

ANTI-CANCER EFFECT OF *CURCUMA LONGA* ON LEUKEMIC CELL LINES EVALUATED BY APOPTOSIS AND COMET ASSAY

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ABSTRACT

Objective: The aimed of this study was to evaluate Ethanolic turmeric extracts (ETE) as anticancer agent by detect the apoptotic induction and DNA damage activity of ETE which were investigated against two human leukemic cell lines, U937 (human monocytic leukemia cell line) and Molt4 (human lymphoblastic cell line).

Methods: ETE obtained by extraction of turmeric by ethanol solvent, apoptosis activity was performed by using 4, 6-diamidino-2-phenylindole dihydrochloride [DAPI] stain; also comet assay which is sensitive method of detection double strand break (DSB) of DNA was used.

Results : showed that apoptotic activity of ETE against U937, Molt4 cell lines were 58.06% and 39.07% respectively while apoptotic activity of Melphalan were 92.04% and 89.03% respectively by using DAPI stain, otherwise apoptotic percentage of ETE were 92.44% and 61.95% against U937, Molt4 respectively while by Melphalan were 100% against both of leukemic cell lines which evaluated by comet assay.

Conclusion: Ethanolic turmeric extracts (ETE) showed that have apoptogenic and DNA damage activity against two human leukemic cell lines. Otherwise DAPI stain and comet assay prove to be suitable tools to detect DNA damage of U937 and Molt4 leukemic cell lines by ETE.

Keywords: *Curcuma longa*, Apoptosis, Comet assay, U937, Molt4, Leukemia.

INTRODUCTION

Curcuma longa which is a perennial herb classified to the Zingiberaceae family which categorized under the class Monocotyledons. The dried rhizome of this erect herb is called turmeric. Curcuminoids consider as major active compound of turmeric, which responsible for the yellow color of it recognized as a rich source of phenolic compounds. curcuminoid complex consist of three main components namely, curcumin, demethoxycurcumin, and Bisdemethoxycurcumin [10]. Curcumin which is major component of curcuminoids has the ability to inhibit the proliferation of many cancer cells, such as leukemia, it may inhibit proliferation of tumor cells by inducing apoptosis [8]. Otherwise a chemotherapy drug which named melphalan is categorized under the class of nitrogen mustard alkylating agents, can hinders the growth of cancer cells [4]. Apoptosis is a programmed cell death and. It considered as a one of the main protective mechanisms against cancer cells by play an essential role in eliminate cancer cells which genetically damaged cells both of initiated cells or cells progressed to malignancy [13]. And the tools for the detection of apoptosis include: DAPI staining (a DNA-binding fluorochrome, is widely used to stain DNA of permeabilized cells) and comet assay (is a method used for determined or measuring DNA damage at the level of each individual cell), There are two condition used in comet assay alkaline and neutral conditions, alkaline comet assay enable to detect wide range of DNA lesions including, single-strand breaks (SSBs) with alkali labile sites (ALSs), DNA-DNA and DNA-protein cross links and specific types of base disorders. While the neutral comet assay will detect mainly double-strand breaks and can be useful for assessing the DNA fragmentation associated with apoptosis [19].

MATERIALS AND METHODS

Ethanolic turmeric extracts (ETE) preparation

Turmeric rhizomes were purchased from a local market in Baghdad. After powdered Dry rhizomes by electrical grinder, 20 grams of the powder were soaked in 100 ml of petroleum-ether for 24 hr. then evaporated and the residue were extracted in 100 ml of the solvent (95% ethanol) using the Soxhlet apparatus at 50 °C. The crude extract evaporated at 45°C using oven and the resultant crude extract was collected and stored at 4°C until use to prepare the required doses and concentrations [16] and [11].

Cell cultures

U937 (human monocytic leukemia cell line) and Molt4 (human lymphoblastic cell line) were obtained from the Center for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Thailand. The cells were cultured and routinely maintained in RPMI 1640 medium supplemented with Penicillin-streptomycin (1% v/v) (Gibco, UK) and 10% heat inactivated fetal bovine serum and were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ inside a CO₂ incubator.

DAPI staining

In order to investigate apoptosis induction, U937 and Molt4 were treated with ETE and melphalan (used as a positive control) then detected by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining techniques according to the method described by Jang [5] with some modifications. One hundred microliters of cells were cultured in 24 well tissue culture plate at density 1 x 10⁶ cell/ml for 24 h, and then cells treated with the concentrations of ETE at (38.66 and 93,16µg/ml) for U937 and Molt4 respectively and with standard anti-cancer drug Melphalan at concentration 100 µg /ml depending on our previous cytotoxicity results. After 24 h from treatment, cells were collected by centrifuge at 2,000 rpm for 5 min and fixed by added methanol and incubated at 4 °C, ethanol then removed by centrifuge at 2,000 rpm for 5 min and 100 µl of DAPI solution was added for 60 min in dark. DAPI then removed by centrifuge at 2,000 rpm for 5min and re-suspended with of glycerin: PBS solution in ratio [1:1], after that, the cells were observed at (40×magnify) with inverted fluorescence microscope by place 10 µl of cell suspension onto the center of glass slide and cover with cover-slip. Apoptosis was quantitated by morphological change after DAPI staining with counting approximated 500 cells for each slide which was represented one sample.

Neutral Comet assay (Single cell gel electrophoresis)

The neutral comet assay was carried out by using Trevigen comet assay kit (catalogue No. 4250-050-K) which includes comet slide, low melting agarose, lysis solution, and SYBR green. Briefly, Cells were cultured in 96 well tissue culture plate at density 5 x 10⁵ cell/ml for 24 h, then treated with ETE, standard curcumin and Melphalan (a positive control), concentrations of ETE at (38.66 and 93,16µg/ml) for U937

and Molt4 respectively compared with standard anti-cancer drug Melphalan at concentration 100 µg /ml as suggested by our previous cytotoxicity results for each, After 24 h from treatment, cells were harvested by centrifuge at 2,000 rpm for 5 min. Re-suspended cells with ice-cold 1X PBS (Ca⁺⁺ and Mg⁺⁺ free) in order to prevent reduce the adhesion of agarose on the comet slide by cell culture media. Ten microliter of cell suspension was combined with 100 µl of low melting agarose (low melting agarose was melt in boiling water for 5 mint and then cool in a 37 °C water bath for at least 20 min before use) and transfer to comet slide. Slides were then place flat for 20 min in dark at 4 °C. Then slides were immersed in ice-cold lysis solution for 30 min, after that slide was removed and drain excess lysis buffer from slide and incubate for 5 min in 1X TBE buffer. The slides were subjected to Electrophoresis in TBE buffer at 21 volts for 10 min. Slides were removed from the electrophoresis chamber and fixed for 5 mints with 70% ethanol, followed dried slides at room temperature in the dark. Then slides were stained with diluted SYBR® Green for 5 min at 4 °C, and then slides gently tap to remove excess SYBR® Green and dry completely at room temperature in dark. Then the cells were observed at (40×magnify) with inverted fluorescence microscope. One hundred randomly selected cells were count per sample to quantify the apoptotic cell percentage. Then scored was calculate from the ratio of (L/W) comet to determined the comet index (CI). Scored range from 1.2 to2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage (HD) [2].

RESULTS

DAPI staining

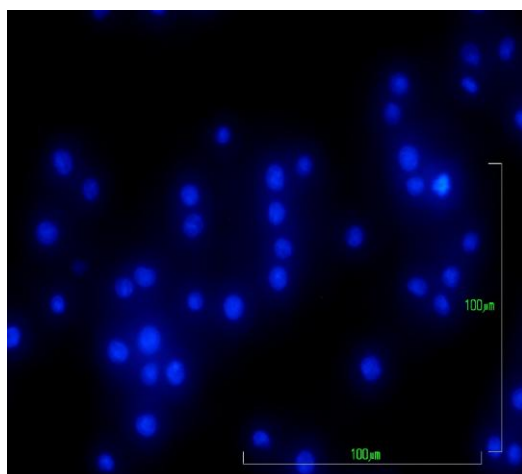
Cancer is considering the most common causative agent of death in the world so it is not surprising that most developed and developing

countries gave significant spot of light focusing on discover drug or agent targeting to treat or prevent cancer and increasing studies in this field since the last forty years until now. The treatment of cancer involves surgery, radiation and chemotherapeutic drugs or often a combination of two or all these three treatment is used. Natural products provide an appreciable percentage of new active lead molecules, clinical candidates and drugs despite competition from different methods of drug discovery [15].

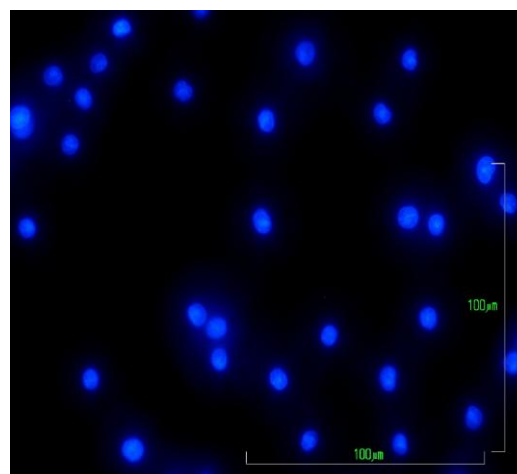
Phenolic compounds have wide range of spreading e in the nature and consuming by humans through diet such as fruits, vegetables and beverages. These phenolics which have human consume are well known as chemopreventive agents against cancer and their mode of action as inducers for apoptotic mechanism in cancer cells suggest their potential effect in a strategy of cancer control [14]. Curcuminoid, a natural coloring agent, is recognized as a rich source of phenolic compounds, consisting of three different compounds: curcumin, demethoxycurcumin and bisdemethoxycurcumin [17].

Curcumin is the major ingredient of *Curcuma longa* rhizomes named turmeric found that inhibit proliferation activities of tumor cells *in vitro* [18].

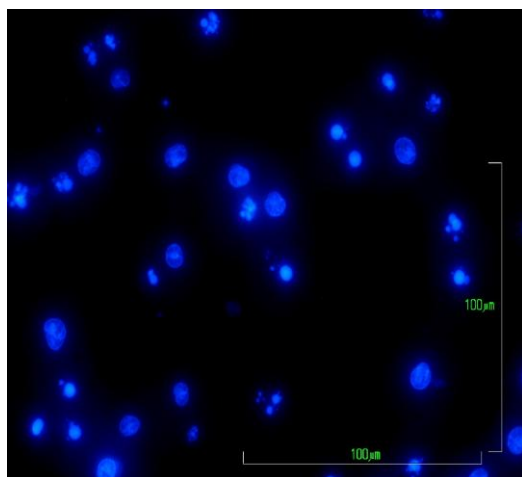
The treatment of ETE caused increased in number of apoptotic cells over the control, Morphology of treated cells suggest that the mode of action of phenolics against tumor cells caused by apoptosis. These morphological properties such as nuclear fragmentation and condensation of chromatin were clearly proved. In this study the apoptotic activity of ETE against U937 and Molt4 cell lines were 58.06% and 39.07% respectively while apoptotic activity of Melphalan were 92.04% and 89.03% against U937 and Molt4 respectively as shows in figures (1).



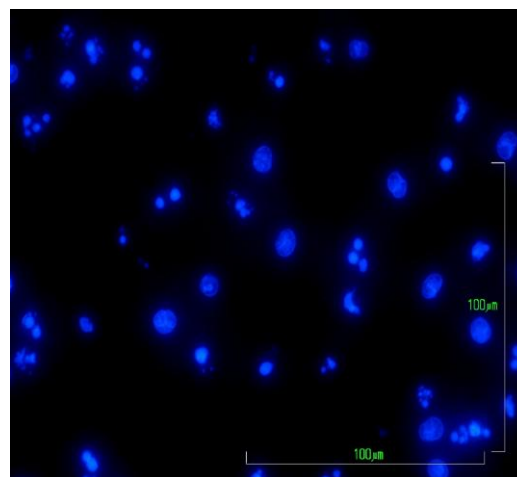
(A)



(B)



(C)



(D)

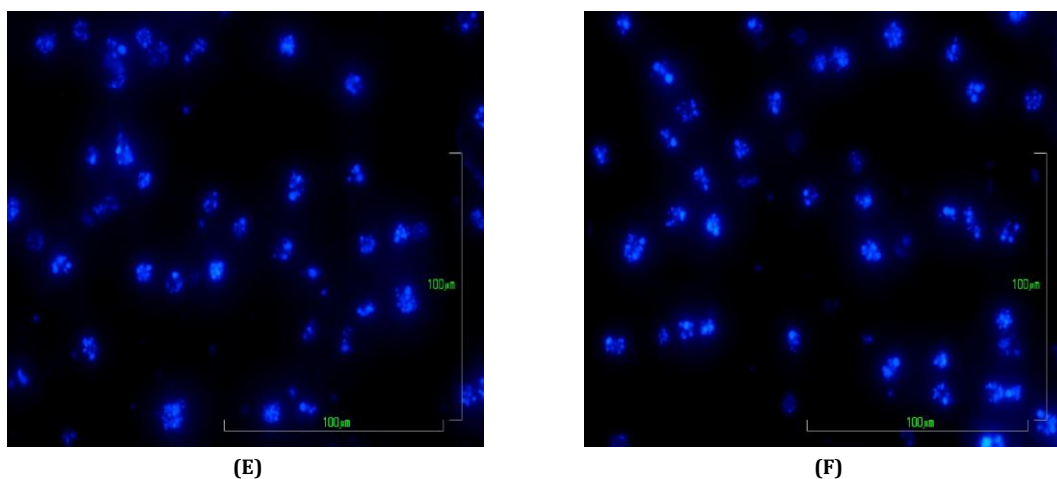


Fig. 1: Fluorescence microscopic images of untreated control U937 (A), Molt4 cells (B) while (C) and (D) ETE treated U937 and ETE treated Molt4 cells respectively, (E) and (F) showed Fluorescence microscopic images of Melphalan (which used as standard reference anti-cancer drug) treated U937 and Molt4 cells respectively. The control cells were with intact nucleus whereas treated cells showed intense comet fragments of nucleus as signs of apoptosis by DAPI staining.

Cytoplasmic aspartate-specific cysteine proteases called Caspases, has an effective role in apoptosis. Otherwise activation of these caspases has been shown direct role for many of the structural and molecular changes in apoptotic cells including degradation of DNA repair enzyme such as poly (ADP) ribose polymerase (PARP) [14].

Neutral Comet assay (Single cell gel electrophoresis)

The comet assay has been growing internationally in use and developed into a recognized precise technique to detect damage of DNA in wide range of various cell types during the last 20 years. It is believed that the comet assay is has high effective tool in clinical and tumor research [12]. The neutral comet assay or electrophoresis for

single cell is a sensitive precise method of detection double strand break (DSB) of DNA caused by apoptosis [7].

In this study followed some investigators to analyze the results by a manual method using ruler in photo of cell on computer monitor to quantify DNA damage caused by apoptosis based on scoring the comets into categories [6]. For categorical data, we followed Cok [3] method. The scoring is conducted in three levels of DNA damage are assigned ranging from 1.2 to 2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage. Results revealed that ETE caused the three levels of DNA damage against two leukemic cell lines as shows in figure (2)

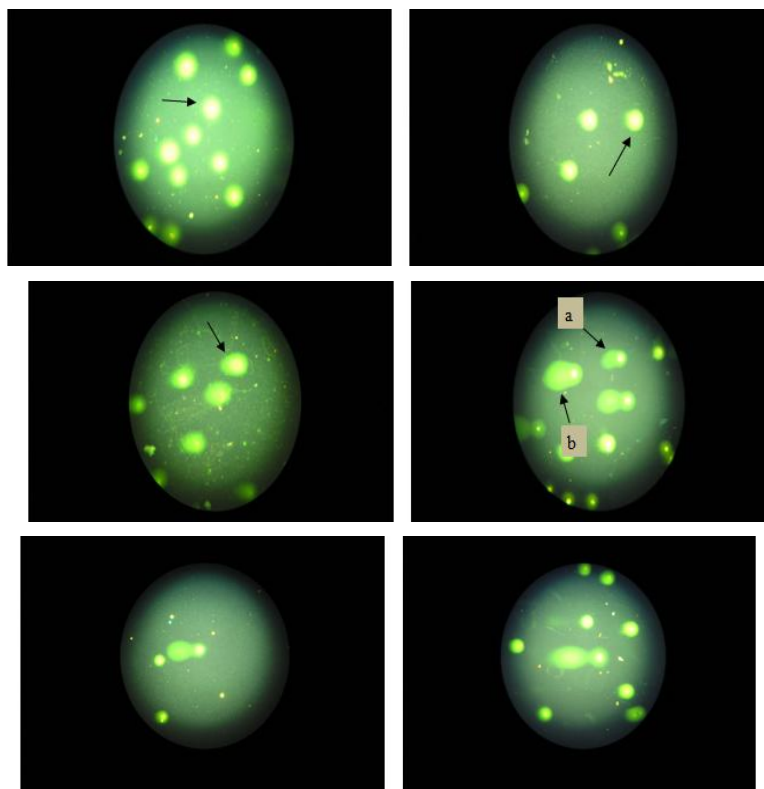


Fig. 1: Fluorescence microscopic images of untreated control U937 (A), untreated control Molt4 cells (B) and ETE treated leukemic cells (U937 and Molt4) which showed low damage (C), medium damage {a} and high damage {b} (D) while (E) and (F) showed Melphalan (which used as standard reference anti-cancer drug) treated U937, Molt4 cells respectively. The control cells were with intact nucleus whereas treated cells showed intense comet of nucleus as signs of DNA damage which evaluated by using Comet assay.

Pathway of apoptosis is related to up- or down-regulation of unique genes including p53 and bcl-2. The chemopreventive property of the natural phenolic compounds is mainly focused to their anti-oxidative, anti-chromosomal aberration and anti-genotoxic activities. Inducing of apoptosis depending on activation of a pre-programmed pathway of biochemical reactions that directly lead to cell death, curcumin induces apoptosis and possess anti proliferative of a various of cancer cells, including breast cancer, colo-rectal carcinoma, hepatocellular carcinoma, leukemia, and lymphoma. Apoptotic mechanism of Malignant cancer cell lines [14] could be either mitochondria-dependent or mitochondria-independent [1]. It is usefulness to combine apoptosis technique by DAPI staining with other genotoxicity assays, such as comet assay, to obtain a more comprehensive understanding.

This study reveals that the comet assay is a rapid and potential method for detection of Deoxy nucleic acid damage, Curcumin was found to be highly inhibition effect in nature to a wide range of several of human leukemic cell lines. Results in table (1) summarized Score range and apoptosis % of ETE; Melphalan on two U937 and Molt4 cell lines by comet assay technique.

Table 1: Score range and apoptosis % of ETE; Melphalan on two leukemic cell lines by comet assay technique

Cell line	Score rang %	(ND)* No damage	(LD)** Low damage	(MD)** Medium damage	(HD)** High damage	Apoptosis %
U93	Non-treat	86.76%	13.24%	0.00%	0.00%	13.24%
	ETE treat	7.56%	71.11%	15.17%	6.16%	92.44%
	Mel. Treat	0.00%	83.76%	4.73%	11.5%	100.00%
Molt4	Non-treat	96.55%	3.45%	0.00%	0.00%	3.45%
	ETE Treat	38.05%	55.59%	2.90%	3.46%	61.95%
	Mel. Treat	0.00%	75.05%	9.45%	15.5%	100.00%

*(ND) expressed as no damage while **(LD) expressed as low damage and ***(MD), ****(HD) expressed as medium damage and high damage respectively.

CONCLUSION

The presented results has been shown that preventive phenolics of ethanolic *Curcuma longa* extract exhibited anti-cancer activity and capable of inducing apoptosis by acting as a potent growth suppressive agent against leukemic cells which evaluated by DAPI stain and comet assay.

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