ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Ginger Oil Effect as Apoptosis Inducer in Colorectal (Caco-2) Cancer Cell Line

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Manuscript Info

Manuscript History:

Received: 12 Februay 2015
Final Accepted: 22 March 2015
Published Online: April 2015

Key words:

ginger oil, Caco-2 cell line, apoptosis, caspase-3

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Abstract

Natural compounds have been used to treat cancer because of their low cytotoxicity and less adverse effects. Ginger has long been used in traditional medicine as it contains active phenolic compounds that have antioxidant, anti-cancer, anti-inflammatory, antiangiogenesis and antiartherosclerotic properties. We investigated the inhibitory activity of GO against colon cancer (Caco-2) cells viability or proliferation. Cells were incubated with GO at concentrations, 0.2%, 0.25, 0.3 to 0.35% of the volume of the media for 72 h at 37°C and 5% CO_2. GO dose-dependently inhibited the viability and proliferation of colon cancer (Caco-2) cells *in vitro*. IC_{50} concentration of GO on treated cells inhibited proliferation and induced apoptosis even by DNA fragmentation besides caspase-3 reaction over expression. Future study will may deal with further investigations of the possible usages of GO as a new alternative chemotherapeutic agent specially for human colon cancer and other types of cancer.

INTRODUCTION

Interest in dietary bioactive compounds to prevent chronic degenerative diseases and enhance health arises from evidence supporting a role of these compounds in human health. Chronic degenerative diseases are of interest because there are no known cure for most of these diseases and currently available treatments usually only provides a temporary reduction of symptoms (Aggarwal, 2004). Natural compounds or dietary agents have been used recently as an important tool to treat cancer because of their low cytotoxicity and less adverse effects. Phytochemicals from natural resources, readily available in our daily diet and are now being utilized as anticarcinogenic compounds. There are various treatments approaches that have been aimed towards the treatment of the disease but the success rate of the chemotherapeutic drugs are reported to be low with high rate of recurrence and various side effects (Nagasawa et al., 2002). Several reports have described the ability of these naturally derived compounds to modulate the process of carcinogenesis; either by blocking or reversing, with nominal cytotoxicity (Ali et al., 2008).

Ginger (*Zingiber officinale*) is one of many dietary substances that are rich in those phytochemicals. For centuries, it has been used as food flavoring agent as well as for traditional medicinal purposes (Bode, 2003). Ginger is known to contain numerous potent phytochemicals including gingerols, shogaols, zingerols and paradols (Grzanna et al., 2005). Current trend in researches has focused their attention to ginger as anti-cancer, anti-oxidant and anti-inflammatory. Of these phenolic constituents, shogaols and gingerols which are the principal active pharmacological components of ginger as they exhibited growth-inhibitory effects against cancer cells namely lung, liver, breast and skin cells. These bioactive compounds can either remain unmodified or undergo metabolic transformation into active or inactive derivatives (Habib et al., 2008) and to fully understand the potential of GO in human health requires an understanding of the effect of bioactive compounds in different regulatory mechanism from molecular and cellular level.

Colorectal cancer is the cause of more than 1/2 million deaths worldwide, and it was ranked as the third leading cause of cancer-related death after lung cancer and stomach cancer (Mayer, 2009). Epidemiological studies have

shown strong evidence that diet and lifestyle play an important role in preventing cancer. In particular, an increased consumption of fruits and vegetables is associated with a decrease in cancer onset and mortality (Murillo et al. 2008). In this study, the apoptotic effects of GO as effective anticarcinogenic and antiproliferation were determined against human colorectal carcinoma (Caco-2 cell line) *in vitro*.

MATERIALS AND METHODS

Chemical reagents: MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, dimethylsulfoxide (DMSO), commercial methanol, commercial ethanol, commercial acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx & E) stain rabbit polyclonal antibodies against cleaved caspase-3, AB reagent, substrate-chromogen mixture and Tween 20 were purchased from Sigma-Aldrich, Egypt.

Ginger Oil (GO): commercially ginger oil was purchased from Sigma, Aldrich. According to the data provided, GO was all natural and obtained by steam distillation. *Zingiber officionale* was the source of GO and it could be tasted in concentration as low as 30 ppm. GO boiling point was listed as 254ºC and density as 0.871g/ml at 25ºC (Grzanna et al., 2005).

Cell line and cell culture: Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were sub-cultured as monolayer according to the instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C, 30 min) fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL Penicillin-Streptomycin and 100 U/mL Amphotericin B at 37°C in a humidified atmosphere of 5% CO_2. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media and supplements were purchased from Invitrogen (Carlsbad, CA).

Methods: 1. Cell Viability Assay: *In vitro* evaluation of antiproliferation effect: growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble formazans (Mosmann, 1983). Viable cell number/well is directly proportional to formazans production. 25×10^3 cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12 h), replaced with fresh medium containing GO. GO was mixed with DMSO in a ratio of 3:1 as GO was at concentrations of 0.2%, 0.25%, 0.3% or 0.35% of the total volume of the used media and were added to the cells. The cells were incubated for 72 h at 37ºC in an incubator with 5% CO_2. After incubation, GO modified medium was replaced by 100 µL of MTT (0.5 mg/mL) medium for incubation (3 h at 37°C and 5% CO_2). MTT medium was then replaced with 100 µL of DMSO and left for 10 min on a platform shaker to solubilize converted formazan. The absorbance values were determined at 570 nm test wavelength and 630 nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage = [1–(net Absorbance of treated well/ net Absorbance of control well)] x 100%, then was plotted against GO concentrations.

2. Determination of DNA fragmentation by DNA laddering assay: cells were seeded in 60-mm petri dishes at density $4x10^5$ cells/plate (treated cells by IC_{50} concentration of GO or positive control cells). Adherent and floating cells were collected by centrifugation at 1000×g/5 min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 10 mmol/L pH 8.0, Triton-X100 0.5%) and kept at 4 ºC/10 min then, lysate was centrifuged at 25.000×g/20 min. Supernatant was incubated with RNase A 40µg/L/1h (37ºC), incubated with proteinase K 40µg/L/1h (37ºC), mixed with NaCl 0.5 mol/L and 50% 2-propanol overnight (-20ºC), then centrifuged at 25.000×g/15 min. After drying, DNA was dissolved in buffer (Tris-HCl 10 mmol/L pH 7.4, edetic acid 1 mmol/L pH 8.0) and separated by 2% agarose gel electrophoresis at 100V for 50 min. DNA was visualized under ultraviolet light after staining with ethidium bromide (Zhang et al., 2003).

3. Cytological changes investigation: detached and trypsinized cells (IC_{50} concentration of GO treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5 min. Cell pellet was re-suspended with 100 µL of PBS (pH 7.3). 10 µL of the suspension was smeared on a glass slide, allowed to air-dry, fixed with cool methanol for 5 min before proceeding by Hx & E stain and examined under light microscope (John and Abraham, 1991).

4. Immunocytochemical investigations: by detection of caspase-3 by immunocytochemistry staining kits. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells (IC_{50} concentration of GO treated cells and positive control cells) on the slide (10 min), followed by blocking solution (10 min). Cells were fixed in ethanol:acetone (9:1) for 30 min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with rabbit polyclonal antibodies against cleaved caspase-3 at 4°C, then AB reagent and substrate-chromogen mixture (30 min). Between each step, the slide was

washed with washing buffer (PBS) with 0.1% Tween 20) (Yoon et al., 2004). The slides were then mounted and examined under light microscope.

5. Statistical analysis: results were presented as means ± standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way-ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc. Statistical significance was set at $P < 0.05$ and high significance was set at $P \leq 0.01$ (Snedecor and Cochran, 1980).

RESULTS

1.Cell Viability Assay: *In vitro* evaluation of antiproliferation effect.

Cytotoxic effect of different concentrations of GO for 72 h on Caco-2 cell line was determined by MTT assay (Figure 1). The cell number started to reduce immediately after treatment with different concentrations of GO and in dose dependent manner. All concentrations were found to be high significantly different ($P \leq 0.01$) in respect to their antiproliferative and apoptotic effects, specially when compared with positive control cells. The percentage of cell inhibition is gradually increased with increasing GO concentration and 95% of cell apoptosis was observed when cells were treated with media containing 0.35% of GO. Cell growth and proliferation were reduced about 25% and 30% when cells were treated with media containing 0.2% and 0.25% of GO, respectively. Caco-2 cells proliferation decreased about 55% when treated with media containing 0.3% of GO.

2.Determination of DNA fragmentation by DNA laddering assay.

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72 h when treated with media containing 0.3% of GO, whereas the untreated cells did not show a typical ladder (Figure 2). The results indicated that GO induced DNA fragmentation which was caused by apoptosis.

3.Cytological changes investigation.

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by media containing 0.3% of GO for 72 h, apoptotic cells were identified by a series morphological changes as an important experimental proof of the underlying processes alterations. Numerous morphological changes indicative of apoptosis appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation as granules, scant vacuolated cytoplasm, degrading nucleus as well as apoptotic bodies formation, were observed (Figure 3b, 3c and 3d).

4.Immunocytochemical investigations.

After Coca-2 cells treatment by media containing 0.3% of GO for 72 h, the reaction for caspase-3 was considered positive (over expression of caspase-3 protein) when over 50% of treated tumor cells had a clear brown cytoplasm staining, with slight degrading in the intensity in the same field (Figures 3f). Specially those fields that had necrotic or apoptotic nucleus as sign for GO treatment effect, but fields of positive control cells have negative reaction of caspase-3 (cytoplasm did not show the brownish reaction stain) (Figure 3e).

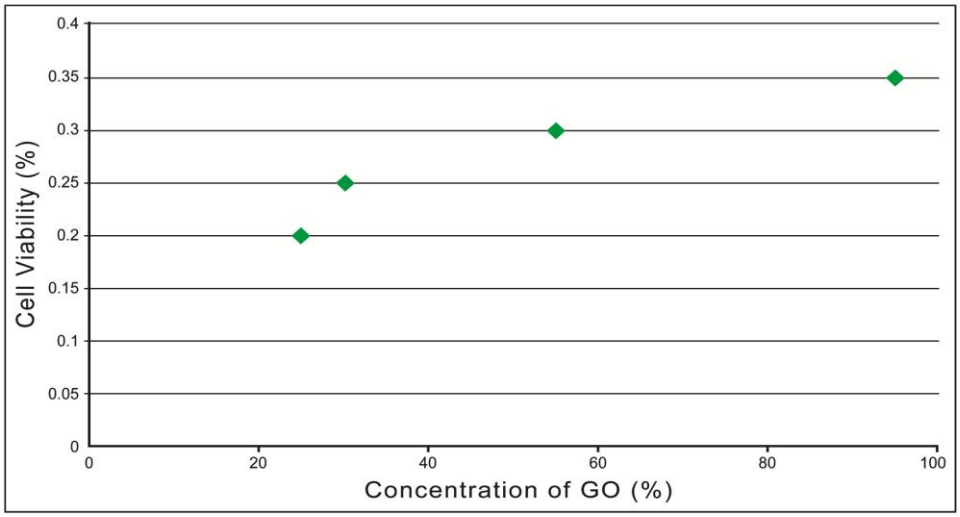

Figure 1: Effect of GO with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean ± SD, n (for each concentration) = 4].

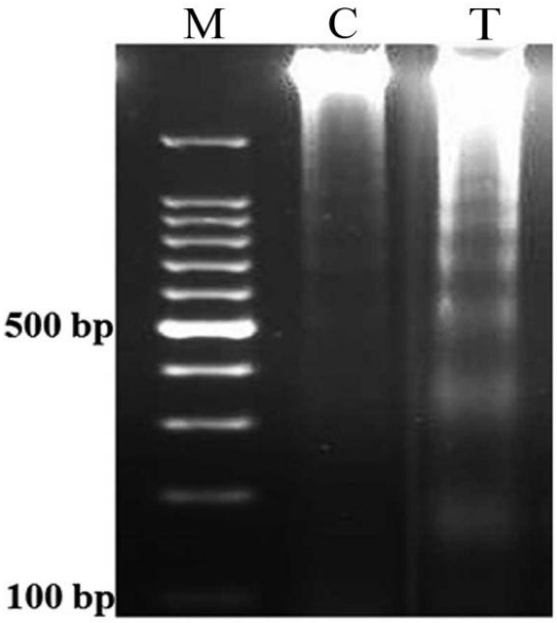

Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from GO treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C) where M indicating to marker.

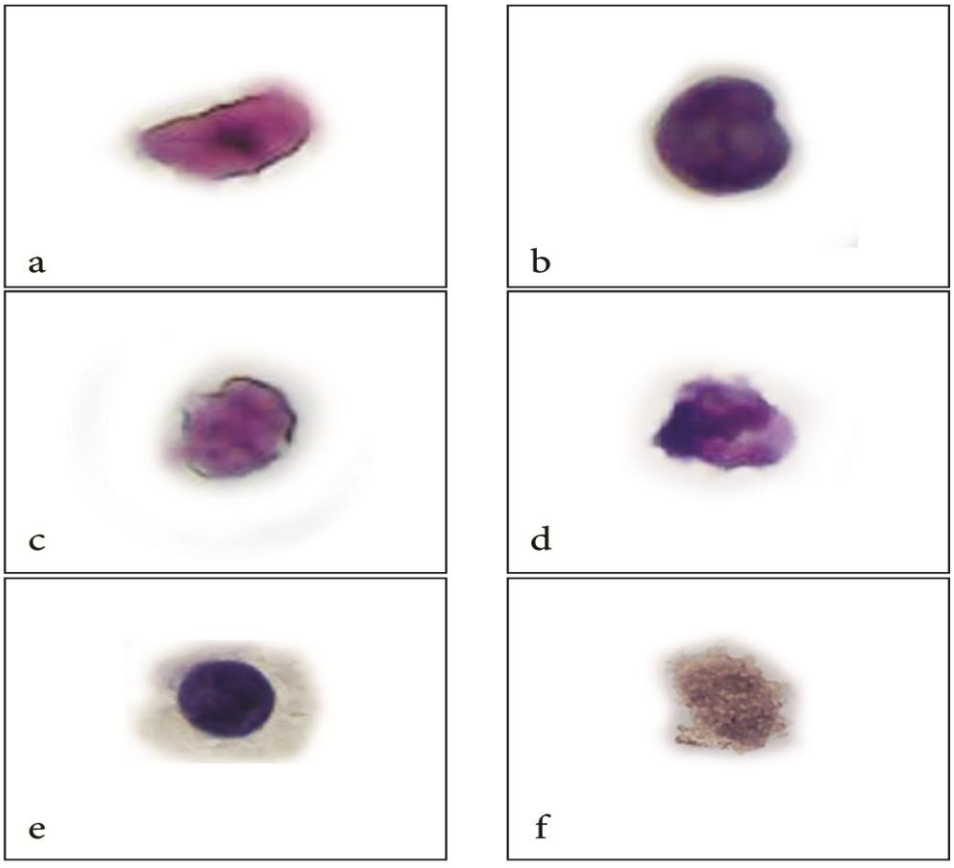

Figure 3: Cells in different stages of apoptosis in treated cells are easily distinguishable. Cell with normal morphology (a). Complete apoptotic cell (b). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (c). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (d). Immunocytochemistry of caspase-3 protein. (e) Control positive cell showing cytoplasm negative reaction for caspase-3 protein. (b) Treated cell showing cytoplasm positive reaction for caspase-3 protein indicating cell apoptosis.

DISSCUTION

Plants are rich in nutrients, vitamins, alkaloids, tannins, polyphenolic compounds and flavonoids. Ginger has long been used in traditional medicine as a cure for some diseases including inflammatory diseases (Bode et al., 2001). Ginger contains active phenolic compounds such as gingerol, paradol and shogoal that have antioxidant, anti-cancer, anti-inflammatory, antiangiogenesis and antiartherosclerotic properties (Mukhopadhyay et al., 2001). It has also been shown to down regulate of NF-κB gene products which were involved in cellular proliferation and angiogenesis, including IL-8 (Lee and Surh, 2001). The recent results confirm these which were obtained (Evan and Vousden, 2005), which showed that saline extract prepared from ginger caused suppression of cell proliferation and marked morphological changes including cell shrinkage and condensation of chromosomes (Meregalli et al., 1998). This is attributed to its anticancer properties (Miyoshi et al., 2003). Literatures suggest that [6]-gingerol, one of the major compounds of ginger is able to inhibit growth and proliferation of colon cancer cells at a concentration of 100μM/L (Jaganathan et al., 2013). Other group of study has shown that after 72 h incubation of [6]-gingerol in colorectal cancer cell, cell growth rate was reduced by 22% and 28% with 150Mm (Jeong et al., 2009).

A dose-dependent increase of DNA fragmentation (by DNA gel-electrophoresis) was observed specially after exposure to the IC_{50} concentration of GO compared with control cells. DNA fragmentation and disintegrating apoptotic cells could be observed. The percentage of both early and late and apoptotic cells was high as was detected by hematoxylin and eosin staining, even there is a suggesting that apoptosis occurred rather slow in colon cancer cells even after treatment (Yusof et al., 2008). Apoptosis of lung epithelium cancer (A549) cells by aqueous extract of ginger is mediated by up regulation of tumor suppressor gene caspase-3 (Vogelstein et al., 2000). Our observation of over caspase-3-expressing is similar to the study (Villunger et al., 2003) which found that [6]-gingerol induced apoptotic death in pancreatic cells (Abdullah et al., 2011). This is consistent with the observation by other researchers whereby colon cancer cells did not undergo apoptosis rapidly (Jaganathan et al., 2011). In our study, we have shown that GO at increasing concentration was not only able to inhibit DNA synthesis but also induced apoptosis specially at high concentrations. GO high concentration induced high percentage of cells that underwent apoptosis and increased GO dose was accompanied by over expression of caspase-3 protein dependently.

CONCLUSION

In this study, we have demonstrated that GO inhibited proliferation and induced apoptosis in colon cancer (Caco-2) cells. Future study will may deal with further investigations of the possible usages of GO as a new alternative chemotherapeutic agent for human colon cancer and other types of cancer.

COMPETING INTERESTS

To our knowledge, previous studies have shown the chemopreventive effect of *Z. officinale* in other cancer cells but less report evidence on GO effect on (Caco-2) colon cancer cells. Thus, our study concerned with represent the mechanism of anticancer effect of GO in colon cancer cells (Caco-2).

ACKNOWLEDGMENTS

We are grateful for all of Nile center for experimental researches team specially Dr. Asmaa M. El-Gharaz (Department of cell culture) for her kindly support in the cell culture and drug induction stage.

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