

Cytotoxic Effect of *trans*-Cinnamaldehyde from *Cinnamomum osmophloeum* Leaves on Human Cancer Cell Lines

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Abstract: Quantitative determination of *trans*-cinnamaldehyde (TCA) was conducted by reversed-phase HPLC from young and mature leaves, and leaf branches of *Cinnamomum osmophloeum*, a Taiwan endemic plant. The results showed that highest yield, 23.79 mg/g of TCA (the tree's age was three years) was obtained in the two year old mature leaves. In addition, cytotoxic and inhibitory effects of TCA was evaluated against selected human cancer cell lines such as Jurkat, U937, and normal cell lines primary purified T cells and macrophages. The results revealed that TCA exhibited potent inhibitory activity against Jurkat and U937 cell viability, and found that the IC₅₀ values were 0.057, 0.076 μ M, respectively. In parallel, no effect on the viability of primary purified T cells and macrophages. Moreover, interestingly at 0.095 μ M, TCA inhibited proliferation of both Jurkat and U937 cell lines approximately 2-fold at 0.057 μ M, compared to controls. In contrast, TCA increases approximately 26% proliferation of mitogen-stimulated human peripheral blood mononuclear cells (PBMCs) during the concentration range studied. Furthermore, by cell cycle analysis, we found that TCA altered the cell cycle phase distribution of Jurkat and U937 cells in a nonlinear concentration-dependent fashion. Taken together our results suggest that TCA may be a useful chemotherapeutic agent for cancer treatment in human.

Keywords: *Cinnamomum osmophloeum*; *trans*-cinnamaldehyde; cytotoxic; Jurkat cell; U937 cell; PBMCs.

1. Introduction

Cancer is one of the major human diseases and causes considerable suffering and economic loss worldwide. Selective destruction of tumor cells without damaging normal cells is an important goal for cancer chemotherapy in the 21 st century. Plants, sources of phytochemicals with anticancer potential to inter-

fere with targets implicated in carcinogenesis and in tumor cell biology makes them interesting tools in cancer research [1]. Among these phytochemicals, phenolic compounds (e.g. flavonoids, catechols, phenylpropanoids, quinones, lignans, stilbenes and derivatives of gallic acid) are the most abundant in our daily diet and have received increasing attention in recent years because of the possible beneficial

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effect in the prevention of some human disease such as heart disease, chronic inflammation, as well as reduction of risk of many types of cancer [2-5], but there is still controversial opinions in this area. There are as yet no extremely effective drugs to treat most cancers; moreover many cancer treatments are very expensive.

Traditional Chinese medicine has been used for pharmaceutical and dietary therapy for several millennia. A number of anticancer Chinese medicinal herbs and many relevant prescriptions have been screened and used for treating and preventing various cancers during long-term folk practice, but it has been mainly practiced through clinical treatment and its chemical and pharmacological bases are not well understood. Because of rapid development and employment of modern analytical equipment and technology, natural active components with anticancer activity have been studied and identified from Chinese medicinal plants [6, 7].

Cinnamomum osmophloeum Kaneh. (Lauraceae) is an endemic tree that grows in Taiwan's natural hart wood forest at an elevation between 400-1500 m. This plant has been interest to researchers because the chemical constituents of its leaf essential oils were similar to those of *Cinnamomum cassia bark* oil, known as cinnamon oil, commonly used in the food and beverages, and was valuable in commerce. The chemical constituents of leaf essential oils were different from various *C. osmophloeum* clones found in different regions of Taiwan [8]. It was found that cinnamaldehyde was the major constituent in the leaf essential oils of some *C. osmophloeum* clones. This plant species were not only important as a spice, but in East Asia was considered to have various medicinal properties such as antipyretic, astringent, carminative, stomachic agents, antitermitic and antibacterial [9-12]. Despite of its excellent use in food industry *C. osmophloeum* currently has no reports on concentration variation of *trans*-cinnamaldehyde (TCA) and its cyto-

toxic and inhibitory effects. The current study was therefore conducted to determine quantitative TCA variation in different leaf samples and its cytotoxic and inhibitory properties.

Cinnamaldehyde was a low molecular weight cinnamic acid analogue with relatively broad distribution in plants and was present in various human foods including beverage, ice cream, sweets and chewing gum. It has been shown various activities such as peripheral vasodilatory, antitumor, antifungal, cytotoxic and mutagenic [13-15]. In addition, cinnamaldehyde showed alcohol dehydrogenases [16], glutathione S-transferase inhibition in human melanoma cells [17], anti-tyrosinase [18], and ATPases inhibition activity [19]. Furthermore, cinnamaldehydes also found to show cyclin dependent kinases (CDKs) inhibition activity [20]. These data have suggested that TCA might be effective chemotherapeutic agent and support the need for further studies on the effects of this molecule in both normal and tumor cells. In this study, we investigated the quantitative determination of TCA in different age leaf samples (young and mature leaves, and leaf branches) from different age of *C. osmophloeum* trees and evaluated its effect on cell viability, cell proliferation and cell cycle analysis in two human cancer cell lines such as Jurkat, U937 and normal cell line primary peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Plant material

The different age leaf samples (young and mature leaves, and leaf branches) from different age trees were collected in March 2003 from the Fenglin, Hualien county in Eastern Taiwan. The authenticity of the plant was confirmed by Forestry Bureau Council of Agriculture, Taiwan. The samples were shade dried and milled to powder form, which were then kept in air-tight brown bottle until use.

2.2. Quantitative determination of TCA

A 1 g of air dried and powder samples (young leaves, mature leaves and leaf branches) were taken for extraction of TCA. The samples were soxhlet extracted with 200 mL of EtOH for 5 h at 80-90 °C. The solid plant material was filtered out using No. 5A quantitative filter papers (Toyo Inc., Japan), and 2 mL aliquots from each filtrate were further filtered using 0.45 µm syringe filters (Whatman). The final filtrates were used for the quantitative determination of TCA by HPLC (high-performance liquid chromatography). Commercially available TCA (Sigma) was used as standard. The standard curve for TCA was prepared using concentrations ranging from 0 to 10 mg/L.

Separation and quantification of TCA was carried out by HPLC. The HPLC system consisted of a Hitachi L-7455 diode array UV detector, an L-7200 auto-sampler, an L-7100 pump. A BDS-C18 column, 100×4 mm (3 µm, Hewlett-Packard), was adapted to the instrument. The solvents used for the separation were as follows: solvent A, acetonitrile; solvent B, HPLC-grade water. The gradient solvent system was as follows: 0 min, 5% A, 100% B; 35 min, 70% A, 30% B. Five microliters of extract was injected into the HPLC system after filtration on a 0.45 µm Millipore membrane. After each analysis, the column was re-equilibrated with phase A for 10 min. Detection of TCA was at 254 nm, and measurements were carried out in duplicate. The concentrations of TCA in different samples are given in Figure 1.

The peak on HPLC chromatogram was observed around 17.33 min after injection of the sample. The compound was isolated, and its chemical structure was determined from its atomic and gas chromatography mass spectral data, and comparing with references as well

[21]. Atomic spectra [¹H nuclear magnetic resonance (NMR) and ¹³C NMR] was performed on a Bruker Avance 500 spectrometer operating at 500.14 MHz, 125.54 MHz, respectively in CDCl₃. GC mass chromatogram was recorded using a Varian saturnm 3800 series GC with a mass sensitive detector (MS 2000). A VA-5 trace analysis, 5% DD Siloxane column of 30 m×0.25 mm×0.25 µm was used. The inlet temperature was set at 250 °C, whereas the oven temperature was programmed as follows: initially at 90 °C then the temperature was raised to 210 °C. Helium was used as a carrier gas at a flow rate of 1 mL/min.

2.3. Cell culture

Human lymphocytic cell line, Jurkat and monocytic cell line, U937 were obtained from American Type Culture Collection (Rockville, MD). All the cells were cultured with complete RPMI-1640 medium (Gibco-BRL) containing 10% (v/v) heat-inactivated fetal calf serum, antibiotics, and HEPES buffer. The cell numbers were determined with a Neubauer hemocytometer and viabilities were assessed by trypan-blue dye exclusion. Blood was collected from healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation as described before [22]. PBMCs were cultured for 2-3h in plastic petri dishes to separate adherent (macrophages-rich) cells, and then primary T lymphocytes were purified by passing through a Nylooon wood column [23]. Such cell preparations were more than 95% CD3+ cells as assessed by flow cytometry. The cells were washed twice with HBSS and resuspended in RPMI 1640 complete medium with 2% human AB serum prior to immunological studies.

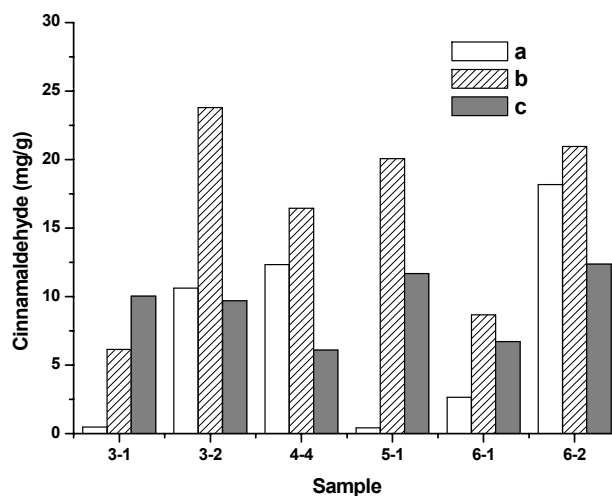


Figure 1. Quantitative determination of *trans*-cinnamaldehyde in *C. osmophloeum* leaves. Different age samples, a: young leaves (open box), b: mature leaves (hatched box) and c: leaf branches (gray box) were collected from different age trees. The x-axis values: 3-1; 3-2; 4-4; 5-1; 6-1; 6-2 represents the trees age followed by the sample age

2.4. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to determine the cytotoxicity [24]. The compound TCA was dissolved in ethanol and the final organic solvent concentration in the cell culture was 1% (v/v). Control cultures were exposed to the solvent only. Cells were seeded in MTT (Sigma) supplemented with PBS (0.1 mg) in the presence and absence (as controls) of TCA was added into each well at different concentrations as indicated and then incubated at 37°C for 4 h. The MTT formazan crystals [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] were dissolved by addition of acid-isopropanol (0.04N HCl in isopropanol) to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes which convert MTT to an insoluble purple formazan in living cells and mixed at room temperature. After 20 min, the level of colored formazan derivative was determined by measuring optical density (OD) with a microplate reader

(BIO-RAD, model 3550, U.S.A.) at 570 nm (OD₅₇₀₋₆₂₀). The mean OD value of the content of four wells was used for assessing the cell viability expressed as percentage of control. Viable cells (%) = [(total cells-dead cells)/ total cells] × 100%.

2.5. Cell proliferation assay

Proliferation was measured by [³H] thymidine incorporation assay described before [25]. Briefly, different concentrations of TCA were added to the culture of 2×10⁶ cells/mL into 96 well microplates for 24 h. PBMCs were stimulated with 5 g/mL phytohemagglutinin-L (PHA) (Sigma) with or without TCA for 72 h. During the last 18 hours cells were pulsed with 1 μCi of [³H] thymidine (Amersham France SA, Les Ulis, France). The result of proliferative response was expressed as (cpm value of experiment) / (cpm value of control) × 100%.

2.6. Cell cycle analysis

For DNA content (cell cycle) analysis cells were plated at a concentration of 2×10^6 cells/mL in their specific medium. After 24 h the medium was replaced with fresh medium containing TCA to be tested or vehicle alone. After 24 h of treatment cells were harvested and gently washed in PBS and stained with 20 g/mL propidium iodide (PI) in 0.1% Triton X-100 and 0.1 mM EDTA. PI was a highly water-soluble fluorescent compound that cannot pass through intact membranes and was generally excluded from viable cells. It binds to DNA by intercalating between the bases with little or no sequence preference. Cell suspensions were analyzed with FAC-Scan flow cytometer and the relative percentages of the cells in G0/G1, S and G2/M phases of the cell cycle were determined using the MODFIT software (Becton Dickinson).

2.7. Statistical analysis

All experimental data were shown as mean \pm SD and accompanied by the number of experiments. For *in vitro* and *in vivo* data, statistical analysis was performed using one-way Analysis of Variance (ANOVA) followed by Dunnetts post-hoc test, and the significant differences were set at $*p < 0.05$; $**p < 0.01$.

3. Results

As an experimental strategy for the determination of TCA in different age leaf samples from different age trees of *C. osmophloeum* were extracted using soxhelt extraction method with alcohol as a solvent. The concentrations of TCA determined by the HPLC method varied from 0.42 to 23.79 mg/g (Figure 1), in different age leaf samples from different age trees. Comparative determination of TCA showed that significantly higher yield, 23.79 mg/g (the tree's age was three years) was obtained from the two years matured

leaves (Figure 1). In a first feasibility study, we aimed to compare the dose-dependent activity of TCA on the cell viabilities of human tumor cell lines with primary human T cells and macrophages. The viability of the cells after treatment with increasing concentrations of TCA was measured after 24 h. The cell numbers were determined with a hemocytometer, and viabilities were assessed by trypan blue dye exclusion (data not shown) and also by MTT assay. The results showed that the decrease in tumor cell number treated with TCA suggested cell death in the cell cultures and the 50% inhibition concentration (IC_{50}) of TCA in Jurkat, U937 cell lines were 0.057, 0.076 μ M, respectively. On the other hand, interestingly no significant effect was observed against the viability of normal T cells and primary macrophages (Figure 2A & 2B).

In order to understand the effect of TCA on the proliferation of Jurkat, U937 cells, and mitogen-stimulated human PBMCs, different concentrations of TCA was added to the cell culture for 24h. As compared with controls, tumor cells treated with TCA exhibited a profound concentration-dependent reduction in their proliferation rate over the 24-h test period. Jurkat cells were reduced by TCA already at 0.057 (32%), 0.076 (42%) and 0.095 μ M (59%), respectively. The stronger decrease of U937 cells proliferation by TCA was of 46% at 0.057, 60% at 0.076 and 75% at 0.095 μ M, respectively (Figure 3). At 0.095 μ M, TCA inhibited proliferation of both Jurkat and U937 cell lines approximately 2-fold at 0.057 μ M, compared to controls. However, interestingly PHA stimulated PBMCs proliferation was increased approximately 26% compared to controls during the concentration range studied (Figure 3).

Cell cycle analysis was performed to explore the mechanism responsible for the anti-proliferative properties of TCA, and its effect on the cell cycle of tumor cells was assessed by FACS analysis. Exponentially growing cultures of each cell line were exposed to a concentration of TCA correspond-

ing to the IC₅₀ and the distribution of cells in the different phases of the cell cycle was de-

termined after 24 h in both treated and parallel untreated cultures.

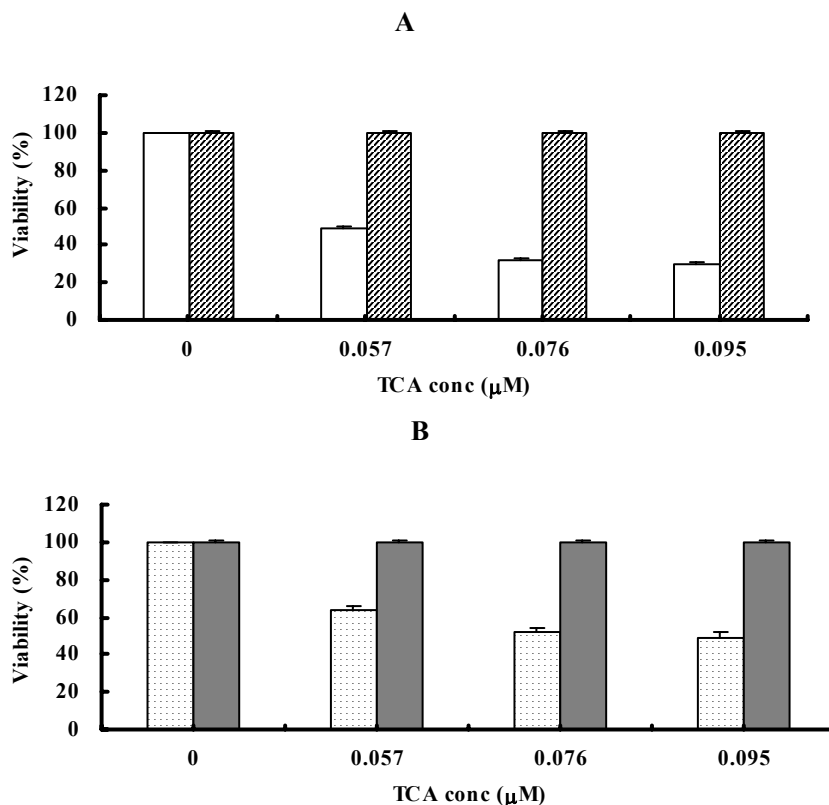


Figure 2. Influence of *trans*-cinnamaldehyde on viability of different human cell lines. Different concentrations of *trans*-cinnamaldehyde were added to the cell culture of Jurkat cells (2A, open box), primary T cells (2A, hatched box), U937 cell line (2B, dotted box), and primary macrophages (2B, gray box) for 24 h. Cell viability was monitored by MTT assay. Data represented as (O.D. value of experiment / O.D. value of control) x100%. * $p < 0.05$; ** $p < 0.01$

As shown in Table 1, TCA altered the cell cycle phase distribution of Jurkat and U937 cells in a nonlinear concentration-dependent fashion. At 0.057 µM, TCA increased the proportion of cells in G0/G1 phase from about 46.8 to 53.8%, 30.4 to 35.2% in Jurkat, U937, respectively. This effect was associated with a decrease in the percentage of cells in the S phase (42.5 to 38.8%) and in G2/M phase

(10.7 to 7.4%) of the Jurkar cells; and in the S phase (48.2 to 46.0) and in the G2/M phase (21.5 to 17.9%) of the U937 cells, respectively. On the other hand, at 0.076 and 0.095 µM, TCA caused decrease the percentage of cells in G0/G1 and S phase, respectively with a concomitant increase the percent of cells in the G2/M phase of the cell cycle (Table 1).

Table 1. Effects of *trans*-cinnamaldehyde at different concentrations (μM) on the cell cycle phase distribution. The data was obtained from three independent experiments and showing the percentage of surviving cells

Test samples	G0/G1	S	G2/M
Jurkat cells			
Control	46.8 ^a	42.5	10.7
0.057 ^b	53.8	38.8	7.4
0.076 ^b	24.5 [*]	29.5 [*]	46.6 [*]
0.095 ^b	26.7 [*]	26.6 [*]	47.7 [*]
U937 cells			
Control	30.4 ^a	48.2	21.5
0.057 ^b	35.2	46.0	17.9
0.076 ^b	21.7 [*]	29.2 [*]	49.1 ^{**}
0.095 ^b	16.4 ^{**}	42.5	41.0 ^{**}

4. Discussion

Well-established methods for the application of HPLC analysis for the quantitative determination of TCA were used in this experiment. The major compound TCA in *C. osmophloeum* clones showed differential distribution temporally and spatially. Our findings were also in agreement with results reported [8], on the availability of TCA. This study revealed the presence of large amount of TCA may be directly related to the bioavailability. Inhibiting tumor growth has been a continuous effort in cancer treatment. A reduction in cell growth and an induction in cell death are two major means to inhibit tumor growth. Epidemiological studies have suggested that dietary factors play an important role in cancer development in humans and the preventive effects of plant-based diets is well documented [26]. Here, we focus on two commonly used and thoroughly studied human

cancer cell lines, viz. Jurkat and U937 cells. In these two cell line models, we showed that cytotoxic effects were caused by TCA. A clear decline in viability was observed at 0.057 M, after 24 h incubation, which became even more pronounced when cultures of the two cell lines, respectively were exposed to higher concentrations (0.076 and 0.095 M), indicating the two human cancer lines are probably affected in a similar manner by TCA. Cinnamaldehyde from *C. cassia* has been shown to inhibit human cancer cell proliferation [27], and the IC₅₀ in HL60, U937 cells were 30.7, 146.5 M, respectively. Although, the chemical constituents of *C. osmophloeum* leaf essential oils were similar to those of *C. cassia bark* oil, statistically significant differences were found in the U937 cells IC₅₀ of TCA and cinnamaldehyde from *C. cassia* [27], where the former (0.076 M) was significantly lower than that of latter (146.5 M).

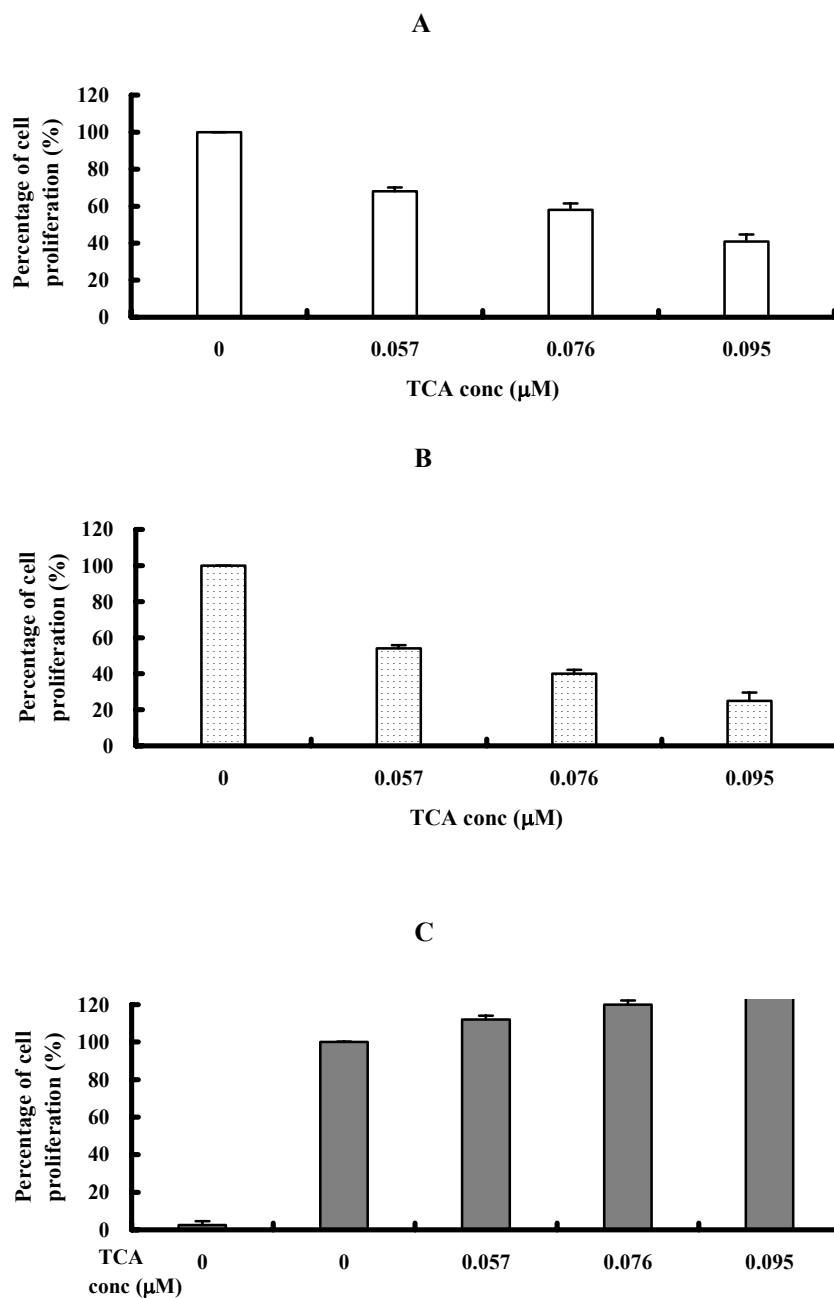


Figure 3. Effect of *trans*-cinnamaldehyde on proliferation of human tumor cell lines and mitogen stimulated primary peripheral human blood mononuclear cells. Different concentrations of *trans*-cinnamaldehyde were added to the cell culture of Jurkat cells (3A), U937 cells (3B), and primary human PBMCs (3C) for 24 h. Cell proliferation was monitored by [³H]-thymidine incorporation assay. Data represented as (cpm value of experiment / cpm value of control) x100%. **p*<0.05; ***p*<0.01

In addition, a component from other plant essential oil, geraniol also inhibits the growth of human colon cancer cells only at relatively high concentration [28]. Furthermore, cinnamaldehyde derivatives, 2'-hydroxy and 2'-benzoxy cinnamaldehydes from the stem bark of *C.cassia*, were showed to inhibit normal mice splenocytes proliferation and attenuate the Con A-triggered progression of cell cycle at G1 phase [13], and Cinnamaldehyde from Rosaceae family showed relatively low tumor-suppressive potencies of B16 melanoma cells [29]. These findings imply that different sources or derivatives of cinnamaldehyde may have quietly different anticancer effects. The inhibitory effect of TCA on cell growth implies that this compound may have a general function in anti-tumor cell growth. This is not unexpected, since cancer cells have developed the capacity of increased proliferation through a variety of signal via integrin, and Ras protein mutation-derived constitutive mitogenic signals, resulting in growing neoplasm, that causes destruction and atrophy of the surrounding tissue and adjacent organs. In a specific tumor, one pathway may play a more important role than the others. The compound TCA may act on more than one pathway and have high potential activity against selected human cancer cell lines.

Normal organ development was controlled by a balance between cell proliferation and apoptosis. In cancer, the balance between proliferation and programmed cell death was disturbed. There was strong evidence that tumor growth was a result of uncontrolled proliferation and reduced apoptosis. Cell cycle control was the major regulatory mechanism of cell growth. This process was regulated by coordinated action of cyclin dependent kinases (CDKs) in association with their specific regulatory cyclin proteins [30]. Many Chinese herbs have been reported to have anticancer activities through inhibiting cell growth by suppressing the expression of cyclin [31,32]. The search for compounds, which are able to act on the specific enzymes,

that regulates the cell division cycle has considerably developed during last years. Cinnamaldehyde derivative CB403 has been shown to have anti-colon and breast tumor activity through the arrest of cell cycle progression in the G2/M phase, which was correlated with a marked increase in the amount of cyclin B1 [33]. In our experiment also support the earlier observations that compound TCA causes marked accumulation of cells in the G2/M phase of the cell cycle occurred with a concomitant decrease of cells in the G0/G1 phase. This finding indicates either (a) an inhibition of progression through the G2 phase or an inhibition of the transition from M into S phase, or (b) that the cells exited from the cell cycle and entered the M phase. G2/M phase progression was regulated by a member of CDK/cyclin family, CDK1/cyclin B. CDK1 protein also known as Cdc2, which was activated by binding to its partner cyclin B during S phase and required for entry into mitosis [34-37]. The mechanism of the inhibition of proliferation and the attenuation of cell cycle progression by TCA needs to be further studied. To our knowledge this is the first study analyzing the ability of TCA to prevent the Jurkat and U937 cancer cell lines.

5. Conclusions

In this present study we found the potent anticancer cinnamaldehyde from the plant origin and its determination will also be important in the future to obtain data on the bioavailability of TCA. The compound TCA showed selective toxicity at concentration as low as 1 μ M against tumor cell lines such as Jurkat and U937 cell lines without being cytotoxic to PBMCs, thereby suggesting that in vivo high doses of TCA may be administered without severe general side effects. Our results suggest that the anticancer effect of TCA was a combination of its effects in inhibiting tumor cell growth and inducing tumor cell apoptosis. It thus has a great advantage over

other types of treatments such as chemotherapy and more recently, hormonal treatments. Most of the chemotherapy treatments fail due to drug resistance. As a natural compound TCA may acts in different pathways on tumor cell growth and survive. We believe that the present investigation together with previous studies [27], provide support to the proposed chemopreventive properties of *trans*-cinnamaldehyde and finally define the possible beneficial outcomes of its use as dietary supplement for the general population toward the development of new therapeutic agents to fight cancer.

Acknowledgements

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