Grape Polyphenols to Arrest *In Vitro* Proliferation of Human Leukemia Cells: A Systematic Review and Meta-analysis

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Abstract

Leukemia is a heterogeneous group of hemopoietic cancers, which accounts for 2.6% of new cases per year of total cancer incidence worldwide. Grapes and grape-derived products, such as grape juice, are naturally rich in polyphenols, bioactive compounds with antioxidant properties. Certain polyphenols have been proved to alter oxidative balance, both in inducing apoptosis in cancer cells and in preventing cancer development via controlling oxidative stress. To assess the therapeutic potential of grape polyphenols in the treatment of leukemia, a systematic review and meta-analysis of the reported data on leukemia was carried out. Following the PRISMA guide, a literature review of published papers on leukemia and polyphenols from the last 50 years was conducted, and 17 scientific articles published from 2002-2017 were included in the study. Resveratrol 50 μm had the highest growth inhibition effect (67%) followed by quercetin (30%). The results also point to a differential effect of polyphenols based on cell lineage; monocytes- and myelocytic-derived cell lines are the most susceptible, with a mean of 85% and 64% proliferation inhibition, respectively. Moreover, results show that growth inhibition cannot be associated with a molecular effect of polyphenols on the cell cycle arrest.

Keywords: grape polyphenols, leukemia, cell proliferation, meta-analysis

Introduction

Several interesting compounds with applications in the field of healthcare can be found in vine, such as polyphenols. Polyphenols are a large family of secondary metabolites that confer selective advantages against ecological stress and are extensively distributed among fruits and vegetables [1]. Polyphenols are compounds that contain at least one aromatic ring bearing one or more hydroxyl substituents (phenols) in their chemical structure. They can be classified into groups according to the complexity of their chemical structure and molecular weight [2-4].

Polyphenols present in grape berries are mainly concentrated in the skin and seeds and constitute between 28-35% and 60-70%, respectively, of the total phenols that can be extracted from grapes [5]. Regarding their structure, the most common polyphenol families in grapes are phenolic acids (such as coumaric, ellagic, caffeic, or gallic), flavonoids (including apigenin, catechin and epicatechin), flavanols (such as quercetin or kaempferol) and stilbenes (which include resveratrol or pterostilbene) [6, 7]. These substances have proved to be important natural antioxidant and anti-inflammatory products that serve as free radical scavengers. As a result of this, grape berries and grape-derivatives have become important compounds with applications in the healthcare and food industries [8]. Several epidemiological studies have shown that a polyphenol-rich diet prevents the development of neurodegenerative and cardiovascular diseases, cancer, diabetes, allergy, and autoimmune disorders [9-11]. Many studies have been developed to analyze the putative beneficial effects of the consumption of grapes, raisins, grape juice, wine and grape extracts in the progression or prevention of specific human diseases [12-15]. Similarly, purified polyphenols such as quercetin, resveratrol and others have been tested in studies carried out *in vitro* and *in vivo* to assess their therapeutic use in the treatment of cancer and other chronic diseases [16, 17].

The molecular mechanisms by which polyphenols act in the cells are not yet well known. Nevertheless, some studies have shown that the protective and therapeutic effect of polyphenols is carried out through different metabolic, cellular and molecular mechanisms that modulate the gene expression, signaling pathways and epigenetic changes in the cells [18,19]. It has been demonstrated that many polyphenols induce apoptosis specifically in pathological cells [20,21], which has resulted in several studies testing polyphenols from different natural sources as chemotherapeutic agents against cancer [17, 28, 29].

Leukemia is a heterogeneous disease resulting from the transformation of hematopoietic stem or progenitor cells, and it is the 10th most prevalent cause of cancer in the world [1]. In the US, from 2009-2015, the number of new cases of leukemia per year was 14.1 per 100,000 inhabitants, and the number of deaths per 100,000 inhabitants was 6.5 [27]. In Spain, 3,419 people died in 2016 due to leukemia, and the incidence of the disease in 2017 was 5,424 cases. There are different types of leukemia that can be divided into acute or chronic in function of the maturity stage of the malignant cells, and into lymphoid or myeloid in relation to the cell lineage. Based on malignant transformation experienced by cell lineages and their clinical characteristics, the main types of leukemia are acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL) [28, 29]. Chronic types of leukemia present higher incidence in adults; however, acute lymphocytic leukemia has become the leading cause of death in children suffering from cancer [31]. Moreover, acute leukemia patients usually require prolonged periods of hospitalization and their quality of life is limited. In the US, incidence rates of acute leukemia (one of the main causes of leukemia morbidity) equaled 5 cases per 100,000 inhabitants in 2018; in 2035, a total of 315,413 cases are expected [27].

Accordingly, many studies are currently being carried out to identify effective treatments against leukemia. Several studies conducted in recent decades have shown that grape pomace and seed extracts, rich in polyphenols, induce apoptosis and inhibit the growth of leukemia cells [31, 32]. However, the natural polyphenol more frequently used in studies with leukemia cells is resveratrol [33]. There is evidence that resveratrol induces apoptosis in the four main types of leukemia (AML, CML, ALL and CLL), but other mechanisms, such as autophagy induction, cell cycle arrest or growth inhibition, are also included in the molecular mechanisms induced by resveratrol in specific cell lines [23, 34-37]. However, to date, no studies have been carried out to integrate all these results and evaluate the putative therapeutic effect of grape polyphenols, as well as their potential use as adjuvants in the treatment of leukemia patients. This could be of great interest for the nutraceutical and food industries, given that grape-derived products have numerous applications in dietary foods and supplements, especially in infant and sports products.

Thus, the aim of this study is to evaluate, based on the available literature, whether grape polyphenols have the same antiproliferative effect on leukemia tumor lines regardless of cell lineage and the type of polyphenol. For this purpose, a systematic review and meta-analysis of research published from 2002-2017 has been carried out.

Materials and Methods

Literature Search

The literature search was conducted following the guidelines included in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guide (PRISMA) [38-40]. Accordingly, a systematic search was carried out using the following databases: the Cochrane library [41], EMBASE [42], MEDLINE [43], CINAHL [44]. Scientific reports

included in the study were obtained using the following search terms: "(grape) AND (polyphenol) AND (leukemia)".

Study Eligibility Criteria

The following inclusion criteria were used for screening: (1) controlled trials, which refers to reports with control assays in every experiment, (2) studies conducted with leukemia tumor cells or leukemia cell lines, and (3) studies using mixtures of polyphenols from grape extracts or purified polyphenols present in grape berries. Exclusion criteria were: (1) uncontrolled trials and (2) use of polyphenols from sources other than grapes.

Study Selection

Both the titles and abstracts of articles that met the criteria were independently screened by two authors of the present study using Covidence Software (Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia). Following this, two authors acted as reviewers by independently assessing full-text articles of potentially eligible studies. In the case of a lack of consensus between the two reviewers involved in the screening or in the assessment of the full-texts, authors discussed the conflict and reached an agreement. We documented the reasons for the exclusion of the full-text articles we assessed.

Quality Assessment

Studies included in the meta-analysis were carried out with established leukemia cell lines cultured *in vitro*. Cell proliferation was analyzed in the presence or absence of specific polyphenols. In this type of study, it is not possible to analyze bias following

the methodology proposed in the Cochrane manual [45], as it is specific to the evaluation of clinical studies. Nevertheless, all studies included encompass reliable selection criteria and there is no risk of bias present. Furthermore, leukemia cell lines had been described, and reagents used were purchased from commercial companies with certified reliability such as Sigma-Aldrich, JF-Natural Technology Co. or ActiVin.

Data Extraction

Data extraction from the selected studies was performed using the standardized procedure developed by PRISMA [39, 40] including the following information: (1) complete author name, (2) year of publication, (3) polyphenol name, (4) concentration of polyphenol, (5) leukemia cell type, and (6) effect on cell proliferation. To perform the sifting, Covidence [46] software was chosen since it facilitates working in collaboration with Cochrane in order to improve the production and use of systematic reviews for healthcare and well-being.

Statistical Analysis

The purpose of this meta-analysis was to analyze the positive or negative effect of grape polyphenols on the proliferation rate of human leukemia cell lines' growth *in vitro*. Data on the effects caused by each polyphenol in the proliferation rate of the different leukemia cell lines were treated separately. The evaluation of the effect of the different polyphenols on cell proliferation, as well as the graphic representation of results was performed using Review Manager 5.3 [47] software. A 95% confidence interval (CI) was established for all outcomes, including cell proliferation and stage of cell cycle arrest. The two main methods used to compare data of a study with dichotomous variables are: analyzing the risk ratio (RR) or comparing the odds ratio

(OR) [48, 49]. The OR analysis is the recommended method to evaluate dichotomous variables, but as the relative risk was higher than 10% in the present study, RR analysis was used. Under these conditions, the OR analysis overestimates the relative risk because the random frequency of the effect of the evaluated compound is not correctly handled, and false positives or negatives can be added to the study, which leads to a biased final result [48]. The RR analysis [50] was therefore used for risk estimation. Data heterogeneity was measured by the chi-square (χ^2) test and the I^2 statistic. The level of significance was defined as $p<0.10$. The I^2 statistic represents the total variation due to the heterogeneity, and I^2 values higher than 50% indicate significant heterogeneity [50]. A random effect (RE), instead of a fixed effect (FE), was used for the analysis because the effect of polyphenols can change depending on the compound used and leukemia cells treated. The fixed parameters in the study were the concentration of each polyphenol and the leukemia cell lines used.

Results

The flow chart describing the process of selecting publications to be included in the study is presented in Figure 1. Once duplicate results were removed, 132 articles were selected as a result of the search carried out. The Titles and Abstracts of these articles were reviewed by the authors against the previously established eligibility criteria. Accordingly, 74 articles were excluded from the study, as they did not fulfill the criteria. The remaining 58 articles were screened by full text. Overall, 24 studies met the criteria, but only 17 articles, which included quantitative data useful for the statistical analysis, were selected to conduct the meta-analysis [31, 32, 37, 51-64].

[Figure 1 near here]

Study Characteristics

The above-mentioned 17 articles included in the study were thoroughly analyzed to obtain quantitative data regarding the viability of leukemia cells based on the type and concentration of polyphenols added to the culture media. Culture conditions for the different cell types used in the studies were the same: cells were grown at 37ºC in an incubator with 5% CO_2 atmosphere using an RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum, L-glutamine, penicillin and streptomycin. The experimental procedures to determine the effect of polyphenols in cell viability and proliferation were carried out in a very similar way by different researchers. When cultured cells reached a cell density of 10^4-10^6 cells/mL, polyphenols were added to the culture media and incubated for 24-72 hours before measuring cell viability.

Outcomes of Interest

The three outcomes pursued in the present study were:

1. Analyze the effect of specific grape polyphenols in cell proliferation of leukemia cells cultured *in vitro*.
2. Determine whether the effect of grape polyphenols in leukemia cell proliferation depends on the type of leukemia cell line.
3. Determine whether cell cycle arrest is the molecular target of grape polyphenols on leukemia cells.

Table 1 summarizes the included articles and the information extracted to evaluate the three outcomes stated above.

[Table 1 near here]

Polyphenols Analyzed

There was a large variability in the type of polyphenols included in the articles selected for the meta-analysis; 13 pure polyphenolic compounds and grape polyphenol

198 extract (GPE) were analyzed. The data included in the meta-analysis were obtained 48
199 hours after treatment with polyphenols at fixed concentrations: 50 μM resveratrol,
200 apigenin, cyanidin glucoside, delphinidin, ellagic acid, kaempferol, malvidin, myricetin,
201 petunidin and pterostilbene, 25 μM viniferin and vineatrol, 10 μM quercetin and 50/100
202 μg/mL GPE. It must be noted that the origin and polyphenol composition of the extracts
203 used in the studies were different, which is the reason why interventions with GPE are
204 always analyzed in a different subgroup. Espino et al.[32] used Tempranillo grape seed
205 extracts at 6,160.01±160.14 mg/L gallic acid equivalents (GAE); León-González et
206 al.[31] used grape pomace extracts at 365 mg/g GAE; Sharif et al.[62] used red wine dry
207 powder extracts at 471 mg/g GAE; Mertens-Talcott et al.[63] used Muscadine and
208 Sauvignon wine extracts to 10,269±36 and 9,932±22 mg/L GAE, respectively; Wang et
209 al.[64], Gao et al.[54] and Hong et al.[57] used commercial grape seed extracts enriched
210 with proanthocyanidins, but there was no reference to GAE. The viable cells (called
211 "events" in figures 2, 3 and 4) following treatment were measured using different
212 methods. However, direct quantification using flow cytometry was the most common.
213 *Cell Lines Used in the Studies*
214 In relation to leukemia cell lines included in the analyzed studies, four of them
215 originated from early myeloid cells (Em): K562, HL60, AML14.3D10, KCL22; two
216 from monocytes (Mc): U937 and THP1; four from B lymphocytes (LB): WSU-CLL,
217 ESKOL, NALM-6, 232B4, and two from T lymphocytes (LT): MOLT-4 and JURKAT.
218 Moreover, HL60, U937, THP1 and AML14.3D10 are acute promyeloid and monocytic
219 leukemia cells, while K562 and KCL22 are chronic myeloid cell lines. In the
220 lymphocytic lineages, NALM-6, MOLT-4 and JURKAT are models of acute leukemia,
221 and 232B4, WSU-CLL and ESKOL, are chronic leukemia cells.

The number of experiments in the articles selected for meta-analysis that were useful for the data extraction process is shown in Table 2, sorted by type of polyphenol and cell line.

[Table 2 near here]

Outcome 1. The Effect of Specific Grape Polyphenols on the Proliferation of Leukemia Cell Lines

Results obtained through this meta-analysis on the effect of grape polyphenols on cell proliferation are presented in five different subgroups. Three of the subgroups show the effects of resveratrol, quercetin and viniferin. The "others" subgroup clusters the effect of other grape polyphenols (where only the results of one experiment are available for each one), and the other subgroup shows the effect of GPE (Figure 2). It is important to highlight that maximum heterogeneity (I^2) was obtained through statistical analysis in all cases. This could be due to the different leukemia cell lines used at variable cell concentrations across the different experiments and laboratories (Table 2). In this case, risk ratio is employed as an indicator.

[Figure 2 near here]

As a preliminary conclusion, a positive overall effect can be considered regarding the use of these chemical compounds as inhibitors of tumor line growth. This can be seen through the presence of diamond-shaped symbols within every cluster to the left side of the vertical line, showing the absence of a null effect in all cases. Furthermore, following a deeper analysis, we infer that both quercetin and viniferin cause a slight inhibitory effect, particularly when compared to resveratrol. The higher number of studies including the use of resveratrol should be considered in the discussion of these results. Similarly, a significant dispersion caused by the wide range

of cell lines used must also be considered. Upon comparing the results when using pure polyphenols, resveratrol shows a high variability in the inhibition of cell proliferation when different leukemia cells are analyzed. It must be noted that 13 interventions and 10 different cell lines have been assessed in the specific case of resveratrol, as opposed to the 3 interventions and 3 cell lines evaluated for quercetin, and the 2 interventions and 2 cell lines for viniferin. Nevertheless, results of the meta-analysis indicate that resveratrol presents the greatest capacity to inhibit cell proliferation with a mean value of 67% (95% CI, 56%-75%), which is significantly higher than the mean inhibition found when quercetin (30%, 95% CI, 22%-38%) and viniferin (15%, 95%CI, 5%-23%) are used. The subgroup "others", which encompasses results of individual studies, was evaluated using 10 different grape polyphenols (apigenin, myricetrin, delphinidin, kaempferol, petunidin, malvidin, pterostilbene, cyanidin glucoside, vineatrol and ellagic acid) and four leukemia cell lines. In this case, the average growth inhibition percentage is 53% (95% CI, 40%-63%). However, the limitation of this subgroup, which includes data from single experiments carried out with different polyphenols, must be taken into account.

According to the number of studies meta-analyzed, 50 µg/mL GPE was the second most effective treatment. The average growth inhibition is 58% (95% CI, 46%-67%), lower than those obtained with resveratrol (67%, 95% CI, 56%-75%). This is an important point considering the GPE used in the studies have different origins and polyphenol compositions; however, only six different cell lines were analyzed in contrast to the ten lines used with resveratrol. No data coming from B-lymphocytes-derived leukemia cells are available.

The results as a whole show the highest value of growth inhibition is the 67% obtained upon exposure to 50 µM resveratrol, followed by the 53% obtained with the

group of other polyphenols used at the same final concentration. It is true that when leukemia cells are treated with GPE the percentage of growth inhibition is 58%, but in these cases the polyphenol composition and concentration are unknown, so this value can be used only as a reference, and not as an individual comparison.

Outcome 2. The Effect of Grape Polyphenols on the Proliferation of Human Leukemia Cells of Different Origin

Results compiled in relation to outcome 2 refer to the influence of grape polyphenols on the cell proliferation of leukemia cells from different lineages. The 12 cell lines used in the studies included in this meta-analysis can be subdivided into four groups according to their source of origin: monocytes (U937 and THP1), early myeloid cells (HL60, K562, KCL22 and AML14.3D10), B lymphocytes (WSU-CLL, ESKOL, NALM-6 and 232B4) and T lymphocytes (MOLT-4 and JURKAT).

Studies performed with leukemia cell lines from lymphocytic origin represent 71% of the total and, as can be seen in Figure 3A, the average percentage of growth inhibition due to the effect of pure polyphenols is 53% (95% CI, 41%-63%) and 44% (95% CI, 28%-57%), for both T-and B-lymphocytic-derived leukemia cells, respectively, which are lower than the values observed for early myeloid (64%, 95% CI, 49%-75%) and monocyte (85%, 95% CI, 65%-94%) derived-leukemia cells. When cells were treated with pure polyphenols, the overall growth inhibition percentage obtained in this outcome was 56% (95% CI, 47%-64%) (Figure 3A), similar to the 58% (95% CI, 46%-67%) obtained when cells were treated with GPE (Figure 3B).

Both cell lines derived from T lymphocytes were acute lymphoid leukemia models, and it is necessary to highlight the effect of apigenin [53] and resveratrol [37] on JURKAT cells, with growth inhibition percentages of 84% and 82%, respectively, as

297 well as the 92% growth inhibition demonstrated by pterostilbene [63] on MOLT-4 cells
298 (Figure 3A). These data are the only ones misaligned with the trend followed by other
299 polyphenols [53, 58, 61], which ranged between 7% and 42% growth inhibition. The mean
300 effect among all the polyphenols tested on this type of leukemia cells is 53% (95% CI,
301 41%-63%), similar to outcome 2's overall effect (56%, 95% CI, 47%-64%) (Figure 3A).
302 The average growth inhibition increased to 58% (95% CI, 37%-72%) when MOLT-4
303 [60] and JURKAT [31, 37] cells were treated with GPE (Figure 3B).
304 [Figure 3 near here]
305 The subgroup of B-lymphocyte-derived leukemia cells includes data obtained
306 using four different polyphenols, three of which (except quercetin) belong to the
307 stilbene family. Four cell lines were also used, as most of the data was obtained from
308 chronic leukemia models (WSU-CLL, ESKOL, 232B4) and only one from the acute
309 NALM-6 leukemia cells. The average inhibition of proliferation in B-lymphocyte-
310 derived leukemia cells is 44% (95% CI, 28%-57%), indicating that these cells are less
311 sensitive to the effect of grape polyphenols than cell lines derived from T lymphocytes.
312 Importantly, resveratrol shows a 17% growth inhibition on NALM-6 cells [55], while on
313 WSU-CLL, 232B4 and ESKOL cells the percentage of growth inhibition is 46%, 64%
314 and 67%, respectively [31, 51, 56]. It was also noted that viniferin exerts little inhibition on
315 the growth of both ESKOL (19%) and WSU-CLL (10%) cells [53].
316 The subgroup of early myeloid leukemia cells includes five studies carried out
317 with three different cell lines and resveratrol. It is worth highlighting the 88% growth
318 inhibition rate obtained with resveratrol in HL60 cells [37], which is the only acute
319 myelocytic leukemia line included in this subgroup (Figure 3A). The mean inhibitory
320 effect of resveratrol on the growth of these cells is 64% (95%CI, 49%-75%), higher than
321 the 59% (95%CI, 33%-75%) obtained with GPE (Figure 3B). Nevertheless, the three

interventions with GPE were carried out by employing commercial polyphenol extracts of unknown composition, thus making it difficult to draw reliable conclusions.

Finally, the subgroup of monocyte-derived cell lines includes experimental data from a study carried out with resveratrol and two leukemia cell lines [37]. The result of the meta-analysis indicates that these leukemia cells experience an average growth inhibition of 85% (95% CI, 65%-94%), making them the most sensitive to the inhibitory effect of pure grape polyphenols; precisely, to resveratrol (Figure 3A). Nevertheless, only two values are included in this subgroup. The only study carried out on these cells with GPE [54] shows a growth inhibition percentage of 47% (Figure 3B), much lower than the effect of resveratrol.

Outcome 3. The Effect of Grape Polyphenols in Cell Cycle Arrest

There was a large variability of targets and techniques used to assess cell death and the growth inhibition of leukemia cells in the articles included in this meta-analysis. Nevertheless, eight articles were detected that included quantitative data related to cell cycle arrest in G_0 phase after the treatment of leukemia cells with grape polyphenols. Therefore, the third outcome seeks to determine whether grape polyphenols inhibit leukemia cell proliferation by arresting the cell cycle in G_0 phase. To achieve this, data from experiments conducted with four grape polyphenols were used: resveratrol (50 μM, 24 hours), quercetin (10 μM, 48 hours), ellagic acid (10 μM, 48 hours), and pterostilbene (44 μM, 48 hours). Data from five experiments performed using GPE (100 μg/mL, 24 hours) were also included.

The results obtained indicate that grape polyphenols are not directly involved in cell cycle arrest of *in vitro* cultured leukemia cell lines (Figure 4). In the subgroup including treatment with pure polyphenols, the mean effect observed was 1% (95% CI, -

0,7%-8%); thus, it can be concluded that the analyzed polyphenols affect cell viability through a different mechanism than G_0 cell cycle arrest (Figure 4). Four out of six data sets show no effect, and when pterostilbene was added to MOLT-4, the opposite effect to what was expected occurred. Only one intervention carried out with quercetin on MOLT-4 cells showed induction of cell cycle arrest. In addition, quercetin shows a small positive effect on 232B4 cells, while resveratrol shows no effect on cell cycle arrest either on 232B4 or on K562 cells.

The subgroup of the data obtained with GPE showed no effect on cell cycle arrest on neither early myeloid [37, 64] nor on T-lymphocytes [60, 62] derived-leukemia cells (Figure 4). The induction of cell cycle arrest in the G_0 phase was only observed once, in the study carried out with HL60 cells treated with *Tempranillo* grape seed extracts at 6,160±160 mg/L GAE [32]. In contrast, the rest of the studies indicate the opposite effect, stimulating the cell cycle progression.

[Figure 4 near here]

In conclusion, as can be seen in Figure 4, the dispersion of results regarding the influence on the cell cycle is greater with GPE than with pure polyphenols. The subgroup of pure polyphenols shows that 4 out of the 6 observations have a value of almost 1, which implies that polyphenols influence neither the promotion nor the impediment of cell-cycle arrest. In contrast, GPE shows that 3 of the 5 experiments assessed are negative for cell cycle arrest. Moreover, the addition of these extracts does not stop the cell cycle, but rather it encourages it.

Discussion

There are systematic reviews and meta-analysis studies that analyze the effect of polyphenols in the prevention and treatment of some types of cancer, such as breast,

lung, prostatic or cervical cancer [24, 26, 65-68]. However, no studies related to leukemia have been carried out. The majority of the studies support the positive effects of these antioxidant compounds in the treatment of cancer; hence the interest in this study to determine whether grape polyphenols have antiproliferative effects in leukemia. The study has been conducted with experimental data on different human leukemia cell lines cultured *in vitro,* given the lack of data in relevant clinical trials of leukemia patients treated with polyphenols.

17 studies were included in the meta-analysis and the results obtained lead to the conclusion that grape polyphenols have an antiproliferative effect on human leukemia cell lines cultured *in vitro,* although this effect is dependent on the type of polyphenol and cell line studied. It must be noted that results indicate there is a large heterogeneity among the analyzed data, which can be explained by the type of studies evaluated in which the cell concentration changed in each study, and by the variety of polyphenols and cell lines used. Therefore, we consider the inability to perform a risk of bias (because all tools available are not suitable for experimental studies like this) a limitation of this study. On the other hand, despite all heterogeneity found in the metanalyses, we could only explore it by subgrouping studies depending on the concentration of each polyphenol and the leukemia cell lines used.

Outcome 1 indicates a global antiproliferative effect of grape polyphenols on leukemia cells, with an average value of growth inhibition of 57% (95% CI, 49%-63%). Among the pure polyphenols analyzed, resveratrol shows the greatest effect, with a 67% (95%CI, 56%-75%) growth inhibition. This result agrees with previous reports indicating that resveratrol could be a good bioactive compound for the prevention and therapeutic use against different types of cancer [16, 28]. Quercetin and viniferin also show a positive effect, but the mean growth inhibition is 30% (95% CI, 22%-38%) and

15% (95% CI, 5%-23%), respectively. It should be highlighted that the number of studies including resveratrol is much higher than the studies carried out with other polyphenols. However, it is noteworthy that the overall effect of the subgroup that includes other grape polyphenols results in a 53% (95% CI, 40%-63%) inhibition of growth. This subgroup includes data coming from single experiments carried out with different polyphenols on leukemia cell lines of various lineages, so the variability of the effect is high. It is interesting to highlight the effect of apigenin (flavonoid) and pterostilbene (stilbene) on JURKAT and MOLT-4 cells, both acute T-lymphoblastic-leukemia lines, that inhibit cell growth by 84% and 92%, respectively. Apigenin has been proposed as a potential natural anti-cancer agent promoting apoptosis through activation of extrinsic caspase-dependent pathways in cancer cells from different origins [69-71]. Moreover, *in vivo* studies carried out with mouse models of certain cancers showed a potential therapeutic use of apigenin at 25 mg /Kg [70]. Pterostilbene belongs to the stilbene family, of which resveratrol is the main representative, and it has been demonstrated that these compounds interfere with metabolic pathways directly linked to senescence and induction of apoptosis in tumor cells [16, 72-74].

The growth inhibition observed when treating leukemia cells with GPE is 58% (95% CI, 46%-67%), higher than the value observed using other polyphenols (53%, 95% CI, 40%-63%), but lower than the 67% (95% CI, 56%-75%) obtained with 50 µM resveratrol. The interpretation of these data is difficult because the specific polyphenol composition of GPE is unknown. However, these results would support the hypothesis that a combination of natural polyphenols rather than a single compound may be more beneficial and effective in the treatment and prevention of leukemia [8].

The results of the effect of grape polyphenols on leukemia cells grouped by cellular lineage (outcome 2) indicate an antiproliferative effect of these compounds,

422 regardless of the cellular origin. The mean value of growth inhibition is 56% (95% CI,
423 47%-64%) with pure polyphenols and 58% (95% CI, 37%-72%) using GPE.
424 The subgroup of the monocyte-derived cells shows the highest percentage of
425 growth inhibition at 85% (95% CI, 65%-94%), followed by the early myeloid-derived
426 cells at 64% (95% CI, 49%-75%). In all of them, the treatment was carried out with 50
427 µM resveratrol, and the highest inhibitory effect is shown in the acute leukemia cells
428 from monocytic (TPH1, U937) and promyelocytic origin (HL60). The chronic myeloid
429 leukemia cells KCL22 and K562 show more variability and lower inhibition
430 percentages (21%-69%). The few data available with GPE rich in proanthocyanidins
431 show variable percentages of growth inhibition and different behaviors (47% in U937
432 cells and 80% in K562). Nevertheless, there is evidence that demonstrates the
433 antiproliferative effect of proanthocyanidins, either alone or in combination with other
434 polyphenols like resveratrol in colorectal, prostate and breast cancer cells [75-78].
435 The leukemia cells of lymphocytic lineages showed the lowest inhibitory effects.
436 The largest number of studies was carried out with T lymphocyte cell lines and the
437 average growth inhibition was 53% (95% CI, 41%-63%), whilst in the cell lines derived
438 from B lymphocytes a 44% (95% CI, 28%-57%) growth inhibition rate was obtained.
439 This result indicates that B-lymphocyte-derived cells are less sensitive to the
440 antiproliferative effect induced by grape polyphenols. Importantly, there are three
441 polyphenols that produce outstanding values of growth inhibition on T-lymphocyte-
442 derived cells; apigenin on JURKAT and the stilbenes, and resveratrol and pterostilbene
443 on MOLT-4.
444 The results allow us to affirm that the antiproliferative effect of grape polyphenols
445 in leukemia cell lines derived from early myeloid cells and monocytes is higher than in
446 cells derived from T and B lymphocytes. Moreover, resveratrol has a higher effect in the

447 acute myeloid- and monocyte-derived cells than in the chronic cell lines. This is a very
448 important aspect to consider for the development of future *in vitro* and *in vivo*
449 experiments. The molecular differences in the intracellular metabolic and signaling
450 pathways in acute and chronic leukemia cells can be directly involved in the
451 effectiveness of the treatment with resveratrol or other polyphenols.

452 Outcome 3 indicates that polyphenols have heterogeneous effects regarding the
453 cell cycle arrest. Only interventions with quercetin can induce the arrest of the cell cycle
454 in B- and T-derived-leukemia cells. In contrast, resveratrol and ellagic acid show no
455 effect, and the treatment with pterostilbene promotes the cell cycle. When cells were
456 treated with GPE, four out of five observations show no effect. These results indicate
457 that the growth inhibition promoted by grape polyphenols on leukemia cells is not
458 produced by arresting the cell cycle in the G_0 phase.

459 The overall conclusion of the meta-analysis is that grape polyphenols have a
460 detrimental effect in the growth of leukemia cells *in vitro.* The degree or magnitude of
461 this effect depends on the source of the cell lines and the specific polyphenol or GPE
462 used. Recent studies have shown an antiangiogenic activity of polyphenol extracts on
463 cancer cells [22] and the ability to modulate epigenetic changes directly involved in
464 cancer development [78, 79]. More specifically, resveratrol has been proved to modulate
465 autophagy and induce apoptosis in leukemia cells [23, 35, 36]. Furthermore, studies with
466 animal models lead to propose that the consumption of polyphenols in childhood and
467 even in the maternal diet could prevent the development of chronic diseases in
468 adulthood [18]. These data, together with the results obtained through this meta-analysis
469 showing the antiproliferative effect of grape polyphenols (mainly resveratrol) on
470 leukemia cells, support the development of *in viv*o studies to assess their usefulness in
471 the treatment and prevention of leukemia.

However, it must be noted that the concentration of polyphenols used in the *in vitro* assays is almost 25 times higher than the average estimated concentration of resveratrol present in grape juice [16]. Moreover, the bioavailability and pharmacokinetics of polyphenols mainly depend on the doses ingested, the food matrix and the gut microbiota. In the case of resveratrol, a safe and efficient dose has been proved between 1g and 5 g per day, although adverse effects can be detected in some individuals [88]. There are studies working on new formulations to enhance the bioavailability of resveratrol, mostly by means of increasing its hydrophilicity, stability and bioaccesibility. Studies carried out with resveratrol and pterostilbene in animal models showed that both can be rapidly absorbed and metabolized in their glucuronidated and sulfated forms. Moreover, due to its chemical structure of a dimethyl ether analog of resveratrol, pterostilbene showed a better membrane permeability and metabolic stability, which consequently increases its bioavailability and pharmacokinetic properties [92]. In a recent study, polyphenolic derivatives with increased lipophilicity have shown better cellular absorption, greater antioxidant capacity and greater antiproliferative effect on tumor cells grown *in vitro* [94]. Also, new procedures to protect polyphenols from digestive degradation have been studied, in order to allow these bioactive compounds to reach their therapeutic targets through food [94]. However, it is important to highlight that resveratrol has shown a dual action pattern depending on the dose; low concentrations increase expression of cell survival proteins, whereas higher doses stimulate cell apoptosis or necrosis regardless of whether the cell is healthy or pathological [91].

A recent study in humans showed that consumption of a phenolic-rich red grape pomace drink produced an increase in the phenolic metabolites in plasma at different times. Nevertheless, there was a high inter-individual variability in the absorption and

497 excretion of phenolic derivatives, which is a key point to be considered [93]. Gut
498 microbiota and phase II metabolic pathways play an important role in the biochemical
499 transformations of polyphenols and hence, more studies in humans are needed to
500 demonstrate the safety and therapeutic properties of dietary polyphenols. Furthermore, it
501 has been proposed that dietary intake of polyphenols derived from grape juice has a
502 beneficial effect on microbiota, which in turn can improve their bioavailability and
503 therefore contribute to enhancing human health benefits [95]. It is well known that the
504 polyphenol content in grape seed, skin or pomace extracts is higher than in grape juice,
505 so new procedures such as enzymatic treatment with exo-1,3-β-glucanase and
506 pectinases during juice obtention, could be used to improve the transfer of polyphenols
507 from the grape pomace [80, 81]. Other methods, such as microwave and ultrasound-
508 assisted extraction, pressurized solvent extraction, osmotic and membrane distillation,
509 heat and enzymatic digestion, nano and ultrafiltration, etc., have been also proved
510 effective to obtain extracts rich in polyphenols from grape and vine by-products [82-85].
511 Thus, it would be interesting to test the antileukemia properties of grape juice enriched
512 with polyphenol extracts [12, 86, 87].

513 The conclusion of this study is that grape polyphenols have a marked
514 antiproliferative effect on leukemia cells grown *in vitro*. However, further *in vivo*
515 studies are needed to improve the bioavailability of the compounds and to avoid the
516 toxicity produced at high concentrations. The interactions between the different
517 components present in natural or supplemented grape derived products could induce
518 antagonistic, synergistic, or additive effects that make it difficult to differentiate
519 between prevention and therapy for the treatment of leukemia. Once these important
520 points have been addressed and safety procedures standardized, clinical trials with

leukemia patients should be conducted in order to determine whether grape polyphenols can be used as a preventive or therapeutic anti-leukemic natural product.

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Conflicts of Interest

The authors declare no conflict of interest.

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843

844 Table 1. Scientific articles included in the meta-analysis.

Authors	Year	O1	O2	O3
Billard et al. [51]	2002	**X**	**X**	
Can et al. [52]	2012	**X**	**X**	
Chen et al. [53]	2005	**X**	**X**	
Espino et al. [32]	2013			**X**
Ferry-Dumazet et al. [37]	2002	**X**	**X**	
Gao et al. [54]	2009	**X**	**X**	
Ghorbani et al. [55]	2015	**X**	**X**	
Gokbulut et al. [56]	2013	**X**	**X**	**X**
Hu et al. [57]	2006	**X**	**X**	
Katsuzaki et al. [58]	2003	**X**	**X**	
León-González et al. [31]	2017	**X**	**X**	
Liao et al. [59]	2012	**X**	**X**	**X**
Mertens-Talcott et al. [61]	2003	**X**	**X**	**X**
Mertens-Talcott et al. [60]	2008	**X**	**X**	**X**
Sharif et al. [62]	2010	**X**	**X**	
Siedlecka-Kroplewska et al. [63]	2012	**X**	**X**	**X**
Wang et al. [64]	2012	**X**	**X**	**X**

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846 O1 (outcome 1): Effect of specific grape polyphenols on the proliferation of leukemia
847 cell lines. O2 (outcome 2): Effect of grape polyphenols on the proliferation of human
848 leukemia cells of different origin. O3 (outcome 3): Effect of grape polyphenols on cell
849 cycle arrest.

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851 Table 2. Articles included in the meta-analysis showing the number of useful
852 experiments for the data extraction, sorted by the type of leukemia cells and
853 polyphenols.

854

Articles	T lymphocytes cell lines	Number of experiments		Articles	Grape polyphenols	Number of experiments
[58, 60, 61, 63]	MOLT-4	9		[31, 32, 54, 57, 60, 62, 64]	GPE	7
[31, 37, 53, 54]	JURKAT	8		[37, 51, 53, 55, 56, 59]	Resveratrol	6
	B lymphocytes cell lines			[53, 56, 61]	Quercetin	3
[37, 51]	WSU-CLL	4		[51]	Viniferin	3
[51]	ESKOL	3		[53]	Apigenin	1
[55]	NALM-6	1		[58]	Cyanidin glucoside	1
[54]	232B4	1		[58]	Delphinidin	1
	Early myeloid cell lines			[61]	Ellagic acid	1
[37, 52, 59, 64]	K562	4		[53]	Kaempferol	1
[32, 37, 54]	HL60	3		[58]	Malvidin	1
[57]	AML 14.3D10	1		[53]	Myricetin	1
[37]	KCL22	1		[58]	Petunidin	1
	Monocytic cell lines			[63]	Pterostilbene	1
[37, 54]	U937	2		[51]	Vineatrol	1
[37]	THP1	1				

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Figure 1. Meta-analysis of the effect of grape polyphenols on leukemia cells' proliferation, based on the PRISMA literature search guide [39].

Figure 2. Outcome 1's results of the effect of specific grape polyphenols on the proliferation of human leukemia cells, grouped by type of polyphenol. Abbreviations: Em, early myeloid cell lines; Mc, monocytic cell lines; LB, B lymphocytes cell lines; LT, T lymphocytes cell lines.

Figure 3. Results of the effect of grape polyphenols on the proliferation of specific human leukemia cells, grouped by cellular lineage. A. Effect of pure polyphenols. B. Effect of GPE. Abbreviations: Em, early myeloid cell lines; Mc, monocytic cell lines; LB, B lymphocytes cell lines; LT, T lymphocytes cell lines.

Figure 4. Effect of grape polyphenols on G_0 cell cycle arrest in leukemia cells. Abbreviations: Em, early myeloid cell lines; LB, B lymphocytes cell lines; LT, T Lymphocytes cell lines.