RESEARCH ARTICLE

The Alcoholic Bark Extract of *Terminalia arjuna* Exhibits Cytotoxic and Cytostatic Activity on Jurkat Leukemia Cells

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Abstract: Background: Natural products are characterized by a complex chemical composition and are capable of concurrently modulating several signalling pathways. Considering the biological complexity of carcinogenesis, natural products represent key components of the therapeutic armamentarium for oncological diseases. The bark of *Terminalia arjuna* is used in traditional Ayurvedic medicine for its astringent, expectorant, cardiotonic, styptic, and antidysenteric properties. Alongside its traditional uses, *Terminalia arjuna* exhibits different biological activities including anti-tumorigenic and anticarcinogenic.

Objective: This study was designed to evaluate the toxic effects of an alcoholic extract obtained from the bark of *T. arjuna* on a human T-lymphoblastic cell line (Jurkat). We explored the phytochemical composition and investigated the cytotoxic, cytostatic, genotoxic, and anti-genotoxic effects.

Methods: The phytochemical composition was analyzed using spectrophotometric methods; all the biological endpoints were assessed through flow cytometry.

Results: The phytochemical screening showed that polyphenols represent about 64% of the extract. Moreover, the extract was cytotoxic on Jurkat cells by inducing both apoptosis and necrosis, and blocked the cell cycle in the G2/M phase. Additionally, it was found that the extract lacks any genotoxic effect, but was not effective in protecting Jurkat cells from the DNA damage induced by H₂O₂ and etoposide.

Conclusion: The results of our study show the toxic effects of *Terminalia arjuna* on Jurkat cells and confirm the pivotal role played by natural compounds in the oncological field. Further studies should be performed to better understand its clinical potential and deepen its toxicological profile.

Keywords: *Terminalia arjuna*, leukemia cells, cytotoxicity, cell cycle, genotoxicity, antigenotoxicity.

1. INTRODUCTION

Cancer is one of the worst plagues of this new millennium. In 2018 it was responsible for an estimated 9.6 million deaths and about 18 million new cases of cancer were diagnosed [1].

Carcinogenesis involves a series of definable and reproducible stages that lead to the transformation of normal cells into cancerous cells [2]. The first phase, called initiation, is characterized by the acquisition of mutations in several different genes (oncogenes); the second stage, i.e. promotion, is characterized by the selective clonal expansion of initiated cells to create a pre-neoplastic lesion, which could phenotypically evolve in a malignant lesion during the final stage of carcinogenesis called progression [2]. Because cancer is a multifactorial disease, the shortcoming of many therapeutic drugs that interact with a single target could be overcome by using multi-target agents [3], which interact simultaneously with different biological targets.

Since ancient times, natural products have played a central role in the treatment and prevention of human diseases [4]. Moving on to the last century, many plant compounds have a key role in anticancer therapy. Vincristine and vinblastine, for example, are two alkaloids with anticancer properties isolated from the Madagascar periwinkle (*Catharanthus roseus*) in the 1960s, which are still used to treat various cancers [5]. Another important anticancer drug is taxol, isolated from the bark of Pacific yew (*Taxus brevifolia*) by the National Cancer Institute (NCI) in the year 1971. Taxol has become one of the most used drugs for breast and ovarian cancers worldwide [6]. Not only in the past but also
nowadays nature continues to be a great wealth of natural molecules with beneficial activities, especially in the oncological area: indeed, 175 small molecules were approved between 1940s and the end of 2014, of which 49% are either natural or directly derived from natural products [7]. The reason why natural products are so widely studied as potential anticancer drugs could be found in their great biodiversity and, in particular, in their complex chemical composition. Since they are composed of countless molecules, they are able to interact with many different molecular targets and modulate various biological pathways [8-13]. Considering the multiple steps involved in the tumorigenesis process, natural products represent key components of the therapeutic armamentarium for oncological diseases. Moreover, given that the occurrence of resistance remains a major obstacle to tumour management [14], compounds capable of concurrently modulating several signalling pathways, as natural products, could help overcoming chemoresistance.

Terminalia arjuna (T. arjuna), also called “Arjun” in Hindi and “Bengali” and “Arjuna” in Sanskrit [15], is an indigenous large woody plant belonging to the family Combretaceae [16], commonly found throughout the Indian peninsula. T. arjuna is an important traditional plant widely used in Ayurvedic medicine and mentioned in many ancient Indian medicinal texts [17]. In particular, the bark was traditionally used for its astringent, demulcent, expectorant, cardiotonic, styptic, and antisydneretic properties; additionally, it has been shown to be useful in the treatment of fractures, ulcers, leukorrhea, diabetes, anemia, cardiopathy and cirrhosis [18]. The bark decoction has also been used for snakebite and scorpion sting [19]. Besides its traditional uses, a large and growing body of literature has investigated the pharmacological activities of T. arjuna. The plant exhibits antimicrobial [20, 21], antiviral [22], hypolipidemic [23, 24], antioxidant [21, 24, 28-30], antitumorigenic, and anticarcinogenic activities [30-36].

In this framework, the aim of this study was to deepen the therapeutic potential of this medicinal plant evaluating whether it is toxic for cancer cells. In particular, we analyzed the phytochemical composition and explored the cytotoxic, cytostatic and antigenotoxic activities of an alcoholic extract obtained from the bark of T. arjuna on a human T-lymphoblastic cell line (Jurkat).

2. MATERIALS AND METHODS

2.1. Plant Extract Preparation

The bark of T. arjuna was collected during the balsamic period (winter) and authenticated by Dr. Paolo Scartezzini, Maharishi Ayurveda Product Ltd., Noida, India. The quality control was performed by Vedic Herbs s.r.l. (Calderio, VR, Italy), which gifted us with a sample of bark powder (voucher #12/11). Ten g of T. arjuna powder was mixed with 100 mL of 100% ethanol. To remove insoluble material, the T. arjuna ethanolic extract (TAAE) was centrifuged.

2.2. Determination of Total Phenolic Content

The Total Phenolic Content (TPC) of TAAE was determined using Folin-Ciocalteu reagent. It was performed in triplicate using a ThermoSpectronic Helios-γ spectrophotometer (Waltham, Massachusetts, USA), according to a previously described method [37]. The results of TPC were expressed as milligram gallic acid equivalents (GAE) per g of dry extract using a calibration curve of gallic acid.

2.3. Total Flavonoids Determination

The Total Flavonoid Content (TFC) was measured by a colorimetric assay, as previously described by Lamaison & Carnet [38]. Briefly, an aliquot (1 mL) of the diluted sample or standard solution of hyperoside (0, 10, 20, 30, 40, 50, 60 µg/mL) was added to a 1 mL of AlCl3·6H2O (2% w/v in methanol). After 10 min under stirring into the dark, the absorbance of the mixture was determined at 394 nm with a ThermoSpectronic Helios-γ spectrophotometer (Waltham, Massachusetts, USA). The evaluation was performed in triplicate, and the total flavonoids content was expressed as mg hyperoside equivalents (HE) per g of dry extract, using a calibration curve of hyperoside.

2.4. Total Proanthocyanidins Determination

The Total Proanthocyanidins Content (TPrC) was measured by a colorimetric assay, as previously described by Porter et al. [39]. Briefly, an aliquot (1 mL) of the diluted sample or standard solution of cyanidin chloride (0, 10, 20, 30, 40, 50, 60 µg/mL) was added to a 6 mL of n-butanol/HCl (95:5) and 0.2 mL of a 2% solution of NH4Fe(SO4)2·12H2O in HCl 2M. The solution was kept 40 min under stirring at 95°C. After cooling, the absorbance of the mixture was determined at 550 nm with a ThermoSpectronic Helios-γ spectrophotometer (Waltham, Massachusetts, USA). The evaluation was performed in triplicate, and the total proanthocyanidins content was expressed as mg cyanidin chloride equivalents (CCE) per g of dry extract, using a calibration curve of cyanidin chloride.

2.5. Cell Cultures and Treatments

Human T-lymphoblastic cells (Jurkat) and human lymphocyte cells (TK6) were provided from LGC standards (LGC Group, Middlesex, UK). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated bovine serum, 1% penicillin-streptomycin solution, and 1% l-glutamine solution (All obtained from Sigma Aldrich). Cells were cultured at 37°C and 5% CO2 in a humidified atmosphere. To maintain exponential growth, Jurkat and TK6 cells were cultured without exceeding the maximum cell density of 3 X 10⁶ mL and 1 X 10⁸ mL, respectively.

Cells were treated with increasing concentration (0 - 1.00 mg/mL) of TAAE for 1, 3, 6, or 24 h depending on experimental conditions. Etoposide (10 µM or 10 µg/mL) and H2O2 (1 mM) (All obtained from Sigma Aldrich) were used as positive controls.

2.6. Analysis of Cell Viability

The determination of cell viability was performed using Guava ViaCount Reagent (Merck Millipore, Burlington, MA, USA) according to manufacturer’s instructions. Briefly, cells were incubated with the reagent containing 7-aminoactinomycin D (7-AAD) and, after incubation at room temperature in the dark for 5 minutes, cells were analyzed by flow cytometry.
2.7. Analysis of Cell Death Mechanisms

To discriminate between apoptotic and necrotic events, Guava Nexin Reagent (Merck Millipore) was used. This reagent, containing 7-AAD and annexin V-phycoerythrin (PE) is able to discriminate apoptotic and necrotic events. After incubation of 20 minutes at room temperature in the dark cells were analyzed by flow cytometry. Three cell populations can be detected: live cells (annexin V −/−7-AAD −), early apoptotic cells (annexin V +/−7-AAD −), and late apoptotic or necrotic cells (annexin V +/−7-AAD +).

2.8. Cell-Cycle Analysis

The percentages of cells in G0/G1, S, and G2/M phases were quantified by the analysis of DNA content using Guava Cell Cycle Reagent (Merck Millipore). After treatment with TAEE for 24 h, cells were fixed with 70% ice-cold ethanol; after washing, cells were suspended in 200 μL Guava Cell Cycle Reagent, containing propidium iodide, and incubated 30 minutes at room temperature in the dark before analysis by flow cytometry.

2.9. Analysis of DNA Damage

In order to assess the genotoxic potential of TAEE, phosphorylation of histone γ-H2A.X was evaluated as marker of DNA double strand breaks. In brief, after treatment of 6 h with TAEE, cells were fixed, permeabilized and incubated for 30 minutes in the dark at room temperature with an anti-γ-H2A.X-Alexa Fluor® (Merck Millipore). Etoposide 10 μM was used as a positive control. Samples were analyzed via flow cytometry.

Furthermore, in addition to the primary damage to the DNA, the mutational effects of TAAE were evaluated through the micronucleus assay. Jurkat cells were treated with TAEE (0 – 0.40 mg/mL) for 24 h. At the end of the treatment, 0.5 × 10⁶ cells were stained following the manufacturer’s instruction of the In Vitro Microflow kit (Litron Laboratories, Rochester, NY, USA). Cells were firstly stained with nucleic acid dye A solution containing ethidium monoazide (EMAS). EMA, after photoactivation, is able to cross the damaged membrane of dead cells. Afterwards, complete lysis solution 1 and then lysis solution 2, which also contains SYTOX green, a chromatin dye, were added in order to digest the cytoplasmatic membrane and have the complete release of nuclei and micronuclei (MN). The double staining of chromatin with EMA and SYTOX green allows the discrimination between nuclei and MN in living cells (SYTOX green−) from fragments derived from damaged chromatin of dead/dying cells (EMAS / SYOTX green−). At the end of the incubation, cells were analyzed by flow cytometry. The discrimination between nuclei and MN has been performed through the analysis of their size (MN are smaller than nuclei) and their fluorescence (MN display 1/10⁶ to 1/10⁸ of the intensity of duplicated nuclei [40]).

2.10. Analysis of Antigenotoxic Activity

To assess the ability of TAEE to protect against DNA damage induced by different genotoxic agents, two different experimental settings were used. In the first one, cells were treated with TAEE before the exposure to the genotoxic agent (pre-treatment protocol); in the second one, cells were treated with TAEE during the exposure to the genotoxic agent (co-treatment protocol), as reported below:

- In the pre-treatment protocol, cells were treated with TAEE 0.40 or 0.80 mg/mL. After 3 h, the culture medium was eliminated, and cells were treated with the genotoxin. After 1 h, cell samples were analyzed.
- In the co-treatment protocol, cells were treated at the same time with TAEE 0.40 or 0.80 mg/mL plus the genotoxin for 1 h; then, cell samples were analyzed.

For H2O2 treatment, cells were treated in Phosphate Buffered Saline (PBS) 1X. The analysis of histone γ-H2A.X phosphorylation was performed as described above.

2.11. Flow Cytometry

All flow cytometric analyses were performed using an EasyCyte 5HT flow cytometer (Guava Technologies-Millipore, Hayward, CA, USA).

2.12. Statistical Analysis

All results are expressed as mean ± SEM of at least three independent experiments. Differences between treatments were assessed by one-way ANOVA, using Dunnet or Bonferroni as post-tests. All statistical analyses were performed using GraphPad InStat 5.0 version (GraphPad Prism, San Diego, CA, USA). P < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. TAEE Contains Phenols, Flavonoids and Proanthocyanidins

*T. arjuna* bark alcoholic extract (TAEE) is known to contain a large amount of phenols with important biological activities. Among them, the flavonoid luteolin and oligomeric proanthocyanidins [41] are known to be responsible for the multi-target potential of the extract towards carcinogenesis [42-45]. Moreover, the phenols contained in TAEE are also important because of their recognised anti-mutagenic activity [35]. Therefore, to assess the bioactivity of the phytocomplex in relation to the content of these molecules, we measured the total phenolic content (TPC), total flavonoids content (TFC) and total proanthocyanidins content (TPPrC) through spectrophotometric methods. The TPC (Table 1), expressed as gallic acid equivalent (GAE)/g of dried extract, has been shown to represent the 64.46 % of the extract. The value of TPC reported in this study is lower than that reported by Viswanatha *et al*., who found about 90% for the alcoholic extract [35]. This discrepancy could be due to the different extraction methods used. As stated above, among the phenolic compounds, literature reports the presence of flavonoids and proanthocyanidins [41]. Accordingly, we found a noteworthy presence of proanthocyanidins, expressed as cyanidin chloride equivalent (CCE)/g of dried extract. In particular, proanthocyanidins represent about one third of the TPC of TAEE (235.49±6.58 mg CCE/g dried extract). Saha *et al.* [46] report a TPPrC lower than that measured in our study (7.5 % against 23.5 %), but they analyzed a different extract (aqueous instead of
alcoholic extract). Finally, the quantification of the TFC, expressed as hyperoside equivalents (HE)/g of dried extract, evidenced a low flavonoids content, which represents the 0.7% of the *T. arjuna* dried extract. Comparing the results of our study with those reported in literature, we can observe a noteworthy increase in proanthocyanidins in TAE (from 7 to 23%), which could be critical for the expression of the multi-target potential of *T. arjuna* towards carcinogenesis.

3.2. TAAEE Exhibits Cytotoxic Activity Against Jurkat Cells But Not Against TK6 Cells

Jurkat cells were treated with increasing concentrations of TAAEE (0 - 1.00 mg/mL) for 24 h. After this treatment period, we observed a dose-dependent decrease of cell viability, reaching 56% at the highest tested concentration (Fig. 1A).

One of the major limitations of current anticancer chemotherapy is the inability to selectively induce cytotoxic effects on cancer cells. The cytotoxicity of antitumor drugs for non-tumorigenic cells is associated with causing systemic toxicity and significant side effects [47, 48]. Hence, the identification of new anticancer agents that are selective towards cancer cells, thus with a better toxicological profile, is crucially important. The activity of TAAEE was also tested on non-tumorigenic cells in order to preliminary assess its selectivity towards tumour cells. No cytotoxic effect was observed on TK6 cells (Fig. 1B) after treatment for 24 h with increasing concentrations of TAAEE. Those results indicate a favourable toxicological profile of the alcoholic extract.

3.3. TAAEE Induces Apoptosis and Necrosis

In order to investigate the mechanism responsible for the cytotoxic effect of TAAEE, we used the annexin V/7-AAD assay. Phosphatidylserine (PS) exposure represents a crucial stage during the apoptotic process. Annexin V binding to PS allows detecting apoptotic cells (annexin V+/7-AAD− cells) [49]. 7-AAD can detect necrotic cells (annexin V+/7-AAD+ cells) due to its ability to permeate only cells with cell membrane damaged [50].

The treatment with increasing concentrations of TAAEE increased the fraction of apoptotic cells. An increase in the fraction of the necrotic cells was also observed (Fig. 2). The percentage of apoptotic cells started to increase from the concentration 0.20 mg/mL (7% versus 1.7% in untreated cells), and further increased up to the highest tested concentrations, where they reached about 29%. Alongside the increase in apoptotic cells, an increment in the fraction of necrotic cells was observed as well, starting from the concentration of 0.20 mg/mL. The percentage of annexin V+/7-AAD+ cells was comparable to the apoptotic percentage: at the 0.80 mg/mL, for example, it was 25% compared to 29% of apoptotic cells (Fig. 2).

Cell death is a fundamental process that regulates several processes and is essential to maintain tissue homeostasis and to wipe out potentially harmful cells [51]. Cell death is generally classified into two main categories: 1) apoptosis, where cells exhibit cytoplasmic shrinkage, chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), and plasma membrane blebbing. Apoptotic cells are efficiently eliminated by neighbouring cells with phagocytic activity and lysosomal degradation [52]; 2) necrosis is a process characterized by the swelling of organelles, the rupture of the plasma membrane, and the lysis of the cell, which culminates with cell corpse removal without obvious phagocytic and lysosomal activities [52]. Of note, apoptosis represents the most characterized form of programmed cell death, while necrosis is considered an uncontrolled type of cell death.

### Table 1. Quantification of total polyphenols, total flavonoids and total proanthocyanidins of *T. arjuna* ethanolic extract.

<table>
<thead>
<tr>
<th>Total Phenols mg GAE/g dry extract</th>
<th>Total Flavonoids mg HE/g dry extract</th>
<th>Total Proanthocyanidins mg CCE/g dry extract</th>
</tr>
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<tr>
<td>644.57±23.21</td>
<td>71.07±0.82</td>
<td>235.49±6.58</td>
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**Fig. (1).** Percentage (%) of viable cells after 24 h treatment of Jurkat cells (A) or TK6 cells (B) with increasing concentrations of TAAEE. **p < 0.01; ***p < 0.001 versus untreated cells.
death [53]. However, in recent years several other forms of cell death have been discovered revealing that a cell can die via a number of different pathways [54].

Anticancer therapeutic approaches based exclusively on apoptosis induction are frequently unsuccessful due to the activation of resistance mechanisms. Moreover, tumour pathology is characterized by high genetic and genomic instability, which contribute to the mutation of some of the molecular actors involved in the apoptotic pathway and their inactivation. Accumulated evidence suggests that necrosis, especially when in concomitance with apoptosis, could represent an alternative mechanism to induce cancer cell death [55]. Furthermore, necrotic cell death is characterized by an inflammatory component that could stimulate an immune response raising the efficacy of tumour cell eradication [55]. Given that inflammatory response induced by necrotic cell death can be related to the onset of toxic effects, a balance between apoptosis and necrosis induction may thus be modulated to enhance the immune response in cancer cells [55]. For instance, many chemicals have been found to induce cancer cell eradication through necrosis, such as β-lapachone, apoptolidin, and honokiol [56-59]. Nevertheless, a concomitant induction of apoptosis and necrosis seems to be induced especially by plant extracts, since they are composed of a complex mixture of multiple active compounds that can act in a synergistic way to impair different biological pathways.

Our research findings reveal that TAEE treatment induced both apoptosis and necrosis in a similar extent. Even if the removal of cancer cells in a regulated manner is more desirable, we could consider TAEE as an anticancer strategy potentially capable of overcoming an eventual failure in apoptotic induction.

A number of recent studies focused on the potential anticancer effects of *T. arjuna* extract and its isolated compounds. In this regard, methanol and acetone extracts obtained from the bark of this medicinal plant were investigated on osteosarcoma cells (U2OS) and glioblastoma cells (U251), where they showed growth inhibition by p53-dependent and -independent pathways [60]. Moreover, Shalini and colleagues observed an antiproliferative activity for *T. arjuna* bark methanolic extract and its phytosome on human breast adenocarcinoma cells (MCF-7) [61]. Two other studies report the cytotoxic activity of the petroleum-ether bark extract of *T. arjuna* against hepatocellular carcinoma (HEPG2) and colorectal adenocarcinoma (HT-29) cell lines [62], and the cytotoxic effects of an ethanolic extract of *T. arjuna* on HEPG2 cells through apoptosis induction [63]. To the best of our knowledge, this latter investigation is the only work which explored the anticancer potential of an ethanolic extract from the bark of *T. arjuna* and reported research findings similar to that of our study, even if in a different tumour cell line.

Furthermore, other studies investigated the antitumor potential of different compounds isolated from the bark of *T. arjuna*, such as casuarin, a hydrolysable tannin, arjunic acid, and arjunolic acid, two triterpenoids. Casuarin has been shown to induce apoptosis in A549 human non-small lung carcinoma cells and in MCF-7 human breast adenocarcinoma cells by activating the Fas/APO-1 apoptotic pathway [64, 65]. Arjunic acid, instead, showed significant cytotoxicity in human non-small lung carcinoma cells (NCI-H460), colorectal adenocarcinoma cells (HT-29), and acute lymphoblastic leukaemia cells (CEM) [66]. Additionally, Joo and colleagues reported that arjunic acid cleaves Poly ADP-ribose polymerase (PARP), activates Bax and p-JNK (c-Jun N-terminal kinase), and reduces the expression of procaspase 3 and Bcl-2 on A549 and NCI-H460 cells [67]. Moreover, *in vitro* and *in vivo* apoptotic induction by arjunolic acid was observed on Ehrlich ascites carcinoma cells by
Elsherbiny and coworkers [68]. For casuarinin, arjunic acid, and arjunolic acid no induction of necrosis was recorded.

We investigated the antitumor properties of a crude plant extract, representing a phytocomplex, wherein a mixture of different compounds contributes to its biological activity. Many natural products could result in activities that later vanished when they are separated into individual chemical components [69]. It is interesting to note that in our study we observed that TAEE induces necrosis, not only apoptosis. The different activity of TAEE could be due to the fact that other components may lead to additional biological activities, such as TAEE-induced necrosis, which has not been observed for casuarinin, arjunic acid and arjunolic acid isolated from *T. arjuna* bark.

### 3.4. TAEE Causes Cell-Cycle Perturbations

In the following experiments, we highlighted the cytostatic effect of TAEE. The treatment with increasing concentrations of TAEE increased the number of cells in the G2/M phase. Starting from the concentration 0.80 mg/mL, the accumulation of cells in G2/M phase appeared to be statistically significant, with 42% versus 29% of untreated cells; at the highest tested concentration, the percentage further increased to 47%. This observed increase was accompanied, at all tested concentrations, by a slight compensatory decrease in the G0/G1 phase (from 55% of untreated cells to 38% at 1.00 mg/mL) (Fig. 3).

Cell cycle takes place over four phases: G1 (gap), S (synthesis), G2 (gap) and M (mitosis), and is finely regulated by cyclins and cyclin-dependent kinases (CDKs), which facilitate chromosome replication and separation, and cytokinesis [70]. CDKs bind cyclins, their regulatory sub-components, which are synthesized and destroyed at specified times throughout the cell cycle, thereby controlling kinase activity [71]. Normally, the G1/S and G2/M checkpoints control cell proliferation accurately; cell-cycle arrest is thus considered one of the most common triggers of cell proliferation inhibition [72].

Even if the replication system is highly preserved, in the last two decades, a broad range of literature highlighted the importance of cell-cycle deregulation over human cancer [71]. Cancer cells are characterized by genomic and chromosomal instability; accumulation of mutations leads to cell-cycle deregulation, uncontrolled proliferation, and tumor progression [71, 73, 74]. Thus, agents able to perturb cell-cycle progression could be considered as effective therapeutic drugs for cancer treatment. Additionally, cancer cells can survive mitotic arrest induced by antimitotic drugs and prevent subsequent cell death, which is considered an active mechanism of chemoresistance [75]. Thus, the cytotoxic effect exhibited by TAEE along with cell-cycle arrest could result in an improved strategy to eradicate tumour cells.

As far as we know, no other authors have investigated the ability of *T. arjuna* extract to arrest cell cycle. Kuo and colleagues, however, found that casuarinin blocks cell-cycle progression in the G0/G1 phase in MCF-7 cells [65] and in A-549 cells [64]. As we observed that TAEE induces a cell-cycle block in the G2/M phase, our findings are in contrast with the aforementioned studies. This different result could be due to the fact that the extract contains many active compounds other than casuarinin or could be a cell-type-specific effect.

### 3.5. TAEE Is Not Genotoxic

In order to evaluate the ability of TAEE to induce DNA damage, H2A.X analysis was performed. Phosphorylation of H2A.X (P-H2A.X) at Ser 139 is considered an early cellular response to DNA double-strand breaks. Thus, the analysis of this event is useful to detect the ability of a compound to induce DNA damage. After 6 h of treatment with TAEE, no significant increase in H2A.X phosphorylation was observed at any tested concentration, thus excluding any genotoxic activity of the extract (Fig. 4).

Hence, to definitely establish the non-genotoxic potential of TAEE, we analyzed the frequency of MN after TAEE treatment. Unlike the phosphorylation of H2A.X assay, through which it is possible to observe only clastogenic ef-
genotoxicity of two different genotoxic agents: hydrogen peroxide (H₂O₂) and etoposide. H₂O₂ causes oxidative DNA damage by the formation of hydroxyl peroxide (·OH). Hydroxyl peroxide attacks the deoxyribose moiety of DNA and generates DNA single-strand and double-strand breaks, and the formation of 8-oxo-7,8-dihydroguanine and abasic sites [95, 96]. Etoposide induces DNA damage by forming a complex with topoisomerase II and DNA, which results in an increase in double-stranded DNA breaks [97]. In order to investigate the antigenotoxic effects of TAEE, two different experimental settings were used: pre-treatment of Jurkat cells with TAEE for 3 h and then treatment with genotoxicants; co-treatment of 1 h with TAEE and genotoxicants. As shown in Fig. 5, the results obtained indicate that TAEE did not exert any protective effect against the genotoxicity of etoposide and H₂O₂, either using pre-treatment or co-treatment experimental protocol.

Fig. (4). Relative expression of phosphorylated H2A.X (P-H2A.X) induced by TAEE in Jurkat cells after 6 h of treatment. Etoposide (10 µM) was used as positive control. *** p < 0.001 versus untreated cells.

However, very few studies have been carried out on the antigenotoxic activity of T. arjuna. Kaur and colleagues [98], for example, studied the protective effect of a tannin fraction isolated from T. arjuna against the genotoxicity of 4-nitro-o-phenylenediamine, sodium azide, and 2-aminofluorene using the Ames assay. The tannin fraction was highly effective only towards 2-aminofluorene, thus showing that the protective effect is dependent on the genotoxic tested [98]. Additionally, Pasquini et al. investigated the antigenotoxic potential of T. arjuna bark against the 4-nitroquinoline-N-oxide genotoxicant, using different in vitro tests (i.e. Salmonella/microsome test, comet assay and micronucleus test) [99]. In this study, the bark of T. arjuna was extracted sequentially with six solvents with decreasing hydrophobicity (i.e. chloroform, acetone, methanol, methanol+HCl, diethyl ether, and ethyl acetate). Among the six tested extracts, authors found a significantly different activity that has been shown to be related to the extraction solvent used: indeed, among them, the acetone extract was the most effective in reducing genotoxic activity while the methanol, ethyl acetate, and chloroform extracts exhibited a different inhibitory activity depending on the test used; conversely, the diethyl ether and the acid methanol extracts lacked or showed the lowest inhibitory effect against the tested genotoxicant [99].

A substantial difference that can be observed between our paper and the above mentioned papers is the different type of extract used in the studies: we tested a raw extract in vitro while the above mentioned papers is the different type of extract used in the studies: we tested a raw extract in vitro which the presence of any active molecules could be much less concentrated than, for example, the tanninic fraction tested by Kaur and colleagues [98]; in addition, the study by Pasquini et al. [99] tested different types of extracts of T. arjuna, but not the ethanolic. Different solvents extract different phytochemicals, which can be responsible for the biological activity of the extract.

Moreover, we have tested the antigenotoxic activity of TAEE against two genotoxicants acting with a mechanism of action different from those used in the studies reported above (i.e. 4-nitroquinoline-N-oxide [99] and 4-nitro-o-phenylenediamine, sodium azide, and 2-aminofluorene [98]). This means that the extract can exert protective effects only against compounds with a specific genotoxic mechanism.

However, although TAEE has not shown any genoprotective activity, the lack of genotoxic effects demonstrates its
Genotoxicity is a dose-independent event for many chronic degenerative diseases such as cancer, cardiomyopathies and atherosclerosis, glaucoma, and neurodegenerative diseases. Moreover, genotoxicity is a dose-independent event for which it is not possible to define a range of concentrations where the effect does not occur.

CONCLUSION

Despite significant advances, current anticancer therapies still have limited effectiveness, mainly due to their low therapeutic index and the frequent appearance of chemoresistance. Hence, there is a compelling need to develop new intervention strategies. Considering their unique features, natural compounds could be considered excellent candidates for this purpose. Taken together, the results of this study suggest that the ethanolic extract obtained from the bark of T. arjuna exerts toxic effects on human leukaemia cells. These toxic effects have been shown to be related to the ability of the extract to induce cell death by both apoptosis and necrosis and block cell-cycle progression in the G2/M phase. Additionally, it was found that TAEE lacks any genotoxic effect and is not cytotoxic for non-tumorigenic cells, hence assuming a favourable toxicological profile. Our findings increase the growing body of literature on the antitumor activity of this medicinal plant, thus confirming the pivotal role played by natural compounds in the oncological field. Further studies should be performed to better understand its clinical potential and deepen its toxicological profile.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABLE DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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