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Evidence for the role of differentiation in the antiproliferative effects exerted by a flavonoid-rich extract of *Citrus bergamia* juice in an *in vitro* model of acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by the arrest of normal differentiation of immature myeloid cells (blasts), which proliferate unceasingly, accumulating in the bone marrow and interfering with the normal process of hematopoiesis. In recent years, the use of drugs capable of inducing blast differentiation has brought significant improvements in the survival of leukemia patients. However, the so-called "differentiation therapy" is still limited to a few subtypes of leukemia and it is often associated with serious adverse effects as well as the onset of drug chemoresistance. Therefore, the search for innovative and safer therapeutic solutions, such as natural products, is absolutely necessary.

In light of these considerations, the purpose of my PhD project was to evaluate the ability of a flavonoid-rich extract from bergamot juice (BJe) to exert antileukemic effects and to induce the differentiation of THP-1 leukemic monocytes, as well as the molecular mechanisms underlying these activities.

The results of our study showed that BJe is capable of reducing THP-1 cell proliferation with a concentration- and time-dependent trend, accompanied by S-phase cell cycle blockage and induction of both extrinsic and intrinsic apoptotic pathways, as witnessed by cleavage of both caspase-8 and -9, which in turn activated caspase-3 and PARP, together with the modulation of BAX, Bcl-2 and p53 levels.

The exposure of THP-1 cells to BJe induced the differentiation of leukemic cells, as shown by changes in cell adhesion, nitro blue tetrazolium (NBT)

assay, and increased expression of differentiation-associated surface antigens such as CD11b, CD14, and CD68. In addition, we observed that the extract is able to modulate protein levels of autophagy-associated markers, including LC3 and Beclin-1, as well as to induce phosphorylation of mitogen-activated protein kinases (MAPKs), ERK, JNK and p38, suggesting a potential mechanism of action underlying the biological effect of BJe.

In conclusion, the results of our study suggest that BJe reduces the proliferation and induces the differentiation of THP-1 cells by inducing both the apoptotic and autophagic machinery, suggesting MAPKs as possible cross-talk between the two processes and highlighting its potential in the area of differentiation therapy in AML.

Keywords: acute myeloid leukemia; bergamot juice extract; flavonoids; nutraceuticals; differentiation; autophagy.

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1. INTRODUCTION

1.1. Hematopoiesis

Hematopoiesis is a highly complex and finely regulated biological process that leads to the formation of different blood cells, whose functions are widely diversified and range from oxygen transport to immunity (Jagannathan-Bogdan and Zon, 2013). Hematic cells originate in the bone marrow from hematopoietic stem cells (HSC), a small population of multipotent primitive cells that have the ability to self-renew and differentiate into highly specialized blood cells with lower proliferating activity, thus rendering them able to endure the hematopoiesis throughout the lifetime of an individual (Mosaad, 2014). Hematopoietic differentiation into diverse lineages follows a hierarchical order (Figure 1). Multipotent progenitors (MMP), derived from HSC, give rise to oligopotent progenitors of both lymphoid (CLP) and myeloid (CMP) lineages from which immune system effector cells (B-, T-, NK-, dendritic cells) and granulocyte-monocyte progenitors (GMP), as well as erythrocyte/megakaryocyte progenitors (MEP) originate. GMP finally give rise to mature granulocytes (neutrophils, eosinophils and basophils) and monocyte/macrophages, while MEP subsequently produce erythrocytes and platelets (Akashi et al., 2000; Galy et al., 1995; Manz et al., 2002).

Despite several studies have suggested that this hematopoietic differentiation process may be way more complex than previously described (Kanji et al., 2011; Zhang et al., 2018), it is well-known that a correct functioning of the hematopoietic machinery is important for the

maintenance of human health, so that abnormalities in this developmental program can often result in hematological diseases, such as leukemia.

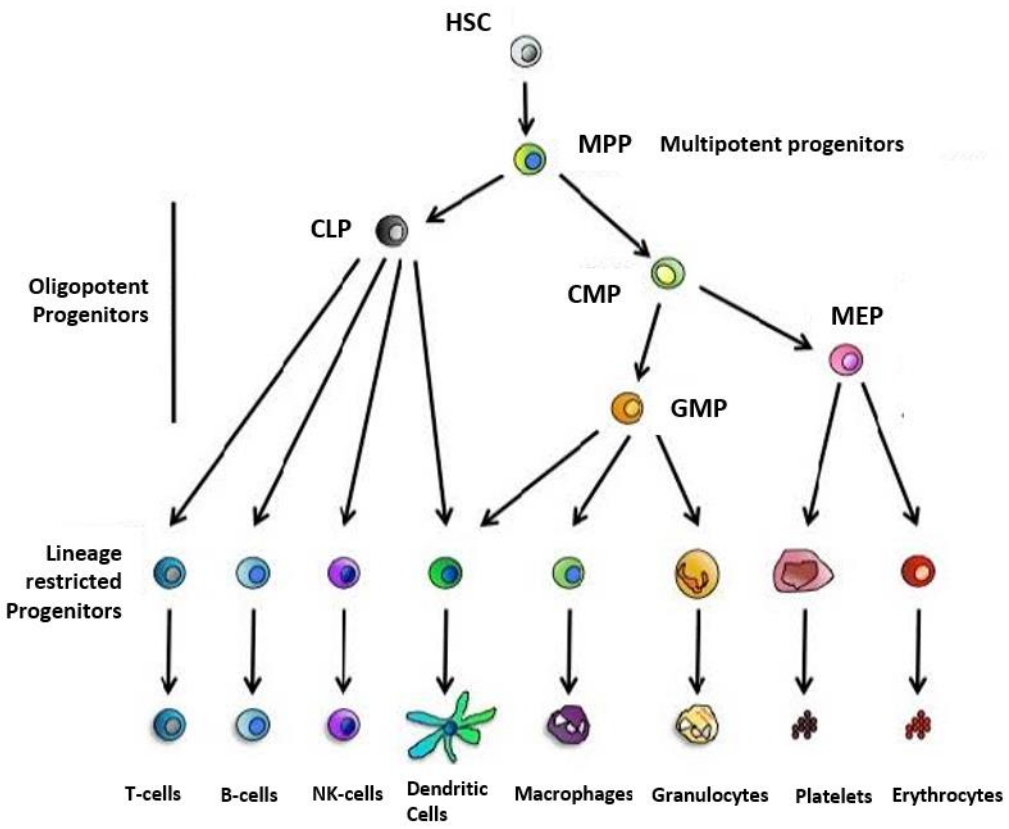


Figure 1. The hematopoietic system. Abbreviations: Hematopoietic stem cells (HSC); multipotent progenitors (MPP); common lymphoid progenitor (CLP); common myeloid progenitor (CMP); megakaryocyte–erythroid progenitors (MEP) granulocyte-monocyte progenitors (GMP).

1.2. Acute myeloid leukemia (AML)

Leukemia is a complex group of hematologic malignancies characterized by uncontrolled proliferation, aberrant regulation in apoptosis and improper differentiation of immature blood cells (blasts), which accumulate in the bone marrow and, subsequently, in the peripheral blood (Brady, 2003; Lazarevic et al., 2018; Nowak et al., 2009). Despite the wide

heterogeneity of this pathology (Wang and Dick, 2005), leukemia is primarily divided into four main types, depending on the cell lineage (myeloid or lymphoid) and the progression rate of the disease (acute or chronic) (Castillo et al., 2019). Acute leukemia arises abruptly and is more aggressive than the chronic one, hence an immediate treatment is required (Martelli et al., 2012). Acute myeloid leukemia (AML) is one of the most common types of leukemia in adults (De Kouchkovsky and Abdul-Hay, 2016; Dores et al., 2012; Yamamoto and Goodman, 2008) with poor prognosis, in which the bone marrow produces too many immature myeloid cells, replacing normal marrow tissue and hematopoietic cells, hence inducing anemia, thrombocytopenia and granulocytopenia (Lowenberg et al., 1999; Tamamyan et al., 2017).

AML incidence is around 4 cases per 100.000 of population in Europe and the United States (US), and it increases with age, with a median age, when first diagnosed, of around 70 years (Dong et al., 2020; Juliusson et al., 2017; Shallis et al., 2019); however, it can also affect children (15-20 % of the cases).

Despite its exact etiology remains elusive, myelodysplastic syndrome is considered one of the most common risk factors for its onset (Chen et al., 2019). Other conditions that can increase the risk for AML include myelofibrosis, aplastic anemia, as well as different congenital disorders, such as Bloom syndrome and Down syndrome. AML can arise also following “leukemogenic agent exposure”, among which tobacco smoke, benzene, radiation, but also former exposure to chemotherapeutic agents.

Nevertheless, it can occur in patients that have not been previously exposed to any risk factor.

Depending on etiology, immunophenotype, morphology and genetics, there are diverse classification systems for this hematological neoplasm (Boddu and Zeidan, 2019; Hartmann and Metzeler, 2019).

The French-American-British (FAB) classification and the newer World Health Organization (WHO) classification represent two of the principal systems that have been utilized to categorize AML. In the 1970s, a team of French, American, and British leukemia experts classified AML into eight subtypes, from M0 to M7, based on cell morphology to define specific immunotypes and how mature the cells are (Table 1).

Table 1. FAB classification of AML.

FAB	Definition
M0	Undifferentiated AML
M1	AML with minimal maturation
M2	AML with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryocytic leukemia

In 2016, the WHO renewed the classification system by relying it on evidence of dysplasia and chromosome translocations and divided this cancer in: AML with changes related to myelodysplasia, AML with recurrent genetic abnormalities, AML related to previous radiation or

chemotherapy, AML not otherwise specified (NOS), myeloid proliferations related to Down syndrome and myeloid sarcoma (Dohner et al., 2017).

AML is diagnosed after a complete blood cell count, together with a bone marrow biopsy, blood smear, but also by morphology, immunophenotyping and cytogenetic studies (Betz and Hess, 2010). Due to impaired hematopoiesis and bone marrow failure, some of the most common symptoms of AML include anemia, thrombocytopenia and granulocytopenia. Depending on the degree of anemia, it may manifest with generalized weakness, dyspnea, fatigue, pallor and chest tightness. Thrombocytopenia may provoke excess bleeding, including frequent or severe nosebleeds, bleeding gums and heavy menstrual bleeding in women, but also bruising and epistaxis. Patients can manifest spontaneous hemorrhage, including intra-abdominal or intracranial hematomas (Webert et al., 2006). Granulocytopenia (neutropenia) can eventually lead to an increased risk of severe or recurrent infections (Cannas et al., 2012).

1.3. Treatment of AML

Currently, AML treatments include stem cell transplantation, radiotherapy and chemotherapy, although the latter represents the backbone of AML management (Roboz, 2012). It is worth to note that treatment of AML highly depends on general medical conditions of the patient. Indeed, for patients in a better general medical condition, the first step for AML treatment consists of an induction therapy (also known as "7+3" regimen) with a continuous infusion of cytarabine for seven days, combined with

anthracycline on days 1 to 3, with the aim of eliminating as many blasts in the blood and bone marrow as possible, and restore the physiological production of white blood cells. Percentages of complete remission, which is achieved when < 5% blasts are present in the bone marrow, are variable and depend on patient's initial condition and AML subtype. Usually, this phase is quite aggressive, and it is often accompanied by severe side effects, highlighting the importance of a meticulous supporting therapy (Sekeres et al., 2020). In certain cases, a target-based therapy is also used, however it is applicable only to a small portion of patients.

Induction therapy is commonly followed by a consolidation phase, initiated with high dose of cytarabine, with or without allogeneic hematopoietic stem cell transplantation. Contrarywise, more clinically debilitated patients are addressed with less intensive therapies, such as hypomethylating agents (azacytidine or decitabine).

As mentioned above, a major problem in the pathophysiology of AML is the blockage of differentiation of immature cells, which continue to proliferate unceasingly. In addition, the arrested differentiation impedes progenitor cells from maturing, hence undergoing apoptosis (Tenen, 2003). For this reason, rather than killing cells by employing cytotoxic and unselective drugs, one area of research is now addressed to the so-called "differentiation therapy", which aims to reprogram malignant leukemic cells into functional ones, as well as to force them to enter the apoptotic pathway as a result of terminal differentiation (Nowak et al., 2009). For these reasons, differentiation therapy might be an attractive solution for AML patients (Gocek and Marcinkowska, 2011), in particular for those who

are ineligible for intensive cytotoxic protocols, due to their poor tolerability. Moreover, the restored differentiation mechanisms of blasts should ameliorate, at least in principle, the immune status of patients without extensive lysis of blood cells typical of some cytotoxic procedures (Mughal et al., 2010).

A clear example of the success of differentiation therapy has been achieved by using pharmacological doses of all-trans retinoic acid (ATRA) and/or arsenic trioxide in acute promyelocytic leukemia (APL), which brought huge improvements in terms of outstanding remission rates and long-term survival (between 80 and 90%) (Stahl and Tallman, 2019).

Unfortunately, ATRA and ATO are only clinically successful for the APL subtype, which represents only 5–10% of total AML cases, showing no efficacy for the other ones. Thereby, the search of novel differentiation inducers for AML therapy, in particular for other AML subtypes, is absolutely necessary.

1.4. Nutraceuticals in cancer

Nowadays, it is commonly known that plant kingdom represents a precious source of potentially useful substances in the prevention and therapy of various diseases, including neoplasms. Albeit there is still no official definition for “nutraceutical”, the term has been used for the first time by Stephen L. Defelice, in 1989, as a merging of the words "Nutrition" and "Pharmaceutical". Nutraceuticals are substances present in food that exert beneficial effects on health that could help in the management of both acute and chronic disorders (Kalra, 2003). Several studies assessed their

positive activities in different pathological conditions, including neurodegenerative and cardiovascular diseases, atherosclerosis, hypertension, inflammation, obesity, diabetes and cancer (Nasri et al., 2014). Effectiveness of nutraceuticals and their general safety have been also supported by different clinical studies (Télessy, 2019).

Among natural compounds that have been extensively studied for their health-promoting properties, a central role is occupied by flavonoids (Figure 2), secondary plant metabolites, very abundant in *Citrus* fruits (Garcia-Lafuente et al., 2009).

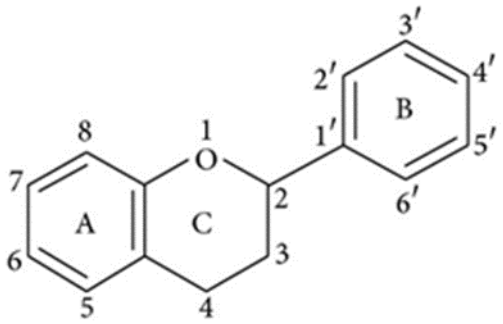


Figure 2. Basic flavonoid structure. The general structure of flavonoids consists of a benzopyran (rings A and C) linked to a phenyl group (ring B). Flavonoids are divided in different classes depending on variations in the C ring as well as in the linkage between the phenyl and benzopyran moiety. Flavonoids can be found either in their glycosylated or aglycone forms.

Indeed, increasing evidence has demonstrated that flavonoids present in vegetables and fruits possess antioxidant and anti-inflammatory effects, as well as anti-tumor properties (Benavente-Garcia and Castillo, 2008). Flavonoids have been shown to interfere with carcinogenesis process, through the modulation of different molecular pathways, by hampering proliferation, metastasis, angiogenesis, as well as by inducing apoptotic

machinery (Abotaleb et al., 2018).

Inhibition of carcinogenesis by *Citrus* juices (Cirmi et al., 2017) and their single flavonoids has been observed both *in vitro* and *in vivo* (Cirmi et al., 2016b). In particular, Matsui et al. (2005) have shown that several flavonoids could induce apoptosis in leukemia and lymphoma cell lines in a concentration-dependent manner. Other reports demonstrated the ability of *Citrus* flavonoids to target differently AML, either by directly interacting with key pathways involved in cancer, or by modulating of gene expression. These include hesperidin (Desai et al., 2015), quercetin (Kawahara et al., 2009; Lee et al., 2015), nobiletin (Chen et al., 2018), diosmetin (Roma et al., 2018; Rota et al., 2016) and naringenin (Park et al., 2008; Shi et al., 2015). Furthermore, several studies asserted that various flavonoids are able to promote differentiation in different leukemia cells (Chen et al., 2013; Hui et al., 2016; Takahashi et al., 1998; Yang et al., 2019), thus suggesting these polyphenolic compounds as attractive candidates for the differentiation-inducing therapy in the context of the aforementioned promising approach for the treatment of leukemia (Sak and Everaus, 2017).

1.5. *Citrus bergamia* Risso et Poiteau

Among flavonoids, those retrieved from *Citrus* fruits stand out among the others for their broad spectrum of biological effects. This because, these fruits are acknowledged to be embedded with antioxidant and anti-inflammatory properties (Ferlazzo et al., 2016b; Maugeri et al., 2019a; Musumeci et al., 2020), apart from exerting a protective effect against

neurodegenerative (Cirmi et al., 2016c; Cirmi et al., 2021) and cardiovascular diseases (Testai and Calderone, 2017), different microbial infections (Cirmi et al., 2016a), and various types of cancer (Cirmi et al., 2016b; Cirmi et al., 2017; Cirmi et al., 2018).

In this context, *Citrus bergamia* Risso & Poiteau (Figure 3), commonly known as bergamot has drawn the attention of many researchers due to the wide plethora of pharmacological effects showed by its derivatives, such as the essential oil and the juice.

Figure 3. *Citrus bergamia* Risso & Poiteau.

Bergamot is a small tree belonging to the Rutaceae family and genus *Citrus*, that is cultivated almost exclusively (95% of worldwide production) along the southern coast of Calabria region (Italy), where the particular environmental conditions are suitable for its cultivation. The rest of the total production is settled in Morocco, Ivory Coast, Iran, Brazil and Argentina. It is considered a hybrid between *Citrus aurantium* L. (sour orange) and either *Citrus aurantiifolia* (lime) or *Citrus limon* L. (lemon), or a mutation of the latter. Its exact geographical and botanical origins are not completely certain, though it has been found in the Mediterranean area for centuries. It may have been imported by Christopher Columbus or it may have originated from Calabria region, deriving from a mutation of other species. Its ethno-pharmacological and botanical aspects have been issued by Rapisarda and Germanò (2013).

This *Citrus* is commonly used for the extraction of its essential oil (BEO), obtained by cold pressing its peel or by steam distillation and widely exploited in perfume industry and in aromatherapy (Mannucci et al., 2017; Navarra et al., 2015b), while the juice (BJ), obtained by squeezing the endocarp of bergamot fruits, has long been considered a by-product. The remaining part from both BEO extraction and BJ retrieving is commonly known as “bergamot pastazzo” and employed on organic soil fertilization, animal feeding and pectin extraction.

BEO is composed of about 95 % by a volatile portion comprising linalyl acetate, linalool, γ-terpinen, α-pinene and β-pinene and by a non-volatile fraction composed mainly by coumarins and psoralenes, such as citropten, bergamottin, bergapten and 5-geranil (Costa et al., 2010).

This phytocomplex has been studied also for its anti-inflammatory (Lombardo et al., 2020) antimicrobial (Cirmi et al., 2016a) cardioprotective (Mollace et al., 2008) and anti-cancer properties (Celia et al., 2013; Maugeri et al., 2021; Navarra et al., 2015a).

Despite being considered for long time a waste product, in the last decades, BJ has been revaluated for its remarkable pharmacological value. In particular, it has been shown that BJ reduced growth rate of different cancer cell lines, by mechanisms that, in SH-SY5Y neuroblastoma cells, are linked to an early impairment in cell adhesive and migratory capability (Delle Monache et al., 2013). The anti-metastatic effect of BJ was also observed in a spontaneous metastatic neuroblastoma severe combined immunodeficient (SCID) mouse model (Navarra et al., 2014). Interestingly, it has been documented that the antiproliferative effect of BJ is due to its flavonoid fraction, because it was able to inhibit proliferation of HT-29 human colon cancer cells, as well as inducing apoptosis through multiple molecular mechanisms (Visalli et al., 2014). Consequently, the flavonoid-rich extract of BJ (BJe) was further studied. Indeed, BJe was shown to possess antioxidant effects (Ferlazzo et al., 2016b; Ferlazzo et al., 2015) and inhibited both gene expression and secretion of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines in a model of LPS-stimulated THP-1 cell line (Risitano et al., 2014), as well as in an *in vitro* model of neuroinflammation (Currò et al., 2016). Moreover, BJe exerted anti-inflammatory and antioxidant effects in *in vivo* models (Gugliandolo et al., 2018; Impellizzeri et al., 2015; Impellizzeri et al., 2016), interacting also with the AMPK/SIRT1 axis (Maugeri et al., 2019b)

and suggesting its potential role in the management of inflammation-based diseases (Marino et al., 2015).

2. AIM OF THE RESEARCH

Based on the evidence supporting the anti-cancer effects of BJe, the first aim of my PhD project was to evaluate whether this extract was able to exert antileukemic effects in an *in vitro* model of AML.

Afterwards, considering the richness of BJe in flavonoids and acknowledged their differentiation potentialities, we further investigated the capability of this extract to differentiate leukemic cells employing the same *in vitro* model, as well as the mechanisms underlying these activities.

3. MATERIALS AND METHODS

3.1. *Citrus bergamia* juice extract

Bergamot fruits, harvested in Reggio Calabria (Italy), were bought from the local markets. Upon peel removal, fruits were pressed, and the primary juice was removed. The remaining pulp, usually discarded, was further processed by using a pressing machine to retrieve the secondary juice, that was extracted and concentrated, following a solid-phase extraction (SPE) extraction by using a SupelcleanTM LC-18 SPE cartridge (Supelco Ltd., Bellefonte, Pennsylvania, USA) according to manufacturer's instructions. In order to extract the flavonoid-rich bergamot fraction, we used ethanol to carry out the final elution and the eluate was transformed into a dry powder by lyophilization. The obtained BJe powder was stored at 4° C in the dark prior to utilization.

3.2. Chemical analysis of BJe

3.2.1. Reagents

Naringenin, neoeriocitrin, neohesperidin, neodiosmin, rhoifolin and vicenin-2, supplied by Extrasynthèse (Genay, France) were used as standards. The Iso-Disc P-34, 3 mm diameter polytetrafluoroethylene (PTFE) 0.45 µm membrane was from Supelco (Bellefonte, PA, USA). All the other chemicals and reagents utilized in the study were of analytical grade and bought from Sigma (Milan, Italy).

3.2.2. Sample preparation

The powder of the extract was solubilized in a phosphate saline buffer (PBS)/dimethylformamide (0.9:0.1 v/v) solution to reach a final concentration of 1.25 mg/mL, then it was centrifuged at 3200 rpm for 5 minutes and filtered by using an Iso-Disc P-34, 3 mm diameter PTFE 0.45 µm membrane and used for reverse phase high performance liquid chromatograph (RP-HPLC) coupled with a diode array detector (RP-HPLC-DAD) separation.

3.2.3. RP-DAD-HPLC separation and identification

The quali-quantitative analysis of flavonoids present in BJe was carried out by injecting the filtered solution into a RP-HPLC-DAD, following Cirmi and collaborators (2021), by using a Shimadzu system (Shimadzu Ltd., Canby, OR, USA). Each sample was separated three times and gave overlapping chromatograms. Flavonoids were identified by matching retention time and UV spectra against reference compounds, as well as by spiking the samples with pure reference compounds. The calibration lines were gained employing known concentrations of pure compounds and were chosen to match the concentration of the tested samples. Quantitative analysis was performed by integrating peaks areas from the chromatograms detected at 280 and 325 nm for flavanone and flavone derivatives, respectively. The calibration curves were constructed, and linear regression equations were retrieved by plotting the peak areas ratios of compounds to those of the external standard, versus the known concentrations of pure compounds.

3.2.4. Acid hydrolysis

The acid hydrolysis has been performed on BJe based on a previously published procedure (Gattuso et al., 2006). In brief, 10 mL of HCl (6 M) in a methanol (25 mL)/water (10 mL) solution was added to 5 mL of BJe to give a solution of 1.2 M HCl in 50% aqueous methanol. As an antioxidant, we added ascorbic acid (50 mg). After refluxing for 20 h at 90°C under constant stirring, the solution was kept at room temperature to cool, vacuum-dried and the residue suspended in 10 mL water/DMF (1:1). Subsequently, the mixture was filtered with an Iso-Disc P-34 membrane and analyzed by HPLC.

3.3. Cell cultures and treatments

The experiments were performed using the human leukemia monocytic THP-1 cells, obtained originally from ATCC (Rockville, MD, USA), employed as an *in vitro* model of AML. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), 2-mercaptoethanol (0.05 mM), glucose (2.5 g/l), penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37°C in a 5% CO_2 in air atmosphere. Each reagent for cell culture was purchased from Gibco (Life Technologies, Monza, Italy). Medium was renewed every 2 to 3 days and cells were subcultured when they reached maximum density (1×10^6 cells/mL). For all experiments, THP-1 cells were treated with different concentration of BJe up to 5 mg/mL, as previously published

(Visalli et al., 2014), that has been solubilized in medium, for different time points.

3.4. MTT assay

In order to determine cell viability in presence of BJe, we performed the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test following (Maugeri et al., 2021). MTT assay is a colorimetric test used to assess cell mitochondrial activity as an indicator of cell viability. It is based on the enzymatic reduction of a yellow MTT salt to formazan crystals (purple) by cells that are metabolically active. THP-1 cells were seeded onto 96-well plates (5×10^4 cells/well) and, after 24 h, they were incubated with fresh growth medium (untreated cells) or with medium supplemented with increasing dilution of BJe, up to 5 mg/mL. After 24, 48 and 72 h, plates were centrifuged, and the supernatant was substituted with fresh medium devoid of phenol red containing 0.5 mg/mL of MTT (Sigma-Aldrich, Milan, Italy) at 37°C for four hours. Upon MTT solution incubation, crystals of formazan were dissolved in 0.1 N HCl/isopropanol solution. Then, the absorbance of each well was recorded by a microplate reader (Bio-Rad Laboratories, Milan, Italy) at 570 nm wavelength with reference at 630 nm. Results were expressed as percentage of cell viability *vs* untreated cells. MTT test was carried out using octuplicates and repeated at least three times. We also determined the half-maximal inhibitory concentration (IC50) for each treatment after selected time points by GraphPAD Prism 8 software.

3.5. Propidium iodide exclusion assay

Propidium iodide (PI) is a dye that enters cell membranes that are not intact and binds to double stranded DNA by intercalating among base pairs. Through fluorescence-activated cell sorting (FACS) techniques, we performed PI exclusion assay to investigate whether BJe possessed cytotoxic properties and was able to affect the membrane integrity in THP-1 cells, as described (Currò et al., 2016). In brief, THP-1 cells were seeded onto 24-well plate at a cellular density of 5×10^5 cells/well and treated with 1, 2.5 and 5 mg/mL of BJe for 24, 48 and 72 h. After treatment, cells were collected and centrifuged at 1200 rpm for 10 minutes, washed and resuspended in 100 µL of cold 1X PBS. Then 10 µL of PI labeling solution (Sigma-Aldrich) was added to the cell suspension and incubated at room temperature for 30 minutes in darkness. Stained cells were analyzed with FL-2 channel by a cytofluorimeter Novocyte 2000 (ACEA Bioscences Inc., San Diego, CA, USA). A minimum of 10,000 events were recorded per sample and the percentage of dead cells compared to non-treated ones was calculated.

3.6. Cell cycle analysis

The ability of PI to bind stoichiometrically to DNA can also be exploited to precisely quantify and discriminate cell phases. We therefore evaluated the role of the extract in interfering with cell cycle progression in THP-1 cells by cytofluorimetric analysis as described by Maugeri and co-workers (2021). In brief, THP-1 cells were plated at a density of 2×10^5 cells/mL

onto 6-well plates and treated accordingly for 24, 48 and 72 h with 1, 2.5 and 5 mg/mL of BJe. Then, leukemic cells were collected, washed with 1X PBS and, in order to fix the cells, 1 mL of ice-cold 70% ethanol was added drop by drop to the cell pellet, while gently vortexing. Samples were therefore kept at 4°C for 2 h and then washed twice with 1X PBS to eliminate any trace of alcohol. Cell pellet was then resuspended in 250 µL of 1X PBS, together with 5 µL of Ribonuclease A (10 mg/mL; Sigma-Aldrich) and incubated for 1 h at 37 °C. Thereafter, 10 µL of PI (1 mg/mL) were added to samples, which were rapidly acquired by Novocyte 2000 cytofluorimeter. At least 10,000 events were analyzed per sample.

3.7. Annexin V/PI staining

Staining with Annexin V-fluorescein isothiocyanate (FITC)/ PI is an acknowledged technique used to determine different type of cell death, such as necrosis and apoptosis. Annexin V is a protein that possesses great affinity to phosphatidylserines, which are usually present in the inner side of the cell membrane and are externalized during the early stages of apoptosis. PI, as described above, is able to penetrate cells with compromised cell membranes (characteristic of both necrosis and late apoptosis) and binds to DNA. In brief, leukemic cells were plated onto 6-well plates at a cellular density of $2x10^5$ cells/mL and, after 24 h, were treated with different concentration of BJe (1, 2.5 and 5 mg/mL) for further 24, 48 h and 72 h. Following incubation period, cells were collected, washed with ice-cold 1X PBS, and resuspended in 200 µl of 1X Binding Buffer, together with 5µl of Annexin-V-FITC, as suggested by kit guidelines

(BD Biosciences, Milan, Italy). Subsequently, samples were gently vortexed and incubated at room temperature for 15 minutes in darkness. Thereafter, 10 µl of PI (20 µg/mL) were added and samples were run on a Novocyte 2000 cytofluorimeter, with a minimum of 10,000 events being analyzed.

3.8. Nitroblue tetrazolium (NBT) reduction assay

In order to evaluate whether BJe was able to induce differentiation in THP-1 cells, we performed the nitroblue tetrazolium (NBT) reduction analysis as described (Gatt et al., 2021; Stoica et al., 2016). NBT test is a colorimetric assay that relies on the ability of differentiated phagocytic cells to produce superoxide anion O_2^- upon stimulation with phorbol 12-myristate 13-acetate (PMA), thus reducing yellow NBT salt to form diformazan, a dark blue insoluble precipitate.

For this assay, THP-1 cells (3×10^4 cells/well) were plated onto a 96 well/plate and exposed to 1 and 2.5 mg/mL of BJe for 72 and 96 h (by renewing treatment after 48 h), we used only medium for negative controls and 10 nM of PMA, a common differentiation inducer, for the positive ones. At the end of incubation period, plates were centrifuged and washed with 100 µl of PBS, then we resuspended cells with 100 µl of PBS containing 2 mg/mL of NBT and 100 ng/mL of PMA for 30’ at 37°C. Thereafter, we centrifuged the plates and diformazan crystals were dissolved with 1:3 KOH 2M/DMSO solution and the absorbance of each well was recorded by a microplate reader (Bio-Rad Laboratories, Milan, Italy). We also performed a parallel MTT test, at the same time, in order to normalize the amount of NBT reduction for the relative number of living cells, therefore calculating the

degree of differentiation as a ratio of NBT absorbance values over MTT ones. All the experiments were repeated at least three times with octuplicates.

3.9. Morphological analysis

With the aim of determining whether the extract was able to modify cell adhesion, we plated THP-1 cells in 100-mm dishes at a density of 1×10^6 per dish. After overnight incubation, cells were treated with BJe (1 and 2.5 mg/mL) up to 96 h or with 10 nM of PMA, used as positive control. We therefore investigated cell morphology by examining the cells with a Zeiss Primo Vert (Carl Zeiss, Milan Italy) inverted phase-contrast microscope at 40X magnification, equipped with AxioCam ERc5s camera, by which pictures were captured.

3.10. Cell surface antigen detection

Flow cytometry techniques were used to detect cell surface markers of differentiation. In brief, THP-1 cells, at a cellular density of 3×10^5 cells/mL, were plated onto 6 well/plates. After 24 h, they were exposed to only medium (negative control), BJe 1 and 2.5 mg/mL or 10 nM of PMA up to 96 h (by renewing treatment after 48 h). At the end of the incubation period, cells were harvested, centrifuged (1200 rpm for 10 minutes at 4°C) and washed with 1X pre-cooled PBS. Cell pellets were resuspended in 100 µl of PBS with either anti-CD68 antibody conjugated with allophycocyanin (APC) (Miltenyi Biotec, Bergisch Gladbach, Germany) or co-stained with anti-CD11b antibody conjugated with APC (Miltenyi Biotec) and anti-CD14 antibody conjugated with FITC (Beckman Coulter, Milan, Italy) and incubated for 30 min in darkness at room temperature. Subsequently

samples were run on a Novocyte 2000 cytofluorimeter, with a minimum of 10,000 events being recorded.

3.11. Real-Time PCR

To evaluate gene expression, THP-1 cells were seeded in 100 mm Petri dishes at a cellular density of 1×10^6 cells per dish and, after 24 h, incubated with fresh medium (negative control) or with BJe (1, 2.5 and 5 mg/mL), for 12 h at 37°C. Then, total RNA was extracted from untreated and treated cells, employing TRIzol reagent (Invitrogen, Carlsbad, CA, USA), by following manufacturer's instructions. Afterwards, equal amounts of extracted RNA (2 µg) from each sample were reverse transcribed into cDNA, using a High-Capacity cDNA Archive Kit (Applied Biosystems, Thermo Fisher, Foster City, CA, USA. Subsequently, the mRNA levels of caspase (CASP) 3, 8 and 9, B-cell lymphoma 2 (Bcl-2), (Bcl-2)-associated X protein (BAX), p53 were determined by real-time PCR (RT-PCR). RT-PCR was performed in a 96-well plate, in a total volume of 20µl with 1x SYBR® Select Master Mix (Applied Biosystems), 0.2 µM of specific primers and 25 ng of RNA that was converted into cDNA.

We used a 7300 RT-PCR System (Applied Biosystems) to carry out the reaction with the following profile: one cycle at 95 °C for 10 min, then 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. A standard dissociation stage was added to check the primer specificity. β-Actin was utilized as housekeeping control. Data collected were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification method (Maugeri et al., 2019b) and values were presented as fold change relative to untreated cells. Primer sequences used for RT-PCR are listed in Table 2.

Table 2. Sequences of oligonucleotide primers used for RT-PCR.

Gene	NCBI Ref. Seq.	Primer sequence
CASP3	NM_004346.4	Forward: 5'-AGCACCTGGTTATTATTCTTGG-3' Reverse: 5'-GCTTGTCGGCATACTGTT-3'
CASP8	NM_001228.4	Forward: 5'-GTCTGTACCTTTCTGGCGGA-3' Reverse: 5'-CTCAGGCTCTGGCAAAGTGA-3'
CASP9	NM_001229.5	Forward: 5'-GCTCAGACCAGAGATTCG-3' Reverse: 5'- ATCCTCCAGAACCAATGTC-3'
p53	NM_000546.6	Forward: 5′-GTGTGGAGTATTTGGATGAC-3′ Reverse: 5'- ATGTAGTTGTAGTGGATGGT-3'
BAX	NM_138764.5	Forward: 5′-GGACGAACTGGACAGTAACATGG-3′ Reverse: 5′-GCAAAGTAGAAAAGGGCGACAAC-3'
Bcl-2	NM_000657.3	Forward: 5′-ATCGCCCTGTGGATGACTGAG-3′ Reverse: 5′-CAGCCAGGAGAAATCAAACAGAGG-3′
β-actin	NM_001101.5	Forward: 5′-TTGTTACAGGAAGTCCCTTGCC-3′ Reverse: 5′-ATGCTATCACCTCCCCTGTGTG-3′

3.12. Western blotting analysis

With the aim of evaluating protein expression, we performed Western blotting analysis. THP-1 cells were seeded onto 100 mm Petri dishes at a cellular density of 1×10^6 cells and, after 24 h, treated with 1-5 mg/mL of BJe for 24 h or with 0.5-2.5 mg/mL of BJe for 48 h. Then, cells were collected, washed with cold PBS and lysed for protein retrieval by adding RIPA buffer (Sigma-Aldrich) containing 1% of protease and phosphatase inhibitor cocktail (Sigma-Aldrich). The lysed cells were subsequently centrifuged at 12,000 g at 4°C for 15 minutes and the supernatant was gently aspirated and placed in a fresh tube kept on ice. Protein concentration was determined as described before (Celano et al., 2015), employing a Bio-Rad Protein Assay (Bio-Rad Laboratories), while bovine serum albumin was used as standard. Thereafter, 30 µg/well of proteins were subjected

to 10% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electro-transferred onto 0.45 μm nitrocellulose (Merck Millipore, Darmstadt, Germany) or polyvinylidene difluoride (PVDF; Merck Millipore) membranes. Non-specific binding sites were blocked with PBS containing 5% (w/v) non-fat dry milk for 1 h at room temperature and membranes were incubated at 4°C overnight with the following primary antibodies: rabbit monoclonal anti-cleaved caspase-8, anti-caspase-9, anti-caspase-3, anti-poly ADP ribose polymerase (PARP), anti-phospho-JNK, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho p38, anti-p38, anti-LC3 a/B, anti-beclin-1, anti-phospho-AKT and anti- AKT, (Cell Signaling Technology, Danvers, USA), diluted 1:1,000 in milk or BSA, mouse monoclonal anti-p53 (Thermo-Fisher Scientific Rockford, IL, USA), diluted 1:200 and mouse monoclonal anti-actin-peroxidase (Sigma-Aldrich), diluted 1:50,000.

After washing thrice with Tris-buffered saline with 0.1% Tween® 20 detergent (TBST), the membranes were incubated, for 2 h at room temperature, with the indicated horseradish peroxidase-conjugated goat anti- rabbit or anti- mouse IgG secondary antibodies (1:5,000; Sigma-Aldrich). Chemiluminescence of protein bands was achieved after incubating blots with appropriate amount of Luminata Forte Western HRP Substrate (Merck Millipore) for 5 minutes and captured by a chemiluminescent detection system C-Digit Blot Scanner (Li-COR Bioscience, Lincoln, NE, USA). Image Studio software (Li-COR Bioscience) was used to quantify protein bands. For each sample, β-actin was used as housekeeping protein to normalize signals intensity.

3.13. Statistical analyses

The one-way or two-way analyses of variance (ANOVA) were employed, according to the assay. Results are expressed as mean ± standard error of the mean (SEM). Multiple comparisons of the means of the groups were performed by the Dunnett's multiple comparison test (GraphPAD Software). P values less than or equal to 0.05 were considered significant.

4. RESULTS

4.1. Quali-quantitative analysis of BJe

The identification of the flavonoids present in the lyophilized powder of BJe was carried out by RP-HPLC-DAD separation. The preliminary results from chromatographic separation at 280 and 325 nm allowed us to distinguish between flavanone and flavone basic skeletons present in the extract. The two chromatograms displayed in Figure 4 showed a prevalence of compounds 2, 4, 6 belonging to flavanone class than compounds 1, 3, 5 with a flavone skeleton. Treatment with aqueous HCl indicated that compounds 2, 4, 6 were not resistant to acidic hydrolysis, suggesting the presence of O-glycoside compounds in their aglycone form (data not shown). Considering the retention time, together with UV spectra and spiking the samples with pure reference molecules, the main peaks of the chromatogram corresponded to neoeriocitrin (2), naringin (4) and neohesperidin (6). These two latter represented the most abundant flavonoids of the extract (0.59 ± 0.037 and 0.44 ± 0.017 mg/mL for compound 4 and 6, respectively), accounting for about 83% of all the BJe utilized, while neoeriocitrin was present at the concentration of 0.11 ± 0.011 mg/mL. The remaining three peaks were identified in the chromatogram as vicenin-2 (1), rhoifolin (3) and neodiosmin (5), present below 0.1 mg/mL. Flavonoids amounts and their chemical structures are presented in Table 3.

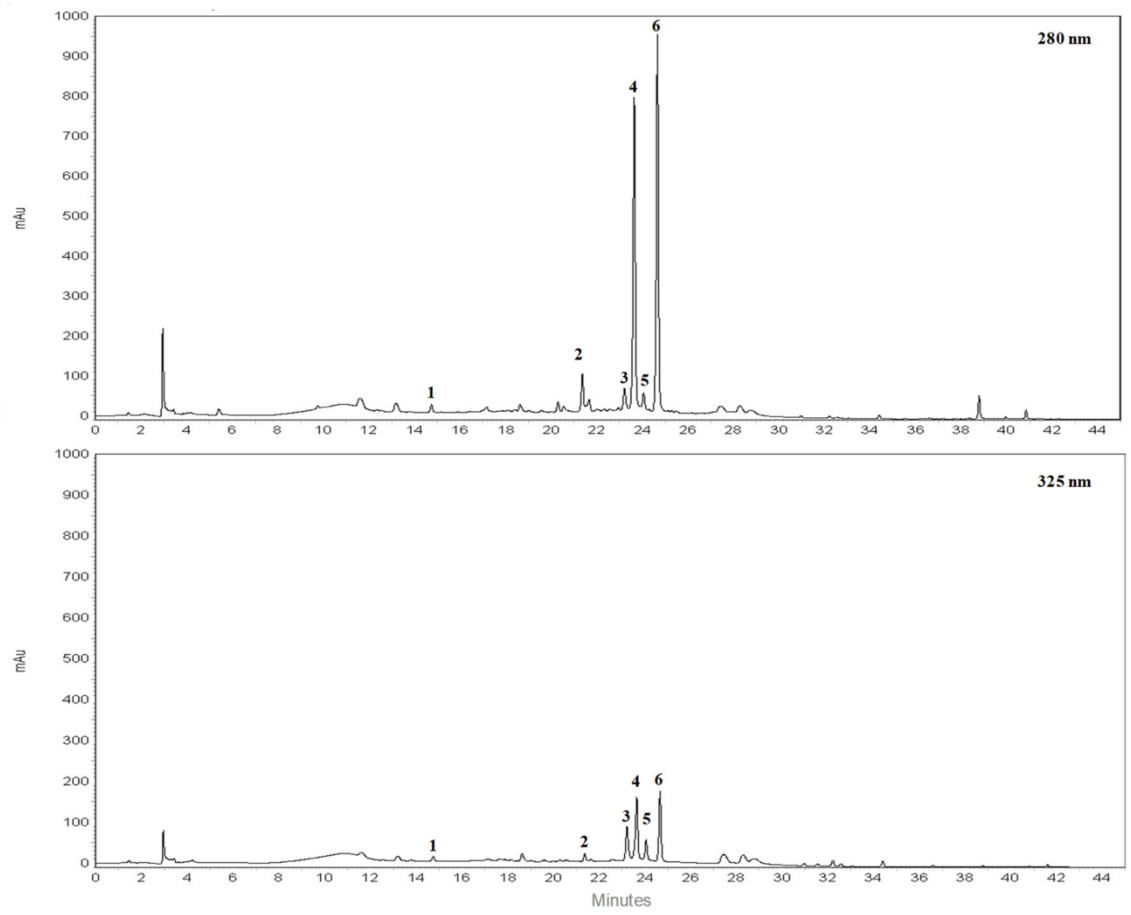
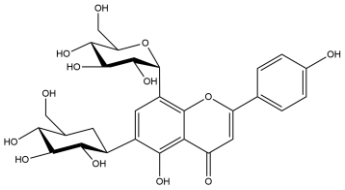
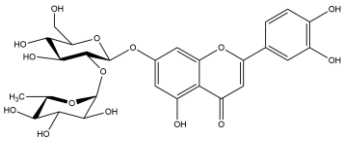
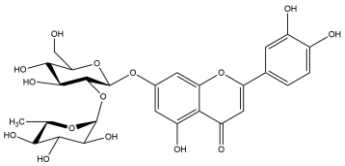
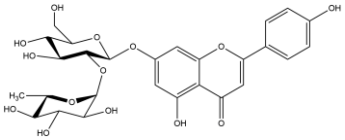
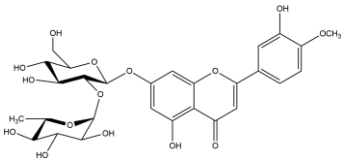
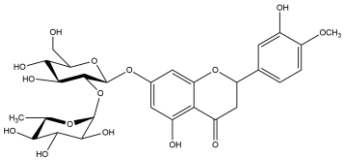


Figure 4. Representative Chromatogram of BJe. Reverse phase high performance liquid chromatograph coupled with a diode array detector (RP-HPLC-DAD) separation of flavonoids found in the lyophilized powder of BJe recorded at 280 nm and 325 nm. Peaks 1–6 correspond to flavonoids, expressed in milligrams (mg) per milliliter (mL) of liquid extract, are the following: vicenin-2 (1), neoeriocitrin (2), rhoifolin (3), naringin (4), neodiosmin (5) and neohesperidin (6). Peak identification was carried out by comparing retention time, UV spectra and spiking the samples with pure reference compounds.

Table 3. Chemical composition of BJe and structural formulas of flavonoids found in the extract.

Peak	Compound	mg/mL (liquid extract)	
		Mean	SD
1	 **Vicenin-2**	< 0.1	-
2	 **Neoeriocitrin**	0.11	0.011
3	 **Rhoifolin**	< 0.1	-
4	 **Naringin**	0.44	0.017
5	 **Neodiosmin**	< 0.1	-
6	 **Neohesperidin**	0.59	0.037

4.2. Cytotoxic activity of BJe in THP-1 cells

4.2.1. BJe decreased cell growth of THP-1 Cells

The effects on cell proliferation of BJe were determined by the MTT test (Figure 5). As shown in Figure 5, BJe was able to hamper THP-1 cell proliferation after 24, 48 and 72 h of treatment. In particular, after 24 h, only the highest concentration of BJe utilized (5 mg/mL) induced a significant decrease of cell viability (-48.4 ± 4.6%; $p < 0.0001$ *vs*. CTRL). Rather, despite to different extent, both 2.5 and 5 mg/mL of the extract significantly reduced THP-1 cell proliferation, after 48 h (-22 ± 4%, $p<0.001$ and -71.7 ± 5%, $p<0.0001$, *vs* CTRL respectively) and the latter time point (-39 ± 3%, $p<0.0001$ and -82.8 ± 5%, $p<0.0001$, *vs* CTRL respectively) of incubation. The IC50s extrapolated from the curves were 3.75 ± 0.4 mg/mL and 2.92 ± 0.32 mg/mL after 48 and 72 h of treatment, respectively. After 24, 48 and 72 h, the 1 mg/mL of BJe did not significantly lessen cell viability.

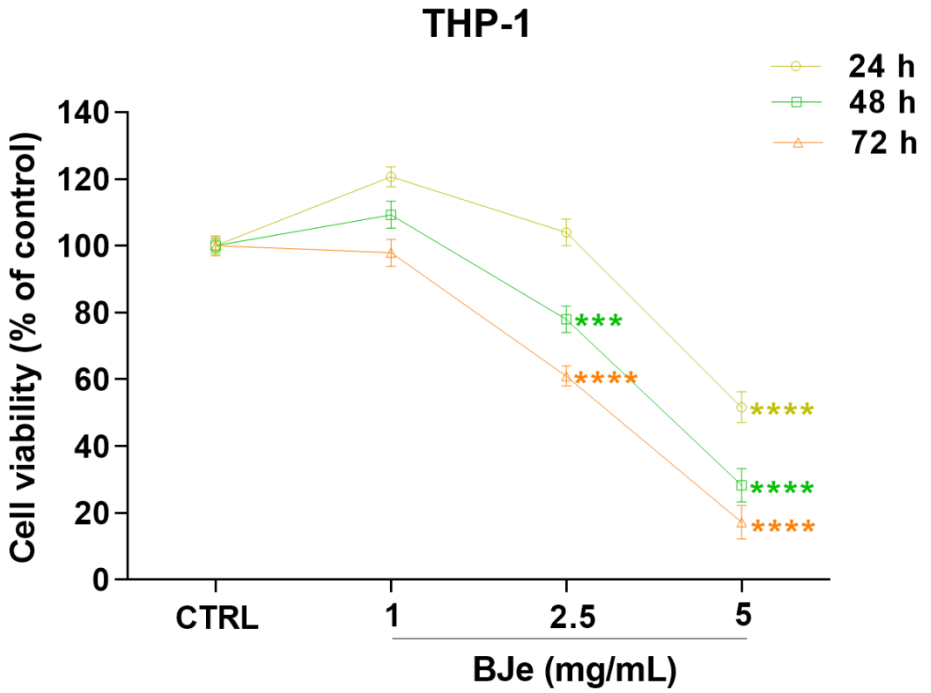


Figure 5. Effect on cell growth of BJe in THP-1 cells. Cells were treated with BJe (1–5 mg/mL) for 24, 48 and 72 h. Cell proliferation was determined by MTT assay. Data are reported as percentages ± SEM of absorbance compared to values detected in the untreated cultures (control, CTRL). Three independent experiments were performed by testing each concentration eight-fold (N= 24). *** $p < 0.001$ and **** $p < 0.0001$ *vs* CTRL.

4.2.2. BJe induced cytotoxic effect in THP-1 Cells

With the aim of evaluating whether the anti-proliferative activity of BJe observed in THP-1 cells was due to a potential cytotoxic effect, we performed PI staining. As presented in Figure 6, after 24 h, 5 mg/mL of the extract provoked a cytotoxic effect in THP-1 cells (48.6 ± 2%, $p<0.0001$ *vs* CTRL), while the 2.5 mg/mL concentration induced a very slight cell death (21.2 ± 3.1%, $p<0.001$ *vs* CTRL) only after 48 h. The concentration of 1 mg/mL did not exert any cytotoxicity in THP-1 cells, supporting the outcome obtained by MTT test.

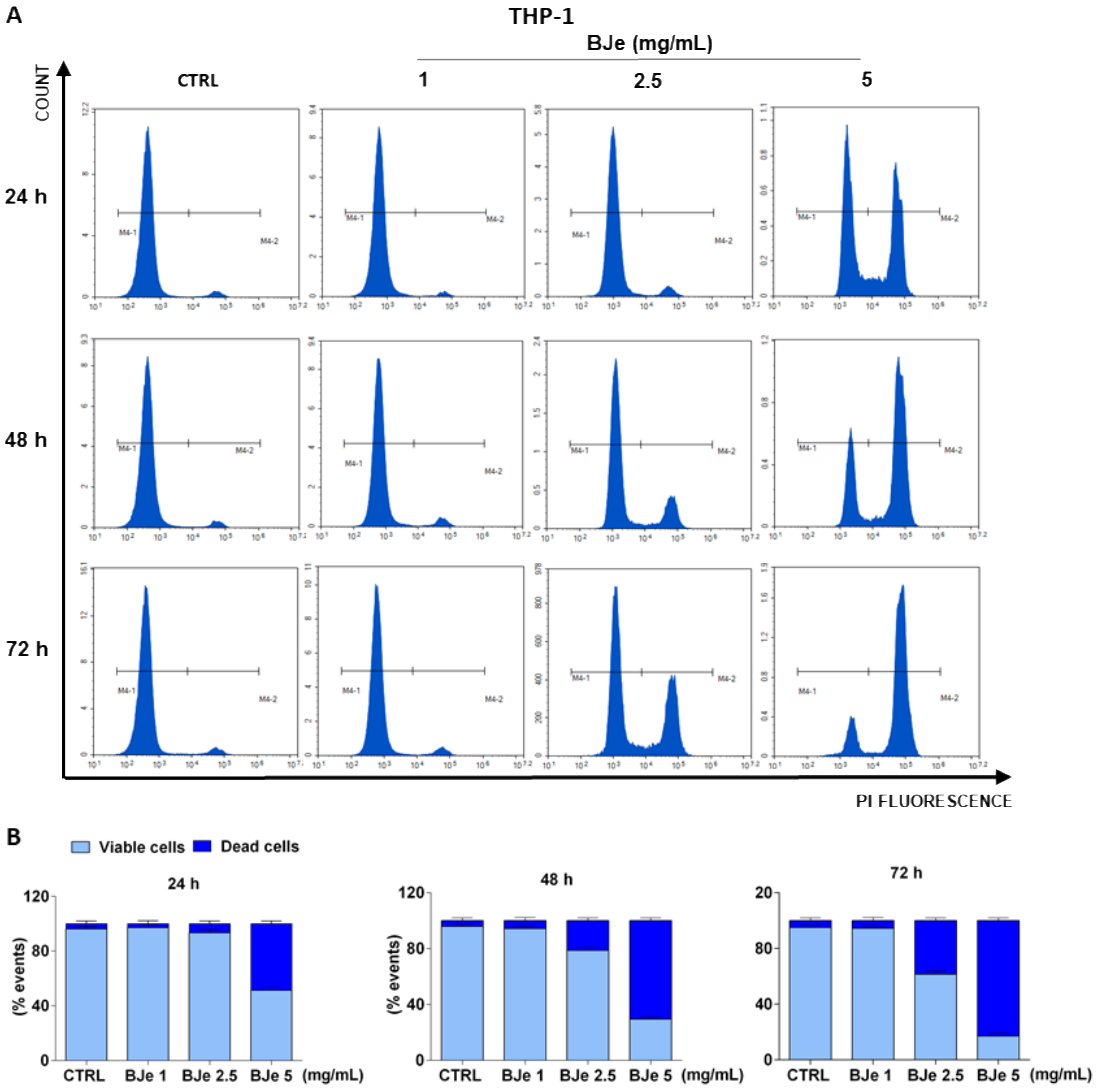


Figure 6. Cytotoxic effects of BJe in THP-1 cells. The cytotoxic activity of BJe (1-5 mg/mL) for 24, 48 and 72 h in THP-1 cells was evaluated by PI staining through flow cytometry. Representative plots of three different experiments performed in triplicate (N = 9) are displayed in A, where the first peak represents viable cells, while the second one, dead cells. In B, histograms show percentages of both viable and dead cells ± SEM of three different experiments carried out in triplicate (N = 9).

4.3. BJe altered cell cycle progression in THP-1 cells

We evaluated whether the anti-proliferative activity of BJe may be correlated to its capacity to interfere with cell cycle progression in THP-1, through PI staining. As shown in Figure 7 the incubation with 2.5 and 5 mg/mL of BJe altered the ratio among G0/G1, S and G2/M phases, indeed it augmented the cell population in S-phase (up to 38.3 ± 3% and 31.2 ± 2.8% vs CTRL after 72 h, respectively), while decreased the number of those in the G0/G1 phase (down to 29.7± 1.9% and 41.7± 2% vs CTRL after 72 h, respectively), together with those in G2/M phase (down to 9.4 ± 2% and 7.7 ± 2.2 % *vs* CTRL after 72 h, respectively).

Although exposure to 5 mg/mL of BJe perturbed cell cycle distribution already after 24 h, BJe 2.5 mg/mL influenced cell cycle only after 48 h, whose S-phase cell cycle arrest was more pronounced at longer times. Treatment with 1 mg/mL of BJe did not impair cell cycle progression in THP-1 cells.

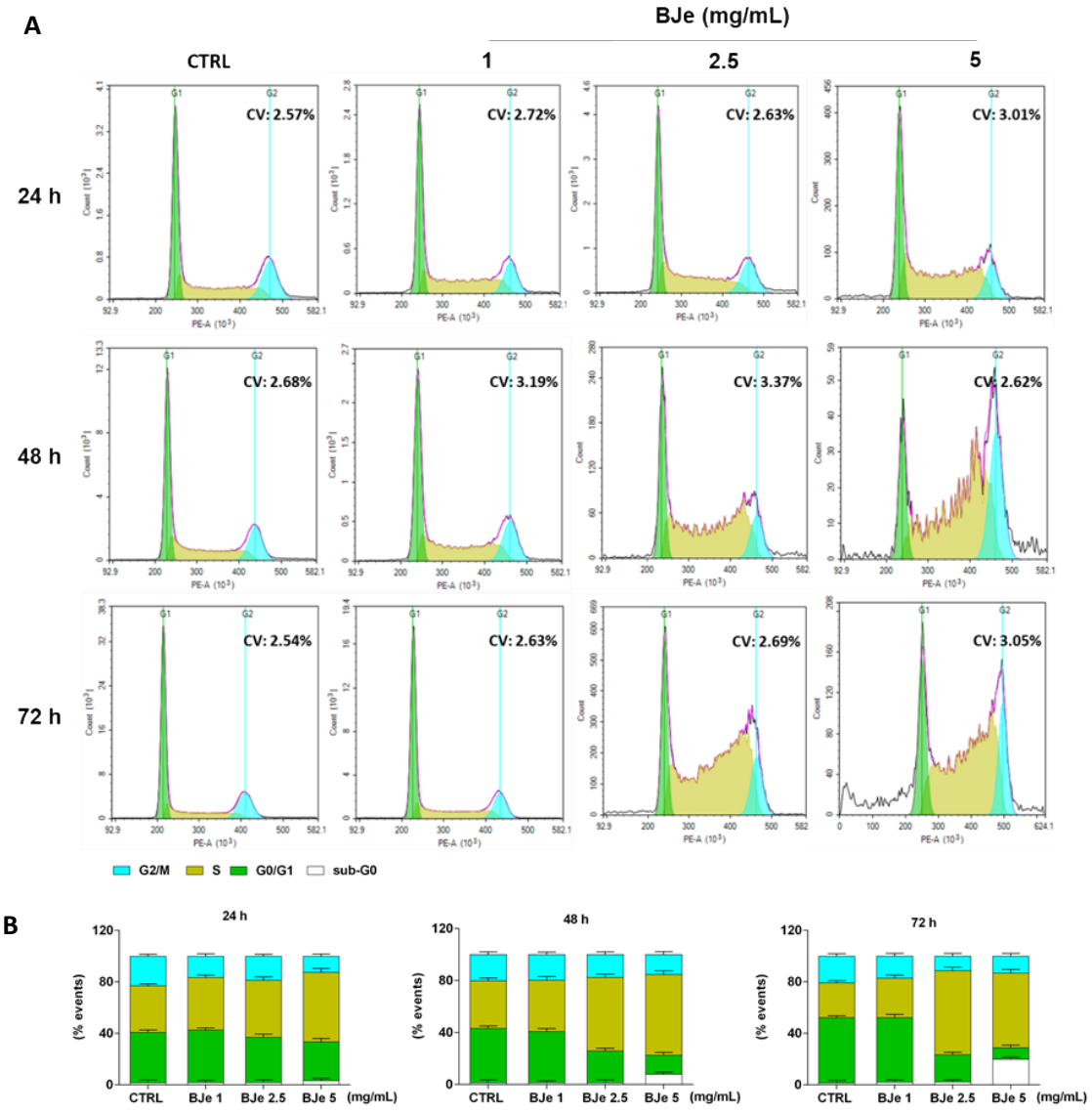


Figure 7. Influence of BJe on cell cycle progression of THP-1 cells. The effects of the treatment for 24, 48 and 72 h with BJe (1–5 mg/mL) on cell cycle distribution of THP-1 cells were assessed by cytofluorimetric analysis. The displayed plots (A) are representative of three independent experiments carried out in triplicate (N = 9). The histograms (B) represent the percentage of events in sub G0 (white bar), G0/G1 (green bar), S (dark yellow bar), and G2/M (cyan bar) phases and are the mean ± SEM of three different experiments.

4.4. BJe promoted apoptosis in THP-1 cells

In order to determine whether apoptosis was involved in the cell death induced by BJe, Annexin V-FITC/PI cytofluorimetric assay was carried out. The treatment with BJe at 2.5 and 5 mg/mL augmented the percentage of cells undergoing apoptosis, both early and late, up to 11.6 ± 2.1% and 47.8 ± 2.3% (24 h) and up to 17.5 ± 2.1% and 64.4 ± 2.2 (48 h), respectively (Figure 8). In addition, treatment with 2.5 and 5 mg/mL of BJe induced apoptosis up to 17.5 ± 2.1% and 64.4 ± 2.2% after 72 h, respectively. Contrariwise, the 1 mg/mL concentration of BJe did not induce cell death after 24, 48 and 72 h, thus supporting results from cell viability tests.

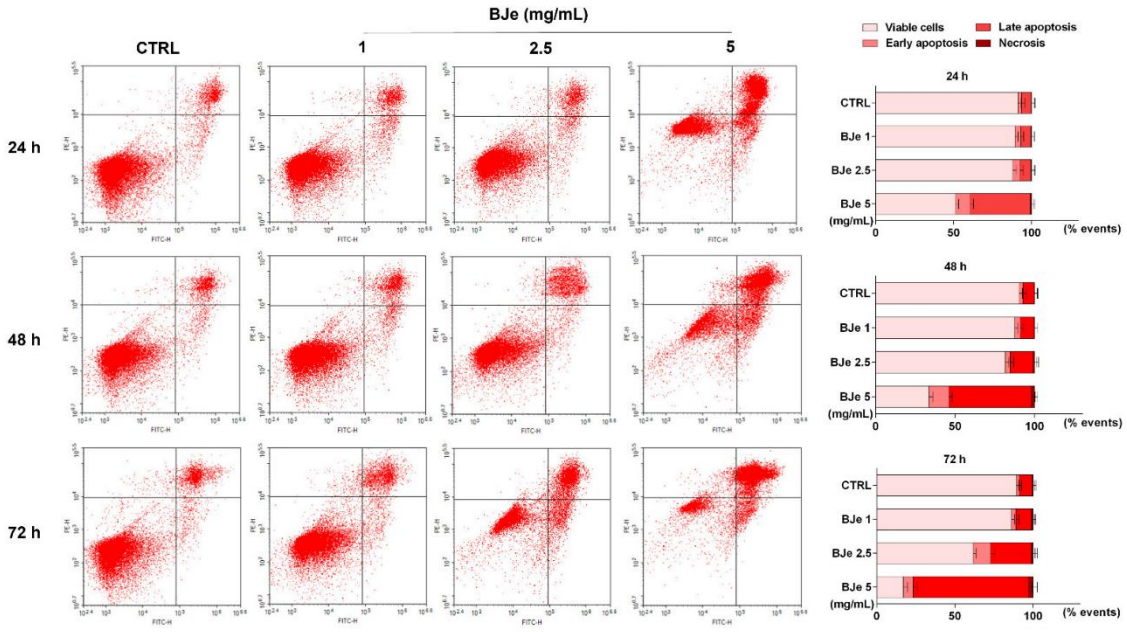


Figure 8. Flow Cytometric assessment of apoptosis in THP-1 cells exposed to BJe. Annexin V-FITC/PI assay was performed to evaluate apoptosis triggered after BJe exposure. Representative Annexin V *vs* PI dot plots of THP-1 cells treated with BJe at different concentrations (1-5 mg/mL), for 24, 48 and 72 h, are shown on the left. Lower left quadrant (Q3) contains the viable cells (Annexin V-/PI-), Lower right one (Q4) comprehends the cells in early apoptosis (Annexin

V+/PI-), upper right one (Q2) presents the cells in late apoptosis (Annexin V+/PI+), while upper left one (Q1) contains the necrotic ones (Annexin V-/PI+). On the right, histograms show the percentages of cells for each quadrant ± SEM of three experiments independently performed in triplicate (N = 9).

4.5. Effect of BJe on apoptotic pathways

In order to investigate the pathways involved in the pro-apoptotic effect of BJe in THP-1 cells, observed after Annexin V-FITC/PI staining assay, we evaluated both gene and protein levels of key factors linked to apoptosis. As shown in Figure 9A, treatment with BJe at 2.5 and 5 mg/mL for 12 h brought an increase of gene expression of the pro-apoptotic p53 by 1.2 ± 0.04-fold and 1.48 ± 0.06-fold ($p<0.05$ and $p<0.001$ vs CTRL), respectively, BAX by 1.3 ± 0.06-fold and 1.5 ± 0.05-fold ($p<0.01$ and $p<0.001$ vs CTRL), as well as a decrease of Bcl-2. CASP8 mRNA levels increased by 1.38 ± 0.06-fold and 1.5 ± 0.05-fold ($p<0.01$ and $p<0.001$ vs CTRL), after treatment with 2.5 and 5 mg/mL for 12 h of BJe, respectively. In addition, both concentrations significantly augmented gene expression of CASP9 by 1.48 ± 0.06-fold and 1.55 ± 0.08 (for both $p<0.001$ vs CTRL), respectively, while CASP3 mRNA levels were increased by 1.4 ± 0.06-fold and 2.06 ± 0.05-fold ($p<0.01$ and $p<0.0001$ vs CTRL), respectively.

These results reflected also at protein level, as determined by Western blotting analysis (Figure 9B). Indeed, the treatment of THP-1 with both concentrations of BJe for 24 h showed a significant increase of p53 and BAX and a decrease of Bcl-2 protein levels and induced the cleavage of both caspase-9 and -8 proteins, initiators of intrinsic and extrinsic apoptotic pathway, respectively. In particular, 2.5 mg/mL and 5 mg/mL of

BJe provoked an increase of cleaved caspase-9 of 1.58 ± 0.08-fold and 1.37 ± 0.07-fold ($p<0.001$ and $p<0.01$ vs CTRL), as well as cleaved caspase-8 of 13 ± 0.29-fold and 14.8 ± 0.29-fold ($p<0.0001$ vs CTRL). We also observed the cleavage of caspase-3 after 2.5 mg/mL (2.2 ± 0.19-fold, $p<0.01$ vs CTRL) and 5 mg/mL (5.05 ± 0.25-fold, $p<0.0001$ vs CTRL), which subsequently induced the cleavage of PARP, a well-known downstream caspase-3 effector, by 13.8 ± 0.22-fold for BJe 2.5 mg/mL ($p<0.0001$) and 26.8 ± 0.45-fold for BJe 5 mg/mL ($p<0.0001$ vs CTRL; Figure 9B).

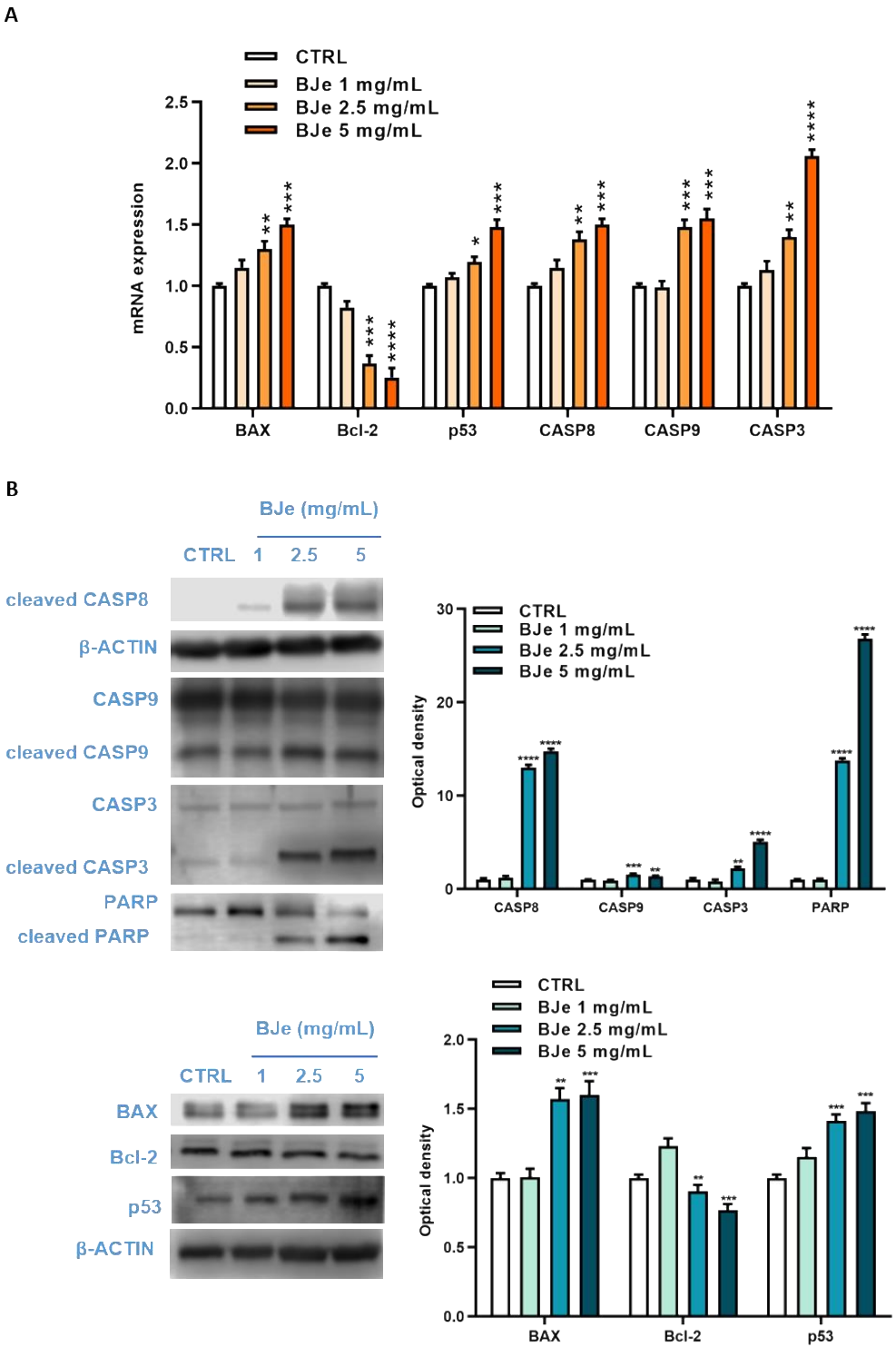


Figure 9. Apoptosis-related gene and protein levels modulation in THP-1 cells treated with BJe. Cells treated with diverse concentrations (1-5 mg/mL) of BJe for 12 and 24 h were processed for mRNA (A) and protein (B) expression studies. Relative quantities of mRNA were determined by the $2^{-\Delta\Delta CT}$ method and were normalized against β-actin. Results are reported as fold change vs unexposed cells (A). In (B) immunoblots of corresponding proteins are displayed

along with their densitometric assays, on the right. The expression of p53, BAX, CASP 8 and Bcl-2 was normalized to β-actin, while that of CASP3, CASP9 and PARP was reported as ratio of the cleaved form with respect to its zymogen. Results are reported as fold change respect to control (untreated) cells and expressed as mean ± SEM of three independent experiments (N = 3), while performed in triplicate for RT-PCR (N = 9). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 *vs* control (CTRL).

4.6. BJe promoted cell differentiation in THP-1 Cells

4.6.1. Effect of BJe on NBT-reducing activity

When myeloid cells undergo maturation, a respiratory burst occurs following activation by PMA, therefore being able to reduce the NBT salt into blue insoluble formazan deposits. This phenomenon is considered as an early sign of differentiation; however, it has been shown that a higher number of immature cells could, with a lesser extent, partly reduce NBT salt, thus hiding the high absorption produced by a fewer number of differentiated cells, since they stopped proliferating as part of their maturation process. For this reason, we coupled a viability cell assessment test, such as MTT assay, with the NBT one, hence using the NBT/MTT ratio to esteem differentiation induction in THP-1 cells exposed to BJe, normalized to their viability.

As shown in Figure 10, the treatment with BJe increased NBT/MTT ratio in THP-1 cells after 72 and 96 h. The highest concentration used (2.5 mg/mL) yielded significant results already after 72 h (1.49 ± 0.009-fold-increase, $p < 0.0001$ *vs* CTRL) with a more pronounced effect after 96 h of treatment (1.86 ± 0.03-fold-increase, $p < 0.0001$ *vs* CTRL). Interestingly,

treatment with 2.5 mg/mL of BJe, for indicated time points, strongly hampered cell growth, thus suggesting that most of the cells that survived after treatment with 2.5 mg/mL of BJe were mature. Nonetheless, exposure to 1 mg/mL of BJe increased NBT/MTT ratio only after 96h (1.29 ± 0.099-fold-increase, $p<0.01$ vs. CTRL). As expected, treatment with 10 nM of PMA significantly augmented NBT/MTT ratio (1.54 ±0.03-fold increase, $p < 0.0001$ vs CTRL after 72 h and 2.03 ± 0.03-fold increase, $p < 0.0001$ *vs* CTRL after 96 h).

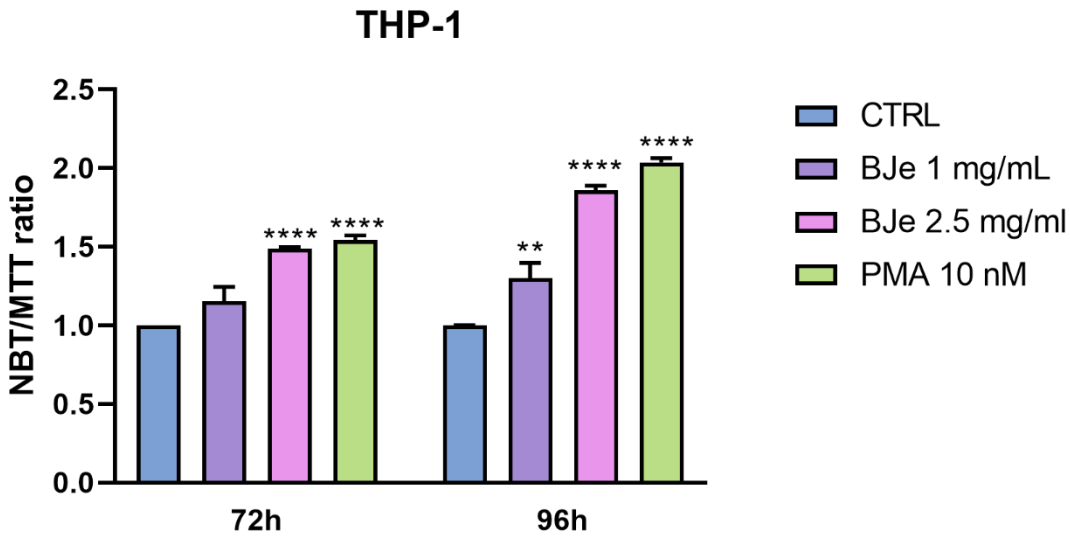


Figure 10. NBT/MTT ratio in THP-1 cells, 72 h or 96 h following exposure to BJe. Cells were treated with 1 and 2.5 mg/mL of BJe for 72 and 96 h. Cell differentiation was determined by NBT assay. Data are reported as mean ± SEM of the ratio between NBT reduction and MTT activity, and compared to untreated cells (control, CTRL). Three independent experiments were performed by testing each concentration eight-fold (N = 24). ** $p < 0.01$ and **** $p < 0.0001$ *vs* CTRL.

4.6.2. BJe induced morphological changes and cell adherence in THP-1 cells

THP-1 cells treated with BJe were examined by light microscopy to observe cell morphology (Figure 11). THP-1 cells were seeded in 100 mm dish at a density of 1×10^6 cells per dish. After overnight incubation, cells were treated with BJe up to 2.5 mg/mL or with 10 nM PMA, and observed after 24, 48, 72 h and 96 h.

During lower treatment times (24–48 h) BJe treated and unexposed cells presented similar morphology (smooth cell surface and round shape; data not shown). Instead, after longer exposure time, with a more pronounced effect after 96 h of treatment, BJe (2.5 mg/mL) and PMA induced the morphologic changes of THP-1 cells into macrophage-like cells, with different cell shapes and ability to adhere to the surface of Petri dishes, respect to untreated cells. It is interesting to note that after 96 h the 1 mg/mL concentration brought an early sign of differentiation, since some cells started to change their shape (Figure 11).

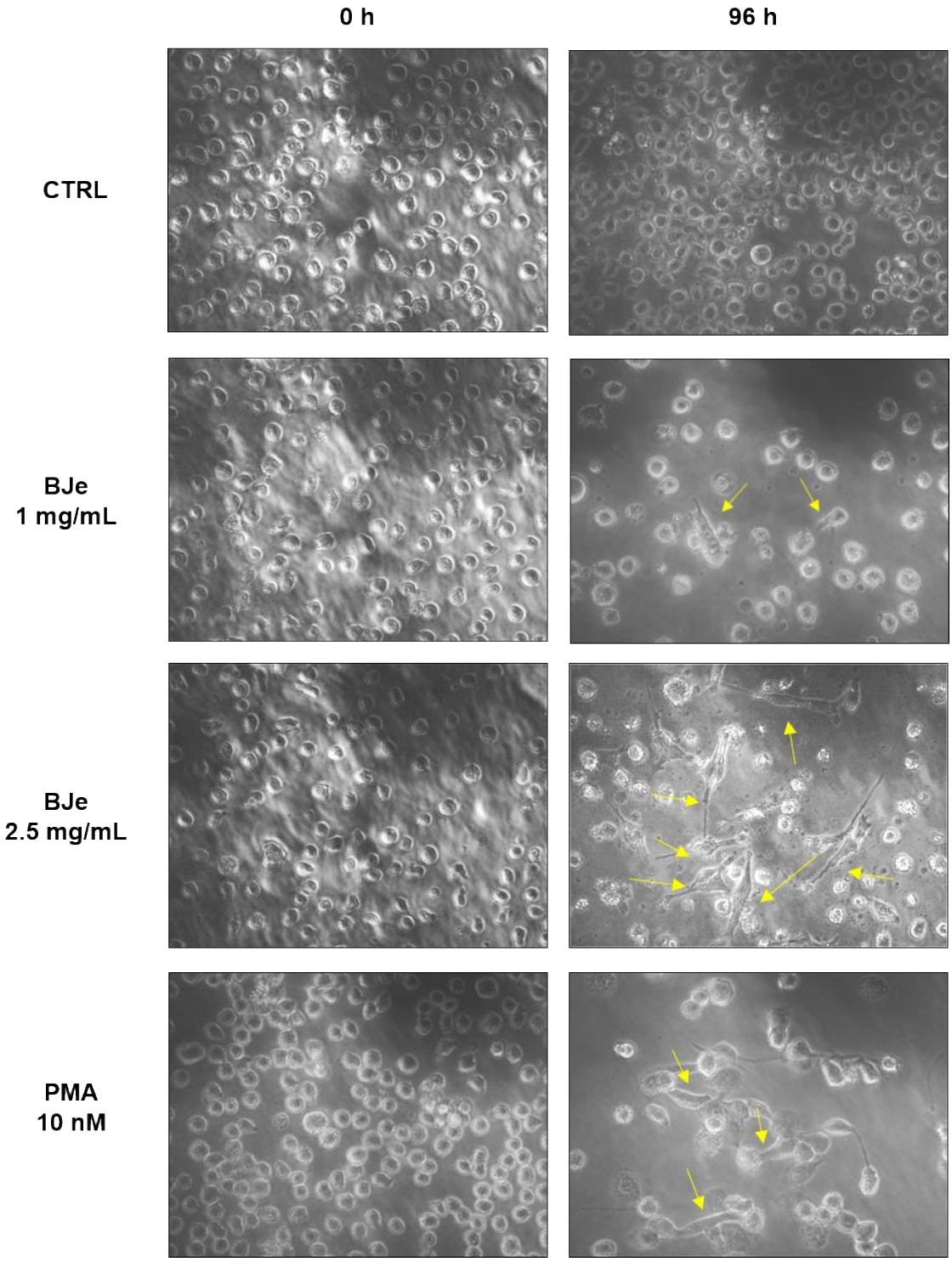


Figure 11. Influence on morphological changes of THP-1 cells by BJe. Cells were treated up to 96 h with BJe up to 2.5 mg/mL or with 10 nM of PMA. In comparison with untreated monocytes, differentiated macrophage-like cells tend to adhere to the surface of the cultivation plates, as indicated with yellow arrows. Cells were observed by light microscopy (magnification 40×). Photos are representative of three independent experiments

4.6.3. BJe increased levels of cell surface differentiation antigens

In order to further confirm THP-1 cell differentiation induced by BJe, we detected cell surface markers of differentiation through flow cytometry. To this aim, we determined the expression of monocytic maturation marker CD14, myeloid differentiation marker CD11b and monocyte to-macrophage differentiation marker CD68. As displayed in Figure 12, after 96 h, both 1 and 2.5 mg/mL of BJe significantly increased the expression of CD68 (11.29 ± 0.0002%, $p<0.0001$ *vs* CTRL and 24.79± 0.002%, $p<0.0001$ *vs* CTRL, respectively) and CD11b (8.69 ± 0.03%, $p<0.0001$ *vs* CTRL and 51.21 ± 0.02%, $p<0.0001$ *vs* CTRL, respectively). Exposure to 2.5 mg/mL of BJe for 96 h significantly increased also CD14 levels (19.17 ± 0.05%, $p<0.0001$ *vs* CTRL), while treatment with 1 mg/mL of BJe did not produce any significant effect. As expected, 10 nM of PMA significantly enhanced CD68, CD11b and CD14 surface antigen expression (42.18 ± 0.002%, $p<0.0001$ *vs* CTRL; 22.53 ± 0.02%, $p<0.0001$ *vs* CTRL; 39.19 ± 0.05%, $p<0.0001$ *vs* CTRL, respectively).

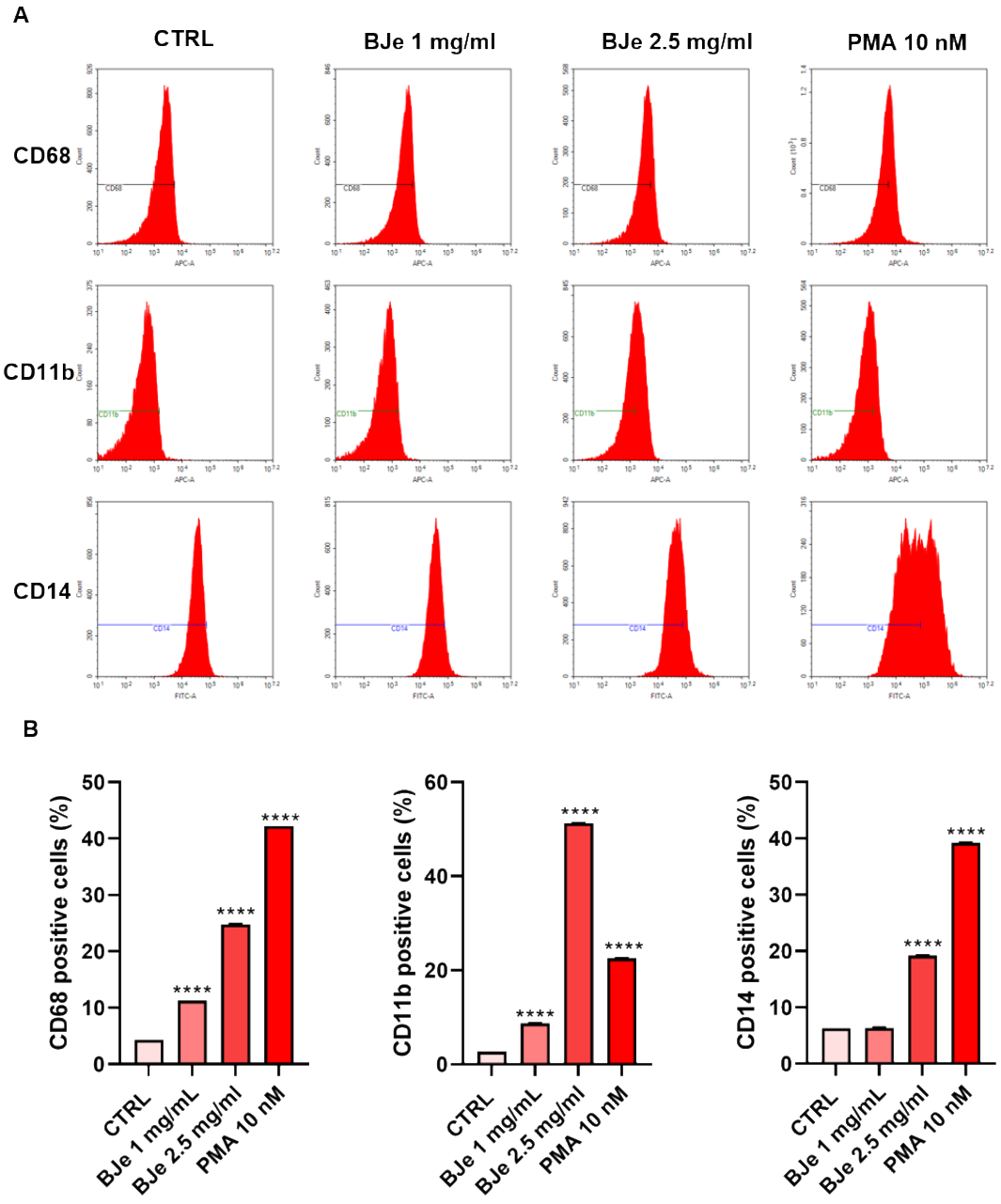


Figure 12. BJe promotes THP-1 cell differentiation by enhancing cell surface antigen expression of differentiation markers. (A) THP-1 cells were treated with BJe (1- 2.5 mg/mL) for 96 h and CD11b, CD14 and CD68 expression were analyzed by flow cytometry. (B) Percentages of $CD11b^{+}$, $CD14^{+}$ and $CD68^{+}$ cells in (A) are expressed as the mean ± SEM of three independent experiments ****$p < 0.0001$ *vs* CTRL.

4.7. BJe induced autophagy in THP-1 cells

Since induction of autophagy is essential for monocyte differentiation to macrophage (Zhang et al., 2012), and the induction of autophagy represents a key component of an effective differentiation-inducing treatment in myeloid leukemia cells (Benjamin et al., 2022), we investigated whether BJe was able to modulate autophagy-related proteins in THP-1 cells. Levels of Beclin-1 (also known as ATG6), a protein involved in the early stage of the autophagic process, were significantly changed after treatment with both 1 and 2.5 mg/mL of BJe for 48 h (Figure 13) by by 1.85 ± 0.28-fold and 2.14 ± 0.18-fold ($p<0.05$ and $p<0.01$ vs. CTRL, respectively). Treatment with 0.5 mg/mL BJe did not induce any significant increase in Beclin-1 levels.

LC3 (microtubule-associated protein light chain 3) protein is a component of the autophagosome; its cytosolic soluble form (LC3-I) is processed, lipidated, and recruited to the autophagosome membrane (LC3-II) during the final stages of autophagy. Immunoblotting analyses, using an antibody that recognizes both forms of the protein, revealed a clear increase in expression levels of both LC3 I and II after BJe exposure (Figure 13). In particular, exposure to 1 and 2.5 mg/mL of BJe significantly increased LC3-I levels by 4.11 ± 0.29-fold ($p<0.001$, vs. CTRL) and 3.70 ± 0.75-fold ($p<0.01$, vs. CTRL), respectively. Levels of LC3-II were augmented of 2.05 ± 0.34-fold ($p<0.05$, vs. CTRL) and 2.16 ± 0.41-fold ($p<0.05$, vs. CTRL) after treatment for 48 h with 1 and 2.5 mg/L, respectively. The 0.5 mg/mL concentration did not exert any significant change. To confirm activation of the autophagic process, we also assessed levels of AKT, a kinase

inhibitor of the autophagic process. Western blotting analysis showed that treatment with BJe decreased the phosphorylation levels of AKT after 48 h of incubation, while total AKT protein levels remained constant. This occurred with both 1 and 2.5 mg/mL concentrations, where p-AKT levels were lessened of 1.47 ± 0.08-fold and of 2.1 ± 0.06-fold ($p < 0.05$ and $p < 0.01$ vs. CTRL), respectively.

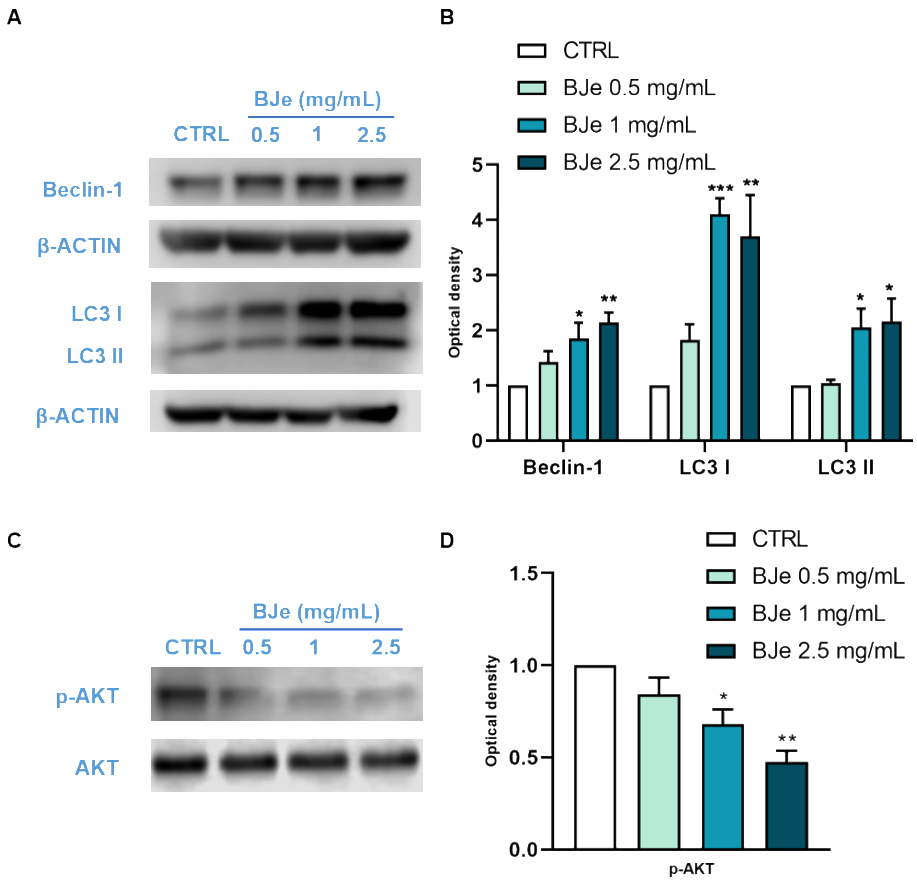


Figure 13. Autophagy-related protein levels modulation in THP-1 cells treated with BJe. Cells were exposed to BJe at 0.5-2.5 mg/mL for 48 h prior being processed for protein expression studies by Western blotting. Representative immunoblots of three independent experiments are displayed together with their densitometric assays, reported on the right. The expression of Beclin-1, LC3 I and LC3 II was normalized to β-actin (A-B), while levels of pAKT were expressed as a ratio of phosphorylated form over total protein (p-AKT/AKT) (C-D). Results are extrapolated as fold change compared to untreated

cells and expressed as mean ± SEM, reporting the values gained in at least three diverse sets of experiments (N = 3 for Beclin-1 and p-AKT; N = 4 for LC3 I-II). *p<0.05, **p<0.01 and ***p<0.001 *vs* control (CTRL).

4.8. BJe triggered MAPKs phosphorylation in THP-1 cells

Recent studies revealed that MAPKs are able to modulate autophagy and consequently induce differentiation in leukemic cells (Mandic et al., 2022), therefore, we studied the effect of BJe on protein expression of phospho-JNK, phospho-ERK1/2 and phospho-p38.

As displayed in Figure 14, treatment with 0.5, 1 and 2.5 mg/mL of BJe for 48 h enhanced the levels of phospho-ERK of 3.30 ± 0.4-fold, 3.87 ± 0.52-fold and 7.52 ± 0.57-fold (p<0.05, p<0.01 and p<0.0001, vs. CTRL respectively) respect to the untreated cells. Treatment with 0.5 mg/mL of BJe significantly increased the levels of phospho-JNK of 2.73 ± 0.18-fold, (p<0.01 vs. CTRL), while 1 mg/mL of 2.98 ± 0.27-fold (p<0.001, vs. CTRL) and 2.5 mg/mL of 3.94 ± 0.31-fold (p<0.0001, vs. CTRL). With regard to phospho-p38 levels, we observed a significant increase in its protein levels with both 1 and 2.5 mg/mL concentrations of BJe utilized by 2.39 ± 0.28-fold (p<0.01 vs. CTRL) and 2.34 ± 0.25-fold (p<0.01 vs. CTRL), respectively mL(Figure 14).

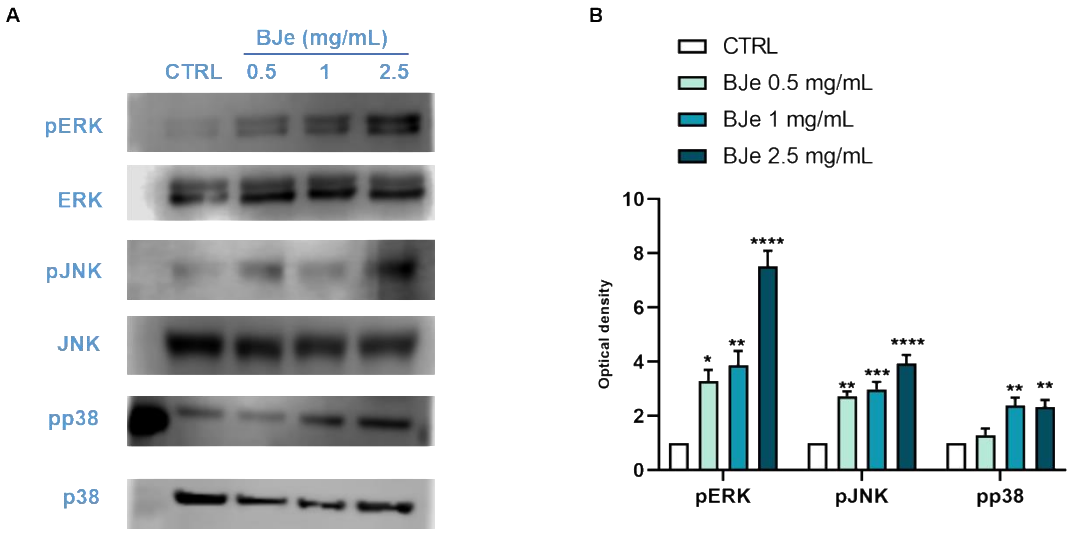


Figure 14. Effects of BJe on MAPKs protein expression in THP-1 cells. Cells were treated with BJe at 0.5, 1 and 2.5 mg/mL for 48 h, then were processed and their protein levels were assessed by Western blotting. Immunoblots representative of three different experiments are displayed on the left, while their densitometric analysis is shown on the right. Levels of phosphorylated ERK, JNK and p38 were reported as a ratio of phosphorylated forms with respect to total proteins. Data were extrapolated as fold change compared to untreated cells, which are arbitrarily assigned as 1 and expressed as mean ± SEM, of three independent experiments (N=3). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 *vs* control (CTRL).

5. DISCUSSION

From Hippocrates who enunciated: "Let thy food be thy medicine and thy medicine be thy food" to the German philosopher Ludwig Feuerbach who claimed: "We are what we eat", until present day, the relationship between nutrition and human health has always stimulated the interest of general population and, in particular, of the scientific community. This because natural substances, usually retrieved from the diet, have been shown to play a key role in the prevention and/or treatment of various diseases, including cancer. On this line, *Citrus* fruits (i.e. lemons, oranges, bergamots, tangerines, limes and grapefruits), which are main constituents of the Mediterranean diet, represent a precious source of these valuable compounds (Russo et al., 2021). It is, indeed, thanks to its bioactive molecules that *Citrus* fruits have been considered over the centuries such important allies to counteract or prevent diverse pathologies. Regarding cancer, ever-increasing studies have highlighted their potentiality as anti-tumor agents, thus suggesting *Citrus* derivatives and extracts as co-adjuvants in cancer therapy (Cirmi et al., 2018; Montano et al., 2022). In particular, as mentioned before, these fruits represent one of the main sources of flavonoids which seem to be responsible of their anti-cancer activities. Flavonoids have been indeed shown to counteract different process of tumorigenesis (Abotaleb et al., 2018) that can underlie their antileukemic activities observed both *in vitro* (Russo et al., 2022) and *in vivo* (Yu et al., 2016).

Despite advances in AML therapy in recent decades, the search for novel and safer therapeutic paths is still necessary, as conventional therapy is often associated with various side effects.

On these lines, natural compounds able to target leukemic cells are considered a conceivable help for AML management (Cotoraci et al., 2021). In this context, the *Citrus* flavonoid luteolin was observed to hamper the proliferation of MV4–11 and MOLM-13 AML cells by lessening eIF4E phosphorylation and blocking the cell cycle in G0/G1 phase (Chen et al., 2020). In addition, exposure to diosmetin delayed tumor growth in AML mouse xenografts (Roma et al., 2018), while nobiletin was shown to induce differentiation and to exert antileukemic activities in THP-1 cells by down-regulating mRNA levels of c-KIT gene (Chen et al., 2018).

Amongst *Citrus* fruits, *Citrus bergamia* Risso & Poiteau has been studied for its anti-cancer properties. In particular, the research group coordinated by Prof. Michele Navarra demonstrated that BJ was capable of reducing the growth rate of diverse cancer cell lines through different molecular mechanisms. In human neuroblastoma SH-SY5Y cells, BJ induced cell cycle arrest in G1 phase without inducing apoptosis and provoked a modification in cell morphology together with a loss of adhesive capacity. This latter was associated with an impairment of actin filaments induced by BJ as well as with a decrease of focal adhesion kinase (FAK) expression, that in turn inhibited cell migration (Delle Monache et al., 2013). Conversely, in human hepatocellular carcinoma HepG2 cells, BJ hampered their growth rate by acting on p21, p53, and NF-κB pathways, as well as by activating both extrinsic and intrinsic apoptotic machinery

(Ferlazzo et al., 2016a). In addition, BJ exerted anti-metastatic effect in a spontaneous metastatic neuroblastoma severe combined immunodeficient (SCID) mouse model (Navarra et al., 2014). With the aim of determining which bioactive compounds of BJ were responsible for its biological effects, Visalli et al., (2014) focused on its flavonoid-rich fraction, BJe. In human colorectal carcinoma HT-29 cells, BJe inhibited their proliferation and triggered apoptosis by multiple mechanisms. These include the increase of reactive oxygen species production, which caused a fall of the mitochondrial membrane potential together with oxidative damage to DNA at high concentrations, while the inhibition of MAPKs pathways and the modulation of cell cycle and apoptosis-related proteins occurred at low concentrations (Visalli et al., 2014). *In vivo*, BJe was shown to prevent spontaneous tumorigenesis in Pirc rats (F344/NTac-Apc^{am1137}), a genetic model of colorectal cancer, by mechanisms related to its anti-inflammatory and pro-apoptotic activities (Navarra et al., 2020).

Considering the anti-cancer properties of BJe observed in solid tumors and the current evidence on antileukemic potentiality of flavonoids, one of the aims of my PhD project was to investigate the role of BJe in the field of hematological diseases, such as AML.

The quali-quantitative profile of flavonoids present in the extract identified neohesperidin and naringin among the most representative compounds. Albeit both components were claimed not to be effective in exerting anti-proliferative activities in THP-1 cells at micromolar concentrations (Chen et al., 2003), this did not happen when we utilized the extract. Indeed, the high solubility in water of BJe allowed us to employ high concentrations of

the extract that corresponded to testing those flavonoids in the millimolar range. This may explain, at least in part, our strong results, bearing in mind that, in BJe, there were also other flavonoids, together with neohesperidin and naringin, which contributed to the anti-proliferative effects we witnessed, probably caused by a synergistic interaction. Similar results were also obtained with PI exclusion assay.

Mechanistically, natural compounds were shown to hamper proliferation, migration, and tumor progression by triggering cell cycle blockage and apoptosis or autophagy in leukemic cells (Cotoraci et al., 2021). In this frame, we evaluated which type of cell death BJe induced in THP-1 cells and the mechanisms underlying its antiproliferative effect. Firstly, it was investigated whether the extract was able to interfere with the progression of THP-1 cells during cell cycle through cytofluorimetric analyses. Cell cycle is characterized by checkpoints that are firmly complied by normal cells and in case of genomic alterations or defects, cells may undergo cell cycle arrest (Stewart et al., 2003). In AML, recurrent aberrations during S phase result to an enhanced and accelerated genome replication, that in turn augments cell growth and renders cells susceptible to acquire mutations, therefore restraining the efficacy of chemotherapies (Schnerch et al., 2012). In our study, BJe altered the ratio among the G0/G1, S and G2/M phases respect to controls, causing an accumulation of cell population in S phase, therefore highlighting its influence on cell cycle progression of THP-1 cells. The blockage of the cell cycle was accompanied also by an increase of cells populating sub-G0/G1 phase, characterized by hypodiploidy, and generally undergoing apoptosis.

Therefore, we verified whether our extract was able to trigger apoptosis through Annexin-V/PI staining. Apoptosis is a process finely ordered and orchestrated in normal cells, and, consequently, its impairment represents a well-known hallmark of cancer (Hanahan and Weinberg, 2011). In this experimental model, BJe induced apoptosis in THP-1 cells already at 24 h of treatment. The apoptotic machinery can also be triggered by an irreversible DNA impairment that subsequently provokes the activation of downstream proteins that can regulate this process. Among these proteins, p53 plays an important role in the promotion of apoptosis, since it can activate downstream proteins such as BAX and Bad, which are well-known pro-apoptotic factors, and hampers the anti-apoptotic ones belonging to the Bcl family (i.e., Bcl-2 and Bcl-XL) following DNA damage. These factors cooperate to either induce or hinder the caspase cascade, thus resulting in apoptosis (Reed, 2001). In our study, we observed at both gene and protein level a decrease of Bcl-2, while BAX and p53 increased, as a clear sign of cells undergoing apoptosis. Moreover, we investigated whether apoptosis caused by BJe followed the intrinsic or extrinsic apoptotic pathway, so we evaluated its effects on caspase cascade. The extrinsic machinery is mediated by caspase-8, a cysteine protease that initiate an apoptotic event in response to extracellular stimulations, which are recognized and propagated by specific cell surface receptors (Muzio et al., 1996). The intrinsic, or mitochondrial pathway, involves the initiator caspase-9 and can be induced by different intracellular stimuli. Both pathways determine the cleavage of the downstream executioner proteins, caspase-3 and -7, followed by the cleavage of PARP (Wu et al., 2016).

Notably, our extract affected both receptor and mitochondria-mediated apoptosis, as observed by the augmented cleavage of caspase-8 and -9, respectively. This was witnessed at both gene and protein levels. These caspases subsequently process caspase-3, which in turn cleaves PARP, therefore unleashing apoptosis. Interestingly, activation of both intrinsic and extrinsic apoptotic machinery was also observed in HepG2 cells treated with BJ (Ferlazzo et al., 2016a).

In AML, the normal differentiation machinery is spoiled, and the cells unceasingly proliferate without undergoing terminal apoptosis (de The, 2018). The principle of the differentiation therapy lies on eliminating differentiation blockage, thus forcing cells to decrease their proliferation (Ferrero et al., 1983; Tsiftsoglou et al., 2003). Malignancy could be therefore more easily monitored, since hematopoietic cells possess specific morphological features and have a wide range of surface markers (Sachs, 1996). The success of differentiation therapy evidenced by ATRA regimen is only applicable to one subtype of leukemia (Mi et al., 2012), the M3 subtype of AML (APL) that accounts for 5-10% of AML cases, and it is often associated with the so-called “differentiation syndrome”, characterized by different unfavorable symptoms, such as fever, respiratory distress, weight gain, dyspnea, lower-extremity edema, pericardial or pleural effusions, low blood pressure, and/or acute renal failure (Cardinale et al., 2014; Frankel et al., 1992; Larson and Tallman, 2003), thus decreasing the overall patient’s compliance. Hence, the search for new differentiation inducers is necessary, especially for leukemia types other than APL, such as AML presenting the mixed lineage

leukemia (MLL) gene rearrangements (MLLr), belonging to subtypes M4 and M5 of AML, that are commonly associated with poor prognosis (Gole and Wiesmuller, 2015; Wuchter et al., 2000).

Several studies have highlighted the pro-differentiating properties of flavonoids, including those from *Citrus*, therefore, considering the richness of BJe in flavonoids, we investigated whether it was able to induce differentiation in THP-1 cells, a cell line belonging to the M5 subtype.

A hallmark of differentiation is the ability of maturing cells to undergo respiratory burst in presence of an external “stimulus”. In normal conditions, this occurs when monocytes or granulocytes encounter harmful microorganisms so that they produce reactive oxygen species (ROS) in order to eliminate them. (Johnston et al., 1975; Saito et al., 1988; Sedgwick et al., 1988). *In vitro*, it is instead required an external stimulus, such as bacterial LPS, N-formyl-methionyl-leucyl-phenylalanine or, more commonly PMA, to mimic the presence of pathogens.

Therefore, the resulting superoxide anion production by the oxidative burst enzymes, is capable of reducing the soluble yellow NBT salt into blue insoluble formazan deposits (Choi et al., 2006). Hence, we evaluated whether our extract was able to reduce NBT after stimulation with PMA. However, since it has been shown that immature cells can, with a lesser extent, partly reduce NBT, we coupled a parallel MTT assay in order to normalize our results with the relative number of cells. Considered the long-time exposure points used for this assay, we tested the least cytotoxic concentrations of BJe and observed that only the higher concentration was

able to significantly increase the NBT/MTT ratio in THP-1 cells, with a more pronounced effect at longer exposure.

Our results are in accordance with what was observed on the extracts from various *Citrus* juices, whereas extracts from bergamot, sweet lime, and Shiikuwasha have been shown to possess an NBT reducing activity in APL cells, HL-60 (Kawaii et al., 1999).

Another important feature of maturing blood cells is their ability to express specific antigen markers, called clusters of differentiation (CDs), that correspond to a specific hematopoietic stage on their membrane surface. Interestingly, our extract was able to increase the expression of monocytic maturation marker CD14, myeloid differentiation marker CD11b and monocyte to- macrophage differentiation marker CD68 in THP-1, therefore suggesting macrophage differentiation (Fan and Edgington, 1991; Holness and Simmons, 1993) and strengthening results obtained from NBT assay. Differentiation of leukemic cells, after BJe treatment, was further confirmed by morphological assays, indeed differentiated cells adhered to the surface of Petri dishes and showed macrophage-like shapes becoming flat and presenting long pseudopodia.

Autophagy from Greek ‘auto’ that means oneself and ‘phagy’ that means to eat, firstly coined by Christian de Duve in 1963 (Klionsky, 2007), is a catabolic process involved in the lysosomal degradation of cytoplasmic material, important for the maintenance of cellular homeostasis (Levine and Kroemer, 2008; Mizushima and Komatsu, 2011). It can be divided into three types: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy; however, the latter is the most studied, since it involves

the “self-digestion” of large structure components. During autophagy macromolecular aggregates, proteins and damaged organelles are sequestered in double-membrane bounded vesicles called autophagosomes. These fuse with lysosomes (thus forming autophagolysosomes) for degradation and recycling of the material (Jin and Klionsky, 2014).

Current literature considers autophagy as a double-edged sword in acute leukemias, since, depending upon the cell context and functional status, it can either promote or suppress tumor growth and survival. Therefore, pharmacological approaches aimed to modulate autophagy may represent a new ground for drugs development (Evangelisti et al., 2015). A putative explanation of an autophagy-mediated cell death is that its stimulation in cancer cells, following anticancer treatments, can be responsible of the degradation of large parts of the cytosol and organelles, therefore causing irreparable cellular atrophy and collapse of vital cell functions (Bursch et al., 2000; Hoyer-Hansen et al., 2005; Inbal et al., 2002; Paglin et al., 2001; Wang et al., 2008).

In AML, autophagy plays an ambivalent role in chemotherapies resistance or targeted therapies, depending on the drug utilized (Joffre et al., 2021); however, ever-increasing evidence claimed it a key factor of an effective differentiation-inducing regimen in myeloid leukemia cells (Benjamin et al., 2022). Indeed, several studies asserted that one of the mechanisms responsible of ATRA and/or ATO induced APL cells differentiation is the activation of autophagic machinery (Moosavi and Djavaheri-Mergny, 2019). In particular, in APL cells, ATRA-induced autophagy promotes

granulocytes differentiation, by a mechanism involving, among the others, the disruption of the oncogenic fusion protein PML-RARα (Isakson et al., 2010).

However, as mentioned before, there is still urgent need to find differentiating agents in non-APL cells, such as THP-1, that is why Benjiamin et al. (2022) studied that the combination of ATRA and valproic acid was able to induce autophagy and differentiation not only in APL and ATRA resistant APL cell lines, but also in THP-1 cells. They further confirmed the involvement of autophagy in their combination regimen because shRNA knockdown of the autophagy regulators ATG7 and TFEB impaired both autophagy and differentiation, thus highlighting its importance.

In our study, results from Western blot analysis showed that BJe was capable of modulating some autophagy-related markers in THP-1 cells, such as Beclin-1 and LC3. In particular, treatment with BJe for 48 h increased levels of Beclin-1 (also known as ATG6), a molecule involved in the early stage of the autophagic process. Genetic studies have demonstrated the tumor suppressor role of Beclin-1 protein in a high percentage of breast, ovarian, and prostate cancers; analysis of human tissue samples derived from breast cancer indicate reduced Beclin-1 expression compared with healthy tissue (Aita et al., 1999; Liang et al., 1999; Vega-Rubin-de-Celis, 2019).

Several proteins are involved during the autophagic process, but LC3 is the only one, among those known, to form a stable association with the membranes of autophagosomes. LC3 exists in two forms: LC3-I, which is

present in the cytoplasm, and LC3-II, which arises from the former and it is processed, lipidated, and recruited to the autophagosome membrane to promote its elongation.

Being a component of the autophagosome, LC3 is, indeed, commonly used as a specific marker to monitor autophagy. Therefore, through Western blotting analyses we used an antibody that recognizes both forms of the protein to assess autophagy in our model and, as expected, we observed a clear increase in expression levels of both LC3 I and II, thus highlighting BJe-induced autophagy in THP-1 cells.

In order to further confirm activation of the autophagic process, we also evaluated levels of AKT, a kinase inhibitor of the autophagic process (Karim et al., 2020). Western blotting analysis showed that treatment with BJe decreased the phosphorylation levels of AKT after 48 h of incubation, while total AKT protein levels remained constant.

Recent studies revealed that MAPKs are able to modulate autophagy and consequently induce differentiation in leukemic cells (Mandic et al., 2022). The way MAPKs induce autophagy is complex and still elusive. In addition, similarly to autophagy, the exact role of MAPKs in cancer is still under debate. This because, on one hand MAPKs can be responsible of activation of survival mechanisms, while, on the other one, they can promote tumor cell death through multiple cellular mechanisms (Low and Zhang, 2016). In particular, the cellular response of the MAPKs cascades seems to be influenced by the nature of the stimulus, as well as the duration of the signal (Engelberg, 2004). For instance, Ventura JJ and collaborators (2006) discovered that transient JNK activation sustains cell

survival, whereas prolonged JNK activation can mediate proapoptotic signaling.

It was pointed out by Mandic and co-workers (2022) how MAPKs, autophagy, and differentiation can mutually correlate. More in details, they suggested that MAPKs can regulate PMA-induced macrophage differentiation of HL-60 APL cells by modulating autophagy through TFEB and FOXO1/3 nuclear translocation, as well as dissociation of beclin-1/Bcl2 complex.

For these reasons, we decided to investigate the effect of BJe on protein expression of pJNK, pERK and pp38. In our study, BJe significantly increased protein levels of pERK, pJNK and pp38 in THP-1 cells, in accordance with its pro-autophagic and pro-differentiating properties. It is interesting to note that BJe exerted a different effect on MAPKs in colon cancer cells (Visalli et al., 2014), and this can be explained, at least in part, because the function of MAPKs in tumor may also depend on cell type (Yue and Lopez, 2020). For instance, in colon cancer cells, activation of ERK/MAPK signaling pathway can be associated with an increase in their cell growth (Fang and Richardson, 2005; Shan et al., 2009), while, conversely it may induce cell death in certain leukemia cells, as a result of a terminal differentiation process (Hong et al., 2005; Marchwicka et al., 2014; Yang et al., 2018).

In conclusion, we showed that BJe induced anticancer and pro-differentiating effects in human leukemia monocytic THP-1 cells, causing cell cycle arrest in S phase and triggering both the apoptotic and autophagic machinery, suggesting MAPKs as possible cross-talk between

the two processes and highlighting its potential in the area of differentiation therapy of AML.

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