

"Cytotoxic and DNA-Damaging Effects of Curcuminoids on Daudi and Jurkat Leukemic Cell Lines: Investigating Potential Alkylating Activity"

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ABSTRACT

This study aimed to assess novel role of curcuminoids as an alkylating agent against leukemic cells (Daudi and Jurkat cell lines). Cytotoxic activity was assessed using an MTT assay. Also, 4-(4'-nitrobenzyl) pyridine (NBP) was used as a DNA model for in vitro DNA alkylation. Results showed curcuminoids possess cytotoxic activity against both the Daudi and Jurkat cell lines. They showed 21.44 µg/ml and 48.82 µg/ml IC50 values, compared to treatment with Melphalan at 22.37 µg/ml and 56.51 µg/ml for the Daudi and Jurkat cell lines, respectively. The curcuminoids were found to possess some alkylating activity, similar to that of Melphalan. In conclusion, chemical screening revealed an intrinsic property of curcuminoids with regard to their in vitro alkylating-like effects; however, these chemicals exert their cytotoxic functions on Daudi and Jurkat cells through a combination of DNA injury mechanisms. This indicates that alkylating-like chemical properties may act synergistically with other forms of cellular stress in activating the process of apoptosis, which expands the understanding of curcuminoids in treating hematological malignancies.

Keywords: Anticancer; Apoptosis; Alkyl group; DNA fragmentation; Dapi Stain.

INTRODUCTION

Alkylation is a form of DNA damage. It occurs by disrupting DNA structure and function by binding alkyl groups (carbon-containing groups) to the DNA molecule. Alkylation usually occurs in the nitrogen bases, such as at the nitrogen atom in adenine and guanine, as well as the oxygen atom in the guanine base, or it can simply be produced via alkylating agents. If these changes are not repaired through the DNA-repairing system, cell alterations can arise. Compounds causing alkylation can form a covalent bond of the alkyl group to the biomolecule(s) based on optimal physiological circumstances, i.e., an aqueous solution at 37 °C and pH 7.4. Factors causing alkylation interfere with both the resting and dividing phases of the cell life cycle, but maximum cytotoxicity occurs in the late G1 and S phases, due to a lack of time to fix DNA errors¹

For therapeutic purposes, alkylating chemicals utilized as anticancer treatments can interfere with DNA and protein biomolecules in various phases of the cell life cycle. They can cause cell death or inhibit growth. Therefore, DNA alkylation can be used to remove malignant cells. The National Cancer Institute defines alkylating substances as a group of compounds employed to treat cancers by reacting with cellular genetic material to kill tumor cells.

Alkylating factors affect DNA proliferation and can cause cell damage. This is primarily observed among rapidly replicating cells, such as tumors. Nitrogen-dependent alkylating agents are usually utilized during the therapy of leukemia as well as for solid tumors. These agents apply their

antineoplastic activity during various stages of cell division in addition to hindering the replication process of leukemic cells¹

The distinguishing cytotoxic aspects of chemical-based alkylation are (I) toxicity to blood cells, (II) digestive system toxicity, as well as (III) central nervous system and gonadal toxicity. Therefore, these drugs represent specific systems of toxic effects and are evaluated through the potency, uptake, and spreading of an agent². Even though they are toxic, medicines causing alkylation of DNA are a prominent malignancy treatment. The alkylating group is one of the main anticancer treatments and is still the most frequently applied in chemotherapy plans.

Although substance-induced alkylation can be applied to control many kinds of malignant cells, these agents are generally most beneficial in treating slow-growing tumors via inducing apoptosis. Finding an eco-friendly, safe, and daily consumable herb with a potent alkylating effect will contribute to developing an anticancer tool derived from natural sources.

Induction of the p53 gene increases the sensitivity of malignant cells to apoptosis triggered by alkylation³. Curcumin, which constitutes %60–50 of the curcuminoids found in *C. longa*, exhibits suppression efficacy against the replication of a wide range of cancer cells. Anticancer potency of curcumin was revealed via inhibited expression of the anti-apoptotic gene yields, activation of caspases, and promotion of tumor-suppressor genes such as p53⁴.

Curcuminoids are phytoactive constituents obtained from turmeric (rhizomes of *Curcuma longa*), an annual herb belonging to the Zingiberaceae family⁵.

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In Asian countries, particularly in the middle and southeastern regions, turmeric is consumed as a food flavoring agent, a seasoning, a food preservative, and a colorant. It is regularly used in many foods⁶. Additionally, turmeric has medicinal properties. Curcuminoids are a family that contains many highly investigated phytochemical compounds. The phytochemical content of turmeric ranges from %6–1 by weight. It is comprised of curcumin (Cur), de-methoxycurcumin (DMC), and bis-demethoxycurcumin (BDMC)^{7,8}. Curcuminoids are lipid-soluble bioactive compounds that have a wide diversity of biological activities. Commonly, they work as natural complexes with a peroxisome proliferator-activated receptor- γ , which exhibits the capability to decrease the creation of cytokines. Curcuminoids have anti-inflammatory, antioxidant, antimicrobial, and antitumor properties⁹. Various studies have established that curcumin (C21H20O6) from turmeric dyes was isolated in the 19th century. It has a wide spectrum of in vivo pharmacological activities, such as cardio-vascular and hepatoprotective activities. Other medicinal functions including anti-inflammatory, anti-angiogenic, anti-diabetic, antiviral, and anticancer properties¹²⁻¹⁰. Despite the fact that the pleiotropic anticancer action of curcuminoids is recognized to work through some established molecular pathways—like modulation of oxidative stress, mitochondrial irregularities, inhibition of NF- κ B, and activation of caspase cascade—it is worth noting their possible chemical interactions with intracellular macromolecules that need further investigation. At this point, the emphasis should be put on the investigation of DNA-alkylating properties of curcuminoids caused by their active electrophilic centers—this is an exciting chemical approach. The objective of the current study is not to challenge or replace the established biochemical processes of apoptosis but to find out whether these alkylating-like chemical characteristics can be used as a preliminary upstream regulator initiating DNA damage and subsequent apoptotic process in leukemic cell cultures.

Apoptosis is programmed cell death, which is a vital defense mechanism against malignant diseases, playing a crucial role in reducing tumor cells. Tumor cells are considered hereditary, as cells with genetic material lesions may initiate tumors, or the cells may become malignant¹³. Both mechanisms are detected using DNA staining with DAPI (genetic material-binding fluorochrome), which is commonly applied in testing for DNA damage. The comet assay is an effective tool for assessing DNA damage in individual cells. Both are commonly used apoptosis detection techniques. Two conditions can be applied in the comet test, neutral or alkaline conditions. The alkaline comet assay can identify a wide spectrum of nucleic acid abnormalities such as breaks of DNA single-strands (SSBs) with alkali labile sites (ALSs), DNA-protein and DNA-DNA crosslinks, in addition to particular types of base lesions. The comet assay under neutral conditions can be used to investigate double-strand breaks and to evaluate the DNA fragmentation related to apoptosis¹⁴. This study aimed to evaluate curcuminoids as an efficient inducer of DNA damage by activating the apoptotic pathway in leukemic cells via alkylation.

MATERIALS AND METHODS

Curcuminoids

Curcuminoids (purified from *Curcuma longa* L. rhizome, Product No. C7722) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The reagent was obtained as a crystalline powder with an analytical purity of %98 as determined by High-Performance Liquid Chromatography (HPLC). The chemical composition of this standardized curcuminoid mixture consists of approximately %77 curcumin, %17 demethoxycurcumin, and %6 bisdemethoxycurcumin. The identity and structural integrity of the compounds were verified prior to biological assays using ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS).

Daudi and Jurkat cell cultures

Two human leukemic cell lines, Daudi (containing B lymphoblasts collected from the peripheral blood of a 16 year old Burkitt's lymphoma patient - passage no. 11) and Jurkat (human acute T cell leukemia) (passage no. 14), were purchased from the American Type Culture Collection (ATCC®), CRL2266™, Manassas, VA, USA. All cells were grown in RPMI 1640 medium supplemented with fetal bovine serum (FBS, %10 v/v), with penicillin-streptomycin (%1 v/v) (Gibco, UK). Both cultures were grown at 37 °C under humidified conditions with %5 CO₂ in T24 flasks (Corning, USA). Cells were re-cultured two to three times weekly.

Cytotoxicity of Curcuminoids on Daudi and Jurkat leukemic cell lines

A curcuminoid solution was investigated for cell toxicity on two types of leukemic cells and compared with cytotoxicity data of Melphalan, a potent chemotherapeutic drug. Cytotoxicity testing was accomplished by employing a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann¹⁵ and Hashim et al.¹⁶. Tested concentrations (10 to 500 μ g/ml) of curcuminoids and Melphalan were prepared individually with RPMI medium. Cells (100 μ l) were placed in each well of a 96-well plate at a density of 10⁵×5 cells/ml. They were then incubated at 37 °C in %5 CO₂, %95 air for 24 h. After that, cells were subjected to treatment with different concentrations of the tested samples at a 200 μ l/well final volume for 24 h. At 21 h, both cell lines were centrifuged at 2, 000x for 10 min (Hunan Kecheng Instrument Equipment Co., model GT216C, China) and re-suspended in 180 μ l of culture medium to wash the treated cells. MTT solution (20 μ l) at 5 mg/ml was added to each well, then incubated at 37 °C for 3 h. After that, 180 μ l of the culture medium was aspirated from each well. The produced violet formazan crystals were liquefied using 180 μ l of DMSO. An optical density (OD) of formed crystals was assessed using UV spectrometry at a λ max of 570 nm. The percent of cell toxicity compared with the -ve control (untreated cells, assumed to be %100 viable) was determined according to the equation:

$$\text{Cytotoxicity \%} = \frac{\text{Ab. of control cells} - \text{Ab. of treated cells}}{\text{Ab. of control cells}} \times 100\% \quad (1)$$

The plot of percent cytotoxicity versus sample concentration was used to calculate the extract concentration that killed %50 of the cells (IC₅₀).

Alkylation test based on nitrogen in 4, (4'-nitrobenzyl) pyridine (NBP)

The 4, (4'-nitrobenzyl) pyridine (NBP) test was employed as a DNA model due to its nucleophilic similarity to DNA¹⁷. NBP can indicate the anticancer alkylation mechanism effect in vitro. The alkylating effect of the sample could be detected from the colored compound formed by the chemical reaction between the tested sample and the NBP under specific conditions. This colored product can be quantitatively measured at 600 nm. The NBP assay was modified from Thomas et al.¹⁷. Briefly, various concentrations of the tested solutions (i.e., curcuminoids at concentrations of 19.339 and 46.58 μ g/ml or Melphalan at 20.747 and 54.48 μ g/ml against both the Daudi and Jurkat cell lines, respectively) were prepared with the Reagecon™ Buffer Solution, pH 4.00, purchased from Fisher Scientific. This mixture was incubated in a pre-heated oven at 70 °C with a 4-(4-nitrobenzyl) pyridine solution in centrifuge tubes with gentle mixing. The process of incubating at 70 °C in the NBP assay must comply with prescribed chemical protocol rather than serve as simulation of physiological conditions. In this in vitro process, NBP behaves as a nucleophile and plays its role of mimicking the N-7 of guanine. The applied elevated temperature is an indispensable means of

achieving successful substitution reactions over reasonable time period owing to the necessity to overcome elevation of activation energy. After 30 min, about 90 μ l of the mixed solutions were pipetted and placed in a 96-well plate containing a mixture of absolute ethanol (EtOH) and 0.1 N NaOH, followed by incubation in an ice bath. The absorbance of the alkylation reaction's blue color was detected at each time under UV-VIS spectrophotometry at a 600 nm wavelength.

DAPI staining technique

For detection of apoptosis induction, both the Daudi and Jurkat cells were subjected to treatments with curcuminoids and Melphalan (a chemotherapeutic drug used as a positive control). DNA damage was assessed with the DAPI staining technique (4, 6-diamidino-2-phenylindole dihydrochloride) following Jang¹⁸ with some modifications. Tested leukemic cells (100 μ l) at a density of 1×10^6 cells/ml were cultured in a 24-well plate for 24 h. Then, the Daudi and Jurkat cells were subjected to treatment with curcuminoids at 19.339 and 46.58 μ g/ml, respectively, based on the cytotoxicity test results. In separate wells, Daudi and Jurkat cell cultures were treated with Melphalan (a standard chemotherapeutic drug) at 20.747 μ g/ml and 54.48 μ g/ml, respectively, according to cytotoxicity test outcomes. Cells were harvested by centrifugation at 2000 rpm for 5 min after 24 h of treatment. They were fixed by ethanol addition, then incubated at 4 °C. After that, they were sedimented by centrifugation at 2000 rpm for 5 min. The DAPI solution (100 μ l) was added to each well, and the plates were kept for 1 h under dark conditions. Then, the DAPI reagent was eliminated by centrifugation at 2000 rpm for 5 min and re-suspended in a solution containing glycerin and PBS in a 1:1 ratio. Finally, the cells were examined using an inverted fluorescence microscope at 40x by placing 10 μ l of cell suspension on a slide and adding a coverslip. Apoptosis was estimated by morphological alterations of approximately 500 cells per slide, which was considered a single sample.

Comet assay under neutral conditions (gel electrophoresis of single cells)

A comet assay with neutral conditions was conducted using a Trevigen comet assay kit, which consists of a comet assay slide, lysis solution, low-melting-point agarose, and SYBR green stain. Both types of leukemic cells were grown in 96-well plates at a density of 5×10^5 cells/ml for 24 h and then subjected to curcuminoids at 19.339 μ g/ml and 46.58 μ g/ml, respectively, against both Daudi and Jurkat cells. Melphalan (a standard chemotherapeutic drug) was tested as a positive control at 20.747 μ g/ml and 54.48 μ g/ml against Daudi and Jurkat cells, respectively. After 24 h, both treated and untreated cells were harvested by centrifugation at 2000 rpm for 5 min. Then, all cells were resuspended in ice-cold 1X PBS (free of Ca²⁺ and Mg²⁺) to avoid reduction of agarose, which adheres to the comet slide. A cell suspension (10 μ l) was mixed with 100 μ l of a low-melting-type agarose (melted for 5 min, then cooled to 37 °C in a water bath for at least 20 min before use), then transferred to a slide. All comet slides were placed horizontally for about 20 min at 4 °C under dark conditions. After that, the slides were soaked in a cold lysis buffer for 30 min, followed by decanting the lysis buffer, and the excess buffer solution was removed from each slide. After that, the slides were incubated for about 5 min in a (1X-TBE) buffer solution. Prepared slides were subjected to gel electrophoresis in TBE-buffer for 10 min at 21 volts. After removing the slides from the electrophoresis chamber, they were fixed with 70% EtOH for 5 min, followed by drying under dark conditions at room temperature. Diluted SYBR® Green was used to stain the slides for about 5 min at 4 °C. The slides were gently tapped to eliminate excess stain. Then, they were completely dried at room temperature under a dark condition. The stained cells were assessed using an inverted fluorescence microscope at a 40x magnification. A minimum of 50 cells on each comet slide were observed for DNA damage, including the tail moment, tail length, tail intensity, and the

percentage of DNA tails. Comet software was used on the micrographs for image analysis to investigate the DNA damage in the tested cells¹⁹.

Statistical analysis

In this study, tests were conducted in triplicate ($n = 3$), while data were obtained as mean $\pm$ SD values. Analysis of variance (ANOVA) was applied to assess the differences among the treatments by Duncan's multiple range tests (DMRTs) with a p -value of 0.05 using SPSS Statistics Base version 19 program for Windows (IBM Corp, Chicago, IL, USA).

RESULTS

Cytotoxicity effect

Cytotoxicity results of curcuminoids and Melphalan chemotherapeutic drugs on the Daudi and Jurkat cell lines are summarized in Figure 1. Our results show that the IC₅₀ values of curcuminoids are 19.339 μ g/ml and 46.58 μ g/ml for Daudi and Jurkat cells, respectively (Figure 1a-b). The toxicity data for Melphalan showed IC₅₀ values of 20.747 μ g/ml and 54.48 μ g/ml for the Daudi and Jurkat cell lines (Figures 1c-d).

Alkylation assay

Alkylating activity is a mechanism that indicates anticancer activity of a bioactive compound. 4, (4'-nitrobenzyl) pyridine (NBP) serves as a DNA model due to its similar reactivity with the guanine base in DNA. Additionally, NBP lacking reactive oxygen as DNA NBP is susceptible to alkylation in the presence of a suitable agent²⁰. During alkylation, NBP interacts with the alkylating agent to produce a pyridinium salt.

In alkaline media, this is converted to a blue colored hybrid product through loss of a proton. It exhibits maximum absorption at 600 nm (Figure 2).

The alkylating activity of the curcuminoids and Melphalan were compared. These results are shown in Figure 3. Melphalan was used as a +ve control as it alkylates DNA, leading to DNA strand breakage. The alkylating activity of Melphalan was time-dependent. Its activity increased with the reaction time. The curcuminoid solution was found to have some alkylating activity. However, its alkylating activity did not increase with reaction time.

DNA damage via DAPI staining

The apoptotic activity of curcuminoids on both the Daudi and Jurkat cell lines was 68.17 and %49.72, respectively. Additionally, the apoptotic potency of Melphalan was %93.08 against Daudi cells and %96.53 against Jurkat cells, as presented in Figure 4

Treatment of Daudi and Jurkat cells with curcuminoids increased the number of apoptotic cells compared to control cells. The morphological characteristics of treated cells show apoptotic activity. These morphological changes include chromatin condensation and DNA fragmentation. The control cells (both Daudi and Jurkat) treated with PBS show intact nuclei (Figures 4A and B), while treated cells (curcuminoids or Melphalan) showed clear nuclear fragments as signs of apoptosis, evidenced by DAPI staining.

Comet assay under neutral conditions (gel electrophoresis of single cells)

Comet test procedures are increasingly used worldwide for detection purposes. They are an accurate and sensitive tool to identify DNA impairment in a wide spectrum of cell categories. The comet assay is an efficient technique in medical and cancer research²⁸. The neutral condition-based comet assay or electrophoresis of single cells is an accurate and sensitive technique for evaluating double-strand breakage (DSB) of DNA during apoptosis²⁹.

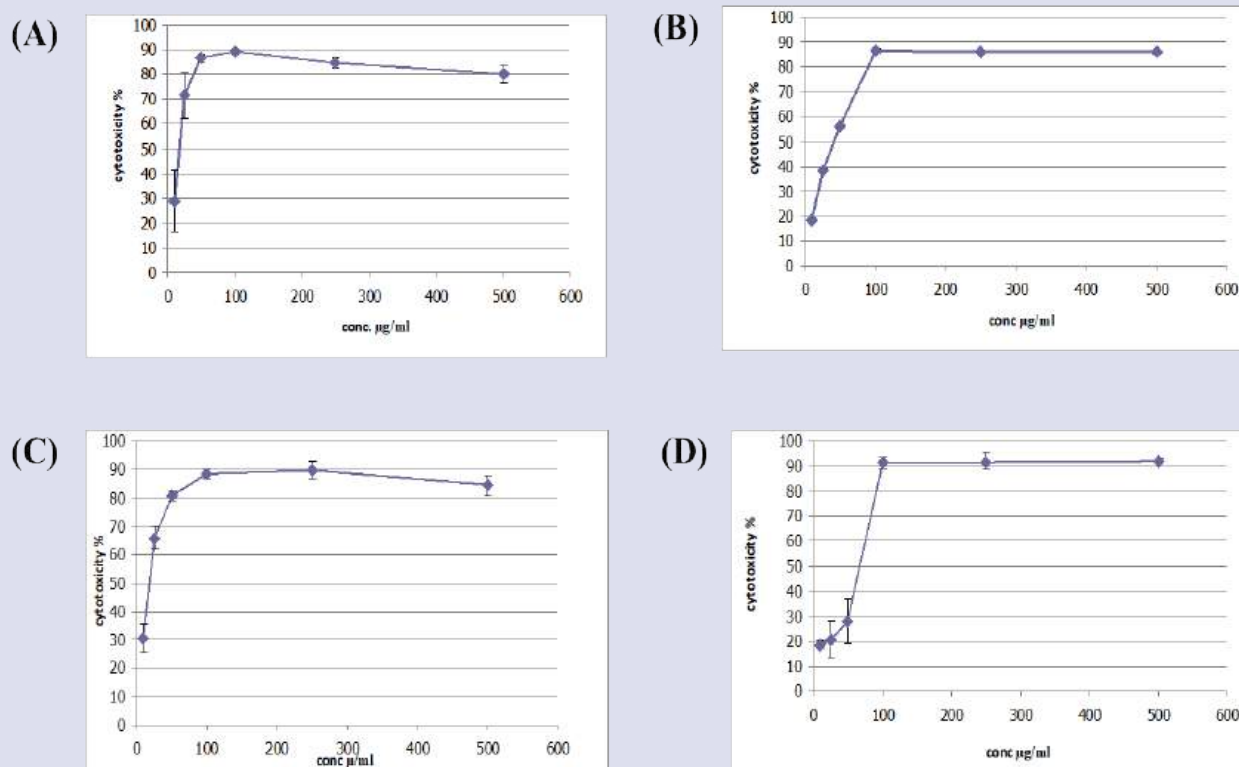


Figure 1. Cytotoxic activity (IC₅₀) of curcuminoids and Melphalan using an MTT assay; (a) 20.747 µg/ml against Daudi cells treated with curcuminoids, (b) 54.48 µg/ml against Jurkat cells treated with curcuminoids, (c) 19.339 µg/ml against Daudi cells treated with Melphalan, and (d) 46.58 µg/ml against Jurkat cells treated with Melphalan.

4-(4'-nitrobenzyl)pyridine (NBP)

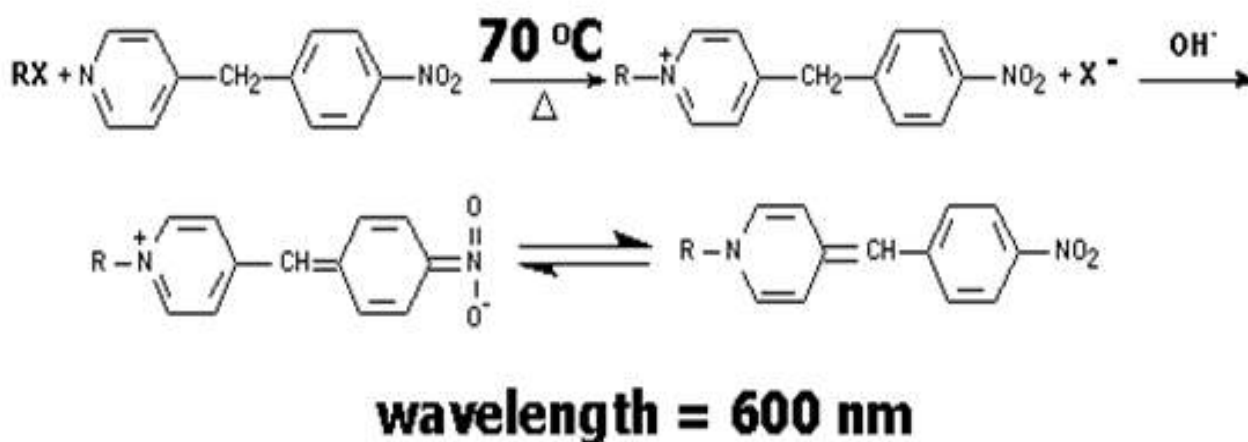


Figure 2. Schematic diagram representing the alkylation reaction.

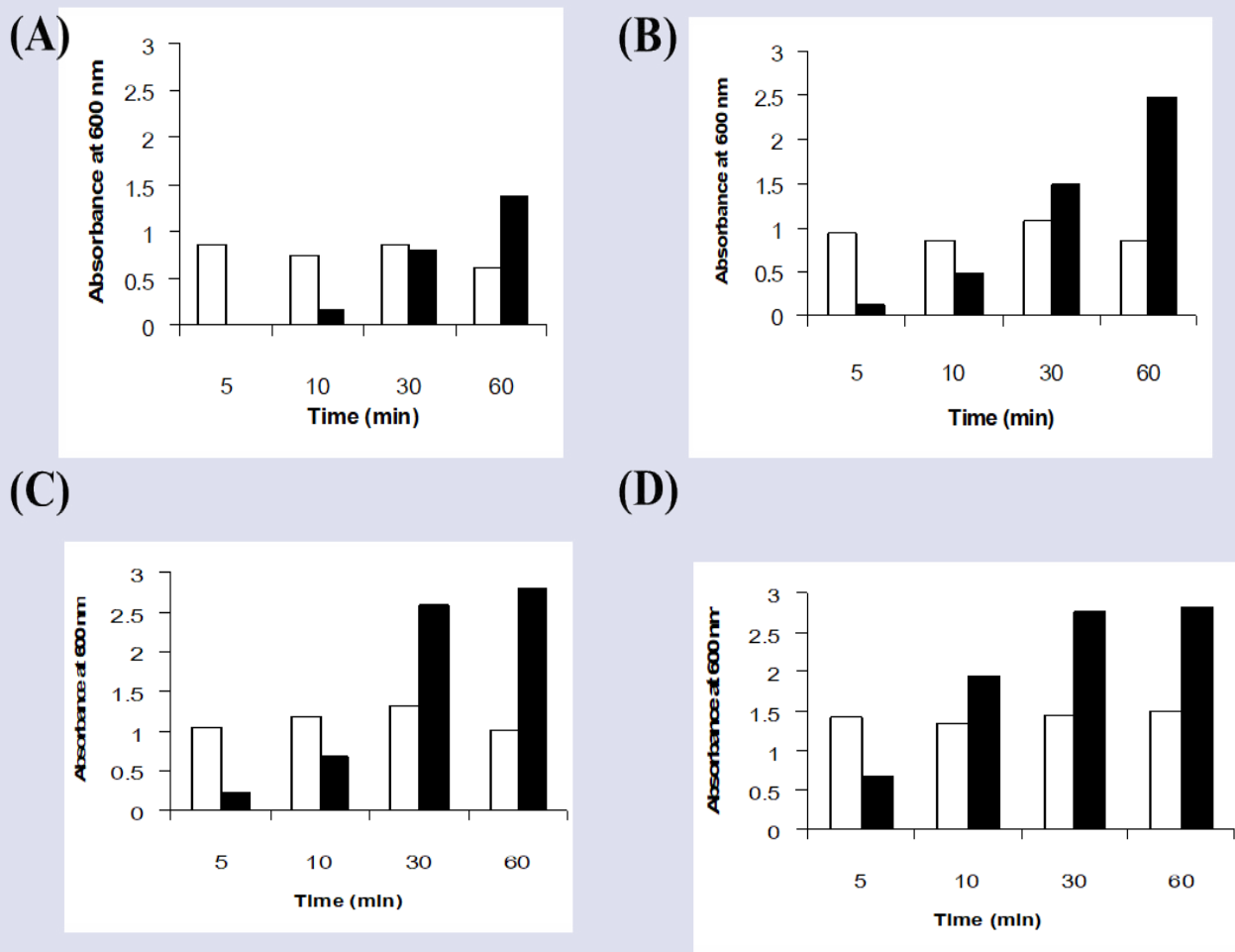


Figure 3. Alkylating activity of curcuminoids (?) and Melphalan (■) at various concentrations. (a) 50 g/ml. (b) 100 g/ml. (c) 250 g/ml. (d) 500 g/ml. The alkylating activity was determined using an NBP assay for various times (5, 10, 30, 60 min).

Table 1. Levels of DNA damage (mean± SD) evaluated by the comet assay in Daudi and Jurkat cells treated with curcuminoids, phosphate buffered saline (PBS), and Melphalan.

Cell line	Parameters	Tail intensity	Tail length	Tail moment	Tail DNA%	Apoptosis (%)
Daudi	Control -ve (PBS-treated)	7.15±0.91	3.11±0.90	1.2±0.45	727±12.83	4.49
	Curcuminoids-treated	18.45±2.15	9.85±2.90	4.87±1.16	2546±14.66	72.44
	Mel. treated	28.42±4.89	17.33±3.66	7.25±3.22	3101.34±20.51	100
Jurkat	Control -ve (PBS-treated)	5.25±0.61	4.01±1.10	2.02±0.67	607±10.63	3.45
	Curcuminoids-treated	16.25±1.18	8.45±1.95	4.39±0.89	1746±19.63	67.95
	Mel. treated	26.32±3.11	18.65±4.02	7.07±2.11	2878.34±17.31	100

The mean tail intensity, tail length, and tail moment values were 2.15±18.45, 2.90±9.85, and 1.16±4.87 for Daudi cells, and 1.18±16.25, 1.95±8.45, and 0.89±4.39 for Jurkat cells treated with curcuminoids after 24 h, respectively. Statistically significant differences ($p<0.05$) were found between curcuminoids (tested sample) and Melphalan (positive control), which is used in the current study as an alkylating chemotherapeutic drug, as shown in Table 1. However, no statistically significant differences ($p>0.05$) were detected between DNA damage profiles of both types of tested leukemic cell lines (Daudi and Jurkat) treated with curcuminoids. Figures 4 and 5 show DNA damage by the DAPI stain and comet assay for treated Daudi and Jurkat cells. There

were some specimens with no comet cell nucleus (tail) for both cell lines treated with curcuminoids. Most cells treated with Melphalan showed actual comet cells and DNA damage. The data presented in Table 1 summarizes the apoptosis results of both the Daudi and Jurkat cells by curcuminoids and Melphalan using the comet assay.

DISCUSSION

DNA-alkylating factors are a recognized class of agents that cause DNA damage in a DNA-targeting alkylation process. They add alkyl groups to particular bases, leading to alkylation products, including

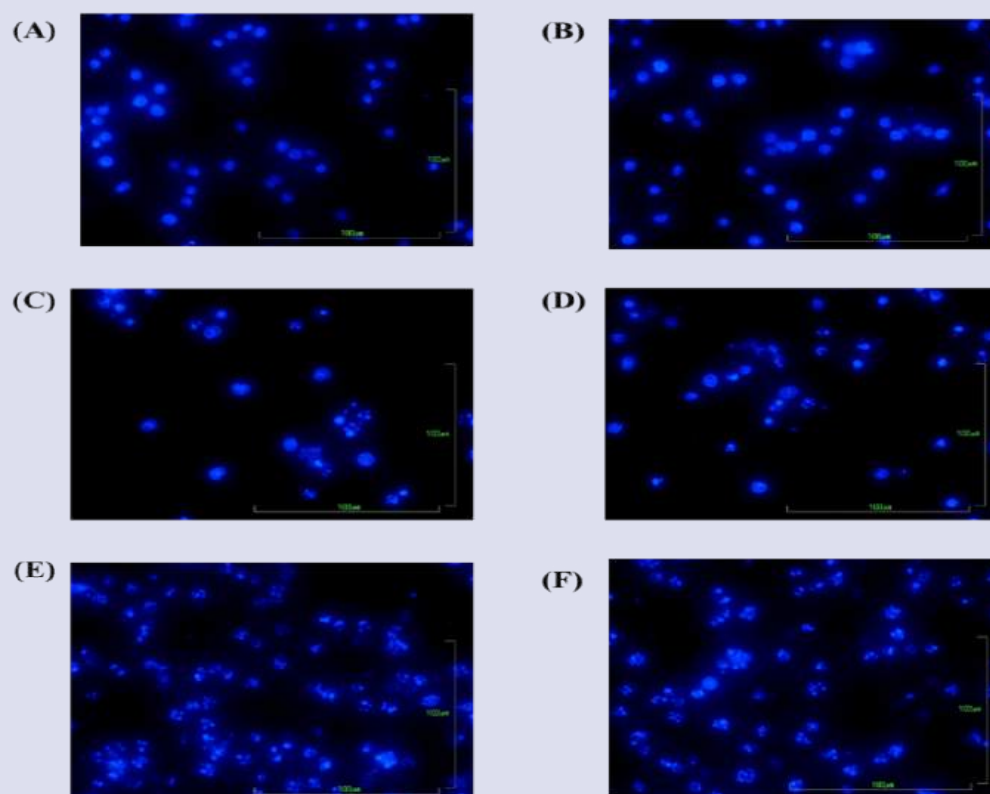


Figure 4. Cells stained with DAPI under fluorescence microscopy: (A) untreated Daudi ; (B) Jurkat cells; (C) Daudi cells treated with curcuminoids; (D) Jurkat cells treated with curcuminoids; (E) Daudi cells treated with Melphalan; (F) Jurkat cells treated with Melphalan.

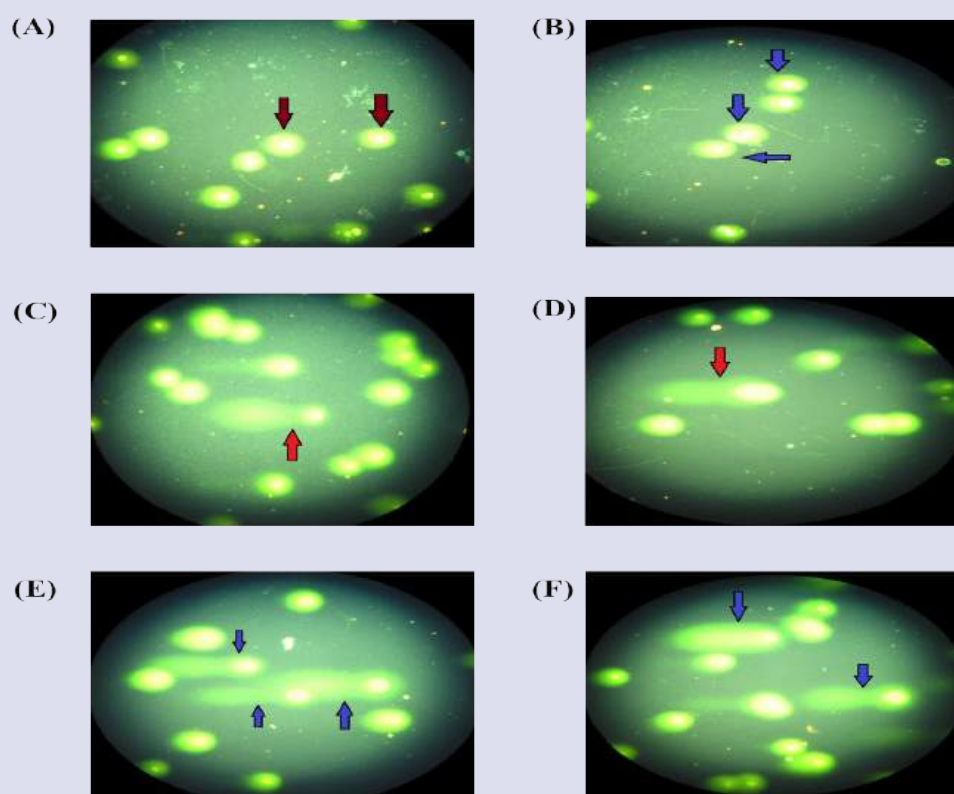


Figure 5. Fluorescence microscopy imagery. (A) PBS-treated Daudi . (B) Jurkat cells. (C) Daudi cells treated with curcuminoids show cells with comets. (D) Jurkat cells treated with curcuminoids show cells with comets. (E) Daudi treated with Melphalan. (F) Jurkat treated with Melphalan. The -ve control cells have intact nuclei compared to treated cells that possess comet nuclei as a sign of DNA damage.

O2-alkylthymine and O4-alkylthymine. This therapeutic mechanism induces DNA damage in leukemia cells due to DNA alteration. Alkylation mechanism-based chemotherapeutic drugs act in all phases of the cancer cell cycle. They are employed to inhibit various cancers, such as those of the lung and breast, in addition to leukemia, lymphoma, multiple myeloma, and sarcoma.¹

While the findings from the NBP assay offer preliminary proof of the alkylation capacity of various curcuminoids, it is necessary to consider certain limitations of this method. The NBP assay is an extremely rudimentary cell-free experimental setup which employs a very simple nucleophilic target, thus missing many aspects of real cellular machinery – e.g., the double-helical steric hindrance of DNA, the protective histone conformation, and the chromatin organization. This means that once the positive outcome of the test was achieved, the chemical interaction was noticed, but there is no assurance that this alkylation process will still occur in the live cell nucleus. This fact demonstrates the necessity of conducting additional experiments in regard to live cells, such as Comet assay and tests for cytotoxicity.

Various alkylating agents are used in chemotherapy including Chlorambucil, Cisplatin, Mechlorethamine, Oxaliplatin, Temozolomide, Thiopeta, Trabectedin, and Melphalan. Melphalan binds to DNA, promoting DNA lesions, leading to cancer cell death.

Melphalan is still frequently used at regular doses to treat multiple myeloma and leukemia. When used together with the inhibitors of CRM1 (KOS-2464, KPT-185, KPT-249) or the medical compound KPT-330, Melphalan, synergistically encourages toxicity in myeloma and leukemia cells²¹.

Melphalan has severe side effects, including lung disorders such as coughing, wheezing, and breathing problems, as well as allergic reactions that lead to skin rashes, irritated skin, and swelling. This can occur when administering Melphalan via the bloodstream, and it is usually more frequent in the first or second dose. Some allergic reactions are life-threatening. They include skin and/or eyes becoming yellow as well as the patient feeling exhausted, short of breath, and dizzy. For these reasons, researchers are working to find alternative alkylating agents with the same effect on cancer cells, but are eco-friendly and cause no health problems.

Curcumin, the main active compound in turmeric, has shown an alkylating effect against leukemic cells. It contains phenolic compounds that work by interfering with the genetic material of malignant cells. It adds an alkyl group to the DNA of cancer cells, thereby destroying them. Alkylating substances have an electrophilic character with covalent binding to electron-abundant functional groups, including particular positions of guanine, such as O-6, N-1, and N-7.^{1,22}

Di-thiolethiones, chalcones, curcuminoids, quinones, coumarins, and terpenoids are a class of Michael acceptors. These acceptors are a set of molecules that possess double bonds close to electron-removing groups. A specific molecule containing an α , β -unsaturated carbonyl group is a Michael acceptor. Curcumin, a natural polyphenolic molecule, is a key compound of curcuminoids obtained from curcuma rhizomes. Curcumin has two α , β -unsaturated residues linked to a carbonyl, thus, it is considered a Michael acceptor. Curcumin usually presents two tautomers, the enol and keto forms. The keto form is solid, while the enol is liquid.^{12,23}

The configuration and dynamics of genetic material are sensitive to the alkylation process of its nitrogen bases, with several consequences including (i) inhibition of DNA proliferation, (II) fragmentation of DNA, (iii) promoting nucleotide base mismatches by destroying the typical hydrogen bonds between nitrogen bases, (iv) intra-catenary cross-linking caused by a bis-alkylation process, (v) crosslinking between complementary strands of DNA can inhibit their parting

during replication or transcription of DNA, and (vi) cross-linking between DNA and related proteins.²

The association between oncogenic DNA and the alkylation activity of curcuminoids can be employed to provide strong cytotoxicity against acute leukemic cell lines through induction of the apoptotic pathway. This serves as a protective barrier.

Finally, DNA-targeting alkylation is a process involved in treating cancer. Employing curcuminoids to induce apoptosis in cancer cells through alkylation activity is a promising and novel method to identify protective agents against cancer cells in early stages.

However, since curcuminoids create various types of DNA lesions in cancer cells, it is reported to possess different anticancer mechanisms that employ DNA damage. So, it has been difficult to determine which particular lesion causes signal apoptosis. However, this study suggests that alkylating may be a probable apoptosis inducer.

Curcuminoid analogues may promote apoptosis of LN-18 cells by initiating an early induction of DNA damage, then directly affecting the mitochondria, resulting in mitochondrial dysfunction²⁴. Jiang. et al., 2015 found that curcumin (the main component of curcuminoids) triggers apoptosis via a mitochondrial pathway and caspase activation. Caspases (cytoplasmic aspartate-specific cysteine proteases) play a role in apoptosis.

From our experimental findings, it appears that curcuminoids have potent cytotoxic properties, present an ability to chemically alkylate, and induce physical breaks in the DNA strands of both Daudi and Jurkat leukemic cell types. It is highly probable that the damage brought about by the curcuminoids in this study may be the first in a series of causative processes resulting in apoptosis. The available literature provides ample evidence that the reported kind of physical damage to DNA and the genomic instability would be responding with the activation of the p53 protein which has a reputation of being a vital protector of the genome.

We propose that a similar downstream molecular pathway is triggered in lymphoid leukemia cells after primary DNA damage caused by curcuminoid treatment. However, it is necessary to distinguish our specific experimental evidence—specifically, direct cytotoxicity, chemical alkylating ability, and physical DNA damage—from hypothesized downstream processes. Although the morphological and genetic damage observed seems to correlate with known pathways, further molecular confirmation (e.g. Western blots for p53 or caspase levels or specific pathway inhibitors) will need to be conducted in future research in this experimental system²⁶. Phenolics are recognized as anti-cancer agents. Their mode of action triggers apoptosis for malignancy control. Phenolic compounds are widely distributed in nature. They are frequently consumed as fruits, vegetables, and beverages.

Malignant diseases frequently cause mortality. Developing countries increasingly conduct studies aiming to find anti-cancer agents or cancer-targeting treatments to control or prevent various types of cancer. The treatment plan for most cancers includes surgery, radiotherapy, and chemotherapy or combinations of these strategies. Natural bioactive products offer new active and eco-friendly biomolecules, with physiological effects²⁷.

The anti-cancer character of natural phenolics is most related to their biological activities, including the quenching effects against oxidative stress, genotoxicity, and chromosomal aberrations. Apoptosis induction is achieved by activation of some biochemical reactions that directly lead to cell death.

The apoptotic mechanism in cancer cells has been either mitochondrial-dependent or mitochondrial-independent³⁰. It is helpful to combine the apoptosis test via the DAPI staining technique with other nucleus-

toxicity assays, such as the comet assay. The comet assay is rapid and sensitive. It is a viable *in vitro* strategy for the investigation of DNA lesions. Curcumin (the main compound of curcuminoids) possesses high anti-cancer activity against various types of leukemic cells.

When interpreting the results regarding cytotoxicity and DNA damage, it is important to see curcuminoids as multi-targeting agents of their pharmacological activity. The *in vitro* chemical observations particularly from the NBP assay imply an alkylating-like property of the substance; however, this chemical property should not be considered to act independently of other signaling pathways in inducing cellular apoptosis. For instance, it is well known that curcuminoids cause oxidative stress via ROS induction and induce inhibition of topoisomerases, both leading to double-strand breaks and genomic instability, which is evident from the Comet assay. Thus, the alkylating potential of curcuminoids should be considered as one of the contributing chemical insults participating in combination with the mitochondrial and enzymatic biochemical pathways in the induction of apoptosis in Daudi and Jurkat cells.

To investigate the extent of physical genomic damage induced by curcuminoids, the Comet assay was performed. The results from this assay clearly confirm that treatment with curcuminoids causes considerable DNA breaks and extensive genomic instability in Daudi and Jurkat cell lines, as evident from the increased values of tail length and tail moment. However, it is important to consider that even though the Comet assay is a very sensitive method for detecting DNA breaks, it cannot differentiate between the underlying mechanisms.

Genomic damage cannot be attributed solely to chemical alkylation or to DNA adduct formation; rather, such damage is likely the sum of various overlapping effects such as alkylation, increased oxidative stress due to increased ROS levels, and active cleavage by enzymes during apoptosis.

When assessing the anticancer properties of the curcuminoids, Daudi (a human B-lymphoblastoid cell line) and Jurkat (a human T-lymphocyte cell line) were deliberately chosen based on their relevance in regards to human hematologic cancers such as leukemias and lymphomas. In general, lymphoid malignancies tend to have high susceptibility to DNA-damaging agents and to chemical damage, which allows for faster evaluation of new agents that are capable of inducing apoptosis.

Nevertheless, we should mention a weakness of our present experimental set-up: the lack of a healthy human cell line as a control sample, such as peripheral blood mononuclear cells (PBMCs), which will definitely strengthen the conclusions of the experiments made in the present work.

Although this logistical limitation exists, it is important to point out that the documented evidence related to the selective cytotoxic effects of curcuminoids on cancer cell lines compared to healthy cell lines is abundant in the literature. Previous studies that utilize hematological models consistently show that curcumin and its derivatives have a good selectivity index by activating apoptotic cascades in leukemic cells while leaving normal lymphocytes unaffected. Hence, while our data fully characterizes the active anti-leukemic effects against Daudi and Jurkat cells, the future clinical application of the obtained data will surely gain from the conducted *ex vivo* safety experiments on normal human cell lines.

The effectiveness of curcumin depends on the concentration, category of cells, and length of treatment. Curcumin can liberate mitochondrial cytochrome-c, induce both caspases and Bax, in addition to down-regulating the Bcl-2 level. Additionally, curcumin inhibits the expression of some genes, such as the Wilms' tumor-1 (WT-1) gene, which is one of the highly expressed genes in leukemia of both lymphoid and myeloid origins. This protein is an indicator of leukemic cells³¹.

In order to understand these findings, one must recognize that although the NBP assay points toward its chemical alkylating properties *in vitro*, the damage to genomic DNA in a live system is a very dynamic process that occurs through multiple, overlapping pathways. Curcuminoids, which have found extensive use in medicine as a pleiotropic agent, impact the generation of double- or single-strand breaks that we have detected in the Comet assay by other means than through chemical alkylation itself.

The most important among these pathways is the production of ROS. Cancer cells, including lymphoma cells, demonstrate altered metabolic processes that contribute to their vulnerability to oxidative stress. Curcuminoids were shown to cause an increase in the level of ROS in the cells almost immediately after exposure to curcuminoids, resulting in oxidative DNA damage including generating 8-oxo-7,8-dihydroguanine that destabilizes the double-stranded structure of DNA and results in the occurrence of single-strand breaks.

In addition, research reveals that curcuminoids may function as inhibitors of Topoisomerase II, a key enzyme in relieving torsional strain during DNA replication and transcription. By sequestering the cleaved topoisomerase-DNA complexes, curcuminoids inhibit DNA relegation and convert temporary single-strand breaks to permanent, lethal double-strand breaks.

Consequently, the cytotoxicity detected in Daudi and Jurkat cell lines in the study can be interpreted as an accumulation of synergistically acting insults. The possible alkylating-like activity detected in the compounds might work together with ROS-related oxidation and inhibition of the enzyme activity to create an overload of the cellular DNA repair machinery.

Curcuminoids are a group of active compounds in turmeric. They present several health advantages, including chemopreventive properties. It is crucial to determine whether curcumin is more effective for humans if consumed individually or as a mixture of curcuminoids. Bioavailability and anti-proliferative activity against cancer cells, as well as phytotherapeutic efficacy of curcuminoids can be significant.

CONCLUSIONS

In conclusion, the present research has provided evidence that curcuminoids have powerful cytotoxic properties towards both Daudi (B-lymphoblastoid) and Jurkat (T-lymphocyte) leukemia cell lines, the latter being specifically chosen as representative and clinically relevant models for human lymphoid cancers that are sensitive to DNA damage. The anticancer effects observed, which are manifested via DNA damage and indicated by apoptosis, as supported by DAPI staining and Comet assays, appear to take place through a multi-target mechanism.

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