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THE APOPTOTIC PROPERTIES OF *CINNAMOMUM ZEYLANICUM* AND *CYMBOPOGON CITRATUS* ON HUMAN HEPATOCELLULAR CARCINOMA CELL LINE (HEPG2)

Sobhy Hassab El-Nabi , Islam El-Garawani and *Aya El-Berry

Department of Zoology, Faculty of Science, Menofia University, Menofia, Egypt.

***Correspondence for Author: Aya El-Berry**

Department of Zoology, Faculty of Science, Menofia University, Menofia, Egypt.

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ABSTRACT

In Egypt, hepatocellular carcinoma (HCC) is considered the third most frequent cause of cancer-related death. The study goal is the discovery of safe, efficacious and cheap alternative natural medicines with less side-effects of chemotherapy. In this study, the apoptotic properties of Cinnamon, *Cinnamomum zeylanicum*, (CWE) and lemongrass, *Cymbopogon citratus* (LgWE) aqueous extracts on human hepatocellular carcinoma cells (HepG2) were investigated. Antioxidant potential of both extracts was evaluated by using DPPH assay in addition to flavonoids and total phenolic contents. Cytotoxic activities of both extracts were assessed using neutral red uptake method and the results revealed (600 and 630µg/ml) for CWE and LgWE respectively. Morphological changes were evaluated using Giemsa and dual acridine orange / ethidium bromide fluorescent staining methods. As a result, both cytoplasmic and nuclear morphological examinations of treated cells showed significant ($P \leq 0.05$) dose dependant apoptotic features at both doses after 24 hr as compared to control. Agarose gel electrophoresis of both extracts-treated cells showed apoptotic laddering pattern at dose dependant manner. In conclusion, cinnamon and lemongrass could be a source of natural antioxidants as they induced growth inhibition and apoptosis in HepG2 cells leading to further investigations for new anticancer drug discovery.

KEYWORDS: Apoptosis - *Cinnamomum zeylanicum* – *Cymbopogon citratus* - HepG2 cells.

1-INTRODUCTION

Hepatocellular carcinoma (HCC), a primary malignancy of the hepatocyte, accounts for 85% to 90% of all primary liver cancer.[1] Current therapeutic interventions mostly involve surgery, radiotherapy and chemotherapy. Traditional cancer treatments are toxic to normal cells because of non-selectivity and often cause serious side effects. Therefore, plant extracts are considered as chemical libraries of structurally diverse compounds, consequently their investigation constituting a promising approach in drug discovery.[2] Bioactive phytochemicals exhibiting the ability to inhibit cancer cytogenesis by suppressing the tumor initiation, promotion and progression are being considered as potential biocompatible anticancer agents.[3] Several studies have been conducted on herbs that possess anticancer properties and have been used as potent anticancer drugs.

Cinnamon *(Cinnamomum zeylanicum)*, is one of the most popular and oldest spices that belonging to the family Lauraceae.[4] Cinnamon has many biological functions such as anti-oxidant, anti-inflammatory and anti-tumor activity.[5,6,7] Cinnamon is a reliable and safer herbal drug that can be used in pharmaceutical preparations for malignant diseases because it has antitumor activity against several human cancer cell lines including hepatocellular carcinoma (HepG2), human cervical tumor (SiHa), human ovarian cancer (A-2780).[8.9.10] *Cymbopogon citratus* (lemongrass) is a member of the Poaceae family. It has many therapeutic applications[11,12] including its antitumor activity against cervical cancer cell lines (HeLa and ME-180).[13]

In this study, an attempt to evaluate anticancer potential of *Cinnamomum zeylanicum* and *Cymbopogon citratus* on human hepatocellular carcinoma cell line (HepG2) was in concern.

2- MATERIALS AND METHODS

Plants

Fresh leaves of lemon grass (*Cymbopogon citratus*, family: Poaceae) were collected freshly from Applied Research Center of Medicinal Plant (ARCMP), Giza, Egypt.

Dried fine powder of cinnamon barks (*Cinnamomum zeylanicum* sp, family: Lauraceae) was purchased from local markets. Both plant materials were identified by a taxonomist, Applied Research Center of Medicinal Plant, Giza, Egypt.

Preparation of water extracts (cinnamon, CWE) and (lemon grass, LgWE)
Two hundred grams fine pieces of both plant materials were mixed with hot distilled water (55°C) for 6 hours. The supernatant was filtered, centrifuged and finally air-dried at 55°C. The dried powders of both extracts were stored at -20°C until use.

Phytochemical screening
Determination of total flavonoids
Total flavonoids content was determined depending on the method of.[14] Results were expressed as mg/ml quercetin equivalents (QE) per 100 g sample.

Determination of total polyphenolic compounds
Total phenolic content was determined depending on the Folin-Ciocalteau method.[15] Results were expressed as mg/ml of gallic acid equivalents (GAE) per 100 g sample.

Determination of antioxidant activities using DPPH radical-scavenging assay
Determination of the DPPH radical-scavenging activity depends on the method of.[16] Results were expressed as µg/ml of gallic acid equivalents (GAE) per 100 g sample. All phytochemical determinations were performed in triplicates.

Cell culture and maintenance
HepG2 cells were purchased from Holding Company for Biological Products and Vaccines (VACSRA) Giza, Egypt. Cells were trypsinized then sub cultured into 25 cm2 tissue culture flasks. 5×10^5 cells were grown in each flask containing 7 ml of complete DMEM growth medium supplemented with 10% fetal bovine serum, 1% (100U/ml penicillin and 100 µg/ml streptomycin) at 37°C. All culture reagents were obtained from (Lonza) supplier, Egypt.

Study design
HepG2 cells were divided as the following:
GroupI served as control without any treatment.
Group II incubated with CWE at 250 and 450 µg/ml.
Group III incubated with LgWE at 250 and 450 µg/ml.
Each treatment was done in triplicates for 24 hr of incubation.

Cytotoxicity using neutral red uptake assay
HepG2 cells were exposed to neutral red dye (4 mg/ml) in serum free DMEM after treatment periods for 3 hours. Cells were washed with PBS then distained using (50%EtOH and 5% glacial acetic acid in distilled water). The absorbance was measured at 540 nm using spectrophotometer (Hewlett Packard, USA). All determinations were performed in triplicates.[17]

Acridine orange/ ethidium bromide dual fluorescent staining
Treated and control cells were smeared on a glass slide and air-dried, smeared cells were fixed in methanol/acetic acid (3:1) for 5 min, cells were hydrated with PBS for 1 min, Stained with a mixture (1:1) of acridine orange (50 µg/ml)/ethidium bromide (5 µg/ml) for 5 min and they were immediately washed with PBS andviewed under fluorescent microscope (Olympus BX 41, Japan). Two hundred of cells per flask were evaluated (400x) and the damaged (apoptotic and necrotic) nuclei were recorded according to the affinity and fluorescent staining pattern. Then representative photos were digitally photographed.[18]

Giemsa staining
Treated and control cells were smeared on a glass slide and air-dried, smeared cells were fixed in methanol/acetic acid (3:1) for 5 min, Cells were washed with PBS for 1 min then stained in Giemsa solution for 15 min and washed with PBS. Light microscope (Olympus BX 41, Japan) is used for examination. Two hundred cells were evaluated and then representative photos were digitally photographed.[19]

Total genomic DNA extraction and apoptosis detection
DNA extraction and detection of apoptosis (DNA fragmentation assay) were done according to "salting out extraction method" of.[20] with some modifications by.[21] Cells were incubated in lysing buffer two hours at 37°C then; proteins were precipitated using 4M NaCl. The resultant supernatant was transferred to a new tube then DNA was precipitated using cold isopropanol. The pellets of nucleic acids were washed with 70% ethyl alcohol. The pellets were resuspended in TE buffer and were incubated with loading mix (0.1% RNase + loading buffer) and then loaded directly into 1.8% normal melting electrophoretic grade agarose in 1X Tris borate EDTA buffer. The apoptotic bands of DNA fragmentation appeared and located at 180 bp and its multiples 360, 540 and 720 bp against thirteen bands of DNA marker (100–3000 bp). The intensity of DNA apoptotic bands were measured by (ImageJ software) as a maximum optical density values.

3-STATISTICAL ANALYSIS
Data were expressed as mean ± standard deviation. Statistical analysis was performed using the student's t-test to detect statistical significance according to untreated group ($P\leq0.05$).

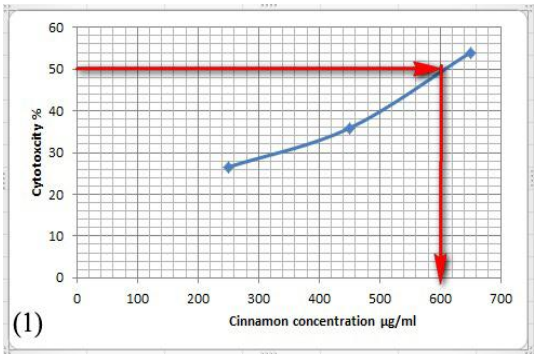
4- RESULTS
Flavonoids and total polyphenolic content
Cinnamon extract possessed flavonoid and total phenolic content (0.214± 0.008 mg/ml, 0.764±0.087 mg/ml, respectively) higher than that of lemongrass (0.105±0.007 mg/ml, 0.626±0.125 mg/ml, respectively).

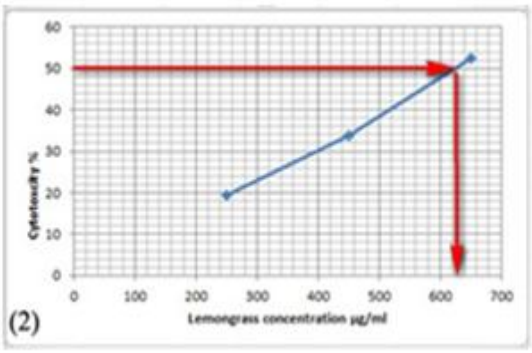
Antioxidant activities using DPPH
It was observed that DPPH radical scavenging activities, at the concentration inhibiting 50% of free radical generation (IC_{50}) of CWE and LgWE were (70.3 µg/ml and 96.8 µg/ml respectively). All these results indicate

that cinnamon extract has antioxidant activity more than that of lemongrass extract.

Cytotoxicity of plant extracts using neutral red assay

The IC_{50} mean values obtained by the neutral red assay were 600 and 630 µg/ml for CWE and LgWE respectively. The lower value of IC_{50} indicates the greater antioxidant activity. Cinnamon has cytotoxic effect on HepG2 cells more than that of cinnamon more than that of lemongrass (Graphs 1, 2).





Graph 1, 2: cytotoxicity assay using neutral red uptake method for CWE and LgWE on HepG2 cells.

Morphological changes in HepG2 cells

The nuclear morphological changes as the formation of apoptotic bodies and nuclear condensation with bright orange color after fluorescent staining with acridine orange/ethidium bromide (Figure 1-1) and cytoplasmic membrane blebbing after Giemsa staining (Figure 1-2) are the characteristic features of dead and apoptotic cells that were significantly ($P \leq 0.05$) increased in dose dependent manner among CWE and LgWE treated groups (Table 1).

Table 1. The effect of CWE and LgWE on the morphological changes of HepG2 cells after acridine orange/ethidium bromide and Giemsa staining at low and high doses.

	Giemsa		AO/EBr
	% Dead	% Blebbing	Nuclear changes
Control	10.0±3.4	6.6±3.3	5.0± 1.0
250 µg/ml (Cin)	31.3±5.1*	16.4±1.9*	50.6 ± 3.2*
450 µg/ml (Cin)	38.8±3.4*	22.2±1.9*	82.3± 2.5*
250 µg/ml (Lg)	29.4±2.3*	22.7±4.6*	51.3± 4.0*
450 µg/ml (Lg)	32.2±4.3*	16.5±3.9*	85.0± 3.0*

*Data were presented as mean±SD; *: significant with respect to untreated cells ($P \leq 0.05$).Cin: cinnamon, Lg: lemongrass.*

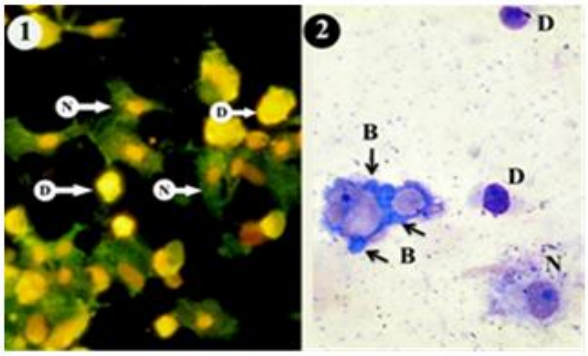


Figure 1. Showing the effect of CWE and LgWE treatments on HepG2 cells morphology. (1) Nuclear morphological changes in HepG2 cells after acridine orange / ethidium bromide fluorescent staining. (2) Cytoplasmic morphological changes in HepG2 cells after Giemsa staining. Where, n: normal cell, b: bleebing cell, d: dead cell.

The effect of plant extracts on total genomic DNA fragmentation of HepG2

The extracted total genomic DNA of control HepG2 cells were found to be intact (undamaged) as seen in (lane: 1-Figure 2) with no release of DNA fragments as control has negligible value of DNA fragments. While, the treatments with CWE and LgWE indicated the presence of damage by the migration of released DNA fragments in a dose dependant manner as apoptotic laddering pattern (lane: 2-5, Figure 2).

CWE and LgWE at high dose recorded significant optical density values of 59.1±5.94 and 32±1.7 respectively, when compared with control DNA content, 5.85±0.49. In addition, CWE and LgWE at low dose recorded significant optical density values of 16.55±1.77 and 15.8±0.71 respectively, when compared with control DNA content, 5.85±0.49. Moreover, CWE at high dose recorded significant optical density values of 59.1±5.94 when compared with LgWE at high dose 32±1.7.

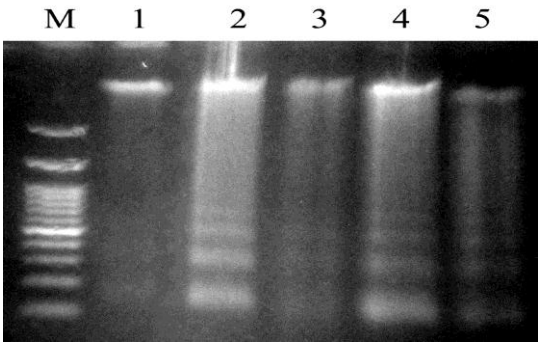


Figure 2. Total genomic DNA fragmentation of control and treated HepG2 cells where lanes (1-5) represent: untreated, 450µg/ml of CWE, 250µg/ml of CWE, 450µg/ml of LgWE, 250µg/ml of LgWE respectively. M: 100bp DNA marker.

5- DISCUSSION

Non-invasive cancer treatments development is needed and often required because (chemotherapy) traditional cancer treatments are toxic to normal cells because of non-selectivity and often cause serious side effects therefore, plant extracts are considered as chemical libraries of structurally diverse compounds, consequently their investigation constituting a promising approach in drug discovery.[2] Searching for new agents as natural products is playing an important role in finding of candidates for the chemotherapeutic agents' development.[22] They offer a valuable source of compounds with a large variety of chemical structures with biological activities and provide many prototypes for novel drugs development.[23]

The modulation of toxic side effects using natural origin extracts has been also paid a great attention. Plants have the ability to synthesize aromatic substances such as polyphenolic compounds, mainly flavonoids and phenolic acids, which have potent antioxidant activities due to their hydrogen-donating and metal-chelating capacities and possess the ability to scavenge several oxidizing species such as hydroxyl radicals.[24,25] In this study, the phytochemical analysis of CWE and LgWE revealed the presence of a large number of bio active compounds including flavonoids and polyphenols which have been shown to have antioxidant and cytotoxicity towards tumor cells.[5,26] Results of DPPH test proved the antioxidant activity of using extracts which reflects their antitumor potential.[27, 28]

Our result revealed that, CWE and LgWE causes the formation of apoptotic bodies and nuclear condensation which gives an indication of inhibiting proliferation of HepG2 cells. Cell proliferation and cell death are carefully and continuously balanced in multi-cellular organisms. Imbalances in the coordination of these processes can result in diseases such as cancer due to over proliferation and reduced cell death.[29]

Cytotoxicity analysis revealed the effectiveness of both extracts which may be due to the presence of a large number of bio active compounds in the CWE and LgWE including flavonoids and phenols which have been shown to have antioxidant and cytotoxicity towards tumor cells.[30, 26] In the light of the present results, CWE treatment can exert anti-cancer effects by inducing cell apoptosis, activating autophagy and inhibiting cell proliferation. There are many reports point to several mechanisms of Cinnamon cytotoxicity on cancer cells such as the effect on proliferation and/or growth inhibition and induction of apoptotic and necrotic cell death through many cell death mechanisms including the caspases and matrix metalloproteinases.[31]

The study results was compatible with result of the study that proved the antitumor activity of cinnamon and lemon grass which inhibited the proliferation of several human cancer cell lines including breast, leukemia, ovarian and lung tumor cells.[8,12, 7]

The treatment with CWE and LgWE indicated the presence of cells damage by the migration of released DNA fragments in dose dependent manner.[7] Most anticancer drugs of plant origin have been known to cause DNA damage or suppress its replication, not necessarily killing the cells directly but inducing apoptosis.[32] During apoptosis, a specific nuclease (now known as caspase-activated DNase or CAD and pre-existed in living cells as an inactive complex) cuts the genomic DNA between nucleosomes generating DNA fragments. This ladder has been used extensively as a marker in studies on apoptotic cell death.[32] There were many studies explained the apoptotic effect of CWE on HepG2 cells.[33,31] It was found that, treatment with CWE has been demonstrated to significantly induce apoptosis by activating JNK and p38.[33, 31] *Cymbopogon citratus* exhibited potential cytotoxic and apoptotic effects on carcinoma cells.[34]

6-CONCLUSION

Cinnamomum zeylanicum and *Cymbopogon citratus* can exert anti-cancer effects by inducing cell apoptosis in addition to Cytotoxicity with increasing dose concentration.

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