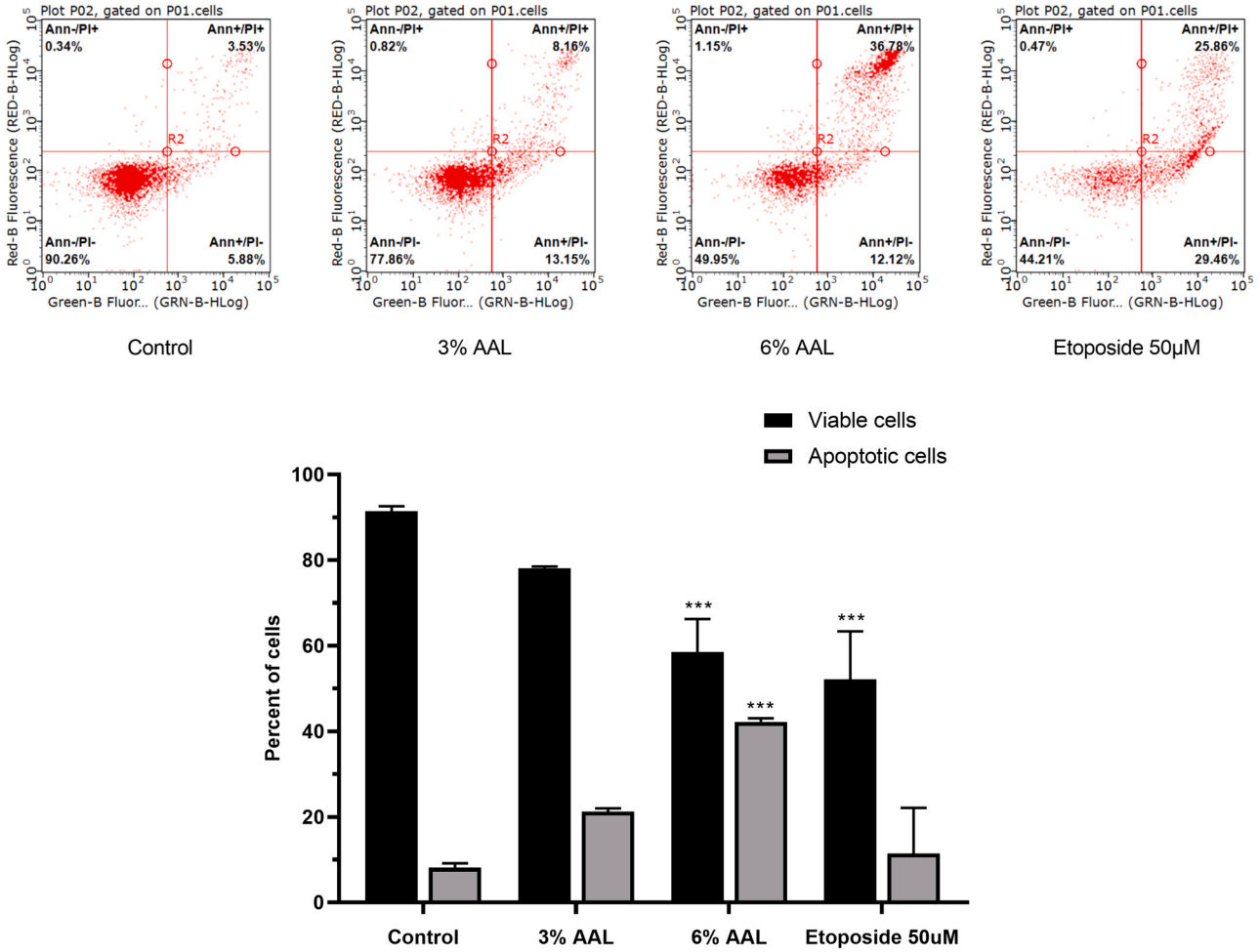

Fig. 4. Dual Annexin V/ PI staining of KG-1 cells treated with increasing concentrations of AAL (3% and 6%) and a positive control treated with etoposide for 24 h through flow cytometry with a shift from double negative staining to double positive staining. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

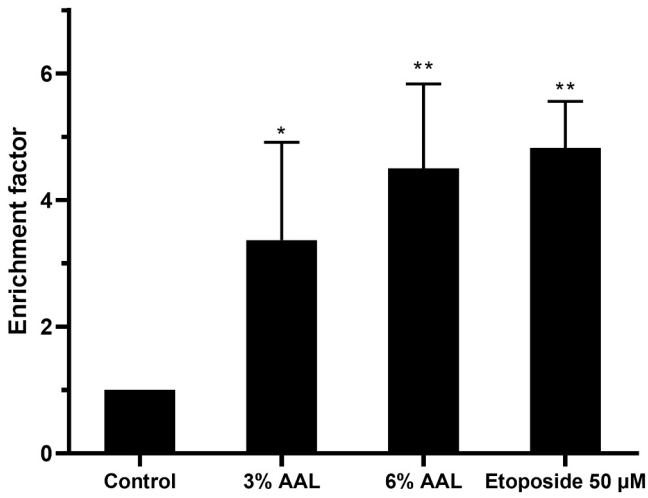

Fig. 5. Quantified DNA fragmentation through Cell Death detection ELISA analysis in KG-1 cells treated with increasing concentrations of AAL (3% and 6%) and a positive control treated with etoposide for 24 h. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

namely D-(-)- Quinic acid and 3-Feruloylquinic acid (Retention time- = 3.013 and 14.588 min, respectively). Moreover, flavonoids such as Astragalin (Retention time = 18.569 min), and hydroxycinnamic acids namely caffeic acid and sinapinic acid (Retention time = 9.249 and 12.774, respectively) were also observed (Tables 1 and 2). Details about the peak areas are included in Supplementary Tables 2 and 3.

4. Discussion

Natural compounds are widely investigated for cancer chemoprevention because of their wide availability and low toxicity compared to the standard chemotherapeutic drugs [1]. In fact, a new definition of chemoprevention focuses nowadays on targeting specific cancer molecular pathways to prevent cancer development and metastasis. Plant-based infusions have been historically considered for their activities in disease prevention treatment and studies also show their efficacy in preventing and treating cancer [39]. In fact, the anti-cancer effects of the ethanolic leaf extract of *Annona cherimola* were previously studied [24], but this study focuses on examining the effect of an aqueous extract which is equivalent to the leaves decoction consumed in the Azores [40].

The antiproliferative effect of the aqueous extract of *Annona cherimola* leaves was assessed in this study. The IC_{50} was found to be 5.03% at 24 h for KG-1% and 3.25% at 48 h for Monomac-1 highlighting that KG-1 are more sensitive to AAL than Monomac-1, which is why further experiments were performed on KG-1 cells. According to Asare et al., the aqueous extract of *Annona muricata* leaves exhibited antiproliferative effects upon treatment of Benign Prostatic Hyperplasia cell line BPH-1

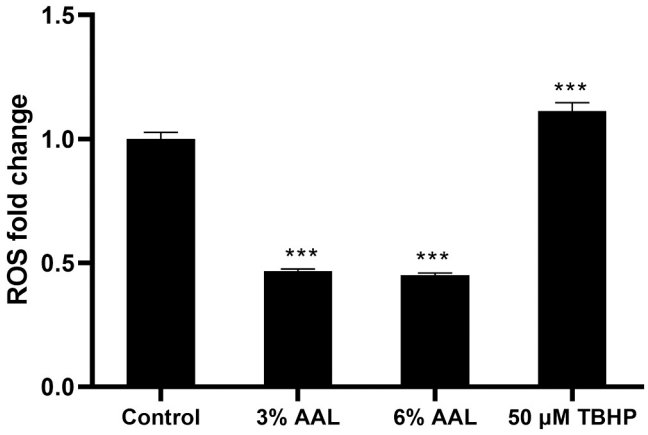

Fig. 7. Measurement of Reactive Oxygen Species in KG-1 cells treated with increasing concentrations of AAL (3% and 6%) and a positive control treated with TBHP using Cellular ROS Detection Assay kit showing a significant decrease in ROS of 0.45 folds at 6% AAL. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

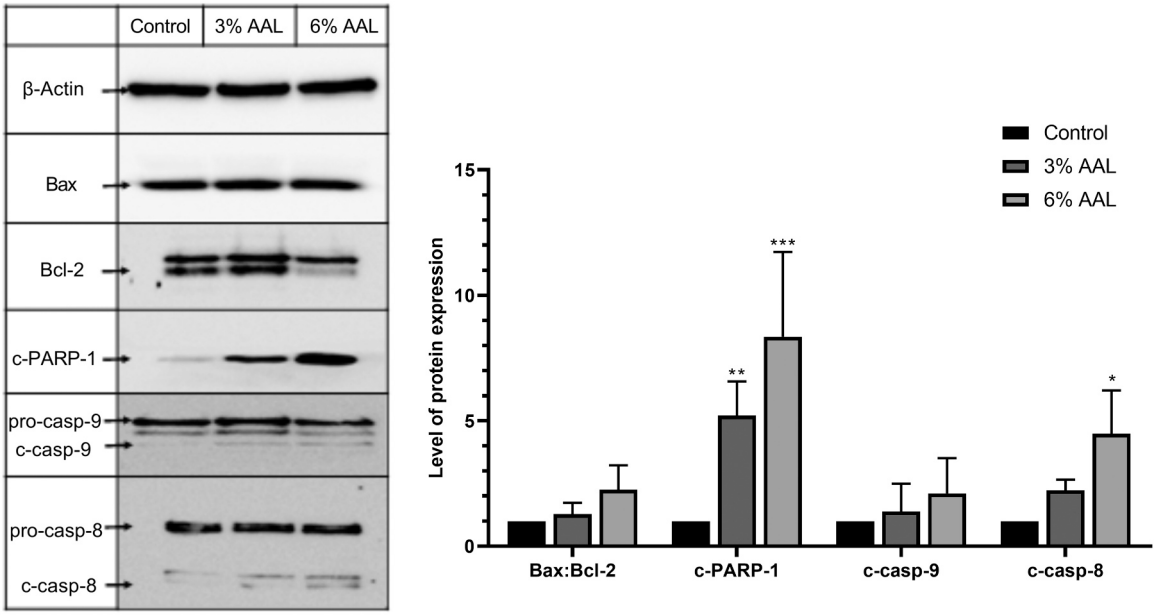

Fig. 6. Western Blot analysis of apoptosis-regulating proteins in KG-1 cells treated with increasing concentrations of AAL (3% and 6%) for 24 h. Upregulated Bax-Bcl2 ratio, upregulated pro-apoptotic protein expression (c-PARP-1, c-cas-9, c-cas-8), and downregulated anti-apoptotic protein expression (Bcl-2) in treated cells. Significant differences are reported, with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, and *** indicating a p-value: $0.0001 < p < 0.001$.

Table 1
Compounds elucidated on the negative run of the LCMS of AAL.

Compound name	Formula	*Mw*	*RT*
Corchorifatty acid f	C18 H32 O5	328.225	26.417
D-saccharic acid	C6 H10 O8	210.037	9.243
Catechin	C15 H14 O6	290.079	10.736
D-(-)-quinic acid	C7 H12 O6	192.063	3.013
Neochlorogenic acid	C16 H18 O9	354.095	11.828
Chlorogenic acid	C16 H18 O9	354.095	12.754
D-(+)-tryptophan	C11 H12 N2 O2	204.089	5.687
N-acetyl-dl-tryptophan	C13 H14 N2 O3	246.100	16.764
3-butene-1,2,3-tricarboxylic acid	C7 H8 O6	188.031	3.078
Dodecanedioic acid	C12 H22 O4	230.151	18.579
12-Oxo phytodienoic acid	C18 H28 O3	292.204	39.788
4-(2,7-Dihydroxy-6-methyl-2-heptanyl)- 3-hydroxybenzoic acid	C15 H22 O5	282.146	12.591
Laricresinol 4-O-glucoside	C26 H34 O11	522.210	15.506
L-phenylalanine	C9 H11 N O2	165.078	2.725
Guanosine	C10 H13 N5 O5	283.092	2.478
4-acetyl-3-hydroxy-5-methylphenyl β-d-glucopyranoside	C15 H20 O8	328.115	11.821
3-feruloylquinic acid	C17 H20 O9	368.111	14.588
Methyl 2-{[2-O-(6-deoxy-α-l-mannopyranosyl)-β-d-glucopyranosyl]oxy} benzoate	C20 H28 O12	460.158	10.452
Uridine monophosphate (ump)	C9 H13 N2 O9 P	324.036	2.514
5-[2-(3-Furyl)ethyl]- 8a-(hydroxymethyl)-5,6-dimethyl-3,4,4a,5,6,7,8,8a-octahydro-1-naphthalenecarboxylic acid	C20 H28 O4	332.199	33.713
Astragalin	C21 H20 O11	448.101	18.569
Endothal	C8 H10 O5	186.052	12.134
Indole-3-lactic acid	C11 H11 N O3	205.073	20.822
D-(-)-salicin	C13 H18 O7	286.105	7.564

Table 2
Compounds elucidated on the positive run of the LCMS of AAL.

Compound name	Formula	*Mw*	*RT*
2,3,4′-Trimethoxychalcone	C18 H18 O4	298.121	11.551
Caffeic acid	C9 H8 O4	180.042	9.249
4-Coumarylalcohol(4-hydroxycoumarin)	C9 H6 O3	162.032	5.108
Dl-α-aminocaprylic acid	C8 H17 N O2	159.126	2.658
(-)-Caryophyllene oxide	C15 H24 O	220.183	23.408
4-Coumaric acid	C9 H8 O3	164.047	7.459
N-methylhernagine	C20 H23 N O4	341.163	11.354
D-(+)-camphor	C10 H16 O	152.120	14.506
Bis(4-ethylbenzylidene)sorbitol	C24 H30 O6	414.205	37.864
Hypoxanthine	C5 H4 N4 O	136.039	2.598
6-Methylquinoline	C10 H9 N	143.074	3.588
Quercetin	C15 H10 O7	302.043	16.285
2-Hydroxycinnamic acid	C9 H8 O3	164.047	14.757
Ferulic acid	C10 H10 O4	194.058	9.155
Jasmonic acid	C12 H18 O3	210.126	15.747
4-Methoxycinnamaldehyde	C10 H10 O2	162.068	8.190
Indole-3-acetic acid	C10 H9 N O2	175.064	2.479
Inosine	C10 H12 N4 O5	268.081	2.599
Salsolinol	C10 H13 N O2	179.095	2.115
4-Phenylbutyric acid	C10 H12 O2	164.084	3.144
Esculin	C15 H16 O9	340.080	9.242
Nootkatone	C15 H22 O	218.167	10.987
Naringenin	C15 H12 O5	272.069	25.634
Sinapinic acid	C11 H12 O5	224.069	12.774

with an IC_{50} of 1.36 mg/mL at 48 h, much higher compared to the IC_{50} obtained in the current study (5.03% AAL is equivalent to only 0.478 mg/mL) [41]. Moreover, AAL did not show any significant anti-proliferative effects on normal cells, namely human MNCs, indicating that the extract's cytotoxicity is selective for AML cell lines. These findings encouraged further experiments to investigate its mode of action in inhibiting AML cell proliferation.

Apoptosis is the natural cell death mechanism targeted by many anticancer therapies [42]. Many natural compounds such as camptothecins and Vinca Alkaloids were found to induce programmed cell death in cancer cells making them potential candidates for future treatments [43]. Hallmarks of AAL extract induced apoptosis included DNA fragmentation in addition to flipping of phosphatidyl serine which were detected by cell death ELISA and cytometric analysis, respectively.

To further elucidate the pathway by which AAL induced this apoptosis, western blot analysis was performed to detect the levels of several proteins pertaining to this pathway. Poly [ADP-ribose] polymerase-1 [PARP-1] is a strand break sensor involved in DNA repair. The cleavage of PARP-1 results in the failure of PARP-1 to repair these strand breaks, therefore leading to apoptosis [38]. Capsase 8, an initiator caspase was found to be activated upon treatment of KG-1 with AAL. In fact, the cleavage of caspase 8 leading to its activation triggers downstream effectors such as caspase 3 [44]. Moreover, an increase in the Bax/Bcl-2 ratio indicated that AAL induced apoptosis via the mitochondrial pathway. In fact, the proapoptotic Bax and antiapoptotic Bcl-2 are both involved in the regulation of mitochondrial permeability which leads to the release of cytochrome c which will in turn lead to the cleavage of procaspase 9 hence its activation [37]. Our study shows that AAL induces cell death via both the extrinsic and the intrinsic pathway of apoptosis since an upregulation of PARP-1, caspase 9, caspase 8 and Bax was observed alongside a decrease in Bcl-2. In another study, the treatment of AML cell lines with ethanolic extract of *Annona cherimola* seeds activated both the intrinsic and extrinsic pathway of apoptosis [21].

At the cellular level, antioxidant networks play a role in scavenging excessive ROS production which causes oxidative stress leading to damage and cell death [45]. Upon treatment of KG-1 with AAL a decrease in ROS production was observed highlighting its antioxidant properties. In fact, many phytochemicals known to be antioxidant were recently shown to possess anticancer properties, which lead to a conclusion that a certain correlation exists between the antioxidant and anticancer properties of these phytochemicals [46]. Although this correlation is not yet fully explained, studies showed that inhibiting ROS can decrease their harmful effects on signal transduction pathways controlling proliferation as well as carcinogenesis induction; however, these processes need to be further investigated [47].

LCMS analysis of AAL extract revealed the presence of many compounds that may contribute to the anticancer effect observed in AML cell lines treated with the extract. Among the many fatty acids present, 12-Oxo-phytodienoic acid, an oxylipin involved in the stress response of plants, was previously studied for its anticancer properties. In fact, this linolenic acid derivative was found to inhibit the proliferation of breast cancer cells by targeting cyclin D1. 12-Oxo-phytodienoic acid was also found to suppress ROS induced cytotoxicity in neuroblastoma SH-SY5Y cells, highlighting its antioxidant potential [48,49]. Amino acids and their derivatives were also present in AAL in addition to many nucleic acids however, they were not previously investigated for their anticancer potential.

Two quinic acids and derivatives were found in AAL: D-(-)-Quinic acid, a cyclic polyol was previously found to inhibit the proliferation of oral carcinoma cells alone by downregulating the expression of cyclin D1 and Akt signaling in addition to having a synergistic inhibitory effect when combined with cisplatin [50]. 3-Feruloylquinic acid is a quinic acid derivative previously described in *Solanum torvum* fruit extract that

was found to be rich in quinic derivatives and exhibited a potent anticancer potential in T47D human breast cancer cells [51].

Several other phytochemicals were found in the AAL extract. Interestingly, many previous studies highlighted the importance of these plant-derived components for chemoprevention and treatment of cancer [52]. A variety of hydroxycinnamic acids and their derivatives are present in AAL, many of which were previously investigated for various properties including anticancer and antioxidant potential. For instance, sinapinic acid, a compound with strong antioxidant activity, was found to have anticancer effects on human prostate cancer cell lines PC-3 and LNCaP [53,54]. Other hydroxycinnamic acids like neochlorogenic acid and chlorogenic acid may decrease cancer risk by scavenging ROS and altering the uptake and metabolism of carcinogens. In addition, these phenolic compounds were found to inhibit CaCo-2 cell proliferation [55]. In a study performed by Fang et al., the antitumor effect of neochlorogenic acid was reported in human gastric carcinoma cells through apoptosis induction, ROS generation and loss of mitochondrial membrane potential (MMP) [56]. Moreover, chlorogenic acid reduced the proliferation of HCT116 and HT-29 in a dose-dependent manner in addition to cell cycle arrest at the S phase [57]. Another hydroxycinnamic acid found in AAL is caffeic acid. In fact, this compound previously showed antiproliferative and apoptotic effects in vitro on several cell lines (HCT115 cells, cutaneous carcinoma cells and HT-1080) in a dose-dependent manner in addition to DNA damage and matrix metalloproteinases loss in the latter [58,59]. Caffeic acid was also studied in combination with paclitaxel in small lung cancer cells where it enhanced the apoptotic effect of the drug both in vitro and in vivo [60]. Similar effects were detected with ferulic acid [61]. Other hydroxycinnamic acids found in AAL were not previously investigated in the scope of cancer research, namely, 2-hydroxycinnamic acid, and 4-methoxycinnamaldehyde.

Phenolic compounds are secondary metabolites present in plants and are involved in their growth and reproduction. These phenolic compounds, which include flavonoids, tannins, coumarins and phenolic acids possess anti-inflammatory, antioxidant and antiviral abilities alongside their anticancer potential [52]. One of the phenolics found in AAL is catechin which is a major phytochemical in tea. Previous studies showed that catechins are important antioxidants. It was reported that the most studied catechin, (−)-epigallocatechin-3-gallate (EGCG), was involved in many anticancer mechanisms, namely receptor tyrosine kinase downregulation, epigenetic alterations, and anti-inflammatory activities. Moreover, studies have shown that EGCG can both scavenge free radicals and enhance ROS production [62]. Astragalin, a flavonoid present in AAL was reported to have several pharmacological properties including anticancer. In fact, a study performed by Burmistrova et al. showed that astragalin inhibits the proliferation of HL-60 leukemic cell line and induces apoptosis via a caspase-dependent mechanism in addition to the release of cytochrome c and ROS production [63]. Another flavonoid, quercetin was also previously studied for its anticancer and antioxidant properties, reducing the proliferation of a variety of cell lines and inducing apoptosis in vitro, in addition to reducing tumor growth in vivo [64,65]. Naringenin a flavonoid belonging to the flavanone subclass has shown anticancer potential by inhibiting metastasis and invasion of glioblastoma cells [66].

4-coumaric acid is a plant metabolite found in AAL. Studies have shown that this coumarin possesses antioxidant properties in vitro and in vivo. Moreover, it exhibited anticancer abilities in MDA-MB-231 breast cancer bone metastatic cells [67]. 2,3,4′-Trimethoxychalcone found in AAL is a chalcone, part of the flavonoids. Among their many pharmacological abilities, chalcones possess anticancer and chemopreventive properties [68]. The lignan glycoside, lariciresinol 4-O-glucoside belongs to a family of lignans and neolignans heavily researched for their anticancer properties [69].

Interestingly, a previous study on *A.* cherimola leaves ethanolic extract reported the abundance of terpenes upon GC-MS analysis; the current study further reports the presence of terpenes and sesquiterpenes in the aqueous extract of these leaves as detected by LC-MS [24]. In fact, D-(+)-Camphor, a monoterpenoid found in AAL was previously examined for its antitumor properties, alone or in combination with other drugs. In fact, a study showed that the use of camphor in combination with immunotherapy might inhibit the growth of YC8 lymphoma cells in mice [70]. The detected sesquiterpene nootkatone was found to sensitizes non-small-cell lung cancer A549 cells to adriamycin by activating the AMPK pathway, leading to growth inhibition and cell cycle arrest at the G1 phase [71]. Another sesquiterpene (-)-Caryophyllene oxide was detected in the AAL extract. Derivatives of this sesquiterpene, β-caryophyllene and β-caryophyllene oxide (BCP and BCPO) were reported to have antiproliferative effects on several type of cancer cells. Moreover, BCP and BCPO were shown to ameliorate the activity of anticancer drugs, namely paclitaxel and doxorubicin [72].

D-Saccharic acid, also known as glucaric acid was also found to be present in AELE on LC-MS. This compound previously demonstrated chemopreventive and anticancer abilities by decreasing β-glucuronidase activity, an enzyme linked to high cancer risk, especially breast and prostate cancer [73]. D-(-)-Salicin, the precursor of aspirin was also reported to possess antioxidant and antiangiogenic and anticancer properties. In fact, a decrease in viability and DNA fragmentation were observed when AML and ALL cells were treated with willow extract [74]. Another compound, 6-Methylquinoline, belongs to the quinoline family. This family of compounds is present in many plants including Annonaceae and is involved in many anticancer mechanisms of action including apoptosis, cell cycle arrest, anti-angiogenesis [75]. Monocarboxylic acids, namely jasmonic acid and 4-phenylbutyric acid were found in the aqueous extract. In fact, jasmonic acid and its derivatives demonstrated selective toxicity on cancer cells and reduced proliferation both in vitro and in vivo [76] while 4-phenylbutyric acid exhibited antiproliferative effects in a dose and time dependent manner in colon cancer cell lines [77].

Many other compounds like Bis(4-ethylbenzylidene)sorbitol, salsolinol, N-Methylhernagine, 3-Butene-1,2,3-tricarboxylic acid, endothal and many others molecules, were not shown to possess anticancer properties in the literature but were detected in AAL by LC-MS. This provides future interest in examining their individual effects on AML cell lines.

5. Conclusion

In conclusion, the data presented in this study confirms the promising pro-apoptotic effect of *Annona cherimola* aqueous leaf extract on acute myeloid leukemia cell lines in vitro, through cleavage of PARP, activation of caspases 8 and 9, and upregulation of the Bax/Bcl-2 ratio, with potent intracellular antioxidant activity. This reveals the protective effects of a tea prepared from A. cherimola leaves in cancer treatment, as a simple water-based leaf extract showed potent anti-proliferative and pro-apoptotic effects on AML cell lines. Future work needs to be done to test these effects on animals in vivo and examine any effects of absorption and bioavailability on the promising effects of the extract. Chemical analysis of the extract shows that it contains many compounds that are anti-cancer compounds as well as others that are potential targets for future investigation. Since plants are considered an important source for novel chemotherapeutic drug discovery, this work paves the way for finding many natural compounds that could be playing a role, either alone or together, in cancer chemoprevention and therapy.

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Authors contributions

Data curation, project administration, formal analysis and first draft preparation: T.H, M.Y, M.E.K, C.A, S.T and M.H.H. Chemical characterization of the extract: R.S, N.G and L.M. Funding acquisition, supervision, methodology and final revision: S.R. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

All authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111592.

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