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Research Article

Comparative Effects Between the Commercial and Homemade Curcuma on HCT116 Adenocarcinoma Cell Line

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Abstract

Background: Curcuma longa is widely used for its ability to inhibit carcinogenesis and the capacity to exert a chemo-preventive effect in different types of cancers including colorectal cancer. While our food habits have changed, the prevalence of colorectal cancer have not stopped increasing.

Materials and methods: We investigated the anti-cancerous effect of the aqueous extract of a commercial Curcuma (cur C) compared to the traditional one (cur T) on the adenocarcinoma cell line HCT116 in order to evaluate if the increase in colorectal cancer rates is related to changes in dietary habits.

Results: Cur T exhibits a physiological activity on colon cancer, assuming its possible use in prevention and cancer therapy.

Conclusion: The change in our eating habits from traditional to commercial can be a direct cause of the increase of cancer in the world and more precisely in Tunisia.

Keywords: Curcuma, HCT 116 cells, adenocarcinoma colon cancer, anticancer treatment.

Introduction

Cancer is the major leading cause of morbidity and mortality in the world. In fact, colorectal cancer (CRC) is the most frequent type of cancer in the world and it is the second most common type of cancer in both genders (women: 10.1%; men: 12.4%) [1]. Several data showed that the number of newly diagnosed CRC cases continues to grow especially in the developing countries [2]. For several years, Tunisia has had a low incidence of CRC that seems very far from developed countries, but significant variation in colorectal cancer incidence rates and trends have been observed in our country, without a consistent screening program. The predicted CRC incidence rate by 2024 should be 39.3/100,000 for males and 22.9/ 100,000 for females [3]. Natural products from plants have been intensively explored to find compounds providing against a large number of diseases associated to cancer. The Mediterranean kitchen is one of the most known, and contains a unique mixture of flavors, tastes and nutrients that have no equal around the world [4]. The Tunisian kitchen is a good example. Actually, the Romans and Carthaginians, and well before them the Greeks, brought a lot of recipes and left for us a huge traditional food heritage. This kitchen is reputed for its proper combination that has never been elaborated. It is characterized by a special use of herbs and aromatic plants [5], and Curcuma is one of these spices. It is widely

used in our traditional dishes [6] and it seems to have anticancer activity due to its active ingredient: the Curcumin, which is present in its rhizome and rootstock [7]. Previous studies have shown that Curcumin has an inhibitory action in all phases of cancer growth, which are initiation, promotion, and propagation [8]. However, some studies report that many traditional dishes using certain spices became absent from the table of many families, and even forgotten over time [9]. We have also noted that Tunisians use more frequently the commercial Curcuma instead of mashing the dried roots as did our ancestors. Moreover, the incidence of colorectal cancer in Tunisia is increasing and it is important for us to know if we have also lost the preventive power of our kitchen. Would the change in the origin of these spices be associated with the increase in cancer?

Thus, the aim of this study was to evaluate the association between the origin of spices that we use in our diet and the prevalence of CRC. In this context, we tried to describe the effects of such diet on the incidence of CRC and we aimed to elucidate the comparative effects between the commercial curcuma and the homemade one on HCT116 adenocarcinoma cell line.

Materials and Methods

Cell Culture

Human colorectal carcinoma cell lines (HCT116) were cultured in DMEM medium containing 10% heat- inactivated fetal bovine serum, a mixture of penicillin (100 U/ml) and streptomycin (100 µg/ml) and incubated at 37ºC in a humidified atmosphere with 5% CO2. The cell numbers were counted by a hematocytometer, and the viability was always greater that 95% in all experiments as assayed by the 0.025% trypan blue exclusion method. Further dilutions of stock solutions of curcuma were made in culture media just before use. In all experiments, the final concentration of DMSO did not exceed 0.3% (v/v), a concentration which is non-toxic to the cells. The same concentration was present in control experiments.

Plant Material

We bought turmeric in two forms, the first was dry rhizomes, and the second was the powder of a local market. We referred to the recipes of our grandmothers and their home-made spice preparations. We milled the rhizomes to have fine powder without any additives. In this way, we had the traditional Curcuma (*cur T*),and the purchased powder was defined as the commercial Curcuma (*cur C*). We also used Curcumine from Sigma Aldrich as a positive control.

Preparation of Extracts

Decoctions were performed by adding 200 ml of distilled water to the sample (1g), heated and boiled for 5 min. Then, the mixture was filtered under reduced pressure then frozen and lyophilized. The obtained filtrate was deeply frozen at −70° C and lyophilized (freeze dried) using lyophilizer to collect crude aqueous extract. For the treatment, turmeric was dissolved in the culture medium.

DPPH Radical Scavenging Assay

The antioxidant activity of extracts was performed using the stable DDPH (1,1-diphenyl-2picrylhydrazyl radical) according to the method reported by Kartal N et al [10]. A volume of 180 µl of various concentrations of extracts (0,009-0,312 µg/ml) was added to 1620 µl of DDPH. The absorbance was observed at 515nm and correlated to the ability of extract to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. The antiradical activity was expressed as IC_{50}, and corresponded to the extract dose required to induce 50% inhibition. The lower IC_{50} value corresponds to a higher antioxidant activity of plant extract. The ability to scavenge the DPPH radical was calculated using the following equation: Scavenging activity (%)=[(A0-A1)]/A0*100

Phase Contrast Microscopy

Cell morphology and cell density was observed after 48H of treatment with *cur T* or *cur C* under an inverted-phase contrast microscope (Axiovert 40 CFL,Zeiss).

Anti-proliferative activity assay

The cytotoxicity of samples in HCT116 cells was analyzed by colorimetric 3-(4,5-dimethyl-2-thiazolyl-)-2,5-diphenyl-2Htetrazolium bromide (MTT assay). Briefly, 1×104 exponentially growing cells were seeded in 96-well micro-culture plates with various samples concentrations. After being incubated for 24 h, the cells were treated with different concentrations of samples for 24h. After the addition of MTT (0.5 mg/ml), cells were incubated at 37 °C for 4 h. Cells for negative control were treated only with vehicle dimethyl sulfoxide (DMSO).After 4h incubation, the culture medium was discarded by gentle aspiration and replaced by 200 µL of DMSO to dissolve the formazan crystals. The amount of formazan product was measured at 570 nm and 650nm as a background using a microplate reader. The number of viable cells is corresponding to the production of formazan. Cell viability was calculated and expressed as percentages according to the following equation: Cell viability (%) = [Sample /Control] x 100

Nuclear Morphology Detection using Hoechst 33342

HCT116 cells (5×104cells) were plated in 60 mm^2 culture dishes and incubated in 5% CO_2 incubator at 37 °C for 24 h. After incubation, the cells were treated with different concentrations of samples while negative control was treated with vehicle DMSO for 48h. The cells were then harvested and washed with PBS and subsequently stained with Hoechst 33342 (40 µg/mL) at room

temperature in the dark for 30 min. Then, the cells were observed under inverted fluorescence microscope. For each sample, 300 cells were examined.

Western Blot

Cell extracts purifications and Western blotting analyses were performed as previously described (Desantis etal., 2015) using the following antibodies: p53 (DO7) (Santacruz Biotechnology: sc-47698), PUMA(B-6) (Santacruz Biotechnology: sc-377015), p85 (Millipore ABS-234). Secondary used antibodies were goat anti-mouse (Biorab 1706516) and goat anti-rabbit (Biorad 1706515), conjugated to horseradish peroxidase. α-β-actin actin (Sigma Aldrich, #A5441). Immuno-stained bands were detected by the chemiluminescent method (Pierce RPN2232).

LDH-Cytotoxicity Assay

Cells were seeded in 96-well culture plates (5 x 104 cells/well). After being incubated for 24 h, cells were treated with different concentrations of samples for 48 h. Lactate dehydrogenase (LDH) activity was determined using the LDH-Cytotoxicity Colorimetric Assay Kit from BioVision (Milpitas, CA, USA) following the manufacturer's protocol. The absorbance of LDH in the culture medium was measured at 340nm as a background using a microplate.

4.0. Statistical Analysis

SPSS Version 16 was used to perform the statistical analysis. Testing of parameters was carried-out using Student's t-test and one-way variance analysis. $p<0.05$ was considered statistically significant.

Results

Comparison of the Antioxidant Activity

All tested extracts showed a promising DPPH scavenging effect in a concentration-dependent manner. The lower IC_{50} corresponds to the highest radical scavenging effectiveness. This was observed with *cur T* (3,45±0,1). This result highlights the greater antioxidant activity of the *cur T* than *cur C*. The results of the DPPH scavenging activity of *cur T* and *cur C* are shown in Table1.

Table 1: IC_{50} values of the extracts of cur T and cur C in DPPH assay. Data are means ±SD

DPPH IC_{50} Scavenging (µg/ml)	
Cur T	3,45±0,010
Cur C	11,5±0,015

IC_{50} values correspond to the sample concentration providing 50% of antioxidant activity. Data are means ± SD of the two independent extract.

Cytotoxicity effect of cur T and cur C on the morphology of HCT116 cell line

After the treatment of HCT116 with *cur C* and *cur T* we observed a plasma membrane blebbing, cell shrinkage and cell detachment only in the sample treated with *cur T*. It clearly showed the apoptotic mechanisms that started through the altered cell membranes (Figure 1).

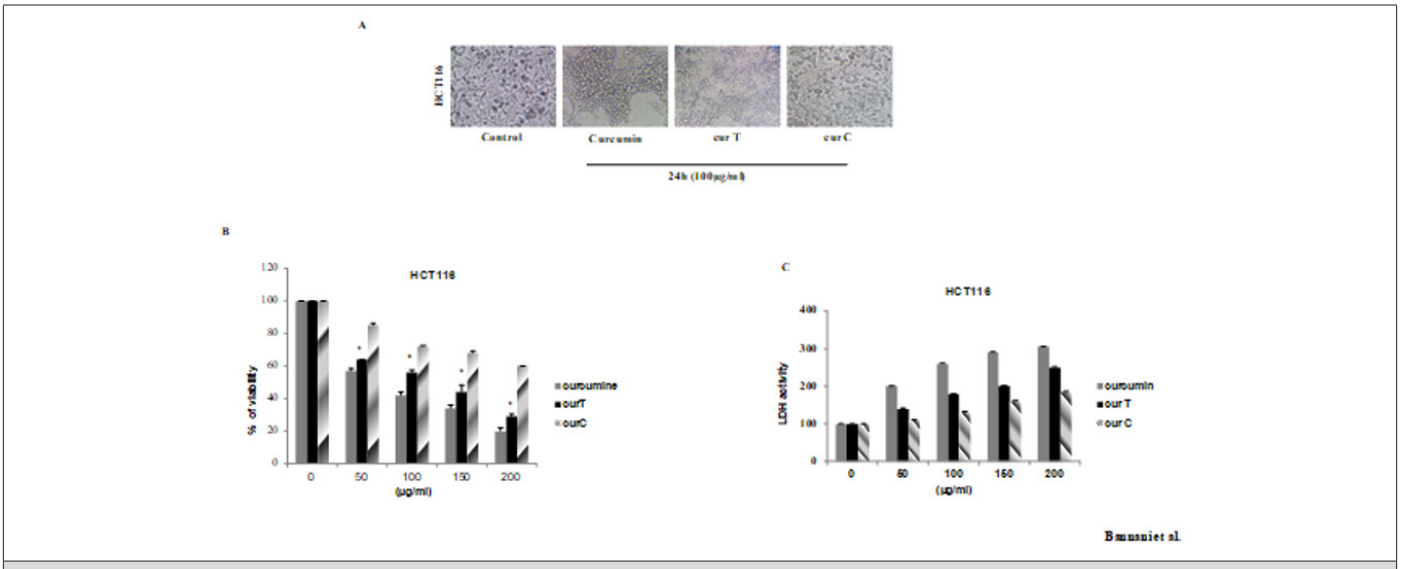

Figure 1: Effects of *cur T*, *cur C* and **Curcumin on cell morphology and growth in HCT116. (A)** HCT116 cells were seeded all night and day and latert reated with100µg/ml of spices for 24h. Cell shrinkage and cell detachment were observed **(B)** Effect of spices on proliferation on HCT116 cell line by MTT assay. Cells were treated witch *Cur T* and *Cur C* at the indicated conditions The inhibition of cell proliferation was in a dose dependent manner(C) Effect of spices on the release of LDH in HCT116 cells. The cells were seeded in 96-well culture plates and treated the day after with *Cur T* and *Cur C* and the medium was collected after 48h of treatment with the indicated concentrations of spices. Light absorptions were analyzed at 340 nm using Tecan infinite M200 spectrophotometer. Data are expressed as means ± standard error of mean, n = 3. *Indicates a significant difference ($p < 0.05$) from Curcumin, *cur T* and *cur C*.

To investigate the potential inhibitory activity of these compounds on HCT116 cells proliferation, we used the MTT assay. As shown in (Figure1B), both treatments with *cur T* and *cur C* were able to inhibit cell proliferation in a dose-and time-dependent manner, but the results also show a higher effect of the *cur T* compared to the *cur C* because, at the lowest doses of *cur T*, strong cytotoxicity is achieved. To further confirm this cytotoxic effect, we performed a colorimetric assay to quantitatively measure lactate dehydrogenase (LDH) released into the media from damaged cells as a biomarker of cytotoxicity and cytolysis. This assay was originally used to quantify cell death occurring via necrosis but has been also shown to accurately measure apoptosis. As we can see in (Figure 1C), there was an increase of LDH presence in the culture medium of cells treated with *cur T* at all used doses, while a similar, but lower effect was obtained with the *cur C*. These results further demonstrated the best activity of *cur T*inHCT116 adenocarcinoma cell line.

Cur T Induces Apoptosis

The staining by Hoechst 33342 exhibited numerous cells with blebbing, condensation and DNA fragmentation, after the treatment with *cur T* and *cur C* for 48H, but no significant nuclear fragmentation in the control group was observed (Figure 2A). These results demonstrate that curcuma induces apoptosis but the best results were obtained through the treatment of cells by *cur T*. These differences are clearly noticeable in the graph in (Figure2B). As a further demonstration of the major apoptotic effect of *cur T* in the studied cell line we performed a FACS analysis after treatment with both spices. As a result, the treatment with *cur T*has shown a greater quantity of cells in the sub G1 phase, characteristic of apoptosis, even with the fewest concentration (25µg/ml) (Figure 2C).

Based on the observation by FACS analysis, we investigated whether these compounds were capable of regulating some of the key players of cell cycle progression. After treatment with *cur T* and *cur C*, we evaluated the expression of proteins which are usually involved in apoptosis and in DNA damage response (Figure 2D). The most important alteration in human tumurogenesis is the deletion or the alteration of p53 gene which is a tumor suppressor. This gene can be activated by several stress condition or DNA damage. As shown in (Figure 2D), treatment with *Cur T* significantly increase in a dose dependent manner. The induction of p53 started as early with 50µg/ml and reached the highest level with 100µg/ml. In addition, we have observed an increase of the level of the apoptosis-inducing factor PUMA (with 100µg/ml) which is an essential mediator of the apoptotic function of p53. Finally, we demonstrated an increase of p85 PARP fragment that is the result of the caspase-3 cleavage protein typical marker of apoptosis.

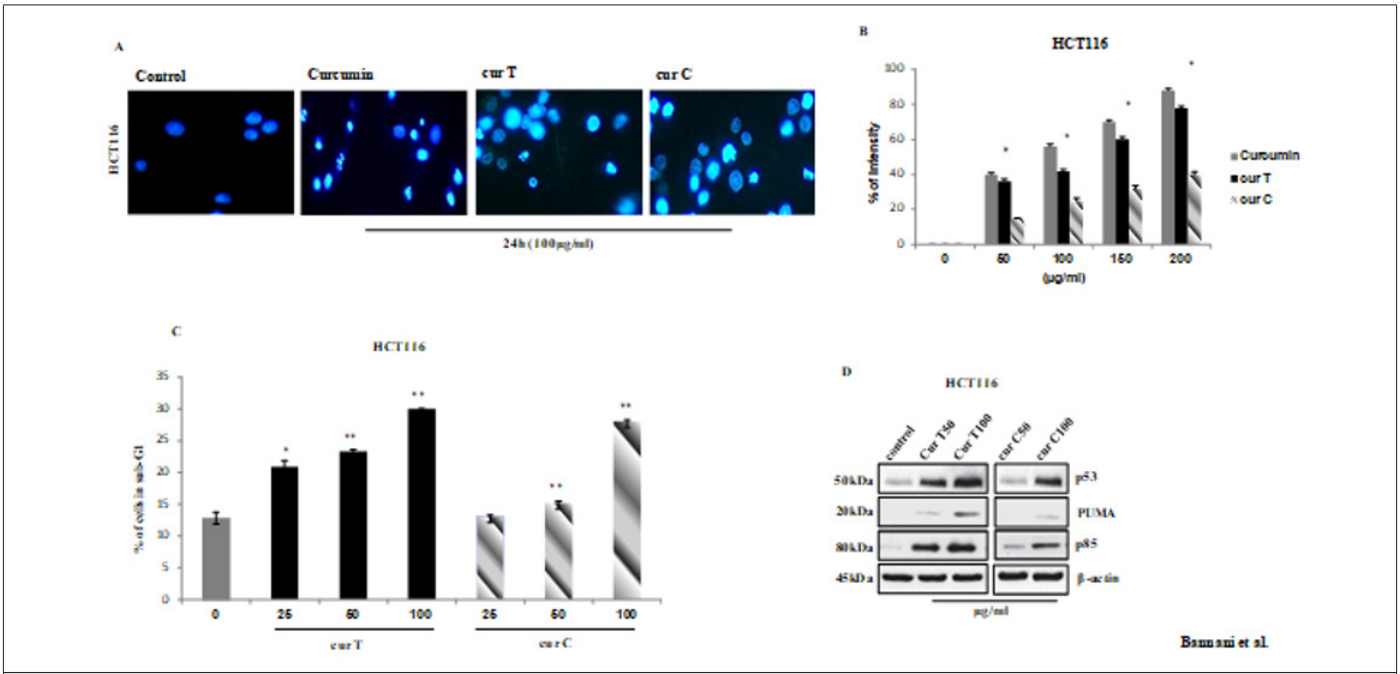

Figure 2: Induction of apoptosis in HCT116 cells by spices treatment.(A) Nuclei of HCT116 cells have been stained with fluorescent DNA-binding dye (Hoechst 33342)and analyzed under fluorescence microscopy using automated analytical software. The detected intensity is represented in the graph. **(B)** Data are expressed as means ± standard error of mean, *Indicates a significant difference ($p < 0.05$) from control. *cur T, cur C* and Curcumin. The DNA contents of the cells after the treatment with spices for 24h were evaluated by FACS analysis using propidium iodide staining. Representative data from the cell cycle analyses are shown. The percentage of sub-G1 phase cells from three different FACS analyses were calculated and presented as the mean ± standard error of the mean. *P<0.05. **(C)** Equal amounts of the proteins extracted from SCE treated cells were subjected to western blot analysis for detection of apoptotic signaling molecules, including p53, PUMA and p85. **(D)**

Discussion

Numerous studies have shown that there is a correlation between regular consumption of fruits and vegetables and prevention of developing lifestyle disorders, such as cancer [11]. Diet and nutrient factors are widely believed to act as pro- and antitumor risk modifiers across the entire multistep process of colorectal tumorigenesis [12]. Curcuma longa seems to be an antiproliferative agent and seems to possess many of the desirable qualities for preventing cancer and could have great potential as chemopreventive [13].

The present research study was directed to check if the change of spices used in our kitchen is related to the increase of CRC cancer rate, that's why we compared the antioxidant and anti-proliferative effects of traditional homemade Curcuma (*cur T*) and commercial Curcuma (*cur C*) on colorectal cells HCT116.

Our results show that *cur T* display the strongest DDPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity comparing it to *cur C*. The lower IC_{50} corresponds to the highest radical scavenging effectiveness observed with *cur T*. A lot of studies showed an important antioxidant effect of curcuma [14] and others concluded that this effect is due to Curcumin [13] but Curcuma contains other molecules that have the same effect [15]. Indeed, in our study, *cur T* showed a promising DPPH scavenging effect in a concentration-dependent manner. These results provide convincing proof of the protective effect of *cur T* treatment against HCT116, which suggests that *cur T* has an anti-proliferative action in HCT116 cells and can potentially serve as an anti-proliferative agent for cancer. A few studies have also revealed that the antioxidant proprieties may directly cause the cytotoxic effect of Curcuma [14]. Indeed, other studies prove the pro-apoptotic [16] and anti-proliferative effect of Curcuma and Curcumin [14], which was also proved by our results by several techniques. In order to evaluate whether our plants extracts are safe for a possible therapeutic use or not, we tested their potential in vitro cytotoxicity in confluent monolayers of human HCT116 cells using the MTT cell viability assay. Our results revealed that, even when we used the fewest dose of *cur T*, they induce more than 50% of mortality. According to the literature, curcuma possesses potent antitumor activity, which may be due to its direct cytotoxic effect or antioxidant properties. This test was consolidated by the LDH release test. Such assay was originally used to quantify cell death occurring via necrosis but also has been shown to accurately measure apoptosis [16] and *cur T* was cytotoxic as assessed biochemically by an increase in LDH content in the supernatant in a dose-dependent manner. We also found with Hoechst typical ultrastructural modifications such as dense and/or fragmented nuclei of cells treated by *cur T* but no significant nuclear fragmentation in the control group was observed. In addition, the investigation of cell cycle demonstrated that the population of sub-G1 phase cells was increased by *cur T* treatment in a dose-dependent manner as it was reported [17]. G1phase arrest correlated with the regulation of tumor protein. Tumor suppressor genes, including p53 and PUMA are the main molecular targets for several herbal drugs. Many studies reported that these genes caused arrest of cell cycle at the G0/G1 phase of human colorectal carcinoma (HCT116 cells) by up regulating the p53. It was recognized as the protein which controls G1 phase which will both induce cell arrest in G1 phase and induce apoptosis in order to maintain tissue homoeostasis [18]. Curcuma has been shown to increase the expression of these tumor suppressor [19,20]. Our finding demonstrated that *Cur T* increases the p53, p21 and PUMA protein expression more than did *Cur C*. As it was reported, Curcuma and its compounds could induce apoptosis of all cancer cells tested in this study in a dose-dependent manner, indicating possibility of curative and preventive windows [21]. Previous studies have evaluated the anti-oxidant and pro-apoptotic effect of Curcuma root. Our results are comparable to these studies. However, the *cur C* effects were not previously evaluated in literature. According to our findings the *cur T* has better effect on HCT116 cells. Culinary habits have dramatically changed with the emergence of commercially proceeded food [22]. The impact of these changes has increased the risk of CRC. The ratios of bioactive molecules are decreased in commercial food leading to the loss of preventive effect against cancer of natural nutrients. This fact is reinforced by our findings.

All taken together, the commercial Curcuma lacks much of its benefits and the use of traditional Curcuma is encouraged. Further investigations should be performed to identify all the bioactive molecules in Curcuma (besides Curcumin). This would allow the complementation of commercial food by the bioactive molecule. Commercial products must undergo controls to validate the health effects. Nevertheless, replication of these findings in other studies is required in order to confirm the reported associations and to be able to make public health recommendations.

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